Understanding molecular mechanism and structural properties of small GTPase mediated-effector activation

Dissertation

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Verständnis des molekularen Mechanismus und der strukturellen Eigenschaften der durch kleine GTPasen vermittelten Effektoraktivierung

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So remember to look up at the stars and not down at your feet. Try to make sense of what you see and hold on to that childlike wonder about what makes the universe exist.

Stephen Hawking

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Abstract

RAS and RHO families of small GTPases are vital elements of signal transduction, which control different biological functions, such as polarity, adhesion, contraction, migration, and differentiation. Abnormal activation of small GTPases cause different diseases, including cancer, neurological disorders, and cardiovascular diseases. These proteins, which act as molecular switches, interact with a variety of effector proteins in their active GTP-bound form and consequently control diverse signaling pathways and biological functions. The molecular mechanism of effector-mediated activation of this superfamily is still not well understood. The aims of this dissertation were to obtain new insight into yet unresolved questions regarding effector activation by RAS/RHO GTPases, and the interaction selectivity of a variety of effectors for different GTPases. Deciphering new functional mechanisms and refining novel targets are important for development of selective drugs, which attenuate signal transduction pathways rather than inhibiting them.

In the first part of this dissertation, we investigated the interaction between RAS association (RA) domain family (RASSF), and different members of the RAS family, such as HRAS, RRAS, RHEB, RALA, RAP1B, and RAP2A. The RASSF family act as non-enzymatic effectors, known as putative tumor suppressors, which are frequently downregulated in cancers. This family contains two groups including RASSF1-6 as group one and RASSF7-10 as group two. However, the mechanism of interaction between this family and RAS proteins is still not clear. By using fluorescence polarization, equilibrium dissociation constants for their interaction were determined. Obtained quantitative results in combination with *in silico* modeling led to the determination of interaction selectivity between different RAS proteins and some members of this effector family, particularly RASSF1 and RASSF5. We found that RASSF group one has higher binding affinities with different RAS proteins as RASSF group two. Especially, RASSF1 and RASSF5 proteins revealed highest binding affinities and sequence similarities among RASSF members in interaction with selected RAS proteins.

In the second part of this thesis, we studied the structure and activation mechanism of ROCK, an effector for the RHO family member RHOA. This protein is an essential regulator of the actin cytoskeleton and stress fiber formation, and is involved in different stages of cardiovascular diseases and is thus a therapeutic drug target. This dissertation provided structural insight into an elongated parallel dimer of purified ROCK full-length protein via electron microscopy. Its kinase activity in phosphorylating its substrate MYPT1 was studied in depth in presence and absence of RHOA. Results suggested that purified ROCK is fully active independent of RHOA. However, we proposed that scaffold proteins might mechanistically modulate ROCK autoinhibition in the cellular context.

In the third part, the interaction of the RHO GTPases, RAC1 and RHOA, with the homology region 1 (HR1) a, b and c of the protein kinase N (PKN) was investigated. PKN is a key effector protein involved in cytoskeleton reorganization and migration. The data indicated that HR1 domains of PKN proteins (HR1a-c) exhibit, in spite of high sequence similarity, different binding properties for RAC1 and RHOA. Therefore, the combination of their binding to RHO proteins appears to control the conformational change and subsequent activation of PKN.

Finally, last part covers a short summary of three papers which focused on comprehensive studies over RAS family proteins, RAF structural properties, and RRAS2 mutations which cause a Noonan syndrome disease.

Zusammenfassung

Die RAS- und RHO-Familien der kleinen GTPasen sind wichtige Elemente der Signaltransduktion, die verschiedene biologischen Funktionen steuern, wie Apoptose, Migration, Stoffwechsel, Kontraktion, Proliferation und Differenzierung. Eine abnormale Aktivierung kleiner GTPasen verursacht verschiedene Erkrankungen, wie zum Beispiel Krebs, neurologische Störungen und Herz-Kreislauf-Erkrankungen. GTPasen fungieren als molekulare Schalter und interagieren mit einer Vielzahl von Effektor-Proteinen. Der molekulare Mechanismus der Effektor-vermittelten Aktivierung dieser Superfamilie ist noch nicht gut verstanden. Die Ziele dieser Dissertation sind es, neue Erkenntnisse über die molekularen Mechanismen der Effektor-Aktivierung durch RAS/RHO GTPasen und ihre Interaktion mit verschiedenen Effektoren zu gewinnen. Das Verständnis der molekularen Mechanismen zur Aktivierung kleiner GTPase-Effektoren und die damit verbundene Identifizierung neuer Zielproteine ist wichtig für die Entwicklung von zielgerichteten Medikamenten, die Signalwege abschwächen, anstatt sie zu hemmen. Im ersten Teil der Doktorarbeit wurde die Interaktion zwischen der *RAS Association (RA) Domain Family* (RASSF) und verschiedenen Mitgliedern der RAS-Familie analysiert. Die Mitglieder der RASSF-Familie wirken als nicht-enzymatische Effektoren und sind mutmaßliche Tumorsuppressoren, die bei Krebserkrankungen häufig herunterreguliert werden. Diese Familie lässt sich in zwei Gruppen unterteilen, RASSF1-6 als erste Gruppe und RASSF7-10 als zweite Gruppe. Allerdings ist der Interaktionsmechanismus zwischen RASSF und RAS-Proteinen unklar. Mit Hilfe der Fluoreszenzpolarisation wurden Gleichgewichtsdissoziationskonstanten für ihre Wechselwirkungen bestimmt. Die quantitativen Ergebnisse in Kombination mit *in silico*-Modellierung wiesen auf eine Interaktionsselektivität zwischen verschiedenen RAS-Proteinen und einigen Mitgliedern dieser Effektor-Familie hin, insbesondere RASSF1 und RASSF5. Wir fanden generell heraus, dass die RASSF Gruppe eins eine höhere Bindungsaffinität mit verschiedenen RAS-Proteinen aufwies als Gruppe zwei. In diesem Kontext zeigten RASSF1- und RASSF5-Proteine aus der ersten Gruppe die höchsten Bindungsaffinitäten und Sequenzähnlichkeiten in ihrer Interaktion mit ausgewählten RAS-Proteinen. Im zweiten Teil haben wir die Struktur und der molekulare Mechanismus des ROCK-Proteins, eines Effektors für das RHO-Familienmitglied RHOA untersucht. Dieses Protein ist ein wesentlicher Regulator des Aktin-Zytoskeletts und der Stressfaserbildung, ist an verschiedenen Stadien von Herz-Kreislauf-Erkrankungen beteiligt und bietet sich damit als neue therapeutische Zielstruktur an. Die Struktur-Funktionsbeziehung der ROCK-Aktivierung durch RHOA ist nicht vollständig verstanden. Die Arbeit gibt strukturelle Einblicke in ein ausgestrecktes, parallel angeordnetes Dimer aus aufgereinigtem, volllängen ROCK mittels Elektronenmikroskopie. Im Detail wurde die Aktivität dieses Proteins überprüft. Hierbei wurde die Phosphorylierung des Substrates MYPT1 in Anwesenheit und Abwesenheit von RHOA mit Hilfe eines Kinase-Assay gemessen. Unsere Daten deuten darauf hin, dass das gereinigte ROCK voll aktiv ist und das Vorhandensein von RHOA seine Aktivität nicht weiter erhöht. Im zellulären Kontext könnten jedoch *Scaffold*-Proteine mechanistisch die ROCK-Autoinhibition modulieren. Im dritten Teil wurde die Interaktion der RHO GTPasen, RAC1 und RHOA, mit der Homologie-Region 1 (HR1) a, b und c der Proteinkinase N (PKN) untersucht. PKN ist ein Schlüsseleffektor, der an der Reorganisation des Zytoskeletts und der Migration beteiligt ist. Unsere Daten zeigen, dass die HR1-Domänen von PKN (HR1a-c) trotz hoher Sequenzähnlichkeiten unterschiedliche Bindungseigenschaften für RAC1 und RHOA aufweisen. Möglicherweise kontrollieren die RHO-Proteine durch ihre unterschiedliche Bindungsaffinitäten die Konformationsänderung und damit einhergehende Aktivierung von PKN. Schließlich umfasst der letzte Teil eine kurze Zusammenfassung von drei Papieren, die sich auf umfassende Studien über Proteine der RAS-Familie, RAF-Struktureigenschaften und RRAS2-Mutationen konzentrierten, die ein Noonan-Syndrom-Erkrankung verursachen.

List of Abbreviations

aa	amino acids
AID	auto inhibitory domain
APS	ammonium persulfate
°C	degree Celsius
CBB	coomassie brilliant blue
Cdc42	cell division cycle 42
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECM	extraceullar matrix
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia, for example
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
Fig.	figure
FL	Full-length
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GST	glutathione S-transferase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
h	hour
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HMEC	human dermal microvascular endothelial cell

HR1	homology region 1
HRAS	Harvey rat sarcoma
IPTG	isopropyl-β-D-thiogalactopyranoside
ITC	isothermal titration calorimetry
kDa	kilo Dalton
KRAS	Kirsten rat sarcoma
lit	litre
M	molar
MAPK	mitogen-activated protein kinase
mg	milligram
µg	microgram
MEK	MARK/ERK kinase
min	minute
ml	milliliter
MLCP	myosin light chain phosphatase
mM	millimolar
mV	millivolt
MW	molecular weight
MYPT1	myosin phosphatase target subunit 1
Ni-NTA	nickel nitrilotriacetic acid
nm	nano meter
Nore1	novel RAS effector
NRAS	neuroblastoma rat sarcoma
OD	optical density
PAGE	polyacrylamide electrophoresis
PH	pleckstrin homology
PIP_2	phosphatidylinositol (3,4)-bisphosphate
PI3K	phosphoinositide 3-kinase
PKN	protein kinase N

RA	RAS associating
RAC	RAS-related C3 botulinum toxin substrate
RAF	rapidly accelerated fibrosarcoma
RAS	rat sarcoma
RASSF	RAS association domain family
RALA	RAS-like protein A
RB	RAS binding
RHEB	RAS homolog enriched in brain
RHO	RAS homology
ROCK	RHO-associated coiled-coil kinase
rpm	rotations per minute
RRAS	RAS-related protein
RT	room temperature
SARAH	Salvador/Rassf/Hipoo
SDS	sodium dodecyl sulfate
sec	second
TBS	tris buffered saline
TBST	tris buffered saline-tween 20
TEMED	Tetramethyldiethyldiamine
TEV	tobacco etch virus
Tris	tri (hydroxymethyl) aminomethane
µM	micromolar
WB	western blot

Amino acid abbreviations

Ala (A)	Alanine
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic Acid
Cys (C)	Cysteine
Gln (Q)	Glutamine
Glu (E)	Glutamic acid
Gly (G)	Glycine
His (H)	Histidine
Ile (I)	Isoleucine
Leu (L)	Leucine
Lys (K)	Lysine
Met (M)	Methionine
Phe (F)	Phenylalanine
Pro (P)	Proline
Se (S)	Serine
Thr (T)	Threonine
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
Val (V)	Valine

I dedicate this thesis to all scientists who sacrificed their life to boost safety and wellbeing of humanity.

Chapter I: General Information

1. RAS superfamily

The RAS superfamily of small guanosine triphosphatases (GTPases) consists of 167 human members, which based on their sequence similarities and function are divided into five major groups: RAS, RHO, RAB, RAN and ARF (Rojas, Fuentes, Rausell, & Valencia, 2012; Wennerberg, Rossman, & Der, 2005; Wittinghofer, 2014) (Fig. 1.1). The RAS-like proteins in the brain family (RAB family) comprise the largest group of this superfamily and is involved in intracellular vesicular transportation and trafficking (Zerial & McBride, 2001). The ADP-ribosylation factor proteins (ARF), similar to the RAB family regulate vesicular transportation (Nie, Hirsch, & Randazzo, 2003). The RAS-like nuclear family (RAN family), is involved in nucleocytoplasmic transportation of RNAs and proteins as well as mitotic spindle organization. This group is known as one of the most abundant small GTPase within the cell. RAS homologous proteins (RHO) are well-known for their impact on the regulation of intracellular actin organization and cytoskeleton, and gene expression. The RAS sarcoma family (RAS family) regulates a wide range of signaling pathways, gene expression, and is also involved in cell survival, proliferation and differentiation (Eckert et al., 2004; Takai, Sasaki, & Matozaki, 2001). In these studies, we focused mainly on the structural-functional relationship of two families of RAS and RHO proteins, because of their prominent roles in tumorigenesis and migration, respectively.

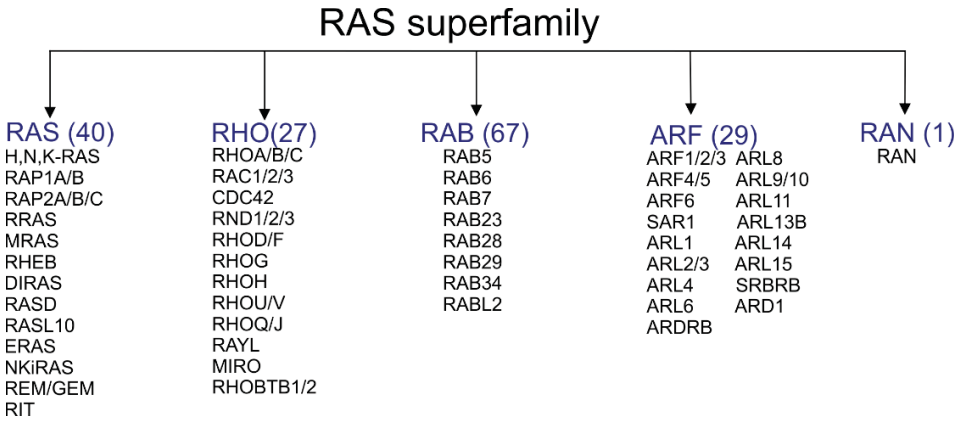

Figure 1.1: RAS superfamily of small GTPases. RAS superfamily proteins consist of 167 human proteins which are divided into five subfamilies, including RAS, RHO, RAB, RAN, ARF, and the numbers indicate the members of each subfamily. Modified from (Wittinghofer, 2014).

Small GTPase proteins act as a molecular switch between inactive GDP-bound form and active GTP-bound form. This cycle is conserved in most small GTPases and achieved by two biochemical reactions; The GDP/GTP exchange reaction and the GTP hydrolysis reaction (Vigil, Cherfils, Rossman, & Der, 2010) (Fig. 1.2). The Guanine nucleotide exchange proteins (GEFs) promote the formation of GTP bound form. The GTPase activating proteins (GAPs) that accelerate the intrinsic GTP hydrolysis (Vetter & Wittinghofer, 2001). Small GTPase proteins, in their active GTP-bound state, interact specifically with a large variety of effector proteins and lead to different biological functions. These proteins share common G domains (G1-G5) and they are around 20 kDa. The majority of them have additional carboxy-terminal hyper-variable (HVR) which undergo post-translational modification and is important for membrane interaction (Konstantinopoulos, Karamouzis, & Papavassiliou, 2007; Vigil et al., 2010).

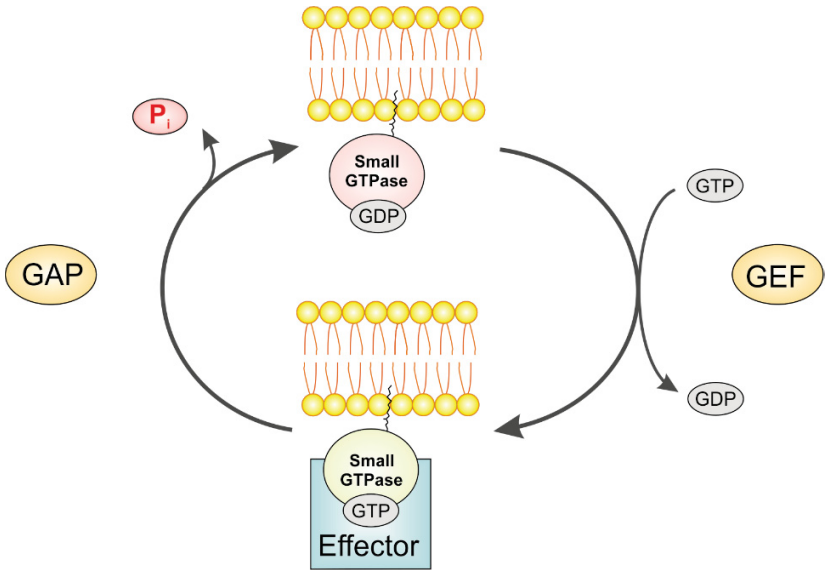

Figure 1.2: Small GTPase cycle. GDP-bound form of GTPases is inactive. A GDP/GTP exchange results in their activation, where the GTP-bound GTPase specifically interacts with its effectors. GEFs and GAPs are regulatory proteins, which stimulate this cycle.

1.1 RAS family

1.1.1 Historical background

The history of the RAS protein family dates back in 1960s, when the highly oncogenic Harvey and Kirsten murine sarcoma viruses (Ha-MSV and Ki-MSV) were discovered by Jennifer Harvey and later Werner Kirsten to cause rapid tumor formation in rats (Harvey, 1964; Malumbres & Barbacid, 2003) (Fig. 1.3). These viral oncogenes, named Harvey and Kirsten RAS (HRAS and KRAS), along with their neuroblastoma RAS (NRAS) viral oncogene homolog, are activated versions of genes encoding 21-kDa phosphor-protein (p21) with guanine nucleotide (GDP and GTP) binding and GTP hydrolyzing activities (Malumbres & Barbacid, 2003; Nakhaei-Rad et al., 2018).

More recent studies have provided evidences for the existence of specific regulators (guanine nucleotide exchange factors or GEFs and GTPase activating proteins or GAPs) and effector proteins activating individual pathways (Cherfils & Zeghouf, 2013; Hennig, Markwart, Esparza-Franco, Ladds, & Rubio, 2015; Keeton, Salter, & Piazza, 2017; Upadhyaya, Bedewy, & Pei, 2016). As the founding members and prototypes of the RAS superfamily proteins (Rojas et al., 2012; Wennerberg et al., 2005; Wittinghofer & Vetter, 2011), HRAS, KRAS and NRAS have become the subject of intense investigations due to their central involvements in signal transduction and their critical contribution to human disease and disorders (Hobbs, Der, & Rossman, 2016; Simanshu, Nissley, & McCormick, 2017).

Later on, other members of RAS proteins (RRASs, RAPs, RHEB, RALs, etc.), which have sequence similarity, have been investigated (Fig. 1.3). The RAS family contains 23 genes encoding for at least 25 RAS paralogs. Additionally , phylogenic analysis identified 25 members of the RAS family out of 35 sequences and they can be divided into eight groups: RAS, RRAS, RAL, RAP, RIT, RHEB, RASD and DIRAS family (Nakhaei-Rad et al., 2018; van Dam, Bos, & Snel, 2011). RASL, RERG, and NKIRAS proteins exhibit strong sequence deviation and thus, excluded from the list (van Dam et al., 2011).

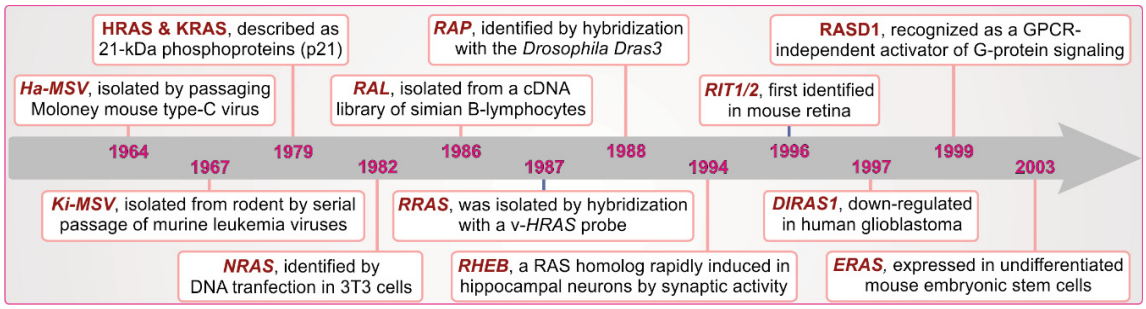

Figure 1.3: Historical timeline of the discovery of various members of the RAS family. Modified from (Nakhaei-Rad et al., 2018).

1.1.2 Structural properties of the RAS GTPases

The RAS family proteins share a highly conserved GDP/GTP binding domain (G domains), which is responsible for nucleotide-dependent conformational changes (Vetter & Wittinghofer, 2001). The structural differences between the two states are primarily confined to two highly mobile regions, designated as a switch I (residues 28-39) and switch II (residues 59-74). In the active state, Tyr-32 and Thr-35 in the switch I and Gly-60 in switch II form main chain hydrogen bonds with the ɣ-phosphate of GTP. GTP hydrolysis triggers drastic rearrangements of the switch regions, resulting in the reorientation of these three residues away from the active site (Fig. 1.4).

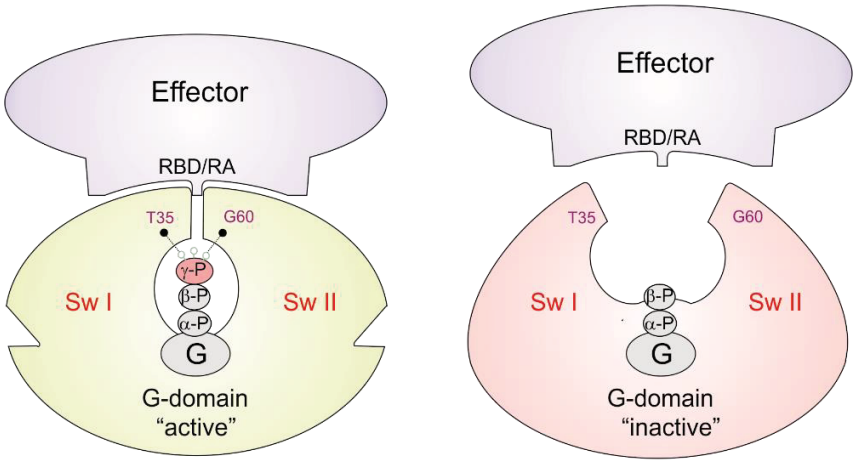

Figure 1.4: The molecular mechanism of GDP-bound form and GTP-bound form of RAS. The left panel shows the active form of RAS (green), and the right panel (pink) shows an inactive form. The exchange of GDP/GTP leads to conformational changes in switch regions, which are responsible for effector binding (blue). Hydrogen bonds between Tyr-35 in the switch I and Gly-60 in switch II with γ-phosphate (red) of GTP cause rearrangement in structure and effector binding.

Although the G domain uses a universally conserved switching mechanism (Wittinghofer & Vetter, 2011), its structure, function and GTP hydrolysis (or GTPase) reaction are adapted to many different signaling pathways and processes (Nakhaei-Rad et al., 2018). The G domains contain five conserved motifs named G1-G5, which are essential for nucleotide and magnesium binding (Bourne, Sanders, & McCormick, 1991) (Fig 1.5). G1 is known as the phosphate-binding loop or P-loop ($P\text{-}_{10}GXXXXGK(S/T)_{17}$; HRAS numbering), as it is responsible for the binding of the phosphate groups of GDP and GTP. P-loop exists not only in GTP-binding proteins but also in ATP-binding proteins (Saraste, Sibbald, & Wittinghofer, 1990). This region contains several important residues followed by a conserved lysine and a serine or threonine. Gly-12 and Gly-13 (HRAS numbering) which are frequently mutated codons in human tumors (Malumbres & Barbacid, 2003) leading to impairment of the GTPase reaction (Ahmadian et al., 1999). The majority of RAS family members contain a glycine at position 12, except ERAS, RASD1/2 and DIRAS3. Therefore, they are constitutively active and are GAP insensitive (Kontani et al., 2002; Nakhaei-Rad et al., 2015). RHEB1 and RHEB2 have an extremely slow GTPase reaction due to an arginine and a serine or a cysteine instead of Gly-12 and Gly-13, respectively, but is interestingly switched off by RHEBGAPs, such as tuberin (also called TSC2) (Scrima, Thomas, Deaconescu, & Wittinghofer, 2008). In the case of ERAS and RASD1/2, there is Ser-12 instead of glycine, and DIRAS3 harbors alanine in this position. In contrast to Gly-12 mutation, another critical residue is Ser-17 (HARS numbering). If this residue is mutated to asparagine, RAS proteins are mainly captured in their inactive form. Overexpressed RAS (S17N) tightly binds to endogenous RASGEFs and sequesters them from endogenous RAS proteins, and thus, interferes with RAS activation (Feig, 1999). G2 (also called effector loop) is an integral part of effector-binding site and contains the highly conserved Tyr-32 and an invariant Thr-35 (HRAS numbering), which are critical for the conformational rearrangement of switch I. RIT1/2 contain histidine at the corresponding position of Tyr-32, which may be the reason for an accelerated nucleotide dissociation (Shao, Kadono-Okuda, Finlin, & Andres, 1999). G3 is a part of switch II and contains the critical catalytic Gln-61 position (HRAS numbering). Similarly to Gly-12 mutations, replacement of Gln-61 by virtually any other amino acid significantly reduces

the intrinsic hydrolysis rate, prevents the GAP-mediated inactivation and thus, induces oncogenic transformation by constitutive activation of RAS (Malumbres & Barbacid, 2003). There is a threonine in RAP paralogs instead of Gln-61, asparagine in RASD1/2, glycine in DIRAS3 and serine in DIRAS1/2. In contrast to RASD1/2 and DIRAS3, which seem to have an impaired GTPase activity (Kontani et al., 2002). Thr-61 in RAP paralogs and most interesting Ser-65 in DIRAS1 and DIRAS2 (Gln-61 in HRAS1), do not compromise the GTPase reaction especially in the presence of RASGAPs (Scrima et al., 2008). GTPase deficiency of RASD and DIRAS paralogs may even be strengthened by an additional amino acid deviation at position 59 (Fig. 1.5). G4 and G5 contain invariant residues and are responsible for the guanine base recognition and contain invariant residues (Paduch, Jeleñ, & Otlewski, 2001). Mutation of Asp-119 in RAS changes the nucleotide specificity from guanosine to xanthosine nucleotide (Schmidt et al., 1996) and acts as dominant negative in a dose dependent manner. G5 provides Ser-145 that stabilizes Asp-119 of G4. Ala-146 binds the guanine base and is another determinant for the guanine-binding ability of the RAS proteins. Lys-147 in replaced in RIT1/2 by alanine and may affect, together with the deviation in G2, the nucleotide binding affinity (Shao et al., 1999).

RAS proteins associate with membranes via series of post-translational modification at the very C-terminal CAAX motif (C is the cysteine, A is any aliphatic amino acid and X is any amino acid)(Lane & Beese, 2006). If the amino acid in the X position of CAAX box is a leucine, as in the case of RALA/B, RRAS1/3, RAP1A/B, RAP2A, then geranylgeranyl transferase modifies the protein with a geranylgeranyl moiety (Benetka, Koranda, Maurer-Stroh, Pittner, & Eisenhaber, 2006), otherwise the protein is modified with a farnesyl moiety by farnesyl transferase (Ahearn, Haigis, Bar-Sagi, & Philips, 2012; Berndt et al., 2011). Two post-prenylation enzymetic steps are critical for proper localization, including proteolytic cleavage of the AAX residues by the endopeptidase REC1 and methylation of the terminal isoprenylcysteine by the methyltransferase ICMT (Ahearn et al., 2012; Berndt et al., 2011; Winter-Vann & Casey, 2005). Due to relatively weak affinity of isoprenylated proteins for cellular membranes (Silvius & l'Heureux, 1994), additional motifs in the hypervariabel region (HVR) are engaged in fine-tuning membrane association with RAS proteins and their functions (Abankwa, Gorfe, & Hancock, 2007; Hanzal-Bayer & Hancock,

2007; Omerovic & Prior, 2009). Some RAS proteins, e.g. KRAS4B, RALA, RRAS3, and RIT1/2, contain a stretch of positively charged amino acids (called polybasic region or PBR), which has been implicated to contact negatively charged phospholipids of the cell membrane (Banerjee, Jang, Nussinov, & Gaponenko, 2016; Nussinov, Tsai, Chakrabarti, & Jang, 2016). Membrane association of KRAS4B is modulated in different ways (Bhagatji, Leventis, Rich, Lin, & Silvius, 2010). PDEδ binds to farnesylated KRAS4B (Dharmaiah et al., 2016) and transport it from perinuclear membranes to plasma membrane (Schmick et al., 2014). ERK1/2 phosphorylates RRAS1/2 at Ser-186 and Ser-201, but not RRAS3, and does not affect their subcellular localization but rather stimulates their activation (Frémin et al., 2016). A further way of increasing the affinity of isoprenylated proteins for cellular membranes is an addition of one or more lipid anchors. KRAS4A, NRAS, HRAS1, ERAS, RRAS1, RAP2A/B, and RALA/B are palmitoylated by acyl protein transferases at cysteine prior to the CAAX motif (Beranger & Tavitian, 1991; Gentry, 2015; Hancock, Magee, Childs, & Marshall, 1989; Schroeder et al., 1997; Tabaczar, Czogalla, Podkalicka, Biernatowska, & Sikorski, 2017; Y. Takahashi et al., 2005; Uechi et al., 2009). In contrast to HRAS1, HRAS2 does not have any C-terminal sites for post-translational modifications, and appears to be distributed between cytosol and nucleus (Guil et al., 2003) (Fig 1.5). Another emerging concept in the field in based on physical interaction of the G domain itself with lipid membrane. A membrane-based, nucleotide-dependent conformational switch operates through distinct regions on the surface of RAS proteins, including the HVR, which reorient with respect to the plasma membrane (Abankwa, Gorfe, Inder, & Hancock, 2010; Cirstea et al., 2010). G domain-membrane interaction may contribute to the specificity of signal transduction and may underlay additional control elements. A critical aspect in this content is the organization of RAS proteins into protein-lipid complexes. These so-called nanoclusters concentrate RAS at the plasma membrane. They are the sites of effector recruitment and activation, and are essential for signal transmission (Abankwa et al., 2007; Zhou & Hancock, 2015).

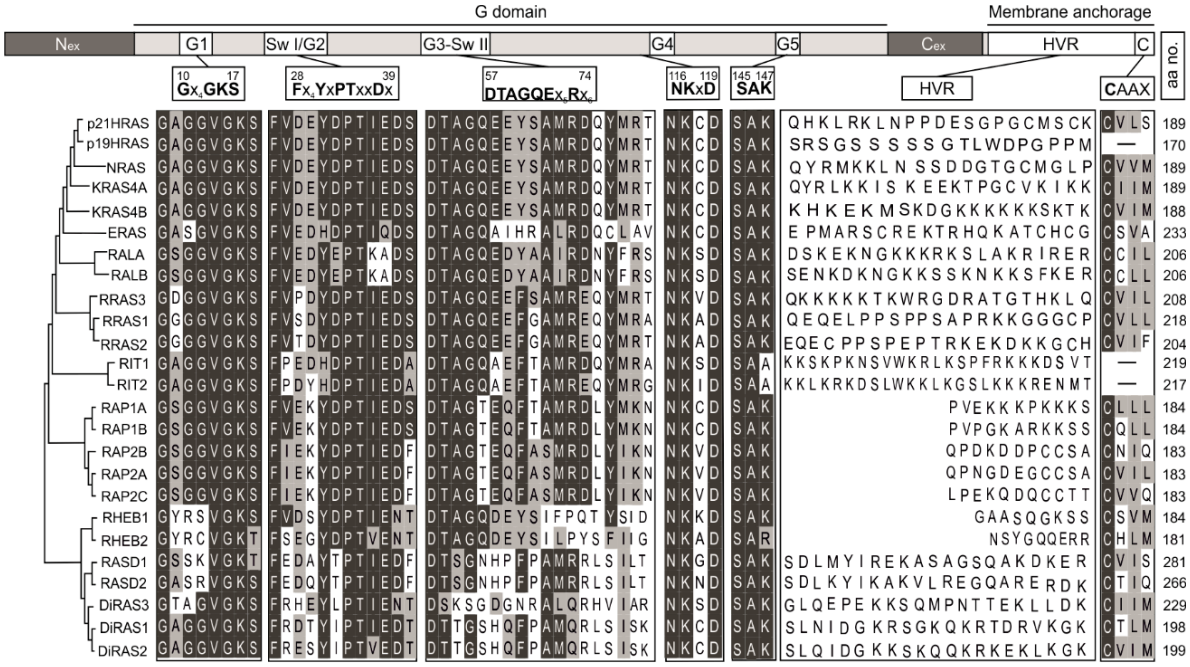

Figure 1.5: Evolutionary conservation of RAS family members. Signature motifs of 25-related proteins are presented according to their phylogenetic categorization. These proteins consist of a G domain with five conserved motifs and a variable C-terminal membrane anchorage region, divided into the hypervariable region (HVR) and CAAX motif. HVR contains several cysteines and series of post-translational modifications, positively charged residues, and other putative motifs. In G domains and CAAX box, conserved amino acids are shown in dark grey, homologous residues in white and variable amino acids in light grey. Modified from (Nakhaei-Rad et al., 2018).

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1.1.3 RAS effectors and signaling pathways

Signal transduction implies physical association of RAS proteins and activation of a spectrum of functionally diverse downstream effectors. These effectors specially interact with the active, GTP-bound form of the RAS proteins, usually, in response to extracellular signals, and link them to downstream signaling pathways in all eukaryotes (Gutierrez-Erlandsson et al., 2013; Karnoub & Weinberg, 2008). They act as protein or lipid kinases, phospholipase, GEFs, GAPs and scaffold proteins (Bunney et al., 2006; Castellano & Downward, 2011; Chan et al., 2013; Ferro & Trabalzini, 2010; Herrmann, 2003; Nakhaei-Rad et al., 2016; Nakhaeizadeh, Amin, Nakhaei-Rad, Dvorsky, & Ahmadian, 2016; Rajalingam, Schreck, Rapp, & Albert, 2007). Two major groups of effectors contain RAS binding (RB) and RAS association (RA) domains, respectively (Nakhaeizadeh et al., 2016; Repasky, Chenette, & Der, 2004; Wohlgemuth et al., 2005). Notably, both types of domains (RB and RA domains) use critical determinants for the interaction with different

RAS proteins, particularly the intermolecular β-sheets (Nakhaeizadeh et al., 2016). Structural studies have provided deep insights into the binding modes and interaction specificities (Mott & Owen, 2015) and yet, the precise mechanism, through which effector association with activated RAS proteins results in effector activation, is still unclear. However, it is generally, accepted that RAS proteins participate directly in the activation of their downstream effectors and do not simply mediate recruitment to specific sites of the membrane. The RAS paralogs share similar effector binding regions with other members of the RAS family but also show distinct deviations (residues 30 and 31 in switch I, and 64, 65, 71, 72, and 73 in switch II) suggesting that they may share downstream effectors with different affinities. (Cox & Der, 2003; Gentry, Martin, Reiner, & Der, 2014; Nakhaeizadeh et al., 2016; Nassar et al., 1996). Here some of the well-known effectors have been described (Fig. 1.6).

CRAF was investigated as a first RAS effector which contains RB domain and belongs to the serine/threonine protein kinase (Kiel et al., 2005; Rezaei Adariani et al., 2018). Later on, BRAF and ARAF which are other members of this family, were investigated. RAF kinases (CRAF, BRAF and ARAF), constitute a small family of serine/threonine kinases, which control evolutionarily conserved pathways and display essential roles during development (Su An et al., 2015; Théodora S Niault & Manuela Baccarini, 2010; Sanges et al., 2012). Thus, it is not surprising that their dysregulation is associated with progression of a variety of human cancers (Su An et al., 2015; Downward, 2003; G Maurer, Bartek Tarkowski, & Manuela Baccarini, 2011; Michael Roring & Tilman Brummer, 2012), pathogenesis of developmental disorders including Noonan, LEOPARD, and cardiofaciocutaneous syndromes (Allanson et al., 2011; Tartaglia, Gelb, & Zenker, 2011), and cardiovascular diseases, such as pulmonary arterial hypertension and heart failure. Works from many laboratories have shown that RAF kinases are integral elements of the RAS–MAPK pathway, which is involved in different signaling pathways (Amardeep Singh Dhillon, Hagan, Rath, & Kolch, 2007; Karnoub & Weinberg, 2008; D. K. Morrison & Cutler Jr, 1997; Rauch, Rukhlenko, Kolch, & Kholodenko, 2016; P. J. Roberts & Der, 2007; Drieke Vandamme, Ana Herrero, Fahd Al-Mulla, & Walter Kolch, 2014). Activation of RAF kinases at the plasma membrane by RAS (Moodie, Willumsen, Weber, & Wolfman, 1993; Van

Aelst, Barr, Marcus, Polverino, & Wigler, 1993; Vojtek, Hollenberg, & Cooper, 1993; Warne, Vician, & Downward, 1993; X.-F. Zhang et al., 1993), together with the identification of their substrates MEK1/2 (MAPK/ERK kinase 1/2) (Kyriakis et al., 1992) has provided the missing link between growth factor signals and MAPK cascade activation (Matallanas et al., 2011). The activities of RAF kinases toward MEK differ widely, with BRAF being the strongest MEK activator, followed by CRAF and ARAF (Angela Baljuls, Boris N Kholodenko, & Walter Kolch, 2013; Deborah T Leicht et al., 2013; Marais, Light, Paterson, Mason, & Marshall, 1997). These proteins obviously underlay different regulatory mechanisms, including binding to membrane-associated RAS proteins, phosphorylation, and dephosphorylation along with homodimerization and heterodimerization (Angela Baljuls et al., 2013; Freeman, Ritt, & Morrison, 2013; Matallanas et al., 2011; Rodriguez-Viciana, Oses-Prieto, Burlingame, Fried, & McCormick, 2006; Linda K Rushworth, Alison D Hindley, Eric O'Neill, & Walter Kolch, 2006; Ünal, Uhlitz, & Blüthgen, 2017).

The second best-characterized RAS effector family, PI3K (class I PI3K), phosphorylates phosphoinositide (4,5) bisphosphate (PIP_2) and generates the second messenger phosphoinositide (3,4,5) trisphosphate (PIP3) that recruits the wide range of protein effectors through their pleckstrin homology (PH) domain to the membrane. Target proteins could be kinases (e.g. AKT and PDK1), adaptor proteins, GEFs, or GAPs that regulate different cellular processes. PI3K-AKT pathway is very well known in controlling cell cycle entry, cell growth, survival, and metabolism (Castellano & Downward, 2011). HRAS1, NRAS, KRAS4B, ERAS, RRAS, and RAP1A activate PI3Ks. AKT or protein kinase B (PKB) belongs to the AGC subfamily of protein kinases. AKT is one of the key proteins downstream of PI3K-PIP3 involved in a wide range of the cellular processes, such as cell proliferation, metabolism, growth, autophagy inhibition, and survival (Franke, Kaplan, & Cantley, 1997; Hers, Vincent, & Tavaré, 2011). Upon extracellular stimuli and the tyrosine receptor activation, class I PI3K generates the PIP3 that engages both PDK1 and AKT through PH domain to the plasma membrane. PDK1 phosphorylates AKT at position Thr-308 that is located on the catalytic domain of AKT (Dario R Alessi et al., 1997). This phosphorylation triggers the inhibitory phosphorylation of TSC1/2 that is a well-known

GAP for RHEB protein. Phosphorylation of TSC1/2 suppresses its inhibitory effect on mTORC1 (Inoki, Li, Zhu, Wu, & Guan, 2002). The second key phosphorylation site for AKT is located on the hydrophobic motifs of AKT Ser-473 that will be phosphorylated through the second mTOR complex (mTORC2).

Phospholipase C epsilon (PLCε) contains C-terminal RA domains, RASGEF domain, and PIP_2 lipase C activities, which controls endocytosis, exocytosis, and cytoskeletal reorganization (Bunney et al., 2006; Erijman & M Shifman, 2016; Kelley, Reks, Ondrako, & Smrcka, 2001). RAL guanine nucleotide dissociation stimulator (RALGDS) links RAS with RALA/B and regulates cellular processes such as vesicular trafficking, endocytosis, and migration (Ferro & Trabalzini, 2010; Neel et al., 2011). RASSF family is responsible for inhibition of cell growth as well as induction of apoptosis (Chan et al., 2013; Katz & McCormick, 1997), and are known as a tumor suppressor which their binding affinities with different RAS proteins, were investigated in this thesis. In the following section more details about this family of the RAS effector is presented.

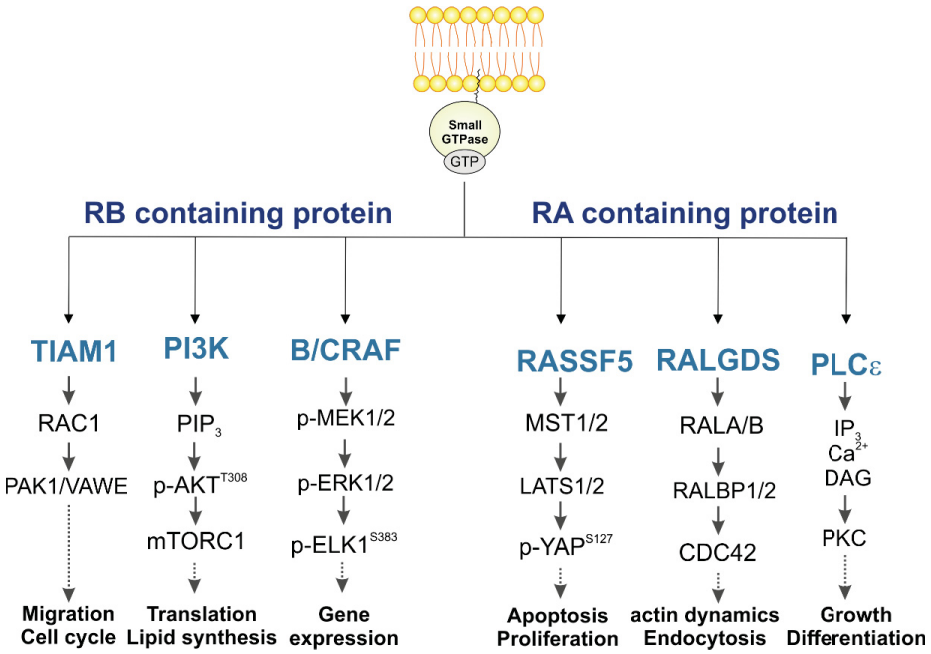

Figure 1.6: RAS effectors and downstream pathways. RAS proteins in active form are capable of interaction with a variety of effectors (blue) and lead to different signaling pathways (black). The majority of these pathways are involved in differentiation, proliferation, apoptosis and gene expression.

1.1.3.1 RAS association containing protein family (RASSF family)

RASSF family comprises as a group of ten proteins, which interact with RAS proteins via their RA domain. Based on the position of their RA domain, they are divided into two groups; RASSF1-6 which is known as group one, and their RA domain is located in C-terminus. Also, they have SARAH domain (Salvador-RASSF-Hippo), which involves in hetero- and homo- dimerization of the RASSF isoforms and interacts with other proteins such as MST1/2. The second group is RASSF proteins (RASSF7-10) with an N-terminal RA domain (Chan et al., 2013) (Fig. 1.7).

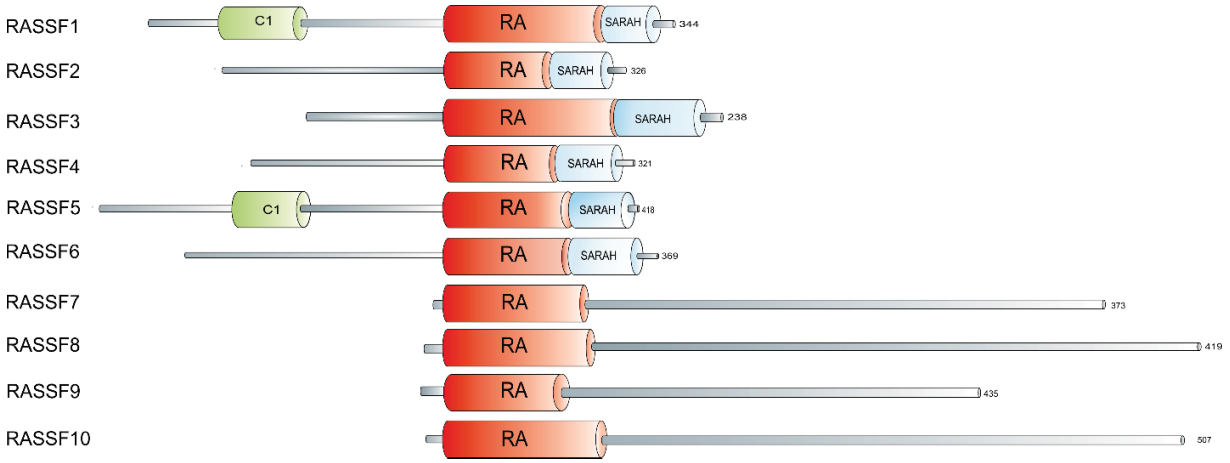

Figure 1.7: Domain organization of RASSF family proteins. Different domains are highlighted, including RAS association domain (RA) in red, C conserved region (C1) in green, the Salvador-RASSF-Hippo domain (SARAH) in blue. Group one contains RASSF1-6 and group two with N-terminal RA domains includes RASSF7-10.

It has been shown that group one of RASSF family, especially RASSF1 and RASSF5 often promote activation of pro-apoptotic kinases, such as MST1/2 which are the mammalian ortholog of the *Drosophila* Hippo kinase, a serine/threonine kinase that plays important roles in cell proliferation, organ size control and apoptosis and exists in two forms: 36kDa caspase cleaved version and 54 kDa full-length protein (Bitra, Sistla, Mariam, Malvi, & Anand, 2017). The full-length protein contains a kinase domain, a C-terminal regulatory region and SARAH domain; however, the truncated version of MST1 only has kinase domain. Crystal structure of SARAH-SARAH domains of MST1 and RASSF showed their interaction leads to form helical antiparallel homo- or hetero-dimers and hydrophobic residues stabilizing the interface (Sanchez-Sanz et al., 2016). MST1/Hippo kinases are

vital in activation of the downstream kinase LATS1/2, as well as warts in *Drosophila*. LATS1/2 stimulates inactivating phosphorylation and cytoplasmic sequestration of the YAP/Yorkie transcription factor which leads to a reduction in proliferation of the cell and increases apoptosis and also interaction with P53 family member (Ferraiuolo, Verduci, Blandino, & Strano, 2017). The RASSF family genes are frequently inactivated by promoter hypermethylation in different human tumors such as lung cancer or hepatocellular carcinoma (L. van der Weyden & D. J. Adams, 2007). They are involved in post-transcriptional inactivation via calpain-mediated proteolysis. Moreover, aberrant epigenetic modifications of RASSF family is the most common aberration of the signaling pathway in human tumors. For example, RASSF6 is strongly reduced in sporadic colorectal cancer tissues, gastric cancer and pancreatic cancer (Barnoud, Schmidt, Donninger, & Clark, 2017; Younesian et al., 2017). Additionally, *RASSF4* overexpression inhibits proliferation and signaling pathways in osteosarcoma cells, which is the most prevalent bone tumor (M. Zhang, Wang, Zhu, & Yin, 2017). RASSF proteins are involved not only in tumorigenesis, proliferation, and apoptosis but also in other cellular functions. For example, RASSF1A protein is involved in the regulation of cardiac function, and RASSF5 protein applies lymphocyte adhesion and trafficking (Pfeifer, Dammann, & Tommasi, 2010).

There are many aspects of RASSF proteins, which require further investigation. The activation mechanism of RASSF family via RAS proteins is still unclear. Among all the members of this family, the X-ray structure of RA RASSF5 (also is known as NORE1 and RAPL) with HRAS has been investigated. The RA domain in this protein is two times larger (around 160 aa) than the enzymatic effectors such as RAF RBD, which explains the long lifetime of the complex between this domain and HRAS (Stieglitz et al., 2008). In this complex formation, both electrostatic and hydrophobic interactions are involved (Chan et al., 2013). This feature is a specific attribute characterizing RASSF function as a scaffold protein. There are various studies that indicated the binding of RASSFs to different RAS family proteins, such as ITC measurement, pull-down assay and *in vivo* studies on different cell lines and etc. (Chan et al., 2013; Dallol et al., 2009; Miertzschke et al., 2007; Vos, Ellis, Bell, Birrer, & Clark, 2000).

Although, by definition, all RASSF proteins contain RA domain, but the presence of this domain does not guarantee that this protein directly interacts with RAS proteins with the same affinity. Therefore, we need to understand, how RAS proteins regulate the RASSF family activation and how activated RASSF proteins modulate downstream signaling pathways.

1.2 RHO family

RHO (RAS homologue) GTPases, are another member of RAS superfamily and are involved in different cellular processes such as modulation of cytoskeletal organization, transcription, cell cycle progression, and cell polarity (Zong, Kaibuchi, & Quilliam, 2001). Additionally, it has been indicated that dysregulation and dysfunction of RHO proteins result in different diseases; for example, cardiovascular disease, neurological disorders such as Alzheimer's disease or Parkinson's disease, tumor invasion, and human immunodeficiency syndrome (Jaffe & Hall, 2005; Raftopoulou & Hall, 2004). In human, this family has 22 protein members, which can be divided into six subgroups: (1) RHO-related proteins such as RHOA, RHOB and RHOC; (2) the RAC-related proteins for example, RAC1, RAC2, RAC3, and RHOG; (3) the CDC42-related proteins including, CDC42, TC10, TCL, RHOV; (4) RHOD-related proteins such as, RHOD, RIF; (5) RND proteins including RND1, RND2, RND3; (6) RHOBTB group, for example, RHOBTB1, THOBTB2, RHOH (Piekny, Werner, & Glotzer, 2005). In terms of structure, RHO proteins compared to the RAS family have an extra α-helix between α3 and β5, which is known as an insert region and is required for RHO kinase effector activation but not for binding (Zong et al., 2001).The best characterized members of this family are: RHOA, RAC1, and CDC42 (Etienne-Manneville & Hall, 2002). Activation of RHOA protein results in the formation of actin stress fibers and focal adhesion assembly, while RAC1 leads to the formation of lamellipodia and membrane ruffling, and CDC42 promotes the formation of filopodia (Jaffe & Hall, 2005; Nobes & Hall, 1999; Wherlock & Mellor, 2002). Moreover, other RHO GTPase proteins including, RHOU, RHOD, RHOF, and RHOQ also lead to filopodia formation. Additionally, RHO GTPases are involved in different aspects of neuronal development, such as axon guidance, axon specification, and neurite extensions,

and disturbed RHO GTPase signaling might lead to cognitive disorders (Aspenström, Fransson, & Saras, 2004; Govek, Newey, & Van Aelst, 2005; Neudauer, Joberty, Tatsis, & Macara, 1998; Tao, Pennica, Xu, Kalejta, & Levine, 2001).

Similar to RAS proteins, RHO GTPase also cycle between an inactive GDP-bound form and an active GTP-bound form. However, they are regulated by three groups of proteins: RHOGAPs, enhance the RHO intrinsic activity to its inactive form, RHOGEFs promote the exchange between the inactive to active form, and GDP-dissociation inhibitors (GDIs) block RHO GTPase activity by sequestering the GDP-bound form from the membrane (Dovas & Couchman, 2005). When RHO proteins are activated, they interact with a variety of target proteins (effectors) which leads to activation of downstream signal transduction.

1.2.1 RHO effectors

RHO proteins interact with different effectors and regulate variety of cellular pathways (Bishop & Alan, 2000). So far, more than 100 effectors for the RHO family have been investigated which are either Kinase proteins or scaffold proteins. Kinase proteins including, RHO-associated coiled-coil kinase (ROCK), protein kinase novel (PKN), citron kinase (CRIK), and P21-activated kinase (PAK) are involved in different downstream phosphorylation cascades (Amin et al., 2013; Dvorsky, Blumenstein, Vetter, & Ahmadian, 2004; Jaiswal, Fansa, Dvorsky, & Ahmadian, 2013; Narumiya, Tanji, & Ishizaki, 2009; Zhao & Manser, 2005). Additionally, scaffold proteins, such as IQ motif containing GTPase activating protein1 (IQGAP1), Wiskott-Aldrich syndrome protein (WASP), Drosophila diaphanous (mDia1), and Rhotekin (RTKN), are important interacting partner for RHO family and they are involved in coordinating many signaling pathways (Hedman, Smith, & Sacks, 2015; Liu, Wang, Chi, Wu, & Chen, 2004). However, the exact function of many of these effectors is still not clear. In the following parts, some of them are described in detail.

1.2.1.1 RHO-associated coiled-coil kinase (ROCK)

ROCK proteins are one of the best investigated groups of the RHO effector proteins which contain two isoforms, ROCK1 (is also named ROKβ and p160ROCK) and ROCK2 (also is called ROKα), they share 65% in their full-length sequence and in their kinase domain

95% identity (Matsui, Yonemura, Tsukita, & Tsukita, 1999; Nakagawa et al., 1996). They act as key regulators of actin cytoskeleton reorganization, cell morphology, motility, division, contraction, polarity and gene expression (Amin et al., 2013). Although, both ROCK isoforms are important in different biological functions, ROCK1 is expressed mainly in lung, testes, liver, spleen, and kidneys, whereas the expression of ROCK2 is mostly limited to the brain and heart (Morgan-Fisher, Wewer, & Yoneda, 2013). It has been shown that ROCK proteins are involved at different stages of cardiovascular diseases, such as cerebral and coronary vasospasm, hypertension and heart failure. Therefore, ROCK proteins are considered as a therapeutic target in cardiovascular medicine (Satoh, Fukumoto, & Shimokawa, 2011). ROCK proteins contain the N-terminus kinase domain, followed by a central amphipathic α-helical segment and a C-terminus pleckstrin homology (PH) domain which is split by insertion of C1 domain (Wen, Liu, Yan, & Zhang, 2008). However, this insertion does not change the structure of the PH domain, which has the ability to interact with 3-phosphate phosphinositides and regulates the localization of the protein. Central coiled-coil region contains, an N-terminal homology region 1 (HR1), RHO interaction domain (RID) and RHO binding domain (RBD), which are responsible for RHOA binding. RID overlaps with the shroom binding domain (SBD) which mediates ROCK-shroom complex formation and has been proposed that regulate ROCK cellular distribution and morphology (Mohan et al., 2013) (Fig. 1.8).

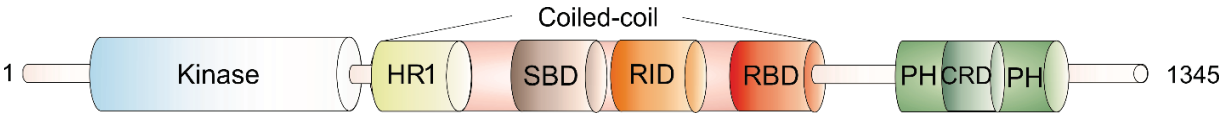

Figure 1.8: Domain organization of ROCK protein. Different domains of ROCK are highlighted in different colors, including kinase domain in blue, coiled-coil region in pink, which contains HR1 (light green), SBD (brown), RID (orange) and RBD (red) and PH domain in green, which is split via CRD (dark green).

It has been shown that ROCK is in the autoinhibited state which inhibits the activity of the kinase domain of a dimeric protein (Couzens, Saridakis, & Scheid, 2009). Interaction of RHOA-GTP to ROCK-RBD has been proposed to release this autoinhibited state and therefore, the kinase domain is able to interact with the substrate (Schofield, Gamell,

Suryadinata, Sarcevic, & Bernard, 2013). So far, different phosphorylation sites on ROCK have been reported but the exact roles of them are not well understood.

1.2.1.2 Protein kinase N1 (PKN)

PKN is another type of effector for the RHO family which belongs to the family of serine/threonine kinase and has three isoforms; PKNα/PRK1, PKNβ and PKNγ/PRK2 (Ono & Mukai, 2002). PKN protein is involved in various biological functions such as regulation of the cytoskeleton, control of transcription factor, migration and apoptosis (Matsuzawa et al., 1997; Mukai et al., 1997). The N-terminal region of PKN protein contains three homology domains which are called HR1a, HR1b and HR1c and each of them is relatively rich in charged amino acids, followed by a Leu zipper-like sequence. Structural analysis of the HR1a shows that it contains two long α helices, which form an antiparallel coiled-coil (ACC finger) structure which is able to interact with RHOA protein (Ono & Mukai, 2002). The homology regions, are followed by C2 like domain and C-terminal kinase domain (Mukai, 2003) (Fig. 1.9). Among other RHO family effectors, HR1 domains of PKN (HR1a, HR1b, and HR1c) have been first described to interact with active RHO proteins (Palmer, Ridden, & Parker, 1995). Later on, this domain has been investigated in other RHO effector proteins such as Rhotekin, Rhophilin, Citron kinase and Kinectin, which have only one HR1 domain. In contrast to the intermolecular parallel coiled-coil structure of HR1 in ROCK protein, the HR1 domains in PKN are form an intermolecular antiparallel coiled-coil (Dvorsky et al., 2004; Flynn, Mellor, Palmer, Panayotou, & Parker, 1998; Hutchinson, Lowe, McLaughlin, Mott, & Owen, 2011). Both HR1a and HR1b form anti-parallel coiled-coil dimer (ACC), but HR1a interacts with RHOA via two different contact sites (I and II) which only contact site II overlap with switch II of RHOA (Maesaki et al., 1999). It has been shown HR1b is also able to interact with RAC1, to the region that is corresponding to the contact site I of RHOA (Owen et al., 2003).

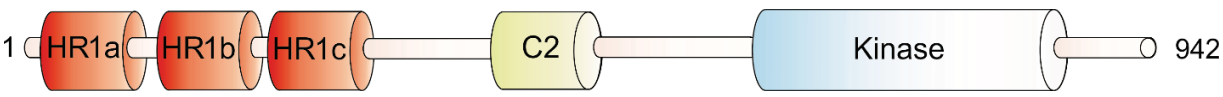

Figure 1.9: Schematic representation of PKN1 protein. The domains of PKN are homology region domains (HR1a-c) in red, protein kinase C conserved region (C2) in green, and the kinase domain in blue.

2. Aims of this study

Small GTPases are key factors in diverse cellular processes and the progression of various human diseases, such as neurological disorders, cardiovascular disease, and cancer. They act as molecular switches between inactive GDP-bound form and active GTP-bound form. The formation of the active form leads to conformational changes in switch regions and provide selectivity effector interaction, which activates different signalling pathways and biological function. The aim of this thesis is to better understand the mechanism of effector interaction with different GTPases including, RAS and RHO proteins.

As a prerequisite to achieve, this aim was a selection of representative proteins from different subfamilies: HRAS from classical RAS protein, RRAS from RRAS family, RALA from RAL proteins, RAP2A and RAP1B from RAP family and RHEB1 from RHEB family. Moreover, the RAS association domain family (RASSF) has been investigated as the first RAS effector with non-enzymatic function, which interacts with RAS proteins via RA domain. They act as key apoptotic activators and tumor suppressors. They are downregulated in many human cancers, although their exact regulatory roles are still unclear. Therefore, the interaction of RAS proteins with RASSF1-10 proteins have been studied.

Furthermore, the molecular mechanism by which ROCK activity is regulated, is not understood. Therefore, the activity of ROCK protein and its kinase domain to phosphorylate MYPT1, one of its substrate, in the present and absence of RHOA was studied. Full-length ROCK was subjected to electron microscopy (EM) studies, which together with biochemical analysis should provide insights into the structure-function relationship of ROCK structure and activity.

The binding mode of how RHO GTPases interact with the homology region1 (HR1) PKN, which has been proposed to regulated PKN activation, is unknown. Therefore, a detailed study of various HR1 domains of PKN with RHOA and RAC1 was the focus of this work.

Moreover, despite the long history, investigations of the fundamental mechanisms of RAF kinase activation have substantially lagged far behind the development of kinase inhibitors and inhibitor technologies. In this review, we summarized all the emerging mechanism

gained from structural, biochemical and computational studies on functional interaction networks of RAF proteins.

RAS proteins are essential factor in activation of multiple signaling pathways and dysregulation from these pathways leads to different diseases such as cancer, developmental disorders, and Noonan syndrome. We described in this review, the current understanding of the regulatory mechanisms of individual RAS proteins and their signaling networks beyond the RAS paralogs.

The last but not the least, Noonan syndrome is one of the most common developmental disorders and it is genetically heterogeneous. The mechanism of this disease is not well understood. We provided structural, biochemical, and functional data support the causal link between RRAS2 mutations and Noonan Syndrome, and characterized the clinical phenotype associated with these gene lesions.

Chapter II: Material and Methods

2.1 Materials

2.1.1 Antibody

2.1.1.1 Primary Antibody

GST (26H1)	Cell signalling technology
His (27E8)	Cell signalling technology
p-MYPT1 (THR853)	Cell signalling technology
p-ROCK	Sigma Aldrich
RHOA	Cell signalling technology

2.1.1.2 Secondary antibody

Licor IRDye 680 RD	Bioscience
Licor IRDye 800CW	Bioscience
Alexa fluor 488-conjugated goat anti-mouse IgG	Invitrogen
Alexa fluor 488-conjugated donkey anti-rabbit IgG	Invitrogen
Alexa fluor 488-conjugated donkey anti-goat IgG	Invitrogen

2.1.2 Chemicals

ATP	Merck
Dithiothreitol (DTT)	Gerbu
DMEM	Thermo Fisher Scientific
DNaseI	Roche
Falcon Tubes	Becton Dickinson Labware
FBS	Biological Industries
GDP	Jena Bioscience
GppNHp	Jena Bioscience
Glutathion (Reduced)	Merck
Glycerol	Roth

Glycine	Merck
Guanidium/HCl	Roth
Yeast -Extract	Roth
HEPES	Carl-Roth
Imidazole	Fluke Chemika
IPTG	Gerbu
KCl	Roth
K_2HPO_4	Roth
KH_2PO_4	Jena Bioscience
mantGppNHp	Merck
Methanol	Merck
$MgCl_2$	Merck
Odyssey Blocking Buffer	Abcam
Page Ruler Prestained Protein Lader	Fermentas
Petri dish	Merck
ROCK inhibitor (Y-27634)	Sigma Aldrich
Sf-900 medium	Thermo fisher
Sodium Azide	StarLab
Sodium Chloride	Roth
SDS	Roth
TEMED	Roth
TEV	MPI
Tris	Merck
Triton X-100	Merck
Tween-20	Sigma Aldrich
DNA and protein standards	Fermentas

2.1.3 Enzyme

phusion polymerase	NEB
Taq DNA polymerase	Qiagen
T4 DNA ligase	New England Biolabs

Restriction endonuclease	Fermentas
Thrombin	Serva
Alkaline Phosphatase	Roche Diagnostics
Phosphodiesterase	Roche Diagnostics

2.1.4 kits

Taq PCR kit	Qiagen
QIAprep spin miniprep kit	Qiagen
QIAprep spin gel extraction kit	Qiagen
QIAprep spin PCR purification kit	Qiagen
QuikChange mutagenesis kit	Stratagene

2.1.5 Buffers and solutions

Acrylamide solution	30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide
Exchange buffer (10x)	2 M $(NH_4)_2SO_4$, 10 mM $ZnCl_2$
dNTP solution	dGTP, dCTP, dATP, dTTP (0.5 mM respectively)
Destaining solution (SDS-PAGE)	40% (v/v) ethanol, 10% (v/v) acetic acid
Staining solution (SDS-PAGE)	40% (v/v) methanol, 10% (v/v) acetic acid 0.4% (w/v) Coomassie brilliant blue R250 0.4% (w/v) Coomassie brilliant blue G250
HPLC buffer	10 mM tetrabutylammonium, 100 mM potassium phosphate, pH 6.5, 7. 5% - 25% (v/v) acetonitrile
Laemmli sample buffer (5x)	5M Tris/HCl pH 8.8, 50% (v/v) glycerol, 500 mM DTT, 20% (w/v) SDS, 5% (w/v) bromophenol

Resolving buffer (SDS-PAGE)	30 mM Na phosphate pH 7.5, and 8.5, 100 mM NaCl
Stacking buffer (SDS-PAGE)	500 mM Tris/H_3PO_4 pH6.8, 4% (w/v) SDS
Running buffer (SDS-PAGE)	25 mM Tris pH 8.3, 192 mM glycine, 2% (w/v) SDS
TAE buffer	40 mM Tris/acetate pH 8.5, 1 mM EDTA
TBS-T buffer	20 mM Tris/HCl pH 7.4, 150 mM NaCl 0.1% (v/v) Tween-20
Transfer buffer	25mM Tris, 190 mM Glycine, 20% Methanol
Tris buffer (standard buffer for GTPase protein)	30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 3 mM DTT, 0.1 mM GDP
Tris buffer (high-salt for GTPase protein)	30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 500 mM NaCl, 3 mM DTT, 0.1 mM GDP
Tris buffer (Glutathione)	30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 3 mM DTT, 20 mM Glutathion (pH 7.5 adjusted with NaOH)
Tris buffer (Standard buffer for His-tag protein)	30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 2 mM ß-ME
Tris buffer (high-salt for His-tag protein)	30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 500 mM NaCl, 2 mM ß-ME
Tris buffer (Imidazole)	30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 2 mM ß-ME, 500 mM Imidazole (pH 7.5 adjusted)

2.1.6 Chromatography materials

GSH-Sepharose fast flow	Amersham Biosciences
Ni-NTA fast flow	Amersham Biosciences
Hi-Load Superdex	Amersham Biosciences

Hi-Load Superdex S200	Amersham Biosciences
Superdex 75 HR 10 / 30	Amersham Biosciences
Superdex 200 HR 10 / 30	Amersham Biosciences
PD 10	Amersham Biosciences
NAP columns	Amersham Biosciences

2.1.7 Expression vectors

pGEX-4T1	Amersham Biosciences
pGEX-4T1-N-Tev	EMBL
pMal-c5X-His	NEB
pFastBac HTb	Invitrogen

2.1.8 Expression strains

Strains	**Genotype**	**References**
BL21 (DE3)	ompT, hsdS$_B$ (r_B^-,m_B^-), gal(λcIts857 ind1, Sam7, nin5, lacUV5-T7gene1), dcm (DE3)	(Studier & Moffatt, 1986)
pLysS	F–, *ompT*, *hsdS*$_B$ (r_B–, m_B–), *dcm*, *gal*, λ(DE3), pLysS, Cm^r	(Studier & Moffatt, 1986)
Rosetta	ompT, hsdS$_B$ (r_B^-,m_B^-), gal(λcIts857 ind1, Sam7, nin5, lacUV5-T7gene1), dcm (DE3), pRARE^2 (Cm^R)	Novagen, Product Information
CodonPLUS RIL	ompT, hsdS$_B$ (r_B^-,m_B^-), gal(λcIts857 ind1, Sam7, nin5, lacUV5-T7gene1), dcm (DE3), Tet^r, endA, HTE (argU, ileY, leuW, Cam^r)	(Carstens & Waeshe, 1999)

2.1.9 Culture media

Luria-Bertani (LB) full medium	10 g/l Bacto-Tryptone, 10 g/l NaCl, 5 g/l yeast extract, 1 tablet NaOH
LB-agar plates	10 g/l Bacto-Tryptone, 10 g/l NaCl, 5 g/l yeast extract, 7.5 g/l Bacto-agar

2.1.10 Antibiotics

Ampicillin	100 mg/l
Chloramphenicol	25 mg/l
Kanamycin	50 mg/l
Penicillin	100 mg/l
Streptomycin	100 mg/l

2.1.11 Instruments

Äkta Prime FPLC	Amersham Pharmacia
Äkta Purifier	Amersham Pharmacia
Biophotometer (G131)	Eppendorf
Centrifuge (5810R, 5317R, 5415D)	Eppendorf
Centrifuge (3K30)	Sigma
Centrifuge Optima LE-80 K, Avanti J-20 XP	Beckman
Centrifuge LaboFuge 400R	Heraeus
HPLC System Gold 166	Beckman
Fluoromax 4	Horiba
Isotermal titration calorimetry	MicroCal
Licor Odyssey	Bioscience
LSM 510-Meta microscopy	Carl Zeiss
Millipore Water System	Millipore
PAGE Chamber	BioRad
PCR-Master cycler	Eppendorf

pH-meter	NeoLab
Pippetts	Eppendorf
Precision quartz cells	Hellma
Rotors (JLA 8.1, JA 10, 14 and Type 45Ti)	Beckman
Stirrer (SB-161)	Stuart
Sonicator UW-70	Bandelin

2.2 Molecular genetics methods

2.2.1 Isolation of plasmid DNA

In order to isolate the DNA plasmid from *E. coli*, around 5 ml of an overnight culture was used. The preparation of the plasmid was done with the Plasmid Mini-Prep Kit system from Qiagen as given by the manufacturer's instructions. The isolation is based on the principle of the alkaline lysis (Birnboim, 1992), and then the plasmids precipitate by water and the concentration measured.

2.2.2 Agarose gel electrophoresis

Horizontal gel electrophoresis is able to analytically and preoperatively separate DNA fragments (McDonell, Simon, & Studier, 1977). By applying the electric field, the negatively charged DNA migrates through the pore-like gel material. The DNA molecules that are shorter move faster and migrate farther than longer ones. The length of fragments is determined, based on a DNA standard marker. 1% agarose gel in 1x TAE buffer was used for analytical separation. For the detection of DNA fragments, in the agarose solution 0.75 mg/l ethidium bromide was added. The DNA samples were mixed with electrophoresis with 20% (v/v) DNA sample buffer (6xdye), and then electrophoresis was performed at a constant voltage of 100 mV. The detection of the bands was performed using a UV illuminator (excitation at 302 nm). By using the QIAquick Gel Extraction Kit, the DNA fragments were isolated.

2.2.3 Polymerase chain reaction (PCR)

For selective amplification of DNA fragments *in vitro*, the polymerase chain reaction can be used (De Noronha & Mullins, 1992). The heat should be stable at the beginning of reaction DNA (template) and two oligonucleotides (primers) that match to replicating DNA sequence and are complementary to each one strand of the desired DNA segment (De Noronha & Mullins, 1992). A typical protocol for RCR consists of 20µl PCR buffer (5x), 1 µl dNTP solution (25 mM each), 20-200 ng template DNA, 100 pmol from forward and reverse primers and 2.0 U Phusion polymerase. The PCR program contains a three-step process, that runs through 20 cycles, thus leading to exponential amplification of DNA. 60s denaturation at 94°C, 30 s hybridization at 60 °C and 30 s elongation at 72 ° C for each kbp length of our template and an initial denaturation at 94 ° C for 2 min and final elongation at 72 ° C for 10 min. The hybridization temperature depends on the melting temperature (Tm) of the primers. Then, the samples were stored at 4 ° C until further use. It is important to consider, that Phusion polymerase has a proofreading function (3 '5' exonuclease activity); therefore, it has very low error rates and it is more suitable for preparative PCR. In order to check positive *E. coli* clones after a transformation, the colony-PCR (also called analytical quick-PCR) used. Instead of template DNA, cells from individual colonies were used.

2.2.4 Site-specific mutagenesis

By using the Quik change protocol from the Strata gene, direct replacement of individual amino acids at the DNA level is performed. In this method, circular plasmid DNA is amplified from *E. coli* and a mutagenic primer pairs with the PCR method. Since the PCR product is unmethylated, it compared to the DNA template. Therefore, the methylation-dependent restriction endonuclease DpnI can remove the non-mutated template (Kunkel, 1985). After restriction digestion for 2 hours, at 37° C, the samples heated for 20 min at 72° C to deactivate the enzyme and then the plasmids transformed into *E. coli* BL21 and then, their sequences were checked by the Seqlab Company.

2.2.5 Hydrolysis of DNA with restriction endonucleases

For the cleavage of double-stranded DNA, the restriction enzyme was used; however, for cleavage with two enzymes, by providing identical or similar condition the reaction was

carried out simultaneously. By running agarose gel electrophoresis on samples, the cleavage products separated.

2.2.6 Ligation of DNA fragments

In order to ligate the DNA, enzyme T4 DNA ligase from Fermentas was used. 20 ng completely restricted vector DNA was mixed with restricted Insert (5-8 molar excess), and also with the 1/10 of the 10-fold concentrated T4 ligase buffer and distilled water to make a total volume of 20 µl. The following ligation with 1 U T4 DNA ligase for an incubation period of overnight at room temperature. The next day, the sample incubated at 72°C for 20 min to inactivate the enzyme and then the ligation mixture transformed into bacteria.

2.2.7 Preparation of electrocompetent bacterial strains

For preparing competent cells, a method described by Chung et al. (Chung, Niemela, & Miller, 1989) was followed with a few modifications: 5 ml of an overnight culture of *E. coli* strain was added to 500 ml LB medium, which was grown at 37 ° C with the appropriate antibiotic, when the cell density reached OD_{600} = 0.5 -0.6. The culture was cooled down for 20 min by keeping the flask in ice and then cells were centrifuged (4000 rpm, 10 min, 4°C). The sedimented cells then resuspended in 250 ml of ice-cold, distilled water solution. Again, cells centrifuged and resuspended in 20 ml of ice-cold, distilled water. Finally, pelleted cells were aliquoted (50µl) and kept on dry ice, and the aliquots were stored at -80 °C.

2.2.8 Transformation of E. coli cells with circular DNA

For transformation, 5 µl of the ligated plasmid (or 20 ng of purified plasmid) was mixed with 50 µl of competent bacteria and the mixture was incubated on ice for 20 min. The cells were then treated with a heat pulse for 1 min at 42˚C and again put on ice for 5 min. 200 µl of LB medium (without antibiotics) was added to the cells and was incubated for 90 min at 37°C shaking. After that, sedimented by centrifugation at 13000 rpm for 5 min and 50 µl of the supernatant was resuspended (the rest of the supernatant was discarded) and then plated on the selective antibiotic containing agar plate under sterile conditions and kept overnight at 37°C.

2.2.9 Storage of transformed bacteria

For storage of transformed bacteria, from a single colony on the LB plate, a fresh culture was made. 1000 µl of the grown overnight grown culture was mixed with 500 µl of glycerol and stored at -80^{o}C.

2.3 Protein biochemical method

2.3.1 Analytical expression test

For finding suitable conditions for the expression of a recombinant protein, an analytical expression test was done on a small scale; 100 ml expression cultures inoculated with a pre-culture 1:100 and incubated at 37°C until the OD_{600} = 0.5 -0.6 and then it was induced by 0.1mM IPTG. After four hours at 37°C or over-night at 20°C induced culture, 1 ml samples were taken, and cells were harvested by centrifuging 5 min at 13,000 rpm and resuspended in an appropriate buffer. 20 µl SDS sample buffer (5x) was mixed with 80 µl of the cells and boiled for 5 minutes at 99 °C. The total cell lysate, pellet, and supernatant then were then loaded on an SDS-PAGE to check the protein expression and solubility.

2.3.2 Expression of recombinant proteins

By using the determined optimal conditions, the expression of proteins was determined. Then, culture volume was scaled up to 5 liters of culture medium as the bacterial cells were harvested by centrifugation for 15 min 5000 rpm, and the pellet was washed in the standard buffer based on the type of protein which was described in the material section and then resuspended in an appropriate buffer. The bacterial cell suspension was then frozen at -20°C in order to help lysis.

2.3.3 Analytical expression test for insect cells

The insect cells method was used for proteins such as kinase protein, which needs post-translational modifications. ROCK protein and its kinase domain were cloned in pFastBac HTb vector containing an N-terminal His6 tag and expressed via baculovirus expression system which is one of the most prominent viruses to affect insect population. In order to test expression of these proteins, *BTI-Tnao38* cells that delivered from *Trichoplusia ni*

were used, and then they were grown in Sf-900 express medium which contains 50 unit penicillin/streptomycin. After the cells reached 70% confluency, they were infected by a produced virus with the ratio of 1:40 (Yoshifumi Hashimoto, Zhang, & Blissard, 2010; Yoshi Hashimoto, Zhang, Zhang, Chen, & Blissard, 2012). Then, they grew for 4 days and each day, 1 ml sample was taken and prepared for western blot to check the expression.

2.3.4 Expression of recombinant proteins in insect cells

When the expression of proteins in an insect cell was checked. The culture volume was then scaled up to 350 ml. The cells were infected with baculoviruses, and after 4 days they were collected by centrifugation for 10 min 5000 rpm at 4°C. The pellet was washed in the standard buffer for His tag proteins and then resuspended in buffer. The bacterial cell suspension was then frozen at -20°C.

2.3.5 Cell lysis

The cells were thawed, to extract the soluble protein, and then one tablet of protease inhibitor (Cocktail), 10 µg/ml Lysozyme, and 1 µg/ml DNAase were added. The cells were on ice and subsequently subjected to lysis by sonication (3 times each time for 2 min).

2.3.6 Affinity chromatography

This technique is based on the binding of a biomolecule to its binding partner, which is immobilized into a stationary phase (a polymeric carrier). In this way, the molecule of interest can be selectively captured by passing through a column. Then, it is eluted by changing external parameters, such as solvents, pH, temperature, and ionic strength; which affect the complex stability, and release of the molecule from the complex and elute in a purified form (Wilchek & Chaiken, 2000). Since not all isolated proteins have a specific ligand, so we can use molecular biological methods to create a vector that has the gene for such an anchor group (which is also called tag) between the promoter and multiple cloning site. The most common systems are the GST-tag or His-tag. In this work, both GST-tag and His-tag proteins are isolated and purified.

2.3.7 GST- and His- fusion system

In this work, the gene of interest was cloned as a fusion protein with N-terminal GST-anchor in pGEX vector or with N-terminal His-anchor in PMal vector. The glutathione S-

transferase from *Schistosoma japonicum*, specifically interacts with its natural ligand glutathione (GSH) and in the case of His-tag protein binds to Ni-NTA column. In the case of GST, since the tag is big, it was removed by a protease recognition (factor Xa, thrombin, PreScission protease, Tev protease or IgA protease). The supernatant (after centrifugation of cell lysate at 40,000 rpm) loaded on the appropriate column, which had equilibrated with standard buffer, which was described in the method's part. Since only a few non-specific components from the lysate could interact with the column. Therefore, most of the cellular proteins removed by rinsing the column with the buffer. Proteins, which bond nonspecifically to the column material, are usually removed with a high-salt buffer. In this way, we can obtain a purity of over 90% in the first steps. The fusion protein is then eluted by 20 mM glutathione or imidazole 500 mM in the buffer (depends on the protein tag). After elution of the protein, for GST-tag proteins the protease thrombin was used to eliminate GST. Then, by passing protein through the GSH column, the protein and GST-tag separated.

2.3.8 Gel filtration

By using gel filtration or size exclusion chromatography, molecules separated according to their size. This column contains the pore size of a covalently cross-linked polymer polyacrylamide agarose or Dextran material. The smaller the molecules are, the further they were prevented from passing through the column. However, particles with a radius above the pore size cannot enter and remain in the exclusion volume; therefore, the molecular weight is inversely proportional to the elution volume. The column needs to calibrate with proteins of known size to help for estimating the sizes of the eluted proteins on the retention volume. In this study, Sephadex S75 and Sephadex S200 columns were used. Based on the amount of protein and volume, column size 16/60, 26/60 were used. This number code indicates the diameter in mm and the length of the column in cm. Adequate buffer for equilibration and elution, filtered and degassed. Depending on the column size flow rate and the volume of the collected fractions as well as the maximum protein loading capacity was determined. The fractions were collected and subjected to SDS-PAGE and then pure fractions were pooled up and concentrated by Amicon filters.

2.3.9 Determination of protein concentration

2.3.9.1 In the Visible range

In order to determine, the total protein concentration of a solution the color reaction with the dye, Coomassie Brilliant Blue G250 was prepared (Bradford, 1976). Through the interaction with the side chains of arginine, and also histidine, lysine, tyrosine, tryptophan and phenylalanine (Compton & Jones, 1985), the dye is stabilized in its anionic form which leads to a bathochromic shift of the absorption maximum of 465 to 595 nm. The protein concentration is determined by using an equation that is created by a BSA standard solution. The absorbance (OD= 595 nm) of the Bradford solution was used as a blank, and then protein absorption was measured to compare to the blank. Only absorbance values between 0.2 and 0.8 were evaluated from the calibration.

2.3.9.2 In the UV range

For highly pure protein solutions, the UV absorption method had been used which the diluted protein solution at 280 nm and 234.5 nm (for a buffer) were measured, and the protein concentration according to Ehresman *et al.* (Ehresman) provides:

$$(A_{280} - A_{234.5})/\varepsilon_{\text{extinction coefficient of protein}} = \text{mg protein/ml}$$

2.3.10 Concentration of proteins

After purification steps, usually, a concentrated protein solution was carried out for further experiments. The most common method is ultra-filtration by using an Amicon filter from Millipore, which a protein solution by centrifugation passes through a membrane with a defined pore size. The solvent and low molecular weight components can pass through the membrane; however, proteins and other high molecular substances were retained over a certain size of the membrane. The pore size of the membrane defines the exclusion limit (molecular weight cut-off, MWCO). The protein centrifuged at 3700 rpm and 4 °C until it reaches the desired final volume and the desired protein concentration.

2.3.11 Nucleotide exchange of small GTPases

The nucleotide exchange of small GTPase is based on the degradation of the present nucleotide (usually GDP form), and binding of an excess (1.5-fold) nucleotide analogue

(John et al., 1990). In the first step, the protein was incubated with synthetic nucleotide (GppNHp, mant-GppNHp), with alkaline phosphatase (1.5 U/mg protein), and exchange buffer, which increases the exchange rate of the nucleotide and enzymatically degradation to Guanine, GMP or monophosphate. The GTPase protein has a much higher binding affinity to the synthetic nucleotides and compares to the monophosphate and guanine. After the quantitative digestion of the original nucleotide, the protein was passed over a NAP5 column to separate nucleotide from protein-containing fractions. Then, 1µl from each fraction was added to 20 µl Bradford solution and the positive fractions were collected.

2.3.12 Reverse-phase HPLC

To determine the activity of small GTPase after purification or nucleotide exchange the reverse-phase high-performance liquid chromatography (reversed-phase HPLC) was used. The separation was carried out under isocratic ion-pair bond using hydrophobic Solid-phase matrix (C-18) (Tucker et al., 1986). By calibration of HPLC flow-photometer with samples of known composition and concentration, then the composition and the nucleotide can be determined quickly and accurately. This method was used for both qualitative and quantitative studies of protein-nucleotide complexes. The acetonitrile concentration was used as a mobile phase (7.5 to 25%) with a flow rate of 1.8 ml/min.

2.3.13 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is one of the common methods for separating proteins according to their molecular masses using a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins (Laemmli, 1970). This method was used for the estimation of the relative molecular weight of proteins. Furthermore, SDS-PAGE is useful to determine the purity of proteins in the fractions from the purification. The protein samples mixed with Laemmli buffer. SDS is an anionic detergent, which denatures proteins and creates a negative charge on the polypeptide in proportion to its length. DTT or β-mercaptoethanol is a reducing agent that prevents the formation of disulfide bonds since disulfide bonding is covalent and do not disrupt by SDS. The electrophoresis was carried out in running buffer. Proteins run at 80 V in the stacking gel, which can concentrate them, and then they separate in the running gel at 100V.

2.3.14 Coomassie staining and destaining of SDS-polyacrylamide gels

Coomassie Blue staining is a nonspecific method, in which the dye Coomassie Brilliant Blue R250 binds to almost all proteins. The gel is soaked in a solution of the dye for at least 15 min and then it destained using a destaining solution (which was explained in the method part) to remove the background color.

2.3.15 Western blot (WB)

This is a common method to detect and analyze a specific protein of interest by using the specific antibody. In the first step, just like normal SDS-PAGE, the protein of interest and marker are loaded to the gel and separated by electrophoresis. After that, instead of staining and destaining by Coomassie solution in the SDS-PAGE method, the protein was transferred from gel to the membrane (Towbin, Staehelin, & Gordon, 1979). For transformation, the wet method was used. In this method, the gel, polyvinylidene difluoride (PVDF) membrane, and filter papers were soaked in transfer buffer for 5 min. In order to transfer the proteins from gel to the membrane, between the gel and positive electrode, sponges, filter papers and the membrane were placed, and voltage 100V for 60 min was applied. After transformation, the blot was dried for 1 hour at room temperature. In order to prevent unspecific binding, the membrane was blocked with Odyssey Blocking Buffer (TBS without tween 20) for one hour. Then, the first antibody, which is specific for each protein, diluted in Odyssey Blocking Buffer (which contains tween 20 to a final concentration of 0.2%) and added to the membrane overnight at 4 °C shaking. Later on, membrane washed with TBS-T buffer 3 times, each time 5 min, and then the secondary antibody, which was diluted similar to the first antibody, was added to the membrane and shaken for 2 hours at room temperature. Finally, the membrane was washed again with TBS-T buffer, 3 times for 5 min and scanned with a Licor system (Eaton et al., 2014).

2.4 Cell Culture method

2.4.1 Cell culture

In this work, a human mammary epithelial cells (HMEC), which grow in Dulbecco's modified eagle's medium (DMEM) media with 10% Fetal Bovine Serum (FBS) and 50 units

of penicillin/streptomycin under 5% CO_2 at 37 °C was used. When the cells are grown confluently, they were detached from the plate by trypsinization. Cells were diluted 1:5 and subcultured 2 times per week, and all cell culture works were performed under sterile conditions.

2.4.2 Cell freezing and recovery

When the cells had grown to 85% confluency, they were transferred into 50 ml falcon tubes and then centrifuged at 1000 rpm for 5 min. The pellet of the cells was resuspended into 1 ml of cryotubes with 92% FBS and 10% DMSO, and they were stored in the liquid nitrogen. For the recovery of the frozen cells, they were thawed in 25 cm Petri dishes with fresh medium. After one day, their medium was changed, and they grew at normal conditions.

2.4.3 Immunocytochemistry

The sterile coverslips placed into a new sterile 24-well culture place. Then the cells had grown in a petri dish, detached and plated normally on to the surface of the coverslip. Then, the cells grew under the same conditions as before (at 37 °C under 5% CO_2), until they reached 70% confluency. In order to treat the cells under different conditions for confocal microscopy, the old medium was removed and a new medium for three different conditions was added including, serum starve (no FBS), 1% serum and normal condition (10% FBS). After that, the cells were returned to the incubator for one day. The next day, the medium on top of the cells was discarded and they washed with PBS. For fixing the cells, the cross-linking method, by using 4% paraformaldehyde, which was diluted in PBS, and can form covalent chemical bonds between the proteins and their surroundings were used. The cells incubated in this solution for 20 min at room temperature. In order to permeabilize the cell membrane, cells were incubated in 0.25% Triton-X-100/PBS for an hour at room temperature. The primary antibodies, which in this study are RHOA and ROCK, were added overnight at 4 °C and then were washed 3 times with PBS. Next, they incubated with secondary antibodies, which were Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa 488-conjugated donkey anti-rabbit IgG, Alexa Fluor 488-conjugated donkey anti-goat IgG with the dilution of 1:500 at room temperature for two hours. After that the slides were washed with PBS three times and then stained with 4’,6-diamidino-2

phenylindole (DAPI) for 5 min and again washed with PBS three times. Finally, the coverslips mounted with Prolong Gold antifade and then the confocal microscopy images were obtained.

2.5 Biophysics methods

2.5.1 Fluorescence polarization

Small GTPase-mant GppNHp interaction with their effectors was performed in standard buffer (50 mM Tris/HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, and 3 mM DTT) at 25 °C using a fluoromax 4 fluorimeter in polarization mode. The effector protein titrated (0.05-300 µM) to 1µM GTPase mant-GppNHp protein increased polarization. By fitting the concentration-dependent binding curve by using a quadratic ligand binding equation the equilibrium dissociation constents (K_d) were calculated (Nakhaeizadeh et al., 2016).

2.5.2 Guanine nucleotide dissociation inhibition measurement

In this method, releasing of mant-GppNHp from RHOA (0.2 µM) in standard buffer (30 mM Tris/HCl pH 7.5, 10 mM Na_2HPO_4/$NaHPO_4$ pH 7.5, and 5 mM DTT) at 25 °C measured. The amount of the respective effector domains (0.05-300 µM) increased, in the presence of unlabeled nucleotide (40 µM). Then, the observed rate constants (k_{obs}) which were single exponentially by using Grafit program (Erithacus software) fitted (Leatherbarrow, 1990), to obtain dissociation contents (k_d) (Blumenstein & Ahmadian, 2004).

2.5.3 Kinase assay

In order to check the activity of ROCK proteins and its kinase domain, in the presence and absence of an active form of RHOA, the kinase assay method was performed. In this method, all conditions contain 400 µM ATP and 5 µM MYPT1, and other compounds (based on the table below) were added. Then, in different time points 0, 5, 15, 30, 60 and 120 min, 30 µl sample was collected and added to the tubes which contain Laemmli buffer (5x) and 10 µl urea (10M) and boiled for 5 minutes at 99 °C to stop the reaction. Furthermore, the samples were leaded on western blot, and the level of MYPT1 phosphorylation was normalized to its total amount.

1	Control (MYPT1 + ATP)
2	KD ROCK (0.02 µM)
3	ROCK FL (0.02 µM)
4	ROCK FL (0.02 µM) + RHOA FL-GppNHp (5µM)
5	ROCK FL (0.02 µM) + ROCK inhibitor (Y-27634) (5µM)

2.5.4 Liposome assay

The liposome sonicated for 2 min. followed by extrusion through 100 nm filters several times. 30 µl of liposome was mixed with 30 µl of sample and kept in room temperature for 20 min. Then, it centrifuged at 20000 g for 30 min at 4 °C. After that, the supernatant was collected and 50 µl buffer was added to the pellet and both samples run in western blot to check the affinity of protein in interaction with liposome.

2.5.5 Cryo-electron microscopy

4 µl of the sample was adsorbed for 1 min on freshly glow-discharged copper grids, which were covered by a thin, continuous carbon film. The grids were then negatively stained with 0.75% uranyl formate for 1 min before blotting with filter paper. All images were taken by an electron microscope equipped with a LaB_6 cathode and operated at 120 kV. Digital electron micrographs were recorded with a 4k × 4k CMOS camera F416 (TVIPS) under minimal dose conditions (15-20 electrons/$Å^2$) at a calibrated magnification of 67,535x, resulting in a pixel size of 2.32 Å. The length and width of 176 individual ROCK protein from 21 images were measured using boxer from the EMAN software package (Ludtke, Baldwin, & Chiu, 1999).

2.6 *In silico* structure analysis

2.6.1 Sequence and Structural modeling

Sequence alignments were performed with the Bioedit program using the ClustalW algorithm (Hall, 1999). By using Chimera the sequence alignments were adjust with superimposed structures (Goddard et al., 2018). In the RASSF-RAS interaction section, the RASSF5-HRAS structure (PDB ID: 3DDC) was used as a template for structural analysis and the structures of RA RASSF members (RASSF1-10 except for RASSF5) were generated

via Swiss-model website. After that, the structural representation was generated using Pymol viewer (DeLano, 2002).

Chapter III: Results

3.1 RASSF-RAS proteins interaction

3.1.1 *In silico* analysis of RAS effector proteins

RAS-GTP can interact with different downstream effectors through two binding domains known as RAS association domain (RA) and RAS binding domain (RB). They contain about 80-100 amino acids and adopt a folding topology related to ubiquitin structure (Nakhaeizadeh et al., 2016). Structural characterizations of different effectors in complex with RAS proteins have revealed that these domains are common to all RAS effectors. They contact RAS through the formation of intermolecular antiparallel β-sheets, which are formed between strand β2 of the canonical ubiquitin fold and the switch I of RAS proteins. This is rather interesting because RA and RB domains associate with different members of RAS family proteins and share the same mode of interaction with RAS proteins. There is no structural difference between RB and RA domains. They are only variant names used for the same ubiquitin fold proteins (Rodriguez-Viciana, Sabatier, & McCormick, 2004). It is not fully understood, how effectors selectively recognize the RAS-GTP form, and how many RA and RB domain-containing proteins exist in the human proteome. In order to get an overview of potential binding partners of RAS GTPases, we first searched in the UniProt database for all RA/RB domain proteins based on the flowchart in Figure 3.1. Using the search tools of UniProt, we found 145 RA and 130 RB domain-containing proteins, respectively. In parallel, the HMMER program was used to search for sequences that are similar to known RA and RB domains which increase the chance to find all proteins containing RA and RB domains. In the HMMER search, a sequence profile is first specified for the domain of interest. Algorithm then scans provided database of protein sequences, which was in our case the UniProt database, and finds all proteins matching this profile. Specification of domain can then be refined, taking all found proteins into account. The whole procedure can be then iteratively repeated until the constant number of identified proteins is reached. We found, very similar results to the search tools of UniProt database, 164 RA and 127 RB domain-containing proteins, respectively (Fig. 3.1). In the next step, many proteins that are abbreviated as “RBD” or “RA”, such as receptor binding proteins,

mitochondrial proton/calcium antiporter protein, and RHO binding proteins, were removed, such that proteins involved in RAS interaction were selected, including 97 RA and 46 RB domain-containing proteins. A multiple sequence alignment using ClustalW algorithm from the Bioedit program revealed many identical sequences of protein isoforms, for example RAF, RASSF, TIAM and RGL isoforms proteins, which were removed. In total, 41 RA in 39 RA domain-containing proteins (Table 1.1) and 16 RB in 14 RB domain-containing effector proteins (Table 1.2) remained in our search.

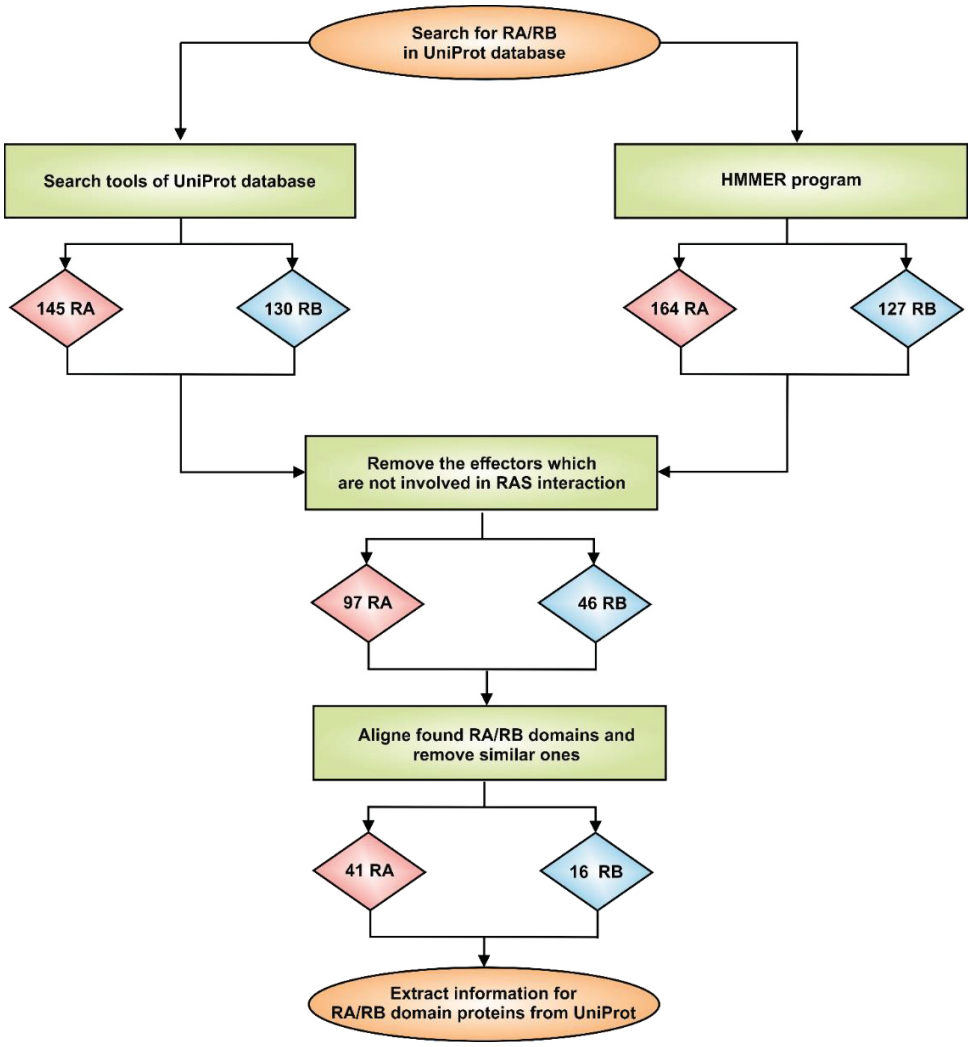

Fig. 3.1: Flowchart of *in silico* RAS effector analysis. RAS effector proteins in human proteome were selected through stepwise search in UniProt database using the search tools and HMMER program. The amino acid sequences were aligned using Bioedit ClustalW algorithm and ultimately not only completely different protein domains were removed (97 RA and 46 RB domain-containing proteins) but also identical sequences to RA/RB effector proteins were omitted (in total, 41 RA in 39 RA domain-containing proteins and 16 RB in 14 RB domain-containing proteins). All proteins are represented in tables 1.1 and 1.2.

Table 1.1. Human proteins containing RA domain

No.	Entry	Gene names	Protein name
1	Q8WWW0	RASSF5	Ras association domain-containing protein 5 (New ras effector 1) (Regulator for cell adhesion and polarization enriched in lymphoid tissues) (RAPL)
2	Q9NS23	RASSF1	Ras association domain-containing protein 1
3	P50749	RASSF2	Ras association domain-containing protein 2
4	Q86WH2	RASSF3	Ras association domain-containing protein 3
5	Q9H2L5	RASSF4	Ras association domain-containing protein 4
6	Q6ZTQ3	RASSF6	Ras association domain-containing protein 6
7	Q02833	RASSF7	Ras association domain-containing protein 7 (HRAS1-related cluster protein 1)
8	Q8NHQ8	RASSF8	Ras association domain-containing protein 8 (Carcinoma-associated protein HOJ-1)
9	O75901	RASSF9	Ras association domain-containing protein 9 (PAM COOH-terminal interactor protein 1) (P-CIP1) (Peptidylglycine alpha-amidating monooxygenase COOH-terminal interactor)
10	A6NK89	RASSF10	Ras association domain-containing protein 10
11	Q5U651	RASIP1	Ras-interacting protein 1 (Rain)
12	Q9P212	PLCE1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon-1 (EC 3.1.4.11) (Pancreas-enriched phospholipase C) (Phosphoinositide phospholipase C-epsilon-1) (Phospholipase C-epsilon-1) (PLC-epsilon-1)
13	Q96JH8	RADIL	Ras-associating and dilute domain-containing protein
14	Q13671	RIN1	Ras and Rab interactor 1 (Ras inhibitor JC99) (Ras interaction/interference protein 1)
15	Q8WYP3	RIN2	Ras and Rab interactor 2 (Ras association domain family 4) (Ras inhibitor JC265) (Ras interaction/interference protein 2)
16	Q8TB24	RIN3	Ras and Rab interactor 3 (Ras interaction/interference protein 3)
17	Q9Y4G8	RAPGEF2	Rap guanine nucleotide exchange factor 2 (Cyclic nucleotide ras GEF) (CNrasGEF) (Neural RAP guanine nucleotide exchange protein) (nRap GEP) (PDZ domain-containing guanine nucleotide exchange factor 1) (PDZ-GEF1) (RA-GEF-1) (Ras/Rap1-associating GEF-1)
18	Q15036	SNX17	Sorting nexin-17
19	Q96L92	SNX27	Sorting nexin-27
20	Q8TEU7	RAPGEF6	Rap guanine nucleotide exchange factor 6 (PDZ domain-containing guanine nucleotide exchange factor 2) (PDZ-GEF2) (RA-GEF-2)
21	P55196	AFDN	Afadin (ALL1-fused gene from chromosome 6 protein) (Protein AF-6) (Afadin adherens junction formation factor)
22	Q7Z5R6	APBB1IP	Amyloid beta A4 precursor protein-binding family B member 1-interacting protein (APBB1-interacting protein 1) (Proline-rich EVH1 ligand 1) (PREL-1) (Proline-rich protein 73) (Rap1-GTP-interacting adapter molecule) (RIAM) (Retinoic acid-responsive proline-rich protein 1) (RARP-1)
23	Q14451	GRB7	Growth factor receptor-bound protein 7 (B47) (Epidermal growth factor receptor GRB-7) (GRB7 adapter protein)
24	Q13322	GRB10	Growth factor receptor-bound protein 10 (GRB10 adapter protein) (Insulin receptor-binding protein Grb-IR)
25	Q14449	GRB14	Growth factor receptor-bound protein 14 (GRB14 adapter protein)
26	Q12967	RALGDS	Ral guanine nucleotide dissociation stimulator (RalGDS) (Ral guanine nucleotide exchange factor) (RalGEF)

27	O15211	RGL2	Ral guanine nucleotide dissociation stimulator-like 2 (RalGDS-like 2) (RalGDS-like factor) (Ras-associated protein RAB2L)
28	Q9NZL6	RGL1	Ral guanine nucleotide dissociation stimulator-like 1 (RalGDS-like 1)
29	Q9BSI0	RGL2	RGL2 (Ral guanine nucleotide dissociation stimulator-like 2)
30	Q3MIN7	RGL3	Ral guanine nucleotide dissociation stimulator-like 3 (RalGDS-like 3)
31	Q70E73	RAPH1	Ras-associated and pleckstrin homology domains-containing protein1
32	P52824	DGKQ	Diacylglycerol kinase theta (DAG kinase theta)
33	Q96P48	ARAP1	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1
34	Q8WZ64	ARAP2	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 2
35	Q8WWN8	ARAP3	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 3
36	B2RTY4	MYO9A	Unconventioal myosin-IXa (Unconventioal myosin-9a)
37	Q13459	MYO9B	Unconventioal myosin-IXb (Unconventioal myosin-9b)
38	Q9HD67	MYO10	Unconventioal myosin-X (Unconventioal myosin-10)
39	Q9P2F6	ARHGAP20	RHO GTPase-activating protein 20 (RHO-type GTPase-activating protein 20)

Table 1.2. Human proteins containing RB domain

No.	Entry	Gene names	Protein name
1	P04049	RAF1	RAF proto-oncogene serine/threonine-protein kinase (EC 2.7.11.1) (Proto-oncogene c-RAF) (cRaf) (Raf-1)
2	P15056	BRAF	Serine/threonine-protein kinase B-raf (EC 2.7.11.1) (Proto-oncogene B-Raf) (p94) (v-Raf murine sarcoma viral oncogene homolog B1)
3	P10398	ARAF	Serine/threonine-protein kinase A-Raf (EC 2.7.11.1) (Proto-oncogene A-Raf) (Proto-oncogene A-Raf-1) (Proto-oncogene Pks)
4	O14924	RGS12	Regulator of G-protein signaling 12 (RGS12)
5	O43566	RGS14	Regulator of G-protein signaling 14 (RGS14)
6	Q13009	TIAM1	T-lymphoma invasion and metastasis-inducing protein 1 (TIAM-1)
7	Q8IVF5	TIAM2	T-lymphoma invasion and metastasis-inducing protein 2 (TIAM-2) (SIF and TIAM1-like exchange factor)
8	P48736	PIK3CG	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform (PI3-kinase subunit gamma) (PI3K-gamma) (PI3Kgamma) (PtdIns-3-kinase subunit gamma) (EC 2.7.1.153) (Phosphatidylinositol 4,5-bisphosphate 3-kinase 110 kDa catalytic subunit gamma) (PtdIns-3-kinase subunit p110-gamma) (p110gamma) (Phosphoinositide-3-kinase catalytic gamma polypeptide) (Serine/threonine protein kinase PIK3CG) (EC 2.7.11.1) (p120-PI3K)
9	P42336	PIK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform (PI3-kinase subunit alpha) (PI3K-alpha) (PI3Kalpha) (PtdIns-3-kinase subunit alpha) (EC 2.7.1.153) (Phosphatidylinositol 4,5-bisphosphate 3-kinase 110 kDa catalytic subunit alpha) (PtdIns-3-kinase subunit p110-alpha) (p110alpha) (Phosphoinositide-3-kinase catalytic alpha polypeptide) (Serine/threonine protein kinase PIK3CA) (EC 2.7.11.1)
10	P42338	PIK3CB	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform (PI3-kinase subunit beta) (PI3K-beta) (PI3Kbeta) (PtdIns-3-kinase subunit beta) (EC 2.7.1.153) (Phosphatidylinositol 4,5-bisphosphate 3-kinase 110 kDa catalytic subunit beta) (PtdIns-3-kinase subunit p110-beta) (p110beta)

11	O00329	PIK3CD	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform (PI3-kinase subunit delta) (PI3K-delta) (PI3Kdelta) (PtdIns-3-kinase subunit delta) (EC 2.7.1.153) (Phosphatidylinositol 4,5-bisphosphate 3-kinase 110 kDa catalytic subunit delta) (PtdIns-3-kinase subunit p110-delta) (p110delta)
12	O00443	PI3KC2A	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit alpha (PI3K-C2-alpha) (PtdIns-3-kinase C2 subunit alpha)
13	O00750	PI3KC2B	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit beta (PI3K-C2-beta) (PtdIns-3-kinase C2 subunit beta)
14	O75747	PI3KC2G	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit gamma (PI3K-C2-gamma) (PtdIns-3-kinase C2 subunit gamma)

To determine the binding capability between effector domains and diverse proteins of the RAS family, we selected the RA domain-containing RASSF family as representative effector proteins and studied in more detail their interaction with different paralogs of the RAS family, including HRAS, RRAS, RALA, RAP1B, RAP2A, and RHEB.

3.1.2 Expression and purification of RASSF proteins

In order to investigate the biochemical and biophysical properties of RASSF interaction with different members of RAS proteins, all RA domains of RASSF family were cloned in PMal vector, which contains maltose-binding protein (MBP, 42 kDa). This protein increases the molecular weight of RASSF RA domains, and it helps to study the interaction between RASSF-RAS via the polarization method. The expression of the proteins in different bacterial strains, including PLysS, Rosetta and CodonPLUS under the same conditions were checked. The expression conditions were 1 mM IPTG at an optical density (OD) between 0.5 and 0.7 at 20°C overnight. Bacterial pellet was collected by centrifugation (14000 rpm, 10 min, 4°C), and suspended in lysis buffer (30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 2 mM βME, 10 µg/ml Lysozyme, 1 µg/ml DNase-I and one tablet of protease cocktail inhibitor), and were subjected to mild sonication (70% duty cycle, 80% power for 20 sec) for 3 times (each time 2 min). The lysate was centrifuged (30000 rpm, 30 min, 4°C) and then was purified in two steps using affinity and size exclusion chromatography. In the first step, the soluble fraction applied to a Ni-NTA affinity column. After that the unbounded proteins were washed with high salt, then the His-tag protein was eluted in a buffer that contains 500 mM imidazole. Samples from elution, pellet, supernatant, and flow trough were run on SDS-PAGE. All RASSF proteins were soluble in the eluted fractions. Next, the eluted fractions were concentrated by Amicon 30 kDa cut-

off. In the second step, the concentrated protein was loaded on size exclusion chromatography (SEC; Superdex 75 26/60 column) to remove impurities and other components, such as imidazole, using 100 mM NaCl, 30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 3 mM DTT. The eluted fraction of RASSF protein was checked with SDS-PAGE and highly purified fractions were collected and concentrated with Amicon 30 kDa cut-off. All RASSF proteins were purified under similar conditions as described above, and they run on SDS-PAGE, which is shown in Figure 3.2. The concentrated proteins (RASSF1=418 µM, RASSF2=467µM, RASSF3= 7663 µM, RASSF4= 587 µM, RASSF5= 432 µM, RASSF6= 340 µM, RASSF7= 237 µM, RASSF8= 346 µM, RASSF9= 363 µM, RASSF10= 259 µM) were stable in standard buffer (30 mM Tris/HCl pH 7.5, 100 mM NaCl, 3 mM DTT) and they were snapped frozen in liquid nitrogen and stored in -80°C.

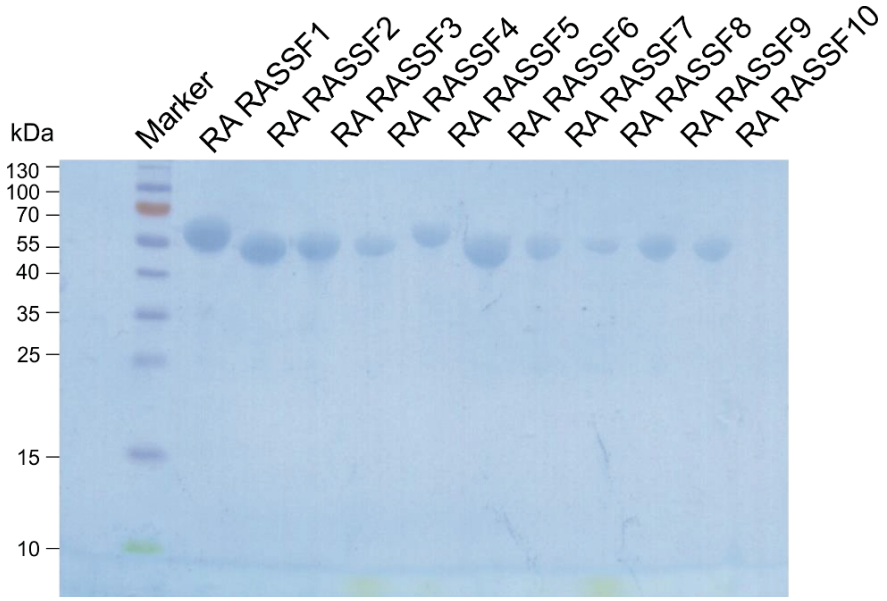

Figure 3.2: SDS-PAGE of purified MBP-RA domain fusion proteins of RASSF 1-10. The standard protein marker and 5 µg of each purified fusion proteins were separated on the gel.

3.1.3 Expression and purification of RAS proteins

In parallel to RASSF proteins, different members of the RAS family have been selected, which include HRAS from classical RAS family, RALA from RAL family, RRAS1 from RRAS proteins, RHEB1 from RHEB family, RAP1B and RAB2A from RAP family, and RIT1 from RIT family. They all cloned in PGEX vector that contains GST-tag. For each one, the expression test was performed as described above and after expression in *E. coli* in large

scale, the pellet resuspended in lysis buffer (30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 3 mM DTT,0.1 mM GDP, 10 µg/ml lysozyme, 1 µg/ml DNase-I and one tablet protease cocktail inhibitor) and sonicated (70% duty cycle, 80% power for 20 sec) for 3 times (each time 2 min). Then, the cell lysate was centrifuged (similar conditions as for RASSF proteins) and supernatant loaded on a GSH affinity column. After washing with high salt buffer (for GST-tag protein) which is described in the method and material chapter, the GST-fusion protein was eluted in a buffer contains 20 mM glutathione. The samples from different steps of purification as well as pellet and supernatant were analyzed via SDS-PAGE. Later on, the protein was concentrated via Amicon 30 kDa cut-off. In the next step, the GST-tag from RAS protein was cleaved by applying 2 units (U) of the TEV protease per mg GST-fusion protein that incubated overnight at 4°C. In the next day, the sample before and after cleavage via TEV enzyme loaded on SDS-PAGE, and if all GST was cleaved from the protein of interest, then the sample was applied on GSH reverse column. In this step, the protein was eluted through column via standard buffer and GST-tag was bonded to the GSH column. Here the SDS-PAGE for cleavage of GST-tag from RHEB protein is shown in Figure 3.3A, and the same protocol was applied for other RAS proteins.

Next, the protein was applied on size exclusion chromatography (SEC; Superdex 75 26/60 column) for exchanging the buffer and also for removing impurities, and the fraction checked via SDS-PAGE. Highly pure fractions were collected and concentrated. The other RAS family proteins also were purified in similar conditions. All purified proteins which were considered in this study are shown in Figure 3.3B.

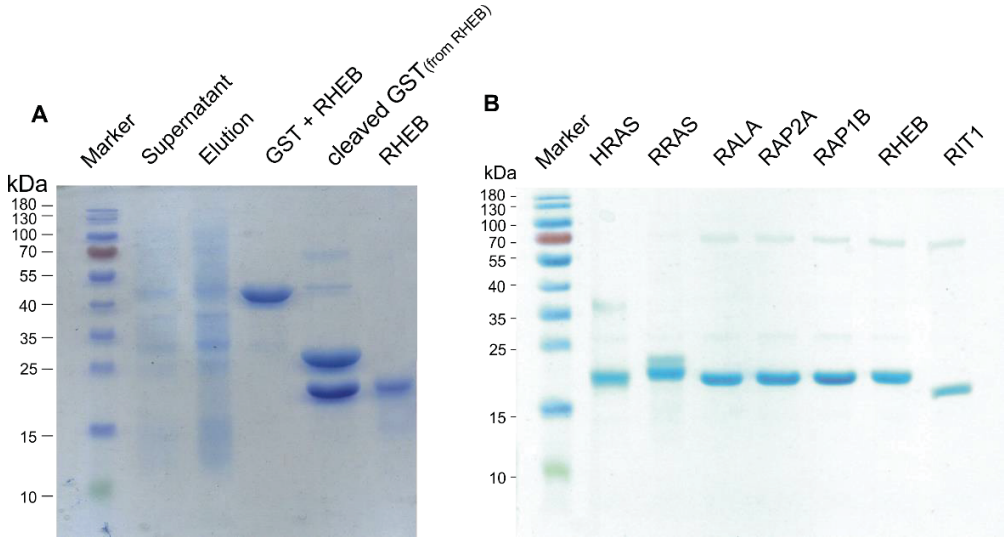

Figure 3.3: SDS-PAGE gel of RAS proteins purification. A) Purification steps of RHEB. Different steps of purification of GST-RHEB and cleavage of the GST-tag are shown. B) Purified RAS proteins members. Sample from the final purification step was applied on SDS-PAGE and was shown in representative lanes for each protein members and standard molecular marker.

After purification of RAS proteins, their activity was checked with HPLC (2.3.12). All RAS proteins except RIT were more than 95% active, expression and purification of RIT1 was done several times, but since it was not more than 70% active then, RIT1 was not considered for measurements. All RAS proteins were stable in standard buffer (30 mM Tris/HCl pH 7.5, 100 mM NaCl, 3 mM DTT), and the concentrated proteins (HRAS, 140 µM; RRAS 250 µM; RALA 173 µM; RAP2A 316 µM; RAP1B 268 µM; RHEB 120 µM) were snapped frozen in liquid nitrogen before storing in -80°C.

3.1.4 Fluorescence polarization

For investigating the binding affinity of RASSF family proteins with RAS proteins, the fluorescence polarization method was used. In the first step, 1 mg of each RAS protein was used for the exchange to mantGppNHp, which is a non-hydrolysable fluorescent GTP analog (2.3.11). The efficiency of nucleotide exchange was checked with HPLC, and it showed RAS proteins were more than 90% in the form of mantGppNHp. Next, 1µM of RAS-mantGppNHp was added to 200µl buffer (30 mM Tris/HCl pH 7.5, 100 mM NaCl, 3 mM DTT) in cuvette which was placed in Fluoromax 4 in polarization mode. When the baseline was constant, then the effector was titrated on RAS protein which contains maltose-binding protein (MBP, 42 kDa). It increased the molecular weight of small-sized RA domains of RASSF family, leads to amplification fluorescence signal in the monomeric

form of fusion proteins. Due to the complex formation between RASSF as effector and RAS proteins the equilibrium titration experiments cause sufficient polarization signal changes upon binding, which were used to determine dissociation constant (K_d) for RASSF-RAS interaction. The binding affinities of RASSF family with RAS protein members are diverse (K_d was between 0.52 μM for HRAS-RASSF5 interaction and no binding for HRAS-RASSF10). MBP-RA RASSF1 and MBP-RA RASSF5 have higher binding affinities toward different RAS paralogs compared to other members of this family. The binding affinity effector proteins towards proteins of the same group, in this case, RAP2A and RAP1B, were also similar. Group two of RASSF family, the affinities for RA RASSF7 and RA RASSF8 are higher compared to the other members of this group. The binding affinities of RASSF proteins with vary RAS proteins were blotted via Grafit software in Figure 3.4.

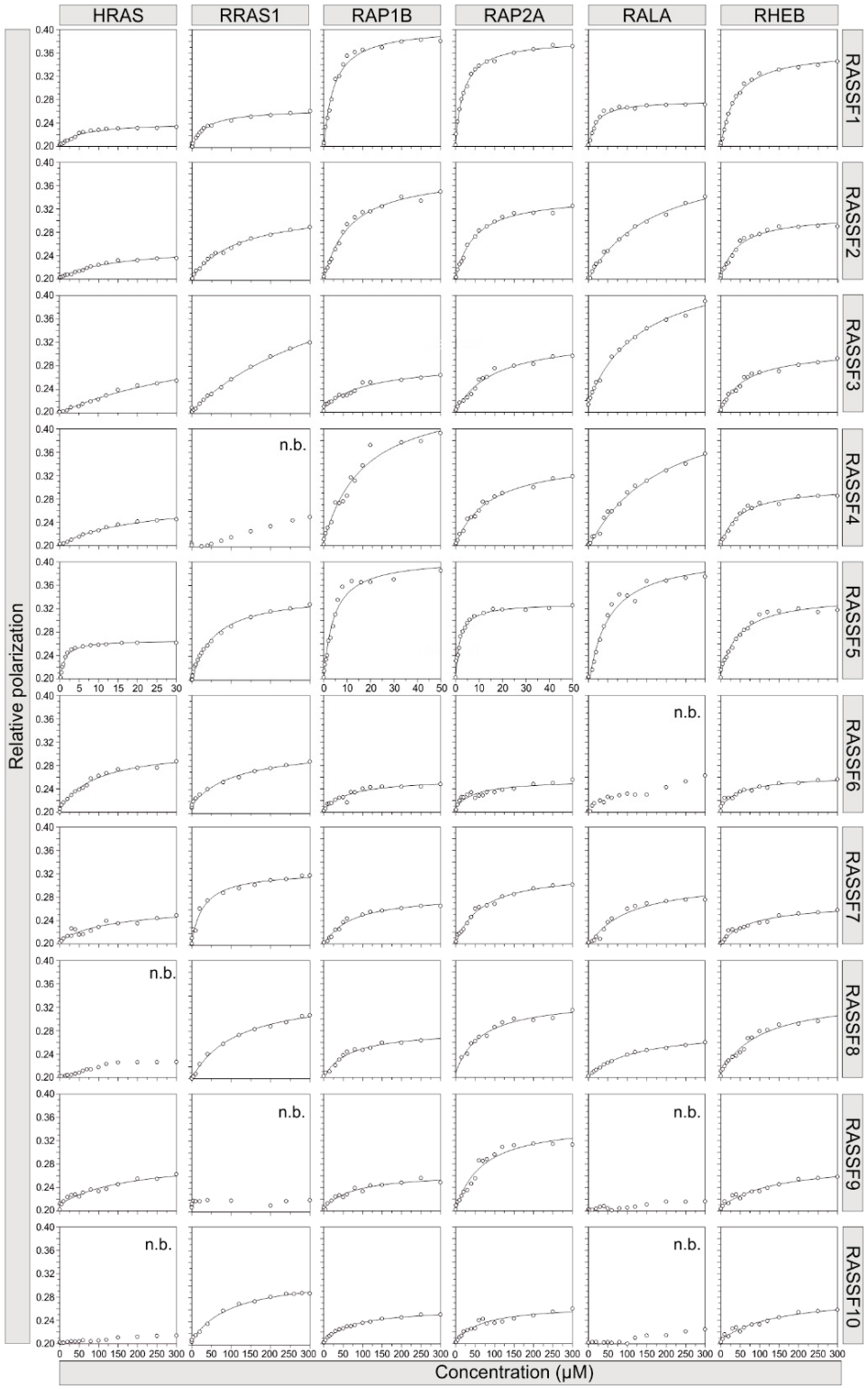

Figure 3.4: Equilibrium dissociation constants for RASSF-RAS interaction. Fluorescence polarization experiments were conducted to determine the dissociation constants (K_d) by titrating the mant-GppNHp form of RAS proteins (1 µM) with increasing concentrations of the respective effector domains. The x-axis shows the concentration of the effector domain as MBP fusion proteins in µM and y-axis represents fluorescence polarization.

The data is summarized in bar charts and K_d values are shown above each bar in Figure 3.5. The red bars represent very strong binding to RAS-mantGppNHp, and green bars also indicate intermediate binding with RAS proteins. However, grey bars show the non-significant binding with RAS proteins.

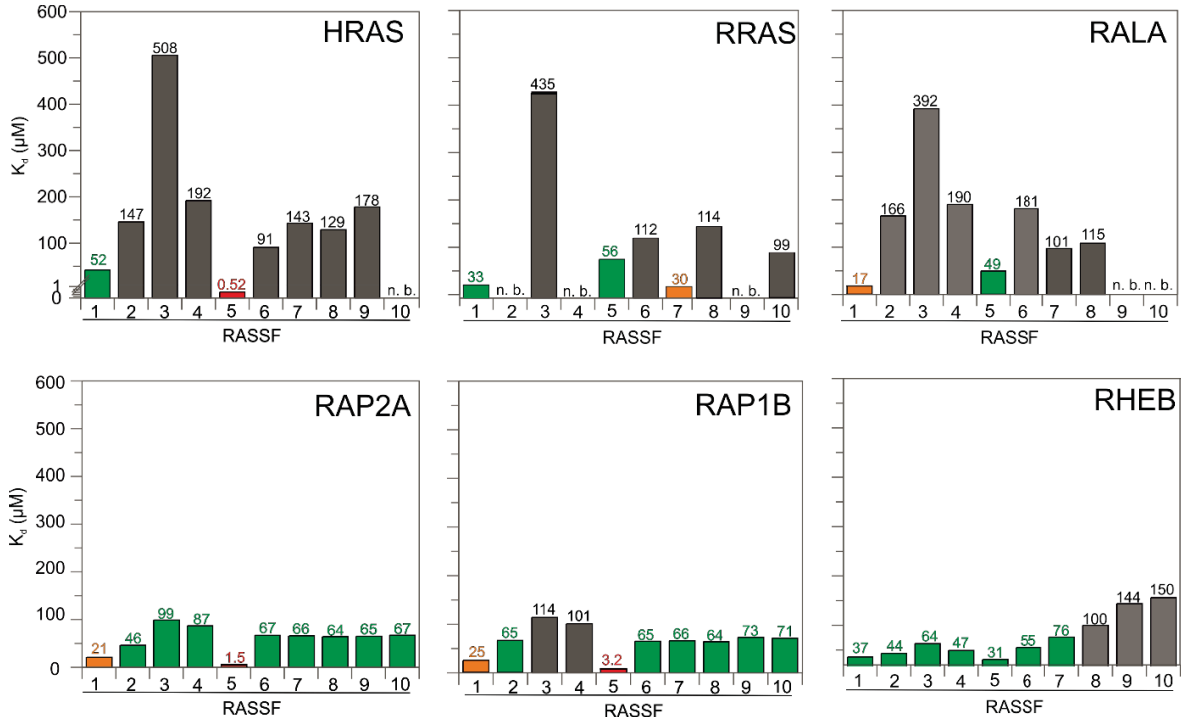

Figure 3.5: Equilibrium dissociation constants for RAS proteins interaction with the RASSF family determined via fluorescence polarization. Grey bars represent the non-significant binding to RAS-mantGppNHp; red bars indicate very strong binding, and green bars represent intermediate binding with RAS proteins. Dissociation rate constant (K_d) values are shown above each bar.

3.1.5 *In silico* analysis of the RAS-RASSF interactions

Previous studies focused mainly on RASSF5 interaction with RAS proteins, but there is a lack of information for interaction between majorities of RASSF members with a variety of RAS proteins under the same conditions. For this reason, we performed further analysis on RASSF5 and HRAS structure (PDB ID: 3DDC), which is the only known domain structure from this family. The main regions of RA RASSF5 that are involved in the interaction with RAS proteins are β2 and α1. The amino acids that are important in this interaction are highlighted in sequence alignment in red in Figure 3.7. There are several vital residues including P283, K308, and F309, that are conserved among the group one (RASSF1-6).

However, other important residues such as L282, D285, A286, I287, K288, Q289, and H291 are almost conversed between RASSF5 and RASSF1 (Fig. 3.6).

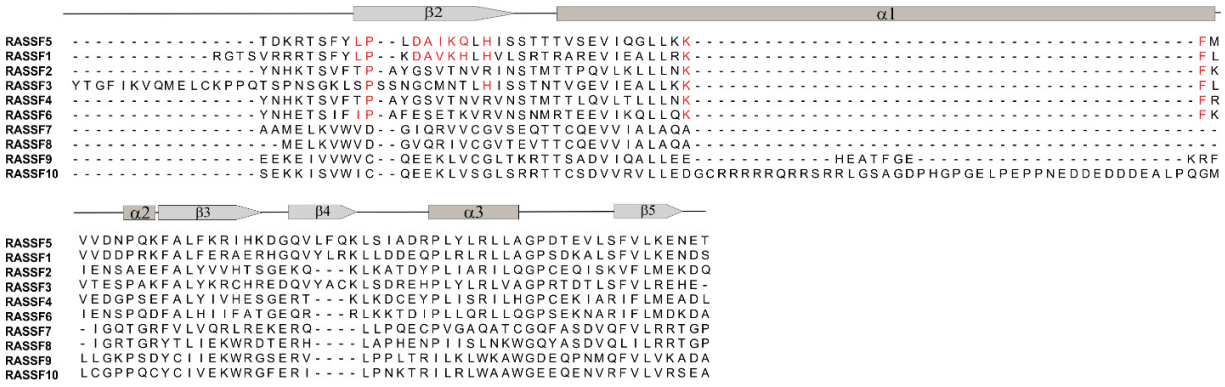

Figure 3.6: Sequence-structure analysis of RASSF RA domains. Amino acid sequence alignment of the RASSF family is shown using the ClustalW algorithm from the Bioedit program. Hotspot residues involved in RAS interaction are highlighted in red, and the secondary structure of RA RASSF5 is shown on top.

The structure models of RA RASSFs were generated, using the Swiss-model website. The sequence of the protein of interest was added to this website, and it found a template from the published PDB structure and provided a structure model. The obtained models for each group were next overlaid. For group one of RASSF proteins, all models except some regions of RASSF3, are overlapping (Fig. 3.7). However, group two models are not completely aligned with RA RASSF5, which are also different in sequence alignments (Fig. 3.6). The amino acids that are important in the interaction between RASSFs and HRAS, are highlighted in Figure 3.7. they are also shown in the left panels in Figure 3.8. These regions are critical for RAS binding and it suggested a high possibility in the interaction between group one of RASSF family and HRAS protein, apart from group two of this family members.

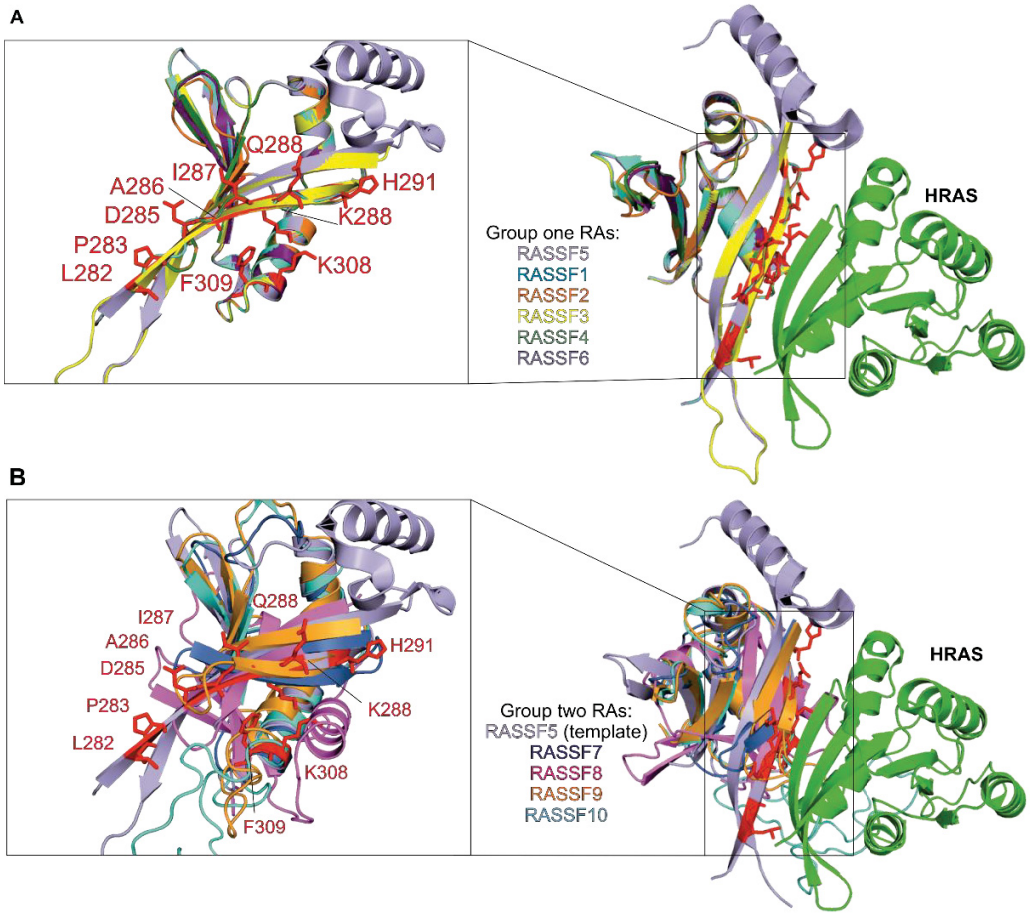

Figure 3.7: Structural modeling of RASSF RA domains. Structural models of RA RASSF proteins (expect for RASSF5) were generated using the Swiss-model website. RA RASSF5-HRAS (PDB ID:3DDC) was used as a template. HRAS structure is shown in green. Critical amino acid residues in the RA RASSF5 domain, which are involved in the interaction with HRAS, are shown in the left panels. (A) RA domain models of group one (RASSF5 (purple), RASSF1 (cyan), RASSF2 (orange), RASSF3 (yellow), RASSF4 (green), RASSF6 (blue), which aligned specially in the interaction regions together. (B) RA domains of group two (RASSF7 (dark purple), RASSF8 (magenta), RASSF9 (orange), RASSF10 (cyan)) that are not completely overlapped with RA RASSF5 as a template.

3.2 Structure-function relationship of ROCK1

3.2.1 Expression and purification of ROCK full-length

ROCK1 full-length contains 1345 amino acids and has a MW of 160 kDa. *ROCK1* was cloned into a pFastBac HTb vector, containing an N-terminal His_6 tag, and was used for a baculovirus-insect cell expression system. *ROCK1* expression was performed in BTI-*Tn*ao38 cells, which were transduced by baculoviruses at 70% confluency (1.5×10^6 cells/ml) in a culture volume of 350 ml. After four days, cells were collected by centrifugation (8000 rpm, 15 min, 4°C), washed twice in buffer (40 mM HEPES pH 7.5, 150 mM NaCl, 3 mM β-mercaptoethanol), and lysed by sonication (70% duty cycle, 80% power for 20 secs; 3 times for 1 min, respectively) in buffer (40 mM HEPES pH 7.5, 150 mM NaCl, 3 mM β-mercaptoethanol, 100 µl DNase-I and one protease inhibitor cocktail tablet). After centrifugation (16000 rpm, 30 min, 4°C), the supernatant was loaded on a Ni-NTA column. Using high salt buffer, the unbound proteins were washed out, and ROCK1 full-length was eluted in buffer containing 500 mM imidazole. The eluted fractions were concentrated using Amicon 100 kDa cut-off, and the concentrated protein was loaded on size exclusion chromatography (SEC; Superdex 200 26/60 column) to remove impurities using 100 mM NaCl, 30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 3 mM DTT. Eluted ROCK1 fractions were separated on SDS-PAGE and fractions with purified protein were collected and concentrated with Amicon 100 kDa cut-off, which reached the concentration of 15 µM. Then, the purity of the sample was checked with western blot using two different antibodies. The anti-His antibody binds to the His-tag in N-terminus of ROCK and the anti-ROCK antibody binds to the kinase domain of the protein (Fig. 3.8). The purified protein was stable in standard buffer (100 mM NaCl, 30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 3 mM DTT) and then it was snap frozen in liquid nitrogen and stored in -80°C.

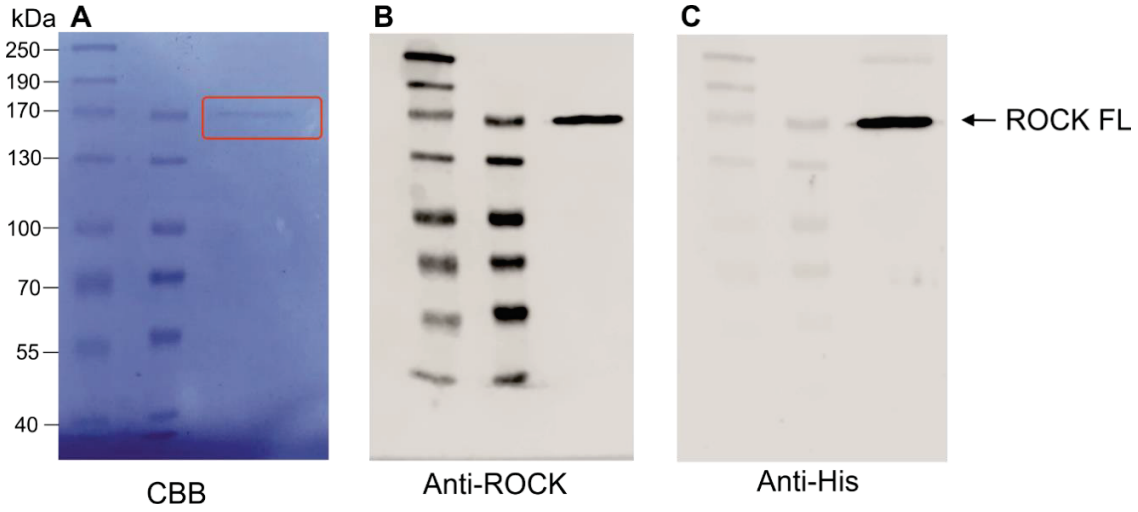

Figure 3.8: SDS-PAGE and western blot of ROCK full-length. The protein sample was separated on a gel with two different markers (the first one is a marker for high range molecular weight and the second one is standard marker). (A) The sample was run on SDS-PAGE and stained with comassive brilliant blue (CBB). (B), (C) The purity of the sample was checked on western blot with two different antibodies (anti-His which recognize N-terminus of protein and anti-ROCK, which interact with the kinase domain).

3.2.2 Guanine nucleotide dissociation inhibition measurements

By using the guanine nucleotide dissociation method (GDI method), the interaction between RHOA and ROCK domains was analyzed. Since the binding of this effector does not affect the environment of the fluorophore mantGppNHp, no changes in fluorescence could be observed upon mixing RHOA-mantGppNHp with ROCK proteins (Blumenstein & Ahmadian, 2004). Therefore, the release of mantGppNHp from RHOA in the presence of 200-fold excess of unlabeled GppNHp was measured. For this purpose, 0.2 µM RHOA-mantGppNHp was used and by adding 40 µM unlabeled GppNHp to the solution the exchange of nucleotide was accomplished within 5 hours using a buffer, containing 30 mM Tris/HCl pH 7.5, 10 mM Na_2HPO_4/$NaHPO_4$ pH 7.5, and 5 mM DTT. However, in the presence of (5 µM) ROCK RBD in similar conditions, after 5 hours the rate of nucleotide dissociation decreased significantly. Due to the binding of RHOA-ROCK RBD, and nucleotide was not able to exchange fast. Additionally, this experiment was monitored in the presence of (5 µM) ROCK full coiled-coil domain (ROCK FCC), which contains HR1, RID and RBD domains of ROCK and as it had been shown previously, each of them has the ability to interact with the active form of RHOA. The GDI measurements represent, ROCK domains bind to RHOA in its active form and also in the presence of ROCK FCC domain, the dissociation rate is lower compared to ROCK RBD (Fig. 3.9).

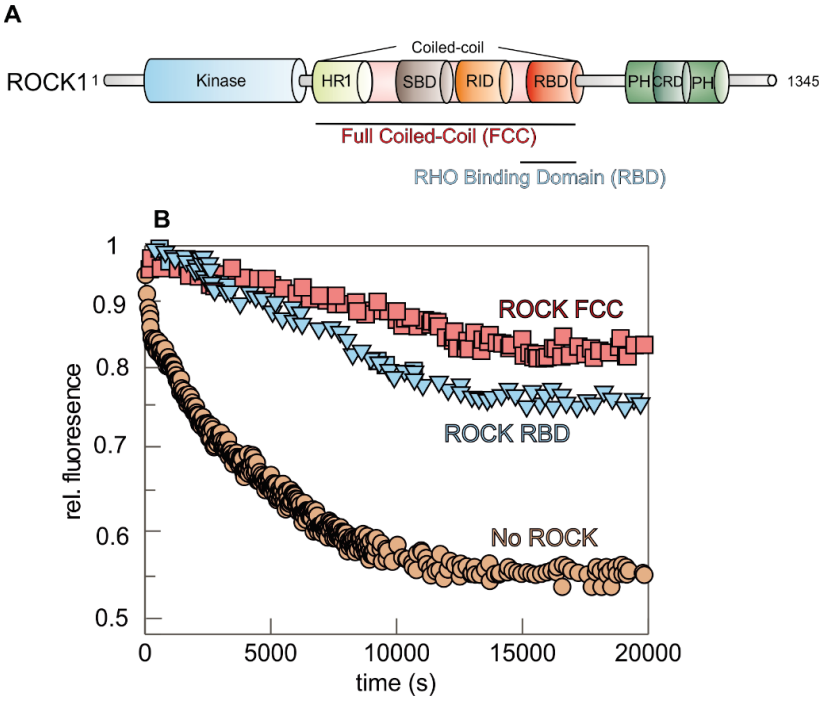

Figure 3.9: Guanine nucleotide dissociation inhibition (GDI) measurement of the various ROCK domains with RHOA-mantGppNHp. (A) Schematic representation of ROCK1 protein. The black bars indicate the full coiled-coil domain (FCC) and RHO binding domain (RBD) which were used in this study. (B) Inhibition of mantGppNHp dissociation from RHOA (0.2 µM) was monitored in both absence and presence of 5µM ROCK domains (RBD, FCC) and 40 µM RHOA-GppNHp. This data indicated that the nucleotide dissociation from RHOA significantly decreased in the presence of ROCK domains.

3.2.3 Kinase assay

Activation of ROCK signaling leads to phosphorylation of different substrates and a variety of biological functions. In this work, in order to check the activity of ROCK full-length and its kinase domain, myosin phosphatase-targeting subunit 1 (MYPT1) was used. MYPT1 is a ROCK substrate and its phosphorylation by ROCK results in inhibition of its enzymatic activity (Amin et al., 2013; Hagerty et al., 2007; Hudson, Heesom, & López Bernal, 2011). GST-MYPT1 purified from *E. coli* can be visualized in western blot with anti-GST antibody. When this protein phosphorylated by ROCK stained by an anti-phospho-MYPT1 antibody. In this assay, 400 µM ATP and 5 µM MYPT1 were used for all conditions at different time points 0, 5, 15, 30, 60 and 120 min. 30 µl of each sample was denatured in 6 µl Laemmli buffer (5x) and 10 µl urea (10M) at 99 °C for 5 min. In the first step, the phosphorylation of MYPT1 was tested in the absence of ROCK protein as a control, it indicated that MYPT1

is not phosphorylated at all (Fig. 3.10A). In the next step, MYPT1 phosphorylation was measured by adding 0.02 µM of ROCK full-length, which indicates that ROCK protein is active even in the absence of RHOA as upstream activator (Fig. 3.10A). Adding 5 µM RHOA-GppNHp to 0.02 µM of ROCK full-length showed that the phosphorylation level of MYPT1 slightly increased but it is not very significant compared to the condition without RHOA (Fig. 3.10A, B). Unexpectedly, the kinase domain (0.02 µM) exhibited a lower activity compare to the ROCK full-length. As a negative control, ROCK inhibitor (Y-27634 (5µM) was added to the reaction, in the presence of 0.02 µM of ROCK full-length. As expected, no substrate phosphorylation was observed. All samples were run on western blot and checked with GST antibody and p-MYPT1 antibody, which are showed separately, and also merged in Figure 3.10A.

In the next step, the C-terminus of ROCK1, which is known as an auto-inhibitory region (AID; aa: 918-1345) was cloned in PET11a vector which contains His-tag and this protein was produced in *E. coli* as described in part 3.1.1. It has been proposed that this region enables to block of the active site of the kinase domain and inhibits its activity (Hartmann, Ridley, & Lutz, 2015; Julian & Olson, 2014). Therefore, the kinase assay was performed in different concentration of ROCK protein from 0.2 µM to 14 µM in the present of 20 µM AID. However, in our measurements, no reduction in phosphorylation level was observed and AID region does not seem to have any effect on the activity of ROCK1 and is represented in Figure 3.10C.

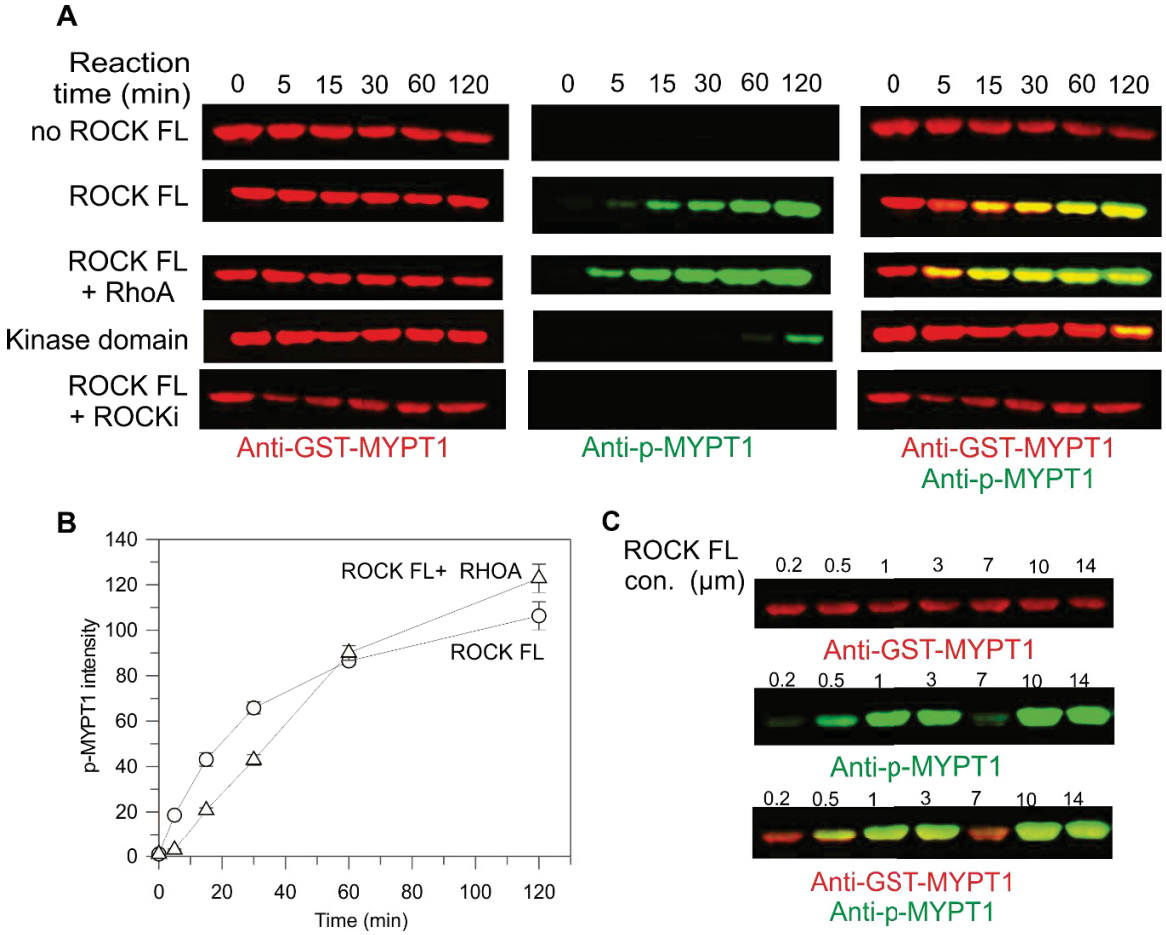

Figure 3.10: Kinase assay analysis of MYPT1 phosphorylation. (A) Western blot analysis of MYPT1 phosphorylation. The samples in various conditions were collected at different time points 0, 5, 15, 30, 60 and 120 min. They were checked via the GST-MYPT1 antibody and p-MYPT1 antibody, which merged in the last row together. As a control, conditions without ROCK protein and ROCK inhibitor in the presence of ROCK protein had checked, and the results showed that no phosphorylation for MYPT1. (B) Statistical analysis of MYPT1 phosphorylation by ROCK full-length in the presence and absence of RHOA-GppNHp. The kinase assay was performed five independent times and the results of western blot analyzed and shown in the above curves. It indicated ROCK full-length is able to phosphorylate MYPT1 as its substrate and by adding RHOA in active form, the level of phosphorylation slightly increased. (C) Western blot analysis of MYPT1 phosphorylation in the presence of AID. Different concentration of ROCK full-length were checked with a high molar excess of AID. However, the phosphorylation level of MYPT1 remained constant.

3.2.4 Structural analysis via cryo-electron microscopy

According to kinase assay, the ROCK protein seems active and may not exist in an autoinhibited state. To analyze the structure of ROCK1 in more detail, purified ROCK FL was subjected to cryo-electron microscopic analysis in the present and absence of liposome in collaboration with Dr. Sabrina Pospich and Prof. Stefan Raunser at the Max-Planck institute of Molecular Physiology in Dortmund, Germany. After the sample were absorbed on copper grids, they were negatively stained with 0.75% uranyl formate before

blotting with filter paper. The cryo-electron microscopy images revealed that ROCK1 adopts a fully open, elongated structure with a central segment (called full coiled-coil or FCC), that forms a parallel coiled-coil dimer with the flanking globular structures of the kinase at the N-terminus and the PH-C1 domains at the C-terminus (Fig. 3.11).

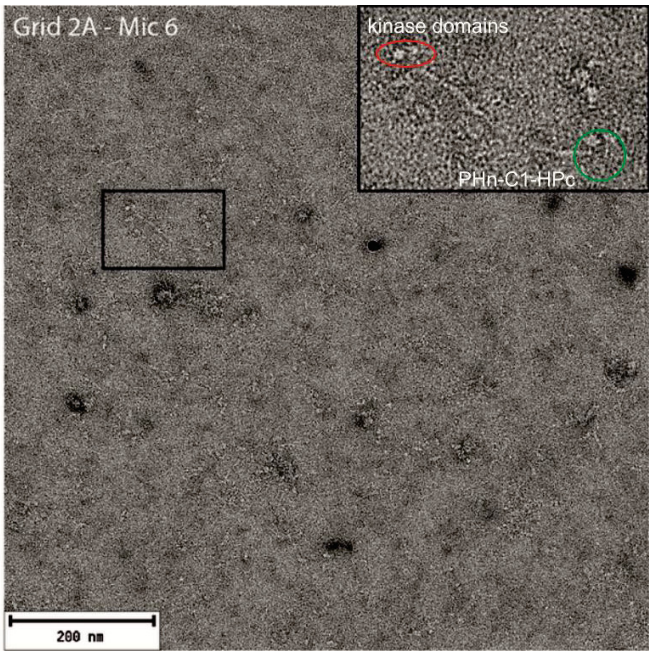

Figure 3.11: Cryo-electron microscopy images of ROCK full-length. Purified ROCK protein was sprayed on microscopy grids and the conformation of ROCK protein by electron microscopy has been recorded. The terminal kinase and PH-C1 domains are highlighted in two different colors, which are separated from each other by fully elongated coiled-coil region (120 nm).

This observation raised an important issue regarding the nature and physical properties of ROCK FL. To gain additional insights into the structural properties of this protein, the liposome assay was performed. In this method, different concentrations of Folch I liposomes added to 30 µl ROCK protein (0.2 mg/ml), and centrifuged for 30 min, 4˚C at 20,000g after incubation for 20 min at room temperature. Immunoblotting of the supernatant and the pellet fractions were performed using an anti-ROCK antibody. Figure 3.12 shows that ROCK1 FL bound to and remained on liposomes after centrifugation.

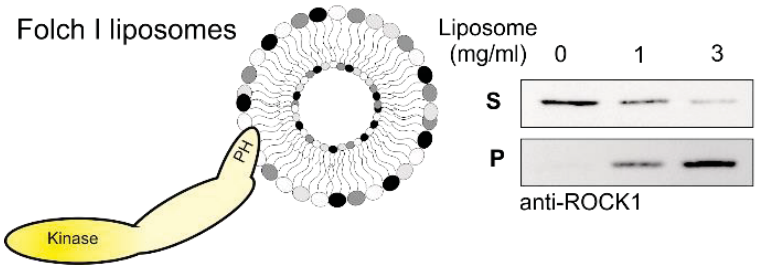

Figure 3.12: Interaction of ROCK1 to the liposome. Different concentrations of FolchI liposome have been tested to examine liposome binding ability of ROCK1 full-length. Sedimentation assay separated ROCK fraction, which is bound to liposome (pellet) from non-bonded protein (supernatant).

Moreover, electron microscopy was performed using a preformed ROCK FL in complex with Folch I liposomes (3mg/ml). These data shown in Figure 3.13 clearly indicates that the central amphipathic region of ROCK remained as an elongated α-helical coiled-coil dimer while the C-terminal regions, containing PH-C1 domains, were associated with liposomes.

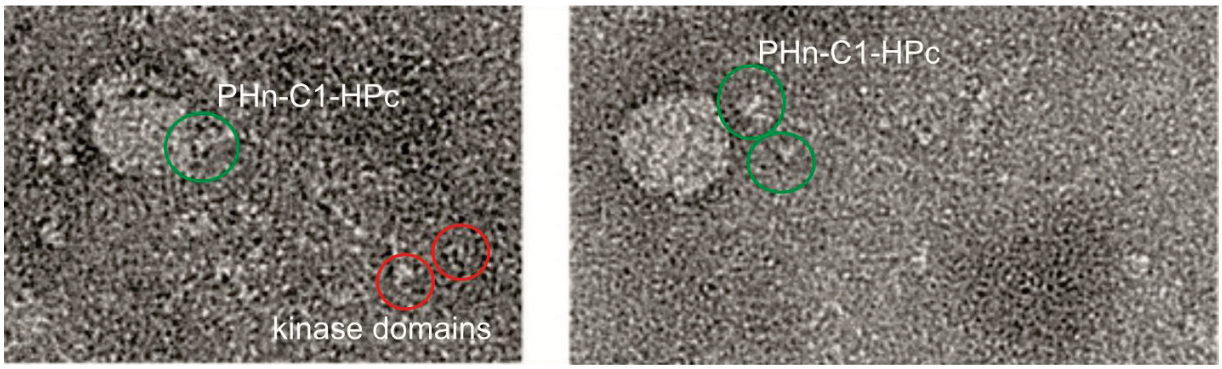

Figure 3.13: Cryo-electron microscopy imaging on ROCK FL associated with liposomes. Liposome assay was performed on ROCK protein, and then the sample applied on microscopy grids and ROCK FL by electron microscopy has been recorded. The kinase domain and C-terminus regions are shown in two different colors, indicating the association of the PH-C1 domains of ROCK FL with the liposomes.

3.2.5 Localization of RHOA and ROCK

In order to investigate the role of RHOA in activation of ROCK protein, localization of RHOA and ROCK proteins was studied with a cell-based assay at different conditions, including serum-starved, 1% FBS and 10% FBS. For this purpose, HMEC-1 (Human Mammary Epithelial Cell 1) cell line was cultured in DMEM media with 10% Fetal Bovine Serum (FBS). The cells were seeded on the glass coverslip and grown for 24 hours with three different serum conditions. The cells were fixed with 4% PFA, permeabilzed with to

0.25% Triton-X-100/PBS for an hour at room temperature and stained with anti-RHOA antibody and anti-ROCK antibody. DAPI was used as a marker for nuclei and phalloidin for F-actin visualization. Figure 3.14 shows RHOA and ROCK localized in cytoplasm in all conditions, and the morphology of the cells did not change remarkably.

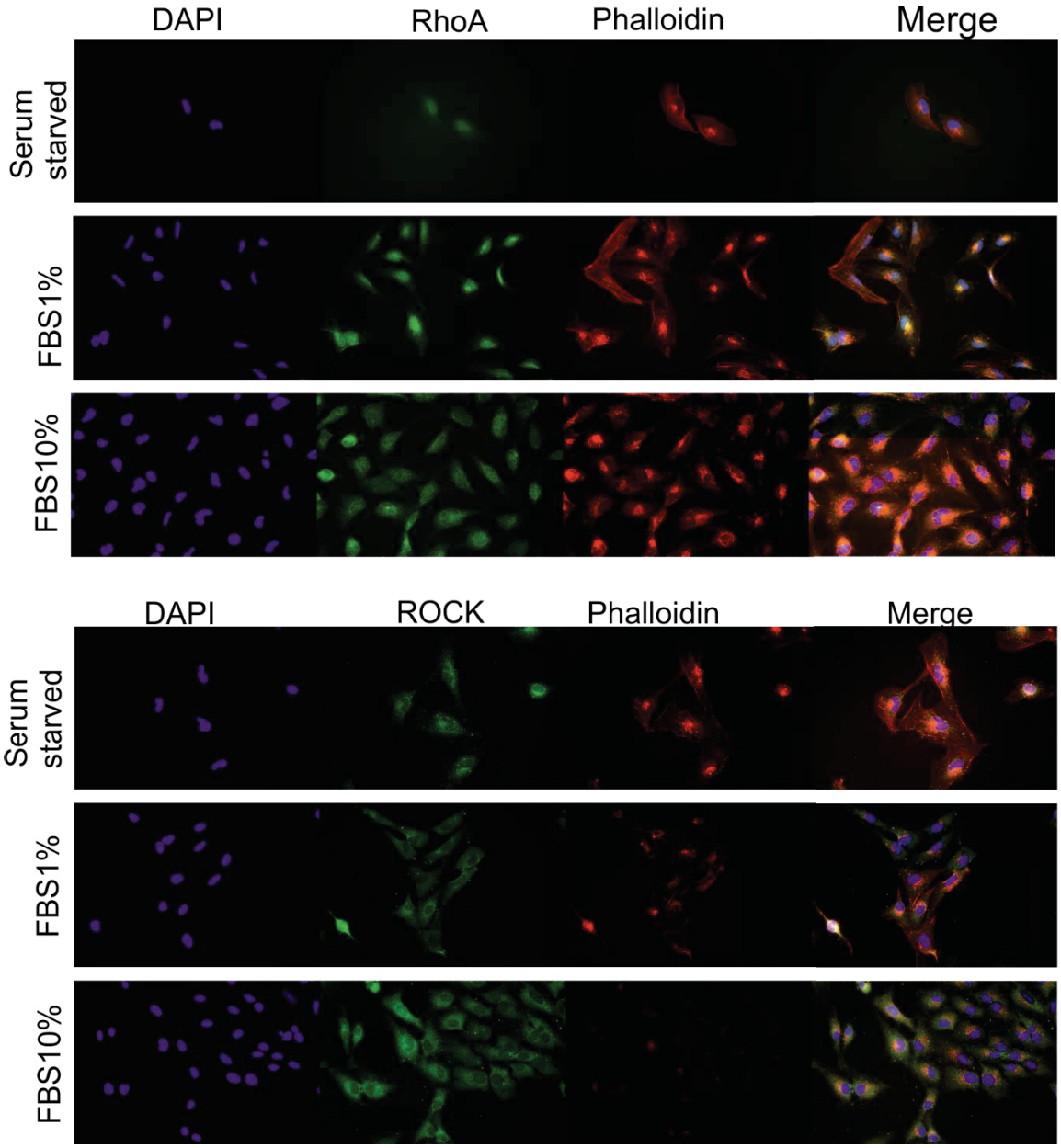

Figure 3.14: Localization of ROCK and RHOA. Cells treated in three different conditions: Serum starved, 1% FBS, and 10% FBS for 24 hours. The localization and morphology of the cells for all conditions are the same. DAPI (blue) used as a marker for nuclei and phalloidin (red) for F-actin. Both RHOA and ROCK localized on the cytoplasm (green). Scale bar = 10 μm.

3.3. PKN-RHO proteins interaction

3.3.1. Expression and purification of PKN1 HR1 domains

PKN1 contains three homology region-1 (HR1) domains, called HR1a, HR1b, and HR1c which are located at its N-terminus. It has been shown that HR1a and HR1b domains bind to the RHO family GTPases (Flynn et al., 1998; Shibata et al., 1996). In order to investigate the molecular mechanism of PKN protein interaction with RHO proteins, we performed different analysis. HR1a, HR1b, HR1c, HR1ab, and HR1abc domains were cloned in pMAL vector, which contains maltose-binding protein (MPB, 42 kDa). MBP increases the overall molecular weight of HR1 domains and it helps to measure their interaction via fluorescence polarization. These domains were expressed in *E. coli* and purified based on the protocol, which is described in part 3.1.2. In order to check the purity of the purified proteins, they were loaded on SDS-PAGE (Fig. 3.15). All MBP-HR1 domains of PKN1 protein were stable in standard buffer (30 mM Tris/HCl pH 7.5, 100 mM NaCl, 3 mM DTT), and the concentrated proteins (HR1a: 500 µM, HR1b: 262 µM, HR1c: 800 µM, HR1ab: 539 µM, HR1abc: 373 µM) were snapped frozen in liquid nitrogen and stored at -80°C.

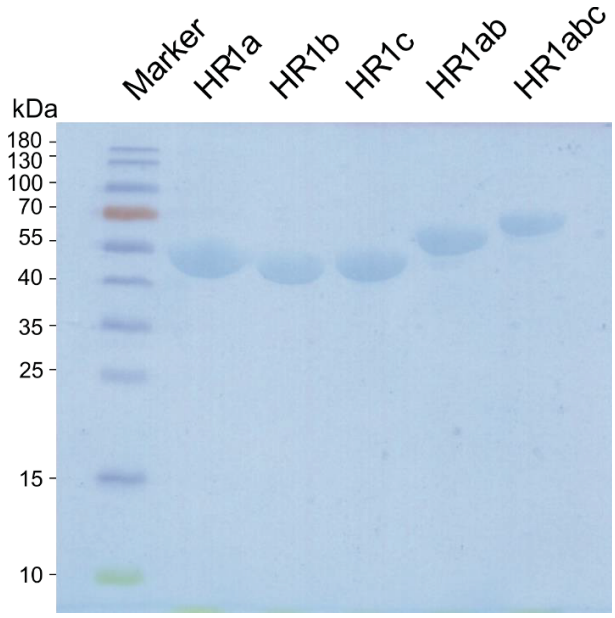

Figure 3.15: Purified MBP-HR1 domains of PKN protein. Coomassie stained SDS-gel showed Samples from the final purification step for each MBP-HR1 domain (5 µg) and the standard molecular marker in kDa.

3.3.2. Fluorescence polarization of PKN-RHO proteins

In this study, RAC1 and RHOA proteins were used and they were purified and labeled with appropriate fluorescent nucleotides as describe in parts 3.1.3 and 3.1.4. Previous studies have described RHOA involved in the interaction with HR1a and HR1b (Shibata et al., 1996) and RAC1 interact with HR1b of PKN (Owen et al., 2003). Therefore, mantGppNHp-bound form of RHOA and RAC1 proteins (1 µM respectively) were added to cuvette with 200 µl buffer (30 mM Tris/HCl pH 7.5, 100 mM NaCl, 3 mM DTT), and fluorescence polarization was monitored using a Fluoromax 4 fluorimeter. Increasing concentrations of MBP-HR1 fusion proteins were titrated on RHO protein. Polarization change, as a signal for complex formation (Fig. 3.16), was used to calculate the equilibrium dissociation constant (K_d) for the interaction between the HR1 domains and the RHO proteins (Fig. 3.17).

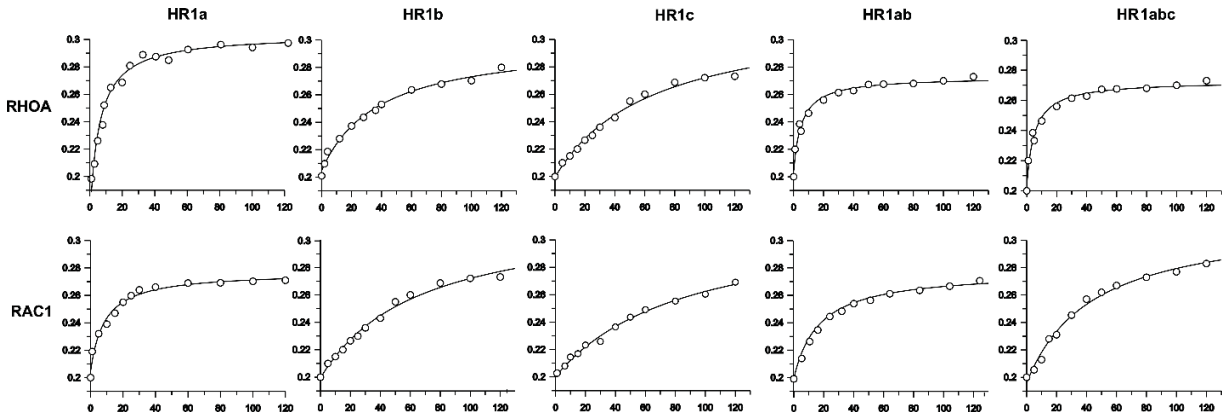

Figure 3.16: Interactions of the PKN1 HR1 domains with RHO proteins monitored by fluorescence polarization. Increasing amounts of MBP fusion PKN1 proteins (as indicated on the x-axis) were titrated on 1 µM mantGppNHp-bound RHOA or RAC1. The interaction was measured using fluorescence polarization.

The results summarized in Figure 3.17 show that binding affinities of HR1b and HR1c for RHOA and RAC1 are very similar and contrast those of HR1a, HR1ab and HR1abc, which are 3 to 6-fold higher for RHOA than RAC1. Among the individual HR1 domains, HR1a has the highest binding affinity toward RHOA, which increased significantly for HR1ab and HR1abc (Fig. 3.17), and it suggests a cooperative association of at least two HR1 domain with RHOA.

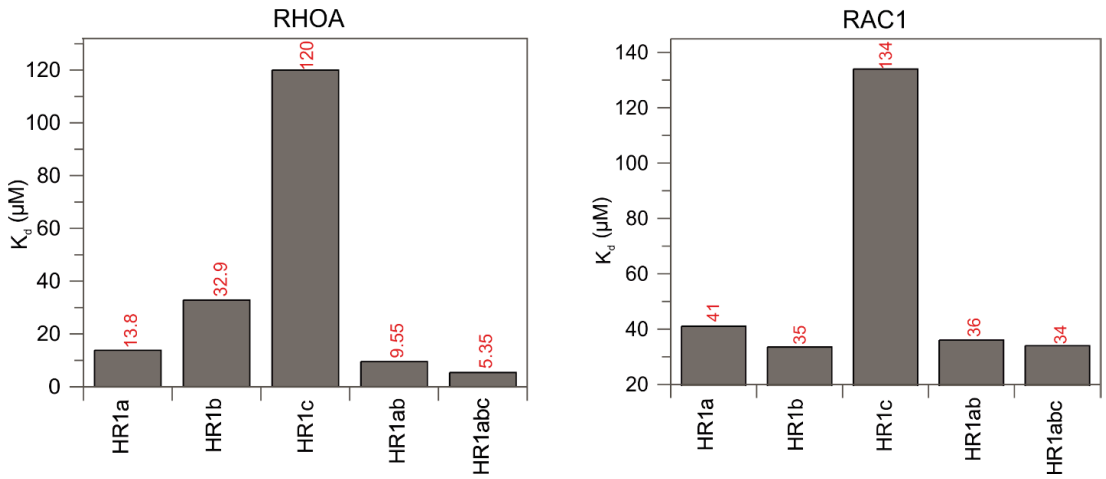

Figure 3.17: Summarized equilibrium dissociation constants (K_d) as a bar chart for RHOA and RAC1 interactions with HR1 domains of PKN1. Grey bars represent the K_d values for the interaction of the PKN HR1 domains with RHOA and RAC1, respectively, and they determined by fluorescence polarization shown in Figure 3.17.

Chapter IV: Discussion

4.1 The selectivity of RAS-RASSF protein interactions

RAS family proteins are involved in multiple signaling pathways and in a variety of cellular processes, including survival, apoptosis, migration, differentiation, and proliferation (Nakhaei-Rad et al., 2018; Vetter & Wittinghofer, 2001). Therefore, physical interaction with and consequently the activation of various downstream effectors is required. It is not completely clear how the binding affinities of the isolated effector domains for RAS proteins determine the specificity of interaction in the cell. Some studies have shown that the biological roles of the RAS family proteins are diverse, while interacting with the common set of effector domains. However, the effectors and their activation mechanisms by the members of the RAS family, such as RAP, RAL, and RHEB, are not well understood (Wohlgemuth et al., 2005). Based on the phylogenic analysis of 25 members of the RAS family (Nakhaei-Rad et al., 2018) (Fig. 1.5), we have selected one representative for each subfamily: HRAS for the classical RAS proteins, RRAS1 for RRAS subfamily, RALA for RAL subfamily, RAP2A and RAP1B for RAP subfamily (to compare interaction in two different subfamilies of RAS proteins), and RHEB1 for RHEB subfamily. This selection considerably extended the number of RAS proteins for the effector interaction analysis, which have not been reported to date.

RASSF proteins were determined as the first RAS effector with non-enzymatic function, which control apoptosis and thus known as tumor suppressors. Thus, they are downregulated in many human cancers, such as lung and breast cancers (Joseph Avruch et al., 2009; Moshnikova, Frye, Shay, Minna, & Khokhlatchev, 2006; Sherwood, Recino, Jeffries, Ward, & Chalmers, 2010; Takenaka, Inoue, Takeshima, Kakura, & Hori, 2013; Louise van der Weyden & David J Adams, 2007). The crystal structure of the RA domain of RASSF5 with HRAS has been determined (Stieglitz et al., 2008), but other members of the RASSF family are still poorly characterized. For example the interaction of endogenous RASSF1C with an active variant of HRAS (G12V) has been shown to promote apoptosis in 293-T cells (Vos et al., 2000). RASSF7 have been reported to interact with NRAS (G12V) in Hela cell after UV irradiation, and thus to be important in apoptotic pathways and

tumorigenesis (S. Takahashi et al., 2011). Among the RASSF1-9, the RA domains of RASSF5, RASSF6, and RASSF7 have been shown, in a GST pull-down assay, to bind HRAS. However, it should be considered that the pull-down method can mainly detect typically binding affinities up to a dissociation constant of 10 μM (Bunney et al., 2006). Therefore, low-affinity interactions cannot be determined by this method (Chan et al., 2013). Additionally, the interaction of some members of the RAS family, including HRAS, RRAS3, and RAP1B with two members of RASSF family (RASSF5 and RASSF1C) has been studied using ITC (Kiel et al., 2005). Accordingly, RASSF1C RA interacts weakly with RAS (39 μM) and there was no interaction with RAP1B. RASSF5 RA interacts with high affinity with HRAS, RRAS3 and RAP1B (0.32, 0.31 and 2.8 μM, respectively). Thus, it was necessary to comparatively study the interaction of all RASSF family members with the respective representatives of the RAS family under the same conditions and an appropriate method, such as fluorescence polarization.

Our measurements revealed that the interaction of the RA domain of the RASSF family proteins with the representatives of the RAS family is diverse. Among the members of RASSF family, RASSF1 and RASSF5 share the highest sequence homology in their RA domain and the residues (*e.g.*, L282, D285, A286, I287, K288, Q289, and H291), involved in RASSF-RAS interaction, are almost identical (Fig. 3.6). In the group 1, RASSF2 RA and RASSF4 RA share 60% identity to each other and ca. 40% identity to RASSF6 RA (Fig. 3.6). There are several critical, highly conserved residues among the group one of RASSF family, which are involved in the interaction with RAS proteins, such as P283, K308, and F309. However, RAS-binding residues are not conserved in group two of RASSF family and our data showed a very low binding affinity between RAS and RA domains of group two (Fig. 3.5). Overall, the RA domains of these two RASSF groups are about 25% identical. Additionally, RA RASSF7 and RA RASSF8 sequences are aligned together especially in residues that are involved in RAS binding and they revealed, with a few exceptions, comparable Kd values for different representatives of the RAS family (Fig. 3.5). Our models showed that group one has a high capacity to interact with RAS proteins, especially HRAS with RASSF1 and RASSF5, respectively. Nevertheless, structural models for group two of RASSFs could not properly align with the RASSF5 structure (Fig. 3.7).

Overall, our experimental data exhibited high selectivity for RASSF1 and RASSF5 compare to other members of the RASSF family (Fig. 3.5), which is in agreement with the *in silico* modeling (Fig. 3.7). Moreover, previous studies showed among the RASSF family members, RASSF1 and RASSF5 are involved in numerous signaling pathways which enable them to regulate different biological functions such as senescence, cell migration, apoptosis, and protein stability (Donninger, Schmidt, Mezzanotte, Barnoud, & Clark, 2016).

Furthermore, our measurements revealed the same effectors have a different binding affinities for the RAS family proteins. RAS proteins share a conserved GDP/GTP binding domains (G1-5 domains), which is important for nucleotide-dependent conformational changes, but their sequences are not identical and show distinct differences (Fig. 1.5) (Nakhaei-Rad et al., 2018; Wittinghofer & Vetter, 2011). High affinity binding of RAP2A and RAP1B to RASSF1 and RASSF5 (Fig. 3.6) is in-line with the proposed biological functions in tumor suppression (Katagiri, Maeda, Shimonaka, & Kinashi, 2003; Praskova, Khoklatchev, Ortiz-Vega, & Avruch, 2004; Vos, Martinez, Ellis, Vallecorsa, & Clark, 2003). Our data showed a lower binding affinities for RASSF1 and RASSF5 with RHEB1 compared to HRAS, RRAS, and RAP proteins. This difference can happen because RHEB1 proteins have some differences in the switch II regions compared to RAS proteins. In RAS, an α-helical conformation leads to changes upon GTP/GDP cycling. However, it has been investigated that switch II region of RHEB proteins undergoes minor changes in response to this cycle and Q64 in this region does not contribute to GTP hydrolysis (Parmar & Tamanoi, 2010). Furthermore, RALA interestingly binds with intermediate affinity to RASSF1. RALA contains Lys-47 and Ala-48 in the switch I region instead of Ile-36 and Glu-37 (HRAS numbering; Fig. 1.5), which are known to critical for the RAS-effector interactions (Bauer et al., 1999). RALA-RASSF1 interaction has not been reported to this date.

In conclusion, our data exhibited RASSF members interact differently with various RAS proteins. This leads to specificity in the signaling properties and biological function of different RAS proteins. These insights help us to understand the specificity of protein-protein interactions in potential tumor suppressor, apoptosis, and protein stability

properties of RASSFs. Moreover, the consequences of RAS proteins with RA domain of RASSF proteins could involve in conformational changes that translocate the SARAH domain of RASSF protein to the membrane. Also it can influence SARAH-mediated dimerization which regulate other downstream proteins (including MST kinases) and it may provide a greater understanding on the molecular mechanism of this family. Therefore, further interaction and structural studies on full-length RASSF proteins and functional reconstitution of RAS interaction networks by using appropriate liposomes may eventually help to determine the functional characterization of multiprotein complexes of RAS and the complete identification of regulatory mechanisms.

4.2 Structure-function relationship of ROCK protein

The RHO-ROCK interaction controls fundamental cellular functions, thus serves as distinguished therapeutic targets in the treatment of a wide variety of diseases, particularly cardiovascular diseases. Using different methods, such as EM, GDI measurement, kinase assay and confocal imaging, we studied the physical and structural properties of ROCK protein and its interaction with RHO GTPases. As previous studies have proposed, ROCK RBD domain has a dimeric structure, but ROCK HR1a domain resembles like PKN1 HR1a domain which has an anti-parallel coiled-coil monomeric structure (Blumenstein & Ahmadian, 2004). However, some studies showed that the structures of different domains of this protein such as RBD, CCC, and SBD switch between dimer and tetramer (Mohan et al., 2013; Shimizu et al., 2003; Tu et al., 2011). The most obvious feature for coiled-coil region of ROCK is its ability to form oligomers and dimers. This feature may allow the combination of interaction between head and tails (Amano, Fukata, & Kaibuchi, 2000; Chen et al., 2002; Craft Jr et al., 2013; Doran, Xun, Taslimi, Saadat, & Ted, 2004; Jacobs et al., 2006; Wen et al., 2008). Some of these studies were based on the proposed autoinhibitory loop, formed by binding of the C-terminus of ROCK to its N-terminal kinase domain that negatively regulates its kinase activity (Amano et al., 2000). The kinase domain includes a dimerization region (aa 47-78) and a hydrophobic motif (aa 370-420), responsible for the dimer formation of ROCK (Chen et al., 2002; Couzens et al., 2009; Doran et al., 2004; Wen et al., 2008). Furthermore, it has been

suggested that the C-terminal PH-C1 domains of ROCK associate with the cell membrane even in an inactive form (Wen et al., 2008). Therefore, in order to understand the functional role of such an elongated coiled-coil protein (the length of globular kinase domains to PH-C1 domains is more than 100 nm long), we performed different structural and functional analyses.

It has been shown that ROCK protein interacts through three different binding domains (RBD, RID and HR1) with the active form of RHOA (Dvorsky et al., 2004). In the first step, the RBD dimeric coiled-coil domains interact with switch regions of two RHOA proteins. Then RID domains might approach the loop six area of the two RHOA proteins, which leads to rearrangement in the structure and cancelation of the autoinhibitory inhibition of this protein. Finally, the HR1 domains interact with the site I of RHOA protein and induce the dimerization of the kinase domains, which lead to autophosphorylation of this protein (Blumenstein & Ahmadian, 2004). Therefore, we performed further experiments and analyzed the interaction of the full coiled-coil region which contains all these three RHOA-binding domains. Moreover, we used the RBD domain as the control for interaction with RHOA protein for the GDI measurements. The data revealed in the presence of ROCK domains, the rate of nucleotide exchange of RHOA protein decreased which is likely due to the interaction of effector domains to RHOA. We showed that full coiled-coil region binds tighter to RHOA as compared to isolated RBD domain (Fig. 3.9).

In vitro experiments have been shown that ROCK protein phosphorylates MYPT1 at two positions (Thr-696 and Thr-853) (Hagerty et al., 2007; Kimura et al., 1996; Loirand, Guérin, & Pacaud, 2006; Somlyo & Somlyo, 2003). Phosphorylation in both positions lead to autoinhibition of myosin light chain phosphatase (MLCP), which is involved in calcium sensitization of smooth muscle contraction (Jeon et al., 2012; Khromov, Choudhury, Stevenson, Somlyo, & Eto, 2009). In order to investigate the activity of ROCK protein, kinase assay is performed. In this method, the level of phosphorylation of MYPT1 as one of ROCK substrate was investigated at different time points. The results showed that ROCK protein was able to phosphorylate MYPT1 in the absence of RHOA. Addition of RHOA to the assay only slightly increased the ROCK activity. As a control, ROCK activity was successfully inhibited in the presence of the ROCK inhibitor (Fig. 3.10). Moreover, the

level of MYPT1 phosphorylation by the kinase domain was considerably lower as compared to the full-length protein, which might be due to the lack of its dimerization, as determined by analytical gel filtration.

Furthermore, the ROCK full-length protein was modeled using the program CHARMm. Accordingly, ROCK HR1 domain was aligned with other structures, including ROCK1 CC structure (PDB ID: 3O0Z)(Tu et al., 2011), ROCK SBD structure (PDB ID: 4L2W)(Mohan et al., 2013), ROCK RBD structure (PDB ID: 1S1C)(Dvorsky et al., 2004; Shimizu et al., 2003), kinase dimer structure (PDB ID: 2ESM)(Jacobs et al., 2006), and PH-C1 structure (PDB ID: 2ROW)(Wen et al., 2008). This structure proposed that full-length ROCK protein contains a parallel α-helical coiled-coil structure that may undergo dynamical changes upon binding to RHOA and membrane (Fig. 4.1).

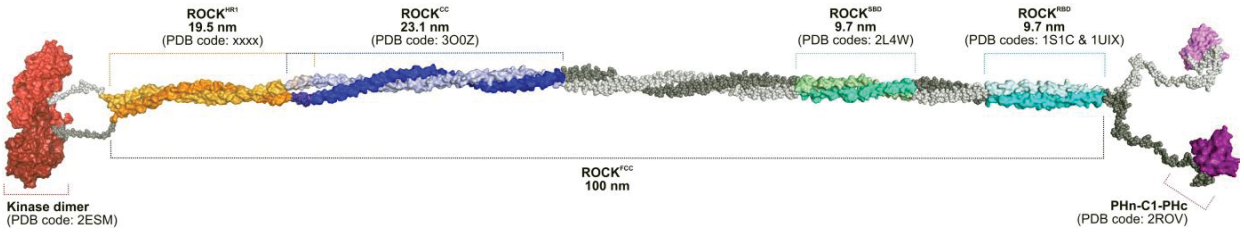

Figure 4.1: Structure properties of ROCK full-length protein. ROCK model structure is built by combing various available structures of different domains, including kinase domain (red), ROCK HR1 (orange), ROCK CC (blue), ROCK RBD (turquoise) and ROCK PH-C1-PH domain (purple), as indicated by the protein database (PDB) codes.

To understand the structure of ROCK protein in more detail, we performed EM measurement using purified ROCK full-length, in absence and presence of the liposomes (Fig. 3.11, 3.13). Under both conditions, ROCK protein has an elongated structure with the length of about 100 nm. This structure strongly suggests that ROCK does not exist in an autoinhibited state, where the PH-C1 contact the kinase domain; instead PH-C1 domains are associated with liposomes as examined by liposome sedimentation assay. Our model is in agreement with the proposed model by Truebestein et al. for ROCK2 protein, which shares 65% sequence identity with ROCK1. Accordingly, it has been suggested that ROCK2 accesses to its substrate as a molecular ruler and it is constitutively active on the cell membrane (Truebestein, Elsner, Fuchs, & Leonard, 2015) (Fig. 3.13).

The localization of ROCK and RHOA protein in Human Mammary Epithelial Cells (HMEC) under different conditions, such as serum starved, 1% FBS and 10% FBS. The results determined in all the conditions both RHOA and ROCK localized in the cytoplasm as both proteins associate with the cell membrane (Fig. 3. 14). Previous studies have been shown, geranylgeranylation of RHOA localized to the cell membrane which is essential for its biological function and interaction with downstream effectors (X. Li et al., 2002). Also, ROCK protein binds to membrane via C-terminal PH-C1 domains (split PH domains) (Wen et al., 2008).

Additionally, it has been shown RHOA interacts with three different domains of ROCK protein, including HR1, RID, and RBD. Thus it has been suggested that RHOA activates ROCK protein through an allosteric association with these domains, that induces conformational changes and activation of ROCK (Blumenstein & Ahmadian, 2004). Moreover, abnormal activation of this pathway observed in numerous human diseases, and this pathway is interested as a new drug target (Mardilovich, Olson, & Baugh, 2012; Narumiya et al., 2009; Schofield & Bernard, 2013). Some studies focused on targeting directly RHO proteins, which leads to blocking the RHO-ROCK signaling pathways (Olson, 2008; Riento & Ridley, 2006). For example, phosphorylation of the cell cycle inhibitor p27 by RSK1, which is downstream of RAS protein, has been analyzed. The results indicated that it directly binds to RHOA and leads to inhibition of ROHA-ROCK pathway (Larrea, Wander, & Slingerland, 2009).

Overall, the RHOA-ROCK pathway has emerged as one of the key signal transduction pathway. It regulates a wide variety of cell functions, such as apoptosis, motility, contraction, and proliferation. As we mentioned above, abnormal activation of this pathway had been shown in cancer, cardiovascular diseases. In our measurement, the interaction between RHOA-ROCK and localization of both on the cytoplasm is determined. On the other hand, our biophysical analysis and electron microscopy measurement, did not support the autoinhibited state for this protein. Therefore, they might be other scaffold protein or ligands, which are not known yet. They may be important to maintain ROCK in the autoinhibited state. By interaction of this protein with RHOA, it might activate and lead to activation of substrate and signaling transduction.

4.3 Molecular Mechanism of PKN-RHO family interaction

PKN protein has been investigated as one of the RHO family effectors. It is involved in the regulation of downstream target proteins such as vimentin, α-actinin, and subunits of neurofilaments (Matsuzawa et al., 1997; Mukai et al., 1997; Watanabe et al., 1996). Additionally, PKN protein regulates cellular processes, including vesicle transport (Gampel, Parker, & Mellor, 1999; Ridley, 2001), cell cycle regulation (Misaki et al., 2001), and control of transcription factor (Kitagawa, Mukai, Takahashi, & Ono, 1998; Takanaga, Mukai, Shibata, Toshimori, & Ono, 1998).

For the first time, the homology region 1 (HR1) was investigated at the N-terminus of the serine/threonine protein kinase PKNα/PRK1, which contains three HR1 domains (HR1a-c). These domains have been shown as RHO binding domain in many effectors (Flynn et al., 1998; Palmer et al., 1995). Based on the sequence analysis of different HR1 domains in the RHO effector proteins, they can be divided into two groups, the PKN-type and the ROCK-type HR1 domains. The sequence of ROCK-type HR1, which include ROCK, Citron, and Kinectin proteins are longer and do not share the same homology as compared to the PKN-type HR1, including PKN, Rhophilin, and Rhotekin (Fig. 4.2).

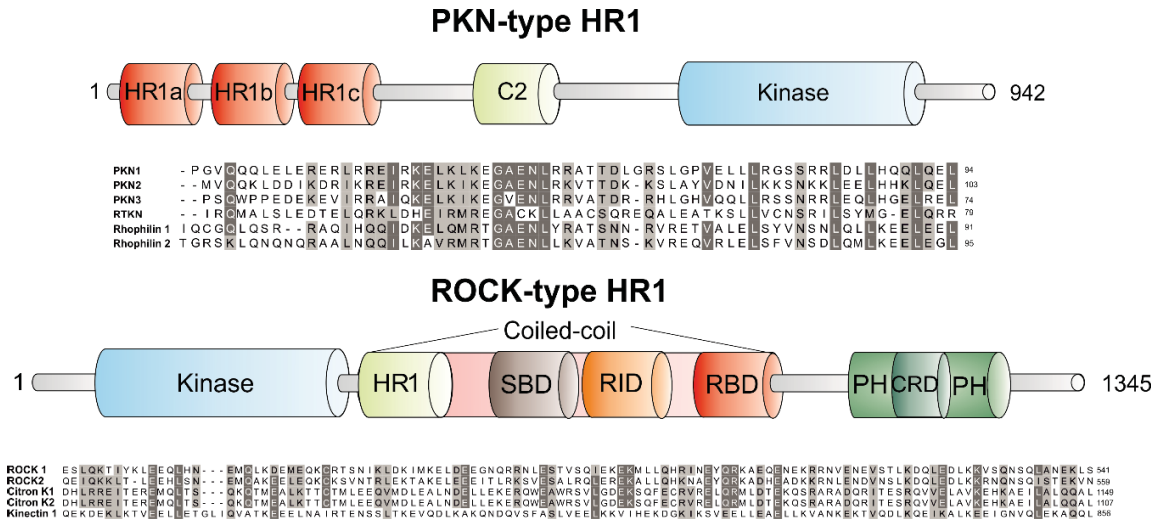

Figure 4.2: Sequence comparison studies of ROCK-type and PKN-type HR1 domains. The sequence alignments of different HR1-like domains were generated using ClustalW. In each group, the amino acids, which are identical (dark gray) and similar 80% (light gray), are determined with different colors. This indicates ROCK-type HR1 domains are longer and do not share the same homology compared to PKN-type HR1 domains.

This indicates a principal difference between the two HR1 types, which leads to different modes of interaction with RHO proteins. PKN contains three homology region domains, which are called HR1a, HR1b, and HR1c. HR1a and HR1b adopt a monomeric antiparallel coiled-coil (ACC) fold. In contrast, HR1 domains of ROCK protein indicates a canonical parallel coiled-coil dimer as describe above.

From the three HR1 domains of PKN, only first two have been shown to bind to the active form of RHOA (Flynn et al., 1998). The crystal structure of HR1a in complex with RHOA-GTPγS has been determined (Maesaki et al., 1999). Accordingly, HR1a binds to two different sites to RHOA, which are known as the first contact site (aa 25-28, 43-54, and 164-172), and second contact site (aa 38-41, 65-69, and 76) (Maesaki et al., 1999). However, in another study, it has been suggested HR1a interacts with RHOA via one contact site, and HR1b does not contribute directly in binding to RHOA (Flynn et al., 1998). Moreover, it has been investigated that HR1b is more thermally stable as compared to HR1a, which might be due to the difference in the biophysical properties of these two domains (Hutchinson et al., 2011). Furthermore, the NMR structure of HR1b in complex with RAC1 has been determined. HR1b has an anti-parallel coiled-coil structure, which binds to residues in the switch I, switch II, α5, and the C-terminal basic amino acids of RAC1 (Owen et al., 2003). It has been proposed that RHO GTPases facilitates the release of the kinase domain of PKN from an autoinhibitory state. However, the molecular mechanism of PKN activation by RHOA and RAC1 remains unclear.

In order to understand more about the mechanism of PKN protein, the interaction of different PKN domains, including HR1a, HR1b, HR1c, HR1ab, and HR1abc, with RHOA and RAC1 proteins was studied using fluorescence polarization. Our data showed that, among individual homology region domains, HR1a has higher affinity for RHOA as compared to the other two domains (HR1b, HR1c). Additionally, HR1b has a similar binding affinity to both RHOA and RAC1, and is also in agreement with previous studies. However, HR1c does not interact with these two members of RHO family. Remarkably, HR1abc has the highest affinity toward interaction with RHOA and RAC1. The binding affinities of the majority of PKN domains with RHOA are higher as compared to RAC1 (Fig. 3.16, 3.17). Our results supported the proposed cooperative binding model, suggesting that the HR1a

domain recognizes RHOA in an active form and facilitates HR1b binding to contact site I, which in turn induces a conformational change and subsequent release of the kinase domain and activation of PKN (Blumenstein & Ahmadian, 2004; Dvorsky et al., 2004). Moreover, HR1c might associate with HR1a and HR1b and stabilize the complex between PKN and the RHO proteins. Nevertheless, it is evident that GTPase interaction is a prerequisite for the activation of effector protein, but many cofactors might be needed to ensure the full activity. For example, some members of the kinase family can be activated through phosphorylation in its activation loop by phosphoinositide-dependent protein Kinase1 (PDK1). For PKN, it has been shown that activated RHO protein binds to PKN and induces a conformational change that is permissive for binding to PDK1. Then PDK1 phosphorylates PKN in the activation loop and stimulates its protein kinase activity (Mukai, 2003).

Chapter V: Short summaries of the supplementary articles

5.1 Structural snapshots of RAF kinase interactions

This section is based on the manuscript by Rezaei Adariani *et al.* (Supplement A).

Summary:

RAF (rapidly accelerated fibrosarcoma) kinases belong to the serine/threonine kinases and they link the RAS GTPase family proteins with the MAPK (mitogen-activated protein kinase) pathway. The three human RAF paralogs (including ARAF, BRAF, and CRAF) regulate a large number of biological processes such as, differentiation, aging, tumorigenesis, survival, proliferation, and apoptosis (J. Avruch et al., 2001; Desideri, Cavallo, & Baccarini, 2015; D. T. Leicht et al., 2007; D. Morrison, 1990; Osborne, Zaganjor, & Cobb, 2012; Wellbrock, Karasarides, & Marais, 2004). Thus, it is not surprising that their dysregulation is associated with progression of a variety of human cancers (S. An et al., 2015; Downward, 2003; G. Maurer, B. Tarkowski, & M. Baccarini, 2011; M. Roring & T. Brummer, 2012), pathogenesis of developmental disorders including Noonan, LEOPARD, cardiofaciocutaneous syndromes (Allanson et al., 2011; Tartaglia et al., 2011), and cardiovascular disease, such as pulmonary arterial hypertension and heart failure (D. Vandamme, A. Herrero, F. Al-Mulla, & W. Kolch, 2014). These proteins are evolutionary conserve across different species and have essential roles during development (Mark, MacIntyre, Digan, Ambrosio, & Perrimon, 1987; T. S. Niault & M. Baccarini, 2010; Sanges et al., 2012). In this review, we summarized emerging mechanistic insights gained from structural, biochemical, and computational studies on functional interaction networks.

Human RAF proteins share evolutionally conserved regions, which are divided into a regulatory N-terminal half, comprising a RAS binding domain, a cysteine-rich domain, and a serine/threonine region and the catalytic C-terminal half representing the kinase domain (Fig. 5.1A).

CRAF RB domain consists of a five-strand mixed β-sheet (β1-β5) with an interrupted α-helix (α1) and two additional 3_{10}-helices (α2 and α3). Consistent with an earlier NMR determination (Patel & Côté, 2013), the RB domain of CRAF has an ubiquitin fold (β1, β2,

α1, β3, β4, α2, and β5). The β-strands are nearly identical with ubiquitin-like protein and α-helices are packed diagonally against a part of β-sheet. To date, several RB domain structures of all three human RAF paralogs have been determined. Superposition of all three RB domain structures revealed a high structural identity. RAF RB domain bind to switch I region of the RAS proteins by forming an intermolecular, antiparallel β-sheet (β1 and β2 of the RB domain of RAF and β2 and β3 of RAS), which establishes a high degree of electrostatic complementarity across the binding interface (Erijman & M Shifman, 2016; Mott & Owen, 2015; Sprang, 1995) (Fig. 5.1B; red residues). Moreover, RAF RB domain has been shown that it interacts with lipid bilayer membrane (Nekhoroshkova, Albert, Becker, & Rapp, 2009; Linda K Rushworth et al., 2006)(Fig. 5.1B; blue residues).

The second domain following RB domain in the conserved region 1 (CR1) is a CRD (cysteine-rich region or C-kinase homologous domain 1), which is connected through a short flexible linker (Z.-L. Li, Prakash, & Buck, 2018). This domain shows high conservation among different species and appears to bind membrane lipids via residues 143-160, which are conserved among different species (Fig. 5.1C; blue residues).

Conserved region 2 (CR2) is a central module in negative regulation of RAF function. Its phosphorylation at Ser-259 (CRAF numbering) followed by 14-3-3 binding locks RAF kinases in a so-called autoinhibited state (Dumaz & Marais, 2003) that blocks both RAS binding and RAF kinase activity (Amardeep S Dhillon, Meikle, Yazici, Eulitz, & Kolch, 2002; Sendoh et al., 2000). CR2 is the substrate of PKA (protein kinase A) and PKB (protein kinase B)/ AKTs (Amardeep S Dhillon, Pollock, et al., 2002; Rommel et al., 1999; Zimmermann & Moelling, 1999). Phosphorylation of RAF paralogs at Ser-259 (CRAF numbering) leads to the association of 14-3-3 proteins and the stabilization of RAF paralogs in their inactive state (Molzan et al., 2010; Pandit et al., 2007; Razzaque et al., 2007). 14-3-3 proteins are ubiquitous adaptor proteins, which serve as scaffold proteins in many cellular functions (Muslin, Tanner, Allen, & Shaw, 1996; Stevers et al., 2017). They bind selectively to the peptide motifs, such as RSXpSXP (single amino acids cods, pS, phosphor-serine; X, any amino acids); arginine, serine, and proline residues, which are important for high-affinity interactions. This motif is identical in RAF kinases regardless of the binding site (Fig. 5.1D).

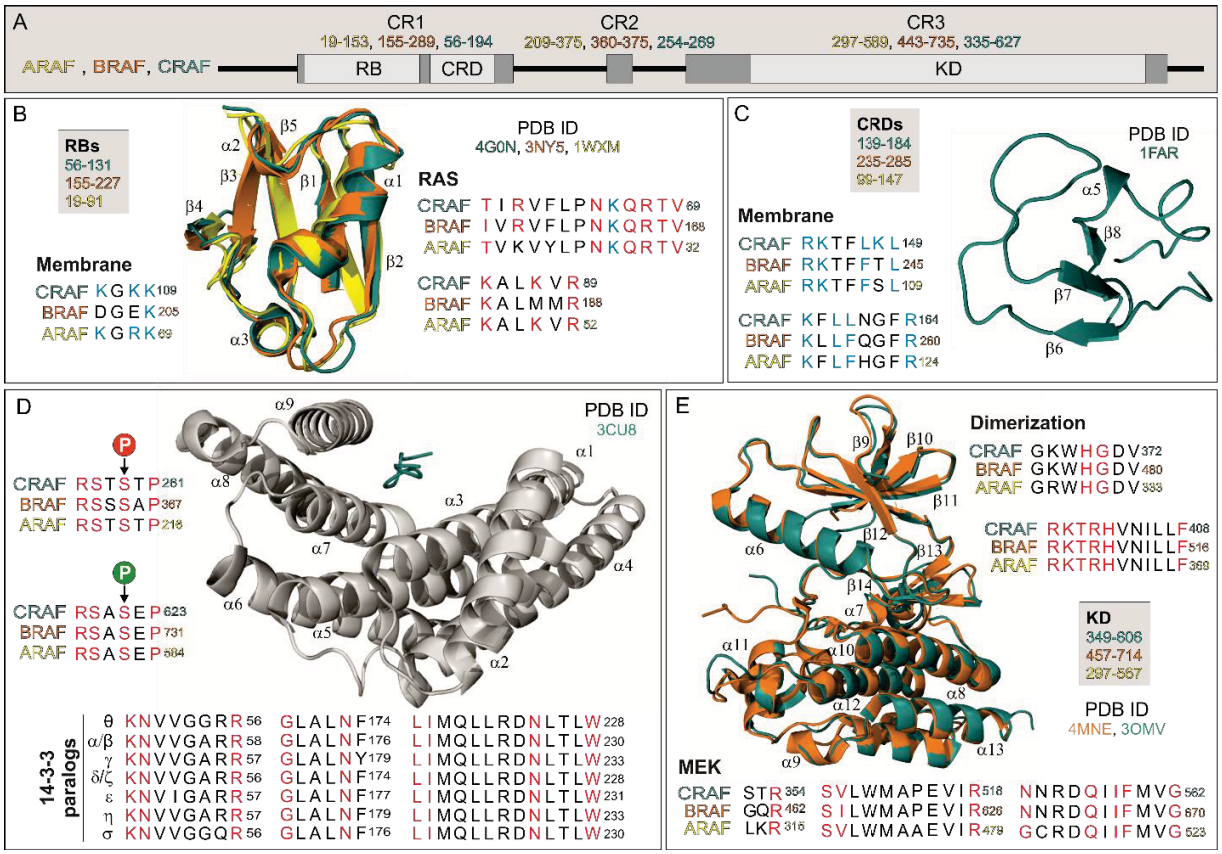

Figure 5.1: Structure-function relationship of individual RAF and RAS domains and motifs. Visualization of the common structure of the RAF proteins. Residues that are proposed to be critical for binding with other domains or proteins are shown in red. The blue amino acids are involved in binding to the membrane. A) Global domain organization of CRAF with numbers on top revealing the domain positions for all three isoforms (CRAF, blue; BRAF, orange; ARAF, yellow). B) RB domain structure of all RAF paralogues interacting with RAS and the membrane followed by a short linker to CRD. C) CRD structure of CRAF can undergo interaction with the membrane by its positively charged amino acids. In contrast BRAF CRD exhibit negative charge residues in positions 202 and 204 which unable the interaction with the membrane. D) Ribbon plot of 14-3-3 binding motifs in RAFs. 14-3-3 protein δ/ζ (gray) complex with CR2 peptide of CRAF (blue). The 14-3-3 binding motifs in CR2 and CR3 of RAFs are shown, together with 14-3-3 isoforms alignments. E) Kinase domain of CRAF and BRAF form a face to face dimer with MEK mediated through RAF dimer interface and kinase interaction with N lobe and C lobe of MEK. Modified from (Rezaei Adariani et al., 2018).

RAF kinase domain includes the two lobes moving relative to each other and consequently opening or closing the catalytic cleft. In an open form, the small lobe with an antiparallel β-sheet structure binds and orients ATP. In the closes form, the α-helical large lobe binds the protein substrates, such as ubiquitously expressed MEK1/2. As RAF dimerization is a key step in pathway activation, the RAF kinases activate MEK1/2 by phosphorylation them at two series (Ser-218/Ser-222 in MEK1) in the catalytic domain (D. R. Alessi et al., 1994;

Roskoski, 2012). The CRAF/BRAF dimerization represent the most effective form for MEK phosphorylation when compared with any form of monomers or homodimers (L. K. Rushworth, A. D. Hindley, E. O'Neill, & W. Kolch, 2006) (Fig. 5.1E).

Overall, emerging evidence indicates that sequential RAS binding of the two N-terminal RAF domains, first RB domain followed by CRD, at the membrane induces a conformational change in RAF and results in the release of C-terminal kinase domain. This mechanism obviously requires additional functions (Anderson, 2006; A. Baljuls, B. N. Kholodenko, & W. Kolch, 2013; Blazevits et al., 2016; Chavan et al., 2015; Cseh, Doma, & Baccarini, 2014; W. Li, Melnick, & Perrimon, 1998; Shaul & Seger, 2007; Wortzel & Seger, 2011; Yoon & Seger, 2006). Future analysis of protein interaction networks along with the network reconstitution at liposomes using purified proteins will provide further mechanistic insights into RAS-mediated RAF activation.

5.2 Structural fingerprints, interactions, and signaling networks of RAS family beyond RAS isoforms

This section is based on the manuscript by Nakhaei-Rad *et al.* (Supplement B).

Summary:

Among the signaling molecules indirectly linked to many different cell surface receptors, RAS proteins essentially respond to a diverse range of extracellular cues. They control activities of multiple signaling pathways and consequently a wide range of cellular processes, including survival, growth, adhesion, migration, and differentiation. Any dysregulation of these pathway leads, thus, to cancer, developmental disorders, metabolic, and cardiovascular diseases. The biochemistry of RAS proteins has become multifaceted since the discovery of the first members, more than 40 years ago. Substantial knowledge has been attained about molecular mechanisms underlying post-translational modification, membrane localization, regulation, and signal transduction through diverse effector molecules. However, the increasing complexity of the underlying signaling mechanisms is considerable, in part due to multiple effector pathways, crosstalks between them and eventually feedback mechanisms. In this review, we describe current understanding of the regulatory mechanisms of individual RAS proteins and their signaling networks beyond the RAS paralogs. Phylogenic analysis identified 25 members of the RAS family out of 35 sequences (van Dam et al., 2011)(Fig. 5.2). RASL, RERG, and NKIRAS proteins exhibit strong sequence deviations and thus, excluded from the list. The RAD family proteins, which are also excluded, make up together with RAS, RHO, RAB, ARF, RAN, and RAG the RAS superfamily (Rojas et al., 2012). The RAS family includes 23 genes coding for at least 25 proteins. Based on sequence identity, structure, and function, the RAS proteins were divided into eight paralog groups: RAS, RAL, RRAS, RIT, RAP, RHEB, RASD, and DIRAS.

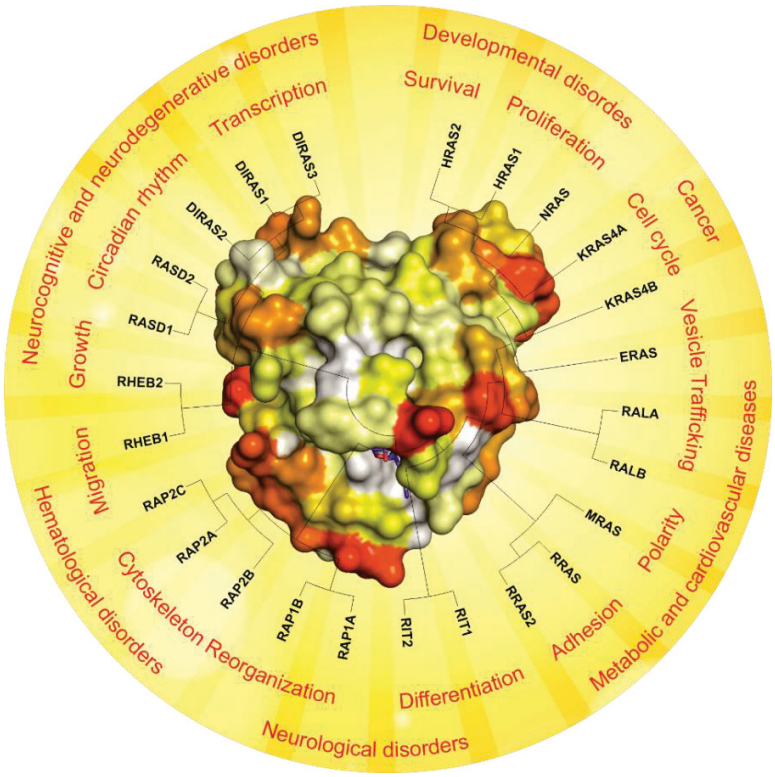

Figure 5.2: The RAS family paralogs. Phylogenic analysis of 25 members of the RAS family are shown. These proteins share a conserved GTP-binding domain (the color spectrum goes from white (identical) through yellow and orange (partially conserved) to red (highly variable)). RAS family proteins control a wide array of signaling pathways and cellular processes distinct from those controlled by RAS paralogs. This review, focused on common features and differences of RAS family proteins regarding their structure, function, signaling and involving in diseases. Modified from (Nakhaei-Rad et al., 2018).

The RAS family proteins share a conserved GDP/GTP binding domains (which has been described in section 1.1.2). However, some members of this family, including ERAS, DIRAS3, and RASD1/2, exhibit distinct amino acids deviation in their G1 and G3 motifs (Fig. 1.5). These proteins accumulate themselves in GTP-bound form due to their impaired GTP hydrolysis and GAP insensitivity (Kontani et al., 2002; Nakhaei-Rad et al., 2015). Moreover, each member of RAS proteins has specific deviation within and additional features outside the G domain that makes them unique in regulation and function. Many members of RAS family exhibit unique amino acid extensions at their N-terminal (N_{ex}) and C-terminal (C_{ex}) ends. For example, the N-terminus of ERAS, which appears to undergo multiple interaction with other proteins (H. Nakhaeizadeh, J. Lissy, S. Rezaei Adariani, S. Nakhaei-Rad, M.R. Ahmadian, unpublished) and contains putative SH3-binding motifs, like RRAS1 and HRAS2/3.

The sequence similarity between RAS proteins, especially in effector binding region was

temping to speculate overlapping functions for related RAS proteins. However, we need to consider the timing, subcellular localization and external stimuli that selectively regulate individual RAS proteins. This complexity comes in part because of their hypervariable region at C-terminus and sequence deviations in the full-length proteins, which provide additional binding sites for various scaffolding and adaptors proteins. Association of RAS proteins with cellular membranes is mediated through a series of post-translational modifications and distinct motifs at their very C-terminal end (Cox, Der, & Philips, 2015; Omerovic & Prior, 2009; Wright & Philips, 2006). RAS proteins, except RIT1/2, serve as substrates for isoprenyl-transferring enzymes, which covalently and irreversibly attach a 15-carbon farnesyl or a 20-carbon geranylgeranyl moiety to the cysteine residue of the very C-terminal CAAX motif (C is cysteine, A is any aliphatic amino acid and X is any amino acids). This motif is present in more than 100 proteins and necessary for diverse cellular processes (Lane & Beese, 2006). Due to a relatively weak affinity of isoprenylated proteins for cellular membranes (Silvius & l'Heureux, 1994), additional motifs in the hypervariable region (HVR) are engaged in fine-tuning membrane association with RAS proteins and their functions (Abankwa et al., 2007; Hanzal-Bayer & Hancock, 2007; Omerovic & Prior, 2009). Some RAS proteins, such as KRAS4B, RALA, RRAS3, and RIT1/2, contain a stretch of positively charged phospholipids of the cell membrane (Banerjee et al., 2016; Nussinov et al., 2016). A further way of increasing the affinity of isoprenylated proteins for cellular membranes is an addition of one or more lipid anchors. KRAS4A, NRAS, HRAS1, ERAS, RRAS1, RAP2A/B, and RALA/B are palmitoylated by acyl protein transferases at cysteines prior to the CAAX motif (Beranger & Tavitian, 1991; Gentry, 2015; Hancock et al., 1989; Y. Takahashi et al., 2005). G domain-membrane interaction may contribute to the specificity of signal transduction and may underlay additional control elements. Acritical aspect in this context is the organization of RAS proteins into protein-lipid complexes. These so-called nanoclusters concentrate RAS at the plasma membrane. They are the sites of effector recruitment and activation, and are essential for signal transmission (Abankwa et al., 2007; Zhou & Hancock, 2015).

RAS family proteins link the extracellular signals, transduced through their receptors, with multiple signaling pathways and consequently control a wide array of cellular processes

(Fig. 5.2). Different RAS paralogs have unique roles in modulating the cellular processes. The specificity comes from several levels: Subcellular localization, upstream stimuli, interaction with scaffolds, regulators and target proteins, and downstream signaling. In this review, we described more precisely the conditions under which individual RAS proteins are activated and how they transduce the signal. In addition, specific regulation of cellular functions by the members of the RAS family depends on selective interaction with downstream targets, the effectors (Mott & Owen, 2015; Nakhaei-Rad et al., 2016), which transduce the signal to distinct pathways (Cox & Der, 2003; Gentry et al., 2014). More than 60 effectors reported for the RAS family proteins and they can activate about 49 pathways which are described in this review. For example, RAF kinases are the major and best studied effectors for this family (Rezaei Adariani et al., 2018).

As RAS family proteins control a wide variety of cellular processes, it is obvious that any dysregulation or dysfunction of the respective signaling pathways results in the development of human diseases, including neurocognitive, hematological, developmental and neurodegenerative disorders, metabolic and cardiovascular diseases, and cancer. For example, somatic mutation frequently identified in KRAS4B, HRAS, NRAS, and RIT1 (COSMIC), contribute to robus gain-of-function (GoF) effects and to various types of cancers as well as leukemia and lymphoma tumors (Simanshu et al., 2017).

To sum up, in this review we discussed unique aspects of each RAS subfamily in term of tissue expression, upstream stimuli, receptor activation, interactions with regulators and effector that collectively fine-tune individual cellular functions under normal and pathological conditions.

5.3 Activating Mutations of RRAS2 Are a Rare Cause of Noonan Syndrome

This section is based on the manuscript by Capri *et al.* (Supplement C).

Summary:

Noonan syndrome (NS [MIM: PS163950]) is one of the most common monogenetic disorders affecting development and growth (A. E. Roberts, Allanson, Tartaglia, & Gelb, 2013). The phenotype of NS comprises a distinctive facies (including hypertelorism, downslanting palpebral fissures, ptosis, and low-set/posteriorly rotated ears), cardiac abnormalities (a wide spectrum of congenital heart defects and cardiomyopathy), postnatally reduced growth, skeletal defects (chest and spine), cryptorchidism, bleeding diathesis, as well as variable neurocognitive impairment and predisposition to malignancies (Tartaglia et al., 2011). A remarkable finding of the molecular genetics of NS and other RASopathies is the occurrence of conserved themes in the mechanism of disease. Through the use of complementary approaches based on 'functional candidacy' (parallel sequencing of selected gene panels containing functionally related candidate genes) or WES, we identified RRAS2 (MIM: 600098; GenBank: NM_012250.5) as a gene implicated in NS. We provide structural, biochemical, and functional data to support the causal link between RRAS2 mutations and NS, outline the mechanisms by which mutations perturb RRAS2 functions, and characterize the clinical phenotype associated with these gene lesions.

Subjects from six unrelated families were included in this study. Clinical data and DNA samples were collected from the participating families and the data reveled three different nucleotide substitutions predicting missense changes of highly conserved amino acids residues (Gly23, Ala70, and Gln72) among RRAS2 orthologs and paralogs. We also identified two small in-frame duplications (p.Gly22_Gly24dup, P.Gly24_Gly26dup) which affect the well-established mutational hotspot of RAS proteins.

In order to decipher the consequences of the observed amino acids changes and the small in-frame duplications on the molecular structure of RRAS2, we performed structural modeling. A closer view into the active site of RRAS2 structure in its active form revealed that the identified RRAS2 mutations affect residues localized around the nucleotide

binding pocket of the GTPase. The corresponding amino acids, including Gly22_Gly26, Ala70, and Gln72, do not only play critical role are involved in stabilization of the switch regions (Fig. 5.3), which are the binding sites for both RRAS2 regulators (GEFs and GAPs) and effectors (Vetter & Wittinghofer, 2001). Specifically, the amino acid stretch encompassing Gly22 to Gly26 constitutes part of the phosphate-binding loop that is responsible for binding to the phosphate groups of either GTP or GDP. These residues play a critical role in nucleotide binding and hydrolysis by contacting both the β-γ phosphates of GTP (shown as GPPNHP, a non-hydrolysable GTP analog in Fig. 5.3) and residues 67 to 69 of the switch II region. Val25 stabilizes the P loop by contacting Val92, Ser94 and Ser100. The Gly22_to_Gly24 and Gly24_to_Gly26 duplicates were predicted to destabilize the P loop and result in increased nucleotide exchange and decreased GTP hydrolysis reactions. Differently, Ala70 and Gln72 are located in the switch II region of the GTPase and are directly involved in Mg^{2+} coordination and GTP hydrolysis reaction. Additionally, Ala70 and Gln72 stabilize the switch I region by contacting Ile47 and Glu48, respectably (Fig. 5.3).

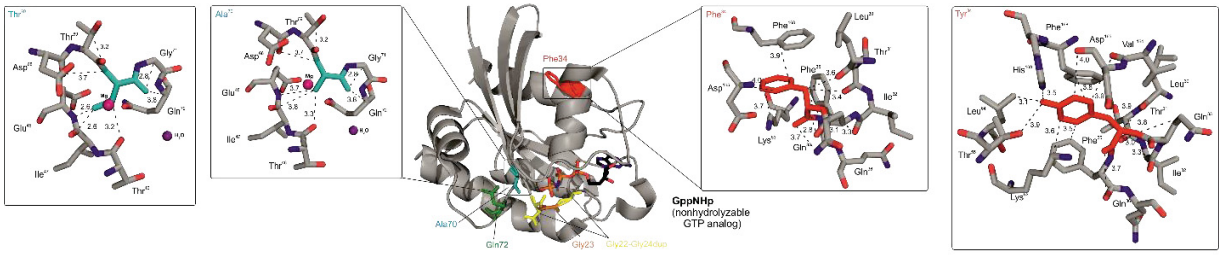

Figure 5.3: Structural modeling of RRAS2 variants. A structural model of the active GTP-bound RRAS2 protein highlights the relative position of the disease-causing missense or insertion mutations. All RRAS2 mutations affect residues that are located in the nucleotide binding active site region, which contains integral elements involved in GTP/GDP binding, GTP hydrolysis, and interactions with regulators (GEFs and GAPs) and effectors. Modified from (Capri et al., 2019).

Based on these considerations, the NS-associated amino acids changes were expected to affect various aspects of RRAS2 biochemical behavior, including a faster nucleotide exchange, an impaired GTP hydrolysis, and a decrease in GEF, GAP, and effector interactions.

Subsequent biochemical analysis of RRAS2$^{p.Ala70Thr}$ clearly confirmed these structural predications, as assessment of the intrinsic and stimulated nucleotide exchange demonstrated a significantly increased response of the RRAS2$^{p.Ala70Thr}$ protein to GEF as compared to wide-type RRAS2. In contrast, the GTP hydrolysis reactions of the mutant were reduced compared to the wide-type protein. Particularly, the GAP-stimulated GTPase activity of RRAS2$^{p.Ala70Thr}$ was significantly decreased (9-fold). Finally, the binding properties to two RRAS2 effectors, CRAF and RASSF5, were assessed. While the affinity of the interaction with CRAF was comparable to that of wide-type RRAS2, binding to RASSF5 was abolished. This suggest the p.Ala70Thr change leads to a structural rearrangement of RRAS2 switch II, which is a key binding site for RASSF5 but not for CRAF. Overall, these data support that the p.Ala70Thr change leads to an accumulation of RRAS2 in its GTP-bound active state, which predicts an increase in signaling activity. The impaired binding to RASSF5, however, suggest a possible differential impact of the missense change on downstream signaling pathways. Taken together, these experimental data suggest that NS-associated RRAS2 mutations variably upregulate MAPK signaling and are likely to affect cellular processes depending on cytoskeleton rearrangement similar to observation of RASopathy-causing KRAS mutation (Gremer et al., 2011).

Our finding establishes RRAS2 germline mutations as a cause of NS. A noticeable finding of this study is the observation of a diverse impact of the p.Ala70Thr on RRAS2 binding to CRAF and RASSF5. These data suggest the possibility that multiple signaling pathways downstream of RRAS2 may contribute to dysfunction of cellular processes, such as cell proliferation. As expected, a variable hyperactivation of the MAPK pathway resulting from the hyperactive state of GTPase and unaltered binding to CRAF was observed for the NS-causing RRAS2$^{p.Ala70Thr}$ protein. Remarkably, impaired binding of this mutant to RASSF5, a known tumor suppressor protein negatively modulating YAP1 levels through activation of the Hippo pathway, was also observed. The impaired binding of RRAS2 to RASSF5 raises the possibility that a less effective Hippo-mediated control of YAP1 levels contribute to disease pathogenesis in NS. Further studies are required to more accurately define the precise mechanisms and circuits linking upregulated RRAS2 function and RAS-MAPK signaling dysregulation.

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Curriculum Vitae

Soheila Rezaei Adariani

➢ Education

Oct. 2016– May 2020	**Research in Biochemistry, and Molecular Biology,** Heinrich-Heine University of Düsseldorf, Germany, Prof. Ahmadian. "Understanding molecular mechanism and structural properties of small GTPase mediated-effector activation".
Jan. 2015- May 2016	**M.Sc. in physics**, Physic and Astronomy department, Clemson University, Clemson, USA, Dr. Sanabria lab. (GPA: 3.5/4), "Conformational selection and submillisecond dynamics of the ligand binding domain of the NMDA receptor"
Sep. 2012- Sep. 2014	**M.Sc. in Organic Chemistry**, Chemistry department, Sharif University of Technology (SUT), Tehran, Iran, Dr. Kalhor lab. (GPA: 17.49/20). "Expression and purification of Amyloid Beta recombinant peptide and the effecs of ultrasmall peptides enriched in branch chain amino acids as inhibitors in Amyloid formation".
July 2013- Oct. 2013	**Visiting Student**, Molecular physical chemistry department, Heinrich-Heine University of Düsseldorf, Germany, Prof. Seidel Lab. "Deciphering folding pathways for multidomain proteins"
Sep. 2008-Sep. 2012	**B.Sc. in Applied Chemistry**, Chemistry department, Isfahan University of Technology (IUT), Isfahan, Iran, Dr. Abdolmaleki lab. (GPA: 18.39/20). "Copper-cationic salphen catalysts for the oxidation of cyclohexene by oxygen"

➢ Skills

Molecular biology	PCR and DNA recombination techniques; site-directed mutagenesis and cloning; RT-PCR
Protein science	Gene expression in insect cells and Escherichia coli; protein purification techniques; analytical HPLC; various chromatographic techniques; Characterization of proteins by using electrophoresis and Western blotting ; Protein folding; enzyme kinetic assays.
Cell biology	Cultures of various eukaryotic cell lines; cell transfection; Immunocytochemistry
Biophysical Techniques	Biomolecular Interaction Analysis (Biacore); florescence polarization; isothermal thermal calorimetery (ITC), mathematical Modeling of biochemical structures.
Chemical Techniques	FT_IR; Synthesis; Organic chemistry synthesis; Amino acid analytical.

Fluorescence Techniques	Fluorescence resonance energy transfer (FRET); Time correlation single photon counting (TCSPC); Spectroscopy; Single molecule detection; Single molecule Spectroscopy; Labeling; Fluorescence Correlation Spectroscopy (FCS).
Computer skills	➢ Softwares: MS-Office, Corel Draw, EndNote, GraFit, Clone manager, Bioedit, Image J, PyMol, Chimera. ➢ Programming: C++, Computational Biology, biological data mining and analysis, Structure modeling.

➢ Conference attendance

Poster

25th International workshop on single molecule spectroscopy and super-resolution microscopy in the life sciences, 2019, Berlin, Germany
German Biophysics Society, 2018, Düsseldorf, Germany
6th International Congress of Biochemistry and Molecular Biology, 2018, Isfahan, Iran
Society for Applied Spectroscopy, 2016, Clemson, USA
10th annual materials and optics in Clemson University, 2015, Clemson, USA
21st Iranian Seminar of Organic Chemistry, 2014, Tehran, Iran

Talk

Biologisch-Medizinisches Forschungszentrum (BMFZ), 2018, Bergisch Gladbach, Germany
Sharif university of technology, 2014, Tehran, Iran
Forschungszentrum Jülich, 2013, Jülich, Germany

➢ Teaching

2015-2016	Teaching in general Physics Lab, Clemson University, Clemson, USA
2013-2014	Teaching in general chemistry lab (I) and (II), Sharif University of Technology, Tehran, Iran

➢ Languages

Persian	Mother tongue
English	Advance (Fluent)
Deutsch	Intermediate (B2)

➢ Awards

2018 Best Poster presenter in 6th International Congress of Biochemistry and Molecular Biology, Isfahan, Iran

2017 Scholarship from European rare disease, NRW Research School, Germany

2016 Honor student in Clemson University, Clemson, USA

2015 Scholarship from Clemson University, Clemson, USA

2014 Ranked #1 among organic chemistry students, Sharif University of Technology, Tehran, Iran

2013 Scholarship from Forschungszentrum jülich as a visiting student

2012 Ranked #3 among Bachelor students in chemistry department, Isfahan University of Technology, Isfahan, Iran

➢ Publications

Research Articles

[1] **Soheila Rezaei Adariani**, Neda S. Kazemein Jasemi, Christoph Wittch, Ehsan Amin, Claus A.M. Seidel, Radovan Dvorsky, Mohammad R. Ahmadian. "Binding selectivity of effectors for RAS proteins". (submitted to JBC)

[2] Yline Capri, Elisabetta Flex, Oliver H. F. Krumbach, Giovanna Carpentieri, Serena Cecchetti, Christina Lißewski, **Soheila Rezaei Adariani**, […]Martin Zenker. "Activating mutations of RRAS2 are a rare cause of Noonan syndrome". *American Journal of Human Genetics,* volume 104, pages1233–1240 (2019).

[3] Björn Hellenkamp, Sonja Schmid, Olga Doroshenko, Oleg Opanasyuk, Ralf Kühnemuth, **Soheila Rezaei Adariani**, […]Thorsten Hugel. "Precision and accuracy of single-molecule FRET measurements—a multi-laboratory benchmark study". *Nature Methods,* 2018, 15 (669).

[4] Inna S. Yanez Orozco, Frank A. Mindlin, Junyan Ma, Bo Wang, Brie Levesque, Matheu Spencer, **Soheila Rezaei Adariani**, George Hamilton, Feng Ding, Mark E. Bowen & Hugo Sanabria . "Identifying weak interdomain interactions that stabilize the supertertiary structure of the N-terminal tandem PDZ domains of PSD-95". *Nature Communications,* 2018, 9 (3724).

[5] Junyan Ma, Inna S.Yanez-Orozco, **Soheila Rezaei Adariani**, Drew Dolino, Vasanthi Jayaraman, and Hugo Sanabria . "High Precision FRET at Single-molecule Level for Biomolecule Structure Determination". *Journal of Visualized Experiments*, 2017, 13 (123).

[6] Drew M Dolino*, **Soheila Rezaei Adariani*,** Sana A Shaikh, Vasanthi Jayaraman, Hugo Sanabria. "Conformational selection and submillisecond dynamics of the ligand-binding domain of the N-methyl-DAspartate receptor". *equal contribution. Journal of Biological Chemistry 2016, 291(31).

[7] Amir Abdolmaleki, **Soheila Rezaei Adariani**. "Copper-cationic salphen catalysts for the oxidation of cyclohexene by oxygen". *Catalysis Communications*, 2015, 59 (97).

Review

[8] **Soheila Rezaei Adariani**, Marcel Buchholzer, Mohammad Akbarzadeh, Saeideh Nakhaei-Rad, Radovan Dvorsky, Mohammad Reza Ahmadian. "Structural snapshots of RAF kinase interactions", *Biochemical Society Transactions,* 2018, 46 (6): 1393-1406.

[9] Saeideh Nakhaei-Rad, Fereshteh Haghighi, Parivash Nouri, **Soheila Rezaei Adariani**, Jana Lissy, Neda S. Kazemein Jasemi, Radovan Dvorsky & Mohammad Reza Ahmadian. "Structural fingerprints, interactions, and signaling networks of RAS family proteins beyond RAS isoforms", *Biochemistry and Molecular Biology*, 2018, 53(2):1-27.

In preparation

[10] Badri Nath Dubey, Ehsan Amin, **Soheila Rezaei Adariani** , Radovan Dvorsky, Lothar Gremer, Jens M. Moll, Martin Wolff, Yan Nie, Melissa Graewert, Britta Tschapek, Ingrid R. Vetter, Lutz Schmitt, Dmitri Svergun, Stefan Raunser, Georg Groth, Luitgard Nagel-Steger, and Mohammad R. Ahmadian. "Structural and functional insights into the p160 Rho-associated coiled-coil-containing protein kinase".

[11] Ehsan Ami, Bardi Nath Dubey, **Soheila Rezaei Adariani**, Radovan Dvorsky, Jens M. Moll, and Mohammad R. Ahmadian. "Molecular mechanism of PKN-RHO proteins".

[12] Silke Pudewell, Jana Lissy,' Hossein Nakhaeizadeh, Mohammed Akbarzadeh, Silke Pudewell, **Soheila Rezaei Adariani** […], Mohammad Reza Ahmadian "Embryonic stem cell-expressed Ras (E-Ras) interacts with Arginase-1 and enhances its activity in quiescent hepatic stellate cells".

[13] Silke Pudewell, Jana Lissy,' Hossein Nakhaeizadeh, **Soheila Rezaei Adariani** , Radovan Dvorsky, Mohammad Reza Ahmadian "Interaction of ERAS and SIN1 promoting signaling towards mTORC2".

[14] Katherina Hemmen, Dmitro Rodnin, Daniel Rohrbeck, **Soheila Rezaei Adariani**, Hugo Sanabria, Claus A.M. Seidel. "Deciphering Folding Pathways of Phage T4 Lysozyme: Influence of Multiple Conformations".

➢ References

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Declaration

I hereby declare, Soheila Rezaei Adariani, that my thesis is presentation of original research performed according to the "Principles of Good Scientific Practice" set out by Heinrich-Heine University of Düsseldorf. This thesis does not contain the data already published in public domain and it has never been submitted or accepted by any other institutions for the award of any other degrees.

Signed: Soheila Rezaei Adariani

Eidesstattliche Erklärung

Hiermit erkläre ich, Soheila Rezaei Adariani, an Eides statt, dass ich die hier vorgelegte Dissertation eigenständig. Es wurden keinerlei andere Quellen und Hilfsmittel, außer den angegebenen, benutzt. Zitate aus anderen Arbeiten wurden kenntlich gemacht. Diese Dissertation wurde in der vorgelegten oder einer ähnlichen Form noch bei keiner anderen Institution eingereicht und es wurden bisher keine erfolglosen Promotionsversuche von mir unternommen.

Signed: Soheila Rezaei Adariani

Reference

Abankwa, D., Gorfe, A. A., & Hancock, J. F. (2007). *Ras nanoclusters: molecular structure and assembly.* Paper presented at the Seminars in cell & developmental biology.

Abankwa, D., Gorfe, A. A., Inder, K., & Hancock, J. F. (2010). Ras membrane orientation and nanodomain localization generate isoform diversity. *Proceedings of the National Academy of Sciences, 107*(3), 1130-1135.

Ahearn, I. M., Haigis, K., Bar-Sagi, D., & Philips, M. R. (2012). Regulating the regulator: post-translational modification of RAS. *Nature reviews Molecular cell biology, 13*(1), 39.

Ahmadian, M. R., Zor, T., Vogt, D., Kabsch, W., Selinger, Z., Wittinghofer, A., & Scheffzek, K. (1999). Guanosine triphosphatase stimulation of oncogenic Ras mutants. *Proceedings of the National Academy of Sciences, 96*(12), 7065-7070.

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., & Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Current Biology, 7*(4), 261-269.

Alessi, D. R., Saito, Y., Campbell, D. G., Cohen, P., Sithanandam, G., Rapp, U., . . . Cowley, S. (1994). Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. *EMBO J, 13*(7), 1610-1619.

Allanson, J. E., Annerén, G., Aoki, Y., Armour, C. M., Bondeson, M. L., Cave, H., . . . Sol-Church, K. (2011). *Cardio-facio-cutaneous syndrome: Does genotype predict phenotype?* Paper presented at the American Journal of Medical Genetics Part C: Seminars in Medical Genetics.

Amano, M., Fukata, Y., & Kaibuchi, K. (2000). Regulation and functions of Rho-associated kinase. *Experimental cell research, 261*(1), 44-51.

Amin, E., Dubey, B. N., Zhang, S.-C., Gremer, L., Dvorsky, R., Moll, J. M., . . . Somlyo, A. V. (2013). Rho-kinase: regulation,(dys) function, and inhibition. *Biological chemistry, 394*(11), 1399-1410.

An, S., Yang, Y., Ward, R., Liu, Y., Guo, X.-X., & Xu, T.-R. (2015). A-Raf: a new star of the family of raf kinases. *Critical reviews in biochemistry and molecular biology, 50*(6), 520-531.

An, S., Yang, Y., Ward, R., Liu, Y., Guo, X. X., & Xu, T. R. (2015). A-Raf: A new star of the family of raf kinases. *Crit Rev Biochem Mol Biol, 50*(6), 520-531. doi:10.3109/10409238.2015.1102858

Anderson, D. H. (2006). Role of lipids in the MAPK signaling pathway. *Prog Lipid Res, 45*(2), 102-119. doi:10.1016/j.plipres.2005.12.003

Aspenström, P., Fransson, Å., & Saras, J. (2004). Rho GTPases have diverse effects on the organization of the actin filament system. *Biochemical Journal, 377*(2), 327-337.

Avruch, J., Khokhlatchev, A., Kyriakis, J. M., Luo, Z., Tzivion, G., Vavvas, D., & Zhang, X. F. (2001). Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade. *Recent Prog Horm Res, 56*, 127-155.

Avruch, J., Xavier, R., Bardeesy, N., Zhang, X.-f., Praskova, M., Zhou, D., & Xia, F. (2009). Rassf family of tumor suppressor polypeptides. *Journal of Biological Chemistry, 284*(17), 11001-11005.

Baljuls, A., Kholodenko, B. N., & Kolch, W. (2013). It takes two to tango--signalling by dimeric Raf kinases. *Mol Biosyst, 9*(4), 551-558. doi:10.1039/c2mb25393c

Baljuls, A., Kholodenko, B. N., & Kolch, W. (2013). It takes two to tango–signalling by dimeric Raf kinases. *Molecular bioSystems, 9*(4), 551-558.

Banerjee, A., Jang, H., Nussinov, R., & Gaponenko, V. (2016). The disordered hypervariable region and the folded catalytic domain of oncogenic K-Ras4B partner in phospholipid binding. *Current opinion in structural biology, 36*, 10-17.

Barnoud, T., Schmidt, M. L., Donninger, H., & Clark, G. J. (2017). The role of the NORE1A tumor suppressor in Oncogene-Induced Senescence. *Cancer letters, 400*, 30-36.

Bauer, B., Mirey, G., Vetter, I. R., García-Ranea, J. A., Valencia, A., Wittinghofer, A., . . . Cool, R. H. (1999). Effector recognition by the small GTP-binding proteins Ras and Ral. *Journal of Biological Chemistry, 274*(25), 17763-17770.

Benetka, W., Koranda, M., Maurer-Stroh, S., Pittner, F., & Eisenhaber, F. (2006). Farnesylation or geranylgeranylation? Efficient assays for testing protein prenylation in vitro and in vivo. *BMC biochemistry, 7*(1), 6.

Beranger, F., & Tavitian, A. (1991). Post-translational processing and subcellular localization of the Ras-related Rap2 protein. *Oncogene, 6*(10), 1835-1842.

Berndt, M., Abrasonis, G., Kovács, G. J., Krause, M., Munnik, F., Heller, R., . . . Möller, W. (2011). Bulk diffusion induced structural modifications of carbon-transition metal nanocomposite films. *Journal of Applied Physics, 109*(6), 063503.

Bhagatji, P., Leventis, R., Rich, R., Lin, C.-j., & Silvius, J. R. (2010). Multiple cellular proteins modulate the dynamics of K-ras association with the plasma membrane. *Biophysical journal, 99*(10), 3327-3335.

Birnboim, H. (1992). [16] Extraction of high molecular weight RNA and DNA from cultured mammalian cells *Methods in enzymology* (Vol. 216, pp. 154-160): Elsevier.

Bishop, A. L., & Alan, H. (2000). Rho GTPases and their effector proteins. *Biochemical Journal, 348*(2), 241-255.

Bitra, A., Sistla, S., Mariam, J., Malvi, H., & Anand, R. (2017). Rassf proteins as modulators of Mst1 kinase activity. *Scientific Reports, 7*, 45020.

Blazevits, O., Mideksa, Y. G., Solman, M., Ligabue, A., Ariotti, N., Nakhaeizadeh, H., . . . Abankwa, D. (2016). Galectin-1 dimers can scaffold Raf-effectors to increase H-ras nanoclustering. *Sci Rep, 6*, 24165. doi:10.1038/srep24165

Blumenstein, L., & Ahmadian, M. R. (2004). Models of the cooperative mechanism for Rho effector recognition implications for RhoA-mediated effector activation. *Journal of Biological Chemistry, 279*(51), 53419-53426.

Bourne, H. R., Sanders, D. A., & McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature, 349*(6305), 117.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry, 72*(1-2), 248-254.

Bunney, T. D., Harris, R., Gandarillas, N. L., Josephs, M. B., Roe, S. M., Sorli, S. C., . . . Ponting, C. P. (2006). Structural and mechanistic insights into ras association domains of phospholipase C epsilon. *Molecular cell, 21*(4), 495-507.

Capri, Y., Flex, E., Krumbach, O. H., Carpentieri, G., Cecchetti, S., Lißewski, C., . . . Piard, J. (2019). Activating mutations of RRAS2 are a rare cause of Noonan syndrome. *The American Journal of Human Genetics, 104*(6), 1223-1232.

Castellano, E., & Downward, J. (2011). RAS interaction with PI3K: more than just another effector pathway. *Genes & cancer, 2*(3), 261-274.

Chan, J. J., Flatters, D., Rodrigues-Lima, F., Yan, J., Thalassinos, K., & Katan, M. (2013). Comparative analysis of interactions of RASSF1-10. *Advances in biological regulation, 53*(2), 190-201.

Chavan, T. S., Muratcioglu, S., Marszalek, R., Jang, H., Keskin, O., Gursoy, A., . . . Gaponenko, V. (2015). Plasma membrane regulates Ras signaling networks. *Cell Logist, 5*(4), e1136374. doi:10.1080/21592799.2015.1136374

Chen, X.-q., Tan, I., Ng, C. H., Hall, C., Lim, L., & Leung, T. (2002). Characterization of RhoA-binding kinase ROKα implication of the pleckstrin homology domain in ROKα function using region-specific antibodies. *Journal of Biological Chemistry, 277*(15), 12680-12688.

Cherfils, J., & Zeghouf, M. (2013). Regulation of small gtpases by gefs, gaps, and gdis. *Physiological reviews, 93*(1), 269-309.

Chung, C., Niemela, S. L., & Miller, R. H. (1989). One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. *Proceedings of the National Academy of Sciences, 86*(7), 2172-2175.

Cirstea, I. C., Kutsche, K., Dvorsky, R., Gremer, L., Carta, C., Horn, D., . . . König, R. (2010). A restricted spectrum of NRAS mutations causes Noonan syndrome. *Nature genetics, 42*(1), 27.

Compton, S. J., & Jones, C. G. (1985). Mechanism of dye response and interference in the Bradford protein assay. *Analytical biochemistry, 151*(2), 369-374.

Couzens, A. L., Saridakis, V., & Scheid, M. P. (2009). The hydrophobic motif of ROCK2 requires association with the N-terminal extension for kinase activity. *Biochemical Journal, 419*(1), 141-148.

Cox, A. D., & Der, C. J. (2003). The dark side of Ras: regulation of apoptosis. *Oncogene, 22*(56), 8999.

Cox, A. D., Der, C. J., & Philips, M. R. (2015). Targeting RAS membrane association: back to the future for anti-RAS drug discovery? : AACR.

Craft Jr, J. W., Zhang, H., Charendoff, M. N., Mindrebo, J. T., Schwartz, R. J., & Briggs, J. M. (2013). Associations between the Rho kinase-1 catalytic and PH domain regulatory unit. *Journal of Molecular Graphics and Modelling, 46*, 74-82.

Cseh, B., Doma, E., & Baccarini, M. (2014). "RAF" neighborhood: protein-protein interaction in the Raf/Mek/Erk pathway. *FEBS Lett, 588*(15), 2398-2406. doi:10.1016/j.febslet.2014.06.025

Dallol, A., Hesson, L. B., Matallanas, D., Cooper, W. N., O'Neill, E., Maher, E. R., . . . Latif, F. (2009). RAN GTPase is a RASSF1A effector involved in controlling microtubule organization. *Current Biology, 19*(14), 1227-1232.

De Noronha, C., & Mullins, J. I. (1992). Amplimers with 3'-terminal phosphorothioate linkages resist degradation by vent polymerase and reduce Taq polymerase mispriming. *Genome Research, 2*(2), 131-136.

DeLano, W. L. (2002). The PyMOL molecular graphics system. *http://www. pymol. org*.

Desideri, E., Cavallo, A. L., & Baccarini, M. (2015). Alike but Different: RAF Paralogs and Their Signaling Outputs. *Cell, 161*(5), 967-970. doi:10.1016/j.cell.2015.04.045

Dharmaiah, S., Bindu, L., Tran, T. H., Gillette, W. K., Frank, P. H., Ghirlando, R., . . . Stephen, A. G. (2016). Structural basis of recognition of farnesylated and methylated KRAS4b by PDEδ. *Proceedings of the National Academy of Sciences, 113*(44), E6766-E6775.

Dhillon, A. S., Hagan, S., Rath, O., & Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene, 26*(22), 3279.

Dhillon, A. S., Meikle, S., Yazici, Z., Eulitz, M., & Kolch, W. (2002). Regulation of Raf-1 activation and signalling by dephosphorylation. *The EMBO journal, 21*(1-2), 64-71.

Dhillon, A. S., Pollock, C., Steen, H., Shaw, P. E., Mischak, H., & Kolch, W. (2002). Cyclic AMP-dependent kinase regulates Raf-1 kinase mainly by phosphorylation of serine 259. *Molecular and cellular biology, 22*(10), 3237-3246.

Donninger, H., Schmidt, M. L., Mezzanotte, J., Barnoud, T., & Clark, G. J. (2016). *Ras signaling through RASSF proteins.* Paper presented at the Seminars in cell & developmental biology.

Doran, J. D., Xun, L., Taslimi, P., Saadat, A., & Ted, F. (2004). New insights into the structure–function relationships of Rho-associated kinase: a thermodynamic and hydrodynamic study of the dimer-to-monomer transition and its kinetic implications. *Biochemical Journal, 384*(2), 255-262.

Dovas, A., & Couchman, J. R. (2005). RhoGDI: multiple functions in the regulation of Rho family GTPase activities. *Biochemical Journal, 390*(1), 1-9.

Downward, J. (2003). Targeting RAS signalling pathways in cancer therapy. *Nature Reviews Cancer, 3*(1), 11.

Dumaz, N., & Marais, R. (2003). Protein kinase A blocks Raf-1 activity by stimulating 14-3-3 binding and blocking Raf-1 interaction with Ras. *Journal of Biological Chemistry, 278*(32), 29819-29823.

Dvorsky, R., Blumenstein, L., Vetter, I. R., & Ahmadian, M. R. (2004). Structural insights into the interaction of ROCKI with the switch regions of RhoA. *Journal of Biological Chemistry, 279*(8), 7098-7104.

Eaton, S. L., Hurtado, M. L., Oldknow, K. J., Graham, L. C., Marchant, T. W., Gillingwater, T. H., . . . Wishart, T. M. (2014). A guide to modern quantitative fluorescent western blotting with troubleshooting strategies. *JoVE (Journal of Visualized Experiments)*(93), e52099.

Eckert, L. B., Repasky, G. A., Ülkü, A. S., McFall, A., Zhou, H., Sartor, C. I., & Der, C. J. (2004). Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. *Cancer research, 64*(13), 4585-4592.

Ehresman, B. P. lmbault and I. H. Weil. 1973. Spectrophotometric determination of protein concentration in cell extracts containing tRNA's and rRNA's. *Anal. Bio-&em, 54*, 454.

Erijman, A., & M Shifman, J. (2016). RAS/effector interactions from structural and biophysical perspective. *Mini reviews in medicinal chemistry, 16*(5), 370-375.

Etienne-Manneville, S., & Hall, A. (2002). Rho GTPases in cell biology. *Nature, 420*(6916), 629.

Feig, L. A. (1999). Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases. *Nature cell biology, 1*(2), E25.

Ferraiuolo, M., Verduci, L., Blandino, G., & Strano, S. (2017). Mutant p53 protein and the Hippo transducers YAP and TAZ: a critical oncogenic node in human cancers. *International journal of molecular sciences, 18*(5), 961.

Ferro, E., & Trabalzini, L. (2010). RalGDS family members couple Ras to Ral signalling and that's not all. *Cellular signalling, 22*(12), 1804-1810.

Flynn, P., Mellor, H., Palmer, R., Panayotou, G., & Parker, P. J. (1998). Multiple interactions of PRK1 with RhoA Functional assignment of the Hr1 repeat motif. *Journal of Biological Chemistry, 273*(5), 2698-2705.

Franke, T. F., Kaplan, D. R., & Cantley, L. C. (1997). PI3K: downstream AKTion blocks apoptosis. *Cell, 88*(4), 435-437.

Freeman, A. K., Ritt, D. A., & Morrison, D. K. (2013). The importance of Raf dimerization in cell signaling. *Small GTPases, 4*(3), 180-185.

Frémin, C., Guégan, J., Plutoni, C., Mahaffey, J., Philips, M., Emery, G., & Meloche, S. (2016). ERK1/2-induced phosphorylation of R-Ras GTPases stimulates their oncogenic potential. *Oncogene, 35*(43), 5692.

Gampel, A., Parker, P. J., & Mellor, H. (1999). Regulation of epidermal growth factor receptor traffic by the small GTPase rhoB. *Current Biology, 9*(17), 955-958.

Gentry, L. R. (2015). *Targeting the Ras-Ral effector pathway for cancer treatment.* The University of North Carolina at Chapel Hill.

Gentry, L. R., Martin, T. D., Reiner, D. J., & Der, C. J. (2014). Ral small GTPase signaling and oncogenesis: more than just 15 minutes of fame. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1843*(12), 2976-2988.

Goddard, T. D., Huang, C. C., Meng, E. C., Pettersen, E. F., Couch, G. S., Morris, J. H., & Ferrin, T. E. (2018). UCSF ChimeraX: Meeting modern challenges in visualization and analysis. *Protein Science, 27*(1), 14-25.

Govek, E.-E., Newey, S. E., & Van Aelst, L. (2005). The role of the Rho GTPases in neuronal development. *Genes & development, 19*(1), 1-49.

Gremer, L., Merbitz-Zahradnik, T., Dvorsky, R., Cirstea, I. C., Kratz, C. P., Zenker, M., . . . Ahmadian, M. R. (2011). Germline KRAS mutations cause aberrant biochemical and physical properties leading to developmental disorders. *Human mutation, 32*(1), 33-43.

Guil, S., Gattoni, R., Carrascal, M., Abián, J., Stévenin, J., & Bach-Elias, M. (2003). Roles of hnRNP A1, SR proteins, and p68 helicase in cH-ras alternative splicing regulation. *Molecular and cellular biology, 23*(8), 2927-2941.

Gutierrez-Erlandsson, S., Herrero-Vidal, P., Fernandez-Alfara, M., Hernandez-Garcia, S., Gonzalo-Flores, S., Mudarra-Rubio, A., . . . Cubelos, B. (2013). R-RAS2 overexpression in tumors of the human central nervous system. *Molecular cancer, 12*(1), 127.

Hagerty, L., Weitzel, D. H., Chambers, J., Fortner, C. N., Brush, M. H., Loiselle, D., . . . Haystead, T. A. (2007). ROCK1 phosphorylates and activates zipper-interacting protein kinase. *Journal of Biological Chemistry, 282*(7), 4884-4893.

Hall, T. A. (1999). *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.* Paper presented at the Nucleic acids symposium series.

Hancock, J. F., Magee, A. I., Childs, J. E., & Marshall, C. J. (1989). All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell, 57*(7), 1167-1177.

Hanzal-Bayer, M. F., & Hancock, J. F. (2007). Lipid rafts and membrane traffic. *FEBS letters, 581*(11), 2098-2104.

Hartmann, S., Ridley, A. J., & Lutz, S. (2015). The function of Rho-associated kinases ROCK1 and ROCK2 in the pathogenesis of cardiovascular disease. *Frontiers in pharmacology, 6*, 276.

Harvey, J. (1964). An unidentified virus which causes the rapid production of tumours in mice. *Nature, 204*(4963), 1104.

Hashimoto, Y., Zhang, S., & Blissard, G. W. (2010). Ao38, a new cell line from eggs of the black witch moth, Ascalapha odorata (Lepidoptera: Noctuidae), is permissive for AcMNPV infection and produces high levels of recombinant proteins. *BMC biotechnology, 10*(1), 50.

Hashimoto, Y., Zhang, S., Zhang, S., Chen, Y.-R., & Blissard, G. W. (2012). Erratum to: BTI-Tnao38, a new cell line derived from Trichoplusia ni, is permissive for AcMNPV infection and produces high levels of recombinant proteins. *BMC biotechnology, 12*(1), 12.

Hedman, A. C., Smith, J. M., & Sacks, D. B. (2015). The biology of IQGAP proteins: beyond the cytoskeleton. *EMBO reports, 16*(4), 427-446.

Hennig, A., Markwart, R., Esparza-Franco, M. A., Ladds, G., & Rubio, I. (2015). Ras activation revisited: role of GEF and GAP systems. *Biological chemistry, 396*(8), 831-848.

Herrmann, C. (2003). Ras–effector interactions: after one decade. *Current opinion in structural biology, 13*(1), 122-129.

Hers, I., Vincent, E. E., & Tavaré, J. M. (2011). Akt signalling in health and disease. *Cellular signalling, 23*(10), 1515-1527.

Hobbs, G. A., Der, C. J., & Rossman, K. L. (2016). RAS isoforms and mutations in cancer at a glance. *J cell Sci, 129*(7), 1287-1292.

Hudson, C. A., Heesom, K. J., & López Bernal, A. (2011). Phasic contractions of isolated human myometrium are associated with Rho-kinase (ROCK)-dependent phosphorylation of myosin phosphatase-targeting subunit (MYPT1). *Molecular human reproduction, 18*(5), 265-279.

Hutchinson, C. L., Lowe, P. N., McLaughlin, S. H., Mott, H. R., & Owen, D. (2011). Mutational analysis reveals a single binding interface between RhoA and its effector, PRK1. *Biochemistry, 50*(14), 2860-2869.

Inoki, K., Li, Y., Zhu, T., Wu, J., & Guan, K.-L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nature cell biology, 4*(9), 648.

Jacobs, M., Hayakawa, K., Swenson, L., Bellon, S., Fleming, M., Taslimi, P., & Doran, J. (2006). The structure of dimeric ROCK I reveals the mechanism for ligand selectivity. *Journal of Biological Chemistry, 281*(1), 260-268.

Jaffe, A. B., & Hall, A. (2005). Rho GTPases: biochemistry and biology. *Annu. Rev. Cell Dev. Biol., 21*, 247-269.

Jaiswal, M., Fansa, E. K., Dvorsky, R., & Ahmadian, M. R. (2013). New insight into the molecular switch mechanism of human Rho family proteins: shifting a paradigm. *Biological chemistry, 394*(1), 89-95.

Jeon, C. Y., Moon, M. Y., Kim, J. H., Kim, H. J., Kim, J. G., Li, Y., . . . Meier, K. E. (2012). Control of neurite outgrowth by RhoA inactivation. *Journal of neurochemistry, 120*(5), 684-698.

John, J., Sohmen, R., Feuerstein, J., Linke, R., Wittinghofer, A., & Goody, R. S. (1990). Kinetics of interaction of nucleotides with nucleotide-free H-ras p21. *Biochemistry, 29*(25), 6058-6065.

Julian, L., & Olson, M. F. (2014). Rho-associated coiled-coil containing kinases (ROCK) structure, regulation, and functions. *Small GTPases, 5*(2), e29846.

Karnoub, A. E., & Weinberg, R. A. (2008). Ras oncogenes: split personalities. *Nature reviews Molecular cell biology, 9*(7), 517.

Katagiri, K., Maeda, A., Shimonaka, M., & Kinashi, T. (2003). RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nature immunology, 4*(8), 741.

Katz, M. E., & McCormick, F. (1997). Signal transduction from multiple Ras effectors. *Current opinion in genetics & development, 7*(1), 75-79.

Keeton, A. B., Salter, E. A., & Piazza, G. A. (2017). The RAS–effector interaction as a drug target. *Cancer research, 77*(2), 221-226.

Kelley, G. G., Reks, S. E., Ondrako, J. M., & Smrcka, A. V. (2001). Phospholipase Cε: a novel Ras effector. *The EMBO journal, 20*(4), 743-754.

Khromov, A., Choudhury, N., Stevenson, A. S., Somlyo, A. V., & Eto, M. (2009). Phosphorylation-dependent autoinhibition of myosin light chain phosphatase

accounts for Ca2+ sensitization force of smooth muscle contraction. *Journal of Biological Chemistry, 284*(32), 21569-21579.

Kiel, C., Wohlgemuth, S., Rousseau, F., Schymkowitz, J., Ferkinghoff-Borg, J., Wittinghofer, F., & Serrano, L. (2005). Recognizing and defining true Ras binding domains II: in silico prediction based on homology modelling and energy calculations. *Journal of molecular biology, 348*(3), 759-775.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., . . . Okawa, K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science, 273*(5272), 245-248.

Kitagawa, M., Mukai, H., Takahashi, M., & Ono, Y. (1998). The Role of PKN in the Regulation of αB-Crystallin Expression via Heat Shock Transcription Factor 1. *Biochemical and biophysical research communications, 252*(3), 561-565.

Konstantinopoulos, P. A., Karamouzis, M. V., & Papavassiliou, A. G. (2007). Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. *Nature reviews Drug discovery, 6*(7), 541.

Kontani, K., Tada, M., Ogawa, T., Okai, T., Saito, K., Araki, Y., & Katada, T. (2002). Di-Ras, a distinct subgroup of ras family GTPases with unique biochemical properties. *Journal of Biological Chemistry, 277*(43), 41070-41078.

Kunkel, T. A. (1985). The mutational specificity of DNA polymerase-beta during in vitro DNA synthesis. Production of frameshift, base substitution, and deletion mutations. *Journal of Biological Chemistry, 260*(9), 5787-5796.

Kyriakis, J. M., App, H., Zhang, X.-f., Banerjee, P., Brautigan, D. L., Rapp, U. R., & Avruch, J. (1992). Raf-1 activates MAP kinase-kinase. *Nature, 358*(6385), 417.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680.

Lane, K. T., & Beese, L. S. (2006). Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. *Journal of lipid research, 47*(4), 681.

Larrea, M. D., Wander, S. A., & Slingerland, J. (2009). p27 as Jekyll and Hyde: regulation of cell cycle and cell motility. *Cell cycle, 8*(21), 3455-3461.

Leatherbarrow, R. J. (1990). *GraFit*: Erithacus Software.

Leicht, D. T., Balan, V., Kaplun, A., Singh-Gupta, V., Kaplun, L., Dobson, M., & Tzivion, G. (2007). Raf kinases: function, regulation and role in human cancer. *Biochim Biophys Acta, 1773*(8), 1196-1212. doi:10.1016/j.bbamcr.2007.05.001

Leicht, D. T., Balan, V., Zhu, J., Kaplun, A., Bronisz, A., Rana, A., & Tzivion, G. (2013). MEK-1 activates C-Raf through a Ras-independent mechanism. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1833*(5), 976-986.

Li, W., Melnick, M., & Perrimon, N. (1998). Dual function of Ras in Raf activation. *Development, 125*(24), 4999-5008.

Li, X., Liu, L., Tupper, J. C., Bannerman, D. D., Winn, R. K., Sebti, S. M., . . . Harlan, J. M. (2002). Inhibition of protein geranylgeranylation and RhoA/RhoA kinase pathway induces apoptosis in human endothelial cells. *Journal of Biological Chemistry, 277*(18), 15309-15316.

Li, Z.-L., Prakash, P., & Buck, M. (2018). A "tug of war" maintains a dynamic protein–membrane complex: molecular dynamics simulations of C-Raf RBD-CRD bound to K-Ras4B at an anionic membrane. *ACS central science, 4*(2), 298-305.

Liu, C.-A., Wang, M.-J., Chi, C.-W., Wu, C.-W., & Chen, J.-Y. (2004). Rho/Rhotekin-mediated NF-κB activation confers resistance to apoptosis. *Oncogene, 23*(54), 8731.

Loirand, G., Guérin, P., & Pacaud, P. (2006). Rho kinases in cardiovascular physiology and pathophysiology. *Circulation research, 98*(3), 322-334.

Ludtke, S. J., Baldwin, P. R., & Chiu, W. (1999). EMAN: semiautomated software for high-resolution single-particle reconstructions. *Journal of Structural Biology, 128*(1), 82-97. doi:10.1006/jsbi.1999.4174

Maesaki, R., Ihara, K., Shimizu, T., Kuroda, S., Kaibuchi, K., & Hakoshima, T. (1999). The structural basis of Rho effector recognition revealed by the crystal structure of human RhoA complexed with the effector domain of PKN/PRK1. *Molecular cell, 4*(5), 793-803.

Malumbres, M., & Barbacid, M. (2003). RAS oncogenes: the first 30 years. *Nature Reviews Cancer, 3*(6), 459.

Marais, R., Light, Y., Paterson, H. F., Mason, C. S., & Marshall, C. J. (1997). Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. *Journal of Biological Chemistry, 272*(7), 4378-4383.

Mardilovich, K., Olson, M. F., & Baugh, M. (2012). Targeting Rho GTPase signaling for cancer therapy. *Future oncology, 8*(2), 165-177.

Mark, G. E., MacIntyre, R., Digan, M., Ambrosio, L., & Perrimon, N. (1987). Drosophila melanogaster homologs of the raf oncogene. *Molecular and cellular biology, 7*(6), 2134-2140.

Matallanas, D., Birtwistle, M., Romano, D., Zebisch, A., Rauch, J., von Kriegsheim, A., & Kolch, W. (2011). Raf family kinases: old dogs have learned new tricks. *Genes & cancer, 2*(3), 232-260.

Matsui, T., Yonemura, S., Tsukita, S., & Tsukita, S. (1999). Activation of ERM proteins in vivo by Rho involves phosphatidyl-inositol 4-phosphate 5-kinase and not ROCK kinases. *Current Biology, 9*(21), 1259-S1253.

Matsuzawa, K., Kosako, H., Inagaki, N., Shibata, H., Mukai, H., Ono, Y., . . . Azuma, I. (1997). Domain-specific phosphorylation of vimentin and glial fibrillary acidic protein by PKN. *Biochemical and biophysical research communications, 234*(3), 621-625.

Maurer, G., Tarkowski, B., & Baccarini, M. (2011). Raf kinases in cancer-roles and therapeutic opportunities. *Oncogene, 30*(32), 3477-3488. doi:10.1038/onc.2011.160

Maurer, G., Tarkowski, B., & Baccarini, M. (2011). Raf kinases in cancer–roles and therapeutic opportunities. *Oncogene, 30*(32), 3477.

McDonell, M. W., Simon, M. N., & Studier, F. W. (1977). Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *Journal of molecular biology, 110*(1), 119-146.

Miertzschke, M., Stanley, P., Bunney, T. D., Rodrigues-Lima, F., Hogg, N., & Katan, M. (2007). Characterisation of interactions of adapter protein RAPL/Nore 1B with Rap GTPases and their role in T cell migration. *Journal of Biological Chemistry*.

Misaki, K., Mukai, H., Yoshinaga, C., Oishi, K., Isagawa, T., Takahashi, M., . . . Ono, Y. (2001). PKN delays mitotic timing by inhibition of Cdc25C: possible involvement of PKN in the regulation of cell division. *Proceedings of the National Academy of Sciences, 98*(1), 125-129.

Mohan, S., Das, D., Bauer, R. J., Heroux, A., Zalewski, J. K., Heber, S., . . . VanDemark, A. P. (2013). Structure of a highly conserved domain of Rock1 required for Shroom-mediated regulation of cell morphology. *PloS one, 8*(12), e81075.

Molzan, M., Schumacher, B., Ottmann, C., Baljuls, A., Polzien, L., Weyand, M., . . . Kuhenne, P. (2010). Impaired binding of 14-3-3 to C-RAF in Noonan syndrome suggests new approaches in diseases with increased Ras signaling. *Molecular and cellular biology, 30*(19), 4698-4711.

Moodie, S. A., Willumsen, B. M., Weber, M. J., & Wolfman, A. (1993). Complexes of Ras. GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science, 260*(5114), 1658-1661.

Morgan-Fisher, M., Wewer, U. M., & Yoneda, A. (2013). Regulation of ROCK activity in cancer. *Journal of Histochemistry & Cytochemistry, 61*(3), 185-198.

Morrison, D. (1990). The Raf-1 kinase as a transducer of mitogenic signals. *Cancer cells (Cold Spring Harbor, NY: 1989), 2*(12), 377-382.

Morrison, D. K., & Cutler Jr, R. E. (1997). The complexity of Raf-1 regulation. *Current opinion in cell biology, 9*(2), 174-179.

Moshnikova, A., Frye, J., Shay, J. W., Minna, J. D., & Khokhlatchev, A. V. (2006). The growth and tumor suppressor NORE1A is a cytoskeletal protein that suppresses growth by inhibition of the ERK pathway. *Journal of Biological Chemistry, 281*(12), 8143-8152.

Mott, H. R., & Owen, D. (2015). Structures of Ras superfamily effector complexes: What have we learnt in two decades? *Critical reviews in biochemistry and molecular biology, 50*(2), 85-133.

Mukai, H. (2003). The structure and function of PKN, a protein kinase having a catalytic domain homologous to that of PKC. *The Journal of Biochemistry, 133*(1), 17-27.

Mukai, H., Toshimori, M., Shibata, H., Takanaga, H., Kitagawa, M., Miyahara, M., . . . Ono, Y. (1997). Interaction of PKN with α-actinin. *Journal of Biological Chemistry, 272*(8), 4740-4746.

Muslin, A. J., Tanner, J. W., Allen, P. M., & Shaw, A. S. (1996). Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell, 84*(6), 889-897.

Nakagawa, O., Fujisawa, K., Ishizaki, T., Saito, Y., Nakao, K., & Narumiya, S. (1996). ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS letters, 392*(2), 189-193.

Nakhaei-Rad, S., Haghighi, F., Nouri, P., Rezaei Adariani, S., Lissy, J., Kazemein Jasemi, N. S., . . . Ahmadian, M. R. (2018). Structural fingerprints, interactions, and signaling networks of RAS family proteins beyond RAS isoforms. *Critical reviews in biochemistry and molecular biology, 53*(2), 130-156.

Nakhaei-Rad, S., Nakhaeizadeh, H., Götze, S., Kordes, C., Sawitza, I., Hoffmann, M. J., . . . Piekorz, R. P. (2016). The role of embryonic stem cell-expressed RAS (ERAS) in the maintenance of quiescent hepatic stellate cells. *Journal of Biological Chemistry*, jbc. M115. 700088.

Nakhaei-Rad, S., Nakhaeizadeh, H., Kordes, C., Cirstea, I. C., Schmick, M., Dvorsky, R., . . . Ahmadian, M. R. (2015). The function of embryonic stem cell-expressed Ras (E-Ras), a unique Ras family member, correlates with its additional motifs and its structural properties. *Journal of Biological Chemistry, 290*(25), 15892-15903.

Nakhaeizadeh, H., Amin, E., Nakhaei-Rad, S., Dvorsky, R., & Ahmadian, M. R. (2016). The RAS-effector interface: isoform-specific differences in the effector binding regions. *PloS one, 11*(12), e0167145.

Narumiya, S., Tanji, M., & Ishizaki, T. (2009). Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. *Cancer and Metastasis Reviews, 28*(1-2), 65-76.

Nassar, N., Horn, G., Herrmann, C., Block, C., Janknecht, R., & Wittinghofer, A. (1996). Ras/Rap effector specificity determined by charge reversal. *Nature structural biology, 3*(8), 723.

Neel, N. F., Martin, T. D., Stratford, J. K., Zand, T. P., Reiner, D. J., & Der, C. J. (2011). The RalGEF-Ral effector signaling network: the road less traveled for anti-Ras drug discovery. *Genes & cancer, 2*(3), 275-287.

Nekhoroshkova, E., Albert, S., Becker, M., & Rapp, U. R. (2009). A-RAF kinase functions in ARF6 regulated endocytic membrane traffic. *PLoS One, 4*(2), e4647. doi:10.1371/journal.pone.0004647

Neudauer, C. L., Joberty, G., Tatsis, N., & Macara, I. G. (1998). Distinct cellular effects and interactions of the Rho-family GTPase TC10. *Current Biology, 8*(21), 1151-1161.

Niault, T. S., & Baccarini, M. (2010). Targets of Raf in tumorigenesis. *Carcinogenesis, 31*(7), 1165-1174. doi:10.1093/carcin/bgp337

Niault, T. S., & Baccarini, M. (2010). Targets of Raf in tumorigenesis. *Carcinogenesis, 31*(7), 1165-1174.

Nie, Z., Hirsch, D. S., & Randazzo, P. A. (2003). Arf and its many interactors. *Current opinion in cell biology, 15*(4), 396-404.

Nobes, C. D., & Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. *The Journal of cell biology, 144*(6), 1235-1244.

Nussinov, R., Tsai, C.-J., Chakrabarti, M., & Jang, H. (2016). A new view of Ras isoforms in cancers. *Cancer research, 76*(1), 18-23.

Olson, M. F. (2008). Applications for ROCK kinase inhibition. *Current opinion in cell biology, 20*(2), 242-248.

Omerovic, J., & Prior, I. A. (2009). Compartmentalized signalling: Ras proteins and signalling nanoclusters. *The FEBS journal, 276*(7), 1817-1825.

Ono, Y., & Mukai, H. (2002). PKN Protein Kinase. *Wiley Encyclopedia of Molecular Medicine*.

Osborne, J. K., Zaganjor, E., & Cobb, M. H. (2012). Signal control through Raf: in sickness and in health. *Cell Res, 22*(1), 14-22. doi:10.1038/cr.2011.193

Owen, D., Lowe, P. N., Nietlispach, D., Brosnan, C. E., Chirgadze, D. Y., Parker, P. J., . . . Mott, H. R. (2003). Molecular dissection of the interaction between the small G

proteins Rac1 and RhoA and protein kinase C-related kinase 1 (PRK1). *Journal of Biological Chemistry, 278*(50), 50578-50587.

Paduch, M., Jeleñ, F., & Otlewski, J. (2001). Structure of small G proteins and their regulators. *ACTA BIOCHIMICA POLONICA-ENGLISH EDITION-, 48*(4), 829-850.

Palmer, R. H., Ridden, J., & Parker, P. J. (1995). Cloning and expression patterns of two members of a novel protein-kinase-C-related kinase family. *European journal of biochemistry, 227*(1-2), 344-351.

Pandit, B., Sarkozy, A., Pennacchio, L. A., Carta, C., Oishi, K., Martinelli, S., . . . Gelb, B. D. (2007). Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. *Nat Genet, 39*(8), 1007-1012. doi:10.1038/ng2073

Parmar, N., & Tamanoi, F. (2010). Rheb G-Proteins and the Activation of mTORC1 *The Enzymes* (Vol. 27, pp. 39-56): Elsevier.

Patel, M., & Côté, J.-F. (2013). Ras GTPases' interaction with effector domains: breaking the families' barrier. *Communicative & integrative biology, 6*(4), e24298.

Pfeifer, G. P., Dammann, R., & Tommasi, S. (2010). RASSF proteins. *Current Biology, 20*(8), R344-R345.

Piekny, A., Werner, M., & Glotzer, M. (2005). Cytokinesis: welcome to the Rho zone. *Trends in cell biology, 15*(12), 651-658.

Praskova, M., Khoklatchev, A., Ortiz-Vega, S., & Avruch, J. (2004). Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. *Biochemical Journal, 381*(2), 453-462.

Raftopoulou, M., & Hall, A. (2004). Cell migration: Rho GTPases lead the way. *Developmental biology, 265*(1), 23-32.

Rajalingam, K., Schreck, R., Rapp, U. R., & Albert, Š. (2007). Ras oncogenes and their downstream targets. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1773*(8), 1177-1195.

Rauch, N., Rukhlenko, O. S., Kolch, W., & Kholodenko, B. N. (2016). MAPK kinase signalling dynamics regulate cell fate decisions and drug resistance. *Current opinion in structural biology, 41*, 151-158.

Razzaque, M. A., Nishizawa, T., Komoike, Y., Yagi, H., Furutani, M., Amo, R., . . . Matsuoka, R. (2007). Germline gain-of-function mutations in RAF1 cause Noonan syndrome. *Nat Genet, 39*(8), 1013-1017. doi:10.1038/ng2078

Repasky, G. A., Chenette, E. J., & Der, C. J. (2004). Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis? *Trends in cell biology, 14*(11), 639-647.

Rezaei Adariani, S., Buchholzer, M., Akbarzadeh, M., Nakhaei-Rad, S., Dvorsky, R., & Ahmadian, M. R. (2018). Structural snapshots of RAF kinase interactions. *Biochem Soc Trans, 46*(6), 1393-1406. doi:10.1042/bst20170528

Ridley, A. J. (2001). Rho family proteins: coordinating cell responses. *Trends in cell biology, 11*(12), 471-477.

Riento, K., & Ridley, A. J. (2006). Inhibition of ROCK by RhoE. *Methods in enzymology, 406*, 533-541.

Roberts, A. E., Allanson, J. E., Tartaglia, M., & Gelb, B. D. (2013). Noonan syndrome. *The Lancet, 381*(9863), 333-342.

Roberts, P. J., & Der, C. J. (2007). Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene, 26*(22), 3291.

Rodriguez-Viciana, P., Oses-Prieto, J., Burlingame, A., Fried, M., & McCormick, F. (2006). A phosphatase holoenzyme comprised of Shoc2/Sur8 and the catalytic subunit of PP1 functions as an M-Ras effector to modulate Raf activity. *Molecular cell, 22*(2), 217-230.

Rodriguez-Viciana, P., Sabatier, C., & McCormick, F. (2004). Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. *Molecular and cellular biology, 24*(11), 4943-4954.

Rojas, A. M., Fuentes, G., Rausell, A., & Valencia, A. (2012). The Ras protein superfamily: evolutionary tree and role of conserved amino acids. *J cell Biol, 196*(2), 189-201.

Rommel, C., Clarke, B. A., Zimmermann, S., Nuñez, L., Rossman, R., Reid, K., . . . Glass, D. J. (1999). Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science, 286*(5445), 1738-1741.

Roring, M., & Brummer, T. (2012). Aberrant B-Raf signaling in human cancer– 10 years from bench to bedside. *Critical Reviews™ in Oncogenesis, 17*(1).

Roring, M., & Brummer, T. (2012). Aberrant B-Raf signaling in human cancer -- 10 years from bench to bedside. *Crit Rev Oncog, 17*(1), 97-121.

Roskoski, R., Jr. (2012). MEK1/2 dual-specificity protein kinases: structure and regulation. *Biochem Biophys Res Commun, 417*(1), 5-10. doi:10.1016/j.bbrc.2011.11.145

Rushworth, L. K., Hindley, A. D., O'Neill, E., & Kolch, W. (2006). Regulation and role of Raf-1/B-Raf heterodimerization. *Molecular and cellular biology, 26*(6), 2262-2272.

Rushworth, L. K., Hindley, A. D., O'Neill, E., & Kolch, W. (2006). Regulation and role of Raf-1/B-Raf heterodimerization. *Mol Cell Biol, 26*(6), 2262-2272. doi:10.1128/MCB.26.6.2262-2272.2006

Sanchez-Sanz, G., Tywoniuk, B., Matallanas, D., Romano, D., Nguyen, L. K., Kholodenko, B. N., . . . Buchete, N. V. (2016). SARAH Domain-Mediated MST2-RASSF Dimeric Interactions. *PLoS Comput Biol, 12*(10), e1005051. doi:10.1371/journal.pcbi.1005051

Sanges, C., Scheuermann, C., Zahedi, R., Sickmann, A., Lamberti, A., Migliaccio, N., . . . Reinders, J. (2012). Raf kinases mediate the phosphorylation of eukaryotic translation elongation factor 1A and regulate its stability in eukaryotic cells. *Cell death & disease, 3*(3), e276.

Saraste, M., Sibbald, P. R., & Wittinghofer, A. (1990). The P-loop—a common motif in ATP-and GTP-binding proteins. *Trends in biochemical sciences, 15*(11), 430-434.

Satoh, K., Fukumoto, Y., & Shimokawa, H. (2011). Rho-kinase: important new therapeutic target in cardiovascular diseases. *American Journal of Physiology-Heart and Circulatory Physiology, 301*(2), H287-H296.

Schmick, M., Vartak, N., Papke, B., Kovacevic, M., Truxius, D. C., Rossmannek, L., & Bastiaens, P. I. (2014). KRas localizes to the plasma membrane by spatial cycles of solubilization, trapping and vesicular transport. *Cell, 157*(2), 459-471.

Schmidt, G., Lenzen, C., Simon, I., Deuter, R., Cool, R. H., Goody, R. S., & Wittinghofer, A. (1996). Biochemical and biological consequences of changing the specificity of p21ras from guanosine to xanthosine nucleotides. *Oncogene, 12*(1), 87-96.

Schofield, A. V., & Bernard, O. (2013). Rho-associated coiled-coil kinase (ROCK) signaling and disease. *Critical reviews in biochemistry and molecular biology, 48*(4), 301-316.

Schofield, A. V., Gamell, C., Suryadinata, R., Sarcevic, B., & Bernard, O. (2013). Tubulin polymerization promoting protein 1 (Tppp1) phosphorylation by Rho-associated coiled-coil kinase (rock) and cyclin-dependent kinase 1 (Cdk1) inhibits microtubule dynamics to increase cell proliferation. *Journal of Biological Chemistry, 288*(11), 7907-7917.

Schroeder, H., Leventis, R., Rex, S., Schelhaas, M., Nägele, E., Waldmann, H., & Silvius, J. R. (1997). S-Acylation and plasma membrane targeting of the farnesylated carboxyl-terminal peptide of N-ras in mammalian fibroblasts. *Biochemistry, 36*(42), 13102-13109.

Scrima, A., Thomas, C., Deaconescu, D., & Wittinghofer, A. (2008). The Rap–RapGAP complex: GTP hydrolysis without catalytic glutamine and arginine residues. *The EMBO journal, 27*(7), 1145-1153.

Sendoh, H., Hu, C.-D., Wu, D., Song, C., Yamawaki-Kataoka, Y., Kotani, J., . . . Kataoka, T. (2000). Role of Raf-1 conserved region 2 in regulation of Ras-dependent Raf-1 activation. *Biochemical and biophysical research communications, 271*(3), 596-602.

Shao, H., Kadono-Okuda, K., Finlin, B. S., & Andres, D. A. (1999). Biochemical characterization of the Ras-related GTPases Rit and Rin. *Archives of biochemistry and biophysics, 371*(2), 207-219.

Shaul, Y. D., & Seger, R. (2007). The MEK/ERK cascade: from signaling specificity to diverse functions. *Biochim Biophys Acta, 1773*(8), 1213-1226. doi:10.1016/j.bbamcr.2006.10.005

Sherwood, V., Recino, A., Jeffries, A., Ward, A., & Chalmers, A. D. (2010). The N-terminal RASSF family: a new group of Ras-association-domaincontaining proteins, with emerging links to cancer formation. *Biochemical Journal, 425*(2), 303-311.

Shibata, H., Mukai, H., Inagaki, Y., Homma, Y., Kimura, K., Kaibuchi, K., . . . Ono, Y. (1996). Characterization of the interaction between RhoA and the amino-terminal region of PKN. *FEBS letters, 385*(3), 221-224.

Shimizu, T., Ihara, K., Maesaki, R., Amano, M., Kaibuchi, K., & Hakoshima, T. (2003). Parallel coiled-coil association of the RhoA-binding domain in Rho-kinase. *Journal of Biological Chemistry, 278*(46), 46046-46051.

Silvius, J. R., & l'Heureux, F. (1994). Fluorometric evaluation of the affinities of isoprenylated peptides for lipid bilayers. *Biochemistry, 33*(10), 3014-3022.

Simanshu, D. K., Nissley, D. V., & McCormick, F. (2017). RAS proteins and their regulators in human disease. *Cell, 170*(1), 17-33.

Somlyo, A. P., & Somlyo, A. V. (2003). Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiological reviews, 83*(4), 1325-1358.

Sprang, S. R. (1995). How Ras works: structure of a Rap-Raf complex. *Structure, 3*(7), 641-643.

Stevers, L. M., Sijbesma, E., Botta, M., MacKintosh, C., Obsil, T., Landrieu, I., . . . Ottmann, C. (2017). Modulators of 14-3-3 Protein-Protein Interactions. *J Med Chem*. doi:10.1021/acs.jmedchem.7b00574

Stieglitz, B., Bee, C., Schwarz, D., Yildiz, Ö., Moshnikova, A., Khokhlatchev, A., & Herrmann, C. (2008). Novel type of Ras effector interaction established between tumour suppressor NORE1A and Ras switch II. *The EMBO journal, 27*(14), 1995-2005.

Studier, F. W., & Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of molecular biology, 189*(1), 113-130.

Tabaczar, S., Czogalla, A., Podkalicka, J., Biernatowska, A., & Sikorski, A. F. (2017). Protein palmitoylation: Palmitoyltransferases and their specificity. *Experimental Biology and Medicine, 242*(11), 1150-1157.

Takahashi, S., Ebihara, A., Kajiho, H., Kontani, K., Nishina, H., & Katada, T. (2011). RASSF7 negatively regulates pro-apoptotic JNK signaling by inhibiting the activity of phosphorylated-MKK7. *Cell death and differentiation, 18*(4), 645.

Takahashi, Y., Inamine, A., Hashimoto, S.-i., Haraguchi, S., Yoshioka, E., Kojima, N., . . . Takemori, T. (2005). Novel role of the Ras cascade in memory B cell response. *Immunity, 23*(2), 127-138.

Takai, Y., Sasaki, T., & Matozaki, T. (2001). Small GTP-binding proteins. *Physiological reviews, 81*(1), 153-208.

Takanaga, H., Mukai, H., Shibata, H., Toshimori, M., & Ono, Y. (1998). PKN interacts with a paraneoplastic cerebellar degeneration-associated antigen, which is a potential transcription factor. *Experimental cell research, 241*(2), 363-372.

Takenaka, M., Inoue, H., Takeshima, A., Kakura, T., & Hori, T. (2013). C. elegans Rassf homolog, rasf-1, is functionally associated with rab-39 Rab GTP ase in oxidative stress response. *Genes to Cells, 18*(3), 203-210.

Tao, W., Pennica, D., Xu, L., Kalejta, R. F., & Levine, A. J. (2001). Wrch-1, a novel member of the Rho gene family that is regulated by Wnt-1. *Genes & development, 15*(14), 1796-1807.

Tartaglia, M., Gelb, B. D., & Zenker, M. (2011). Noonan syndrome and clinically related disorders. *Best practice & research Clinical endocrinology & metabolism, 25*(1), 161-179.

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, 76*(9), 4350-4354.

Truebestein, L., Elsner, D. J., Fuchs, E., & Leonard, T. A. (2015). A molecular ruler regulates cytoskeletal remodelling by the Rho kinases. *Nature communications, 6*, 10029.

Tu, D., Li, Y., Song, H. K., Toms, A. V., Gould, C. J., Ficarro, S. B., . . . Eck, M. J. (2011). Crystal structure of a coiled-coil domain from human ROCK I. *PloS one, 6*(3), e18080.

Tucker, J., Sczakiel, G., Feuerstein, J., John, J., Goody, R. S., & Wittinghofer, A. (1986). Expression of p21 proteins in Escherichia coli and stereochemistry of the nucleotide-binding site. *The EMBO journal, 5*(6), 1351-1358.

Uechi, Y., Bayarjargal, M., Umikawa, M., Oshiro, M., Takei, K., Yamashiro, Y., . . . Taguchi, T. (2009). Rap2 function requires palmitoylation and recycling endosome localization. *Biochemical and biophysical research communications, 378*(4), 732-737.

Ünal, E. B., Uhlitz, F., & Blüthgen, N. (2017). A compendium of ERK targets. *FEBS letters, 591*(17), 2607-2615.

Upadhyaya, P., Bedewy, W., & Pei, D. (2016). Direct inhibitors of Ras-effector protein interactions. *Mini reviews in medicinal chemistry, 16*(5), 376-382.

Van Aelst, L., Barr, M., Marcus, S., Polverino, A., & Wigler, M. (1993). Complex formation between RAS and RAF and other protein kinases. *Proceedings of the National Academy of Sciences, 90*(13), 6213-6217.

van Dam, T. J., Bos, J., & Snel, B. (2011). Evolution of the Ras-like small GTPases and their regulators. *Small GTPases, 2*(1), 4-16.

van der Weyden, L., & Adams, D. J. (2007). The Ras-association domain family (RASSF) members and their role in human tumourigenesis. *Biochim Biophys Acta, 1776*(1), 58-85. doi:10.1016/j.bbcan.2007.06.003

van der Weyden, L., & Adams, D. J. (2007). The Ras-association domain family (RASSF) members and their role in human tumourigenesis. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1776*(1), 58-85.

Vandamme, D., Herrero, A., Al-Mulla, F., & Kolch, W. (2014). Regulation of the MAPK pathway by raf kinase inhibitory protein. *Crit Rev Oncog, 19*(6), 405-415.

Vandamme, D., Herrero, A., Al-Mulla, F., & Kolch, W. (2014). Regulation of the MAPK pathway by raf kinase inhibitory protein. *Critical Reviews™ in Oncogenesis, 19*(6).

Vetter, I. R., & Wittinghofer, A. (2001). The guanine nucleotide-binding switch in three dimensions. *Science, 294*(5545), 1299-1304.

Vigil, D., Cherfils, J., Rossman, K. L., & Der, C. J. (2010). Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? *Nature Reviews Cancer, 10*(12), 842.

Vojtek, A. B., Hollenberg, S. M., & Cooper, J. A. (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell, 74*(1), 205-214.

Vos, M. D., Ellis, C. A., Bell, A., Birrer, M. J., & Clark, G. J. (2000). Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. *Journal of Biological Chemistry, 275*(46), 35669-35672.

Vos, M. D., Martinez, A., Ellis, C. A., Vallecorsa, T., & Clark, G. J. (2003). The pro-apoptotic Ras effector Nore1 may serve as a Ras-regulated tumor suppressor in the lung. *Journal of Biological Chemistry, 278*(24), 21938-21943.

Warne, P. H., Vician, P. R., & Downward, J. (1993). Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. *Nature, 364*(6435), 352.

Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Morii, N., . . . Narumiya, S. (1996). Protein kinase N (PKN) and PKN-related protein rhophilin as targets of small GTPase Rho. *Science, 271*(5249), 645-648.

Wellbrock, C., Karasarides, M., & Marais, R. (2004). The RAF proteins take centre stage. *Nat Rev Mol Cell Biol, 5*(11), 875-885. doi:10.1038/nrm1498

Wen, W., Liu, W., Yan, J., & Zhang, M. (2008). Structure basis and unconventional lipid membrane binding properties of the PH-C1 tandem of rho kinases. *Journal of Biological Chemistry, 283*(38), 26263-26273.

Wennerberg, K., Rossman, K. L., & Der, C. J. (2005). The Ras superfamily at a glance. *J cell Sci, 118*(5), 843-846.

Wherlock, M., & Mellor, H. (2002). The Rho GTPase family: a racs to wrchs story. *Journal of cell science, 115*(2), 239-240.

Wilchek, M., & Chaiken, I. (2000). An overview of affinity chromatography *Affinity Chromatography* (pp. 1-6): Springer.

Winter-Vann, A. M., & Casey, P. J. (2005). Post-prenylation-processing enzymes as new targets in oncogenesis. *Nature Reviews Cancer, 5*(5), 405.

Wittinghofer, A. (2014). *Ras Superfamily Small G Proteins: Biology and Mechanisms*: Springer.

Wittinghofer, A., & Vetter, I. R. (2011). Structure-function relationships of the G domain, a canonical switch motif. *Annual review of biochemistry, 80*, 943-971.

Wohlgemuth, S., Kiel, C., Krämer, A., Serrano, L., Wittinghofer, F., & Herrmann, C. (2005). Recognizing and defining true Ras binding domains I: biochemical analysis. *Journal of molecular biology, 348*(3), 741-758.

Wortzel, I., & Seger, R. (2011). The ERK Cascade: Distinct Functions within Various Subcellular Organelles. *Genes Cancer, 2*(3), 195-209. doi:10.1177/1947601911407328

Wright, L. P., & Philips, M. R. (2006). Thematic review series: lipid posttranslational modifications CAAX modification and membrane targeting of Ras. *Journal of lipid research, 47*(5), 883-891.

Yoon, S., & Seger, R. (2006). The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. *Growth Factors, 24*(1), 21-44. doi:10.1080/02699050500284218

Younesian, S., Shahkarami, S., Ghaffari, P., Alizadeh, S., Mehrasa, R., Ghavamzadeh, A., & Ghaffari, S. H. (2017). DNA hypermethylation of tumor suppressor genes RASSF6 and RASSF10 as independent prognostic factors in adult acute lymphoblastic leukemia. *Leukemia research, 61*, 33-38.

Zerial, M., & McBride, H. (2001). Rab proteins as membrane organizers. *Nature reviews Molecular cell biology, 2*(2), 107.

Zhang, M., Wang, D., Zhu, T., & Yin, R. (2017). RASSF4 overexpression inhibits the proliferation, invasion, EMT, and Wnt signaling pathway in osteosarcoma cells. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics, 25*(1), 83-91.

Zhang, X.-F., Settleman, J., Kyriakis, J., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., . . . Avruch, J. (1993). Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. *Nature, 364*(6435), 308.

Zhao, Z.-s., & Manser, E. (2005). PAK and other Rho-associated kinases–effectors with surprisingly diverse mechanisms of regulation. *Biochemical Journal, 386*(2), 201-214.

Zhou, Y., & Hancock, J. F. (2015). Ras nanoclusters: Versatile lipid-based signaling platforms. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1853*(4), 841-849.

Zimmermann, S., & Moelling, K. (1999). Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science, 286*(5445), 1741-1744.

Zong, H., Kaibuchi, K., & Quilliam, L. A. (2001). The insert region of RhoA is essential for Rho kinase activation and cellular transformation. *Molecular and cellular biology, 21*(16), 5287-5298.

Supplement articles

Supplement A: Structural snapshots of RAF kinase interactions

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Review Article

Structural snapshots of RAF kinase interactions

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RAF (rapidly accelerated fibrosarcoma) Ser/Thr kinases (ARAF, BRAF, and CRAF) link the RAS (rat sarcoma) protein family with the MAPK (mitogen-activated protein kinase) pathway and control cell growth, differentiation, development, aging, and tumorigenesis. Their activity is specifically modulated by protein–protein interactions, post-translational modifications, and conformational changes in specific spatiotemporal patterns via various upstream regulators, including the kinases, phosphatase, GTPases, and scaffold and modulator proteins. Dephosphorylation of Ser-259 (CRAF numbering) and dissociation of 14-3-3 release the RAF regulatory domains RAS-binding domain and cysteine-rich domain for interaction with RAS-GTP and membrane lipids. This, in turn, results in RAF phosphorylation at Ser-621 and 14-3-3 reassociation, followed by its dimerization and ultimately substrate binding and phosphorylation. This review focuses on structural understanding of how distinct binding partners trigger a cascade of molecular events that induces RAF kinase activation.

Introduction

The discovery of the viral oncogene *v-raf* from the transforming murine retrovirus 3611-MSV in 1983 [1] paved the way for the discovery of a cellular homolog CRAF in 1985 [2] and soon after its paralogs ARAF [3] and BRAF [4]. Evolutionary conservation across different species, including worms (Lin-45) [5] and flies (Draf) [6], unequivocally indicates the biological importance of RAF (rapidly accelerated fibrosarcoma) kinases (Figure 1). Lin-45 encodes a BRAF ortholog that is necessary for larval viability, fertility, and induction of vulval cell fates [7]. Draf plays an important role in early embryogenesis [6]. The three human RAF paralogs regulate a large number of biochemical processes, including survival, proliferation, differentiation, stress responses, and apoptosis [8–13]. RAF kinases constitute a small family of serine/threonine kinases, which control evolutionarily conserved pathways and have essential roles during development [14–16]. Thus, it is not surprising that their dysregulation is associated with progression of a variety of human cancers [16–19], pathogenesis of developmental disorders including Noonan, LEOPARD, and cardiofaciocutaneous syndromes [20,21], and cardiovascular diseases, such as pulmonary arterial hypertension and heart failure [22].

Works from many laboratories have shown that RAF kinases are integral elements of the RAS–MAPK pathway, which is involved in different signaling pathways [22–27]. Activation of RAF kinases at the plasma membrane by RAS [1,28–32], together with the identification of their substrates MEK1/2 (MAPK/ERK kinase 1/2) [33] has provided the missing link between growth factor signals and MAPK cascade activation [34]. The activities of RAF kinases toward MEK differ widely, with BRAF being the strongest MEK activator, followed by CRAF and ARAF [35–37]. These proteins obviously underlay different regulatory mechanisms, including binding to membrane-associated RAS proteins, phosphorylation, and dephosphorylation along with homodimerization and heterodimerization [34,35,38–41]. These and other events collectively result in RAF kinase activation [42].

Despite the long history, investigations of the fundamental mechanisms of RAF kinase activation have substantially lagged far behind the development of kinase inhibitors and inhibitor technologies. In this review, we summarize emerging mechanistic insights gained from structural, biochemical, and

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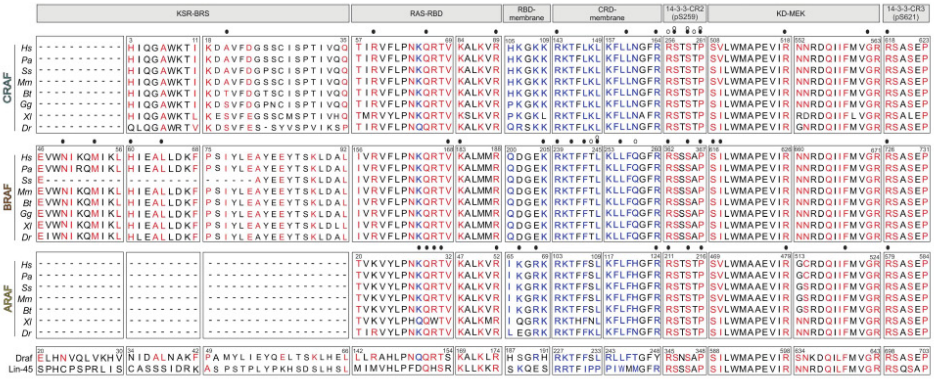

Figure 1. Evolutionary conservation of RAF family members.

Multiple amino acid sequence alignment of RAF family members from different organisms (*Hs*, *Homo sapiens*; *Pa*, *Pongo abelii*; *Ss*, *Sus scrofa*; *Mm*, *Mus musculus*; *Bt*, *Bos taurus*; *Gg*, *Gallus gallus*; *Xl*, *Xenopus laevis*; *Dr*, *Danio rerio*; *Dm*, *Drosophila melanogaster*; *Ca*, *Caenorhabditis elegans*) illustrates selected regions extracted from this figure. Red amino acids are involved in protein interaction, whereas blue amino acids contact membranes.

computational studies on functional interaction networks. Human RAF paralogs share evolutionarily conserved regions (Figure 1), which are functionally split into a regulatory N-terminal half, comprising a RAS-binding domain, a cysteine-rich domain, and a serine/threonine-rich region and a catalytic C-terminal half representing the kinase domain (Figure 2A). In the following, we will discuss the structure–function relationships of individual domains and motifs and their interactions with membrane lipids, RAS, 14-3-3, MEK1/2, and KSR1/2 (kinase suppressor of RAS 1/2).

RAS-binding domains

Signal transduction implies physical association of RAS proteins with their effectors and activation of individual signaling pathways. Effectors specifically interact with the active, GTP-bound form of RAS proteins. These interactions occur usually in response to extracellular signals and link them to downstream signaling pathways in all eukaryotes [26,43]. Effectors act as protein or lipid kinases, phospholipase, GEFs (guanine nucleotide exchange), GAPs (GTPase-activating proteins), and scaffold proteins [44–47]. There are two major groups of effectors: one contains RAS-binding domains (RBDs) and the other RAS association (RA) domains [48,49]. Mining in the UniProt database led us to the identification of 118 distinct human proteins containing RBDs and RA domains (Rezaei Adariani, Dvorsky, et al. unpublished data). Notably, both types of domains utilize critical determinants for the interaction with different RAS proteins, particularly the intermolecular β-sheets (see next section) [50]. Structural studies have provided deep insights into the binding modes and interaction specificities [51–53]. Detailed analysis of 16 RAS structures in complex with different RBD and RA-domain effectors has revealed that, in spite of low sequence similarity, their mode of interaction is well conserved [50]. Yet, the precise mechanism through which effector association with RAS proteins results in their activation is still unclear. It is, however, generally accepted that RAS proteins participate directly in the activation of their downstream effectors and do not simply mediate their recruitment to specific sites at the membrane [54].

A striking feature of RAS proteins is the plethora of possible interactions with a large number of effectors. Notably, RAS proteins change their conformation mainly at two highly mobile regions, designated as switch I (aa 30–40) and switch II (aa 60–68) [53,55]. Mainly in the GTP-bound form, the switch regions of the RAS proteins provide a platform for the association with effector proteins, especially through their RBDs or RA domains. This interaction appears to be a prerequisite for effector activation [49,50,56–58]. However, CRAF RBD and RALGDS (Ral guanine nucleotide dissociation stimulator)-RA domains share a similar ubiquitin-like fold and contact the switch I region via a similar binding mode. In contrast, PI3Kα (phosphoinositide 3-kinase α)-RBD, RASSF5 (RAS association domain-containing protein 5)-RA, and PLCε (phosphatidylinositol

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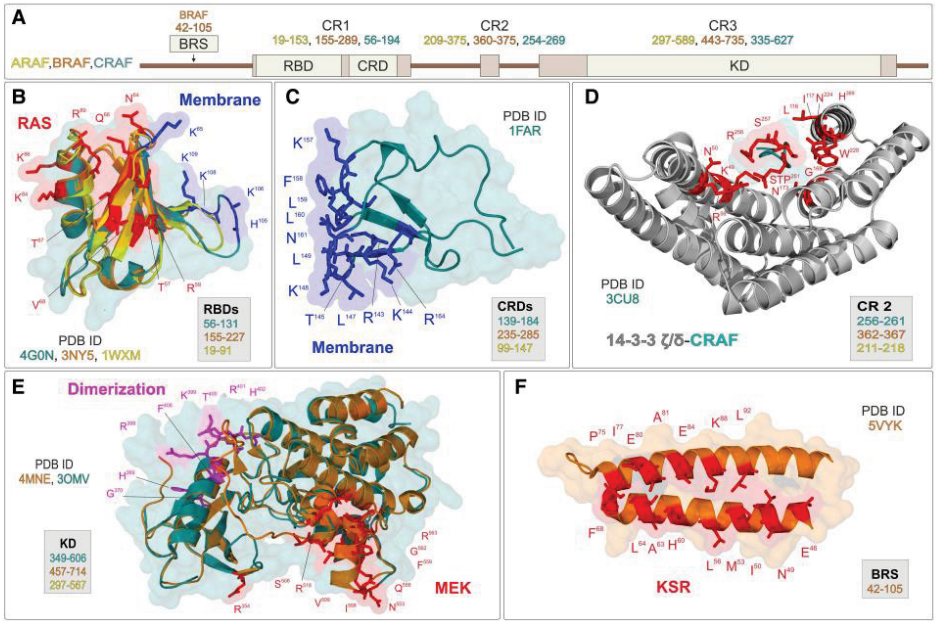

Figure 2. Structural fingerprints for RAF kinase interactions with RAS and the membrane lipids.

Critical residues involved in protein interaction and membrane binding are depicted in red and blue, respectively. CR encompassing amino acids are shown at the upper panel. (**A**) Domain organization of RAF kinases with the typical conserved regions (CR1, CR2, and CR3) along with the functional domains, including the RBD, the CRD, and the kinase domain (KD). (**B**) Overlaid RBD structures of the RAF paralogs and the amino acids interacting with RAS and the membrane. BRAF RBD exhibits negative charges in positions 202 and 204. RAF RBD encompassing amino acids are boxed. (**C**) CRD structure of CRAF and the membrane-binding amino acids. RAF CRD encompassing amino acids are boxed. (**D**) 14-3-3 δ/ζ structure in complex with the CR2 peptide of CRAF along with interacting amino acids of CRAF and 14-3-3 paralogs. (**E**) Overlaid structures of CRAF and BRAF kinase domains' along with MEK-binding amino acids. (**F**) CC-SAM domain of KSR1 in complex with RBS domain of BRAF.

4,5-bisphosphate phosphodiesterase epsilon)-RA domains do not share sequence and structural similarity, but commonly associate with the switch regions, especially switch I [59–63].

RAF–RBD interactions with RAS proteins

Major studies were carried out in the late 1980s and 1990s with regard to RAS interaction with its effectors (reviewed in refs [52,64–69]). An interaction study of CRAF association with RRAS1 led to the identification of the first RBD (aa 51–131) [70]. Soon after CRAF binding to HRAS was reported to be GTP-dependent [28–31,71]. Within a year, the sites of interaction between HRAS and CRAF were determined [72] along with quantitative analysis of the binding affinity between them [73]. All of this occurred before the first structure revealed the CRAF RBD structure and its mode of binding to a RAS family member, RAP1A (RAS-related protein 1A) [74]. CRAF RBD consists of a five-strand mixed β-sheet (β1– β5) with an interrupted α-helix (α1) and two additional 3_{10}-helices (α2 and α3) (Figure 2B). Consistent with an earlier NMR determination [51], the RBD of CRAF has an ubiquitin fold (β1, β2, α1, β3, β4, α2, and β5). The β-strands are nearly identical with ubiquitin-like protein and α-helices are packed diagonally against a part of the β-sheet. To date, several RBD structures of all three human RAF paralogs have been determined (Table 1). Superposition of all three RBD structures revealed a high structural identity (Figure 2B).

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Table 1 Structures related to RAF kinases

Proteins and their complexes	PBD ID	Resolution (Å)	References
BRS			
BRAF-KSR1	5VYK	1.75	[75]
RBD			
RAP1A-GppNHp-CRAF RBD[1]	1C1Y	2.2	[59]
RAP1A(E30D/K31E)-GDP-CRAF RB(A85K/N71R)	3KUC	1.92	[76]
RAS-GDP-CRAF RBD(A85K)	3KUD	2.15	[76]
BRAF RBD	3NY5	1.99	Unpublished data
HRAS-GppNHp-CRAF RBD	4G0N	2.45	[77]
HRAS(Q61L)-GppNHp-CRAF RBD	4G3X	3.25	[77]
KRAS-GppNHp-ARAF RBD	2MSE	NMR	[54]
BRAF RBD	5J17	NMR	[58]
BRAF RBD-Rigosertib	5J18	NMR	[58]
BRAF RBD	5J2R	NMR	[58]
ARAF RBD	1WXM	NMR	Unpublished data
CRD			
CRAF CRD	1FAQ, 1FAR	NMR	[78]
CR2			
14-3-3-CRAF CR2	3CU8, 3NKX	2.4	[79]
14-3-3γ-CRAF CR2	3IQV	1.2	[79]
14-3-3γ-CRAF CR2	3O8I	2.0	[79]
14-3-3-CRAF CR2(S233/S259)	4FJ3	1.95	[80]
14-3-3-CRAF CR2-CN-A	4IHL	2.2	[81]
14-3-3γ-CRAF-CR2(S233/S259)	4IEA	1.7	[81]
14-3-3γ-CRAF CR2	3IQJ	1.15	[79]
Kinase domain[2]			
CRAF KD	3OMV	4	[79]
BRAF KD(V599E)	1UWJ	3.5	[82]
BRAF–MEK1	4MNE	2.84	[83]

[1]GppNHp is a non-hydrolyzable GTP analog.
[2]A large number of kinase structures in complex with small molecules are not included.

RAF RBDs bind to the switch I region (also known as the effector loop) of the RAS proteins by forming an intermolecular, antiparallel β-sheet (β1 and β2 of the RBD and β2 and β3 of RAS), which establishes a high degree of electrostatic complementarity across the binding interface [53,77,84,85]. RAF RBDs are mainly positively charged, whereas switch I regions of RAS proteins bear mainly negative charges. Among the 10 RAS-binding residues of RAF RBD (Figure 2B, red residues), Arg-59, Gln-66, Lys-84, and Arg-89 (CRAF numbering) contribute to the high binding affinity between RAS and RAF [86]. Genetic studies on *Drosophila melanogaster* have shown that Arg-89 is strongly involved in the RAS–RAF interaction both *in vivo* and *in vitro*. Its substitution for leucine (R89L) abolishes RAS association and consequently activation of CRAF [87]. The R89T mutation has been reported in breast cancer [88]. This mutation may impair RAS–CRAF interaction, since a conservative substitution of Arg-89 for lysine (R89K) disabled CRAF RBD binding to HRAS [89]. Collectively, a search in cancer databases showed that among the 10 RAS-binding in RAF paralogs, seven residues are mutated in human cancer (Supplementary Table S1).

Arg-59 represents a point of RAF paralog discrimination as ARAF, in contrast with BRAF and CRAF, contains a lysine (Lys-22) instead of arginine (Figure 1). CRAF(R59K) loses its proper binding to HRAS, whereas

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ARAF(K22R) gains a higher affinity for HRAS [90]. The substitution of the conserved Gln-66 among three RAF paralogs for histidine in CRAF and for proline in ARAF (aa 29) has been reported in breast and colorectal carcinoma [91,92]. Lys-84, which is conserved in all species (Figure 1), is responsible for effector specificity and favors the complex formation of CRAF with HRAS in preference to RAP1A. Its substitution for alanine strongly reduces its binding affinity to RAS proteins [86,93]. An interesting observation is that A85K mutation tremendously increases CRAF binding not only to GTP-bound HRAS [87] but also to GDP-bound HRAS [94].

Membrane association of RAF RBDs

Cellular membranes play a critical role in the localization and orientation of protein complexes and in fine-tuning of protein functions [95]. As outlined above, the activity of RAS and RAF paralogs is regulated through different parameters, including membrane association. Analysis of dynamic interactions between KRAS4B and lipid bilayer membrane has revealed that association of ARAF RBD with active KRAS4B not only reorients KRAS4B at the membrane surface but also facilitates membrane binding of ARAF RBD itself [54]. This is in agreement with previous observation that disrupted RAS-association of ARAF full-length disturbs its membrane localization when substituting Arg-52 for leucine (as well as R89L in CRAF) [96,97]. Four basic residues, Lys-28, Lys-66, Arg-68, and Lys-69 (ARAF numbering), are engaged in lipid binding, two of which are identical in RAF kinases, while the other two are variable (Figure 2B). Notably, mutations of Lys-28, Arg-52, Lys-66, Arg-68, and Lys-69 in ARAF have been reported in human cancer [88,98–100]. BRAF strikingly contains acidic residues at positions equivalent to Lys-66 and Arg-68 (not only in human but also in other species; Figure 1), which most probably repel membrane lipids. BRAF and CRAF studies have shown that they significantly differ regarding their interactions with HRAS [101]. BRAF binds RAS with higher affinities and does not discriminate between farnesylated and nonfarnesylated HRAS when compared with CRAF. The farnesyl moiety of HRAS has been reported to promote CRAF CRD (cysteine-rich domain) association with HRAS (see the next section).

Cysteine-rich domain

The second domain following RBD in the conserved region 1 (CR1) is a CRD (also called cysteine-rich region or C-kinase homologous domain 1), which is connected through a short flexible linker [102,103]. CRD shows high conservation among different species (Figure 1) and appears to bind membrane lipids via residues 143–160 (Figure 2C), which are conserved among different species (Figure 1). Point mutations of Arg-143 to tryptophan, glutamine, or leucine in CRAF and the equivalent Arg-239 in BRAF to glutamine have been identified in breast and lung carcinoma as well as in melanoma [88,104]. Substitution of Arg-103 and Lys-104 in ARAF CRD (Arg-143 and Lys-144 in CRAF, respectively) for alanine has been shown to disrupt ARAF membrane binding and results in its localization in the cytosol [97]. Two very recent computational studies have analyzed dynamic interaction of KRAS4B with the CRAF RBD–CRD tandem at anionic membranes and proposed how the RAF–RAS complex is regulated at the membrane interface [103,105]. Accordingly, RAF association with the membrane starts with direct binding of RBD to GTP-bound RAS followed by CRD association to the phosphatidylserine-containing liposomes. CRD–membrane interaction is stabilized, in addition to basic residues, by four highly conserved hydrophobic amino acids, Thr-145, Leu-147, Leu-149, Phe-158 Leu-159, Leu160, and Asp-161 (Figure 1). Numerous studies have reported that CRD also binds RAS with low affinity [56,101,105–116]. This may lead to a competitive mechanism between membrane binding of CRAF CRD and its association with KRAS4B [103]. Unlike others reports, these two studies have shown that CRD is in the vicinity, but does contact RAS and/or its farnesyl moiety [103,105]. Membrane binding of CRD stabilizes RAS–RAF interaction and, thus, facilitates RAF activation. Farnesylation and carboxymethylation of Cys-186 of HRAS together with hydrophobic amino acids of CRAF CRD have been suggested to strengthen HRAS–CRAF interaction [116]. CRDs contain two functional zinc-binding motifs and bind membrane lipids such as phosphatidic acid and phosphatidylserine [58,117–119]. Substitution of two invariant zinc-binding cysteines for serines (C165S/C168S) [96,120] and three basic residues for alanine (Agr-143, Lys-144, and Lys-148) (Figure 2C) diminishes HRAS-dependent activation of CRAF and CRD association with phosphatidylserine-containing liposomes [121].

Several studies have previously shown that CRAF CRD undergoes direct interaction with HRAS, which appears to be enhanced by the farnesyl moiety if using farnesylated RAS [57,101,103,105,107,113,116]. In contrast with RAF RBD, which binds to GTP-bound RAS, HRAS–CRAF CRD interaction is outside the switch regions of HRAS and thus independent of its nucleotide-bound state. This interaction is compromised if

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Leu-149 and Phe-151 in CRAF CRD were substituted for threonine and glutamine (L149T/F151Q), respectively [113]. L149F substitution in BRAF (L245F) has been detected in melanoma and cardiofaciocutaneous syndrome (NSEuroNet database) (COSMIC database) [122], which may potentiate BRAF CRD interaction with RAS and/or membrane.

RAS–RAF interactions at the membrane interface

Cellular membranes play a critical role in the localization and orientation of protein complexes and in fine-tuning of protein functions [95]. As outlined above, the diversity of RAS and RAF paralogs is regulated through different parameters, including membrane association. For example, orientation of the RAS G domain on the membrane (for more details, see refs [54,123–130]) and intrinsic membrane-binding site of RAF, such as CRD of RAF (see above). In addition, NMR measurements of nanodisc-tethered complexes of isotopically labeled KRAS4B-GTP with ARAF RBD have recently shown that ARAF RBD directly contacts the anionic membrane surface, while KRAS4B-GTP adopts a new semi-exposed orientation intermediate between the exposed and occluded orientations [54]. The only residue of the KRAS4B G domain contacting the membrane is R41, which is conserved in numerous RAS proteins. ARAF residues engaged in membrane binding (Lys-66, Arg-68, and Lys-69; Figure 1B) are highly conserved in ARAF and CRAF proteins from different organisms except *Xenopous laevis* and *Caenorhabditis elegans* (Figure 1). These basic residues remarkably are acidic in BRAF proteins, suggesting distinct mechanistic differences between the RAF paralogs. In contrast, membrane-binding residues of RAF CRD are conserved within various species, which may stabilize RAS–RAF interaction and thus facilitates RAF activation.

Serine/threonine-rich region

This very short region, also called conserved region 2 (CR2; Figure 2A), is a central module in negative regulation of RAF function. Its phosphorylation at Ser-259 (CRAF numbering) followed by 14-3-3 binding locks RAF kinases in a so-called autoinhibited state [131] that blocks both RAS binding and RAF kinase activity [132,133]. CR2 is the substrate of PKA (protein kinase A) and PKB (protein kinase B)/AKTs [134–136]. Gain-of-function mutations in this region are associated with the development of tumors and RASopathies [137,138]. Point mutations in CR2, including R256S, S257L, S259F, and T260R, cause cancer or are associated with developmental disorders (Supplementary Table S2), e.g. hypertrophic cardiomyopathy in Noonan syndrome [79,137–139].

Phosphorylation of RAF paralogs at Ser-259 (CRAF numbering) leads to the association of 14-3-3 proteins and the stabilization of RAF paralogs in their inactive state [79,88,137–143]. 14-3-3 proteins are ubiquitous adaptor proteins, which serve as scaffold proteins in many cellular functions [79,144]. In humans, seven distinct genes encode for nine paralogs (α, β, γ, δ, ε, η, σ, τ, and ζ), which adopt a homo-/heterodimeric [145,146], W-like structure with the two concave surfaces facing the same side of the molecule, whereby the dimer forms a binding groove [147]. They selectively bind peptide motifs, such as RSXpSXP (single amino acids code; pS, phosphor-serine; X, any amino acid); arginine, serine, and proline residues, which are important for high-affinity interactions [148]. This motif is identical in RAF kinases (Figures 1 and 2D) regardless of the binding sites. Phosphorylated serines in CRAF, including Ser-259 and Ser-621, already identified in 1993 [149] are key phosphorylation sites in two distinct motifs in the RAF kinases (Figure 2D) [144]. In contrast to pSer-259, an inhibitory 14-3-3-binding site [79,131,133], 14-3-3 association with pSer-621 in a conserved region (CR3) stabilizes the active state of the RAF kinases [147]. All 14-3-3 paralogs are able to modulate RAF kinase function due to invariant RAF-binding residues and similar tertiary structure of all 14-3-3 proteins (Figure 2D).

Catalytic kinase domain

The molecular mechanism for the RAF activation in the cell involves a series of complex processes that lead to conformational changes, dimerization, and ultimately activation of the kinase domain [150]. The latter constitutes a major part of CR3, which has all known signatures of protein kinases [151], including the two lobes moving relative to each other and consequently opening or closing the catalytic cleft. In an open form, the small lobe with an antiparallel β-sheet structure binds and orients ATP. In the closed form, the α-helical large lobe binds the protein substrates, such as ubiquitously expressed MEK1/2 (Figure 2E). As RAF dimerization is a key step in pathway activation, the RAF kinases activate MEK1/2 by phosphorylating them at two serines (Ser-218/Ser-222 in MEK1) in the catalytic domain [151,152]. An inspection of amino acid sequences of RAF

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kinases from different organisms showed identical MEK-binding residues (Figure 1) [153]. However, it is known that RAF kinases differ in their kinase activities. BRAF followed by CRAF and ARAF exhibits the highest MEK activation [35,36]. This can be attributed to dimerization-induced allosteric regulation of protein kinases [41]. RAF kinases form both homodimers and heterodimers, which is crucial for substrate recognition, catalytic efficiency, and substrate specificity [35]. The CRAF/BRAF heterodimers represent the most effective form for MEK phosphorylation when compared with any form of monomers or homodimers [38]. The structure of BRAF kinase domain and MEK1 is insensitive to BRAF dimerization but sensitive to the active conformation of the BRAF kinase and MEK1 phosphorylation, which in turn leads to destabilization of the RAF–MEK1 heterotetrameric complex [83].

Approximately 200 BRAF mutations have been identified in human tumors (see Supplementary Table S1). Based on their mechanism of activation, they can be categorized into three groups corresponding to their sensitivity to inhibitors. Group one mutations (e.g. V600E/K/D/R) signal as monomers and have been suggested to act in a RAS-independent manner [154,155]. Therefore, they are sensitive to BRAF monomer inhibitors. Group 2 mutations (e.g. K601E or G469A, R509H) signal as constitutive dimers and are RAS-independent; hence, they are resistant to RAF inhibitor vemurafenib and may be sensitive to novel MEK inhibitors or RAF dimer inhibitors [154,156]. However, group three mutations have impaired kinase activity (D594G/N) or have low kinase activity (G466V/E). This group is RAS-dependent, and by increasing their binding to RAS or activation of receptors activate ERK (extracellular signal-regulated kinase) signaling [155].

Scaffolding RAF kinases by KSR1/2

Scaffolding proteins play an essential role in regulating the MAPK pathway activity [157–159]. MAPK scaffold proteins especially are dynamic entities that (i) directly interact with multiple components of the MAPK signaling complex, (ii) consolidate or sequester protein interactions to physically insulate the MAPK pathway to specific cellular locations, and (iii) regulate signal strength and stimulus-specific responses to efficiently transmit MAPK signals in a spatiotemporal manner and narrow its actions [157,160,161]. Scaffold proteins regulating MAPK signaling include KSR1/2 [162–164], MORG1 [165], MP1 [166], paxillin [167], β-arrestin [168], MEKK1 [169], and FHL1 [170]. KSR1/2, which belongs to the best characterized MAPK scaffold proteins, controls the signaling strength and duration of the RAF/MEK/ERK complex at the plasma membrane [157,159].

KSR1/2 are pseudokinases homologous to RAF kinases but lack the ability to interact with RAS proteins [83,171]. KSR co-ordinates the assembly of a multiprotein complex containing RAF, MEK, and ERK and facilitates signal transduction from RAS to ERK [172]. Nguyen et al. [173] did not observe that KSR binds to CRAF or BRAF *in vivo*. However, Lavoie et al. have shown that the selective heterodimerization of BRAF with KSR1 directly binds to a BRAF-specific region (BRS) at the N-terminus of BRAF through the coiled-coil/sterile α-motif (CC-SAM). BRS (∼60 aa) forms an α-hairpin which consists of two antiparallel α-helices connected by a short turn (Figure 2F) [75].

In BRAF, I666R mutation disabled binding to MEK1 as well as prevented MEK1 phosphorylation, and in KSR1, W831R mutation abolished MEK1 binding [75]. The crystal structure of the KSR2 kinase domain bound to MEK1 through activation segments and C-lope αG helix reveals that residues Ser-218 and Ser-222 are located at the heterodimer interface and are masked by KSR2, making them unaccessible for RAF phosphorylation [174]. Isolated MEK1–BRAF–14-3-3 complexes proved the stable BRAF–MEK1 interaction in the presence of 14-3-3 [83]. Interestingly, MEK promotes, independently of its catalytic function, BRAF-KSR1/2 heterodimerization and allosterically activates BRAF [75]. A recent study has shown that a direct binding of tumor suppressor DIRAS3 with KSR1 interferes with RAS-induced cell transformation. DIRAS3 either enhances homodimerization of KSR1 or recruits KSR1 to the RAS–CRAF complex and thereby sequesters CRAF from binding to BRAF [175].

Conclusions

Emerging evidence indicates that sequential RAS binding of the two N-terminal RAF domains, first by RBD and then followed by CRD at the membrane, induces a conformational change in RAF and results in the release of the C-terminal kinase domain. This mechanism requires additional functions, including dimerization [35,95,160,161,176–180]. Lipid membranes act not only as a platform for the assembly of protein complexes but also as a scaffold to stabilize protein–protein interactions and potentiate the signal transduction [35,36,54]. Future analysis of protein interaction networks along with the network reconstitution at liposomes using purified proteins will provide further mechanistic insights into RAS-mediated RAF activation.

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RAF kinases are known to regulate, in addition to MEK1/2, also adenylyl cyclase, ASK1, calcineurin, CDC25, DMPK, MST2, MYPT, Rb, ROCK, troponin T, and vimentin, thereby controlling different processes, such as proliferation, differentiation, apoptosis, and contraction and motility, respectively [13,14,181–183]. However, the mechanisms how RAF kinases regulate these proteins still need to be addressed in greater detail in a cell-type-specific manner.

Abbreviations

BRS, BRAF-specific region; CC-SAM, coiled-coil/sterile α-motif; CR2, conserved region 2; CRD, cyteine-rich domain; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; RA domain, RAS association domain; RAF, rapidly accelerated fibrosarcoma; RAP, RAS-related protein; RAS, rat sarcoma; RASSF, RAS association domain family proteins; RHEB, RAS homolog enriched in brain; RBD, RAS-binding domain; KSR, kinase suppressor of RAS.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

References

1 Rapp, U.R., Goldsborough, M.D., Mark, G.E., Bonner, T.I., Groffen, J., Reynolds, Jr, F.H. et al. (1983) Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. *Proc. Natl Acad. Sci. U.S.A.* **80**, 4218–4222 https://doi.org/10.1073/pnas.80.14.4218
2 Bonner, T.I., Kerby, S.B., Sutrave, P., Gunnell, M.A., Mark, G. and Rapp, U.R. (1985) Structure and biological activity of human homologs of the raf/mil oncogene. *Mol. Cell. Biol.* **5**, 1400–1407 https://doi.org/10.1128/MCB.5.6.1400
3 Huleihel, M., Goldsborough, M., Cleveland, J., Gunnell, M., Bonner, T. and Rapp, U.R. (1986) Characterization of murine A-raf, a new oncogene related to the v-raf oncogene. *Mol. Cell. Biol.* **6**, 2655–2662 https://doi.org/10.1128/MCB.6.7.2655
4 Ikawa, S., Fukui, M., Ueyama, Y., Tamaoki, N., Yamamoto, T. and Toyoshima, K. (1988) B-raf, a new member of the raf family, is activated by DNA rearrangement. *Mol. Cell. Biol.* **8**, 2651–2654 https://doi.org/10.1128/MCB.8.6.2651
5 Han, M., Golden, A., Han, Y. and Sternberg, P.W. (1993) *C. elegans* lin-45 raf gene participates in let-60 ras-stimulated vulval differentiation. *Nature* **363**, 133–140 https://doi.org/10.1038/363133a0
6 Mark, G.E., MacIntyre, R.J., Digan, M.E., Ambrosio, L. and Perrimon, N. (1987) *Drosophila melanogaster* homologs of the raf oncogene. *Mol. Cell. Biol.* **7**, 2134–2140 https://doi.org/10.1128/MCB.7.6.2134
7 Hsu, V., Zobel, C.L., Lambie, E.J., Schedl, T. and Kornfeld, K. (2002) *Caenorhabditis elegans* lin-45 raf is essential for larval viability, fertility and the induction of vulval cell fates. *Genetics* **160**, 481–492 PMID:11861555
8 Morrison, D.K. (1990) The Raf-1 kinase as a transducer of mitogenic signals. *Cancer Cells* **2**, 377–382 PMID:2150916
9 Avruch, J., Khokhlatchev, A., Kyriakis, J.M., Luo, Z., Tzivion, G., Vavvas, D. et al. (2001) Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade. *Recent Prog. Horm. Res.* **56**, 127–155 https://doi.org/10.1210/rp.56.1.127
10 Wellbrock, C., Karasarides, M. and Marais, R. (2004) The RAF proteins take centre stage. *Nat. Rev. Mol. Cell Biol.* **5**, 875–885 https://doi.org/10.1038/nrm1498
11 Leicht, D.T., Balan, V., Kaplun, A., Singh-Gupta, V., Kaplun, L., Dobson, M. et al. (2007) Raf kinases: function, regulation and role in human cancer. *Biochim. Biophys. Acta* **1773**, 1196–1212 https://doi.org/10.1016/j.bbamcr.2007.05.001
12 Osborne, J.K., Zaganjor, E. and Cobb, M.H. (2012) Signal control through Raf: in sickness and in health. *Cell Res.* **22**, 14–22 https://doi.org/10.1038/cr.2011.193
13 Desideri, E., Cavallo, A.L. and Baccarini, M. (2015) Alike but different: RAF paralogs and their signaling outputs. *Cell* **161**, 967–970 https://doi.org/10.1016/j.cell.2015.04.045
14 Niault, T.S. and Baccarini, M. (2010) Targets of Raf in tumorigenesis. *Carcinogenesis* **31**, 1165–1174 https://doi.org/10.1093/carcin/bgp337
15 Sanges, C., Scheuermann, C., Zahedi, R.P., Sickmann, A., Lamberti, A., Migliaccio, N. et al. (2012) Raf kinases mediate the phosphorylation of eukaryotic translation elongation factor 1A and regulate its stability in eukaryotic cells. *Cell Death Dis.* **3**, e276 https://doi.org/10.1038/cddis.2012.16

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Biochemical Society Transactions (2018) **46** 1393–1406
https://doi.org/10.1042/BST20170528

16 An, S., Yang, Y., Ward, R., Liu, Y., Guo, X.-X. and Xu, T.-R. (2015) A-Raf: a new star of the family of raf kinases. *Crit. Rev. Biochem. Mol. Biol.* **50**, 520–531 https://doi.org/10.3109/10409238.2015.1102858
17 Downward, J. (2003) Targeting RAS signalling pathways in cancer therapy. *Nat. Rev. Cancer* **3**, 11–22 https://doi.org/10.1038/nrc969
18 Maurer, G., Tarkowski, B. and Baccarini, M. (2011) Raf kinases in cancer-roles and therapeutic opportunities. *Oncogene* **30**, 3477–3488 https://doi.org/10.1038/onc.2011.160
19 Roring, M. and Brummer, T. (2012) Aberrant B-Raf signaling in human cancer — 10 years from bench to bedside. *Crit. Rev. Oncog.* **17**, 97–121 https://doi.org/10.1615/CritRevOncog.v17.i1.70
20 Allanson, J.E., Annerén, G., Aoki, Y., Armour, C.M., Bondeson, M.-L., Cave, H. et al. (2011) Cardio-facio-cutaneous syndrome: does genotype predict phenotype? *Am. J. Med. Genet. C Semin. Med. Genet.* **157**, 129–135 https://doi.org/10.1002/ajmg.c.30295
21 Tartaglia, M., Gelb, B.D. and Zenker, M. (2011) Noonan syndrome and clinically related disorders. *Best Pract. Res. Clin. Endocrinol. Metab.* **25**, 161–179 https://doi.org/10.1016/j.beem.2010.09.002
22 Vandamme, D., Herrero, A., Al-Mulla, F. and Kolch, W. (2014) Regulation of the MAPK pathway by raf kinase inhibitory protein. *Crit. Rev. Oncogenesis* **19**, 405–415 https://doi.org/10.1615/CritRevOncog.2014011922
23 Morrison, D.K. and Cutler, R.E. (1997) The complexity of Raf-1 regulation. *Curr. Opin. Cell Biol.* **9**, 174–179 https://doi.org/10.1016/S0955-0674(97)80060-9
24 Dhillon, A.S., Hagan, S., Rath, O. and Kolch, W. (2007) MAP kinase signalling pathways in cancer. *Oncogene* **26**, 3279–3290 https://doi.org/10.1038/sj.onc.1210421
25 Roberts, P.J. and Der, C.J. (2007) Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **26**, 3291–3310 https://doi.org/10.1038/sj.onc.1210422
26 Karnoub, A.E. and Weinberg, R.A. (2008) Ras oncogenes: split personalities. *Nat. Rev. Mol. Cell Biol.* **9**, 517–531 https://doi.org/10.1038/nrm2438
27 Rauch, N., Rukhlenko, O.S., Kolch, W. and Kholodenko, B.N. (2016) MAPK kinase signalling dynamics regulate cell fate decisions and drug resistance. *Curr. Opin. Struct. Biol.* **41**, 151–158 https://doi.org/10.1016/j.sbi.2016.07.019
28 Zhang, X.-F., Settleman, J., Kyriakis, J.M., Takeuchi-Suzuki, E., Elledge, S.J., Marshall, M.S. et al. (1993) Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. *Nature* **364**, 308–313 https://doi.org/10.1038/364308a0
29 Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74**, 205–214 https://doi.org/10.1016/0092-8674(93)90307-C
30 Moodie, S.A., Willumsen, B.M., Weber, M.J. and Wolfman, A. (1993) Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* **260**, 1658–1661 https://doi.org/10.1126/science.8503013
31 Warne, P.H., Viciana, P.R. and Downward, J. (1993) Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. *Nature* **364**, 352–355 https://doi.org/10.1038/364352a0
32 Van Aelst, L., Barr, M., Marcus, S., Polverino, A. and Wigler, M. (1993) Complex formation between RAS and RAF and other protein kinases. *Proc. Natl Acad. Sci. U.S.A.* **90**, 6213–6217 https://doi.org/10.1073/pnas.90.13.6213
33 Kyriakis, J.M., App, H., Zhang, X.-F., Banerjee, P., Brautigan, D.L., Rapp, U.R. et al. (1992) Raf-1 activates MAP kinase-kinase. *Nature* **358**, 417–421 https://doi.org/10.1038/358417a0
34 Matallanas, D., Birtwistle, M., Romano, D., Zebisch, A., Rauch, J., von Kriegsheim, A. et al. (2011) Raf family kinases: old dogs have learned new tricks. *Genes Cancer* **2**, 232–260 https://doi.org/10.1177/1947601911407323
35 Baljuls, A., Kholodenko, B.N. and Kolch, W. (2013) It takes two to tango — signalling by dimeric Raf kinases. *Mol. Biosyst.* **9**, 551–558 https://doi.org/10.1039/C2MB25393C
36 Marais, R., Light, Y., Paterson, H.F., Mason, C.S. and Marshall, C.J. (1997) Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. *J. Biol. Chem.* **272**, 4378–4383 https://doi.org/10.1074/jbc.272.7.4378
37 Leicht, D.T., Balan, V., Zhu, J., Kaplun, A., Bronisz, A., Rana, A. et al. (2013) MEK-1 activates C-Raf through a Ras-independent mechanism. *Biochim. Biophys. Acta* **1833**, 976–986 https://doi.org/10.1016/j.bbamcr.2013.01.015
38 Rushworth, L.K., Hindley, A.D., O'Neill, E. and Kolch, W. (2006) Regulation and role of Raf-1/B-Raf heterodimerization. *Mol. Cell. Biol.* **26**, 2262–2272 https://doi.org/10.1128/MCB.26.6.2262-2272.2006
39 Rodriguez-Viciana, P., Oses-Prieto, J., Burlingame, A., Fried, M. and McCormick, F. (2006) A phosphatase holoenzyme comprised of Shoc2/Sur8 and the catalytic subunit of PP1 functions as an M-Ras effector to modulate Raf activity. *Mol. Cell* **22**, 217–230 https://doi.org/10.1016/j.molcel.2006.03.027
40 Freeman, A.K., Ritt, D.A. and Morrison, D.K. (2013) The importance of Raf dimerization in cell signaling. *Small GTPases* **4**, 180–185 https://doi.org/10.4161/sgtp.26117
41 Lavoie, H., Li, J.J., Thevakumaran, N., Therrien, M. and Sicheri, F. (2014) Dimerization-induced allostery in protein kinase regulation. *Trends Biochem. Sci.* **39**, 475–486 https://doi.org/10.1016/j.tibs.2014.08.004
42 Lavoie, H. and Therrien, M. (2015) Regulation of RAF protein kinases in ERK signalling. *Nat. Rev. Mol. Cell Biol.* **16**, 281–298 https://doi.org/10.1038/nrm3979
43 Gutierrez-Erlandsson, S., Herrero-Vidal, P., Fernandez-Alfara, M., Hernandez-Garcia, S., Gonzalo-Flores, S., Mudarra-Rubio, A. et al. (2013) R-RAS2 overexpression in tumors of the human central nervous system. *Mol. Cancer* **12**, 127 https://doi.org/10.1186/1476-4598-12-127
44 Herrmann, C. (2003) Ras-effector interactions: after one decade. *Curr. Opin. Struct. Biol.* **13**, 122–129 https://doi.org/10.1016/S0959-440X(02)00007-6
45 Castellano, E. and Downward, J. (2010) Role of RAS in the regulation of PI 3-kinase. *Curr. Top. Microbiol. Immunol.* **346**, 143–169 PMID:20563706
46 Rajalingam, K., Schreck, R., Rapp, U.R. and Albert, S. (2007) Ras oncogenes and their downstream targets. *Biochim. Biophys. Acta* **1773**, 1177–1195 https://doi.org/10.1016/j.bbamcr.2007.01.012
47 Nakhaei-Rad, S., Haghighi, F., Nouri, P., Rezaei Adariani, S., Lissy, J., Kazemein Jasemi, N.S. et al. (2018) Structural fingerprints, interactions, and signaling networks of RAS family proteins beyond RAS isoforms. *Crit. Rev. Biochem. Mol. Biol.* **53**, 130–156 https://doi.org/10.1080/10409238.2018.1431605
48 Repasky, G.A., Chenette, E.J. and Der, C.J. (2004) Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis? *Trends Cell Biol.* **14**, 639–647 https://doi.org/10.1016/j.tcb.2004.09.014

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https://doi.org/10.1042/BST20170528

49 Wohlgemuth, S., Kiel, C., Krämer, A., Serrano, L., Wittinghofer, F. and Herrmann, C. (2005) Recognizing and defining true Ras binding domains I: biochemical analysis. *J. Mol. Biol.* **348**, 741–758 https://doi.org/10.1016/j.jmb.2005.02.048

50 Nakhaeizadeh, H., Amin, E., Nakhaei-Rad, S., Dvorsky, R. and Ahmadian, M.R. (2016) The RAS-effector interface: isoform-specific differences in the effector binding regions. *PLoS ONE* **11**, e0167145 https://doi.org/10.1371/journal.pone.0167145

51 Patel, M. and Côté, J.-F. (2013) Ras GTPases' interaction with effector domains: breaking the families' barrier. *Commun. Integr. Biol.* **6**, e24298 https://doi.org/10.4161/cib.24298

52 Herrmann, C. and Nassar, N. (1996) Ras and its effectors. *Prog. Biophys. Mol. Biol.* **66**, 1–41 https://doi.org/10.1016/S0079-6107(96)00015-6

53 Mott, H.R. and Owen, D. (2015) Structures of Ras superfamily effector complexes: what have we learnt in two decades? *Crit. Rev. Biochem. Mol. Biol.* **50**, 85–133 https://doi.org/10.3109/10409238.2014.999191

54 Mazhab-Jafari, M.T., Marshall, C.B., Smith, M.J., Gasmi-Seabrook, G.M., Stathopulos, P.B., Inagaki, F. et al. (2015) Oncogenic and RASopathy-associated K-RAS mutations relieve membrane-dependent occlusion of the effector-binding site. *Proc. Natl Acad. Sci. U.S.A.* **112**, 6625–6630 https://doi.org/10.1073/pnas.1419895112

55 Vetter, I.R. and Wittinghofer, A. (2001) The guanine nucleotide-binding switch in three dimensions. *Science* **294**, 1299–1304 https://doi.org/10.1126/science.1062023

56 Drugan, J.K., Khosravi-Far, R., White, M.A., Der, C.J., Sung, Y.-J., Hwang, Y.-W. et al. (1996) Ras interaction with two distinct binding domains in Raf-1 5 be required for Ras transformation. *J. Biol. Chem.* **271**, 233–237 https://doi.org/10.1074/jbc.271.1.233

57 Thapar, R., Williams, J.G. and Campbell, S.L. (2004) NMR characterization of full-length farnesylated and non-farnesylated H-Ras and its implications for Raf activation. *J. Mol. Biol.* **343**, 1391–1408 https://doi.org/10.1016/j.jmb.2004.08.106

58 Athuluri-Divakar, S.K., Vasquez-Del Carpio, R., Dutta, K., Baker, S.J., Cosenza, S.C., Basu, I. et al. (2016) A small molecule RAS-mimetic disrupts RAS association with effector proteins to block signaling. *Cell* **165**, 643–655 https://doi.org/10.1016/j.cell.2016.03.045

59 Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F. and Wittinghofer, A. (1995) The 2.2 A crystal structure of the Ras-binding domain of the serine/threonine kinase c-Raf1 in complex with Rap1A and a GTP analogue. *Nature* **375**, 554–560 https://doi.org/10.1038/375554a0

60 Huang, L., Hofer, F., Martin, G.S. and Kim, S.-H. (1998) Structural basis for the interaction of Ras with RalGDS. *Nat. Struct. Biol.* **5**, 422–426 https://doi.org/10.1038/nsb0698-422

61 Stieglitz, B., Bee, C., Schwarz, D., Yildiz, O., Moshnikova, A., Khokhlatchev, A. et al. (2008) Novel type of Ras effector interaction established between tumour suppressor NORE1A and Ras switch II. *EMBO J.* **27**, 1995–2005 https://doi.org/10.1038/emboj.2008.125

62 Pacold, M.E., Suire, S., Perisic, O., Lara-Gonzalez, S., Davis, C.T., Walker, E.H. et al. (2000) Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma. *Cell* **103**, 931–943 https://doi.org/10.1016/S0092-8674(00)00196-3

63 Bunney, T.D., Harris, R., Gandarillas, N.L., Josephs, M.B., Roe, S.M., Sorli, S.C. et al. (2006) Structural and mechanistic insights into ras association domains of phospholipase C epsilon. *Mol. Cell* **21**, 495–507 https://doi.org/10.1016/j.molcel.2006.01.008

64 Marshall, M.S. (1993) The effector interactions of p21ras. *Trends Biochem. Sci.* **18**, 250–254 https://doi.org/10.1016/0968-0004(93)90175-M

65 Wittinghofer, A. and Herrmann, C. (1995) Ras-effector interactions, the problem of specificity. *FEBS Lett.* **369**, 52–56 https://doi.org/10.1016/0014-5793(95)00667-X

66 Marshall, C.J. (1996) Ras effectors. *Curr. Opin. Cell Biol.* **8**, 197–204 https://doi.org/10.1016/S0955-0674(96)80066-4

67 Wittinghofer, A. and Nassar, N. (1996) How Ras-related proteins talk to their effectors. *Trends Biochem. Sci.* **21**, 488–491 https://doi.org/10.1016/S0968-0004(96)10064-5

68 Wittinghofer, A. (1998) Signal transduction via Ras. *Biol. Chem.* **379**, 933–937 PMID:9792425

69 Corbett, K.D. and Alber, T. (2001) The many faces of Ras: recognition of small GTP-binding proteins. *Trends Biochem. Sci.* **26**, 710–716 https://doi.org/10.1016/S0968-0004(01)01974-0

70 Spaargaren, M., Martin, G.A., McCormick, F., Fernandez-Sarabia, M.J. and Bischoff, J.R. (1994) The Ras-related protein R-ras interacts directly with Raf-1 in a GTP-dependent manner. *Biochem. J.* **300**, 303–307 https://doi.org/10.1042/bj3000303

71 Koide, H., Satoh, T., Nakafuku, M. and Kaziro, Y. (1993) GTP-dependent association of Raf-1 with Ha-Ras: identification of Raf as a target downstream of Ras in mammalian cells. *Proc. Natl Acad. Sci. U.S.A.* **90**, 8683–8686 https://doi.org/10.1073/pnas.90.18.8683

72 Barnard, D., Diaz, B., Hettich, L., Chuang, E., Zhang, X.F., Avruch, J. et al. (1995) Identification of the sites of interaction between c-Raf-1 and Ras-GTP. *Oncogene* **10**, 1283–1290 PMID:7731678

73 Herrmann, C., Martin, G.A. and Wittinghofer, A. (1995) Quantitative analysis of the complex between p21 and the ras-binding domain of the human raf-1 protein kinase. *J. Biol. Chem.* **270**, 2901–2905 https://doi.org/10.1074/jbc.270.7.2901

74 Nassar, N., Horn, G., Herrmann, C., Block, C., Janknecht, R. and Wittinghofer, A. (1996) Ras/Rap effector specificity determined by charge reversal. *Nat. Struct. Biol.* **3**, 723–729 https://doi.org/10.1038/nsb0896-723

75 Lavoie, H., Sahmi, M., Maisonneuve, P., Marullo, S.A., Thevakumaran, N., Jin, T. et al. (2018) MEK drives BRAF activation through allosteric control of KSR proteins. *Nature* **554**, 549–553 https://doi.org/10.1038/nature25478

76 Filchtinski, D., Sharabi, O., Rüppel, A., Vetter, I.R., Herrmann, C. and Shifman, J.M. (2010) What makes Ras an efficient molecular switch: a computational, biophysical, and structural study of Ras-GDP interactions with mutants of Raf. *J Mol Biol.* **399**, 422–435 https://doi.org/10.1016/j.jmb.2010.03.046

77 Fetics, S.K., Guterres, H., Kearney, B.M., Buhrman, G., Ma, B., Nussinov, R. et al. (2015) Allosteric effects of the oncogenic RasQ61L mutant on Raf-RBD. *Structure* **23**, 505–516 https://doi.org/10.1016/j.str.2014.12.017

78 Mott, H.R., Carpenter, J.W., Zhong, S., Ghosh, S., Bell, R.M. and Campbell, S.L. (1996) The solution structure of the Raf-1 cysteine-rich domain: a novel ras and phospholipid binding site. *Proc. Natl Acad. Sci.* **93**, 8312–8317 https://doi.org/10.1073/pnas.93.16.8312

79 Molzan, M., Schumacher, B., Ottmann, C., Baljuls, A., Polzien, L., Weyand, M. et al. (2010) Impaired binding of 14-3-3 to C-RAF in Noonan syndrome suggests new approaches in diseases with increased Ras signaling. *Mol. Cell. Biol.* **30**, 4698–4711 https://doi.org/10.1128/MCB.01636-09

80 Molzan, M. and Ottmann, C. (2012) Synergistic binding of the phosphorylated S233-and S259-binding sites of C-RAF to one 14-3-3ζ dimer. *J. Mol. Biol.* **423**, 486–495 https://doi.org/10.1016/j.jmb.2012.08.009

81 Molzan, M., Kasper, S., Röglin, L., Skwarczynska, M., Sassa, T., Inoue, T. et al. (2013) Stabilization of physical RAF/14-3-3 interaction by cotylenin A as treatment strategy for RAS mutant cancers. *ACS Chem. Biol.* **8**, 1869–1875 https://doi.org/10.1021/cb4003464

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Biochemical Society Transactions (2018) **46** 1393–1406
https://doi.org/10.1042/BST20170528

82 Wan, P.T., Garnett, M.J., Roe, S.M., Lee, S., Niculescu-Duvaz, D., Good, V.M. et al. (2004) Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* **116**, 855–867 https://doi.org/10.1016/S0092-8674(04)00215-6
83 Haling, J.R., Sudhamsu, J., Yen, I., Sideris, S., Sandoval, W., Phung, W. et al. (2014) Structure of the BRAF-MEK complex reveals a kinase activity independent role for BRAF in MAPK signaling. *Cancer Cell* **26**, 402–413 https://doi.org/10.1016/j.ccr.2014.07.007
84 Sprang, S.R. (1995) How Ras works: structure of a Rap-Raf complex. *Structure* **3**, 641–643 https://doi.org/10.1016/S0969-2126(01)00198-8
85 Erijman, A. and Shifman, J.M. (2016) RAS/effector interactions from structural and biophysical perspective. *Mini-Rev. Med. Chem.* **16**, 370–375 https://doi.org/10.2174/1389557515666151001141838
86 Block, C., Janknecht, R., Herrmann, C., Nassar, N. and Wittinghofer, A. (1996) Quantitative structure-activity analysis correlating Ras/Raf interaction in vitro to Raf activation in vivo. *Nat. Struct. Biol.* **3**, 244–251 https://doi.org/10.1038/nsb0396-244
87 Fridman, M., Maruta, H., Gonez, J., Walker, F., Treutlein, H., Zeng, J. et al. (2000) Point mutants of c-raf-1 RBD with elevated binding to v-Ha-Ras. *J. Biol. Chem.* **275**, 30363–30371 https://doi.org/10.1074/jbc.M003193200
88 Zehir, A., Benayed, R., Shah, R.H., Syed, A., Middha, S., Kim, H.R. et al. (2017) Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. *Nat. Med.* **23**, 703 https://doi.org/10.1038/nm.4333
89 Zeng, J., Fridman, M., Maruta, H., Treutlein, H.R. and Simonson, T. (1999) Protein-protein recognition: an experimental and computational study of the R89K mutation in Raf and its effect on Ras binding. *Protein Sci.* **8**, 50–64 https://doi.org/10.1110/ps.8.1.50
90 Weber, C.K., Slupsky, J.R., Herrmann, C., Schuler, M., Rapp, U.R. and Block, C. (2000) Mitogenic signaling of Ras is regulated by differential interaction with Raf isozymes. *Oncogene* **19**, 169–176 https://doi.org/10.1038/sj.onc.1203261
91 Lim, B., Mun, J., Kim, J.-H., Kim, C.W., Roh, S.A., Cho, D.-H. et al. (2015) Genome-wide mutation profiles of colorectal tumors and associated liver metastases at the exome and transcriptome levels. *Oncotarget* **6**, 22179
92 Lefebvre, C., Bachelot, T., Filleron, T., Pedrero, M., Campone, M., Soria, J.-C. et al. (2016) Mutational profile of metastatic breast cancers: a retrospective analysis. *PLoS Med.* **13**, e1002201 https://doi.org/10.1371/journal.pmed.1002201
93 Terada, T., Ito, Y., Shirouzu, M., Tateno, M., Hashimoto, K., Kigawa, T. et al. (1999) Nuclear magnetic resonance and molecular dynamics studies on the interactions of the Ras-binding domain of Raf-1 with wild-type and mutant Ras proteins. *J. Mol. Biol.* **286**, 219–232 https://doi.org/10.1006/jmbi.1998.2472
94 Kiel, C., Filchtinski, D., Spoerner, M., Schreiber, G., Kalbitzer, H.R. and Herrmann, C. (2009) Improved binding of raf to Ras.GDP is correlated with biological activity. *J. Biol. Chem.* **284**, 31893–31902 https://doi.org/10.1074/jbc.M109.031153
95 Chavan, T.S., Muratcioglu, S., Marszalek, R., Jang, H., Keskin, O., Gursoy, A. et al. (2015) Plasma membrane regulates Ras signaling networks. *Cell Logist.* **5**, e1136374 https://doi.org/10.1080/21592799.2015.1136374
96 Bondeva, T., Balla, A., Várnai, P. and Balla, T. (2002) Structural determinants of Ras-Raf interaction analyzed in live cells. *Mol. Biol. Cell.* **13**, 2323–2333 https://doi.org/10.1091/mbc.e02-01-0019
97 Nekhoroshkova, E., Albert, S., Becker, M. and Rapp, U.R. (2009) A-RAF kinase functions in ARF6 regulated endocytic membrane traffic. *PLoS ONE* **4**, e4647 https://doi.org/10.1371/journal.pone.0004647
98 Giannakis, M., Mu, X.J., Shukla, S.A., Qian, Z.R., Cohen, O., Nishihara, R. et al. (2016) Genomic correlates of immune-cell infiltrates in colorectal carcinoma. *Cell Rep.* **15**, 857–865 https://doi.org/10.1016/j.celrep.2016.03.075
99 George, J., Lim, J.S., Jang, S.J., Cun, Y., Ozretić, L., Kong, G. et al. (2015) Comprehensive genomic profiles of small cell lung cancer. *Nature* **524**, 47 https://doi.org/10.1038/nature14664
100 Mouradov, D., Sloggett, C., Jorissen, R.N., Love, C.G., Li, S., Burgess, A.W. et al. (2014) Colorectal cancer cell lines are representative models of the main molecular subtypes of primary cancer. *Cancer Res.* **74**, 3238–3247 https://doi.org/10.1158/0008-5472.CAN-14-0013
101 Fischer, A., Hekman, M., Kuhlmann, J., Rubio, I., Wiese, S. and Rapp, U.R. (2007) B- and C-RAF display essential differences in their binding to Ras: the isotype-specific N terminus of B-RAF facilitates Ras binding. *J. Biol. Chem.* **282**, 26503–26516 https://doi.org/10.1074/jbc.M607458200
102 Chuang, E., Barnard, D., Hettich, L., Zhang, X.F., Avruch, J. and Marshall, M.S. (1994) Critical binding and regulatory interactions between Ras and Raf occur through a small, stable N-terminal domain of Raf and specific Ras effector residues. *Mol. Cell. Biol.* **14**, 5318–5325 https://doi.org/10.1128/MCB.14.8.5318
103 Li, Z.-L., Prakash, P. and Buck, M. (2018) A 'Tug of War' maintains a dynamic protein-membrane complex: molecular dynamics simulations of C-Raf RBD-CRD bound to K-Ras4B at an anionic membrane. *ACS Cent. Sci.* **4**, 298–305 https://doi.org/10.1021/acscentsci.7b00593
104 Abaan, O.D., Polley, E.C., Davis, S.R., Zhu, Y.J., Bilke, S., Walker, R.L. et al. (2013) The exomes of the NCI-60 panel: a genomic resource for cancer biology and systems pharmacology. *Cancer Res.* **73**, 4372–4382 https://doi.org/10.1158/0008-5472.CAN-12-3342
105 Li, S., Jang, H., Zhang, J. and Nussinov, R. (2018) Raf-1 cysteine-Rich domain increases the affinity of K-Ras/Raf at the membrane, promoting MAPK signaling. *Structure* **26**, 513–525.e2 https://doi.org/10.1016/j.str.2018.01.011
106 Brtva, T.R., Drugan, J.K., Ghosh, S., Terrell, R.S., Campbell-Burk, S., Bell, R.M. et al. (1995) Two distinct Raf domains mediate interaction with Ras. *J. Biol. Chem.* **270**, 9809–9812 https://doi.org/10.1074/jbc.270.17.9809
107 Hu, C.-D., Kariya, K.-, Tamada, M., Akasaka, K., Shirouzu, M., Yokoyama, S. et al. (1995) Cysteine-rich region of Raf-1 interacts with activator domain of post-translationally modified Ha-Ras. *J. Biol. Chem.* **270**, 30274–30277 https://doi.org/10.1074/jbc.270.51.30274
108 Hu, C.-D., Kariya, K.-i., Kotani, G., Shirouzu, M., Yokoyama, S. and Kataoka, T. (1997) Coassociation of Rap1A and Ha-Ras with Raf-1 N-terminal region interferes with ras-dependent activation of Raf-1. *J. Biol. Chem.* **272**, 11702–11705 https://doi.org/10.1074/jbc.272.18.11702
109 Roy, S., Lane, A., Yan, J., McPherson, R. and Hancock, J.F. (1997) Activity of plasma membrane-recruited Raf-1 is regulated by Ras via the Raf zinc finger. *J. Biol. Chem.* **272**, 20139–20145 https://doi.org/10.1074/jbc.272.32.20139
110 Luo, Z., Diaz, B., Marshall, M.S. and Avruch, J. (1997) An intact Raf zinc finger is required for optimal binding to processed Ras and for ras-dependent Raf activation in situ. *Mol. Cell. Biol.* **17**, 46–53 https://doi.org/10.1128/MCB.17.1.46
111 Daub, M., Jöckel, J., Quack, T., Weber, C.K., Schmitz, F., Rapp, U.R. et al. (1998) The RafC1 cysteine-rich domain contains multiple distinct regulatory epitopes which control Ras-dependent Raf activation. *Mol. Cell. Biol.* **18**, 6698–6710 https://doi.org/10.1128/MCB.18.11.6698
112 Okada, T., Hu, C.-D., Jin, T.-G., Kariya, K.-i., Yamawaki-Kataoka, Y. and Kataoka, T. (1999) The strength of interaction at the Raf cysteine-rich domain is a critical determinant of response of Raf to Ras family small GTPases. *Mol. Cell. Biol.* **19**, 6057–6064 https://doi.org/10.1128/MCB.19.9.6057

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https://doi.org/10.1042/BST20170528

113 Williams, J.G., Drugan, J.K., Yi, G.-S., Clark, G.J., Der, C.J. and Campbell, S.L. (2000) Elucidation of binding determinants and functional consequences of Ras/Raf-cysteine-rich domain interactions. *J. Biol. Chem.* **275**, 22172–22179 https://doi.org/10.1074/jbc.M000397200
114 Chan, E.Y., Stang, S.L., Bottorff, D.A. and Stone, J.C. (2002) Mutations in conserved regions 1, 2, and 3 of Raf-1 that activate transforming activity. *Mol. Carcinog.* **33**, 189–197 https://doi.org/10.1002/mc.10031
115 Hibino, K., Shibata, T., Yanagida, T. and Sako, Y. (2011) Activation kinetics of RAF protein in the ternary complex of RAF, RAS-GTP, and kinase on the plasma membrane of living cells: single-molecule imaging analysis. *J. Biol. Chem.* **286**, 36460–36468 https://doi.org/10.1074/jbc.M111.262675
116 Ke, H., Matsumoto, S., Murashima, Y., Taniguchi-Tamura, H., Miyamoto, R., Yoshikawa, Y. et al. (2017) Structural basis for intramolecular interaction of post-translationally modified H-Ras*GTP prepared by protein ligation. *FEBS Lett.* **591**, 2470–2481 https://doi.org/10.1002/1873-3468.12759
117 Ghosh, S., Xie, W.Q., Quest, A.F., Mabrouk, G.M., Strum, J.C. and Bell, R.M. (1994) The cysteine-rich region of raf-1 kinase contains zinc, translocates to liposomes, and is adjacent to a segment that binds GTP-ras. *J. Biol. Chem.* **269**, 10000–10007
118 Hekman, M., Hamm, H., Villar, A.V., Bader, B., Kuhlmann, J., Nickel, J. et al. (2002) Associations of B- and C-Raf with cholesterol, phosphatidylserine, and lipid second messengers: preferential binding of Raf to artificial lipid rafts. *J. Biol. Chem.* **277**, 24090–24102 https://doi.org/10.1074/jbc.M200576200
119 Ghosh, S., Strum, J.C., Sciorra, V.A., Daniel, L. and Bell, R.M. (1996) Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-*O*-tetradecanoylphorbol-13-acetate-stimulated Madin-Darby canine kidney cells. *J. Biol. Chem.* **271**, 8472–8480 https://doi.org/10.1074/jbc.271.14.8472
120 Zang, M., Waelde, C.A., Xiang, X., Rana, A., Wen, R. and Luo, Z. (2001) Microtubule integrity regulates Pak leading to Ras-independent activation of Raf-1. Insights into mechanisms of Raf-1 activation. *J. Biol. Chem.* **276**, 25157–25165 https://doi.org/10.1074/jbc.M100152200
121 Improta-Brears, T., Ghosh, S. and Bell, R.M. (1999) Mutational analysis of Raf-1 cysteine rich domain: requirement for a cluster of basic aminoacids for interaction with phosphatidylserine. *Mol. Cell. Biochem.* **198**, 171–178 https://doi.org/10.1023/A:1006981411691
122 Sarkozy, A., Carta, C., Moretti, S., Zampino, G., Digilio, M.C., Pantaleoni, F. et al. (2009) Germline *BRAF* mutations in Noonan, LEOPARD, and cardiofaciocutaneous syndromes: molecular diversity and associated phenotypic spectrum. *Hum. Mutat.* **30**, 695–702 https://doi.org/10.1002/humu.20955
123 Abankwa, D., Gorfe, A.A. and Hancock, J.F. (2007) Ras nanoclusters: molecular structure and assembly. *Semin. Cell Dev. Biol.* **18**, 599–607 https://doi.org/10.1016/j.semcdb.2007.08.003
124 Abankwa, D., Gorfe, A.A., Inder, K. and Hancock, J.F. (2010) Ras membrane orientation and nanodomain localization generate isoform diversity. *Proc. Natl Acad. Sci. U.S.A.* **107**, 1130–1135 https://doi.org/10.1073/pnas.0903907107
125 Cirstea, I.C., Kutsche, K., Dvorsky, R., Gremer, L., Carta, C., Horn, D. et al. (2010) A restricted spectrum of NRAS mutations causes Noonan syndrome. *Nat. Genet.* **42**, 27–29 https://doi.org/10.1038/ng.497
126 Zhou, Y. and Hancock, J.F. (2018) Deciphering lipid codes: K-Ras as a paradigm. *Traffic* **19**, 157–165 https://doi.org/10.1111/tra.12541
127 Kapoor, S., Triola, G., Vetter, I.R., Erlkamp, M., Waldmann, H. and Winter, R. (2012) Revealing conformational substates of lipidated N-Ras protein by pressure modulation. *Proc. Natl Acad. Sci. U.S.A.* **109**, 460–465 https://doi.org/10.1073/pnas.1110553109
128 Vogel, A., Nikolaus, J., Weise, K., Triola, G., Waldmann, H., Winter, R. et al. (2014) Interaction of the human N-Ras protein with lipid raft model membranes of varying degrees of complexity. *Biol. Chem.* **395**, 779–789 https://doi.org/10.1515/hsz-2013-0294
129 Sperlich, B., Kapoor, S., Waldmann, H., Winter, R. and Weise, K. (2016) Regulation of K-Ras4B membrane binding by calmodulin. *Biophys. J.* **111**, 113–122 https://doi.org/10.1016/j.bpj.2016.05.042
130 Erwin, N., Patra, S., Dwivedi, M., Weise, K. and Winter, R. (2017) Influence of isoform-specific Ras lipidation motifs on protein partitioning and dynamics in model membrane systems of various complexity. *Biol. Chem.* **398**, 290–263 https://doi.org/10.1515/hsz-2016-0289
131 Dumaz, N. and Marais, R. (2003) Protein kinase A blocks Raf-1 activity by stimulating 14-3-3 binding and blocking Raf-1 interaction with Ras. *J. Biol. Chem.* **278**, 29819–29823 https://doi.org/10.1074/jbc.C300182200
132 Sendoh, H., Hu, C.-D., Wu, D., Song, C., Yamawaki-Kataoka, Y., Kotani, J. et al. (2000) Role of Raf-1 conserved region 2 in regulation of Ras-dependent Raf-1 activation. *Biochem. Biophys. Res. Commun.* **271**, 596–602 https://doi.org/10.1006/bbrc.2000.2674
133 Dhillon, A.S., Meikle, S., Yazici, Z., Eulitz, M. and Kolch, W. (2002) Regulation of Raf-1 activation and signalling by dephosphorylation. *EMBO J.* **21**, 64–71 https://doi.org/10.1093/emboj/21.1.64
134 Dhillon, A.S., Pollock, C., Steen, H., Shaw, P.E., Mischak, H. and Kolch, W. (2002) Cyclic AMP-dependent kinase regulates Raf-1 kinase mainly by phosphorylation of serine 259. *Mol. Cell. Biol.* **22**, 3237–3246 https://doi.org/10.1128/MCB.22.10.3237-3246.2002
135 Zimmermann, S. and Moelling, K. (1999) Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* **286**, 1741–1744 https://doi.org/10.1126/science.286.5445.1741
136 Rommel, C., Clarke, B.A., Zimmermann, S., Nunez, L., Rossman, R., Reid, K. et al. (1999) Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* **286**, 1738–1741 https://doi.org/10.1126/science.286.5445.1738
137 Pandit, B., Sarkozy, A., Pennacchio, L.A., Carta, C., Oishi, K., Martinelli, S. et al. (2007) Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. *Nat. Genet.* **39**, 1007–1012 https://doi.org/10.1038/ng2073
138 Razzaque, M.A., Nishizawa, T., Komoike, Y., Yagi, H., Furutani, M., Amo, R. et al. (2007) Germline gain-of-function mutations in RAF1 cause Noonan syndrome. *Nat. Genet.* **39**, 1013–1017 https://doi.org/10.1038/ng2078
139 Kobayashi, T., Aoki, Y., Niihori, T., Cavé, H., Verloes, A., Okamoto, N. et al. (2010) Molecular and clinical analysis of *RAF1* in Noonan syndrome and related disorders: dephosphorylation of serine 259 as the essential mechanism for mutant activation. *Hum. Mutat.* **31**, 284–294 https://doi.org/10.1002/humu.21187
140 Hodis, E., Watson, I.R., Kryukov, G.V., Arold, S.T., Imielinski, M., Theurillat, J.-P. et al. (2012) A landscape of driver mutations in melanoma. *Cell* **150**, 251–263 https://doi.org/10.1016/j.cell.2012.06.024
141 Martin, D., Abba, M.C., Molinolo, A.A., Vitale-Cross, L., Wang, Z., Zaida, M. et al. (2014) The head and neck cancer cell oncogenome: a platform for the development of precision molecular therapies. *Oncotarget* **5**, 8906–8923 PMID:25275298
142 Shi, J., Hua, X., Zhu, B., Ravichandran, S., Wang, M., Nguyen, C. et al. (2016) Somatic genomics and clinical features of lung adenocarcinoma: a retrospective study. *PLoS Med.* **13**, e1002162 https://doi.org/10.1371/journal.pmed.1002162

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https://doi.org/10.1042/BST20170528

143 Imielinski, M., Greulich, H., Kaplan, B., Araujo, L., Amann, J., Horn, L. et al. (2014) Oncogenic and sorafenib-sensitive ARAF mutations in lung adenocarcinoma. *J. Clin. Invest.* **124**, 1582–1586 https://doi.org/10.1172/JCI72763
144 Stevers, L.M., Sijbesma, E., Botta, M., MacKintosh, C., Obsil, T., Landrieu, I. et al. (2018) Modulators of 14-3-3 protein-protein interactions. *J. Med. Chem.* **61**, 3755–3778 https://doi.org/10.1021/acs.jmedchem.7b00574
145 Yaffe, M.B., Rittinger, K., Volinia, S., Caron, P.R., Aitken, A., Leffers, H. et al. (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell* **91**, 961–971 https://doi.org/10.1016/S0092-8674(00)80487-0
146 Shen, Y.H., Godlewski, J., Bronisz, A., Zhu, J., Comb, M.J., Avruch, J. et al. (2003) Significance of 14-3-3 self-dimerization for phosphorylation-dependent target binding. *Mol. Biol. Cell* **14**, 4721–4733 https://doi.org/10.1091/mbc.e02-12-0821
147 Tzivion, G. and Avruch, J. (2002) 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. *J. Biol. Chem.* **277**, 3061–3064 https://doi.org/10.1074/jbc.R100059200
148 Muslin, A.J., Tanner, J.W., Allen, P.M. and Shaw, A.S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* **84**, 889–897 https://doi.org/10.1016/S0092-8674(00)81067-3
149 Morrison, D.K., Heidecker, G., Rapp, U.R. and Copeland, T.D. (1993) Identification of the major phosphorylation sites of the Raf-1 kinase. *J. Biol. Chem.* **268**, 17309–17316 PMID:8349614
150 Roskoski, R.J. (2010) RAF protein-serine/threonine kinases: structure and regulation. *Biochem. Biophys. Res. Commun.* **399**, 313–317 https://doi.org/10.1016/j.bbrc.2010.07.092
151 Alessi, D.R., Saito, Y., Campbell, D.G., Cohen, P., Sithanandam, G., Rapp, U. et al. (1994) Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. *EMBO J.* **13**, 1610–1619 PMID:8157000
152 Roskoski, R.J. (2012) MEK1/2 dual-specificity protein kinases: structure and regulation. *Biochem. Biophys. Res. Commun.* **417**, 5–10 https://doi.org/10.1016/j.bbrc.2011.11.145
153 Hatzivassiliou, G., Song, K., Yen, I., Brandhuber, B.J., Anderson, D.J., Alvarado, R. et al. (2010) RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature* **464**, 431–400 https://doi.org/10.1038/nature08833
154 Yao, Z., Torres, N.M., Tao, A., Gao, Y., Luo, L., Li, Q. et al. (2015) BRAF mutants evade ERK-dependent feedback by different mechanisms that determine their sensitivity to pharmacologic inhibition. *Cancer Cell* **28**, 370–383 https://doi.org/10.1016/j.ccell.2015.08.001
155 Yao, Z., Yaeger, R., Rodrik-Outmezguine, V.S., Tao, A., Torres, N.M., Chang, M.T. et al. (2017) Tumours with class 3 BRAF mutants are sensitive to the inhibition of activated RAS. *Nature* **548**, 234 https://doi.org/10.1038/nature23291
156 Karoulia, Z., Wu, Y., Ahmed, T.A., Xin, Q., Bollard, J., Krepler, C. et al. (2016) An integrated model of RAF inhibitor action predicts inhibitor activity against oncogenic BRAF signaling. *Cancer Cell* **30**, 485–498 https://doi.org/10.1016/j.ccell.2016.06.024
157 Brown, M.D. and Sacks, D.B. (2009) Protein scaffolds in MAP kinase signalling. *Cell. Signal.* **21**, 462–469 https://doi.org/10.1016/j.cellsig.2008.11.013
158 Morrison, D.K. and Davis, R.J. (2003) Regulation of MAP kinase signaling modules by scaffold proteins in mammals. *Ann. Rev. Cell Dev. Biol.* **19**, 91–118 https://doi.org/10.1146/annurev.cellbio.19.111401.091942
159 Liang, Y. and Sheikh, F. (2016) Scaffold proteins regulating extracellular regulated kinase function in cardiac hypertrophy and disease. *Front. Pharmacol.* **7**, 37 https://doi.org/10.3389/fphar.2016.00037
160 Shaul, Y.D. and Seger, R. (2007) The MEK/ERK cascade: from signaling specificity to diverse functions. *Biochim. Biophys. Acta* **1773**, 1213–1226 https://doi.org/10.1016/j.bbamcr.2006.10.005
161 Wortzel, I. and Seger, R. (2011) The ERK cascade: distinct functions within various subcellular organelles. *Genes Cancer* **2**, 195–209 https://doi.org/10.1177/1947601911407328
162 Kornfeld, K., Hom, D.B. and Horvitz, H.R. (1995) The ksr-1 gene encodes a novel protein kinase involved in Ras-mediated signaling in *C. elegans*. *Cell* **83**, 903–913 https://doi.org/10.1016/0092-8674(95)90206-6
163 Sundaram, M. and Han, M. (1995) The *C. elegans* ksr-1 gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. *Cell* **83**, 889–901 https://doi.org/10.1016/0092-8674(95)90205-8
164 Therrien, M., Chang, H.C., Solomon, N.M., Karim, F.D., Wassarman, D.A. and Rubin, G.M. (1995) KSR, a novel protein kinase required for RAS signal transduction. *Cell* **83**, 879–888 https://doi.org/10.1016/0092-8674(95)90204-X
165 Vomastek, T., Schaeffer, H.-J., Tarcsafalvi, A., Smolkin, M.E., Bissonette, E.A. and Weber, M.J. (2004) Modular construction of a signaling scaffold: MORG1 interacts with components of the ERK cascade and links ERK signaling to specific agonists. *Proc. Natl Acad. Sci. U.S.A.* **101**, 6981–6986 https://doi.org/10.1073/pnas.0305894101
166 Schaeffer, H.J., Catling, A.D., Eblen, S.T., Collier, L.S., Krauss, A. and Weber, M.J. (1998) MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. *Science* **281**, 1668–1671 https://doi.org/10.1126/science.281.5383.1668
167 Ishibe, S., Joly, D., Zhu, X. and Cantley, L.G. (2003) Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis. *Mol. Cell* **12**, 1275–1285 https://doi.org/10.1016/S1097-2765(03)00406-4
168 McDonald, P.H., Chow, C.-W., Miller, W.E., Laporte, S.A., Field, M.E., Lin, F.-T. et al. (2000) β-Arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* **290**, 1574–1577 https://doi.org/10.1126/science.290.5496.1574
169 Karandikar, M., Xu, S. and Cobb, M.H. (2000) MEKK1 binds raf-1 and the ERK2 cascade components. *J. Biol. Chem.* **275**, 40120–40127 https://doi.org/10.1074/jbc.M005926200
170 Sheikh, F., Raskin, A., Chu, P.-H., Lange, S., Domenighetti, A.A., Zheng, M. et al. (2008) An FHL1-containing complex within the cardiomyocyte sarcomere mediates hypertrophic biomechanical stress responses in mice. *J. Clin. Invest.* **118**, 3870–3880 https://doi.org/10.1172/JCI34472
171 Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D. and Therrien, M. (2002) KSR is a scaffold required for activation of the ERK/MAPK module. *Genes Dev.* **16**, 427–438 https://doi.org/10.1101/gad.962902
172 Ritt, D.A., Daar, I.O. and Morrison, D.K. (2006) KSR regulation of the Raf-MEK-ERK cascade. *Methods Enzymol.* **407**, 224–237 https://doi.org/10.1016/S0076-6879(05)07019-9
173 Nguyen, A., Burack, W.R., Stock, J.L., Kortum, R., Chaika, O.V., Afkarian, M. et al. (2002) Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. *Mol. Cell. Biol.* **22**, 3035–3045 https://doi.org/10.1128/MCB.22.9.3035-3045.2002
174 Brennan, D.F., Dar, A.C., Hertz, N.T., Chao, W.C., Burlingame, A.L., Shokat, K.M. et al. (2011) A Raf-induced allosteric transition of KSR stimulates phosphorylation of MEK. *Nature* **472**, 366 https://doi.org/10.1038/nature09860

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https://doi.org/10.1042/BST20170528

175 Baljuls, A., Dobrzyński, M., Rauch, J., Rauch, N. and Kolch, W. (2016) Stabilization of C-RAF:KSR1 complex by DiRas3 reduces availability of C-RAF for dimerization with B-RAF. *Cell Signal.* **28**, 1451–1462 https://doi.org/10.1016/j.cellsig.2016.06.019
176 Li, W., Melnick, M. and Perrimon, N. (1998) Dual function of Ras in Raf activation. *Development* **125**, 4999–5008 PMID: 9811584
177 Anderson, D.H. (2006) Role of lipids in the MAPK signaling pathway. *Prog. Lipid Res.* **45**, 102–119 https://doi.org/10.1016/j.plipres.2005.12.003
178 Yoon, S. and Seger, R. (2006) The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. *Growth Factors* **24**, 21–44 https://doi.org/10.1080/02699050500284218
179 Cseh, B., Doma, E. and Baccarini, M. (2014) 'RAF' neighborhood: protein-protein interaction in the Raf/Mek/Erk pathway. *FEBS Lett.* **588**, 2398–2406 https://doi.org/10.1016/j.febslet.2014.06.025
180 Blaževitš, O., Mideksa, Y.G., Šolman, M., Ligabue, A., Ariotti, N., Nakhaeizadeh, H. et al. (2016) Galectin-1 dimers can scaffold Raf-effectors to increase H-ras nanoclustering. *Sci. Rep.* **6**, 24165 https://doi.org/10.1038/srep24165
181 Chen, J., Fujii, K., Zhang, L., Roberts, T. and Fu, H. (2001) Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. *Proc. Natl Acad. Sci. U.S.A.* **98**, 7783–7788 https://doi.org/10.1073/pnas.141224398
182 Romano, D., Nguyen, L.K., Matallanas, D., Halasz, M., Doherty, C., Kholodenko, B.N. et al. (2014) Protein interaction switches coordinate Raf-1 and MST2/Hippo signalling. *Nat. Cell Biol.* **16**, 673–684 https://doi.org/10.1038/ncb2986
183 Varga, A., Ehrenreiter, K., Aschenbrenner, B., Kocieniewski, P., Kochanczyk, M., Lipniacki, T. et al. (2017) RAF1/BRAF dimerization integrates the signal from RAS to ERK and ROKalpha. *Sci. Signal.* **10**, eaai8482 https://doi.org/10.1126/scisignal.aai8482

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Supplement B: Structural fingerprints, interactions, and signaling networks of RAS family proteins beyond RAS isoforms

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REVIEW ARTICLE

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Structural fingerprints, interactions, and signaling networks of RAS family proteins beyond RAS isoforms

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ABSTRACT

Among the signaling molecules indirectly linked to many different cell surface receptors, RAS proteins essentially respond to a diverse range of extracellular cues. They control activities of multiple signaling pathways and consequently a wide array of cellular processes, including survival, growth, adhesion, migration, and differentiation. Any dysregulation of these pathway leads, thus, to cancer, developmental disorders, metabolic, and cardiovascular diseases. The biochemistry of RAS family proteins has become multifaceted since the discovery of the first members, more than 40 years ago. Substantial knowledge has been attained about molecular mechanisms underlying post-translational modification, membrane localization, regulation, and signal transduction through diverse effector molecules. However, the increasing complexity of the underlying signaling mechanisms is considerable, in part due to multiple effector pathways, crosstalks between them and eventually feedback mechanisms. Here, we take a broad view of regulatory and signaling networks of all RAS family proteins that extends beyond RAS paralogs. As described in this review, a lot is known but a lot has to be discovered yet.

Graphical abstract: The RAS paralogs, KRAS4B, NRAS, and HRAS, are the best investigated members of the RAS family, not only because of their oncogenic capacity. This protein family, however, contains 22 additional isoforms and paralogs, most of which are distantly related, with typically 20–30% amino acid identity, although they share a conserved GTP-binding domain [the color spectrum goes from white (for identical) through yellow and orange (for partially conserved) to red (for highly variable amino acids). RAS family proteins control a wide array of signaling pathways and cellular processes distinct from those controlled by RAS paralogs. This review focuses on common features and differences of RAS family proteins regarding their structure, function, regulation, signaling, and involvement in diseases.

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Historical background

The history of the RAS protein family dates back in 1960s, when the highly oncogenic Harvey and Kirsten murine sarcoma viruses (Ha-MSV and Ki-MSV) were discovered by Jennifer Harvey and later Werner Kirsten to cause rapid tumor formation in rats (Malumbres and Barbacid 2003) (Figure 1). These viral oncogenes, named Harvey and Kirsten RAS (HRAS and KRAS), along with their neuroblastoma RAS (NRAS) viral oncogene homolog, are activated versions of genes encoding 21-kDa phospho-protein (p21) with guanine nucleotide (GDP and GTP) binding and GTP hydrolyzing activities (Malumbres and Barbacid 2003). Later studies have provided evidences for the existence of specific regulators (guanine nucleotide exchange factors or GEFs and GTPase activating proteins or GAPs) and effector proteins activating individual pathways (Cherfils and Zeghouf 2013; Hennig et al. 2015; Upadhyaya et al. 2016; Keeton et al. 2017). As the founding members and prototypes of the RAS superfamily proteins (Wennerberg 2005; Wittinghofer and Vetter 2011; Rojas et al. 2012), HRAS, KRAS, and NRAS have become the subject of intense investigations due to their central involvements in signal transduction and their critical contribution to human diseases and disorders (Hobbs et al. 2016; Simanshu et al. 2017).

In this review, we describe current understanding of the regulatory mechanisms of individual RAS proteins and their signaling networks beyond the RAS paralogs. Phylogenic analysis identified 25 members of the RAS family out of 35 sequences (van Dam et al. 2011) (Figure 2). RASL, RERG, and NKIRAS proteins exhibit strong sequence deviations and thus, excluded from the list. The RAD family proteins, which are also excluded, make up together with RAS, RHO, RAB, ARF, RAN, and RAG the RAS superfamily (Rojas et al. 2012).

By the time passing, new evidences indicate tissue- and cell-specific function of RAS proteins. The sequence similarity between RAS proteins, especially in effector

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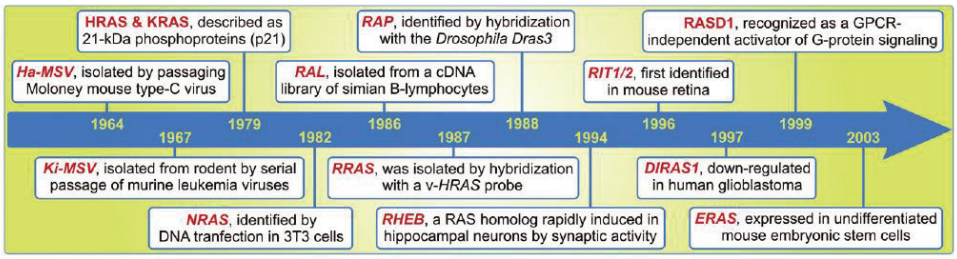

Figure 1. Historical timeline of the discovery of various members of the RAS family.

binding regions (see next section) was tempting to speculate overlapping functions for related RAS proteins. However, we need to consider the timing, subcellular localization and external stimuli that selectively regulate individual RAS proteins. This complexity comes in part because of their hypervariable region at C-terminus and sequence deviations in the full-length proteins, which provide additional binding sites for various scaffolding and adaptors proteins. Therefore, we discuss unique aspects of each RAS subfamily in term of tissue expression, upstream stimuli, receptor activation, interactions with regulators and effector that collectively fine-tune individual cellular functions under normal and pathological conditions. A large number of data, which will not be considered in detail, are summarized in Table 1.

RAS isoforms versus paralogs

The RAS family includes 23 genes coding for at least 25 proteins. Based on sequence identity, structure and function, the RAS proteins were divided into eight paralog groups: RAS, RAL, RRAS, RIT, RAP, RHEB, RASD, and DIRAS (Figure 2). Average sequence homology among paralogs vary between 30% and 60% while exceeds 90% within individual paralog groups. We introduced, for more clarity, names of some members, for example RRAS2 for TC21, RRAS3 for MRAS, RIT2 for RIN, RASD1 for DEXRAS, RASD2 for RHES, and DIRAS1 for RIG.

While majority of RAS proteins corresponds to one unique gene, some RAS family members are transcribed by the same genes. These isoforms, thus, originate from different mRNA transcripts, produced by alternative splicing and mostly differ in their subcellular localization. One example is HRAS with three isoforms p21, p19, and HRAS variant, which are designated HRAS1–3. HRAS1 (generally known as HRAS) has a stop codon in exon 4A and is translated to yield a p21-kDa protein with the canonical sequence with 189 amino acids. An in-frame stop codon in exon IDX leads to a transcript translated to produce a novel 170-amino acid protein called HRAS2 (known as p19HRAS or HRASIDX) (Cohen et al. 1989). HRAS3, a RASopathy-associated gene with a *de novo* 10-nucleotide-long deletion promoting constitutive retention of exon IDX in *HRAS1* gene (Pantaleoni et al. 2017). These three HRAS isoforms share an identical G domain and considerably different amino acids from 152 to 189 (Figure 2). HRAS3 contains an insertion of 24 amino acids between the residues 151 and 152 of HRAS1 (Pantaleoni et al. 2017). The other example is the *KRAS* gene, which encodes two transcripts, KRAS4A and KRAS4B, which are processed by alternative splicing of fourth coding exons 4A and 4B (McGrath et al. 1983). Also in this case, yielded proteins of 189 and 188 residues that significantly differ in their very C-terminal end (Figure 2), which take different ways of membrane trafficking (see below). HRAS and KRAS isoforms are co-expressed widely in human tissues (Guil et al. 2003; Plowman et al. 2006). Until now, no isoform of NRAS has been reported.

Structural fingerprints

The G domain and its molecular switch function

The RAS family proteins are usually known as molecular switches, cycling between an active GTP-bound state and an inactive GDP-bound state (Vetter and Wittinghofer 2001). Accordingly, they share a conserved GDP/GTP-binding domain (or G domain), which is responsible for nucleotide-dependent conformational changes. The structural differences between the two states are primarily confined to two highly mobile regions, designated as switch I (residues 28–39) and switch II (residues 59–74) (Figure 2). In the active state Tyr-32 and Thr-35 in switch I and Gly-60 in switch II form a hydrogen bonding network with the γ-phosphate of GTP. GTP hydrolysis triggers drastic rearrangements of the switch regions, resulting in the reorientation of these three critical residues away from the active site. Although the G domain uses a universally conserved switching mechanism (Wittinghofer and

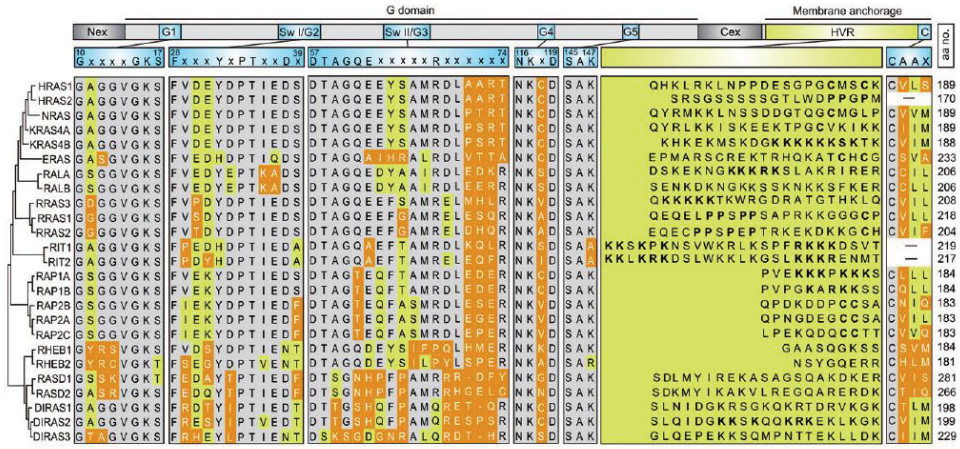

Figure 2. Evolutionary conservation of RAS family members. Signature motifs of 25 RAS-related proteins are presented according to their phylogenetic categorization. These proteins consist of a G domain with the five conserved motifs and a variable C-terminal membrane anchorage region, divided in hypervariable region (HVR) and the CAAX motif[©]. HVR contains several cysteines and serines for post-translational modifications, positively charged residues and other putative motifs, for example PXXP motifs as binding sites for SH3 domain-containing proteins. Certain members exhibit extensions at their N-terminal (Nex) and C-terminal (Cex) ends, which are summarized in Table 2. Conserved residues are shown in gray, homologous residues in orange and variable residues in olive.

Vetter 2011), its structure, function and GTP hydrolysis (or GTPase) reaction are adapted to many different signaling pathways and processes (see below).

The G domain consists of five conserved motifs, termed G1-G5 (Bourne et al. 1991) (Figure 2), which are central in nucleotide and magnesium binding. G1 is also known as the phosphate-binding loop or P-loop, as it is responsible for the binding of the phosphate groups of GDP and GTP. P-loop exists not only in GTP-binding proteins but also in ATP-binding proteins (Saraste et al. 1990) and typically contains several critical residues followed by a conserved lysine and a serine or threonine. Gly-12 and Gly-13 (HRAS numbering) are frequently mutated codons in human tumors (Malumbres and Barbacid 2003) leading to impairment of the GTPase reaction (Ahmadian et al. 1999). The majority of RAS family members contain a glycine at position 12 except ERAS, RASD1/2, and DIRAS3. These GTP-binding proteins do not act as molecular switches as they are GAP insensitive and thus persist in a constitutive active state (Kontani et al. 2002; Nakhaei-Rad et al. 2015). RHEB1 and RHEB2 have an extremely slow GTPase reaction due to an arginine and a serine or a cysteine instead of Gly-12 and Gly-13, respectively, but is interestingly switched off by RHEBGAPs, such as tuberin (also called TSC2) (Scrima et al. 2008). In the case of ERAS and RASD1/2, there is Ser-12 instead of glycine, and DIRAS3 harbors alanine in this position. In contrast to Gly-12 mutation, Ser-17 mutation to asparagine is used as dominant negative RAS mutant. Overexpressed RAS (S17N) tightly binds to endogenous RASGEFs and sequesters them from endogenous RAS proteins, and thus, interferes with RAS activation (Feig 1999). G2 (also called effector loop) is an integral part of effector-binding site and contains the highly conserved Tyr-32 and an invariant Thr-35 (HRAS numbering), which are critical for the conformational rearrangement of switch I. RIT1/2 contain histidine at the corresponding position of Tyr-32, which may be the reason for an accelerated nucleotide dissociation (Shao et al. 1999). G3 is a part of switch II and contains the critical catalytic Gln-61 position. Similarly to Gly-12 mutations, replacement of Gln-61 by virtually any other amino acid significantly reduces the intrinsic hydrolysis rate, prevents the GAP-mediated inactivation and, thus, induces oncogenic transformation by constitutive activation of RAS (Malumbres and Barbacid 2003). There is a threonine in RAP paralogs instead of Gln-61, asparagine in RASD1/2, glycine in DIRAS3 and serine in DIRAS1/2. In contrast to RASD1/2 and DIRAS3, which seem to have an impaired GTPase activity (Kontani et al. 2002), Thr-61 in RAP paralogs and most interesting Ser-65 in DIRAS1 and DIRAS2 (Gln-61 in HRAS1), do not compromise the GTPase reaction especially in the presence of RASGAPs (Scrima et al. 2008) (see "Regulatory proteins" section for more detail). GTPase deficiency of RASD and DIRAS

Table 1. Data summary for the RAS family proteins.

Proteins	Synonyme	Expression pattern	Upstream signals	GEFs	GAPs	Downstream target	Post-translational modifications
HRAS1	p21HRAS	Ubiquitous	Growth factors, phorbol esters	RASGRF, SOS1/2, RASGRP1–4, PLCε	p120RASGAP, NF1, RASA1–3, SynGAP1	C/BRAF, PI3K, RalGDS, PLCε, RASSF, RGL3, FAK	Far, Cm, Palm, Ub, S-Nit
HRAS2	p19HRAS	n. d.	n. d.	n. d.	n. d.	RACK1	n. d.
HRAS3	$HRAS^{fsIDX}$	RASopathy gene	n. d.	n. d.	n. d.	n. d.	Far, Cm, Palm, Ub
NRAS		Ubiquitous	Growth factors, phorbol esters, LI3, CSF1	SOS1/2, RASGRP1–4	p120RASGAP, NF1, RASA1–3, SynGAP1	C/BRAF, PI3K, RALGDS, PLCε, RASSF, Calmodulin (KRAS4B)	Far, Cm, Palm
KRAS4A							Far, Cm, P, Ac, Ub
KRAS4B	HRAS2, RASK2						
ERAS	KRAS2, HRASP	Embryonic stem cells, hepatic stellate cells	n. d.	n. d.	n. d.	PI3Kα/δ, RASSF5	Far, Cm, Palm
RALA		Ubiquitous	Aurora-A, PKA, alpha-thrombin	RALGDS, RALGPS1/2 RGL1–4	RALGAP1/2	RalBP1, SEC5, EXO84, PLD1, PLCδ, ZONAB, TBK1	Ger, Cm Palm, P, Ub
RALB			PKCα, thrombin				
RRAS1	RRAS	Ubiquitous	Sema4D/3E-plexin B1/D1, EphB2; SRC, TCF8, NOTCH1, IL9, ORP3/VAP-A	RASGRF, C3G, CalDAG-GEFI/II/III	p120RASGAP,GAP1, NF1	PLCε, Gridin, FLNa, PI3K, RAP1, RAF, RIN2, VEGF	Ger, Cm, P
RRAS2	TC21	Heart, placenta, kidney, ovaries, skeletal muscle	IL9/IL3			CRAF	
RRAS3	MRAS	Brain		SOS1, RASGRF		SHOC2/PP1C, CRAF, RGL3	Ger, Cm
RIT1	RIBB, RIT, ROC1	Ubiquitous	NGF/EGF, injury, stress, PACAP38, Gαi/s/o	SOS1, GRF	SynGAP, GAP1	PAR6, RALGDS, RGL2/3, MKK3/6, SIN1, BRAF	P
RIT2	RIN, ROC2	Adult brain	NGF/EGF, PACAP38, Gαi/s/o, Forskolin/KCl			PAR6	n. d.
RAP1A	KREV1	Ubiquitous	cAMP, PLC, E-cadherin, ERM, Glucose, FGF2, GLP1, PAR4, integrins	EPAC1/2, Repac, CALDAG-GEF, PDZGEF1/22, C3G, DOCK4, PLCε1	RapGAP-I/II, SIPA1, E6TP1/SPAR, SPA-Ls, CAPR I	B/CRAF, AF6, KRIT1, RAPL, PI3K, ARAP3, RIAM, RGS14, RPIP9	Ger, Cm, P
RAP1B	OK/SW-cl.11	B/T cells					
RAP2A		B/T cells, excitatory synapses	PLC, cAMP	C3G, EPAC, CalDAGGEFI, PDZGEF1		JNK, MAP4K4, PARG1, TNIK, RPIP9, MINK, PLCε	Ger, Cm, Plam, P
RAP2B		Platelet, neutrophils	Thrombin, convulxin				Ger, Far, Cm, Palm
RAP2C		Circulating mononuclear leukocytes, liver, skeletal muscle, prostate, uterus, rectum, stomach, and bladder		PDZGEF1	n. d.	TNIK	
RHEB1		Ubiquitous	EGF, NGF, hypoxia, amino acids, forskolin, Low glocuse, BDNF, insulin, FGF	TCTP	TSC1/2, RGS10	mTOR, FKBP38, PLD1, PERK, BACE1, CRAF, NIX/LC3-II, Dynein, NOTCH1, RASSF1	Far, Cm, P
RHEB2		Ubiquitous, brain	NGF, SPC	n. d.	TSC1/2	mTOR, AKT1, CAD	
RASD1	AGS1, DEXRAS1	Brain, heart, liver, kidney, skeletal muscle, pancreas, placenta	Corticosteroids, estrogen, T3, nNOS	CAPON	n. d.	Gαi/o, PAP7, FE65, PLCδ	F, Cm
RASD2	RHES, TEM2	Corpus striatum, olfactory tubercle				PAP7	
DIRAS1	RIG, GBTS1	Brain, heart	n. d.	n. d.	RAPGAP1/2	CRAF, RAC1, EPAC1, smgGDS	Far, Ger
DIRAS2		Brain			RAPGAP1/2	smgGDS	
DIRAS3	ARHI, NOEY2, RHOI	Ovary, breast epithelial cells			n. d.	STAT3, CRAF	Myr

Ac: acetylation; Cm: carboxymethylation; Far: farnesylataion; Ger: geranylgeranylation; n. d.: not determind; Palm: palmitoylation; P: phosphorylation; Ub: ubiquitination; Myr: N-myristoylation; S-nit: S-nitrosylation.

paralogs may even be strengthened by an additional amino acid deviation at position 59 (Figure 2). G4 and G5 contain invariant residues and are responsible for the guanine base recognition. Mutation of Asp-119 in RAS changes the nucleotide specificity from guanosine to xanthosine nucleotides (Schmidt et al. 1996) and acts as dominant negative in a dose dependent manner (Tuder et al. 1999). G5 provides Ser-145 that stabilizes Asp-119 of G4. Ala-146 binds the guanine base and is another determinant for the guanine-binding ability of the RAS proteins. Lys-147 is replaced in RIT1/2 by alanine and may affect, together with the deviation in G2, the nucleotide binding affinity (Shao et al. 1999).

Membrane anchorage and subcellular distribution

Interactions between signaling proteins and cellular membranes are emerging as important modulators of

cellular signaling. The spatiotemporal organization in cells is largely dependent on both the nature and the dynamics of the association of proteins with specific sites of the cell membranes (Herrero et al. 2016). Association of RAS proteins with cellular membranes is mediated through a series of post-translational modifications and distinct motifs at their very C-terminal end (Wright and Philips 2006; Omerovic and Prior 2009; Cox et al. 2015; Nussinov et al. 2016; Wang and Casey 2016). RAS proteins, except for RIT1/2, serve as substrates for isoprenyl-transferring enzymes, which covalently and irreversibly attach a 15-carbon farnesyl or a 20-carbon geranylgeranyl moiety to the cysteine residue of the very C-terminal CAAX (C is cysteine, A is any aliphatic amino acid and X is any amino acid) motif (Figure 2). This motif is present in more than 100 proteins and necessary for diverse cellular processes (Lane and Beese 2006).

If the amino acid in the X position of CAAX is a leucine, as in the case of RALA/B, RRAS1/3, RAP1A/B, RAP2A (Figure 2), then geranylgeranyl transferase modifies the protein with a geranylgeranyl moiety (Benetka et al. 2006), otherwise the protein is modified with a farnesyl moiety by farnesyl transferase (Ahearn et al. 2011; Berndt et al. 2011). Two post-prenylation enzymatic steps are critical for proper localization, including proteolytic cleavage of the AAX residues by the endopeptidase RCE1 and methylation of the terminal isoprenylcysteine by the methyltransferase ICMT (Winter-Vann and Casey 2005; Ahearn et al. 2011; Berndt et al. 2011).

Due to a relatively weak affinity of isoprenylated proteins for cellular membranes (Silvius and l'Heureux 1994), additional motifs in the hypervariable region (HVR) are engaged in fine-tuning membrane association with RAS proteins (Figure 2) and their functions (Abankwa et al. 2007; Hanzal-Bayer and Hancock 2007; Omerovic and Prior 2009). Some RAS proteins, e.g. KRAS4B, RALA, RRAS3, and RIT1/2 (Figure 2), contain a stretch of positively charged amino acids (called polybasic region or PBR; Figure 2), which has been implicated to contact negatively charged phospholipids of the cell membrane (Banerjee et al. 2016; Nussinov et al. 2016). Membrane association of KRAS4B is modulated in different ways (Ashery et al. 2006; Bhagatji et al. 2010; Alvarez-Moya et al. 2011). PDEδ binds to farnesylated KRAS4B (Dharmaiah et al. 2016) and transport it from perinuclear membranes to plasma membrane (Chandra et al. 2011; Schmick et al. 2014). ERK1/2 phosphorylates RRAS1/2 at Ser-186 and Ser-201, but not RRAS3, and does not affect their subcellular localization but rather stimulates their activation (Fremin et al. 2016).

A further way of increasing the affinity of isoprenylated proteins for cellular membranes is an addition of one or more lipid anchors. KRAS4A, NRAS, HRAS1, ERAS, RRAS1, RAP2A/B, and RALA/B are palmitoylated by acyl protein transferases at cysteines prior to the CAAX motif (Figure 2) (Hancock et al. 1989; Beranger et al. 1991; Schroeder et al. 1997; Takahashi et al. 2005; Uechi et al. 2009; Gentry et al. 2015; Tabaczar et al. 2017). In contrast to HRAS1, HRAS2 does not have any C-terminal sites for post-translational modifications (Figure 2), and appears to be distributed between cytosol and nucleus (Guil et al. 2003). Another emerging concept in the field is based on physical interaction of the G domain itself with lipid membrane. A membrane-based, nucleotide-dependent conformational switch operates through distinct regions on the surface of RAS proteins, including the HVR, which reorient with respect to the plasma membrane (Abankwa et al. 2010; Cirstea et al. 2010). G domain-membrane interaction may contribute to the specificity of signal transduction and may underlay additional control elements. A critical aspect in this context is the organization of RAS proteins into protein–lipid complexes. These so-called nanoclusters concentrate RAS at the plasma membrane. They are the sites of effector recruitment and activation, and are essential for signal transmission (Abankwa et al. 2007; Zhou and Hancock 2015). It is not entirely clear how RAS nanoclustering is regulated (see "Modulatory scaffold proteins" section).

Modulatory post-translational modifications

Trafficking of RAS proteins (Wurtzel et al. 2015) have recently been shown to be highly specific for respective RAS proteins and dependent on specific post-translational modifications beyond prenylation and acylation (Oertli et al. 2000; Berzat et al. 2006; Calvo and Crespo 2009; Jang et al. 2015; Lynch et al. 2015; Schmick et al. 2015), namely, phosphorylation (Bivona et al. 2006; Sung et al. 2013), ubiquitination (Jura et al. 2006; Rodriguez-Viciana and McCormick 2006; de la Vega et al. 2010; Wang et al. 2015), and S-nitrosylation (Shanshiashvili et al. 2011; Chen et al. 2015). The molecular basis of these modifications is mostly still unclear.

Acetylation of KRAS at Lys-104 interferes with GEF-induced nucleotide exchange (Yang et al. 2012, 2013; Knyphausen et al. 2016). S-nitrosylation of Cys-118 of HRAS promotes nucleotide exchange (Lander et al. 1995; Williams et al. 2003; Heo and Campbell 2004). Ubiquitination of HRAS at Lys-117 accelerates intrinsic nucleotide exchange, thereby promoting GTP loading, while KRAS monoubiquitination at Lys-147 leads to an impaired regulator-mediated GTP hydrolysis (Baker et al.

2013a, 2013b; Sasaki et al. 2011). RRAS1 phosphorylation at Tyr-66 by EphB2 receptor and Src blocks its effector interaction, for example with CRAF (Zou et al. 1999, 2002). In contrast, ERK1/2 phosphorylates RRAS1 and RRAS2 at the C-terminal HVR at Ser-186 and Ser-201, respectively and promotes cell adhesion and migration (Fremin et al. 2016). In addition, phosphorylation of RAS proteins also modulates their subcellular localization. KRAS phosphorylation by PKC at the C-terminal Ser-181 promotes its dissociation from the plasma membrane and translocation to intracellular membranes, including the outer membrane of mitochondria (Bivona et al. 2006). A similar scenario is RALA phosphorylation at Ser-194 by Aurora-A, which promotes RALA relocalization from the plasma membrane to mitochondria leading to mitochondrial fission (Kashatus et al. 2011).

The concept of family member selectivity

In spite of sharing a conserved G domain, each RAS family member has specific deviation within and additional features outside the G domain that make them unique in regulation and function. In the following, we compare individual members in the frame of 11 subfamilies with HRAS as a prototype of the family. Many members of the RAS family exhibit unique amino acid extensions at their N-terminal (N_{ex}) and C-terminal (C_{ex}) ends (Figure 2 and Table 2). The N-terminus of ERAS, which appears to undergo multiple interaction with other proteins (H. Nakhaeizadeh, J. Lissy, S. Rezaei Adariani, S. Nakhaei-Rad, M.R. Ahmadian, unpublished) and contains putative SH3-binding motifs, like RRAS1 and HRAS2/3 (Table 2). RRAS1 N-terminus, interestingly is critical for protein targeting and function (Wang et al. 2000). These motifs may provide additional mechanisms for sorting and trafficking to specific subcellular sites, as proposed for ERAS (Nakhaei-Rad et al. 2015). RRAS paralogs contain extended N-termini that seems to be critical for cell migration (Holly et al. 2005). RALA N-terminal extension is involved in SRC-induced PLD activation (Jiang et al. 1995). Signal-induced recruitment of DIRAS3 to the plasma membrane appears to be regulated by its N-terminal extension (Klingauf et al. 2013), which is essential for its interaction with STAT3 and importin (Nishimoto et al. 2005; Huang et al. 2009). Notably, DIRAS3 contains a glycine at position 2, which usually is used as a site for myristoylation (Resh 2004).

Protein interaction networks

RAS proteins are known to undergo interactions with diverse types of proteins, some of which are summarized as follows.

Regulatory proteins

RAS is believed to persist in its inactive form in resting cells. This scenario is based on the assumption that its intrinsic GTPase reaction is faster than its intrinsic GDP/GTP exchange reaction. A further issue is that these very slow reactions require catalysis by GEFs and GAPs, respectively, which are controlled by upstream signals and locally regulate RAS activity. There are, however, several RAS family members, including ERAS, DIRAS3, and RASD1/2, which exhibit distinct amino acid deviations in G1 and G3 motifs (Figure 2). These proteins accumulate themselves in GTP-bound form due to their impaired GTP hydrolysis and GAP insensitivity (Kontani et al. 2002; Nakhaei-Rad et al. 2015; Ogita et al. 2015), and may underlay a different mechanism of regulation. Unlike classical RAS proteins, these GTP binding proteins are not ubiquitously expressed (Table 1) and may be regulated at the level of transcription as recently shown for ERAS (Nakhaeizadeh et al. 2016). All other members of the RAS family appear to act as intracellular switches and to be controlled by GEFs and GAPs (Table 1). However, no RHEBGEF has been identified so far.

Table 2. Amino acid extensions beyond the G domain and HVR (see text for more detail).

N-terminal extensions	
ERAS	[1]MELPTKPGTFDLGLATWSPSFQGETHRAQARRRDVGRQ
RRAS1	[1]MSSGAASGTGRGRPRGGGPGPGDPPP
RRAS2	[1]MAAAGWRDGSG
RRAS3	[1]MATSAVPSDN
RALA	[1]MAANKPKGQNS
RALB	[1]MAANKSKGQSS
RIT1	[1]MDSGTRPVGSCCSSPAGL
RIT2	[1]MEVENEASCSPGSASGG
RASD1	[1]MKLAAMIKKMCPSDSELSIP
RASD2	[1]MMKTLSSGNCTLSVPA
DIRAS3	[1]MGNASFGSKEQKLLKRLRLLPALLILRAFKPHRK
C-terminal extensions	
HRAS2	[152]SRSGSSSSSGTLWDPPGPM
HRAS3	[152]SRSGSSSSSGTPRDPCDPAAPRAG
RASD1	[197]LPSEMSPDLHRKVSVQYCDVLHKKALRNKKLLRAGSGGGGGDPGDAFGIVAPFARR
RASD2	[192]LPHEMSPALHRKISVQYGDAFHPRPFCMRRVKEMDAYGMVSPFARR

Postulated GEF activity of TCTP towards RHEB1 has been disproved (Rehmann et al. 2008b). There are no specific GEFs and GAPs described for RIT1/2 yet (Shi et al. 2013).

There are 30 RASGEFs known in human genome (van Dam et al. 2009) sharing a common catalytic domain, called CDC25 (Crechet et al. 1990; Quilliam et al. 2002; Mitin et al. 2005; van Dam et al. 2011). Consistent with the RHOGEF family (Jaiswal et al. 2013), RASGEFs also exhibit selectivity profile towards distinct groups of the RAS family (Popovic et al. 2013), which is a pivotal step in establishing specific activation of the downstream signaling pathways (Figure 3). The CDC25 domains of SOS1, EPAC2 and RALGDS specifically bind HRAS, RAP2B, dRal, the *Drosophila* ortholog of RALA, respectively and structurally rearrange critical regions of the nucleotide-binding site, including P-loop and switch I/II and consequently catalyze the GDP/GTP exchange (Boriack-Sjodin et al. 1998; Rehmann et al. 2008a; Popovic et al. 2016). They apparently operate by a simple allosteric competitive mechanism (Guo et al. 2005). In the cell, the specificity of the RASGEFs is obviously determined by other domains of the respective proteins, for example SOS1 (Gureasko et al. 2008).

Unlike GEFs, GAPs for different groups of the RAS family are mechanistically rather heterogeneous (Scheffzek and Ahmadian 2005). RASGAPs provide common structural fingerprints (Ahmadian et al. 2003), especially a catalytic arginine, which stabilizes Gln-61 of RAS and RRAS paralogs and stimulate the very slow GTPase reaction (Ahmadian et al. 1997; Scheffzek et al. 1997). RAPGAPs as well as the RHEBGAP, tuberin or TSC2, utilize a catalytic asparagine that substitute for the non-functional threonine of RAP paralogs and glutamine of RHEB1 in the switch II regions (Daumke et al. 2004; Yu et al. 2005; Scrima et al. 2008; Marshall et al. 2009). Tuberin requires for its GAP activity a heterodimerization with non-catalytic hamartin (also called TSC1) (Li et al. 2004). GAP1^{IP4BP}, however, utilizes a catalytic arginine to inactivate RAP1 (Kupzig et al. 2009). RALGAPs share a similar catalytic mechanism as RHEBGAPs. They undergo a complex with a non-catalytic subunit and stimulate the GTPase reaction of RALA/B, most likely by supplying a catalytic asparagine, too (Shirakawa et al. 2009). DIRAS1/2 share GAPs with RAP paralogs, which also have a serine instead of a catalytic glutamine (Figure 2) and can be inactivated by RAPGAPs (Gasper et al. 2010).

Effector selectivity

Signal transduction implies physical association of RAS proteins with and activation of a spectrum of functionally diverse downstream effectors. Effectors specifically interact with the active, GTP-bound form of the RAS proteins, usually, in response to extracellular signals, and link them to downstream signaling pathways in all eukaryotes (Karnoub and Weinberg 2008; Gutierrez-Erlandsson et al. 2013). They act as protein or lipid kinases, phospholipase, GEFs, GAPs and scaffold proteins (Table 1) (Herrmann 2003; Rajalingam et al. 2007; Castellano and Downward 2010; Ferro and Trabalzini 2010; Bunney and Katan 2011; Chan and Katan 2013; Nakhaei-Rad et al. 2016; Nakhaeizadeh et al. 2016). Two major groups of effectors contain RAS binding (RB) and RAS association (RA) domains, respectively (Repasky et al. 2004; Wohlgemuth et al. 2005; Nakhaeizadeh et al. 2016). Mining in the UniProt database led to the identification of 118 distinct human proteins containing RB and RA domains (Rezaei Adariani, Dvorsky et al., unpublished). Notably, both types of domains utilize critical determinants for the interaction with different RAS proteins, particularly the intermolecular β-sheets (Nakhaeizadeh et al. 2016). Structural studies have provided deep insights into the binding modes and interaction specificities (Mott and Owen 2015) and yet, the precise mechanism, through which effector association with activated RAS proteins results in effector activation, is still unclear. It is, however, generally accepted that RAS proteins participate directly in the activation of their downstream effectors and do not simply mediate recruitment to specific sites of the membrane.

The RAS paralogs share a similar effector binding regions with other members of the RAS family but also show distinct deviations (residues 30 and 31 in switch I, and 64, 65, 71, 72, and 73 in switch II) suggesting that they may share downstream effectors with different affinities (Wittinghofer and Nassar 1996). ERAS preferentially interacts with PI3K rather than CRAF as compared to HRAS. Trp-79 of ERAS (Arg-41 in HRAS) turned out to be critical for ERAS binding to PI3K, RALGDS, and PLCε (Nakhaei-Rad et al. 2015). Ser-34 of RHEB1, and Lys-31 in RAP1A (Glu-31 in HRAS1) have been discussed as specificity determining for their effectors (Wittinghofer and Nassar 1996). Notably, residues 70–72 (67–69 in HRAS1) in the switch II region appear to undergo contacts with Arg-15 and Ser-16 (Gly-12 and Gly-13 in HRAS1) in P-loop and may contribute to an alternative mechanism of intrinsic GTP hydrolysis (Karassek et al. 2010).

Modulatory scaffold proteins

Signal transduction of RAS family proteins are maintained by at least three classes of interacting partners. These include regulators (GEFs and GAPs) that control

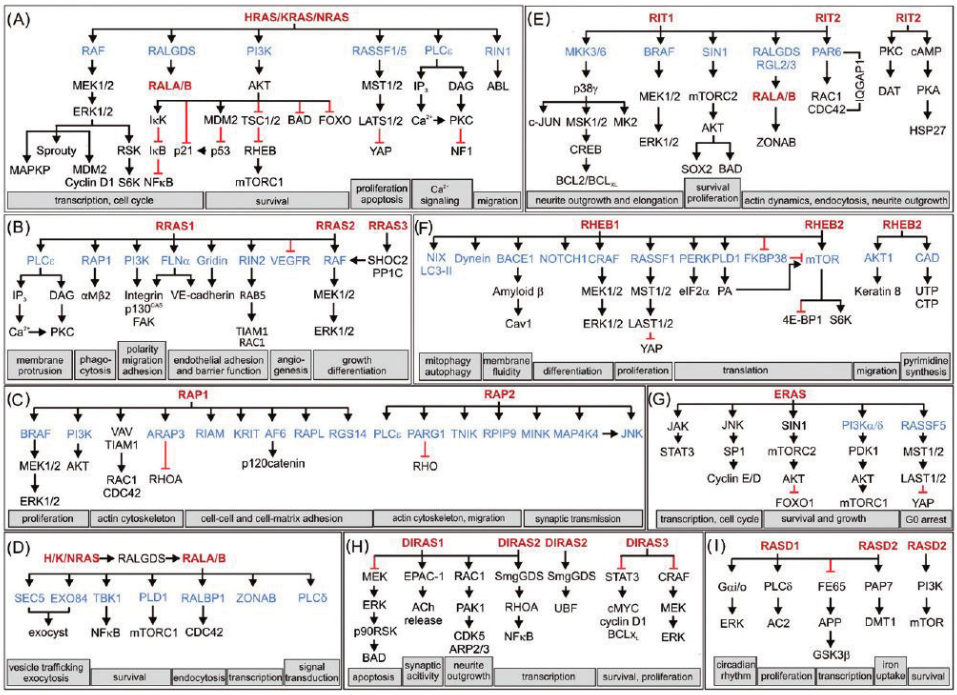

Figure 3. Signal transduction pathways downstream of the RAS family proteins. Signaling schemes are divided in different paralog in red colors (A–I). Reviewed effectors are shown in blue. Other downstream interacting proteins are shown in black. Black arrows indicate activating events and red lines inhibiting events in pathways. See D for missing RALA/B signaling in A.

the GTPase cycle and a wide spectrum of effectors that initiate signaling cascades downstream of the RAS proteins. It has become evident that an increasing number of additional RAS scaffold proteins, including CAM, GAL1, GAL3, IQGAP1, NCL, NPM1, SHOC2, SHP2, SPRY, SPRED1, and GAB1, are critical in modulating and integrating RAS proteins in various signaling networks at the biological membranes. CAM binds to KRAS4B PBR (Wu et al. 2011; Sperlich et al. 2016) and determines activation of distinct downstream pathways (Nussinov et al. 2015; Jang et al. 2017). KRAS4B interaction with CAM leads to the suppression of the non-canonical Wnt/Ca^{2+} pathway that strongly contributes to its tumorigenic properties (Wang et al. 2015). Similarly, CAM binds to RALA and PLCδ and modulates RALA-mediated PLCδ activity (Grujic and Bhullar 2009). RIT2 PBR acting as a docking site for CAM is essential for the EGF dependent RIT2 signal transduction (Lee et al. 1996). A CAM interaction of *Drosophila* Ric, a RIT1/2 ortholog, has been shown, however, to negatively regulate Ric crosstalk to the RAS-MAPK pathway (Harrison et al. 2005). SHOC2 (also called SUR-8) in complex with PP1c links RRAS3 with the inactive CRAF and stimulates CRAF activity by dephosphorylating of SHOC2, thus, promotes the RAS-RAF-controlled MAPK activation to control proliferation and neurite outgrowth (Cordeddu et al. 2009; Motta et al. 2016). SHOC2 also binds p110α subunit of PI3K and regulates cell motility, invasion, and metastasis (Kaduwal et al. 2015). IQGAP1, which contains an inactive RASGAP domain (Kurella et al. 2009; Nouri et al. 2017), binds BRAF and ERK1/2, and potentiates their activity in response to EGF (Ren et al. 2007). An ERK1/2-binding IQGAP1 peptide has been reported to disrupt IQGAP1-ERK1/2 interactions and inhibit RAS- and RAF-driven tumorigenesis (Jameson et al. 2013). GAL1, GAL3, NPM1, and NCL has been suggested to modulate RAS nanocluster formation and activate the MAPK pathway but the molecular nature remains to be determined (Plowman et al. 2008; Inder et al. 2009). GAL1 has recently been shown to form a complex with CRAF and potentiate HRAS nanoclustering (Blazevits et al. 2016). Other scaffold proteins, such as SPRY,

SPRED1, and GAB1 act differently. SPRY2, for example, interrupts signal transduction from FGFR to RAS by binding to GRB2 and disrupting the GRB2-SOS complex if phosphorylated by CK1 (Yim et al. 2015). SPRY2 appears to regulate the specific activation of RAC1 by HRAS, which probably would be mediated by TIAM1 and PI3K (Lito et al. 2009). SPRED1 interferes with the membrane anchorage and signaling of KRAS but not HRAS (Siljamaki and Abankwa 2016) and modulate the activity of NF1, a RASGAP, by binding and recruiting it to the plasma membrane (Dunzendorfer-Matt et al. 2016). GAB1 modulates, together with the tyrosine phosphatase SHP2, p120RASGAP activity by recruiting it to activated EGFR at the plasma membrane (Montagner et al. 2005). Future studies will shed light on the underlying mechanisms of these groups of modulatory proteins, the total number of which may increase.

Signal integration and transduction

RAS family proteins link the extracellular signals, transduced through their receptors, with multiple signaling pathways and consequently control a wide array of cellular processes. Different RAS paralogs have unique roles in modulating the cellular processes. The specificity comes from several levels: Subcellular localization, upstream stimuli, interactions with scaffolds, regulators and target proteins, and downstream signaling. In this part, we describe more precisely the conditions under which individual RAS proteins are activated and how they transduce the signal.

Upstream signals

The convergence of multiple upstream cascades on the RAS proteins mostly underlay a similar mechanism. Different types of extracellular signals, transmitted across the plasma membrane by diverse cell surface receptors are linked with RAS proteins through different, specific GEFs and GAPs (Table 1) (Quilliam et al. 2002; Hennig et al. 2015). Interestingly, activation of different transmembrane receptors, including receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), ion channel receptors (e.g. mGluR or NMDAR), cytokine receptors and adhesion receptors, lead to the activation of distinct RAS proteins in distinct cell types. For example, IL3, CSF1, and EGF preferentially activate KRAS4B and RRAS3 over HRAS or NRAS in B and T lymphocytes (Ehrhardt et al. 2004), GLP1 and PAR4 peptide activate RAP1 in islet cells and platelets, respectively (Trumper et al. 2005), FGF2 activates RAP1A/B in endothelial cells (Yan et al. 2008), IL9/IL3 activate RRAS3 in T-helper cells (Louahed et al. 1999), EGF, and NGF activate RIT1 in non-neuronal and neuronal tissues (Shi and Andres 2005), EGF, NGF, and PACAP38 neuropeptide activate RIT2 in neuronal tissues (Spencer et al. 2002b; Shi et al. 2008), EGF and forskolin activate RHEB in rat pheochromocytoma PC12 cells (Yee and Worley 1997), while insulin, FGF and BDNF activate the RHEB1 in neuronal cells (Yamagata et al. 1994; Zhang et al. 2003; Takei 2004), and glucocorticoid dexamethasone induces a RASD1-mediated adipogenesis in adipocytes (Cha et al. 2013). nNOS activation via NMDR stimulation results in S-nitrosylation and CAPON adaptor acts as a GEF to activate RASD1 (Fang et al. 2000; Cheah et al. 2006). L-DOPA, thyroid hormone and Estrogen regulate RASD2 in striatal tissue (Subramaniam et al. 2011; Ghiglieri et al. 2015).

The upstream signals specifically activate distinct GEFs, which in turn selectively activate distinct members of the RAS family and ultimately control distinct cellular processes (Buday and Downward 2008). A nice example is RAP1-mediated formation of cell-cell junction regulated by five different RAP1GEFs (Kooistra et al. 2007). Prominent examples are EPAC1/2, which is directly activated by cAMP (de Rooij et al. 1998), controls cellular processes ranging from insulin secretion to cardiac contraction and vascular permeability (Gloerich and Bos 2010). A different scenario is CalDAG-GEFIII (also called RASGRP3) that operates on multiple RAS proteins (Rebhun et al. 2000; Yamashita et al. 2000). In endothelial cells, CalDAG-GEFIII activates RRAS1 and interferes with transendothelial permeability and angiogenesis, respectively (Ichimiya et al. 2015), while it affects inflammatory response in macrophages by activating RAP1 (Tang et al. 2014). Other well-studied GEFs are SOS1/2, RASGRP1–4, and RASGRF1/2 (Hennig et al. 2015). Collective binding of multiple SOS1 and GRB2 domains to their protein and phospholipid ligands are finely tuned in order to cooperatively control cellular processes, including pluripotency and differentiation factors (Findlay et al. 2013). RASGRP1 opposes EGFR-SOS1 signals and suppresses proliferation in normal intestinal epithelial cells (Depeille et al. 2015). RASGRF1/2 carry out specific roles in three forms of synaptic plasticity in the CA1 region of the hippocampus (Jin et al. 2014). RALGDS, an effector of different RAS proteins (Ferro and Trabalzini 2010; Yoshizawa et al. 2017), activates RALA to regulate insulin-secretory process in pancreatic β-cells in response to intracellular Ca^{2+} and cAMP (Ljubicic et al. 2009) and to promote exocytosis of endothelial Weibel-Palade bodies (Rondaij et al. 2008). The latter, which are also regulated by cAMP-EPAC-RAP1 (van Hooren et al. 2012), are critical elements of hemostasis, inflammation or angiogenesis (Mourik and Eikenboom 2017).

Contrary to GEFs, only a few reports are known about the signaling cascades, which control recruitment and activation of GAPs. In the thymus, p120RASGAP, the GAP prototype (Trahey and McCormick 1987), acts for example as a negative regulator of the RAS-MAPK pathway during positive selection and survival of naive T cells (Lapinski et al. 2011). The activity of p120 is regulated by ANXA6O, which binds p120 and recruit it to the membrane in a Ca^{2+}-dependent manner (Grewal et al. 2005; Grewal and Enrich 2006). The much larger NF1 regulates for example RAS inactivation in dendritic spines of pyramidal neurons in the CA1 region of the rat hippocampus (Oliveira and Yasuda 2014). Dual-specificity RASGAP paralogs, $GAP1^{IP4BP}$ and CAPRI, coordinate RAS and RAP signaling pathways (Kupzig et al. 2006; Sot et al. 2010). Inhibition of $GAP1^{IP4BP}$ by an integrin $\alpha_{IIb}\beta_3$ outside-in signaling via PI3K leads to sustained RAP1 activation and platelet spreading (Battram et al. 2017). Ca^{2+}-dependent dimerization of CAPRI, a $GAP1^{IP4BP}$ paralog, switches its specificity from RASGAP to RAPGAP (Dai et al. 2011). SynGAP is another dual-specificity GAP, which is a negative regulator of RAS and RAP proteins in dendritic spines (Jeyabalan and Clement 2016). It is one of the most abundant post-synaptic density proteins, where it binds as a homo-trimer to multiple copies of PSD95 (Zeng et al. 2016). SynGAP requires its C2 domain to catalyze RAP inactivation (Pena et al. 2008). Spine-associated, classical RAPGAPs, SPAR1–3, are recruited through their interactions with Fezzin proteins to the post-synaptic SHANK scaffold and regulate dendritic spine morphology (Dolnik et al. 2016).

Semaphorins, the plexin family of semaphorin receptors, exhibit GAP activities towards RRAS paralogs (Hota and Buck 2012). Sema3E-PLXND1 counteracts angiogenesis through RRAS inactivation (Sakurai et al. 2010). However, SEMA4D–PLXNB1–RND1 complex inactivates RRAS in order to induce growth cone collapse in hippocampal neurons (Oinuma et al. 2004), while SEMA4D-PLXNB1 acts on RRAS3 to regulate actin-based dendrite remodeling (Tasaka et al. 2012). As the GAP activities of PLXNs is a matter of debate, a structural, and biochemical study has shown that PLXNs apparently use a non-canonical catalytic mechanism to act as GAPs on RAP but not on RRAS paralogs (Wang et al. 2012). In this study, SEMA3A stimulated the RAPGAP activity of PLXNA1 to induce neuronal growth cone collapse.

TSC1/TSC2 heterodimerization facilitates TSC2 RHEBGAP activity leading to RHEB inactivation and inhibition of the RHEB-induces mTORC1 activation (Tee et al. 2003; Long et al. 2005). VPS34, a class III PI3K, upregulates RHEB and mTOR activities via production of PIP_3 and recruits PIKFYVE to the plasma membrane, where VPS34 forms a complex with PIKFYVE and TSC1 (Mohan et al. 2016). This in turn disengages TSC2 from the TSC1/TSC2 heterodimer, leading to TSC2 ubiquitination and degradation. Arginine, a key activator of mTORC1, cooperates with growth factor signaling, which suppresses lysosomal localization of the TSC complex and interaction with RHEB (Carroll et al. 2016). MCRS1 regulates the lysosome localization of RHEB1 in an amino acid-dependent manner and inhibits TSC2 binding to RHEB1 (Fawal et al. 2015). In myoblasts, however, TSC2 phosphorylation and inactivation by ERK results in activation of the RHEB-mTORC1 axis and regulation of protein synthesis (Miyazaki and Takemasa 2017).

Downstream targets and pathways

Classical RAS signaling

Specific regulation of cellular functions by the members of the RAS family depends on selective interaction with downstream targets, the effectors (Mott and Owen 2015; Nakhaeizadeh et al. 2016), which transduce the signal to distinct pathways (Cox and Der 2003; Bos 2005; Rajalingam et al. 2007; Braun and Shannon 2008; Karnoub and Weinberg 2008; Castellano and Downward 2010; Dodd and Tee 2012; Gentry et al. 2014). More than 60 effectors reported for the RAS family proteins (Table 1) can activate about 49 pathways (Figure 3). RAF kinases (ARAF, BRAF, and CRAF) are the major and best-studied effectors for RAS family. These kinases are critical elements of the MAPK pathway, which control gene expression and thus, different cellular processes including proliferation, apoptosis, and differentiation (Desideri et al. 2015). RAF kinases phosphorylate MEK, which in turn phosphorylates ERK kinases and triggers their translocation into the nucleus, where they activate transcription factors, such as ELK1, ETS1, MYC, FOS, and DUSP1 (Unal et al. 2017). Rarely analyzed are, however, a large number of other CRAF substrates, which are involved in different processes, including adenylyl cycle, vimentin kinase, Rb, CDC25, troponin T, DMPK, and MYPT (Galaktionov et al. 1995; Janosch et al. 2000; Shimizu et al. 2000; Broustas et al. 2001; Hindley and Kolch 2002; Ehrenreiter et al. 2005; Kaliman and Llagostera 2008; Davis and Chellappan 2008; Niault and Baccarini 2010). CRAF directly associates with MST2, ASK1, ROCK, and calcineurin, and controls proliferation, apoptosis, contraction, and motility, respectively (Chen et al. 2001; Niault and Baccarini 2010; Romano et al. 2014; Desideri et al. 2015; Varga et al. 2017).

CRAF and BRAF are apparently downstream of many different members of the RAS family, including HRAS,

KRAS4B, NRAS, RAP1A, RRAS1, RRAS2, RRAS3, RHEB1, RIT1, and DIRAS3 (Figure 3) (Self et al. 2001; Wellbrock et al. 2004; Jin et al. 2006; Karbowniczek et al. 2006; Baljuls et al. 2012; Mott and Owen 2015; Yaoita et al. 2016). CRAF activity is known to be directly dependent on its heterodimer formation with BRAF, which appears to be stabilized by ARAF as a scaffold protein (Rebocho and Marais 2013). Also ARAF homodimer seems to promote MAPK pathway activation (Mooz et al. 2014). However, due to a lower binding affinity for ARAF, HRAS seems to preferentially activate CRAF (Weber et al. 2000). In contrast to HRAS1, HRAS2 does not interact with two known HRAS effectors, CRAF and RIN1 (Guil et al. 2003). HRAS2 interacts with RACK1, a scaffolding protein that forms multiprotein complexes with p120RASGAP, MAP kinases, PKCs, and SRC proteins (Guil et al. 2003). It also regulates telomerase activity through its interaction with p73 and arrest cell cycle at G1/S phase (Camats et al. 2009). The RASopathy-associated HRAS3, which has a 24-amino acid insertion at Gly-151 and Val-152 with partial similarity to the C-terminus of HRAS2 (Table 2), is a weak hyperactive RAS protein with constitutive plasma membrane localization in comparison to HRAS1. It has been suggested that it may, due to its insertion, interact with signaling platforms located at different subcellular compartments (Pantaleoni et al. 2017).

The second best-characterized RAS effector family, PI3K (class I PI3K), phosphorylates phosphoinositide (4,5) bisphosphate (PIP_2) and generates the second messenger phosphoinositide (3,4,5) trisphosphate (PIP_3) that recruits the wide range of protein effectors through their pleckstrin homology (PH) domain to the membrane. Target proteins could be kinases (e.g. AKT and PDK1), adaptor proteins, GEFs, or GAPs that regulate different cellular processes (Vanhaesebroeck et al. 2001). PI3K-AKT pathway is very well known in controlling cell cycle entry, cell growth, survival, and metabolism (Castellano and Downward 2011). HRAS1, NRAS, KRAS4B, ERAS, RRAS, and RAP1A activate PI3Ks. AKT or protein kinase B (PKB) belongs to AGC subfamily of protein kinases. AKT is one of the key proteins downstream of PI3K-PIP3 involved in a wide range of the cellular processes, such as cell proliferation, metabolism, growth, autophagy inhibition, and survival (Andjelkovic et al. 1997; Pearce et al. 2010; Hers et al. 2011). Upon extracellular stimuli and the tyrosine receptor activation, class I PI3K generates the PIP3 that engages both PDK1 and AKT through PH domain to the plasma membrane. PDK1 phosphorylates AKT at position Thr-308 that is located on the catalytic domain of AKT (Alessi et al. 1997). This phosphorylation triggers the inhibitory phosphorylation of TSC1/2 that is a well-known GAP for RHEB protein. Phosphorylation of TSC1/2 suppresses its inhibitory effect on mTORC1 (Inoki et al. 2002, 2003). Second key phosphorylation site for AKT is on the hydrophobic motifs of AKT Ser-473 that will be phosphorylated through the second mTOR complex (mTORC2).

Other RAS effectors are RALGDS, PLCε, and RASSF. RALGDS links RAS with RALA/B, and regulates cellular processes such as vesicular trafficking, endocytosis and migration (Ferro and Trabalzini 2010). RPM/RGL3, another member of the RALGDS family, is an effector for both HRAS and RRAS3, which has inhibitory effects on the MAPK pathway (Ehrhardt et al. 2001). Dual functions of PLCε, activated by RAS proteins (Kelley et al. 2001; Song et al. 2001; Ada-Nguema et al. 2006; Bunney et al. 2006, 2009), include RAPGEF and PIP_2 lipase C activities, which controls endocytosis, exocytosis, and cytoskeletal reorganization (Bunney and Katan 2006). RASSF5 (also called NORE1) forms a complex with MST1/2 kinases, human orthologs of Hippo, and promotes apoptosis and cell cycle arrest (Stieglitz et al. 2008; Chan and Katan 2013). RASSF1 is also potential tumor suppressor and is required for death receptor-dependent apoptosis and mediates activation of STK3/MST2 and STK4/MST1 during FAS-induced apoptosis by preventing their dephosphorylation (Praskova et al. 2004). Notably, there are many more RAS effectors reported, e.g. TIAM1, p120RASGAP, RIN, AF6, IMP, GRB7, and SIN1 (Pamonsinlapatham et al. 2009; Berndt et al. 2011; Stephen et al. 2014; McCormick 2015, 2016).

It is believed that different RAS isoforms can generate specific biological functions. HRAS has a critical role in mediating different cellular effects. Focal adhesion kinase (FAK) is a widely expressed non-receptor tyrosine kinase and is stimulated by PDGF. HRAS plays as an intermediate protein regulating PDGF-induced FAK tyrosine phosphorylation in human hepatic stellate cells (HSCs) (Carloni et al. 2000). Oncogenic HRAS preferentially activates endogenous CRAF compared to ARAF, which is due to the reduced binding affinity of HRAS for ARAF (Weber et al. 2000). In primary hepatocytes, HRAS is the major mediator of ERK induced proliferation and survival, while HRAS and KRAS both mediate PI3K-induced survival (Rosseland et al. 2008). KRAS4A and KRAS4B share the same effectors but some proteins are specific for KRAS4B, such as CAM (Villalonga et al. 2001), which facilitates KRAS4B interaction with CRAF, RASGAP, and plasma membrane. Moreover, it has been shown that KRAS4B binding to CAM will lead to the suppression of non-canonical WNT signaling that strongly contributes to its tumorigenic properties (Wang et al. 2015).

RRAS signaling

RRAS binds FLNa and promotes endothelial barrier function, which is loss if interfering with the RRAS-FLNA interaction (Griffiths et al. 2011). Another RRAS effector is gridin that is associated with VE-cadherin and controls transendothelial permeability (Griffiths et al. 2011; Ichimiya et al. 2015). In response to a wide variety of inflammatory mediators, RRAS also activates, together with RAP1, αMβ2 integrin in macrophages via a pathway involving RAP1 (Caron et al. 2000), stimulates the formation of focal adhesion through FAK and p130CAS (Kwong et al. 2003), activates PLCε and controls the actin cytoskeleton arrangement (Ada-Nguema et al. 2006). The RRAS-RIN2-RAB5 axis recruits the RACGEF TIAM1 to control RAC1-dependent endothelial cell adhesion (Sandri et al. 2012).

RAP signaling

RAP proteins contribute to several biological processes which are often related to the cytoskeleton, adhesion receptors, and cellular trafficking (Frische and Zwartkruis 2010). RAP1 regulates adhesion to ECM via activation of RGS14, PKD1, and RAPL (Nonaka et al. 2008; Plak et al. 2016; Zhang et al. 2017), controls cell–cell junction via interaction with AF6 and KRIT1 (Glading et al. 2007; Kooistra et al. 2007). RAP2 interacts with MAP4K4, MINK, TNIK, RPIP9, PARG1, and PLCε and, thus, participates in different pathways (Rebhun et al. 2000; Ohba et al. 2001; Stork 2003; Stope et al. 2004). In neurons, RAP2 regulates JNK activity leading to depotentiation by mediating synaptic internalization of AMPA receptors (Zhu et al. 2005). The RAP2 effector MAP4K4, but obviously not TNIK, mediates activation of JNK pathway (Machida et al. 2004). RAP2 interaction with TNIK increases the kinase activity and interferes with the cell spreading. TNIK is a specific RAP2 effector and is involved in actin cytoskeleton regulation (Taira et al. 2004). PLCε is activated via RAP2B and its activation increases intracellular level of Ca^{2+}. RAP2B is involved in Lung cancer development through its interaction with PLCε (Nonaka et al. 2008; Tyutyunnykova et al. 2017). PARG1 is a specific effector of RAP2 which induces typical cytoskeletal changes for RHO inactivation in fibroblasts. RAP2 interacts with ZPH region of PARG1 which mediates suppression of PARG1 action (Myagmar et al. 2005). RPIP9 is a RAP2 effector and its activation happens during the malignant breast epithelial transformation and is related to metastatic lymph node invasion (Raguz et al. 2005). Misshapen/NIKs-related kinase (MINK) is a RAP2 interacting protein whose interaction with RAP2 is GTP dependent. MINK is enriched in the brain and activated MINK phosphorylates the postsynaptic scaffold protein TANC1 (Nonaka et al. 2008).

RAL signaling

A well-studied function of RAL proteins is the regulation and assembly of the multiprotein exocyst complex and, therefore, regulation of exocytosis. Activated RALA, but none of the other RAS proteins, interacts with SEC5 and EXO84 in a competitive manner (Moskalenko et al. 2002; Sugihara et al. 2002; Jin et al. 2005). RALA-SEC5 and RALA-EXO84 interactions are critical regulators of vesicle trafficking and exocytosis of adhesion molecules, transporters, and receptors in many cell types and organisms (de Leeuw et al. 2001; Shipitsin and Feig 2004; Kawato et al. 2008; Lopez et al. 2008; Sanchez-Ruiz et al. 2011; Teodoro et al. 2013). RAL-exocyst complex regulates the actin cytoskeletal organization by mediating filopodia formation (Sugihara et al. 2002), cellular motility (Spiczka and Yeaman 2008), autophagosome formation (Bodemann et al. 2011), protein sorting (Shipitsin and Feig 2004), neurite branching (Lalli and Hall 2005), and cytokinesis (Cascone et al. 2008; Shirakawa and Horiuchi 2015). RALBP1 (also called RLIP76), the first RAL effector that have been described, regulates mitotic progression of cytokinesis (Cascone et al. 2008), and endocytosis of EGF and insulin receptors through the interaction with active RALA and RALB (Nakashima et al. 1999; Jullien-Flores et al. 2000). RALA interaction with PLD1 stimulates together with ARF6 mTORC1 signaling (Xu et al. 2011) and modulates localization of the cell cycle inhibitor, p27 (Tazat et al. 2013). This interaction, however, appears to be nucleotide-independent and mediated via the 11 amino acid extension of RALA (Jiang et al. 1995).

RIT signaling

RIT1/2 interact, among known RAS effectors, with AF6 and RALGDS family proteins, which consists of RALGDS, RGL, RGL2/Rlf, and RGL3 (Ferro and Trabalzini 2010), that directly link RIT1 to RAL signaling pathways (Shao et al. 1999; Shao and Andres 2000). RIT2 targets the RAC/CDC42 activation via PAR6 and regulates neurite outgrowth in PC12 cells (Hoshino and Nakamura 2003; Hoshino et al. 2005). RIT1 binds SIN1 and may regulate AKT phosphorylation by mTORC2 (Cai and Andres 2014). This and other studies confirmed the unique role of RIT1 but no other RAS proteins in protection against cellular stress (Shi et al. 2011; Cai et al. 2012). In this context, RIT1 also activates the second survival cascade, p38-MSK1-CREB, which results in expression of anti-apoptotic proteins, such as BCL-2 and BLC-XL (Shi et al. 2012). Activation of the RIT1-MKK3/MKK6-p38γ axis

promotes c-JUN transcriptional activity (Sakabe et al. 2002). RIT1 regulates the p38-MK2-HSP27 axis and by subsequent AKT activation and BAD phosphorylation, leads to the inhibition of apoptosis induced by ROS (Cai et al. 2011).

RIT1/2 are also involved in neuron differentiation, neurogenesis, neurite growth, and branching. RIT1 links NGF signaling to the MEK-ERK signal pathway (Spencer et al. 2002a) and regulates neurite elongation and branching *via* BRAF and p38 but not the AKT pathway (Hynds et al. 2003; Shi and Andres 2005). RIT1, however, modulates the proliferation and differentiation of neuronal progenitor cells *via* SIN1-mTORC2-AKT axis in adult brain, which results, among others, in phosphorylation of SOX2, a stem cell-specific transcriptional factor (Mir et al. 2017). RIT2 has been found in different protein complexes. Downstream of PACAP38-Gαs-SRC axis, RIT2 controls neuronal differentiation via HSP27, which stabilizes the actin cytoskeleton (Shi et al. 2008). In addition, RIT2 participates in regulated, PKC-dependent, endocytosis and internalization of DAT1, and terminates dopamine signaling in the brain (Navaroli et al. 2011).

RHEB signaling

RHEB1 plays an essential role in different organs and regulates various cellular processes ranging from cell growth to apoptosis (Ehrkamp et al. 2013). A well-studied pathway is RHEB1-mTORC1 that regulates translation, autophagy, and cell growth (Heard et al. 2014; Armijo et al. 2016; Potheraveedu et al. 2017). RHEB1 directly binds and activates mTOR (Long et al. 2005). This activity is obviously modulated by different proteins. PLD1 binds RHEB1 and potentiates mTOR activation and presumably leads to cell size regulation (Sun et al. 2008). PLD1-produced phosphatidic acid directly interacts with the mTOR domain that is targeted by rapamycin (Fang et al. 2001). In contrast, PDE4D and GAPDH bind to RHEB1 and sequester it from mTOR activation (Lee et al. 2009; Kim et al. 2010). The latter is regulated by cAMP and Gly-3-P, which binds PDE4D and GAPDH, respectively, and release RHEB1 to bind mTOR and activates mTORC1 (Lee et al. 2009; Kim et al. 2010). Due to its high similarity to HRAS within the switch I region, RHEB1 has been shown to interact with CRAF and BRAF although with a different binding affinity (Karassek et al. 2010). While RHEB1 binding to BRAF inhibits its kinase activity and prevents BRAF-dependent activation of the MAPK pathway (Im et al. 2002; Karbowniczek et al. 2004), it appears to bind CRAF and activates cell transformation and neurite outgrowth (Yee and Worley 1997). In addition, RHEB1 binds dynein and blocks aggresome formation and autophagy (Zhou et al. 2009), interacts with FKBP38 and interferes with the BCL2 family protein association with the pro-apoptotic BAX/BAK proteins (Ma et al. 2010), and RHEB1-NOTCH association is involved in cell-fate decision (Karbowniczek et al. 2010). In addition, RHEB interaction with β-site amyloid precursor protein (APP)-cleaving enzyme1 (β-secretase, BACE1) results in its instability and lower level of amyloid β generation (Shahani et al. 2014). Protein kinase-like ER kinase (PERK) is known as a novel RHEB1 effector and its activation results in an eIF2α phosphorylation and inhibition of protein synthesis again in a mTORC-independent manner (Tyagi et al. 2015). In addition, there is a crosstalk between RHEB1 and Hippo pathway, where RHEB1 stimulates Hippo signaling via binding to RASSF1. However, the RASSF1 binding to RHEB has an adverse effect on mTORC activity (Nelson and Clark 2016).

ERAS signaling

Our knowledge about effector interaction and signal transduction of ERAS as well as DIRAS and RASD paralogs is very limited. The constitutive active ERAS controls growth of mouse embryonic stem cells and maintains quiescence in rat hepatic stellate cells via the PI3K-PDK1-AKT-mTORC1 axis (Takahashi et al. 2003; Nakhaei-Rad et al. 2016). ERAS may also regulate other pathways, including MST1/2-LATS1/2-YAP and SIN1-mTORC2 (Nakhaei-Rad et al. 2016), which remains to be proved.

DIRAS signaling

DIRAS proteins antagonizes RAS signaling (Bergom et al. 2016) leading to decreased levels of phosphorylation of CRAF, MEK, ERK, p90RSK, and BAD (Zhu et al. 2013). In *Caenorhabditis elegans*, DIRas-1 ortholog binds to Epac-1 and modulates the synaptic plasticity in neurons (Tada et al. 2012). Zebrafish DIRas increases the protein levels and activity of Rac1 and regulates via Rac1-Pak1-Cdk5-ARP2/3 axis neurite outgrowth (Yeh and Hsu 2016). DIRAS3 interferes with IL6-induced STAT3 phosphorylation and transcriptional activity towards cMYC, Cyclin D1, and Bcl-xL (Nishimoto et al. 2005). Moreover, DIRAS3 directly binds CRAF probably via its N-terminal extension and interferes with MEK-ERK1/2 activation (Klingauf et al. 2013).

RASD signaling

RASD1 (also called AGS1 or DEXRAS) is a non-receptor activator of $G_{\alpha i}$ and $G_{\alpha o}$ proteins (Cismowski et al. 1999;

Cismowski et al. 2000; Blumer and Lanier 2014). It blocks receptor-mediated sensitization of AC1 in a Gβγ-dependent manner (Nguyen and Watts 2005) and inhibits PMA-induced activation stimulation of AC2 by interfering with PKCδ autophosphorylation (Nguyen and Watts 2006). RASD2 (also called RHES) binds to PAP7 in a PKA-dependent manner and activates DMT1 and iron uptake in the striatum (Choi et al. 2013).

Dysfunctions and diseases

As RAS family proteins essentially control a wide variety of cellular processes, it is obvious that any dysregulation or dysfunction of the respective signaling pathways results in the development of human diseases, including developmental, hematological, neurocognitive and neurodegenerative disorders, metabolic and cardiovascular diseases, and cancer.

Somatic mutations, frequently identified for example in *KRAS4B*, *HRAS*, *NRAS*, and *RIT1* (COSMIC), contribute to robust gain-of-function (GoF) effects and to various types of cancers as well as leukemia and lymphoma tumors (The Cancer Genome Atlas Research Network 2014; Simanshu et al. 2017). Such oncogenes are constitutive active and thus, strongly contribute to neoplastic signal transduction (Hobbs et al. 2016). Similarly, GoF mutations of genes frequently related to *BRAF* and *PI3K*, cause constitutive activation of the MAPK and PDK1-AKT/PKB pathways (Santarpia et al. 2012; Mandal et al. 2016). In contrast, loss-of-function (LoF) mutations of tumor-suppressive DIRAS genes is associated with progression of various cancers, including esophageal, ovarian, breast, and colon cancers and particularly also glioblastoma (Ligon et al. 1997; Ellis et al. 2002; Reif et al. 2011; Zhu et al. 2013; Zheng et al. 2017). A proposed mechanism for the tumor suppressive functions of DIRAS1 is sequestration of SmgGDS from activation of KRAS4B, RAP1A, and RHOA (Bergom et al. 2016). Negative regulation of ERK and p38 by DIRAS1 appears to induce apoptosis and inhibit invasion and metastasis (Zhu et al. 2013). DIRAS3 downregulation may underlay transcriptional mechanisms, involving E2F1 and E2F4, and also loss of DIRAS3 mRNA binding proteins (Guénard and Durocher 2010). LoF somatic mutations in the *NF1* gene, encoding a RASGAP protein, result in dysregulation of the RAS/MAPK pathway and thus, cause neurofibromatosis, a multisystem disorder, and tumor predisposition syndrome (Philpott et al. 2017; Postema et al. 2018). Somatic *NF1* mutations are associated with the development of sporadic tumors in children (Brems et al. 2009; Ratner and Miller 2015; Varan et al. 2016; Philpott et al. 2017).

Mild GoF effects by germline mutations of *KRAS4B*, *HRAS1/2*, *NRAS*, *RIT1*, and *RRAS1/3* genes (NSEuroNet database) cause a class of developmental syndromes. These phenotypically overlapping genetic disorders collectively known as RASopathies are mainly caused by dysregulation of the RAS-MAPK pathway. RASopathies include Noonan syndrome (genes encoding KRAS4B, NRAS, RRAS1/3, RIT1, SOS1, SOS2, RASGAP1M, BRAF, CRAF), cardio-facio-cutaneous syndrome (KRAS4B, BRAF, ERK1/2), Costello syndrome (HRAS1, HRAS2), neurofibromatosis type 1 (neurofibromin), Legius syndrome (SPRED1), Noonan syndrome with multiple lentigines (BRAF, CRAF), and capillary malformation/arteriovenous malformation syndrome (p120RASGAP) (Rauen 2013; Flex et al. 2014; Korf et al. 2015; Lissewski et al. 2015; Aoki et al. 2016; Tidyman and Rauen 2016; Cao et al. 2017; Higgins et al. 2017; Pantaleoni et al. 2017; Simanshu et al. 2017; Ueda et al. 2017). RASopathies have pleiomorphic features, including in part facial anomalies, cognitive impairment, and congenital heart defects (Gelb et al. 2015; Lissewski et al. 2015; Aoki et al. 2016; Cave et al. 2016; Mainberger et al. 2016; Simanshu et al. 2017). Inactivating germline mutations in *NF1* gene are associated with impaired activation of the RAS pathways and increase risk of neoplasms (Alkindy et al. 2012; Ratner and Miller 2015).

RAS proteins are also involved in neuropsychiatric and neurodegenerative disorders, e.g. RIT2 in schizophrenia and autism (Glessner et al. 2010; Navaroli et al. 2011; Liu et al. 2016), RIT2 and DIRAS1 in Parkinson's disease (Latourelle et al. 2012; Pankratz et al. 2012; Nalls et al. 2014), RASD2 and RRAS1 in Huntington's disease (Miller et al. 2012; Ray et al. 2014; Vahatupa et al. 2016). Alterations in the expressional control of DIRAS2 also contribute to the ADHD phenotype of the attention deficit-hyperactivity disorder (Reif et al. 2011; Grunewald et al. 2016). RASD1 plays a role in synchronizing circadian rhythms, as its deletion impairs circadian entrainment to light cycles and alters phase shifts to light (Cheng et al. 2004). The molecular nature of these (dys)functions are not well understood. However, several biochemical studies have provided valuable molecular insights into the roles of RAS protein in these disorders. The RASD2 activity as a SUMO-E3 ligase (Subramaniam et al. 2010) on the polyglutamine-expanded mutant huntingtin protein leading to augmented neurotoxicity and likely to Huntington's disease (Harrison 2012; Thapliyal et al. 2014). S-nitrosylation and activation of RASD1 by NMDA-nNOS pathway induces physiological iron uptake through interaction with PAP7 and activation of DMT1, and may be critical for NMDA neurotoxicity (Cheah et al. 2006; Chen et al. 2013; Choi

et al. 2013). The role of RIT2 in neuropsychiatric disorders may be based on its role in the internalization and downregulation of biogenic amine transporters, which are discussed to be central to autism (Navaroli et al. 2011).

Conclusions and perspectives

More than 30 years intensive research and tens of thousands of published studies have provided valuable insights into biology, biochemistry, and biophysics of the RAS family proteins. We have gained deep knowledge about their membrane trafficking, structure–function relationship, mechanisms of GDP/GTP binding, and accelerated nucleotide exchange by GEFs, intrinsic and GAP-stimulated GTP hydrolysis, interaction with effectors, and activation of diverse signaling pathways. However, these studies have their eligible confinement: Cell-free investigations have been predominantly carried out in the absence of lipid membrane using defined domains rather than full-length proteins, and cell-based studies have been mostly performed using heterologous expression of tagged genes and their variants in a methodologically congenial cell lines. As the omics era is coming to an end and the research becomes decelerated, many new movements are emerged, especially due to the accessibility of new technologies. Several novel mechanisms have been uncovered that have extended our understanding the role of protein–protein/protein–lipid interactions, and various types of post-translational modifications in the modulation of the RAS protein activity. Another issue is the activation mechanism of regulators and effectors. Notably, identification of additional components of the RAS interaction networks is a critical step towards understanding both the relationship between the RAS proteins and the selective activation of respective effectors, and the molecular signatures required for spatiotemporal integration and activation of the GEFs and GAPs. Identification and functional reconstitution of specific interaction networks by using appropriate liposomes and full-length regulators and effector proteins may eventually provide fundamental insights into the functional characterization of multiprotein complexes of RAS and the complete identification of regulatory mechanisms. In this context, an interesting issue, which is increasingly appreciated, is a RAS-membrane interaction that appears to generate RAS isoform specificity with respect to regulator and effector interactions. This is likely achieved by scaffold proteins which may modulate isoform specificity at specific site of the cell. Hence, elucidation of the RAS signal transduction requires not only RAS-effector interactions but also additional structures and interplay of multiprotein complexes. Keeping this in mind, accumulating evidence support a role for cell type-dependent RAS paralog functions that should prompt future efforts to examine the respective pathways in a more context-specific manner. Such efforts could lead to the identification of disease-specific therapeutic opportunities.

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Disclosure statement

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References

Abankwa D, Gorfe AA, Hancock JF. 2007. Ras nanoclusters: molecular structure and assembly. Semin Cell Dev Biol. 18:599–607.

Abankwa D, Gorfe AA, Inder K, Hancock JF. 2010. Ras membrane orientation and nanodomain localization generate isoform diversity. Proc Natl Acad Sci USA. 107:1130–1135.

Ada-Nguema AS, Xenias H, Hofman JM, Wiggins CH, Sheetz MP, Keely PJ. 2006. The small GTPase R-Ras regulates organization of actin and drives membrane protrusions through the activity of PLCepsilon. J Cell Sci. 119:1307–1319.

Ahearn IM, Haigis K, Bar-Sagi D, Philips MR. 2011. Regulating the regulator: post-translational modification of RAS. Nat Rev Mol Cell Biol. 13:39–51.

Ahmadian MR, Kiel C, Stege P, Scheffzek K. 2003. Structural fingerprints of the Ras-GTPase activating proteins neurofibromin and p120GAP. J Mol Biol. 329:699–710.

Ahmadian MR, Stege P, Scheffzek K, Wittinghofer A. 1997. Confirmation of the arginine-finger hypothesis for the GAP-stimulated GTP-hydrolysis reaction of Ras. Nat Struct Mol Biol. 4:686–689.

Ahmadian MR, Zor T, Vogt D, Kabsch W, Selinger Z, Wittinghofer A, Scheffzek K. 1999. Guanosine triphosphatase stimulation of oncogenic Ras mutants. Proc Natl Acad Sci USA. 96:7065–7070.

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol. 7:261–269.

Alkindy A, Chuzhanova N, Kini U, Cooper DN, Upadhyaya M. 2012. Genotype-phenotype associations in neurofibromatosis type 1 (NF1): an increased risk of tumor complications in patients with NF1 splice-site mutations? Hum Genomics. 6:12.

Alvarez-Moya B, Barcelo C, Tebar F, Jaumot M, Agell N. 2011. CaM interaction and Ser181 phosphorylation as new K-Ras signaling modulators. Small GTPases. 2:99–103.

Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq JM, Hemmings BA. 1997. Role of translocation in the activation and function of protein kinase B. J Biol Chem. 272:31515–31524.

Aoki Y, Niihori T, Inoue S, Matsubara Y. 2016. Recent advances in RASopathies. J Hum Genet. 61:33–39.

Armijo ME, Campos T, Fuentes-Villalobos F, Palma ME, Pincheira R, Castro AF. 2016. Rheb signaling and tumorigenesis: mTORC1 and new horizons. Int J Cancer. 138:1815–1823.

Ashery U, Yizhar O, Rotblat B, Kloog Y. 2006. Nonconventional trafficking of Ras associated with Ras signal organization. Traffic. 7:119–126.

Baker R, Lewis SM, Sasaki AT, Wilkerson EM, Locasale JW, Cantley LC, Kuhlman B, Dohlman HG, Campbell SL. 2013a. Site-specific monoubiquitination activates Ras by impeding GTPase-activating protein function. Nat Struct Mol Biol. 20:46–52.

Baker R, Wilkerson EM, Sumita K, Isom DG, Sasaki AT, Dohlman HG, Campbell SL. 2013b. Differences in the regulation of K-Ras and H-Ras isoforms by monoubiquitination. J Biol Chem. 288:36856–36862.

Baljuls A, Beck M, Oenel A, Robubi A, Kroschewski R, Hekman M, Rudel T, Rapp UR. 2012. The tumor suppressor DiRas3 forms a complex with H-Ras and C-RAF proteins and regulates localization, dimerization, and kinase activity of C-RAF. J Biol Chem. 287:23128–23140.

Banerjee A, Jang H, Nussinov R, Gaponenko V. 2016. The disordered hypervariable region and the folded catalytic domain of oncogenic K-Ras4B partner in phospholipid binding. Curr Opin Struct Biol. 36:10–17.

Battram AM, Durrant TN, Agbani EO, Heesom KJ, Paul DS, Piatt R, Poole AW, Cullen PJ, Bergmeier W, Moore SF, Hers I. 2017. The phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) binder Rasa3 regulates phosphoinositide 3-kinase (PI3K)-dependent Integrin alphaIIbbeta3 outside-in signaling. J Biol Chem. 292:1691–1704.

Benetka W, Koranda M, Maurer-Stroh S, Pittner F, Eisenhaber F. 2006. Farnesylation or geranylgeranylation? Efficient assays for testing protein prenylation in vitro and in vivo. BMC Biochem. 7:6.

Beranger F, Tavitian A, de Gunzburg J. 1991. Post-translational processing and subcellular localization of the Ras-related Rap2 protein. Oncogene. 6:1835–1842.

Bergom C, Hauser AD, Rymaszewski A, Gonyo P, Prokop JW, Jennings BC, Lawton AJ, Frei A, Lorimer EL, Aguilera-Barrantes I, et al. 2016. The tumor-suppressive small GTPase DiRas1 binds the noncanonical guanine nucleotide exchange factor SmgGDS and antagonizes SmgGDS interactions with oncogenic small GTPases. J Biol Chem. 291:6534–6545.

Berndt N, Hamilton AD, Sebti SM. 2011. Targeting protein prenylation for cancer therapy. Nat Rev Cancer. 11:775.

Berzat AC, Brady DC, Fiordalisi JJ, Cox AD. 2006. Using inhibitors of prenylation to block localization and transforming activity. Methods Enzymol. 407:575–597.

Bhagatji P, Leventis R, Rich R, Lin CJ, Silvius JR. 2010. Multiple cellular proteins modulate the dynamics of K-ras association with the plasma membrane. Biophys J. 99:3327–3335.

Bivona TG, Quatela SE, Bodemann BO, Ahearn IM, Soskis MJ, Mor A, Miura J, Wiener HH, Wright L, Saba SG, et al. 2006. PKC regulates a farnesyl-electrostatic switch on K-Ras that promotes its association with Bcl-XL on mitochondria and induces apoptosis. Mol Cell. 21:481–493.

Blazevits O, Mideksa YG, Solman M, Ligabue A, Ariotti N, Nakhaeizadeh H, Fansa EK, Papageorgiou AC, Wittinghofer A, Ahmadian MR, Abankwa D. 2016. Galectin-1 dimers can scaffold Raf-effectors to increase H-ras nanoclustering. Sci Rep. 6:24165.

Blumer JB, Lanier SM. 2014. Activators of G protein signaling exhibit broad functionality and define a distinct core signaling triad. Mol Pharmacol. 85:388–396.

Bodemann BO, Orvedahl A, Cheng T, Ram RR, Ou YH, Formstecher E, Maiti M, Hazelett CC, Wauson EM, Balakireva M, et al. 2011. RalB and the exocyst mediate the cellular starvation response by direct activation of autophagosome assembly. Cell. 144:253–267.

Boriack-Sjodin PA, Margarit SM, Bar-Sagi D, Kuriyan J. 1998. The structural basis of the activation of Ras by Sos. Nature. 394:337–343.

Bos JL. 2005. Linking Rap to cell adhesion. Curr Opin Cell Biol. 17:123–128.

Bourne HR, Sanders DA, McCormick F. 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature. 349:117.

Braun BS, Shannon K. 2008. Targeting Ras in myeloid leukemias. Clin Cancer Res. 14:2249–2252.

Brems H, Beert E, de Ravel T, Legius E. 2009. Mechanisms in the pathogenesis of malignant tumours in neurofibromatosis type 1. Lancet Oncol. 10:508–515.

Broustas CG, Grammatikakis N, Eto M, Dent P, Brautigan DL, Kasid U. 2001. Phosphorylation of the myosin-binding subunit of myosin phosphatase by Raf-1 and inhibition of phosphatase activity. J Biol Chem. 277:3053–3059.

Buday L, Downward J. 2008. Many faces of Ras activation. Biochim Biophys Acta. 1786:178–187.

Bunney TD, Baxendale RW, Katan M. 2009. Regulatory links between PLC enzymes and Ras superfamily GTPases: signalling via PLCepsilon. Adv Enzyme Regul. 49:54–58.

Bunney TD, Harris R, Gandarillas NL, Josephs MB, Roe SM, Sorli SC, Paterson HF, Rodrigues-Lima F, Esposito D, Ponting CP, et al. 2006. Structural and mechanistic insights into ras association domains of phospholipase C epsilon. Mol Cell. 21:495–507.

Bunney TD, Katan M. 2006. Phospholipase C epsilon: linking second messengers and small GTPases. Trends Cell Biol.16:640–648.

Bunney TD, Katan M. 2011. PLC regulation: emerging pictures for molecular mechanisms. Trends Biochem Sci. 36:88–96.
Cai W, Andres DA. 2014. mTORC2 is required for rit-mediated oxidative stress resistance. PLoS One. 9:e115602
Cai W, Rudolph JL, Harrison SM, Jin L, Frantz AL, Harrison DA, Andres DA. 2011. An evolutionarily conserved Rit GTPase-p38 MAPK signaling pathway mediates oxidative stress resistance. Mol Biol Cell. 22:3231–3241.
Cai W, Rudolph JL, Sengoku T, Andres DA. 2012. Rit GTPase regulates a p38 MAPK-dependent neuronal survival pathway. Neurosci Lett. 531:125–130.
Calvo F, Crespo P. 2009. Structural and spatial determinants regulating TC21 activation by RasGRF family nucleotide exchange factors. Mol Biol Cell. 20:4289–4302.
Camats M, Kokolo M, Heesom KJ, Ladomery M, Bach-Elias M. 2009. P19 H-ras induces G1/S phase delay maintaining cells in a reversible quiescence state. PLoS One. 4:e8513.
Cao H, Alrejaye N, Klein OD, Goodwin AF, Oberoi S. 2017. A review of craniofacial and dental findings of the RASopathies. Orthod Craniofac Res. 20 Suppl 1:32–38.
Carloni V, Pinzani M, Giusti S, Romanelli RG, Parola M, Bellomo G, Failli P, Hamilton AD, Sebti SM, Laffi G, Gentilini P. 2000. Tyrosine phosphorylation of focal adhesion kinase by PDGF is dependent on ras in human hepatic stellate cells. Hepatology 31:131–140.
Caron E, Self AJ, Hall A. 2000. The GTPase Rap1 controls functional activation of macrophage integrin alphaMbeta2 by LPS and other inflammatory mediators. Curr Biol. 10:974–978.
Carroll B, Maetzel D, Maddocks OD, Otten G, Ratcliff M, Smith GR, Dunlop EA, Passos JF, Davies OR, Jaenisch R, et al. 2016. Control of TSC2-Rheb signaling axis by arginine regulates mTORC1 activity. Elife. 5:e11058.
Cascone I, Selimoglu R, Ozdemir C, Del Nery E, Yeaman C, White M, Camonis J. 2008. Distinct roles of RalA and RalB in the progression of cytokinesis are supported by distinct RalGEFs. EMBO J. 27:2375–2387.
Castellano E, Downward J. 2010. Role of RAS in the regulation of PI 3-kinase. Curr Top Microbiol Immunol. 346:143–169.
Castellano E, Downward J. 2011. RAS interaction with PI3K: more than just another effector pathway. Genes Cancer. 2:261–274.
Cave H, Caye A, Strullu M, Aladjidi N, Vignal C, Ferster A, Mechinaud F, Domenech C, Pierri F, Contet A, et al. 2016. Acute lymphoblastic leukemia in the context of RASopathies. Eur J Med Genet. 59:173–178.
Cha JY, Kim HJ, Yu JH, Xu J, Kim D, Paul BD, Choi H, Kim S, Lee YJ, Ho GP, et al. 2013. Dexras1 mediates glucocorticoid-associated adipogenesis and diet-induced obesity. Proc Natl Acad Sci USA. 110:20575–20580.
Chan JJ, Katan M. 2013. PLCε and the RASSF family in tumour suppression and other functions. Adv Biol Regul. 53:258–279.
Chandra A, Grecco HE, Pisupati V, Perera D, Cassidy L, Skoulidis F, Ismail SA, Hedberg C, Hanzal-Bayer M, Venkitaraman AR, et al. 2011. The GDI-like solubilizing factor PDEdelta sustains the spatial organization and signalling of Ras family proteins. Nat Cell Biol. 14:148–158.
Cheah JH, Kim SF, Hester LD, Clancy KW, Patterson SE, Papadopoulos V, Snyder SH. 2006. NMDA receptor-nitric oxide transmission mediates neuronal iron homeostasis via the GTPase Dexras1. Neuron 51:431–440.
Chen J, Fujii K, Zhang L, Roberts T, Fu H. 2001. Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. Proc Natl Acad Sci USA. 98:7783–7788.
Chen Y, Khan RS, Cwanger A, Song Y, Steenstra C, Bang S, Cheah JH, Dunaief J, Shindler KS, Snyder SH. 2013. Dexras1, a small GTPase, is required for glutamate-NMDA neurotoxicity. J Neurosci. 33:3582–3587.
Chen Y, Mathias L, Falero-Perez JM, Kim SF. 2015. PKA-mediated phosphorylation of Dexras1 suppresses iron trafficking by inhibiting S-nitrosylation. FEBS Lett. 589:3212–3219.
Cheng H-YM, Obrietan K, Cain SW, Lee BY, Agostino PV, Joza NA, Harrington ME, Ralph MR, Penninger JM. 2004. Dexras1 potentiates photic and suppresses nonphotic responses of the circadian clock. Neuron. 43:715–728.
Cherfils J, Zeghouf M. 2013. Regulation of small GTPases by GEFs, GAPs, and GDIs. Physiol Rev. 93:269–309.
Choi BR, Bang S, Chen Y, Cheah JH, Kim SF. 2013. PKA modulates iron trafficking in the striatum via small GTPase, Rhes. Neuroscience. 253:214–220.
Cirstea IC, Kutsche K, Dvorsky R, Gremer L, Carta C, Horn D, Roberts AE, Lepri F, Merbitz-Zahradnik T, Konig R, et al. 2010. A restricted spectrum of NRAS mutations causes Noonan syndrome. Nat Genet. 42:27–29.
Cismowski MJ, Ma C, Ribas C, Xie X, Spruyt M, Lizano JS, Lanier SM, Duzic E. 2000. Activation of heterotrimeric G-protein signaling by a Ras-related protein implications for signal integration. J Biol Chem. 275:23421–23424.
Cismowski MJ, Takesono A, Ma C, Lizano JS, Xie X, Fuernkranz H, Lanier SM, Duzic E. 1999. Genetic screens in yeast to identify mammalian nonreceptor modulators of G-protein signaling. Nat Biotechnol. 17:878–883.
Cohen JB, Broz SD, Levinson AD. 1989. Expression of the H-ras proto-oncogene is controlled by alternative splicing. Cell. 58:461–472.
Cordeddu V, Di Schiavi E, Pennacchio LA, Ma'ayan A, Sarkozy A, Fodale V, Cecchetti S, Cardinale A, Martin J, Schackwitz W, et al. 2009. Mutation of SHOC2 promotes aberrant protein N-myristoylation and causes Noonan-like syndrome with loose anagen hair. Nat Genet. 41:1022–1026.
Cox AD, Der CJ. 2003. The dark side of Ras: regulation of apoptosis. Oncogene. 22:8999–9006.
Cox AD, Der CJ, Philips MR. 2015. Targeting RAS membrane association: back to the future for anti-RAS drug discovery? Clin Cancer Res. 21:1819–1827.
Crechet JB, Poullet P, Mistou MY, Parmeggiani A, Camonis J, Boy-Marcotte E, Damak F, Jacquet M. 1990. Enhancement of the GDP-GTP exchange of RAS proteins by the carboxyl-terminal domain of SCD25. Science. 248:866–868.
Dai Y, Walker SA, de Vet E, Cook S, Welch HC, Lockyer PJ. 2011. Ca^{2+}-dependent monomer and dimer formation switches CAPRI Protein between Ras GTPase-activating protein (GAP) and RapGAP activities. J Biol Chem. 286:19905–19916.
Daumke O, Weyand M, Chakrabarti PP, Vetter IR, Wittinghofer A. 2004. The GTPase-activating protein Rap1GAP uses a catalytic asparagine. Nature. 429:197–201.
Davis RK, Chellappan S. 2008. Disrupting the Rb-Raf-1 interaction: a potential therapeutic target for cancer. Drug News Perspect. 21:331–335.

de la Vega M, Burrows JF, McFarlane C, Govender U, Scott CJ, Johnston JA. 2010. The deubiquitinating enzyme USP17 blocks N-Ras membrane trafficking and activation but leaves K-Ras unaffected. J Biol Chem. 285:12028–12036.

de Leeuw HP, Fernandez-Borja M, Reits EA, Romani de Wit T, Wijers-Koster PM, Hordijk PL, Neefjes J, van Mourik JA, Voorberg J. 2001. Small GTP-binding protein Ral modulates regulated exocytosis of von Willebrand factor by endothelial cells. Arterioscler Thromb Vasc Biol. 21:899–904.

de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. 1998. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature. 396:474–477.

Depeille P, Henricks LM, van de Ven RA, Lemmens E, Wang CY, Matli M, Werb Z, Haigis KM, Donner D, Warren R, Roose JP. 2015. RasGRP1 opposes proliferative EGFR-SOS1-Ras signals and restricts intestinal epithelial cell growth. Nat Cell Biol. 17:804–815.

Desideri E, Cavallo AL, Baccarini M. 2015. Alike but different: RAF paralogs and their signaling outputs. Cell. 161:967–970.

Dharmaiah S, Bindu L, Tran TH, Gillette WK, Frank PH, Ghirlando R, Nissley DV, Esposito D, McCormick F, Stephen AG, Simanshu DK. 2016. Structural basis of recognition of farnesylated and methylated KRAS4b by PDEdelta. Proc Natl Acad Sci USA. 113:E6766–E6775.

Dodd KM, Tee AR. 2012. Leucine and mTORC1: a complex relationship. Am J Physiol Endocrinol Metab. 302:E1329–E1342.

Dolnik A, Kanwal N, Mackert S, Halbedl S, Proepper C, Bockmann J, Schoen M, Boeckers TM, Kuhl SJ, Schmeisser MJ. 2016. Sipa1l3/SPAR3 is targeted to postsynaptic specializations and interacts with the Fezzin ProSAPiP1/Lzts3. J Neurochem. 136:28–35.

Dunzendorfer-Matt T, Mercado EL, Maly K, McCormick F, Scheffzek K. 2016. The neurofibromin recruitment factor Spred1 binds to the GAP related domain without affecting Ras inactivation. Proc Natl Acad Sci USA. 113:7497–7502.

Ehrenreiter K, Piazzolla D, Velamoor V, Sobczak I, Small JV, Takeda J, Leung T, Baccarini M. 2005. Raf-1 regulates Rho signaling and cell migration. J Cell Biol. 168:955–964.

Ehrhardt A, David MD, Ehrhardt GR, Schrader JW. 2004. Distinct mechanisms determine the patterns of differential activation of H-Ras, N-Ras, K-Ras 4B, and M-Ras by receptors for growth factors or antigen. Mol Cell Biol. 24:6311–6323.

Ehrhardt GR, Korherr C, Wieler JS, Knaus M, Schrader JW. 2001. A novel potential effector of M-Ras and p21 Ras negatively regulates p21 Ras-mediated gene induction and cell growth. Oncogene. 20:188–197.

Ehrkamp A, Herrmann C, Stoll R, Heumann R. 2013. Ras and rheb signaling in survival and cell death. Cancers (Basel). 5:639–661.

Ellis CA, Vos MD, Howell H, Vallecorsa T, Fults DW, Clark GJ. 2002. Rig is a novel Ras-related protein and potential neural tumor suppressor. Proc Natl Acad Sci USA. 99:9876–9881.

Fang M, Jaffrey SR, Sawa A, Ye K, Luo X, Snyder SH. 2000. Dexras1: a G protein specifically coupled to neuronal nitric oxide synthase via CAPON. Neuron 28:183–193.

Fang Y, Vilella-Bach M, Bachmann R, Flanigan A, Chen J. 2001. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science. 294:1942–1945.

Fawal MA, Brandt M, Djouder N. 2015. MCRS1 binds and couples Rheb to amino acid-dependent mTORC1 activation. Dev Cell. 33:67–81.

Feig LA. 1999. Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases. Nat Cell Biol. 1:E25–E27.

Ferro E, Trabalzini L. 2010. RalGDS family members couple Ras to Ral signalling and that's not all. Cell Signal. 22:1804–1810.

Findlay GM, Smith MJ, Lanner F, Hsiung MS, Gish GD, Petsalaki E, Cockburn K, Kaneko T, Huang H, Bagshaw RD, et al. 2013. Interaction domains of Sos1/Grb2 are finely tuned for cooperative control of embryonic stem cell fate. Cell. 152:1008–1020.

Flex E, Jaiswal M, Pantaleoni F, Martinelli S, Strullu M, Fansa EK, Caye A, De Luca A, Lepri F, Dvorsky R, et al. 2014. Activating mutations in RRAS underlie a phenotype within the RASopathy spectrum and contribute to leukaemogenesis. Hum Mol Genet. 23:4315–4327.

Fremin C, Guegan JP, Plutoni C, Mahaffey J, Philips MR, Emery G, Meloche S. 2016. ERK1/2-induced phosphorylation of R-Ras GTPases stimulates their oncogenic potential. Oncogene. 35:5692–5698.

Frische EW, Zwartkruis FJ. 2010. Rap1, a mercenary among the Ras-like GTPases. Dev Biol. 340:1–9.

Galaktionov K, Jessus C, Beach D. 1995. Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation. Genes Dev. 9:1046–1058.

Gasper R, Sot B, Wittinghofer A. 2010. GTPase activity of Di-Ras proteins is stimulated by Rap1GAP proteins. Small GTPases. 1:133–141.

Gelb BD, Roberts AE, Tartaglia M. 2015. Cardiomyopathies in Noonan syndrome and the other RASopathies. Prog Pediatr Cardiol. 39:13–19.

Gentry LR, Martin TD, Reiner DJ, Der CJ. 2014. Ral small GTPase signaling and oncogenesis: More than just 15 minutes of fame. Biochim Biophys Acta. 1843:2976–2988.

Gentry LR, Nishimura A, Cox AD, Martin TD, Tsygankov D, Nishida M, Elston TC, Der CJ. 2015. Divergent roles of CAAX motif-signaled posttranslational modifications in the regulation and subcellular localization of Ral GTPases. J Biol Chem. 290:22851–22861.

Ghiglieri V, Napolitano F, Pelosi B, Schepisi C, Migliarini S, Di Maio A, Pendolino V, Mancini M, Sciamanna G, Vitucci D, et al. 2015. Rhes influences striatal cAMP/PKA-dependent signaling and synaptic plasticity in a gender-sensitive fashion. Sci Rep. 5:10933.

Glading A, Han J, Stockton RA, Ginsberg MH. 2007. KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. J Cell Biol. 179:247–254.

Glessner JT, Reilly MP, Kim CE, Takahashi N, Albano A, Hou C, Bradfield JP, Zhang H, Sleiman PM, Flory JH, et al. 2010. Strong synaptic transmission impact by copy number variations in schizophrenia. Proc Natl Acad Sci USA. 107:10584–10589.

Gloerich M, Bos JL. 2010. Epac: defining a new mechanism for cAMP action. Annu Rev Pharmacol Toxicol. 50:355–375.

Grewal T, Enrich C. 2006. Molecular mechanisms involved in Ras inactivation: the annexin A6-p120GAP complex. Bioessays. 28:1211–1220.

Grewal T, Evans R, Rentero C, Tebar F, Cubells L, de Diego I, Kirchhoff MF, Hughes WE, Heeren J, Rye KA, et al. 2005. Annexin A6 stimulates the membrane recruitment of

p120GAP to modulate Ras and Raf-1 activity. Oncogene. 24:5809–5820.
Griffiths GS, Grundl M, Allen JS, III, Matter ML. 2011. R-Ras interacts with filamin a to maintain endothelial barrier function. J Cell Physiol. 226:2287–2296.
Grujic O, Bhullar RP. 2009. Ral GTPase interacts with the N-terminal in addition to the C-terminal region of PLC-delta1. Biochem Biophys Res Commun. 383:401–405.
Grunewald L, Landaas ET, Geissler J, Weber H, Quast C, Roh S, Schartner C, Lesch KP, Romanos M, Kittel-Schneider S, et al. 2016. Functional impact of an ADHD-associated DIRAS2 promoter polymorphism. Neuropsychopharmacology. 41:3025–3031.
Guénard F, Durocher F. (2010). DHX9 (DEAH (Asp-Glu-Ala-His) box polypeptide 9). Atlas Genet Cytogenet Oncol Haematol. 14:548.
Guil S, de La Iglesia N, Fernandez-Larrea J, Cifuentes D, Ferrer JC, Guinovart JJ, Bach-Elias M. 2003. Alternative splicing of the human proto-oncogene c-H-ras renders a new Ras family protein that trafficks to cytoplasm and nucleus. Cancer Res. 63:5178–5187.
Guo Z, Ahmadian MR, Goody RS. 2005. Guanine nucleotide exchange factors operate by a simple allosteric competitive mechanism. Biochemistry. 44:15423–15429.
Gureasko J, Galush WJ, Boykevisch S, Sondermann H, Bar-Sagi D, Groves JT, Kuriyan J. 2008. Membrane-dependent signal integration by the Ras activator Son of sevenless. Nat Struct Mol Biol. 15:452–461.
Gutierrez-Erlandsson S, Herrero-Vidal P, Fernandez-Alfara M, Hernandez-Garcia S, Gonzalo-Flores S, Mudarra-Rubio A, Fresno M, Cubelos B. 2013. R-RAS2 overexpression in tumors of the human central nervous system. Mol Cancer. 12:127.
Hancock JF, Magee AI, Childs JE, Marshall CJ. 1989. All ras proteins are polyisoprenylated but only some are palmitoylated. Cell. 57:1167–1177.
Hanzal-Bayer MF, Hancock JF. 2007. Lipid rafts and membrane traffic. FEBS Lett. 581:2098–2104.
Harrison LM. 2012. Rhes: a GTP-binding protein integral to striatal physiology and pathology. Cell Mol Neurobiol. 32:907–918.
Harrison SM, Rudolph JL, Spencer ML, Wes PD, Montell C, Andres DA, Harrison DA. 2005. Activated RIC, a small GTPase, genetically interacts with the Ras pathway and calmodulin during Drosophila development. Dev Dyn. 232:817–826.
Heard JJ, Fong V, Bathaie SZ, Tamanoi F. 2014. Recent progress in the study of the Rheb family GTPases. Cell Signal. 26:1950–1957.
Hennig A, Markwart R, Esparza-Franco MA, Ladds G, Rubio I. 2015. Ras activation revisited: role of GEF and GAP systems. Biol Chem. 396:831–848.
Heo J, Campbell SL. 2004. Mechanism of p21Ras S-nitrosylation and kinetics of nitric oxide-mediated guanine nucleotide exchange. Biochemistry. 43:2314–2322.
Herrero A, Matallanas D, Kolch W. 2016. The spatiotemporal regulation of RAS signalling. Biochem Soc Trans. 44:1517–1522.
Herrmann C. 2003. Ras-effector interactions: after one decade. Curr Opin Struct Biol. 13:122–129.
Hers I, Vincent EE, Tavaré JM. 2011. Akt signalling in health and disease. Cell Signal. 23:1515–1527.
Higgins EM, Bos JM, Mason-Suares H, Tester DJ, Ackerman JP, MacRae CA, Sol-Church K, Gripp KW, Urrutia R, Ackerman MJ. 2017. Elucidation of MRAS-mediated Noonan syndrome with cardiac hypertrophy. JCI Insight. 2:e91225.
Hindley A, Kolch W. 2002. Extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK)-independent functions of Raf kinases. J Cell Sci. 115:1575–1581.
Hobbs GA, Der CJ, Rossman KL. 2016. RAS isoforms and mutations in cancer at a glance. J Cell Sci. 129:1287–1292.
Holly SP, Larson MK, Parise LV. 2005. The unique N-terminus of R-ras is required for Rac activation and precise regulation of cell migration. Mol Biol Cell. 16:2458–2469.
Hoshino M, Nakamura S. 2003. Small GTPase Rin induces neurite outgrowth through Rac/Cdc42 and calmodulin in PC12 cells. J Cell Biol. 163:1067–1076.
Hoshino M, Yoshimori T, Nakamura S. 2005. Small GTPase proteins Rin and Rit bind to PAR6 GTP-dependently and regulate cell transformation. J Biol Chem. 280:22868–22874.
Hota PK, Buck M. 2012. Plexin structures are coming: opportunities for multilevel investigations of semaphorin guidance receptors, their cell signaling mechanisms, and functions. Cell Mol Life Sci. 69:3765–3805.
Huang S, Chang IS, Lin W, Ye W, Luo RZ, Lu Z, Lu Y, Zhang K, Liao WS, Tao T, et al. 2009. ARHI (DIRAS3), an imprinted tumour suppressor gene, binds to importins and blocks nuclear import of cargo proteins. Biosci Rep. 30:159–168.
Hynds DL, Spencer ML, Andres DA, Snow DM. 2003. Rit promotes MEK-independent neurite branching in human neuroblastoma cells. J Cell Sci. 116:1925–1935.
Ichimiya H, Maeda K, Enomoto A, Weng L, Takahashi M, Murohara T. 2015. Girdin/GIV regulates transendothelial permeability by controlling VE-cadherin trafficking through the small GTPase, R-Ras. Biochem Biophys Res Commun. 461:260–267.
Im E, von Lintig FC, Chen J, Zhuang S, Qui W, Chowdhury S, Worley PF, Boss GR, Pilz RB. 2002. Rheb is in a high activation state and inhibits B-Raf kinase in mammalian cells. Oncogene. 21:6356–6365.
Inder KL, Lau C, Loo D, Chaudhary N, Goodall A, Martin S, Jones A, van der Hoeven D, Parton RG, Hill MM, Hancock JF. 2009. Nucleophosmin and nucleolin regulate K-Ras plasma membrane interactions and MAPK signal transduction. J Biol Chem. 284:28410–28419.
Inoki K, Li Y, Xu T, Guan KL. 2003. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev. 17:1829–1834.
Inoki K, Li Y, Zhu T, Wu J, Guan KL. 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. 4:648–657.
Jaiswal M, Dvorsky R, Ahmadian MR. 2013. Deciphering the molecular and functional basis of Dbl family proteins: a novel systematic approach toward classification of selective activation of the Rho family proteins. J Biol Chem. 288:4486–4500.
Jameson KL, Mazur PK, Zehnder AM, Zhang J, Zarnegar B, Sage J, Khavari PA. 2013. IQGAP1 scaffold-kinase interaction blockade selectively targets RAS-MAP kinase-driven tumors. Nat Med. 19:626–630.
Jang H, Abraham SJ, Chavan TS, Hitchinson B, Khavrutskii L, Tarasova NI, Nussinov R, Gaponenko V. 2015. Mechanisms

of membrane binding of small GTPase K-Ras4B farnesylated hypervariable region. J Biol Chem. 290:9465–9477.
Jang H, Banerjee A, Chavan T, Gaponenko V, Nussinov R. 2017. Flexible-body motions of calmodulin and the farnesylated hypervariable region yield a high-affinity interaction enabling K-Ras4B membrane extraction. J Biol Chem. 292:12544–12559.
Janosch P, Kieser A, Eulitz M, Lovric J, Sauer G, Reichert M, Gounari F, Buscher D, Baccarini M, Mischak H, Kolch W. 2000. The Raf-1 kinase associates with vimentin kinases and regulates the structure of vimentin filaments. FASEB J. 14:2008–2021.
Jeyabalan N, Clement JP. 2016. SYNGAP1: mind the gap. Front Cell Neurosci. 10:32.
Jiang H, Luo JQ, Urano T, Frankel P, Lu Z, Foster DA, Feig LA. 1995. Involvement of Ral GTPase in v-Src-induced phospholipase D activation. Nature. 378:409–412.
Jin A, Kurosu T, Tsuji K, Mizuchi D, Arai A, Fujita H, Hattori M, Minato N, Miura O. 2006. BCR/ABL and IL-3 activate Rap1 to stimulate the B-Raf/MEK/Erk and Akt signaling pathways and to regulate proliferation, apoptosis, and adhesion. Oncogene. 25:4332–4340.
Jin R, Junutula JR, Matern HT, Ervin KE, Scheller RH, Brunger AT. 2005. Exo84 and Sec5 are competitive regulatory Sec6/8 effectors to the RalA GTPase. EMBO J. 24:2064–2074.
Jin SX, Bartolome C, Arai JA, Hoffman L, Uzturk BG, Kumar-Singh R, Waxham MN, Feig LA. 2014. Domain contributions to signaling specificity differences between Ras-guanine nucleotide releasing factor (Ras-GRF) 1 and Ras-GRF2. J Biol Chem. 289:16551–16564.
Jullien-Flores V, Mahe Y, Mirey G, Leprince C, Meunier-Bisceuil B, Sorkin A, Camonis JH. 2000. RLIP76, an effector of the GTPase Ral, interacts with the AP2 complex: involvement of the Ral pathway in receptor endocytosis. J Cell Sci. 113: 2837–2844.
Jura N, Scotto-Lavino E, Sobczyk A, Bar-Sagi D. 2006. Differential modification of Ras proteins by ubiquitination. Mol Cell. 21:679–687.
Kaduwal S, Jeong WJ, Park JC, Lee KH, Lee YM, Jeon SH, Lim YB, Min do S, Choi KY. 2015. Sur8/Shoc2 promotes cell motility and metastasis through activation of Ras-PI3K signaling. Oncotarget. 6:33091–33105.
Kaliman P, Llagostera E. 2008. Myotonic dystrophy protein kinase (DMPK) and its role in the pathogenesis of myotonic dystrophy 1. Cell Signal. 20:1935–1941.
Karassek S, Berghaus C, Schwarten M, Goemans CG, Ohse N, Kock G, Jockers K, Neumann S, Gottfried S, Herrmann C, et al. 2010. Ras homolog enriched in brain (Rheb) enhances apoptotic signaling. J Biol Chem. 285:33979–33991.
Karbowniczek M, Cash T, Cheung M, Robertson GP, Astrinidis A, Henske EP. 2004. Regulation of B-Raf kinase activity by tuberin and Rheb is mammalian target of rapamycin (mTOR)-independent. J Biol Chem. 279:29930–29937.
Karbowniczek M, Robertson GP, Henske EP. 2006. Rheb inhibits C-raf activity and B-raf/C-raf heterodimerization. J Biol Chem. 281:25447–25456.
Karbowniczek M, Zitserman D, Khabibullin D, Hartman T, Yu J, Morrison T, Nicolas E, Squillace R, Roegiers F, Henske EP. 2010. The evolutionarily conserved TSC/Rheb pathway activates Notch in tuberous sclerosis complex and Drosophila external sensory organ development. J Clin Invest. 120:93–102.
Karnoub AE, Weinberg RA. 2008. Ras oncogenes: split personalities. Nat Rev Mol Cell Biol. 9:517–531.
Kashatus DF, Lim KH, Brady DC, Pershing NL, Cox AD, Counter CM. 2011. RALA and RALBP1 regulate mitochondrial fission at mitosis. Nat Cell Biol. 13:1108–1115.
Kawato M, Shirakawa R, Kondo H, Higashi T, Ikeda T, Okawa K, Fukai S, Nureki O, Kita T, Horiuchi H. 2008. Regulation of platelet dense granule secretion by the Ral GTPase-exocyst pathway. J Biol Chem. 283:166–174.
Keeton AB, Salter EA, Piazza GA. 2017. The RAS-effector interaction as a drug target. Cancer Res. 77:221–226.
Kelley GG, Reks SE, Ondrako JM, Smrcka AV. 2001. Phospholipase C(epsilon): a novel Ras effector. EMBO J. 20:743–754.
Kim HW, Ha SH, Lee MN, Huston E, Kim DH, Jang SK, Suh PG, Houslay MD, Ryu SH. 2010. Cyclic AMP controls mTOR through regulation of the dynamic interaction between Rheb and phosphodiesterase 4D. Mol Cell Biol. 30:5406–5420.
Klingauf M, Beck M, Berge U, Turgay Y, Heinzer S, Horvath P, Kroschewski R. 2013. The tumour suppressor DiRas3 interacts with C-RAF and downregulates MEK activity to restrict cell migration. Biol Cell. 105:91–107.
Knyphausen P, Lang F, Baldus L, Extra A, Lammers M. 2016. Insights into K-Ras 4B regulation by post-translational lysine acetylation. Biol Chem. 397:1071–1085.
Kontani K, Tada M, Ogawa T, Okai T, Saito K, Araki Y, Katada T. 2002. Di-Ras, a distinct subgroup of ras family GTPases with unique biochemical properties. J Biol Chem. 277:41070–41078.
Kooistra MR, Dube N, Bos JL. 2007. Rap1: a key regulator in cell-cell junction formation. J Cell Sci. 120:17–22.
Korf B, Ahmadian R, Allanson J, Aoki Y, Bakker A, Wright EB, Denger B, Elgersma Y, Gelb BD, Gripp KW, et al. 2015. The third international meeting on genetic disorders in the RAS/MAPK pathway: towards a therapeutic approach. Am J Med Genet. 167A:1741–1746.
Kupzig S, Bouyoucef-Cherchalli D, Yarwood S, Sessions R, Cullen PJ. 2009. The ability of GAP1IP4BP to function as a Rap1 GTPase-activating protein (GAP) requires its Ras GAP-related domain and an arginine finger rather than an asparagine thumb. Mol Cell Biol. 29:3929–3940.
Kupzig S, Deaconescu D, Bouyoucef D, Walker SA, Liu Q, Polte CL, Daumke O, Ishizaki T, Lockyer PJ, Wittinghofer A, Cullen PJ. 2006. GAP1 family members constitute bifunctional Ras and Rap GTPase-activating proteins. J Biol Chem. 281:9891–9900.
Kurella VB, Richard JM, Parke CL, Lecour LF, Jr., Bellamy HD, Worthylake DK. 2009. Crystal structure of the GTPase-activating protein-related domain from IQGAP1. J Biol Chem. 284:14857–14865.
Kwong L, Wozniak MA, Collins AS, Wilson SD, Keely PJ. 2003. R-Ras promotes focal adhesion formation through focal adhesion kinase and p130(Cas) by a novel mechanism that differs from integrins. Mol Cell Biol. 23:933–949.
Lalli G, Hall A. 2005. Ral GTPases regulate neurite branching through GAP-43 and the exocyst complex. J Cell Biol. 171:857–869.
Lander HM, Ogiste JS, Pearce SF, Levi R, Novogrodsky A. 1995. Nitric oxide-stimulated guanine nucleotide exchange on p21ras. J Biol Chem. 270:7017–7020.

Lane KT, Beese LS. 2006. Thematic review series: lipid post-translational modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. J Lipid Res. 47:681–699.

Lapinski PE, Qiao Y, Chang CH, King PD. 2011. A role for p120 RasGAP in thymocyte positive selection and survival of naive T cells. J Immunol. 187:151–163.

Latourelle JC, Dumitriu A, Hadzi TC, Beach TG, Myers RH. 2012. Evaluation of Parkinson disease risk variants as expression-QTLs. PLoS One. 7:e46199.

Lee CH, Della NG, Chew CE, Zack DJ. 1996. Rin, a neuron-specific and calmodulin-binding small G-protein, and Rit define a novel subfamily of ras proteins. J Neurosci. 16:6784–6794.

Lee MN, Ha SH, Kim J, Koh A, Lee CS, Kim JH, Jeon H, Kim DH, Suh PG, Ryu SH. 2009. Glycolytic flux signals to mTOR through glyceraldehyde-3-phosphate dehydrogenase-mediated regulation of Rheb. Mol Cell Biol. 29:3991–4001.

Li Y, Corradetti MN, Inoki K, Guan KL. 2004. TSC2: filling the GAP in the mTOR signaling pathway. Trends Biochem Sci. 29:32–38.

Ligon AH, Pershouse MA, Jasser SA, Yung WK, Steck PA. 1997. Identification of a novel gene product, RIG, that is down-regulated in human glioblastoma. Oncogene. 14:1075–1081.

Lissewski C, Kant SG, Stark Z, Schanze I, Zenker M. 2015. Copy number variants including RAS pathway genes - How much RASopathy is in the phenotype?. Am J Med Genet. 167A:2685–2690.

Lito P, Mets BD, Appledorn DM, Maher VM, McCormick JJ. 2009. Sprouty 2 regulates DNA damage-induced apoptosis in Ras-transformed human fibroblasts. J Biol Chem. 284:848–854.

Liu X, Shimada T, Otowa T, Wu YY, Kawamura Y, Tochigi M, Iwata Y, Umekage T, Toyota T, Maekawa M, et al. 2016. Genome-wide association study of Autism spectrum disorder in the east Asian populations. Autism Res. 9:340–349.

Ljubicic S, Bezzi P, Vitale N, Regazzi R. 2009. The GTPase RalA regulates different steps of the secretory process in pancreatic beta-cells. PLoS One. 4:e7770.

Long X, Lin Y, Ortiz-Vega S, Yonezawa K, Avruch J. 2005. Rheb binds and regulates the mTOR kinase. Curr Biol. 15:702–713.

Lopez JA, Kwan EP, Xie L, He Y, James DE, Gaisano HY. 2008. The RalA GTPase is a central regulator of insulin exocytosis from pancreatic islet beta cells. J Biol Chem. 283:17939–17945.

Louahed J, Grasso L, De Smet C, Van Roost E, Wildmann C, Nicolaides NC, Levitt RC, Renauld JC. 1999. Interleukin-9-induced expression of M-Ras/R-Ras3 oncogene in T-helper clones. Blood. 94:1701–1710.

Lynch SJ, Snitkin H, Gumper I, Philips MR, Sabatini D, Pellicer A. 2015. The differential palmitoylation states of N-Ras and H-Ras determine their distinct Golgi subcompartment localizations. J Cell Physiol. 230:610–619.

Ma D, Bai X, Zou H, Lai Y, Jiang Y. 2010. Rheb GTPase controls apoptosis by regulating interaction of FKBP38 with Bcl-2 and Bcl-XL. J Biol Chem. 285:8621–8627.

Machida N, Umikawa M, Takei K, Sakima N, Myagmar BE, Taira K, Uezato H, Ogawa Y, Kariya K. 2004. Mitogen-activated protein kinase kinase kinase kinase 4 as a putative effector of Rap2 to activate the c-Jun N-terminal kinase. J Biol Chem. 279:15711–15714.

Mainberger F, Langer S, Mall V, Jung NH. 2016. Impaired synaptic plasticity in RASopathies: a mini-review. J Neural Transm. 123:1133–1138.

Malumbres M, Barbacid M. 2003. RAS oncogenes: the first 30 years. Nat Rev Cancer. 3:459.

Mandal R, Becker S, Strebhardt K. 2016. Stamping out RAF and MEK1/2 to inhibit the ERK1/2 pathway: an emerging threat to anticancer therapy. Oncogene. 35:2547–2561.

Marshall CB, Ho J, Buerger C, Plevin MJ, Li GY, Li Z, Ikura M, Stambolic V. 2009. Characterization of the intrinsic and TSC2-GAP-regulated GTPase activity of Rheb by real-time NMR. Sci Signal. 2:ra3.

McCormick F. 2015. KRAS as a therapeutic target. Clin Cancer Res. 21:1797–1801.

McCormick F. 2016. K-Ras protein as a drug target. J Mol Med. 94:253–258.

McGrath JP, Capon DJ, Smith DH, Chen EY, Seeburg PH, Goeddel DV, Levinson AD. 1983. Structure and organization of the human Ki-ras proto-oncogene and a related processed pseudogene. Nature. 304:501–506.

Miller JP, Yates BE, Al-Ramahi I, Berman AE, Sanhueza M, Kim E, de Haro M, DeGiacomo F, Torcassi C, Holcomb J, et al. 2012. A genome-scale RNA-interference screen identifies RRAS signaling as a pathologic feature of Huntington's disease. PLoS Genet. 8:e1003042.

Mir S, Cai W, Andres DA. 2017. RIT1 GTPase regulates Sox2 transcriptional activity and hippocampal neurogenesis. J Biol Chem. 292:2054–2064.

Mitin N, Rossman KL, Der CJ. 2005. Signaling interplay in Ras superfamily function. Curr Biol. 15:R563–R574.

Miyazaki M, Takemasa T. 2017. TSC2/Rheb signaling mediates ERK-dependent regulation of mTORC1 activity in C2C12 myoblasts. FEBS Open Biol. 7:424–433.

Mohan N, Shen Y, Dokmanovic M, Endo Y, Hirsch DS, Wu WJ. 2016. VPS34 regulates TSC1/TSC2 heterodimer to mediate RheB and mTORC1/S6K1 activation and cellular transformation. Oncotarget. 7:52239–52254.

Montagner A, Yart A, Dance M, Perret B, Salles JP, Raynal P. 2005. A novel role for Gab1 and SHP2 in epidermal growth factor-induced Ras activation. J Biol Chem. 280:5350–5360.

Mooz J, Oberoi-Khanuja TK, Harms GS, Wang W, Jaiswal BS, Seshagiri S, Tikkanen R, Rajalingam K. 2014. Dimerization of the kinase ARAF promotes MAPK pathway activation and cell migration. Sci Signal. 7:ra73.

Moskalenko S, Henry DO, Rosse C, Mirey G, Camonis JH, White MA. 2002. The exocyst is a Ral effector complex. Nat Cell Biol. 4:66–72.

Mott HR, Owen D. 2015. Structures of Ras superfamily effector complexes: what have we learnt in two decades?. Crit Rev Biochem Mol Biol. 50:85–133.

Motta M, Chillemi G, Fodale V, Cecchetti S, Coppola S, Stipo S, Cordeddu V, Macioce P, Gelb BD, Tartaglia M. 2016. SHOC2 subcellular shuttling requires the KEKE motif-rich region and N-terminal leucine-rich repeat domain and impacts on ERK signalling. Hum Mol Genet. 25:3824–3835.

Mourik M, Eikenboom J. 2017. Lifecycle of Weibel-Palade bodies. Hamostaseologie. 37:13–24.

Myagmar BE, Umikawa M, Asato T, Taira K, Oshiro M, Hino A, Takei K, Uezato H, Kariya K. 2005. PARG1, a protein-

tyrosine phosphatase-associated RhoGAP, as a putative Rap2 effector. Biochem Biophys Res Commun. 329:1046–1052.

Nakashima S, Morinaka K, Koyama S, Ikeda M, Kishida M, Okawa K, Iwamatsu A, Kishida S, Kikuchi A. 1999. Small G protein Ral and its downstream molecules regulate endocytosis of EGF and insulin receptors. EMBO J. 18:3629–3642.

Nakhaei-Rad S, Nakhaeizadeh H, Gotze S, Kordes C, Sawitza I, Hoffmann MJ, Franke M, Schulz WA, Scheller J, Piekorz RP, et al. 2016. The role of embryonic stem cell-expressed RAS (ERAS) in the maintenance of quiescent hepatic stellate cells. J Biol Chem. 291:8399–8413.

Nakhaei-Rad S, Nakhaeizadeh H, Kordes C, Cirstea IC, Schmick M, Dvorsky R, Bastiaens PI, Häussinger D, Ahmadian MR. 2015. The function of embryonic stem cell-expressed Ras (E-Ras), a unique Ras family member, correlates with its additional motifs and its structural properties. J Biol Chem. 290:15892–15903.

Nakhaeizadeh H, Amin E, Nakhaei-Rad S, Dvorsky R, Ahmadian MR. 2016. The RAS-effector interface: isoform-specific differences in the effector binding regions. PLoS One. 11:e0167145.

Nalls MA, Pankratz N, Lill CM, Do CB, Hernandez DG, Saad M, DeStefano AL, Kara E, Bras J, Sharma M, et al. 2014. Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. Nat Genet. 46:989–993.

Navaroli DM, Stevens ZH, Uzelac Z, Gabriel L, King MJ, Lifshitz LM, Sitte HH, Melikian HE. 2011. The plasma membrane-associated GTPase Rin interacts with the dopamine transporter and is required for protein kinase C-regulated dopamine transporter trafficking. J Neurosci. 31:13758–13770.

Nelson N, Clark GJ. 2016. Rheb may complex with RASSF1A to coordinate Hippo and TOR signaling. Oncotarget. 7:33821–33831.

Nguyen CH, Watts VJ. 2005. Dexras1 blocks receptor-mediated heterologous sensitization of adenylyl cyclase 1. Biochem Biophys Res Commun. 332:913–920.

Nguyen CH, Watts VJ. 2006. Dexamethasone-induced Ras protein 1 negatively regulates protein kinase C δ: implications for adenylyl cyclase 2 signaling. Mol Pharmacol. 69:1763–1771.

Niault TS, Baccarini M. 2010. Targets of Raf in tumorigenesis. Carcinogenesis. 31:1165–1174.

Nishimoto A, Yu Y, Lu Z, Mao X, Ren Z, Watowich SS, Mills GB, Liao WS, Chen X, Bast RC Jr, Luo RZ. 2005. A Ras homologue member I directly inhibits signal transducers and activators of transcription 3 translocation and activity in human breast and ovarian cancer cells. Cancer Res. 65:6701–6710.

Nonaka H, Takei K, Umikawa M, Oshiro M, Kuninaka K, Bayarjargal M, Asato T, Yamashiro Y, Uechi Y, Endo S, et al. 2008. MINK is a Rap2 effector for phosphorylation of the postsynaptic scaffold protein TANC1. Biochem Biophys Res Commun. 377:573–578.

Nouri K, Timson DJ, Ahmadian MR. 2017. New model for the interaction of IQGAP1 with CDC42 and RAC1. Small GTPases. [2017 Jun 19];[1–7]. [Epub ahead of print]. doi: 10.1080/21541248.2017.1321169

Nussinov R, Muratcioglu S, Tsai CJ, Jang H, Gursoy A, Keskin O. 2015. The key role of calmodulin in KRAS-driven adenocarcinomas. Mol Cancer Res. 13:1265–1273.

Nussinov R, Tsai CJ, Chakrabarti M, Jang H. 2016. A new view of ras isoforms in cancers. Cancer Res. 76:18–23.

Oertli B, Han J, Marte BM, Sethi T, Downward J, Ginsberg M, Hughes PE. 2000. The effector loop and prenylation site of R-Ras are involved in the regulation of integrin function. Oncogene. 19:4961–4969.

Ogita Y, Egami S, Ebihara A, Ueda N, Katada T, Kontani K. 2015. Di-Ras2 protein forms a complex with SmgGDS protein in brain cytosol in order to be in a low affinity state for guanine nucleotides. J Biol Chem. 290:20245–20256.

Ohba Y, Ikuta K, Ogura A, Matsuda J, Mochizuki N, Nagashima K, Kurokawa K, Mayer BJ, Maki K, Miyazaki J, Matsuda M. 2001. Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis. EMBO J. 20:3333–3341.

Oinuma I, Ishikawa Y, Katoh H, Negishi M. 2004. The Semaphorin 4D receptor Plexin-B1 is a GTPase activating protein for R-Ras. Science. 305:862–865.

Oliveira AF, Yasuda R. 2014. Neurofibromin is the major ras inactivator in dendritic spines. J Neurosci. 34:776–783.

Omerovic J, Prior IA. 2009. Compartmentalized signalling: Ras proteins and signalling nanoclusters. FEBS J. 276:1817–1825.

Pamonsinlapatham P, Hadj-Slimane R, Lepelletier Y, Allain B, Toccafondi M, Garbay C, Raynaud F. 2009. p120-Ras GTPase activating protein (RasGAP): a multi-interacting protein in downstream signaling. Biochimie. 91:320–328.

Pankratz N, Beecham GW, DeStefano AL, Dawson TM, Doheny KF, Factor SA, Hamza TH, Hung AY, Hyman BT, Ivinson AJ, et al. 2012. Meta-analysis of Parkinson's disease: identification of a novel locus, RIT2. Ann Neurol. 71:370–384.

Pantaleoni F, Lev D, Cirstea IC, Motta M, Lepri FR, Bottero L, Cecchetti S, Linger I, Paolacci S, Flex E, et al. 2017. Aberrant HRAS transcript processing underlies a distinctive phenotype within the RASopathy clinical spectrum. Hum Mutat. 38:798–804.

Pearce LR, Komander D, Alessi DR. 2010. The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol. 11:9–22.

Pena V, Hothorn M, Eberth A, Kaschau N, Parret A, Gremer L, Bonneau F, Ahmadian MR, Scheffzek K. 2008. The C2 domain of SynGAP is essential for stimulation of the Rap GTPase reaction. EMBO Rep. 9:350–355.

Philpott C, Tovell H, Frayling IM, Cooper DN, Upadhyaya M. 2017. The NF1 somatic mutational landscape in sporadic human cancers. Hum Genomics. 11:13.

Plak K, Pots H, Van Haastert PJ, Kortholt A. 2016. Direct Interaction between TalinB and Rap1 is necessary for adhesion of Dictyostelium cells. BMC Cell Biol. 17:1.

Plowman SJ, Ariotti N, Goodall A, Parton RG, Hancock JF. 2008. Electrostatic interactions positively regulate K-Ras nanocluster formation and function. Mol Cell Biol. 28:4377–4385.

Plowman SJ, Berry RL, Bader SA, Luo F, Arends MJ, Harrison DJ, Hooper ML, Patek CE. 2006. K-ras 4A and 4B are co-expressed widely in human tissues, and their ratio is altered in sporadic colorectal cancer. J Exp Clin Cancer Res. 25:259–267.

Popovic M, Rensen-de Leeuw M, Rehmann H. 2013. Selectivity of CDC25 homology domain-containing guanine nucleotide exchange factors. J Mol Biol. 425:2782–2794.

Popovic M, Schouten A, Rensen-de Leeuw M, Rehmann H. 2016. The structure of the Guanine Nucleotide Exchange Factor Rlf in complex with the small G-protein Ral identifies conformational intermediates of the exchange reaction and the basis for the selectivity. J Struct Biol. 193:106–114.

Postema FAM, Hopman SMJ, Hennekam RC, Merks JHM. 2018. Consequences of diagnosing a tumor predisposition syndrome in children with cancer: a literature review. Pediatr Blood Cancer. 65:e26718.

Potheraveedu VN, Schopel M, Stoll R, Heumann R. 2017. Rheb in neuronal degeneration, regeneration, and connectivity. Biol Chem. 398:589–606.

Praskova M, Khoklatchev A, Ortiz-Vega S, Avruch J. 2004. Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. Biochem J. 381:453–462.

Quilliam LA, Rebhun JF, Castro AF. 2002. A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. Prog Nucleic Acid Res Mol Biol. 71:391–444.

Raguz S, De Bella MT, Slade MJ, Higgins CF, Coombes RC, Yague E. 2005. Expression of RPIP9 (Rap2 interacting protein 9) is activated in breast carcinoma and correlates with a poor prognosis. Int J Cancer. 117:934–941.

Rajalingam K, Schreck R, Rapp UR, Albert S. 2007. Ras oncogenes and their downstream targets. Biochim Biophys Acta. 1773:1177–1195.

Ratner N, Miller SJ. 2015. A RASopathy gene commonly mutated in cancer: the neurofibromatosis type 1 tumour suppressor. Nat Rev Cancer. 15:290–301.

Rauen KA. 2013. The RASopathies. Annu Rev Genomics Hum Genet. 14:355–369.

Ray A, Basu S, Miller NM, Chan AM, Dittel BN. 2014. An increase in tolerogenic dendritic cell and natural regulatory T cell numbers during experimental autoimmune encephalomyelitis in Rras-/- mice results in attenuated disease. J Immunol. 192:5109–5117.

Rebhun JF, Castro AF, Quilliam LA. 2000. Identification of guanine nucleotide exchange factors (GEFs) for the Rap1 GTPase. Regulation of MR-GEF by M-Ras-GTP interaction. J Biol Chem. 275:34901–34908.

Rebocho AP, Marais R. 2013. ARAF acts as a scaffold to stabilize BRAF:CRAF heterodimers. Oncogene. 32:3207–3212.

Rehmann H, Arias-Palomo E, Hadders MA, Schwede F, Llorca O, Bos JL. 2008a. Structure of Epac2 in complex with a cyclic AMP analogue and RAP1B. Nature. 455:124–127.

Rehmann H, Bruning M, Berghaus C, Schwarten M, Kohler K, Stocker H, Stoll R, Zwartkruis FJ, Wittinghofer A. 2008b. Biochemical characterisation of TCTP questions its function as a guanine nucleotide exchange factor for Rheb. FEBS Lett 582:3005–3010.

Reif A, Nguyen TT, Weissflog L, Jacob CP, Romanos M, Renner TJ, Buttenschon HN, Kittel-Schneider S, Gessner A, Weber H, et al. 2011. DIRAS2 is associated with adult ADHD, related traits, and co-morbid disorders. Neuropsychopharmacology. 36:2318–2327.

Ren JG, Li Z, Sacks DB. 2007. IQGAP1 modulates activation of B-Raf. Proc Natl Acad Sci USA. 104:10465–10469.

Repasky GA, Chenette EJ, Der CJ. 2004. Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis?. Trends Cell Biol. 14:639–647.

Resh MD. 2004. Membrane targeting of lipid modified signal transduction proteins. Subcell Biochem. 37:217–232.

Rodriguez-Viciana P, McCormick F. 2006. Ras ubiquitination: coupling spatial sorting and signal transmission. Cancer Cell. 9:243–244.

Rojas AM, Fuentes G, Rausell A, Valencia A. 2012. The Ras protein superfamily: evolutionary tree and role of conserved amino acids. J Cell Biol. 196:189–201.

Romano D, Nguyen LK, Matallanas D, Halasz M, Doherty C, Kholodenko BN, Kolch W. 2014. Protein interaction switches coordinate Raf-1 and MST2/Hippo signalling. Nat Cell Biol. 16:673–684.

Rondaij MG, Bierings R, van Agtmaal EL, Gijzen KA, Sellink E, Kragt A, Ferguson SS, Mertens K, Hannah MJ, van Mourik JA, et al. 2008. Guanine exchange factor RalGDS mediates exocytosis of Weibel-Palade bodies from endothelial cells. Blood 112:56–63.

Rosseland CM, Wierod L, Flinder LI, Oksvold MP, Skarpen E, Huitfeldt HS. 2008. Distinct functions of H-Ras and K-Ras in proliferation and survival of primary hepatocytes due to selective activation of ERK and PI3K. J Cell Physiol. 215:818–826.

Sakabe K, Teramoto H, Zohar M, Behbahani B, Miyazaki H, Chikumi H, Gutkind JS. 2002. Potent transforming activity of the small GTP-binding protein Rit in NIH 3T3 cells: evidence for a role of a p38gamma-dependent signaling pathway. FEBS Lett. 511:15–20.

Sakurai A, Gavard J, Annas-Linhares Y, Basile JR, Amornphimoltham P, Palmby TR, Yagi H, Zhang F, Randazzo PA, Li X, et al. 2010. Semaphorin 3E initiates antiangiogenic signaling through plexin D1 by regulating Arf6 and R-Ras. Mol Cell Biol. 30:3086–3098.

Sanchez-Ruiz J, Mejias R, Garcia-Belando M, Barber DF, Gonzalez-Garcia A. 2011. Ral GTPases regulate cell-mediated cytotoxicity in NK cells. J Immunol. 187:2433–2441.

Sandri C, Caccavari F, Valdembri D, Camillo C, Veltel S, Santambrogio M, Lanzetti L, Bussolino F, Ivaska J, Serini G. 2012. The R-Ras/RIN2/Rab5 complex controls endothelial cell adhesion and morphogenesis via active integrin endocytosis and Rac signaling. Cell Res. 22:1479–1501.

Santarpia L, Lippman SM, El-Naggar AK. 2012. Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. Expert Opin Ther Targets. 16:103–119.

Saraste M, Sibbald PR, Wittinghofer A. 1990. The P-loop – a common motif in ATP-and GTP-binding proteins. Trends Biochem Sci. 15:430–434.

Sasaki AT, Carracedo A, Locasale JW, Anastasiou D, Takeuchi K, Kahoud ER, Haviv S, Asara JM, Pandolfi PP, Cantley LC. 2011. Ubiquitination of K-Ras enhances activation and facilitates binding to select downstream effectors. Sci Signal. 4:ra13.

Scheffzek K, Ahmadian MR. 2005. GTPase activating proteins: structural and functional insights 18 years after discovery. Cell Mol Life Sci. 62:3014–3038.

Scheffzek K, Ahmadian MR, Kabsch W, Wiesmuller L, Lautwein A, Schmitz F, Wittinghofer A. 1997. The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. Science. 277:333–338.

Schmick M, Kraemer A, Bastiaens PI. 2015. Ras moves to stay in place. Trends Cell Biol. 25:190–197.

Schmick M, Vartak N, Papke B, Kovacevic M, Truxius DC, Rossmannek L, Bastiaens PIH. 2014. KRas localizes to the plasma membrane by spatial cycles of solubilization, trapping and vesicular transport. Cell. 157:459–471.

Schmidt M, Bienek C, Rümenapp U, Zhang C, Lümmen G, Jakobs KH, Just I, Aktories K, Moos M, von Eichel-Streiber C. 1996. A role for Rho in receptor-and G protein-stimulated phospholipase C Reduction in phosphatidylinositol 4, 5-bisphosphate by *Clostridium difficile* toxin B. Naunyn-Schmiedeberg's Arch Pharmacol. 354:87–94.

Schroeder H, Leventis R, Rex S, Schelhaas M, Nagele E, Waldmann H, Silvius JR. 1997. S-Acylation and plasma membrane targeting of the farnesylated carboxyl-terminal peptide of N-ras in mammalian fibroblasts. Biochemistry. 36:13102–13109.

Scrima A, Thomas C, Deaconescu D, Wittinghofer A. 2008. The Rap-RapGAP complex: GTP hydrolysis without catalytic glutamine and arginine residues. EMBO J. 27:1145–1153.

Self AJ, Caron E, Paterson HF, Hall A. 2001. Analysis of R-Ras signalling pathways. J Cell Sci. 114:1357–1366.

Shahani N, Pryor W, Swarnkar S, Kholodilov N, Thinakaran G, Burke RE, Subramaniam S. 2014. Rheb GTPase regulates beta-secretase levels and amyloid beta generation. J Biol Chem. 289:5799–5808.

Shanshiashvili L, Narmania N, Barbakadze T, Zhuravliova E, Natsvlishvili N, Ramsden J, Mikeladze DG. 2011. S-nitrosylation decreases the adsorption of H-Ras in lipid bilayer and changes intrinsic catalytic activity. Cell Biochem Biophys. 59:191–199.

Shao H, Andres DA. 2000. A novel RalGEF-like protein, RGL3, as a candidate effector for rit and Ras. J Biol Chem. 275:26914–26924.

Shao H, Kadono-Okuda K, Finlin BS, Andres DA. 1999. Biochemical characterization of the Ras-related GTPases Rit and Rin. Arch Biochem Biophys. 371:207–219.

Shi GX, Andres DA. 2005. Rit contributes to nerve growth factor-induced neuronal differentiation via activation of B-Raf-extracellular signal-regulated kinase and p38 mitogen-activated protein kinase cascades. Mol Cell Biol. 25:830–846.

Shi GX, Cai W, Andres DA. 2012. Rit-mediated stress resistance involves a p38-mitogen- and stress-activated protein kinase 1 (MSK1)-dependent cAMP response element-binding protein (CREB) activation cascade. J Biol Chem. 287:39859–39868.

Shi GX, Cai W, Andres DA. 2013. Rit subfamily small GTPases: regulators in neuronal differentiation and survival. Cell Signal. 25:2060–2068.

Shi GX, Jin L, Andres DA. 2008. Pituitary adenylate cyclase-activating polypeptide 38-mediated Rin activation requires Src and contributes to the regulation of HSP27 signaling during neuronal differentiation. Mol Cell Biol. 28:4940–4951.

Shi GX, Jin L, Andres DA. 2011. A rit GTPase-p38 mitogen-activated protein kinase survival pathway confers resistance to cellular stress. Mol Cell Biol. 31:1938–1948.

Shimizu M, Wang W, Walch ET, Dunne PW, Epstein HF. 2000. Rac-1 and Raf-1 kinases, components of distinct signaling pathways, activate myotonic dystrophy protein kinase. FEBS Lett. 475:273–277.

Shipitsin M, Feig LA. 2004. RalA but not RalB enhances polarized delivery of membrane proteins to the basolateral surface of epithelial cells. Mol Cell Biol. 24:5746–5756.

Shirakawa R, Fukai S, Kawato M, Higashi T, Kondo H, Ikeda T, Nakayama E, Okawa K, Nureki O, Kimura T, et al. 2009. Tuberous sclerosis tumor suppressor complex-like complexes act as GTPase-activating proteins for Ral GTPases. J Biol Chem. 284:21580–21588.

Shirakawa R, Horiuchi H. 2015. Ral GTPases: crucial mediators of exocytosis and tumourigenesis. J Biochem. 157:285–299.

Siljamaki E, Abankwa D. 2016. SPRED1 Interferes with K-ras but Not H-ras Membrane Anchorage and Signaling. Mol Cell Biol. 36:2612–2625.

Silvius JR, l'Heureux F. 1994. Fluorometric evaluation of the affinities of isoprenylated peptides for lipid bilayers. Biochemistry. 33:3014–3022.

Simanshu DK, Nissley DV, McCormick F. 2017. RAS proteins and their regulators in human disease. Cell. 170:17–33.

Song C, Hu CD, Masago M, Kariyai K, Yamawaki-Kataoka Y, Shibatohge M, Wu D, Satoh T, Kataoka T. 2001. Regulation of a novel human phospholipase C, PLCepsilon, through membrane targeting by Ras. J Biol Chem. 276:2752–2757.

Sot B, Kotting C, Deaconescu D, Suveyzdis Y, Gerwert K, Wittinghofer A. 2010. Unravelling the mechanism of dual-specificity GAPs. EMBO J. 29:1205–1214.

Spencer ML, Shao H, Andres DA. 2002a. Induction of neurite extension and survival in pheochromocytoma cells by the Rit GTPase. J Biol Chem. 277:20160–20168.

Spencer ML, Shao H, Tucker HM, Andres DA. 2002b. Nerve growth factor-dependent activation of the small GTPase Rin. J Biol Chem. 277:17605–17615.

Sperlich B, Kapoor S, Waldmann H, Winter R, Weise K. 2016. Regulation of K-Ras4B membrane binding by calmodulin. Biophys J. 111:113–122.

Spiczka KS, Yeaman C. 2008. Ral-regulated interaction between Sec5 and paxillin targets Exocyst to focal complexes during cell migration. J Cell Sci. 121:2880–2891.

Stephen AG, Esposito D, Bagni RK, McCormick F. 2014. Dragging ras back in the ring. Cancer Cell. 25:272–281.

Stieglitz B, Bee C, Schwarz D, Yildiz O, Moshnikova A, Khokhlatchev A, Herrmann C. 2008. Novel type of Ras effector interaction established between tumour suppressor NORE1A and Ras switch II. EMBO J. 27:1995–2005.

Stope MB, Vom Dorp F, Szatkowski D, Bohm A, Keiper M, Nolte J, Oude Weernink PA, Rosskopf D, Evellin S, Jakobs KH, Schmidt M. 2004. Rap2B-dependent stimulation of phospholipase C-epsilon by epidermal growth factor receptor mediated by c-Src phosphorylation of RasGRP3. Mol Cell Biol. 24:4664–4676.

Stork PJ. 2003. Does Rap1 deserve a bad Rap?. Trends Biochem Sci. 28:267–275.

Subramaniam S, Mealer RG, Sixt KM, Barrow RK, Usiello A, Snyder SH. 2010. Rhes, a physiologic regulator of sumoylation, enhances cross-sumoylation between the basic sumoylation enzymes E1 and Ubc9. J Biol Chem. 285:20428–20432.

Subramaniam S, Napolitano F, Mealer RG, Kim S, Errico F, Barrow R, Shahani N, Tyagi R, Snyder SH, Usiello A. 2011. Rhes, a striatal-enriched small G protein, mediates mTOR signaling and L-DOPA-induced dyskinesia. Nat Neurosci. 15:191–193.

Sugihara K, Asano S, Tanaka K, Iwamatsu A, Okawa K, Ohta Y. 2002. The exocyst complex binds the small GTPase RalA to mediate filopodia formation. Nat Cell Biol. 4:73–78.

Sun Y, Fang Y, Yoon MS, Zhang C, Roccio M, Zwartkruis FJ, Armstrong M, Brown HA, Chen J. 2008. Phospholipase D1 is an effector of Rheb in the mTOR pathway. Proc Natl Acad Sci USA. 105:8286–8291.

Sung PJ, Tsai FD, Vais H, Court H, Yang J, Fehrenbacher N, Foskett JK, Philips MR. 2013. Phosphorylated K-Ras limits cell survival by blocking Bcl-xL sensitization of inositol trisphosphate receptors. Proc Natl Acad Sci USA. 110:20593–20598.

Tabaczar S, Czogalla A, Podkalicka J, Biernatowska A, Sikorski AF. 2017. Protein palmitoylation: Palmitoyltransferases and their specificity. Exp Biol Med (Maywood). 242:1150–1157.

Tada M, Gengyo-Ando K, Kobayashi T, Fukuyama M, Mitani S, Kontani K, Katada T. 2012. Neuronally expressed Ras-family GTPase Di-Ras modulates synaptic activity in *Caenorhabditis elegans*. Genes Cells. 17:778–789.

Taira K, Umikawa M, Takei K, Myagmar BE, Shinzato M, Machida N, Uezato H, Nonaka S, Kariya K. 2004. The Traf2- and Nck-interacting kinase as a putative effector of Rap2 to regulate actin cytoskeleton. J Biol Chem. 279:49488–49496.

Takahashi K, Mitsui K, Yamanaka S. 2003. Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. Nature. 423:541–545.

Takahashi K, Nakagawa M, Young SG, Yamanaka S. 2005. Differential membrane localization of ERas and Rheb, two Ras-related proteins involved in the phosphatidylinositol 3-kinase/mTOR pathway. J Biol Chem. 280:32768–32774.

Takei N. 2004. Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent local activation of translation machinery and protein synthesis in neuronal dendrites. J Neurosci. 24:9760–9769.

Tang S, Chen T, Yu Z, Zhu X, Yang M, Xie B, Li N, Cao X, Wang J. 2014. RasGRP3 limits Toll-like receptor-triggered inflammatory response in macrophages by activating Rap1 small GTPase. Nat Commun. 5:4657.

Tasaka G, Negishi M, Oinuma I. 2012. Semaphorin 4D/Plexin-B1-mediated M-Ras GAP activity regulates actin-based dendrite remodeling through Lamellipodin. J Neurosci. 32:8293–8305.

Tazat K, Harsat M, Goldshmid-Shagal A, Ehrlich M, Henis YI. 2013. Dual effects of Ral-activated pathways on p27 localization and TGF-beta signaling. Mol Biol Cell. 24:1812–1824.

Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J. 2003. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol. 13:1259–1268.

Teodoro RO, Pekkurnaz G, Nasser A, Higashi-Kovtun ME, Balakireva M, McLachlan IG, Camonis J, Schwarz TL. 2013. Ral mediates activity-dependent growth of postsynaptic membranes via recruitment of the exocyst. EMBO J. 32:2039–2055.

Thapliyal A, Verma R, Kumar N. 2014. Small G proteins Dexras1 and RHES and their role in pathophysiological processes. Int J Cell Biol. 2014:308535.

The Cancer Genome Atlas Research Network. 2014. Comprehensive molecular profiling of lung adenocarcinoma. Nature 511:543–550.

Tidyman WE, Rauen KA. 2016. Expansion of the RASopathies. Curr Genet Med Rep. 4:57–64.

Trahey M, McCormick F. 1987. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science. 238:542–545.

Trumper J, Ross D, Jahr H, Brendel MD, Goke R, Horsch D. 2005. The Rap-B-Raf signalling pathway is activated by glucose and glucagon-like peptide-1 in human islet cells. Diabetologia. 48:1534–1540.

Tuder RM, Cool CD, Geraci MW, Wang J, Abman SH, Wright L, Badesch D, Voelkel NF. 1999. Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. Am J Respir Crit Care Med. 159:1925–1932.

Tyagi R, Shahani N, Gorgen L, Ferretti M, Pryor W, Chen PY, Swarnkar S, Worley PF, Karbstein K, Snyder SH, Subramaniam S. 2015. Rheb inhibits protein synthesis by activating the PERK-eIF2alpha signaling cascade. Cell Rep. 10:684–693.

Tyutyunnykova A, Telegeev G, Dubrovska A. 2017. The controversial role of phospholipase C epsilon (PLCε) in cancer development and progression. J Cancer. 8:716–729.

Uechi Y, Bayarjargal M, Umikawa M, Oshiro M, Takei K, Yamashiro Y, Asato T, Endo S, Misaki R, Taguchi T, Kariya K. 2009. Rap2 function requires palmitoylation and recycling endosome localization. Biochem Biophys Res Commun. 378:732–737.

Ueda K, Yaoita M, Niihori T, Aoki Y, Okamoto N. 2017. Craniosynostosis in patients with RASopathies: accumulating clinical evidence for expanding the phenotype. Am J Med Genet. 173:2346–2352.

Unal EB, Uhlitz F, Bluthgen N. 2017. A compendium of ERK targets. FEBS Lett. 591:2607–2615.

Upadhyaya P, Bedewy W, Pei D. 2016. Direct Inhibitors of Ras-Effector Protein Interactions. Mini Rev Med Chem. 16:376–382.

Vahatupa M, Prince S, Vataja S, Mertimo T, Kataja M, Kinnunen K, Marjomaki V, Uusitalo H, Komatsu M, Jarvinen TA, Uusitalo-Jarvinen H. 2016. Lack of R-Ras leads to increased vascular permeability in ischemic retinopathy. Invest Ophthalmol Vis Sci. 57:4898–4909.

van Dam TJ, Bos JL, Snel B. 2011. Evolution of the Ras-like small GTPases and their regulators. Small GTPases. 2:4–16.

van Dam TJ, Rehmann H, Bos JL, Snel B. 2009. Phylogeny of the CDC25 homology domain reveals rapid differentiation of Ras pathways between early animals and fungi. Cell Signal. 21:1579–1585.

van Hooren KW, van Agtmaal EL, Fernandez-Borja M, van Mourik JA, Voorberg J, Bierings R. 2012. The Epac-Rap1 signaling pathway controls cAMP-mediated exocytosis of Weibel-Palade bodies in endothelial cells. J Biol Chem. 287:24713–24720.

Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ, Waterfield MD. 2001. Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem. 70:535–602.

Varan A, Sen H, Aydin B, Yalcin B, Kutluk T, Akyuz C. 2016. Neurofibromatosis type 1 and malignancy in childhood. Clin Genet. 89:341–345.

Varga A, Ehrenreiter K, Aschenbrenner B, Kocieniewski P, Kochanczyk M, Lipniacki T, Baccarini M. 2017. RAF1/BRAF

dimerization integrates the signal from RAS to ERK and ROKalpha. Sci Signal. 10:eaai8482.
Vetter IR, Wittinghofer A. 2001. The guanine nucleotide-binding switch in three dimensions. Science. 294:1299–1304.
Villalonga P, Lopez-Alcala C, Bosch M, Chiloeches A, Rocamora N, Gil J, Marais R, Marshall CJ, Bachs O, Agell N. 2001. Calmodulin binds to K-Ras, but not to H- or N-Ras, and modulates its downstream signaling. Mol Cell Biol. 21:7345–7354.
Wang B, Zou JX, Ek-Rylander B, Ruoslahti E. 2000. R-Ras contains a proline-rich site that binds to SH3 domains and is required for integrin activation by R-Ras. J Biol Chem. 275:5222–5227.
Wang M, Casey PJ. 2016. Protein prenylation: unique fats make their mark on biology. Nat Rev Mol Cell Biol. 17:110–122.
Wang MT, Holderfield M, Galeas J, Delrosario R, To MD, Balmain A, McCormick F. 2015. K-Ras promotes tumorigenicity through suppression of non-canonical Wnt signaling. Cell. 163:1237–1251.
Wang Y, He H, Srivastava N, Vikarunnessa S, Chen YB, Jiang J, Cowan CW, Zhang X. 2012. Plexins are GTPase-activating proteins for Rap and are activated by induced dimerization. Sci Signal. 5:ra6.
Weber CK, Slupsky JR, Herrmann C, Schuler M, Rapp UR, Block C. 2000. Mitogenic signaling of Ras is regulated by differential interaction with Raf isozymes. Oncogene. 19:169–176.
Wellbrock C, Karasarides M, Marais R. 2004. The RAF proteins take centre stage. Nat Rev Mol Cell Biol. 5:875–885.
Wennerberg K. 2005. The Ras superfamily at a glance. J Cell Sci. 118:843–846.
Williams JG, Pappu K, Campbell SL. 2003. Structural and biochemical studies of p21Ras S-nitrosylation and nitric oxide-mediated guanine nucleotide exchange. Proc Natl Acad Sci USA. 100:6376–6381.
Winter-Vann AM, Casey PJ. 2005. Post-prenylation-processing enzymes as new targets in oncogenesis. Nat Rev Cancer. 5:405–412.
Wittinghofer A, Nassar N. 1996. How Ras-related proteins talk to their effectors. Trends Biochem Sci. 21:488–491.
Wittinghofer A, Vetter IR. 2011. Structure-function relationships of the G domain, a canonical switch motif. Annu Rev Biochem. 80:943–971.
Wohlgemuth S, Kiel C, Kramer A, Serrano L, Wittinghofer F, Herrmann C. 2005. Recognizing and defining true Ras binding domains I: biochemical analysis. J Mol Biol. 348:741–758.
Wright LP, Philips MR. 2006. Thematic review series: lipid posttranslational modifications. CAAX modification and membrane targeting of Ras. J Lipid Res. 47:883–891.
Wu LJ, Xu LR, Liao JM, Chen J, Liang Y. 2011. Both the C-terminal polylysine region and the farnesylation of K-RasB are important for its specific interaction with calmodulin. PLoS One. 6:e21929.
Wurtzel JG, Lee S, Singhal SS, Awasthi S, Ginsberg MH, Goldfinger LE. 2015. RLIP76 regulates Arf6-dependent cell spreading and migration by linking ARNO with activated R-Ras at recycling endosomes. Biochem Biophys Res Commun. 467:785–791.
Xu L, Salloum D, Medlin PS, Saqcena M, Yellen P, Perrella B, Foster DA. 2011. Phospholipase D mediates nutrient input to mammalian target of rapamycin complex 1 (mTORC1). J Biol Chem. 286:25477–25486.
Yamagata K, Sanders LK, Kaufmann WE, Yee W, Barnes CA, Nathans D, Worley PF. 1994. rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. J Biol Chem. 269:16333–16339.
Yamashita S, Mochizuki N, Ohba Y, Tobiume M, Okada Y, Sawa H, Nagashima K, Matsuda M. 2000. CalDAG-GEFIII activation of Ras, R-ras, and Rap1. J Biol Chem. 275:25488–25493.
Yan J, Li F, Ingram DA, Quilliam LA. 2008. Rap1a is a key regulator of fibroblast growth factor 2-induced angiogenesis and together with Rap1b controls human endothelial cell functions. Mol Cell Biol. 28:5803–5810.
Yang MH, Laurent G, Bause AS, Spang R, German N, Haigis MC, Haigis KM. 2013. HDAC6 and SIRT2 regulate the acetylation state and oncogenic activity of mutant K-RAS. Mol Cancer Res. 11:1072–1077.
Yang MH, Nickerson S, Kim ET, Liot C, Laurent G, Spang R, Philips MR, Shan Y, Shaw DE, Bar-Sagi D, et al. 2012. Regulation of RAS oncogenicity by acetylation. Proc Natl Acad Sci USA. 109:10843–10848.
Yaoita M, Niihori T, Mizuno S, Okamoto N, Hayashi S, Watanabe A, Yokozawa M, Suzumura H, Nakahara A, Nakano Y, et al. 2016. Spectrum of mutations and genotype-phenotype analysis in Noonan syndrome patients with RIT1 mutations. Hum Genet. 135:209–222.
Yee WM, Worley PF. 1997. Rheb interacts with Raf-1 kinase and may function to integrate growth factor- and protein kinase A-dependent signals. Mol Cell Biol. 17:921–933.
Yeh CW, Hsu LS. 2016. Zebrafish diras1 promoted neurite outgrowth in neuro-2a cells and maintained trigeminal ganglion neurons in vivo via Rac1-dependent pathway. Mol Neurobiol. 53:6594–6607.
Yim DG, Ghosh S, Guy GR, Virshup DM. 2015. Casein kinase 1 regulates Sprouty2 in FGF-ERK signaling. Oncogene. 34:474–484.
Yoshizawa R, Umeki N, Yanagawa M, Murata M, Sako Y. 2017. Single-molecule fluorescence imaging of RalGDS on cell surfaces during signal transduction from Ras to Ral. Biophysics. 14:75–84.
Yu Y, Li S, Xu X, Li Y, Guan K, Arnold E, Ding J. 2005. Structural basis for the unique biological function of small GTPase RHEB. J Biol Chem. 280:17093–17100.
Zeng M, Shang Y, Araki Y, Guo T, Huganir RL, Zhang M. 2016. Phase transition in postsynaptic densities underlies formation of synaptic complexes and synaptic plasticity. Cell. 166:1163–1175. e1112.
Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D. 2003. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol. 5:578–581.
Zhang YL, Wang RC, Cheng K, Ring BZ, Su L. 2017. Roles of Rap1 signaling in tumor cell migration and invasion. Cancer Biol Med. 14:90–99.
Zheng R, Gao D, He T, Zhang M, Zhang X, Linghu E, Wei L, Guo M. 2017. Methylation of DIRAS1 promotes colorectal cancer progression and may serve as a marker for poor prognosis. Clin Epigenet. 9:50.
Zhou X, Ikenoue T, Chen X, Li L, Inoki K, Guan KL. 2009. Rheb controls misfolded protein metabolism by inhibiting aggresome formation and autophagy. Proc Natl Acad Sci USA. 106:8923–8928.

Zhou Y, Hancock JF. 2015. Ras nanoclusters: Versatile lipid-based signaling platforms. Biochim Biophys Acta. 1853:841–849.

Zhu Y, Pak D, Qin Y, McCormack SG, Kim MJ, Baumgart JP, Velamoor V, Auberson YP, Osten P, van Aelst L, et al. 2005. Rap2-JNK removes synaptic AMPA receptors during depotentiation. Neuron. 46:905–916.

Zhu YH, Fu L, Chen L, Qin YR, Liu H, Xie F, Zeng T, Dong SS, Li J, Li Y, et al. 2013. Downregulation of the novel tumor suppressor DIRAS1 predicts poor prognosis in esophageal squamous cell carcinoma. Cancer Res. 73:2298–2309.

Zou JX, Liu Y, Pasquale EB, Ruoslahti E. 2002. Activated SRC oncogene phosphorylates R-ras and suppresses integrin activity. J Biol Chem. 277:1824–1827.

Zou JX, Wang B, Kalo MS, Zisch AH, Pasquale EB, Ruoslahti E. 1999. An Eph receptor regulates integrin activity through R-Ras. Proc Natl Acad Sci USA. 96:13813–13818.

Supplement C: Activating Mutations of RRAS2 Are a Rare Cause of Noonan syndrome

REPORT

Activating Mutations of *RRAS2* Are a Rare Cause of Noonan Syndrome

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Aberrant signaling through pathways controlling cell response to extracellular stimuli constitutes a central theme in disorders affecting development. Signaling through RAS and the MAPK cascade controls a variety of cell decisions in response to cytokines, hormones, and growth factors, and its upregulation causes Noonan syndrome (NS), a developmental disorder whose major features include a distinctive facies, a wide spectrum of cardiac defects, short stature, variable cognitive impairment, and predisposition to malignancies. NS is genetically heterogeneous, and mutations in more than ten genes have been reported to underlie this disorder. Despite the large number of genes implicated, about 10%–20% of affected individuals with a clinical diagnosis of NS do not have mutations in known RASopathy-associated genes, indicating that additional unidentified genes contribute to the disease, when mutated. By using a mixed strategy of functional candidacy and exome sequencing, we identify *RRAS2* as a gene implicated in NS in six unrelated subjects/families. We show that the NS-causing *RRAS2* variants affect highly conserved residues localized around the nucleotide binding pocket of the GTPase and are predicted to variably affect diverse aspects of RRAS2 biochemical behavior, including nucleotide binding, GTP hydrolysis, and interaction with effectors. Additionally, all pathogenic variants increase activation of the MAPK cascade and variably impact cell morphology and cytoskeletal rearrangement. Finally, we provide a characterization of the clinical phenotype associated with *RRAS2* mutations.

Noonan syndrome (NS [MIM: PS163950]) is one of the most common monogenic disorders affecting development and growth.[1] The phenotype of NS comprises a distinctive facies (e.g., hypertelorism, downslanting palpebral fissures, ptosis, and low-set/posteriorly rotated ears), cardiac abnormalities (a wide spectrum of congenital heart defects and cardiomyopathy), postnatally reduced growth, skeletal defects (chest and spine), cryptorchidism, bleeding diathesis, as well as variable neurocognitive impairment and predisposition to malignancies,[1,2] most commonly juvenile myelomonocytic leukemia (JMML [MIM: 607785]).[3] NS is generally transmitted as an autosomal-dominant trait and is genetically heterogeneous. So far, pathogenic variants in more than ten genes have been reported as causative events underlying this disorder.[4] While mutations in *PTPN11* (MIM: 176876), *SOS1* (MIM: 182530), *RAF1* (MIM: 164760), and *RIT1* (MIM: 609591) have been documented to occur most frequently,[5–11] a smaller proportion of cases has been ascribed to mutations in other functionally related genes, including *NRAS* (MIM: 164790), *KRAS* (MIM: 190070), *BRAF* (MIM: 164757), *MAP2K1* (MIM: 176872), *SOS2* (MIM: 601247), *LZTR1* (MIM: 600574), *MRAS* (MIM: 608435), and *RASA2* (MIM: 601589).[12–20] Although the causal link between mutations in a subset of these genes and the disorder still remains to be confirmed,[4] the accumulated molecular evidence strongly supports the view that NS is caused by upregulated intracellular traffic through the RAS-mitogen-activated protein kinase (MAPK) signaling pathway.[21,22] Other disorders clinically related to NS (e.g., cardio-facio-cutaneous syndrome [MIM: PS115150], Costello syndrome [MIM: 218040], neurofibromatosis type 1 [MIM: 162200], Legius syndrome [MIM: 611431], Mazzanti syndrome [MIM: 607721], and Noonan syndrome with multiple lentigines

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[MIM: PS151100]) are also caused by mutations in genes encoding key proteins of the RAS-MAPK signaling backbone or upstream regulators (i.e., *CBL*, *HRAS*, *KRAS*, *NF1*, *SPRED1*, *SHOC2*, *BRAF*, *MAP2K1*, and *MAP2K2*).[21,22] In all these related conditions, termed RASopathies, increased signaling through RAS and the MAPK cascade can result from upregulated activity of RAS proteins, enhanced function of upstream signal transducers (e.g., proteins positively controlling RAS function) or downstream RAS effectors, as well as from the inefficient signaling switch-off by feedback mechanisms (e.g., neurofibromin and CBL loss of function). More recently, the use of whole-exome sequencing (WES) has allowed the discovery of RASopathy-associated genes encoding signal transducers or modulators that do not belong to the canonical RAS-MAPK pathway, but when functionally perturbed, are predicted to impact RAS signaling by still poorly characterized circuits.[20,23–29]

A remarkable finding of the molecular genetics of NS and other RASopathies is the occurrence of conserved themes in the mechanism of disease. This applies in particular to mutations affecting genes encoding the various members of the RAS superfamily of GTPases that have been implicated in these disorders, including *KRAS*, *HRAS*, *NRAS*, *RRAS*, *MRAS*, and *CDC42*.[11–14,20,23–26,30] Missense mutations in these genes affect a small number of highly conserved amino acid residues that lead to overactivation of these proteins by decreasing/impairing their GTPase activity in response to GTPase-activating proteins (GAPs), increasing guanine nucleotide exchange factor (GEF)-independent GDP release, altering binding properties to effectors, or a combination of these mechanisms.[31] Notably, while these germline mutations may affect the same residues that are generally mutated in cancer, multiple lines of evidence indicate that RASopathy-causing changes are generally less activating than their respective cancer-associated somatic lesions.[21]

Despite the large number of genes implicated in NS and related phenotypes, about 10%–20% of affected individuals with a convincing clinical diagnosis of NS do not have mutations in currently known RASopathy-associated genes, indicating that other unidentified genes contribute to this disorder. Through the use of complementary approaches based on "functional candidacy" (parallel sequencing of selected gene panels containing functionally related candidate genes) or WES, we identified *RRAS2* (MIM: 600098; GenBank: NM_012250.5) as a gene implicated in NS. We provide structural, biochemical, and functional data to support the causal link between *RRAS2* mutations and NS, outline the mechanisms by which mutations perturb RRAS2 function, and characterize the clinical phenotype associated with these gene lesions.

Subjects from six unrelated families were included in the study. Clinical data and DNA samples were collected from the participating families after written informed consent was obtained. DNA samples were stored and used under research projects approved by the Review Boards of the participating institutions. Because of a suspected RASopathy, subjects 1, 2, 3-III-1, and 5 were referred for diagnostic genetic testing by sequencing of an "extended" panel of RASopathy-associated genes designed to include a set of candidate disease genes selected in the frame of the NSEuroNet Consortium, while subjects 4 and 6 were analyzed by WES (Supplemental Subjects and Methods). In five cases, the *RRAS2* variant (c.68G>T [p.Gly23Val], c.65_73dup [p.Gly22_Gly24dup], c.70_78dup [p.Gly24_Gly26dup], c.208G>A [p.Ala70Thr], c.215A>T [p.Gln72Leu]) arose *de novo* (i.e., it was not identified in parental blood DNA samples). In family 3, mutation scan in one affected family member (3-III-1) identified the heterozygous c.208G>A missense change, and subsequent cosegregation analysis confirmed the occurrence of the variant in three similarly affected relatives. All variants were validated by Sanger sequencing. In all cases, no other candidate variant was identified, further supporting the clinical relevance of this finding. In subject 4, the *RRAS2* variant was detected in both amniocyte and peripheral blood DNA, at 44% and 46% of reads, respectively, indicating the heterozygous mutation was present in the germline of the subject. The clinical data of the affected subjects from the six families are shown in Table 1, facial features of four affected individuals as well as the pedigree of family 3 are presented in Figure 1, and a detailed clinical history is provided in the Supplemental Note. Taken together, the identified *RRAS2* variants included three different nucleotide substitutions predicting missense changes of highly conserved amino acid residues (Gly23, Ala70, and Gln72) among RRAS2 orthologs and paralogs (Figure S1). Alterations to the corresponding positions in other GTPases of the RAS superfamily have already been reported to cause RASopathies or to contribute to oncogenesis (Table S1). In the remaining cases, we identified two small in-frame duplications (p.Gly22_Gly24dup, p.Gly24_Gly26dup) affecting the well-established mutational hotspot of RAS proteins (Figure 2A). Of note, p.Gly22_Gly24dup had previously been reported as somatic event in an uterine leiomyosarcoma specimen,[32] and other similar, but not identical, small in-frame duplications affecting these residues have also been reported in association with different cancers in the Catalogue of Somatic Mutations in Cancer (COSMIC database). The two small in-frame duplications and c.68G>T (p.Gly23Val) and c.215A>T (p.Gln72Leu) substitutions were absent from general population databases, while the c.208G>A (p.Ala70Thr) change had previously been reported in two subjects in gnomAD (heterozygous state, frequency < 0.00001) (Table S2). Multiple *in silico* prediction algorithms uniformly rated these changes as deleterious/pathogenic (Table S2).

RRAS2 (RAS related 2, also known as TC21, teratocarcinoma 21) is a member of the RAS superfamily of GTPases, originally described in 1990.[33] The protein shares the same four conserved functional domains with HRAS, KRAS, and NRAS, and about 55% amino acid sequence homology with HRAS (Figure 2A), which reaches 80%

Table 1. Clinical Features and Genotype of Individuals with *RRAS2* Variants

			Family 3						
	Subject 1	**Subject 2**	**3-II-1**	**3-II-2**	**3-III-1**	**3-III-2**	**Subject 4**	**Subject 5**	**Subject 6**
Origin	Algerian	Sri Lanka	German				Indian	Serbian	South American/ Ashkenazi
Gender	M	M	F	F	F	M	M	F	M
Age at last visit	7 y 11 m	12 y 2 m	32 y	40 y	7 y 1 m	1 y 7 m	2 weeks	8 y 10 m	22 m (last measurement 18 m)
RRAS2 variant	c.65_73dup (p.Gly22_Gly24dup)	c.68G>T (p.Gly23Val)	c.208G>A (p.Ala70Thr)	c.208G>A (p.Ala70Thr)	c.208G>A (p.Ala70Thr)	c.208G>A (p.Ala70Thr)	c.215A>T (p.Gln72Leu)	c.208G>A (p.Ala70Thr)	c.70_78dup (p.Gly24_ Gly26dup)
Inheritance	*de novo*	*de novo*	presumed paternal	presumed paternal	maternal	maternal	*de novo*	*de novo*	*de novo*
Prenatal features	NE, PH	PH	NA	NA	NA	N	NE, fetal ventriculo-megaly and cardiac abnormalities	NE	PH, LGA
Birth measurements: weight, length, OFC (weeks GA)	3,730 g, 50.5 cm, 37 cm (35)	3,180 g, 46.5 cm, 35 cm (35)	NA	3,740 g, 51 cm, 36 cm	3,110 g, 48 cm, 36 cm (39)	2,440 g, 48 cm, 32 cm (35)	2,400 g (33)	NA	3,600 g, 51 cm, 38 cm (35)
Feeding difficulties	PF	PF, TF	NA	NA	PF	N	NA	N	N
Height at last examination	125.5 cm (+0.3 SD)	139.5 (−1.5 SD) 85 cm (−3.3 SD)[a]	160 cm (−1.3 SD)	170 cm (+0.3 SD)	108 cm (−3.0 SD)	78 cm (−1.8 SD)	NA	122 cm (−2.1 SD)	84.5 cm (+0.5 SD)
Weight	27.5 kg (+0.5 SD)	32.5 kg (−1.4 SD)	NA	59 kg (+0.1 SD)	18.6 kg (−1.8 SD)	11 kg (−0.4 SD)	NA	22 kg (−1.9 SD)	12.5 kg (+0.7 SD)
OFC	54 cm (+1.2 SD)	57 cm (+2.5 SD)	52.5 cm (−2.2 SD)	55.5 cm (+0.2 SD)	52 cm (+0.4 SD)	49 cm (+0.2 SD)	NA	52.5 cm (+0.2 SD)	54.5 cm (+5.0 SD)
Cryptorchidism	N	N	NA	NA	NA	N	hypoplastic scrotum	NA	N
Congenital heart defect	SVAoS	VSD	VSD	N	N	N	TOF	AVSD, multiple VSDs	N
Lymphatic anomalies	N	N	N	N	N	N	N	N	N
Facial anomalies	typical NS	typical NS	suggestive NS	very mild in adulthood	typical NS	typical NS	multiple anomalies	suggestive NS	typical NS
Development	N	mild MD, mild LD	N	N	mild MD, mild LD	N	NA	N	mild global delay
Neurology	N	Chiari malformation	N	N	N	N	non-obstructive hydrocephalus	N	mild ventriculomegaly, hypotonia
Skeletal	N	N	N	N	N	N	11 rib pairs, proximally placed thumb, spinal canal stenosis	pectus excavatum	N

(*Continued on next page*)

Table 1. *Continued*

	Subject 1	Subject 2	Family 3				Subject 4	Subject 5	Subject 6
			3-II-1	3-II-2	3-III-1	3-III-2			
Hematology & oncology	N	lymphopenia	N	N	N	N	thrombocytopenia	N	N
Skin and hair	glabellar heamangioma	N	N	N	atopic dermatitis,	N	N	N	glabellar hemangioma
Ocular	N	strabismus	N	strabismus	hyperopia, bilateral ptosis	N	NA	N	strabismic amblyopia, esotropia
Other malformations/ anomalies	N	GH deficiency, GH treatment from age 4 y	unilateral duplex kidney	N	multiple allergies, bronchitis	N	labyrinth dysplasia, anteriorly placed anus	minor hippocampal malformation on brain MRI	N

Abbreviations: AVSD, atrioventricular septal defect; F, female; GA, gestational age; GD, global delay; GH, growth hormone; LD, learning difficulties; LGA, large for gestational age; M, male; m, months; MD, motor delay; N, none/normal; NA, not applicable/not available; NE, nuchal edema; OFC, occipitofrontal head circumference; PF, poor feeding reported; PH, polyhydramnios; SVAoS, supravalvular aortic stenosis; TF, tube feeding (>4 weeks); TOF, Tetralogy of Fallot; y, years.
[a]Before onset of growth hormone treatment at age 3 y 6 m.

when considering the region between residues 5 to 120 (i.e., excluding the hypervariable tail at the C terminus).[34,35] RRAS2 controls multiple cellular processes, including proliferation, survival, and migration, and its functional dysregulation has been documented to contribute to oncogenesis.[34,36,37] Indeed, a number of oncogenic RRAS2 variants have been reported, including the p.Gly23Val, p.Ala70Thr, and p.Gln72Leu changes, in a variety of solid tumors (Table S1). More recently, the p.Gln72Leu change in RRAS2 has been identified in subjects with isolated JMML,[38] which represents the archetypal somatic RASopathy. Notably, germline mutations in other *RAS* genes affecting analogous codons to those observed in the present cases have also been identified (Table S1), including the missense mutation p.Gln87Leu in RRAS (homologous to p.Gln72Leu in RRAS2), previously reported in individuals having features reminiscent of NS.[23]

In order to decipher the consequences of the observed amino acid changes and the small in-frame duplications on the molecular structure of RRAS2, we performed structural modeling. A closer view into the active site of RRAS2 structure in its active form (Figure 2B, left) revealed that the identified *RRAS2* mutations affect residues localized around the nucleotide binding pocket of the GTPase. The corresponding amino acids, including Gly22-Gly26, Ala70, and Gln72, do not only play a critical role in GDP/GTP exchange and GTP hydrolysis but also are involved in stabilization of the switch regions (Figure 2B, right), which are the binding sites for both RRAS2 regulators (GEFs and GAPs) and effectors.[39] Specifically, the amino acid stretch encompassing Gly22 to Gly26 constitutes part of the phosphate-binding loop (P loop; residues Gly21 to Ser28) that is responsible for binding to the phosphate groups of either GTP or GDP. These residues play a critical role in nucleotide binding and hydrolysis by contacting both the β-γ phosphates of GTP (shown as GppNHp, a non-hydrolyzable GTP analog in Figure 2B) and residues 67 to 69 of the switch II region (SwII; Asp68 to Arg84). Val25 stabilizes the P loop by contacting Val92, Ser94, and Ser100. The Gly22-to-Gly24 and Gly24-to-Gly26 duplications were predicted to destabilize the P loop and result in increased nucleotide exchange and decreased GTP hydrolysis reactions. Differently, Ala70 and Gln72 are located in the switch II region of the GTPase and are directly involved in Mg^{2+} coordination and GTP hydrolysis reaction. Additionally, Ala70 and Gln72 stabilize the switch I region (SwI; Phe39-Ser50) by contacting Ile47 and Glu48, respectively. Based on these considerations, the NS-associated amino acid changes were expected to affect various aspects of RRAS2 biochemical behavior, including a faster nucleotide exchange, an impaired GTP hydrolysis, and a decrease in GEF, GAP, and effector interactions. Subsequent biochemical analysis of $RRAS2^{p.Ala70Thr}$ clearly confirmed these structural predictions, as assessment of the intrinsic and stimulated nucleotide exchange demonstrated a significantly increased

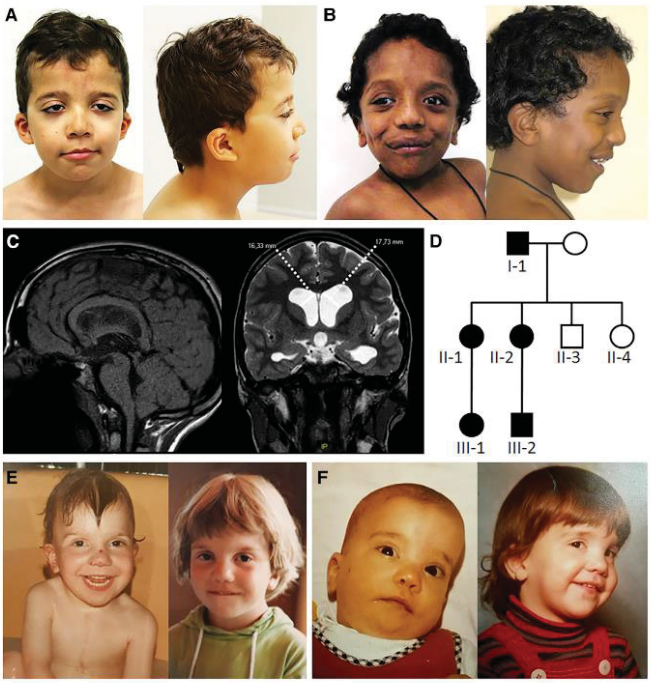

Figure 1. Clinical Features of Individuals with Heterozygous Noonan Syndrome-Causing *RRAS2* Variants
(A) Clinical appearance of subject 1 at 7 years and 11 months. Note the distinctive NS features, including bitemporal narrowing, downslanting palpebral fissures, ptosis, low-set ears, and low posterior hairline.
(B) Facial features of subject 2 at 2 years and 6 months. Facial features overlap those characterizing subject 1, even though a "coarse" face is also observed.
(C) Subject 2 brain MRI at 11 years and 9 months showing Chiari type 1 malformation and bilateral ventricular dilatation.
(D) Pedigree of family 3.
(E) Clinical appearance of subject 3-II-1 at the age of 11 months and 4.5 years.
(F) Facial features of subject 3-II-2 at 9 months and 5 years. The NS facial gestalt of subjects 3-II-1 and 3-II-2 became less obvious in adulthood.

response of the RRAS2$^{p.Ala70Thr}$ protein to GEF as compared to wild-type RRAS2 (Figure 2C). In contrast, the GTP hydrolysis reactions of the mutant were reduced compared to the wild-type protein. Particularly, the GAP-stimulated GTPase activity of RRAS2$^{p.Ala70Thr}$ was significantly decreased (9-fold) (Figure 2C). Finally, the binding properties to two RRAS2 effectors, RAF1 (CRAF) and RASSF5, were assessed. While the affinity of the interaction with CRAF was comparable to that of wild-type RRAS2, binding to RASSF5 was abolished (Figure 2C). This suggests the p.Ala70Thr change leads to a structural rearrangement of RRAS2 switch II, which is a key binding site for RASSF5 but not for CRAF. Overall, these data support that the p.Ala70Thr change leads to an accumulation of RRAS2 in its GTP-bound active state, which predicts an increase in signaling activity. The impaired binding to RASSF5, however, suggest a possible differential impact of the missense change on downstream signaling pathways.

RRAS2 shares downstream effectors with the other members of the RAS subfamily;[35] however, little information exists about the function of this protein in cellular processes and development. Similarly, scant data exist on the specific role of this protein in intracellular signaling as well as on the extent of functional overlap with the other RAS proteins implicated in RASopathies. To explore the consequences of NS-associated *RRAS2* mutations on the intracellular signaling pathways affected in NS, the signaling flows through the MAPK and phosphatidylinostiol-3 kinase (PI3K)-AKT cascades were evaluated using transient expression in HEK293T cells. Expression of all mutants resulted in variably enhanced ERK phosphorylation compared to cells overexpressing the wild-type protein (Figure 3A). Notably, RRAS2$^{p.Ala70Thr}$ and RRAS2$^{p.Gln72Leu}$ were observed to constitutively promote increased ERK phosphorylation, while only a slight increase was observed basally in cells expressing the RRAS2$^{p.Gly22_Gly24dup}$ and RRAS2$^{p.Gly23Val}$ mutants. However, this slight increase substantially strengthened after stimulation with EGF. This activating role of p.Gly22_Gly24dup is in line with previous evidence supporting the gain-of-function role of short insertional mutations in the P loop of other members of the RAS family.[40] Based on previous data indicating that upregulated RRAS2 promotes tumorigenesis in a PI3K-dependent manner,[41] the impact of NS-associated mutants on PI3K-AKT signaling was also assessed. No significant difference in the extent of AKT phosphorylation was documented, indicating a specific functional link between RRAS2 and the MAPK signaling cascade, at least in the present experimental conditions. In line with these findings, *Rras2* KO mice showed a downmodulation of Erk activation and unaltered levels of phosphorylated Akt.[42]

RAS proteins interact with multiple signaling platforms, which allow these proteins to differentially control multiple signaling pathways.[43] Such complex behavior is attained by their dynamic interaction with the plasma membrane and other intracellular membranes (i.e., endosomes, endoplasmic reticulum, and Golgi). To explore any perturbing effect of mutations on the subcellular

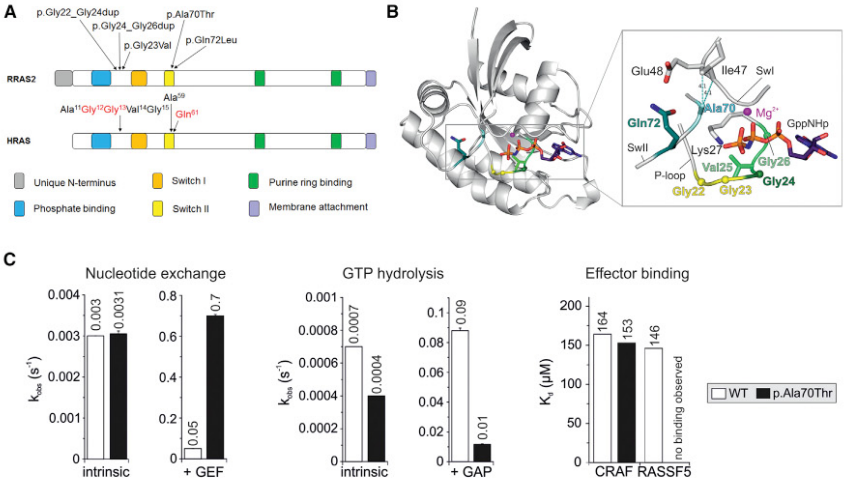

Figure 2. RRAS2 Structure and Location and Functional Impact of Noonan Syndrome-Causing Variants
(A) Schematic representation of RRAS2 and HRAS proteins. Conserved motifs critical for tight guanine nucleotide binding and hydrolysis, and position of the disease-causing RRAS2 variants are illustrated together with the homologous residues of HRAS. The three residues representing the mutational hotspots of oncogenic *HRAS* mutations are shown in red.
(B) Structural modeling of RRAS2 variants. A structural model of the active GTP-bound RRAS2 protein highlights the relative position of the disease-causing missense or insertion mutations. All *RRAS2* mutations affect residues that are located in the nucleotide binding active site region, which contains integral elements involved in GDP/GTP binding, GTP hydrolysis, and interactions with regulators (GEFs and GAPs) and effectors.
(C) Biochemical assessment of RRAS2$^{p.Ala70Thr}$. RRAS2^{WT} and RRAS2$^{p.Ala70Thr}$ proteins were biochemically characterized regarding their nucleotide exchange (left), GTP hydrolysis (middle), and effector binding (right) properties. The nucleotide exchange reaction was measured in the absence (intrinsic) and in the presence of the catalytic RASGEF domain of mouse RASGRF1, while the catalytic activity of the GTPase was assessed in the absence (intrinsic) and in the presence of the p120 RASGAP GAP domain. The RAS-binding and RAS association domains of CRAF and RASSF5 were used to evaluate the binding behavior of the RRAS2$^{p.Ala70Thr}$ mutant to RAS effectors. Overall, the data indicate that the p.Ala70Thr change leads to an accumulation of the protein in its GTP-bound active state, resulting to an increased signaling activity. The missense change, however, is predicted to differentially impact on the diverse downstream signaling pathways.

localization and distribution of RRAS2, including possible preferential targeting to specific intracellular domains, confocal laser scanning microscopy analysis was performed in HEK293T cells transiently expressing Myc-tagged RRAS2 constructs under starved condition. Similarly to the wild-type protein, a fraction of all RRAS2 mutant proteins co-localized with GM130, indicating their targeting to the Golgi apparatus, and the remainder were largely found at the plasma membrane (Figure 3B, left), indicating that mutations do not cause any overt subcellular redistribution of the GTPase. Notably, transient expression of all mutants was found to variably impact cell morphology and cytoskeletal rearrangement, with all mutant proteins promoting spreading and adhesion (Figure 3B, right). Taken together, these experimental data suggest that NS-associated *RRAS2* mutations variably upregulate MAPK signaling and are likely to affect cellular processes depending on cytoskeleton rearrangement similar to observations of RASopathy-causing KRAS mutants.[44]

Our findings establish *RRAS2* germline mutations as a cause of NS. Although previous screening of a cohort of 116 subjects with a clinical diagnosis of NS without a genetic explanation did not identify germline pathogenic *RRAS2* variants,[45] the present collaborative effort allowed to identify six unrelated affected individuals. Of the case subjects reported here, two individuals carrying *de novo* germline NS-causing *RRAS2* variants (subjects 1 and 2) were identified among 1,220 samples addressed to Robert Debré Hospital, Paris, for diagnostic testing for NS, between February 2016 and September 2018. Within the same period, 181 of these subjects were found to carry a *PTPN11* mutation. At the University Hospital of Magdeburg, screening of a multigene panel including *RRAS2* in a cohort of 280 subjects with a tentative diagnosis of NS and negative results for mutations in previously known genes yielded two *RRAS2* mutation-positive cases. Finally, no putative *RRAS2* mutation was identified among 150 case subjects with a clinical diagnosis of NS from Ospedale Pediatrico Bambino Gesù, Rome. Overall, these findings indicate that *RRAS2* mutations are rare events in NS.

The phenotypes associated with the two *RRAS2* mutation hotspots were found to fit well within the clinical spectrum of NS even though they appeared variable in terms of

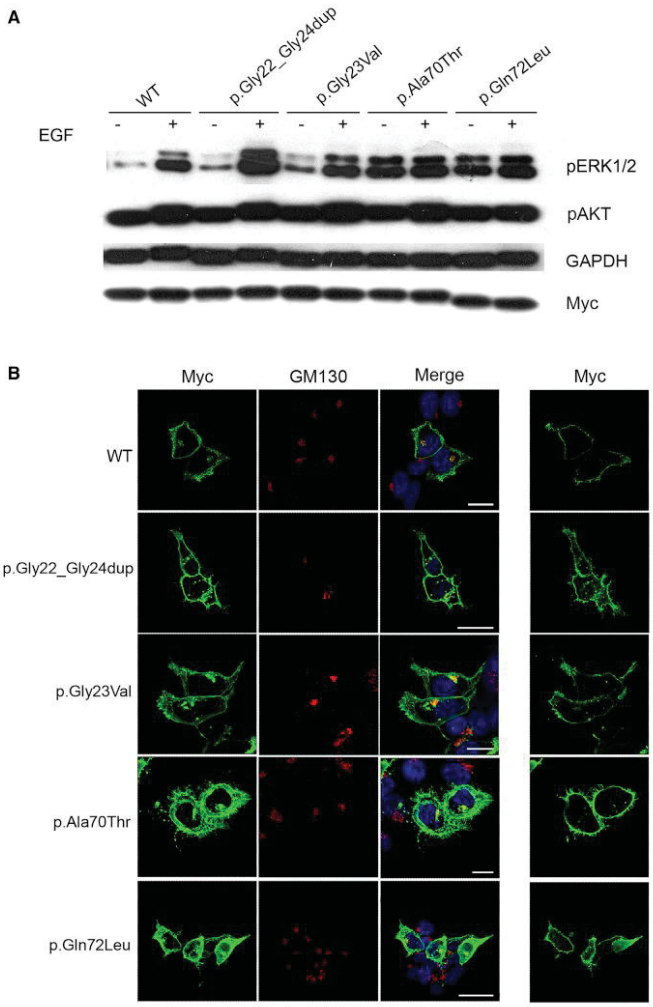

Figure 3. Biochemical and Functional Characterization of Noonan Syndrome-Causing *RRAS2* Variants

(A) ERK and AKT phosphorylation assays. HEK293T cells were transfected with the indicated Myc-tagged RRAS2 constructs. Following starvation (18 h) and EGF stimulation (30 ng/mL for 15 min), ERK and AKT phosphorylation levels were evaluated using a mouse monoclonal anti-phospho-p44/42 ERK (Thr202/Tyr204) antibody and a rabbit polyclonal anti-phospho-AKT (Ser473) antibody, respectively. To assess myc-RRAS2 protein levels, 20 μg of total lysates were immunoblotted with a mouse monoclonal anti-Myc antibody. Membranes were re-probed with mouse monoclonal anti-GAPDH antibody for protein normalization. Representative blot of three performed experiments are shown.

(B) RRAS2 subcellular localization showed by confocal laser scanning microscopy (CLSM) observations (left). Assays were performed on HEK293T cells starved overnight and stained with an anti-Myc mouse monoclonal antibody, followed by goat anti-mouse Alexa Fluor-488 (green), and an anti-GM130 (Golgi marker) rabbit polyclonal antibody, followed by goat anti-rabbit Alexa Fluor-594 (red). Nuclei are visualized by DAPI staining (blue). Co-localization areas were detected in yellow. CLSM observation were also performed at the basal level of cells to show the distinctive pattern of adhesion-like structures and cytoskeletal rearrangement in cells expressing the RRAS2 mutants (right). In all panels, bars correspond to 21 μm.

severity. While individuals 1, 2, 5, and 6 had features fitting typical NS, the phenotype in some affected members of family 3 was relatively mild. On the other hand, subject 4 showed a complex and particularly severe phenotype with multiple congenital anomalies and neonatal lethality. Of note, prenatal features (nuchal edema, polyhydramnios, and/or cardiomyopathy) were reported in five of six subjects, and none showed pulmonary valve stenosis or hypertrophic cardiomyopathy. While the small size of the studied cohort does not allow us to outline specific genotype-phenotype correlations, we hypothesize that such variable expressivity likely reflects the differential strength of individual variants to perturb RRAS2 function and intracellular signaling. Consistent with the collected functional data, p.Gln72Leu (analogous to p.Gln61Leu in HRAS, NRAS, and KRAS) is a strong activating mutation and has not been observed to occur as a germline event in *HRAS*, *KRAS*, or *NRAS*. Similar differences in the biological and phenotypic consequences have previously been reported for *HRAS*, *NRAS*, and *KRAS*,[12–14,30,31,46–53] including the positions corresponding to the presently identified *RRAS2* mutations. The genotype-phenotype correlations in *HRAS* are illustrative and correlate well with the present findings: while p.Ala59Thr has been associated with Costello syndrome and p.Gly12Val has been reported with severe expression of Costello syndrome,[46] p.Gln61Leu and other changes at this codon have only been reported as somatic events in cancer (Table S1).

A noticeable finding of this study is the observation of a diverse impact of the p.Ala70Thr on RRAS2 binding to CRAF/RAF1 and RASSF5. These data suggest the possibility that multiple signaling pathways downstream of RRAS2 may contribute to dysregulation of cellular processes

(e.g., cell proliferation). As expected, a variable hyperactivation of the MAPK pathway resulting from the hyperactive state of the GTPase and unaltered binding to CRAF was observed for the NS-causing RRAS2^{Ala70Thr} protein. Remarkably, impaired binding of this mutant to RASSF5, a known tumor suppressor protein negatively modulating YAP1 levels through activation of the Hippo pathway, was also observed. YAP1 is a transcriptional cofactor promoting cell proliferation, which undergoes RASSF5-mediated phosphorylation and degradation.[54] The impaired binding of RRAS2 to RASSF5 raises the possibility that a less effective Hippo-mediated control of YAP1 levels may contribute to disease pathogenesis in NS.

Among RAS GTPases, RRAS2 exhibits the highest amino acid identity to HRAS, KRAS, and NRAS.[35] Somatic mutations in *RRAS2* have been established to contribute to oncogenesis, even though in a substantially restricted tumor type and less frequently compared to *HRAS*, *KRAS*, and *NRAS*. Consistently, it was originally demonstrated that RRAS2 proteins containing amino acid substitutions analogous to those with oncogenic role in HRAS, KRAS, and NRAS have transforming properties comparable to the strong transforming activity of RAS oncoproteins and similarly promote constitutive activation of the MAPK cascade.[55] Our findings, which are in line with the data presented in an accompanying report by Niihori et al. published in this issue,[56] further extend these observations by demonstrating the clinical relevance of a narrow spectrum of germline pathogenic variants in *RRAS2* as the event underlying a small fraction of NS cases via upregulation of MAPK signaling. Further studies are required to more accurately define the precise mechanisms and circuits linking upregulated RRAS2 function and RAS-MAPK signaling dysregulation.

Accession Numbers

The accession numbers for the five *RRAS2* variants reported in this paper are ClinVar: SCV000902249–SCV000902253.

Supplemental Data

Supplemental Data can be found online at https://doi.org/10.1016/j.ajhg.2019.04.013.

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Declaration of Interests

K.G.M. declares no additional conflicts of interest beyond her employment affiliation. L.M.V. is a former employee of GeneDx. All the other authors declare no competing interests.

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Web Resources

CADD, https://cadd.gs.washington.edu/
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
COSMIC, https://cancer.sanger.ac.uk/cosmic
dbSNP, https://www.ncbi.nlm.nih.gov/snp
Exome Aggregation Consortium (ExAC) Browser, http://exac.broadinstitute.org/
GenBank, https://www.ncbi.nlm.nih.gov/genbank
GeneMatcher, https://genematcher.org
gnomAD, https://gnomad.broadinstitute.org/
Muscle, https://www.ebi.ac.uk/Tools/msa/muscle/
MutationAssessor, http://mutationassessor.org/r3/
MutationTaster, http://mutationtaster.org
MutPred2, http://mutpred.mutdb.org/
OMIM, http://www.omim.org/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
PROVEAN, http://provean.jcvi.org/index.php

References

1. Roberts, A.E., Allanson, J.E., Tartaglia, M., and Gelb, B.D. (2013). Noonan syndrome. Lancet *381*, 333–342.
2. Tartaglia, M., Gelb, B.D., and Zenker, M. (2011). Noonan syndrome and clinically related disorders. Best Pract. Res. Clin. Endocrinol. Metab. *25*, 161–179.
3. Strullu, M., Caye, A., Lachenaud, J., Cassinat, B., Gazal, S., Fenneteau, O., Pouvreau, N., Pereira, S., Baumann, C., Contet, A., et al. (2014). Juvenile myelomonocytic leukaemia and Noonan syndrome. J. Med. Genet. *51*, 689–697.
4. Grant, A.R., Cushman, B.J., Cavé, H., Dillon, M.W., Gelb, B.D., Gripp, K.W., Lee, J.A., Mason-Suares, H., Rauen, K.A., Tartaglia, M., et al. (2018). Assessing the gene-disease association of 19 genes with the RASopathies using the ClinGen gene curation framework. Hum. Mutat. *39*, 1485–1493.
5. Tartaglia, M., Mehler, E.L., Goldberg, R., Zampino, G., Brunner, H.G., Kremer, H., van der Burgt, I., Crosby, A.H., Ion, A., Jeffery, S., et al. (2001). Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat. Genet. *29*, 465–468.
6. Tartaglia, M., Pennacchio, L.A., Zhao, C., Yadav, K.K., Fodale, V., Sarkozy, A., Pandit, B., Oishi, K., Martinelli, S., Schackwitz, W., et al. (2007). Gain-of-function *SOS1* mutations cause a distinctive form of Noonan syndrome. Nat. Genet. *39*, 75–79.
7. Roberts, A.E., Araki, T., Swanson, K.D., Montgomery, K.T., Schiripo, T.A., Joshi, V.A., Li, L., Yassin, Y., Tamburino, A.M., Neel, B.G., and Kucherlapati, R.S. (2007). Germline gain-of-function mutations in *SOS1* cause Noonan syndrome. Nat. Genet. *39*, 70–74.
8. Zenker, M., Horn, D., Wieczorek, D., Allanson, J., Pauli, S., van der Burgt, I., Doerr, H.-G., Gaspar, H., Hofbeck, M., Gillessen-Kaesbach, G., et al. (2007). SOS1 is the second most common

Noonan gene but plays no major role in cardio-facio-cutaneous syndrome. J. Med. Genet. *44*, 651–656.
9. Pandit, B., Sarkozy, A., Pennacchio, L.A., Carta, C., Oishi, K., Martinelli, S., Pogna, E.A., Schackwitz, W., Ustaszewska, A., Landstrom, A., et al. (2007). Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. Nat. Genet. *39*, 1007–1012.
10. Razzaque, M.A., Nishizawa, T., Komoike, Y., Yagi, H., Furutani, M., Amo, R., Kamisago, M., Momma, K., Katayama, H., Nakagawa, M., et al. (2007). Germline gain-of-function mutations in *RAF1* cause Noonan syndrome. Nat. Genet. *39*, 1013–1017.
11. Kouz, K., Lissewski, C., Spranger, S., Mitter, D., Riess, A., Lopez-Gonzalez, V., Lüttgen, S., Aydin, H., von Deimling, F., Evers, C., et al. (2016). Genotype and phenotype in patients with Noonan syndrome and a *RIT1* mutation. Genet. Med. *18*, 1226–1234.
12. Cirstea, I.C., Kutsche, K., Dvorsky, R., Gremer, L., Carta, C., Horn, D., Roberts, A.E., Lepri, F., Merbitz-Zahradnik, T., König, R., et al. (2010). A restricted spectrum of NRAS mutations causes Noonan syndrome. Nat. Genet. *42*, 27–29.
13. Schubbert, S., Zenker, M., Rowe, S.L., Böll, S., Klein, C., Bollag, G., van der Burgt, I., Musante, L., Kalscheuer, V., Wehner, L.-E., et al. (2006). Germline *KRAS* mutations cause Noonan syndrome. Nat. Genet. *38*, 331–336.
14. Zenker, M., Lehmann, K., Schulz, A.L., Barth, H., Hansmann, D., Koenig, R., Korinthenberg, R., Kreiss-Nachtsheim, M., Meinecke, P., Morlot, S., et al. (2007). Expansion of the genotypic and phenotypic spectrum in patients with KRAS germline mutations. J. Med. Genet. *44*, 131–135.
15. Sarkozy, A., Carta, C., Moretti, S., Zampino, G., Digilio, M.C., Pantaleoni, F., Scioletti, A.P., Esposito, G., Cordeddu, V., Lepri, F., et al. (2009). Germline *BRAF* mutations in Noonan, LEOPARD, and cardiofaciocutaneous syndromes: molecular diversity and associated phenotypic spectrum. Hum. Mutat. *30*, 695–702.
16. Nava, C., Hanna, N., Michot, C., Pereira, S., Pouvreau, N., Niihori, T., Aoki, Y., Matsubara, Y., Arveiler, B., Lacombe, D., et al. (2007). Cardio-facio-cutaneous and Noonan syndromes due to mutations in the RAS/MAPK signalling pathway: genotype-phenotype relationships and overlap with Costello syndrome. J. Med. Genet. *44*, 763–771.
17. Yamamoto, G.L., Aguena, M., Gos, M., Hung, C., Pilch, J., Fahiminiya, S., Abramowicz, A., Cristian, I., Buscarilli, M., Naslavsky, M.S., et al. (2015). Rare variants in *SOS2* and *LZTR1* are associated with Noonan syndrome. J. Med. Genet. *52*, 413–421.
18. Cordeddu, V., Yin, J.C., Gunnarsson, C., Virtanen, C., Drunat, S., Lepri, F., De Luca, A., Rossi, C., Ciolfi, A., Pugh, T.J., et al. (2015). Activating Mutations Affecting the Dbl Homology Domain of SOS2 Cause Noonan Syndrome. Hum. Mutat. *36*, 1080–1087.
19. Chen, P.-C., Yin, J., Yu, H.-W., Yuan, T., Fernandez, M., Yung, C.K., Trinh, Q.M., Peltekova, V.D., Reid, J.G., Tworog-Dube, E., et al. (2014). Next-generation sequencing identifies rare variants associated with Noonan syndrome. Proc. Natl. Acad. Sci. USA *111*, 11473–11478.
20. Higgins, E.M., Bos, J.M., Mason-Suares, H., Tester, D.J., Ackerman, J.P., MacRae, C.A., Sol-Church, K., Gripp, K.W., Urrutia, R., and Ackerman, M.J. (2017). Elucidation of *MRAS*-mediated Noonan syndrome with cardiac hypertrophy. JCI Insight *2*, e91225.
21. Tartaglia, M., and Gelb, B.D. (2010). Disorders of dysregulated signal traffic through the RAS-MAPK pathway: phenotypic spectrum and molecular mechanisms. Ann. N Y Acad. Sci. *1214*, 99–121.
22. Rauen, K.A. (2013). The RASopathies. Annu. Rev. Genomics Hum. Genet. *14*, 355–369.
23. Flex, E., Jaiswal, M., Pantaleoni, F., Martinelli, S., Strullu, M., Fansa, E.K., Caye, A., De Luca, A., Lepri, F., Dvorsky, R., et al. (2014). Activating mutations in RRAS underlie a phenotype within the RASopathy spectrum and contribute to leukaemogenesis. Hum. Mol. Genet. *23*, 4315–4327.
24. Gripp, K.W., Aldinger, K.A., Bennett, J.T., Baker, L., Tusi, J., Powell-Hamilton, N., Stabley, D., Sol-Church, K., Timms, A.E., and Dobyns, W.B. (2016). A novel rasopathy caused by recurrent de novo missense mutations in *PPP1CB* closely resembles Noonan syndrome with loose anagen hair. Am. J. Med. Genet. A. *170*, 2237–2247.
25. Martinelli, S., Krumbach, O.H.F., Pantaleoni, F., Coppola, S., Amin, E., Pannone, L., Nouri, K., Farina, L., Dvorsky, R., Lepri, F., et al.; University of Washington Center for Mendelian Genomics (2018). Functional Dysregulation of CDC42 Causes Diverse Developmental Phenotypes. Am. J. Hum. Genet. *102*, 309–320.
26. Young, L.C., Hartig, N., Boned Del Río, I., Sari, S., Ringham-Terry, B., Wainwright, J.R., Jones, G.G., McCormick, F., and Rodriguez-Viciana, P. (2018). SHOC2-MRAS-PP1 complex positively regulates RAF activity and contributes to Noonan syndrome pathogenesis. Proc. Natl. Acad. Sci. USA *115*, E10576–E10585.
27. Motta, M., Fidan, M., Bellacchio, E., Pantaleoni, F., Schneider-Heieck, K., Coppola, S., Borck, G., Salviati, L., Zenker, M., Cirstea, I.C., and Tartaglia, M. (2018). Dominant Noonan syndrome-causing LZTR1 mutations specifically affect the kelch domain substrate-recognition surface and enhance RAS-MAPK signaling. Hum. Mol. Genet. *28*, 1007–1022.
28. Bigenzahn, J.W., Collu, G.M., Kartnig, F., Pieraks, M., Vladimer, G.I., Heinz, L.X., Sedlyarov, V., Schischlik, F., Fauster, A., Rebsamen, M., et al. (2018). LZTR1 is a regulator of RAS ubiquitination and signaling. Science *362*, 1171–1177.
29. Steklov, M., Pandolfi, S., Baietti, M.F., Batiuk, A., Carai, P., Najm, P., Zhang, M., Jang, H., Renzi, F., Cai, Y., et al. (2018). Mutations in LZTR1 drive human disease by dysregulating RAS ubiquitination. Science *362*, 1177–1182.
30. Aoki, Y., Niihori, T., Kawame, H., Kurosawa, K., Ohashi, H., Tanaka, Y., Filocamo, M., Kato, K., Suzuki, Y., Kure, S., and Matsubara, Y. (2005). Germline mutations in *HRAS* proto-oncogene cause Costello syndrome. Nat. Genet. *37*, 1038–1040.
31. Gremer, L., Gilsbach, B., Ahmadian, M.R., and Wittinghofer, A. (2008). Fluoride complexes of oncogenic Ras mutants to study the Ras-RasGap interaction. Biol. Chem. *389*, 1163–1171.
32. Huang, Y., Saez, R., Chao, L., Santos, E., Aaronson, S.A., and Chan, A.M. (1995). A novel insertional mutation in the TC21 gene activates its transforming activity in a human leiomyosarcoma cell line. Oncogene *11*, 1255–1260.
33. Drivas, G.T., Shih, A., Coutavas, E., Rush, M.G., and D'Eustachio, P. (1990). Characterization of four novel ras-like genes expressed in a human teratocarcinoma cell line. Mol. Cell. Biol. *10*, 1793–1798.
34. Bos, J.L. (1997). Ras-like GTPases. Biochim. Biophys. Acta *1333*, M19–M31.
35. Nakhaei-Rad, S., Haghighi, F., Nouri, P., Rezaei Adariani, S., Lissy, J., Kazemein Jasemi, N.S., Dvorsky, R., and Ahmadian, M.R. (2018). Structural fingerprints, interactions, and

signaling networks of RAS family proteins beyond RAS isoforms. Crit. Rev. Biochem. Mol. Biol. *53*, 130–156.

36. Graham, S.M., Oldham, S.M., Martin, C.B., Drugan, J.K., Zohn, I.E., Campbell, S., and Der, C.J. (1999). TC21 and Ras share indistinguishable transforming and differentiating activities. Oncogene *18*, 2107–2116.
37. Erdogan, M., Pozzi, A., Bhowmick, N., Moses, H.L., and Zent, R. (2007). Signaling pathways regulating TC21-induced tumorigenesis. J. Biol. Chem. *282*, 27713–27720.
38. Stieglitz, E., Taylor-Weiner, A.N., Chang, T.Y., Gelston, L.C., Wang, Y.-D., Mazor, T., Esquivel, E., Yu, A., Seepo, S., Olsen, S., et al. (2015). The genomic landscape of juvenile myelomonocytic leukemia. Nat. Genet. *47*, 1326–1333.
39. Vetter, I.R., and Wittinghofer, A. (2001). The guanine nucleotide-binding switch in three dimensions. Science *294*, 1299–1304.
40. Klockow, B., Ahmadian, M.R., Block, C., and Wittinghofer, A. (2000). Oncogenic insertional mutations in the P-loop of Ras are overactive in MAP kinase signaling. Oncogene *19*, 5367–5376.
41. Larive, R.M., Moriggi, G., Menacho-Márquez, M., Cañamero, M., de Álava, E., Alarcón, B., Dosil, M., and Bustelo, X.R. (2014). Contribution of the R-Ras2 GTP-binding protein to primary breast tumorigenesis and late-stage metastatic disease. Nat. Commun. *5*, 3881.
42. Larive, R.M., Abad, A., Cardaba, C.M., Hernández, T., Cañamero, M., de Álava, E., Santos, E., Alarcón, B., and Bustelo, X.R. (2012). The Ras-like protein R-Ras2/TC21 is important for proper mammary gland development. Mol. Biol. Cell *23*, 2373–2387.
43. Hancock, J.F. (2003). Ras proteins: different signals from different locations. Nat. Rev. Mol. Cell Biol. *4*, 373–384.
44. Gremer, L., Merbitz-Zahradnik, T., Dvorsky, R., Cirstea, I.C., Kratz, C.P., Zenker, M., Wittinghofer, A., and Ahmadian, M.R. (2011). Germline KRAS mutations cause aberrant biochemical and physical properties leading to developmental disorders. Hum. Mutat. *32*, 33–43.
45. Ceremsak, J.J., Yu, A., Esquivel, E., Lissewski, C., Zenker, M., Loh, M.L., and Stieglitz, E. (2016). Germline *RRAS2* mutations are not associated with Noonan syndrome. J. Med. Genet. *53*, 728.
46. Quélin, C., Loget, P., Rozel, C., D'Hervé, D., Fradin, M., Demurger, F., Odent, S., Pasquier, L., Cavé, H., and Marcorelles, P. (2017). Fetal costello syndrome with neuromuscular spindles excess and p.Gly12Val HRAS mutation. Eur. J. Med. Genet. *60*, 395–398.
47. Carta, C., Pantaleoni, F., Bocchinfuso, G., Stella, L., Vasta, I., Sarkozy, A., Digilio, C., Palleschi, A., Pizzuti, A., Grammatico, P., et al. (2006). Germline missense mutations affecting KRAS Isoform B are associated with a severe Noonan syndrome phenotype. Am. J. Hum. Genet. *79*, 129–135.
48. Gripp, K.W., Hopkins, E., Sol-Church, K., Stabley, D.L., Axelrad, M.E., Doyle, D., Dobyns, W.B., Hudson, C., Johnson, J., Tenconi, R., et al. (2011). Phenotypic analysis of individuals with Costello syndrome due to HRAS p.G13C. Am. J. Med. Genet. A. *155A*, 706–716.
49. Gripp, K.W., Sol-Church, K., Smpokou, P., Graham, G.E., Stevenson, D.A., Hanson, H., Viskochil, D.H., Baker, L.C., Russo, B., Gardner, N., et al. (2015). An attenuated phenotype of Costello syndrome in three unrelated individuals with a *HRAS* c.179G>A (p.Gly60Asp) mutation correlates with uncommon functional consequences. Am. J. Med. Genet. A. *167A*, 2085–2097.
50. Bertola, D., Buscarilli, M., Stabley, D.L., Baker, L., Doyle, D., Bartholomew, D.W., Sol-Church, K., and Gripp, K.W. (2017). Phenotypic spectrum of Costello syndrome individuals harboring the rare *HRAS* mutation p.Gly13Asp. Am. J. Med. Genet. A. *173*, 1309–1318.
51. Pantaleoni, F., Lev, D., Cirstea, I.C., Motta, M., Lepri, F.R., Bottero, L., Cecchetti, S., Linger, I., Paolacci, S., Flex, E., et al. (2017). Aberrant HRAS transcript processing underlies a distinctive phenotype within the RASopathy clinical spectrum. Hum. Mutat. *38*, 798–804.
52. Altmüller, F., Lissewski, C., Bertola, D., Flex, E., Stark, Z., Spranger, S., Baynam, G., Buscarilli, M., Dyack, S., Gillis, J., et al. (2017). Genotype and phenotype spectrum of NRAS germline variants. Eur. J. Hum. Genet. *25*, 823–831.
53. Stark, Z., Gillessen-Kaesbach, G., Ryan, M.M., Cirstea, I.C., Gremer, L., Ahmadian, M.R., Savarirayan, R., and Zenker, M. (2012). Two novel germline KRAS mutations: expanding the molecular and clinical phenotype. Clin. Genet. *81*, 590–594.
54. Nussinov, R., Zhang, M., Tsai, C.J., Liao, T.J., Fushman, D., and Jang, H. (2018). Autoinhibition in Ras effectors Raf, PI3Kα, and RASSF5: a comprehensive review underscoring the challenges in pharmacological intervention. Biophys. Rev. *10*, 1263–1282.
55. Graham, S.M., Cox, A.D., Drivas, G., Rush, M.G., D'Eustachio, P., and Der, C.J. (1994). Aberrant function of the Ras-related protein TC21/R-Ras2 triggers malignant transformation. Mol. Cell. Biol. *14*, 4108–4115.
56. Niihori, T., Nagai, K., Fujita, A., Ohashi, H., Okamoto, N., Okada, S., Harada, A., Kihara, H., Arbogast, T., Funayama, R., et al. (2019). Germline activating *RRAS2* mutations cause Noonan syndrome. Am. J. Hum. Genet. *104*, this issue, 1233–1240.

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Supplemental Data

Activating Mutations of *RRAS2* Are a Rare Cause of Noonan Syndrome

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SUPPLEMENTAL NOTE: CASE REPORTS

Subject 1 (Figure 1A)

He is the second child of six from a non-consanguineous couple of Algerian descent. During pregnancy, first trimester transient cystic hygroma was observed and third trimester was complicated by polyhydramnios. The prenatal karyotype was normal, 46,XY. At birth after 35 weeks of gestation, birth parameters were increased for gestational age: birth weight 3,730 g (+2.3 SD), length 50.5 cm (+1.4 SD) and head circumference 37 cm (+3.0 SD). He had stridor during the first weeks of life, feeding difficulties, gastroesophageal reflux and failure to thrive in the first months but those symptoms improved spontaneously. Psychomotor development was normal. He was able to walk alone at 13 months, had no speech delay and attended a normal school. He had one episode of febrile seizure in infancy. Supravalvular aortic stenosis was discovered at age 2 years and required cardiac surgery when he was 7.5 years old. He had bilateral ptosis and was prescribed glasses for myopia.

At last examination (7 years and 11 months), his growth parameters were within the normal ranges: his height was 125.5 cm (-0.3 SD), his weight was 27.5 kg (+0.5 SD) and his OFC was 54 cm (+1.2 SD). He had facial features suggestive of NS (i.e., thick low set ears, bilateral ptosis, down slanted palpebral fissures, telecanthus and a deeply grooved philtrum). He also had a glabellar nevus flammeus. Both testes were palpable. The remainder of his examination was unremarkable.

Subject 2 (Figure 1B,C)

He is the second child of four from a non-consanguineous couple of Sri Lankan descent. During pregnancy, third trimester polyhydramnios was observed, otherwise ultrasounds were normal. Prenatal karyotype was normal, 46,XY. Birth weight, length and head circumference at 35 weeks of gestation were 3,180 g (+1.1 SD), 47.5 cm (-0.2 SD), and 35 cm (+1.6 SD), respectively. A RASopathy was suspected in the neonatal period. He had bilateral inguinal hernia. Feeding difficulties, gastroesophageal reflux and failure to thrive required G-tube feeding by the age of 10 months. He had gingival hypertrophy and delayed tooth eruption. A small ventricular septal defect was detected at birth without any consequence on cardiac function. He had strabismus and myopia, Chiari type 1 malformation, bilateral ventricular dilatation (Figure 1C) and macrocephaly. At age 2.5 years, his height was 77.5 cm (-3.8 SD) and growth hormone deficiency was discovered;

under substitutive treatment with growth hormone (started at 3,5 years), his height increased from -3.3 SD to normal values.

Psychomotor development was slightly delayed: he was able to walk alone at 18 months and first words appeared at 24 months. He attended a regular school until the age of 10, and then entered special education school. At the age of 12 years and 2 months, his height was 139.5 cm (-1.5 SD), weight was 32.5 kg (-1.4 SD), and OFC was 57.5 cm (+2.5 SD). CGH array analysis provided normal results.

Family 3 (Figure 1D-F)

The pedigree of this family is presented in Figure 1D. The cousins III-1 and III-2 were referred for genetic evaluation because of suspected Noonan syndrome. III-1 was born at term with a birth weight of 3110 g (-0.8 SD), body length 48 cm (-1.6 SD), and OFC 36 cm (+0.9 SD). She did not have any major malformations. Her motor development was mildly delayed; she started walking at 20 months. She had atopic dermatitis and curly hair in infancy. She also developed multiple allergies and chronic bronchitis. She underwent surgery for ocular ptosis. Her height at the age of 22 months was in the low normal range (81.5 cm; -1.1 SD), her weight was 12 kg (+0.3 SD) and her OFC 50 cm (+1.6 SD). She was noted to have a broad forehead, hypertelorism, ocular ptosis and low set ears. Upon re-evaluation at age 7.1 years she was short statured (height 108 cm; -3.0 SD); her weight was 18.6 kg (-1.8 SD) and her OFC 52 cm (+0.4 SD). She was attending a regular school with minor extra support and was reported to have a reduced attention span. Permission to publish facial photographs was not given, but photos were evaluated by a clinical expert in RASopathies (M.Z.) and confirmed to display typical features of NS.

Her mother II-1 had NS-like facial features more obvious at younger age (Fig. 1E). She had a normal development and her adult height was in the low normal range (160 cm; -1.3 SD). She has a duplicated kidney and a ventricular septal defect that had to be corrected by heart surgery in childhood.

III-2 was born prematurely (due to maternal uterus bicornis) after 35 weeks of gestation with a birth weight of 2440 g (-0.6 SD), body length 48 cm (0 SD), and OFC 32 cm (-0.8 SD). He did not have any major malformations. His motor development was normal. His growth was mildly delayed; at the age of 19 months his height was 78 cm (-1.8 SD), while weight (11 kg) and OFC (49 cm) were within the normal ranges (-0.4 and +0.2 SD, respectively). Apart from a hydrocele testis and inguinal hernia that had to be surgically corrected at the age of 3 years, there are no medical issues. His facial gestalt was strongly suggestive of NS (permission to publish facial photographs was not given). The boy's

mother II-2 had NS-like facial anomalies especially in childhood (Fig. 1F) that dissolved with age. She reported a heart murmur in childhood, but no cardiac abnormality was noted at a recent cardiology exam. She had normal growth, mild pubertal delay (menarche at age 16), and normal intellectual development. Her adult body measurements were normal: height 170 cm (+0.3 SD), weight (59 kg; +0.1 SD), and OFC 55.5 cm (+0.2 SD). The grandfather I-1 was not personally examined, but photographs documented a typical facial appearance of NS with broad forehead, hypertelorism, down-slanting palpebral fissures, palpebral ptosis, and low-set ears. His adult height was reported to be around 172 cm (-1.3 SD). No cardiac issues were known.

Subject 4

A 36 year-old prima gravida female and her 33 year-old non-consanguineous partner, both of Indian descent, were seen in Clinical Genetics in Mississauga, Canada at 20 weeks and 1 day gestation for prenatal findings of bilateral ventriculomegaly (10 mm and 11 mm, respectively), increased nuchal fold of 7 mm and abnormalities of the right outflow tract with only 2 great vessels identified. Fetal echocardiogram confirmed Tetralogy of Fallot. Amniocentesis was done and revealed a normal male array CGH. DNA was banked from amniocytes. The pregnancy was complicated by polyhydramnios requiring amnioreduction. Fetal MRI at 26 weeks gestation showed macrocephaly, markedly enlarged extra-axial CSF spaces, non-obstructive hydrocephalus, labyrinth dysplasia and borderline lateral ventricles. The male proband was born at 33 weeks and 3 days via caesarean section due to maternal pre-eclampsia. Birth weight was 2400 g (+0.4 SD). On physical examination, this baby had dysmorphic facial features (i.e., macrocephaly, low posterior hairline, small downslanting palpebral fissures, low-set posteriorly rotated ears, broad nasal root, micrognathia). He had a wrist flexion contracture, proximal placement of the thumb, hypoplastic toenails, small scrotum and an anteriorly-placed anus. Investigations revealed pulmonary edema, 11 pairs of ribs, abnormal renal morphology, spinal canal stenosis and thrombocytopenia. Diagnostic imaging confirmed the Tetralogy of Fallot and the prenatal brain findings. The baby remained critically ill and was unable to be weaned from the ventilator and as a result, the parents decided to proceed with palliation. The baby died at 2 weeks of age. Facial photographs are not available.

Subject 5

The female subject was born after uneventful delivery to a non-consanguineous couple from Serbia. During pregnancy an increased fetal nuchal fold was noted. Prenatal karyotyping was done with a normal result, 46,XX. Birth measurements at term were

normal (weight 3,050 g, -0.7 SD; length 50 cm, -0.1 SD; head circumference 35 cm, +0.3 SD). A congenital heart defect was diagnosed postnatally consisting of an atrioventricular septal defect with additional multiple ventricular septal defects. The baby developed heart failure and was treated by pulmonary artery banding in infancy. Complete surgical correction of the heart malformation has been postponed, so far. The patient complained of frequent headaches and therefore a brain MRI was performed, which showed only a minor hippocampal anomalies (bilateral mild decrease in volume of posterior parts of the hippocampus). At the age of 8 years her height was 122 cm (-2.1 SD) and her weight was 22 kg (-1.9 SD). She had relative macrocephaly (OFC 52.5 cm, +0.2 SD). She displayed pectus excavatum and craniofacial featured suggestive of NS (mild hypertelorism, downslanting palpebral fissures, and low set ears). Her motor, speech and cognitive development were normal and she also had normal vision and hearing. No family members were clinically affected.

Subject 6

He was born at 35 weeks gestation to a G1P0 mother by c-section due to maternal fever and nonreassuring fetal heart rate. Ultrasounds demonstrated polyhydramnios and large for gestational age parameters since 30 weeks. Birth weight 3.6 kg (+2.1 SD), length 51 cm (+1.4 SD), head circumference 38 cm (+2.7 SD). At 8 months his length was at +0.4 SD and his head was 50 cm (+4.2 SD). Over the first 18 months his length remained in the average range but head circumference climbed to 54.5 cm (+5.0 SD).

Dysmorphic facial features were apparent at birth, and he was noted to have a wide fontanelle, downslanting palpebral fissures, broad nasal bridge, low set and posteriorly rotated ears. At 6 months, he was noted to have a glabellar nevus flammeus, epicanthal folds and downslanting palpebral fissures. Inner canthal distance was 2.9 cm (+2.0 SD) and outer canthal distance 8.4 cm (+2.2 SD). Ears were borderline low-set. Neck was not webbed. On his chest exam the left nipple was fuller than the right and the right nipple angled downward. At 22 months, his anterior fontanelle was still open around 3 cm.

At 2 weeks he was noted to have moderate appendicular hypotonia, proximal muscle weakness and head lag. During his neonatal hospitalization, neurology was consulted for bilateral tonic upper extremity extension/flexion/posturing. At 3 months he had axial hypotonia but no further abnormal movements. At 6 months he was progressing but still with hypotonia noted. He was late with rolling over. At 8 months he started sitting alone. Esotropia was noted and he was diagnosed with strabismic amblyopia for which he was prescribed patching. No structural anomalies were noted by ophthalmologic exam. At 22

months he could crawl well, pull to stand, walk with assistance, speak 3 words and make word approximations for many more words.

Head ultrasound in the neonatal period was unremarkable with upper limit of normal lateral and third ventricles. At 6 months, a repeat head ultrasound showed very mild ventriculomegaly. Metabolic testing was non-diagnostic and chromosomal microarray demonstrated a duplication of 855 kb at Xp21.1 of uncertain significance including the 5' UTR region and exon 1 of the DMD gene, which is associated with either Duchenne or Becker muscular dystrophy. Blood creatine kinase level was non-elevated. A diagnostic RASopathy panel was done due to suspicion for Noonan syndrome and was negative. Targeted *PTEN* testing was negative. Permission to publish facial photographs was not given by parents, but images could be evaluated by a clinical expert in RASopathies (M.Z.) confirming facial anomalies typical of a RASopathy.

SUPPLEMENTAL FIGURES

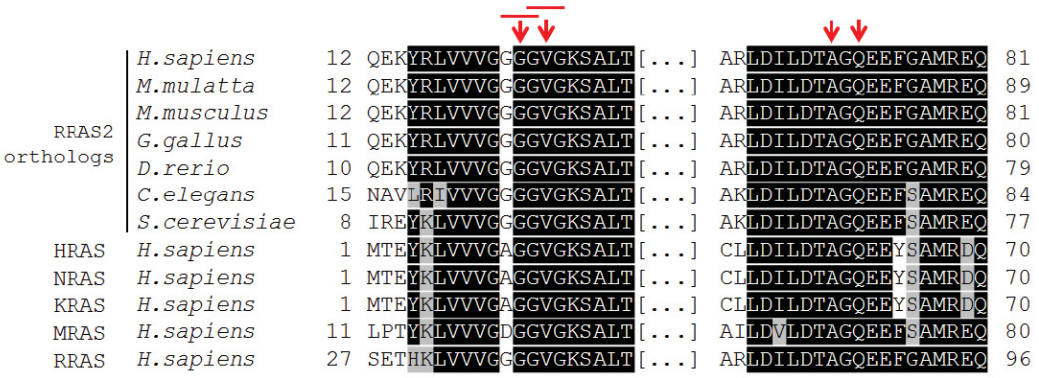

Figure S1. Multiple alignment of RRAS2 orthologs and human paralogs. Invariant residues among proteins are highlighted in black. Amino acids affected by the identified Noonan syndrome-causing missense mutations are indicated (arrows). The red bars encompass the residues implicated in the identified disease-causing in-frame duplications. Gray and white background indicate residues with high (>0.5, Gonnet PAM 250 matrix) and poor (≤0.5, Gonnet PAM 250 matrix) degree of conservation, respectively. Alignment was generated using MUSCLE v3.8. RRAS2: *H.sapiens*, NP_036382.2; *M.mulatta*, XP_002799673.1; *M.musculus*, NP_080122.2; *G.gallus*, NP_001006466.1; *D.rerio*, NP_001017815.1; *C.elegans*, NP_496623.1; *S.cerevisiae*, NP_014301.1; HRAS: *H.sapiens*, NP_005334.1; NRAS: *H.sapiens*, NP_002515.1; KRAS: *H.sapiens*, NP_203524.1; MRAS: *H.sapiens*, NP_001078518.1; RRAS: *H.sapiens*, NP_006261.1

SUPPLEMENTAL TABLES

Table S1. Missense variants in RRAS2 and analogous mutations seen in other RAS superfamily members.

p.Gly23Val

	RRAS2	KRAS	HRAS	NRAS	RRAS	MRAS
Specific variant	Gly23Val	Gly12Val	Gly12Val	Gly12Val	Gly38Val	Gly22Val
ClinVar ID	-	12583	12600, 279921	40470	-	-
ClinVar Classification	-	PATH	PATH	PATH	-	-
NSEuroNet count	-	-	CS (6)	-	-	-
Germline phenotype[1]	NS (this report)	NS spectrum	NP, CS, severe CS, CMEMS	NP	-	-
Mosaic phenotype	-	NS(S), cerebral AVM	NS(S), KEN(S)	-	-	-
Reference	-	ClinVar, [1]	ClinVar, [1]	-	-	-
COSMIC ID	COSM5749115	COSM520	COSM483	COSM566	-	-
FATHMM	0.38 (NEUTRAL)	0.98 (PATH)	0.99 (PATH)	0.92 (PATH)	-	-
Count	2	10321	261	124	-	-
Somatic phenotype[2]	GT, Te	C, P, L	UT, T, S	H, C	-	-
Variant[3]	Gly23Asp	Gly12Asp	Gly12Asp	Gly12Asp	Gly38Asp	Gly22Asp
ClinVar ID	-	12582	12612, 180854	39648	-	-
ClinVar Classification	-	PATH	PATH	PATH	-	-
NSEuroNet count	-	-	CS (5)	NS (1)	-	-
Germline phenotype[1]	-	RASopathy	CS, RASopathy	NP	-	-
Mosaic phenotype	-	NS(S), KEN(S), cerebral AVM	NS(S)	KEN(S)	-	-
Reference	-	ClinVar; [1, 2]	ClinVar, [1]	[1]	-	-
COSMIC ID	COSM6989585	COSM521	COSM484	COSM564	-	-
FATHMM	0.32 (NEUTRAL)	0.98 (PATH)	0.99 (PATH)	0.91 (PATH)	-	-
Count	1	15078	93	865	-	-
Somatic phenotype[2]	Oe	C, P, L	S, UT	H	-	-

Variant[3]	Gly23Cys	Gly12Cys	Gly12Cys	Gly12Cys	Gly38Cys	Gly22Cys
ClinVar ID	-	12578	12613	40468	-	-
ClinVar Classification	-	PATH/LPATH	PATH	PATH	-	-
NSEuroNet count	-	RASopathy (1)	CS (15)	-	-	-
Germline phenotype[1]	-	NO	CS, RASopathy	NP		
Mosaic phenotype	-	-	NS(S), KEN(S)	-	-	-
Reference	-	-	ClinVar, [1]	-	-	-
COSMIC ID	COSM5749961	COSM516	COSM481	COSM562	-	-
FATHMM	0.43 (NEUTRAL)	0.98 (PATH)	0.99 (PATH)	0.92	-	-
Count	1	5116	39	183	-	-
Somatic phenotype[2]	GT	L, C, P	S, UT	H, C	-	-
Variant[3]	Gly23Ala	Gly12Ala	Gly12Ala	Gly12Ala	Gly38Ala	Gly22Ala
ClinVar ID	-	45122	12603, 40430	219097	-	-
ClinVar Classification	-	PATH/LPATH	PATH	PATH	-	-
NSEuroNet count	-	-	CS (37)	-	-	-
Germline phenotype[1]	-	NO	CS	NP	-	-
Mosaic phenotype	-	PKK	NS(S), KEN(S)	-	-	-
Reference	-	[3]	ClinVar	-	-	-
COSMIC ID	COSM6998029	COSM522	COSM485	COSM565	-	-
FATHMM	0.39 (NEUTRAL)	0.98 (PATH)	0.99 (PATH)	0.93 (PATH)	-	-
Count	1	2398	13	119	-	-
Somatic phenotype[2]	H	C, L, P	T, B	H, S	-	-
Variant[3]	Gly23Ser	Gly12Ser	Gly12Ser	Gly12Ser	Gly38Ser	Gly22Ser
ClinVar ID	-	12584	12602	177778	-	-
ClinVar Classification	-	PATH	PATH	PATH	-	-
NSEuroNet count	-	CFC (2)	CS (520)	NS (2)	-	-
Germline phenotype[1]	-	NO	CS, RASopathy, CMEMS	RASopathy	-	-
Mosaic phenotype	-	-	NS(S), KEN(S), WHN	-	-	-
Reference	-	-	ClinVar, [1]	-	-	-
COSMIC ID	COSM6865195	COSM516	COSM480	COSM563	-	-
FATHMM	0.40 (NEUTRAL)	0.98 (PATH)	0.99 (PATH)	0.91 (PATH)	-	-
Count	1	1998	104	269	-	-
Somatic phenotype[2]	Pr	C, L, P	UAT	H, UAT	-	-

Variant[3]	Gly23Arg	Gly12Arg	Gly12Arg	Gly12Arg	Gly38Arg	Gly22Arg
ClinVar ID	-	12579	375961	40469	-	-
ClinVar Classification	-	PATH	PATH/LPATH	PATH	-	-
NSEuroNet count	-	-	-	CS (1)	-	-
Germline phenotype[1]	-	NO	NO	NP	-	-
Mosaic phenotype	-	-	-	-	-	-
Reference	-	-	-	-	-	-
COSMIC ID	-	COSM483	COSM482	COSM561	-	COSM5937099
FATHMM	-	0.98 (PATH)	0.99 (PATH)	0.93 (PATH)	-	0.98 (PATH)
Count	-	1468	16	42	-	1
Somatic phenotype[2]	-	P, C, L	SG, T, Br	H, S	-	S

Legend to Tables S1:
[1]phenotype classification according to ClinVar and NSEuroNet
[2]most frequently associated tumors/affected tissues are listed (order according to frequency)
[3]other observed variants at this position
Abbreviations: AVM, arteriovenous malformation; B, breast cancer; Br, Brain tumor; C, colon cancer; CFC, CFC syndrome; CMEMS, congenital myopathy with excess of muscle spindles; CMN, congenital melanocytic nevus / neurocutaneous melanosis; CS, Costello syndrome; CSHS, Cutaneous-skeletal hypophosphatemia syndrome; E, endometrium cancer; G, gastric cancer; GT, Genital tract malignancies; H, hematopoetic and lymphoid malignancies; KEN(S), keratinocytic epidermal nevus (syndrome); L, lung cancer; NS, Noonan syndrome; NSCMN, Nevus spilus-type congenital melanocytic nevus; NS(S), nevus sebaceous (syndrome) / Schimmelpenning syndrome; NO, not observed; NP: phenotype not provided for an observed variant; O, ovarian cancer; Oe, oesophageal cancer; P, pancreatic cancer; PKK, phakomatosis pigmentokeratotica; Pr, Prostate cancer; S, skin cancer; SG, salivary gland cancer; T, thyroid cancer; Te, testicular malignancies; UAT, upper aerodigestive tract; UT, urinary tract; WHN, wooly hair nevus.
References:
1. Boppudi S, Bögershausen N, Hove HB, Percin EF, Aslan D, Dvorsky R, Kayhan G, Li Y, Cursiefen C, Tantcheva-Poor I, et al. (2016). Specific mosaic KRAS mutations affecting codon 146 cause oculoectodermal syndrome and encephalocraniocutaneous lipomatosis. Clin. Genet. *90*, 334-42. (and literature cited herein)
2. Nagatsuma M, Takasawa K, Yamauchi T, Nakagawa R, Mizuno T, Tanaka E, Yamamoto K, Uemura N, Kashimada K, Morio T. (2019). A postzygotic KRAS mutation in a patient with Schimmelpenning syndrome presenting with lipomatosis, renovascular hypertension, and diabetes mellitus. J. Hum. Genet. *64*, 177-181.
3. Om A, Cathey SS, Gathings RM, Hudspeth M, Lee JA, Marzolf S, Wine Lee L. (2017). Phacomatosis Pigmentokeratotica: A Mosaic RASopathy with Malignant Potential. Pediatr. Dermatol. *34*, 352-355.
4. Park PG, Park E, Hyun HS, Kang HG, Ha IS, Cho TJ, Ko JM, Cheong HI. (2018). Cutaneous Skeletal Hypophosphatemia Syndrome in Association with a Mosaic HRAS Mutation. Ann. Clin. Lab. Sci. *48*, 665-669.

p.Ala70Thr

	RRAS2	KRAS	HRAS	NRAS	RRAS	MRAS
Specific variant	Ala70Thr	Ala59Thr	Ala59Thr	Ala59Thr	Ala85Thr	Ala69Thr
ClinVar ID	-	12581	40435	40473	-	-
ClinVar Classification	-	PATH/LPATH	LPATH	LPATH	-	-
NSEuroNet count	-	-	-	-	-	-
Germline phenotype[1]	NS (this report)	NO	CS	NP	-	-
COSMIC ID	COSM3719717	COSM546	COSM495	COSM578	-	-
FATHMM	0.98	0.98	0.97	0.99	-	-
Count	5	29	7	8	-	-
Somatic phenotype[2]	G, H, GT	C, G, SG	Oe, C	P, H	-	-
Variant[3]	Ala70Gly	Ala59Gly	Ala59Gly	Ala59Gly	Ala85Gly	Ala69Gly
ClinVar ID	-	-	-	-	-	-
ClinVar Classification	-	-	-	-	-	-
NSEuroNet count	-	-	-	-	-	-
Germline phenotype[1]	-	-	-	-	-	-
COSMIC ID	-	COSM28518	-	COSM5878737	-	-
FATHMM	-	0.99	-	0.99	-	-
Count	-	10	-	1	-	-
Somatic phenotype[2]	-	H, C, P	-	H	-	-
Variant[3]	Ala70Glu	Ala59Glu	Ala59Glu	Ala59Glu	Ala85Glu	Ala69Glu
ClinVar ID	-	-	-	-	-	-
ClinVar Classification	-	-	-	-	-	-
NSEuroNet count	-	-	-	-	-	-
Germline phenotype[1]	-	-	-	-	-	-
COSMIC ID	-	COSM547	-	-	-	-
FATHMM	-	0.99	-	-	-	-
Count	-	8	-	-	-	-
Somatic phenotype[2]	-	C, H	-	-	-	-

Variant[3]	Ala70Ser	Ala59Ser	Ala59Ser	Ala59Ser	Ala85Ser	Ala69Ser
ClinVar ID	-	222075				
ClinVar Classification	-	LPATH	-	-	-	-
NSEuroNet count	-	-	-	-	-	-
Germline phenotype[1]	-	NP	-	-	-	-
COSMIC ID	-	COSM1235389		COSM5351687	-	-
FATHMM	-	0.98		0.99 (PATH)	-	-
Count	-	2		1	-	-
Somatic phenotype[2]	-	C		H	-	-
Variant[3]	**Ala70Val**	**Ala59Val**	**Ala59Val**	**Ala59Val**	**Ala85Val**	**Ala69Val**
ClinVar ID	-	132969	-	-	-	-
ClinVar Classification	-	NP	-	-	-	-
NSEuroNet count	-	-	-	-	-	-
Germline phenotype[1]	-	NO	-	-	-	-
COSMIC ID	-	COSM6362494	-	-	COSM2752954	-
FATHMM	-	0.99 (PATH)	-	-	0.97 (PATH)	-
Count	-	2	-	-	1	-
Somatic phenotype[2]	-	T	-	-	C	-
Variant[3]	**Ala70Asp**	**Ala59Asp**	**Ala59Asp**	**Ala59Asp**	**Ala85Asp**	**Ala69Asp**
ClinVar ID	-	-	-	-	-	-
ClinVar Classification	-	-	-	-	-	-
NSEuroNet count	-	-	-	-	-	-
Germline phenotype[1]	-	-	-	-	-	-
COSMIC ID	-	-	COSM5991568	COSM253327	-	-
FATHMM	-	-	0.99 (PATH)	0.99 (PATH)	-	-
Count	-	-	1	5	-	-
Somatic phenotype[2]	-	-	S	S	-	-
Variant[3]	**Ala70Leu**	**Ala59Leu**	**Ala59Leu**	**Ala59Leu**	**Ala85Leu**	**Ala69Leu**
ClinVar ID	-	-	179260	-	-	-
ClinVar Classification	-	-	LPATH	-	-	-
NSEuroNet count	-	-	CS (1)	-	-	-
Germline phenotype[1]	-	-	CS	-	-	-
COSMIC ID	-	-	-	-	-	-
FATHMM	-	-	-	-	-	-
Count	-	-	-	-	-	-
Somatic phenotype[2]	-	-	-	-	-	-

Mosaic phenotype: no RASopathy mosaic phenotype has been reported for mutations at this codon in any gene of the RAS superfamily.

p. Gln72Leu

	RRAS2	KRAS	HRAS	NRAS	RRAS	MRAS
Specific variant	Gln72Leu	Gln61Leu	Gln61Leu	Gln61Leu	Gln87Leu	Gln71Leu
ClinVar ID	9447	45116	376033	375874	-	-
ClinVar Classification	PATH	PATH	LPATH	PATH/LPATH	-	-
NSEuroNet count	-	-	-	-	NS (1)	-
Germline phenotype[1]	Severe RASopathy (this report)	NO	NO	NO		-
Mosaic phenotype	-	-	KEN(S)	NSCMN	-	-
Reference	-	-	[1]	[1]	-	-
COSMIC ID	COSM687135	COSM553	COSM498, 4169862, 52978	COSM583	-	-
FATHMM	0.98	0.98	0.98	0.99	-	-
Count	6	130	206	329	-	-
Somatic phenotype[2]		C, L, P	S, UT	C, L, P	-	-
Variant[3]	Gln72His	Gln61His	Gln61His	Gln61His	Gln87His	Gln71His
ClinVar ID	-	45117, 177881	376318, 376319	373003	-	-
ClinVar Classification	-	PATH/LPATH	LPATH	PATH	-	-
NSEuroNet count	-	-	-	-	-	-
Germline phenotype[1]	NS	NO	NS	NP	-	-
Mosaic phenotype	-	-	-	NSCMN	-	-
Reference	-	-	-	[1]	-	-
COSMIC ID	COSM925280, 925279	COSM554, 555	COSM502, 503	COSM585, 586	-	
FATHMM	0.94	0.93	n/a	0.92/ 0.93	-	-
Count	3	398	22	213	-	-
Somatic phenotype[2]	E, UAT	C, P, H	S, T	H, S	-	-

Variant[3]	Gln72Arg	Gln61Arg	Gln61Arg	Gln61Arg	Gln87Arg	Gln71Arg
ClinVar ID	-	45115	160364, 376320, 376321	13900	-	-
ClinVar Classification	-	PATH	LPATH	PATH	-	-
NSEuroNet count	-	-	-	-	-	-
Germline phenotype[1]	-	NS	NP	NP	-	-
Mosaic phenotype	-	-	KEN(S), PKK, CSHS	CMN, NS(S), CSHS, KEN(S)	-	-
Reference	-	-	ClinVar; [1,4]	ClinVar, [1]	-	-
COSMIC ID	-	COSM552	COSM499, 3736923	COSM584,579, 33693, 28048	-	-
FATHMM	-	0.98	0.97	0.99	-	-
Count	-	150	310, 72	1,690/8/2/405	-	-
Somatic phenotype[2]	-	T, P, C	T, UT, AG	S, T, H, U	-	-
Variant[3]	Gln72Lys	Gln61Lys	Gln61Lys	Gln61Lys	Gln87Lys	Gln71Lys
ClinVar ID	-	177777	12601	73058	-	-
ClinVar Classification	-	PATH	PATH/LPATH	VUS	-	-
NSEuroNet count	-	-	-	-	-	-
Germline phenotype[1]	-	NO	NS	Rasopathy	-	-
Mosaic phenotype	-	-	-	CMN	-	-
Reference	-	-	-	ClinVar, [1]	-	-
COSMIC ID	COSM6560654	COSM549	COSM496, 949379	COSM580, 28049, 12730, 53223	-	-
FATHMM	0.99	0.99	0.99	0.99	-	-
Count	1	59	165	1550	-	-
Somatic phenotype[2]	B	C, L, T	T, S	S, H, T	-	-

Variant[3]	Gln72Pro	Gln61Pro	Gln61Pro	Gln61Pro	Gln87Pro	Gln71Pro
ClinVar ID	-	375966	376322	280409	-	-
ClinVar Classification	-	PATH/LPATH	LPATH	PATH	-	-
NSEuroNet count	-	-	-	-	-	-
Germline phenotype[1]	-	NO	NO	NP	-	-
Mosaic phenotype	-	-	-	-	-	-
Reference	-	-	-	-	-	-
COSMIC ID	-	COSM551	COSM500	COSM582	-	-
FATHMM	-	0.99	0.98	0.99	-	-
Count	-	22	3	43	-	-
Somatic phenotype[2]	-	H, T, C	T	H	-	-
Variant[3]	Gln72Glu	Gln61Glu	Gln61Glu	Gln61Glu	Gln87Glu	Gln71Glu
ClinVar ID	-	376324	376444	375875	-	-
ClinVar Classification	-	LPATH	LPATH	PATH	-	-
NSEuroNet count	-	-	-	-	-	-
Germline phenotype[1]	-	NO	NO	NO	-	-
Mosaic phenotype	-	-	-		-	-
Reference	-	-	-	-	-	-
COSMIC ID	-	COSM483	COSM497	COSM581	-	-
FATHMM	-	0.99	0.99	0.99	-	-
Count	-	261	1	10	-	-
Somatic phenotype[2]	-	UT, T, S	GT	H, UAT	-	-

Table S2. *In silico* prediction of functional effects and presence in databases for the observed *RRAS2* variants.

	c.68G>T p.(Gly23Val)	c.65_73dup p.(Gly22_Gly24dup)	c.70_78dup, p.Gly24_Gly26dup	c.208G>A p.(Ala70Thr)	c.215A>T p.(Gln72Leu)
dbSNP	no entry	no entry	no entry	no entry	rs113954997
ExAC	no entry	no entry	no entry	1/121368 (0.000008239)	no entry
gnomAD	no entry	no entry	no entry	2/251096 (0.000007965)	no entry
Mutation Taster	disease causing (0.999999947459)	polymorphism (0.999973756014)	polymorphism (0.999999898998)	disease causing (0.999999999934)	disease causing (0.999999999994)
PolyPhen-2	probably_damaging (1.000)	n.a.	n.a.	probably_damaging (0.999)	possibly_damaging (0.946)
MutPred2	0.882	n.a.	n.a.	0.776	0.915
Mutation Assessor	medium (3.285)	n.a.	n.a.	medium (3.22)	medium (3.215)
CADD PHRED score	23.6	17.4	17.69	26.7	28.4
PROVEAN score	-8.247	-11.113	-11.106	-3.940	-6.834

RRAS2 reference: NM_012250.6 (ENST00000256196.8)
n.a. – not available
dbSNP (https://www.ncbi.nlm.nih.gov/snp)
ExAC (http://exac.broadinstitute.org/)
gnomAD (https://gnomad.broadinstitute.org/)
MutationTaster (http://www.mutationtaster.org/): The probability value is the probability of the prediction, i.e. a value close to 1 indicates a high 'security' of the prediction.
PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/): PolyPhen-2 calculates the naive Bayes posterior probability that a given mutation is damaging and reports estimates of false positive (the chance that the mutation is classified as damaging when it is in fact nondamaging) and true positive (the chance that the mutation is classified as damaging when it is indeed damaging) rates.
MutPred2 (http://mutpred.mutdb.org/): a score threshold of 0.50 would suggest pathogenicity (a threshold of 0.68 yields a false positive rate (fpr) of 10% and that of 0.80 yields an fpr of 5%).
MutationAssessor (http://mutationassessor.org/r3/): Functional impact combined score (FI score), larger scores indicate more likely functional impact of a mutation. FI ≤ 0.8 neutral impact, 0.8 < FIS ≤ 1.9 low impact, 1.9 < FIS ≤ 3.5 medium impact, FIS > 3.5 high impact.
CADD PHRED score – GRCh37-v1.4 (https://cadd.gs.washington.edu/): PHRED-like (-10*log10(rank/total)) scaled C-score: ranking a variant relative to all possible substitutions of the human genome (8.6 × 10^9). A scaled C-score ≥10: variant is belongs to 10% most deleterious variants; C-score ≥20: variant belongs to 1% most deleterious variants; C-score ≥30: variant belongs to 0.1% most deleterious variants.
PROVEAN v1.1.3. (http://provean.jcvi.org/index.php): Variants with a score equal to or below -2.5 are considered "deleterious", Variants with a score above -2.5 are considered "neutral".

ONLINE METHODS

Subjects. Individuals were referred for diagnostic genetic testing because of a suspected RASopathy or unspecified disorder with multiple congenital anomalies. Four affected families were ascertained by members of the European Network on Noonan syndrome and Related Disorders (NSEuroNet), and two additional cases could be identified through successful GeneMatcher match (Sobreira *et al*. A matching tool for connecting investigators with an interest in the same gene. Hum. Mutat. 2015, *36*:928-30) Written informed consent for diagnostic genetic testing was obtained from patients and/or their parents according to national regulations, and specific written consent from subjects or their legal guardians was obtained for using clinical, genotypic and photographic data for scientific publication. The study was approved by the ethics committees of the Medical Faculty of the Otto-von-Guericke University Magdeburg and Ospedale Pediatrico Bambino Gesù.

Molecular analyses. For subjects 1 and 2, the mutational screening of genes implicated in RASopathies was performed at the French reference center for RASopathy testing, Robert Debré Hospital's molecular lab, Paris. The set of tested genes included the *PTPN11, CBL, SOS1, HRAS, KRAS, NRAS, SHOC2, RAF1, BRAF, MAP2K1, MAP2K2, RIT1, RRAS* and *RRAS2*. The custom targeted panel that is used there contains *RRAS2* gene, as a candidate RASopathy gene, since February 2016. For subjects 3-III-1 and 5, screening of known RASopathy genes and novel RAS-MAPK pathway-related candidates (including *RRAS2*) was performed by multigene panel sequencing carried out at the Institute of Human Genetics, University Hospital, Magdeburg. Enrichment of target genes was achieved using a Nextera Rapid Capture Custom Enrichment kit (Illumina) and an Illumina MiSeq System for sequencing with a minimum depth of 100x for all target regions. Family members of subject 3-III-1 were specifically sequenced for the respective *RRAS2* exon by conventional Sanger sequencing. In subjects 4 and 6, a trio-based WES was performed at GeneDx (Gaithersburg, MD) on banked amniocyte DNA and peripheral blood DNA, respectively. Target enrichment used the Clinical Research Exome kit (Agilent Technologies) or the IDT xGen Exome Research Panel v1.0, and sequenced using an Illumina HiSeq4000 sequencing system with 2×150-bp reads to a mean depth of coverage of 143X and 96X, respectively. Reads were aligned to GRCh37/UCSC hg19, and analyzed using GeneDx's XomeAnalyzer, which is a

custom-developed data analysis tool for variant annotation, filtering, and viewing (Retterer *et. al*. Clinical application of whole-exome sequencing across clinical indications. Genet. Med. 2016, *18*:696-704). For subject 4, heterozygosity for the *RRAS2* mutation (germline non-mosaic status) was additionally confirmed by multigene panel sequencing of amniocyte and peripheral blood DNA. Custom amplicon libraries (Paragon Genomics) were created targeting the following 21 genes: *ASXL1, CBL, DNMT3A, EZH2, GATA2, JAK3, KRAS, MAP2K1, NF1, NRAS, PTPN11, RAC2, RRAS, RRAS2, RUNX1, SAMD9, SAMD9L, SETBP1, SH2B3, SOS1, ZRSZ2*. Libraries were indexed and sequenced on an Illumina MiSeq platform resulting in an average coverage depth of 1017x. Reads were mapped to GRCh37/hg19, and variant calling was completed with VarScan2 and annotated with a custom in-house script. Only those variants with >3x coverage and >3% mutant allele frequency were considered. Finally, the entire *RRAS2* coding sequence was analyzed at the Genetics and Rare Diseases Research Division of the Ospedale Pediatrico Bambino Gesù, Rome, in 150 unrelated patients with clinical features fitting Noonan syndrome or overlapping this disorder, who had been tested negative for mutations in previously identified disease genes, using genomic DNA obtained from circulating leukocytes. A custom gene panel covering the coding exons of a set of candidate genes was designed with the Illumina Design Studio software. Library prep was performed with the amplicon-based TruSeq Custom Amplicon kit (Illumina), following manufacturer's instructions, and sequencing was carried out on a MiSeq sequencer (Illumina) with a 2×150 bp paired-end read protocol. Alignment and variant calling were performed with the MiSeq Reporter software (Illumina). VCF output files were annotated using Variant Studio v.2.2 (Illumina). All reported variants were visualized with the Integrative Genomics Viewer v.2.3 (IGV).

Structural modeling. Structural analysis was made using the homology model of RRAS2, which was obtained starting from the structure of MRAS-GppNHp (PDB ID: 3PIR) as a template, by using SWISS-MODEL. Structural impacts of investigated mutations were explored with the help of program PyMOL.

Constructs. The four NS-associated *RRAS2* mutations resulting in the p.Gly22_Gly24dup, p.Gly23Val, p.Ala70Thr and p.Gln72Leu amino acid changes were introduced by site-directed mutagenesis in an N-terminal Myc-tagged human *RRAS2* cDNA cloned in pcDNA3 vector. pGEX4T-1 vector was used for

overexpression of human *RRAS2* (WT and Ala70Thr), mouse RASGRF1 (residues 201-487) and human p120RASGAP (residues 713-1047), and pMal-c5X vector for overexpression of human CRAF RAS binding-domain (RBD) (residues 51-131), and RASSF5 RAS association (RA) domain (residues 200-358).

Proteins. All proteins, except CRAF RBD and RASSF5 RA, were isolated in a first step as glutathione S-transferase (GST) fusion proteins by affinity chromatography on a glutathione Sepharose column and purified in a second step by size exclusion chromatography after proteolytic cleavage of GST. CRAF RBD and RASSF5 RA were purified as his-tagged proteins. These proteins were isolated from the supernatant via Ni-NTA affinity purification. Nucleotide-free and fluorescent nucleotide-bound RRAS2 variants were prepared using alkaline phosphatase (Roche) and phosphodiesterase (Sigma Aldrich) at 4 °C. Fluorescent nucleotides were methylanthraniloyl (m-) labelled deoxyguanosine diphosphate (mdGDP), tetramethylrhodamine-conjugated GTP (tGTP), and guanosine 5'-β,γ-imidotriphosphate (mGppNHp); the latter is a non-hydrolyzable GTP analog. All proteins were analyzed by SDS-PAGE and stored at -80 °C.

Cell cultures. Human HEK 293T cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 1% l-glutamine and antibiotics.

Fluorescence measurements. The intrinsic and GEF-catalyzed nucleotide exchange reactions of mdGDP-bound RRAS2 proteins (0.2 µM) and 10 µM Cdc25Mm285 (the catalytic domain of mouse RASGRF1) were measured in Hellma Micro-cuvette 115F-QS (10 mm) and a buffer, containing 150 mM NaCl, 30 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, and 3 mM DTT at 20 °C using a luminescence spectrometer LS50B, and an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Observed rate constants (k_{obs}) were fitted single exponentially using the Grafit program (Erithacus software). Intrinsic GTP hydrolysis was measured in Hellma Micro-cuvette 115F-QS (10 mm) and a buffer, containing 30 mM Tris/HCl, pH 7.5, 10 mM K_2HPO_4/KH_2PO_4, 5 mM $MgCl_2$, 3 mM dithiothreitol at 25 °C using a luminescence spectrometer LS50B, and excitation wavelength of 543 nm and emission wavelength of 580 nm. GAP-stimulated reaction of tGTP-bound RRAS2 proteins (0.2 µM) was measured in the presence of the catalytic domain of p120 RASGAP (10 µM) in 30 mM Tris/HCl, pH 7.5, 10 mM K_2HPO_4/KH_2PO_4, 5 mM $MgCl_2$, 3 mM dithiothreitol at 25°C using a Hi-Tech Scientific (SF-61) stopped-flow

instrument. The excitation wavelength was 543 nm. Obtained data are averages of at least four independent measurements. The observed rate constants (k_{obs}) were fitted single exponentially using the Grafit program (Erithacus software). The interaction of mGppNHp-bound RRAS2 proteins (1 µM) with increasing concentration of the RAS-binding domain of CRAF and RA domain of RASSF5 effectors was measured in a buffer containing 30 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, and 3 mM DTT at 25°C using a Fluoromax 4 fluorimeter in polarization mode. Excitation wavelength was 360 nm and emission wavelength 450 nm. The dissociation constants (K_d) were calculated by fitting the concentration dependent binding curve using a quadratic ligand binding equation.

Confocal laser scanning microscopy (CLSM). HEK 293T cells were seeded at the density of 7 x 103 in 24-well cluster plates onto 12-mm cover glasses. After 24 hours of culture in complete medium, cells were transfected with the pcDNA3 constructs expressing wild-type RRAS2 or each of the four mutants. Twenty-four hours after transfection, cells were starved overnight, and fixed with 3% paraformaldehyde, permeabilized with 0.5 % Triton X-100, and stained with a mouse monoclonal anti-Myc (Cell Signaling), rabbit polyclonal anti-GM130 (Abcam) primary antibodies, rinsed twice with PBS, and incubated 1 h with specific secondary antibodies conjugated with Alexa Fluor 488 and 594. After staining, coverslips were extensively rinsed and then mounted on the microscope slide by using Vectashield with DAPI mounting medium (Vector Laboratories). CLSM observations were performed on a Leica TCS SP2 AOBS apparatus (Leica Microsystems), and images were acquired using a dedicated software (Leica).

ERK/AKT phosphorylation assays. HEK 293T cells were seeded in six-well plates the day before transfection. Monolayer were transfected at 70 to 80% confluency with Fugene 6 transfection reagent (Promega), with wild-type or mutant Myc-tagged RRAS2 expression constructs. At 12 h after transfection, cells were serum-starved for 18 h, and then treated with EGF (30 ng/ml, Invitrogen) for 15 minutes or left unstimulated. ERK activation status was assessed by immunoblotting using a mouse monoclonal anti-phospho-p44/42 ERK (Thr^{202}/Tyr^{204}) antibody (Cell Signaling), and levels of phosphorylated AKT were assessed using a rabbit polyclonal anti-phospho-AKT (Ser^{473}) antibody (Cell Signaling). To evaluate myc-RRAS2 protein levels, 20 µg of total lysates were immunoblotted with a mouse

monoclonal anti-Myc antibody (Cell Signaling). Membranes were re-probed with mouse monoclonal anti-GAPDH antibody (Santa Cruz) for protein normalization.

Supplement D: The binding selectivity of effectors for RAS proteins

The binding selectivity of effectors for RAS proteins*

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*Running title: RAS-Effector selectivity

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SUMMARY

RAS effectors specifically interact with the GTP-bound RAS proteins to link extracellular signals to downstream signaling pathways. Physical interactions of the effectors are achieved by two types of sequence domains, located in RAS binding (RB) and RAS association (RA) domains, which share common structural characteristics. Using database searches in the human proteome, we extracted 41 RA domains in 39 proteins and 16 RB domains in 14 proteins in human proteome, which can specifically select one of the 25 members in the RAS family. Most of the RA/RB domain containing proteins remained largely uncharacterized, although the molecular nature of RAS-effector interactions is well-studied for some proteins. Here, we comprehensively investigated the sequence-structure-function relationship between different representatives of the RAS family, including HRAS, RRAS, RALA, RAP1B, RAP2A, RHEB1 and RIT1, and all members of RA domain family proteins (RASSFs) and two RB domain-containing proteins, CRAF and TIAM1. The binding affinity for RAS-effector interactions, determined using fluorescence polarization, broadly range between high (0.3 μM) and very low (500 μM) affinities, which raised a central question about the relevance of highly variable binding affinities in the regulation of signaling events. Our study determined mainly two hotspots throughout the RA/RB domains from an average of 19 RAS-binding residues. Moreover, we found new interactions of RRAS1, RIT1 and RALA for RASSF7, RASSF9 and RASSF1, respectively, which were systematically and closely explored in a sequence-structure-property relationship analysis. Distinct functional properties and possible biological roles of these interactions remain to be investigated on the cellular context.

Keywords: Effectors; GTPase; NORE-1; protein interactions; RAS; RA domain; RAF kinase; RASSF; RASSF1; RASSF5; RAS association domain; RAS binding domain; RB domain; TIAM1

INTRODUCTION

RAS family proteins control activities of multiple signaling pathways and consequently a wide array of cellular processes, including survival, growth, adhesion, migration, and differentiation (1). Any dysregulation of these pathways leads, thus, to cancer, developmental disorders, metabolic, and cardiovascular diseases (2). Signal transduction implies a physical association of RAS proteins with and activation of a spectrum of functionally diverse downstream effectors, *e.g.*, CRAF, PI3Kα, TIAM1, RALGDS, PLCε and RASSF5 (3-11). RAS-effector interaction essentially requires RAS association with membranes (12), and its activation by specific regulatory proteins (*e.g.*, guanine nucleotide exchange factors or GEFs), leading to the formation of GTP-bound, active RAS (13-15). Notably, RAS proteins change their conformation mainly at two highly mobile regions, designated as switch I (residues 30-40) and switch II (residues 60-68) (16-18). Only in GTP-bound form, the switch regions of the RAS proteins provide a platform for the association of the effector proteins (19,20).

To date, two domains, the RAS binding (RB) and RAS association (RA) domains, have been defined for various effectors. They are comprised of 80-100 amino acids and have a similar ubiquitin-like topology (8,21-23). Considering different RAS effectors, RB and RA domain interactions with RAS proteins do not exhibit the same mode of interaction between different RAS effectors. However, CRAF RB and RALGDS RA domains share a similar structure and contact the switch I region *via* a similar binding mode (24,25). In contrast, PI3Kα RB, RASSF5 RA and PLCε RA domains do not share sequence and structural similarity but commonly associate with the switch regions, especially switch I (26-28). RAS-effector interaction strikingly shares a similar binding mode adopted by three components: Two antiparallel β-sheets of the RA/RB domains and the RAS switch I region, respectively, and the first α-helix of the RA/RB domains (29).

In this study, we conducted an in-depth database search in human proteome and extracted 57 RA/RB domains. We used 10 RASSF RA domains to analyze their interactions with 7 representatives of the RAS proteins family, including HRAS, RRAS1, RAP1B, RAP2A, RALA, RIT1 and RHEB1. CRAF and TIAM1 RB domains were used as controls. The binding analysis was performed under the same conditions using fluorescence polarization. Obtained dissociation constants (K_d) with a broad range (0.3 – 500 μM) along with a matrix

for a potential interaction of 25 RAS proteins and 57 RA/RB domains provide us a detailed view of the sequence-structure-property relationships of RAS-effector binding capabilities.

RESULTS

Human proteome contains 39 RA and 14 RB domain-containing proteins–Mining in the UniProt database led to the extraction of 130 RB and 145 RA domain-containing proteins, respectively. In a parallel search using HMMER, 127 RB and 164 RA domain-containing proteins were extracted. These numbers were reduced to 46 RB and 97 RA domain-containing proteins by excluding proteins containing RHO binding domains, mitochondrial proton/calcium antiporter domain, and receptors. In the last step, all isoforms with identical sequences of the RB and RA domains were excluded using multiple sequence alignments generated with the ClustalW algorithm. Such approach ended up with a total number of 16 RB domains in 14 RB domain-containing proteins and 41 RA domains in 39 RA domain-containing proteins, respectively (Fig. S1; Tables S1, S2). Both types of RAS effector domains share high sequence identity 10.5% and 9.2% and sequence similarity of 25.5% and 20.2% (Fig. S2 and S3).

Direct interaction of different RA domain-containing proteins with RAS proteins has been comprehensively analyzed (23,30). However, the majority of proteins with a RA domain, however, remains uncharacterized (Table S1). The RAS association domain family (RASSF), which controls a broad range of signaling pathways (8,31), is the largest RA domain-containing protein family (Fig. 1). Their RA domains differently interact with HRAS (8). From them only the interaction of RASSF1 and RASSF5/NORE1 RA domains have been characterized quantitatively so far (23,30). Other characterized RA domain-containing proteins, including RALGDS-like proteins, PLCε, AF6, RIN1/2, and PDZGEF1/2, regulate diverse cellular processes. They share high structural similarity and exhibit differential selectivity for HRAS and RAP1B (23,30).

RB domain-containing proteins are mostly kinases (Table S2). The serine/threonine RAF kinase family proteins (A/B/CRAF; (32)) activates the MEK-ERK axis and controls cell proliferation and differentiation (33,34). PI3Kα generates phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and regulates cell growth, cell survival, cytoskeleton reorganization, and metabolism (35). RGS12/14, which usually act as inactivators of $G\alpha$ proteins (36), physically interact with various members of the RAS family. They appear to facilitate the assembly of the components of the MAPK pathway through direct association with activated HRAS (37). TIAM1/2, which act specific GEFs for the RHO family proteins and control cell migration (38,39), have been suggested to recognize activated RAS proteins (40). However, their direct interaction with RAS proteins has not been shown to date (23). Moreover, some proteins, reported as RAS effectors, do not apparently contain a RA/RB domain (Table S3).

Variable affinities for the RAS-effector interactions–To determine the binding capability between the effector domains and diverse proteins of the RAS family, the following proteins were selected for this study: (i) All 10 RASSF family proteins as representative RA domain-containing effector proteins; (ii) CRAF and TIAM1 RB domains were included because (Fig. 1) many different RAS proteins have been reported to bind to CRAF RB domain and none to TIAM RB domain, yet; and (iii) The RAS family includes 23 genes coding for at least 25 proteins, which share, considering their G domains, sequence identity of 48.6% and a sequence similarity of 61.5% (Fig. S4). Based on sequence identity, structure and function of their G domains, the RAS proteins were divided into eight paralog groups: RAS, RRAS, RAP, RAL, RIT, RHEB, RASD and DIRAS (41). RAS-related proteins RASLs, RERG, RERGL, NKIRAS1/2 were excluded from this list and study due to their strong sequence deviations.

To monitor binding we applied a fluorescence polarization assay (21) to determine the dissociation constants (K_d) for the RAS-effector interactions. For this, we prepared HRAS, RRAS, RAP1B, RAP2A, RALA, RIT1 and RHEB1 in complex with a non-hydrolysable, fluorescent analog of GTP, Representatives of RASD and DIRAS groups were not applied due to their physical instability. Small-sized RB and RA domains were fused to maltose-binding protein (MBP, 42 kDa) to increase their overall molecular weight, and to ensure a homogeneous

monomeric form of the fusion proteins. Figure 1 shows an SDS gels for all purified proteins used in this study.

Increasing concentrations of MBP-fused effector proteins were titrated to RAS-mGppNHp proteins to assess the binding capability of the respective interaction pairs. We observed a significant change in fluorescence polarization for the majority of the measurements (Fig. S5 and S6). However, evaluated K_d values ranged from 0.3 to more than 500 µM. These data are summarized in Table S5 and illustrated in Figure 2. Under these experimental conditions, CRAF RB domain revealed the highest affinity for HRAS, RRAS1 and RASSF5 RA domain exhibited a relatively high affinity for HRAS, RAP1B, RAP2A (Fig. 2A, B, green bars). The intermediate affinities were obtained for the interaction of CRAF RB domain with RAP1B as well as RASSF1 with RAP1B, RAP2A and RALA, RASSF9 with RIT1 and RASSF7 with RRAS1 (Fig. 2A, B; blue bars). The majority of the interaction pairs showed, however, low and very low affinities (Fig. 2B, red and black bars, respectively). Among them, RHEB notably revealed the majority of low affinity interactions. No binding was observed for twelve pairwise interactions.

Identification of common RAS binding site pattern in RA/RB domains–To understand the atomic interactions between RAS and effector proteins, and explain observed variable affinities, we analyzed various structures of RAS-effector protein complexes. To date, 13 structures of RAS-effector protein complexes exist in the PDB (Table S6). Since some of them contain more than one complex in the unit cell, there were altogether 19 complexes available for the analysis. In order to map atomic interactions responsible for observed variable affinities, we have extracted information about interacting interface from all these complex structures (Fig. 3A and S7) and combined them with their sequence alignments (Fig. S2-S4). It is important to note that some amino acids, aligned according to the sequence, were quite distant in the space. Therefore, we edited the sequence alignment to synchronize it with the structural alignment. Our python code finally took sequence alignments with PDB files of complex structures as inputs and calculated all interaction pairs in analyzed complex structures in the form of an interaction matrix. The resultant matrix comprehensively relates the interacting residues on both sides of complexes, with RAS paralogs as rows and the RA/RB domains as columns (Fig. 3B). All numbering in this study is based on HRAS on the one side and CRAF and RASSF5, for RB and RA domains respectively, on the other side.

Each element of the matrix that can be accounted for a `hotspot´, relates one homologous residue from RAS proteins to one homologous residue from the RA/RB domains. The number value of this element, ranging from 0 to 19, represents the number of complex structures in which these residues interact (Fig. 3B). Thus, zero means that these two residues do not contact each other in any structure while a maximal value 19 means that this particular interaction exists in all analyzed complex structures of the RAS-RA/RB domains. We have sorted the residues at both sides of the matrix according to their conservation *vs.* variability. As can be seen in Figures 3A and S4, the majority of the residues (14 out of 20) on the side of 25 RAS proteins are highly conserved, nine of which (Q/N25, D/E33, I/V36, E37, D38, S/T39, Y40, R/K41 in switch I, and Y64 in switch II; HRAS numbering) account for major hotspots (Fig. 3B). On the other side, and in contrast, the majority of 19 RAS interacting residues in RA/RB domains are highly variable and only 2 distant residues are highly conserved (R/K59 and K/R84; CRAF numbering; R/K241 and K/R308; RASSF5 numbering) (Fig. 3A and 3B).

However, what is striking is the middle cluster of the matrix with the most frequent interactions between the highly conserved residues in the switch I region of the RAS proteins (β2-strand residues 36-41; HRAS numbering) and the highly variable residues of the RA/RB domains (β2-strand residues 64-71; CRAF numbering; residues 284-291; RASSF5 numbering) (Fig. 3A and 3B). This cluster adopts an arrangement of intermolecular β-sheet interactions in an anti-parallel fashion (Fig. S7). A substantial number of these contacts in this cluster are mediated by main-chain/main-chain interactions, which typically involve hydrogen bonds between the N-H group and the carbonyl oxygen of the amino acids 37-39 from the RAS side and positions 66-69 (CRAF numbering) and 286-289 (RASSF5 numbering) from the side of the RA/RB domains.

DISCUSSION

Effector selection and activation by a RAS protein in a proper cellular context and appropriate protein network are known to initiate a cascade of biochemical reactions and thus controls defined cellular functions in all types of cells. It is also increasingly clear that functionalization of the effectors with various modular building blocks, especially the RA/RB domains, is a prerequisite for successful orchestration of a series of spatiotemporal events, including recruitment, subcellular localization, assembly of proactive protein complexes, and ultimately association with and activation *via* the RAS protein. An issue that is investigated in-depth in this study is how many effectors for RAS proteins exist in the human proteome and how they achieve the desired affinity and selectivity for their cognate RAS protein.

The total numbers of RAS effectors differ from study to study. A SMART database search has provided 108 RA and 20 RB domain-containing proteins in one of the early and first comprehensive studies on RAS-effector interactions (23). These numbers have been slightly reduced to 100 RA domains and only a few members of RB domain-containing proteins, including A/B/CRAF, TIAM1/2 and RGS12/14 proteins (30). In a next study, Kiel *et al*. has come to around 70 human proteins, containing RA and RBD domains (42). Ibáñez Gaspar *et al*. have analyzed in their very recent, comprehensive study 56 established and predicted RAS effectors with the potential ability to bind to RAS oncoproteins (43). Our search, using the UniProt database and the program HMMER, alongside with a cross-check of each individual sequence, ended up with 41 RA in 39 RA domain-containing proteins and 16 RB in 14 RB domain-containing proteins (Fig. S1). Thus, our lists contain 53 proteins, also including RALGDSL2 and SNX17 (Tables S1 and S2). SNX17 along with SNX27 and SNX31, which possess a FERM-like domain, have been shown to directly bind to GTP-bound HRAS (44), and may thus be involved in endosomal RAS signaling processes (45). However, we exclude RASGEF3-5, KRIT1 and RGL4. Sequences, related to RA or RB domains, were not found in other proteins (Table S3), such as SIN1, SNX31, HK1 (Hexokinase 1) and SHANK2-3, which have been recently described as new RAS effector proteins (44,46-49).

In order to refine a comprehensive list of RAS proteins and their effectors regarding their capabilities of mutual binding, we have investigated pairwise interaction between selected proteins (Fig. 2), related them to available structural data (Fig. 3), and combined them with data described previous studies (Fig. S8).

The RASSF family contains 10 members and is divided in two groups; RASSF1-6 typically have C-terminal RA and SARAH domains and RASSF7-10 an N-terminal RA domain (Fig. 1) (50). However, RAS-binding residues are not conserved in group two of the RASSF family and overall, the RA domains of these two RASSF groups are about 25% identical. Our data showed a much lower binding affinity between RAS family members and RA domains of group two (Fig. 2).

RASSF1 and RASSF5 RA domains share the highest sequence homology and several residues, including L282, D285, A286, I/V287, K288, H291, K308, V311, V312, and D313 (RASSF5 numbering), involved in RAS interaction (Fig. 3B), are almost identical. These RASSFs have been described in many studies as effectors for H/K/NRAS, RRAS1 and RAP1A (19,31,51,52). Accordingly, we have determined high and intermediate affinities for their association with RAS family members in this study (Fig. 2) and in part also in a previous report (21). In a most recent study, Shifman and coworkers have shown that RASSF1 also interacts with ERAS and DIRAS3 (53), which are atypical members of the RAS family (41). Furthermore, RALA strikingly showed an intermediate affinity for RASSF1 (Fig. 2). RALA, as well as RALB, contains at positions 36 and 37 (HRAS numbering) lysine and alanine, rather different residues then isoleucine and glutamate in other RAS proteins, which are known to be critical for the RAS-effector interactions (54). RALA-RASSF1 interaction has not been reported to date and awaits further cell-based investigations.

Among all RASSF family members only RASSF1 and RASSF5 interact in high or intermediate affinities with all investigated RAS family members, with an exception of RIT1 (Fig. 2). RASSF7-9 RA domains share

high sequence similarity and are different from RASSF10 (Fig. S2). A common signature of the RASSF members is the existence of the K/R241 and K/R308 hotspots (Fig. 3B). They revealed, with a few exceptions, comparable K_d values for different representatives of the RAS family (Fig. 2). RIT1-RASSF9 interaction with an intermediate affinity of 27 µM is quite remarkable, especially because a RASSF9 protein has not been reported yet as an RAS effector. RIT1 contains an alanine instead of the conserved S/T39 (HRAS numbering) and RASSF9 contains two negatively charged glutamic acids instead of the positively charged lysine residues at 307 and 308 (RASSF5 numbering; Fig. S2). These two drastic deviations may be responsible for the very low affinity of RASSF9 for HRAS due to electrostatic repulsion with D33. However, RIT1 contains also an aspartic acid at the corresponding position and yet shows an intermediate affinity for RASSF9.

RHEB broadly exhibited low-affinity interaction with RASSF1-7, especially RASSF1 (Fig. 2), which may be based on a large number of amino acid deviations in both switch regions (Fig. 3B and S4). It has been proposed that RHEB may complex with RASSF1 to coordinate signaling pathways, after processing by MST/LATS and TOR kinases (55). In the presence of RASSF1, RHEB has been shown to stimulate the MST/LATS/YAP pathways, but is suppressed in its ability to activate the TOR pathway. Physical interaction of RHEB with RASSFs remains to be shown in cells, in a way shown for other RAS and RAS-like proteins (53).

CRAF RB domain is one of the most and best-studied RAS effectors with the highest selectivity for the H/K/NRAS paralogs and to a certain extend also for the RRAS proteins (21). CRAF RB domain revealed an intermediate affinity for RAP1B and RHEB1 but not for RIT1 or RAP2A (Fig. 2). The RAP1 and RAP2 subgroups differ at positions 25 and 39 (HRAS numbering), which are in the case of RAP1 proteins occupied by favorable glutamine and serine (Fig. 3B). The two orders of magnitude lower affinity of RAP1B for CRAF RB domain stems from the drastic deviation at position 31 (HRAS numbering). K31 in RAP proteins obviously collides with the K84 in CRAF and disfavors a RAP-CRAF interaction (Fig. S8); this was why RAP1A mutated at this site was used for successful determination of the complex structure between RAP1A and CRAF RB domain (25). Phosphorylation of RAP1A at S11 has been recently proposed to promote RAP1A-CRAF RB domain interaction (56).

An intermediate affinity for CRAF RB domain interaction with RHEB G domain (Fig. 2) points to previous reports of a direct relationship between these two crucial signaling molecules. PKA-dependent phosphorylation of CRAF at S43 has been shown to reciprocally potentiate RHEB-CRAF interaction and to decrease CRAF interaction with HRAS (57). An asparagine instead of D38 (HRAS numbering) in the switch I region seems to be critical for the unique CRAF binding properties of RHEB. In a different study Henske and coworkers have shown that RHEB interacts with and inhibits BRAF (58). In this context, RHEB not only hinders the BRAF association with HRAS but also interferes with BRAF activation and its heterodimerization with CRAF. As the RB domains of the RAF paralogs are highly conserved (32), especially regarding their RAS binding residues (Fig. S3), differences between BRAF and CRAF interactions with RHEB may stem from deviations outside the RB domains or from different phosphorylation states. Heard *et al*. have recently reported a strong interaction between RHEB-GTP and BRAF (but not with CRAF) and that RHEB overexpression decreases and RHEB knockdown increases RAF/MEK/ERK activation (59). They have shown that a variant of RHEB (Y35 to asparagine; Y32 in HRAS) impedes RHEB interaction with BRAF leading to an increased BRAF/CRAF heterodimerization and thus activation of the MAPK pathway. Accordingly, they have proposed a dual function for RHEB, suppression of the MAPK pathway and mTORC1 activation (59).

RIT1-CRAF interaction has been frequently proposed due to their critical roles in developmental disorders, collectively called RASopathy (60), but not directly shown. We observed a very low affinity for these two proteins (Fig. 2), which may stem from the sequence deviation between RIT1 and HRAS in their switch I region (Fig. 3B). In an early study on biochemical characterization of RIT, Andres and coworkers have shown that RIT1 interacts with RA domains of RALGDS and AF6 but not with CRAF RB domain (61). In a different

study, they have shown that RIT1 binds and activates BRAF but not CRAF (62). This may again implicate that additional regions may exist outside the highly conserved RB domains of the RAF paralogs, which differently facilitate the interaction with the RAS proteins, like RIT1 or RHEB.

As there is not published structure for TIAM1 RB domain, we selected also TIAM1 for our study and obtained very low affinity interactions, such as 381 μM for HRAS (Fig. 2). TIAM1 shares only three identical residues with CRAF, namely R59, K84 and L86 (CRAF numbering), which are obviously not sufficient for a tight interaction with HRAS (Fig. 3B). Shirazi Fard *et al*. have shown in a cellular context that HRAS is associated with endogenous TIAM1 using immunoprecipitation and that HRAS-GTP can be pulled down using TIAM1 RB domain (63). They have proposed that HRAS-GTP-TIAM1 interaction is crucial for a TIAM1-catalyzed RAC1 activation.

An ever-present central concern in biophysical investigation of protein-protein interactions is the relevance of low (10-30 μM) to very low (>> 30 μM) affinity interactions in the regulation of signaling events. This type of protein complex relies on weak, transient interactions that are emerging as important components of large signaling complexes at the plasma membrane that are required to response to external stimuli.

A frequently encountered issue in the enhancement of RAS-effector interaction is post translational modification. Thurman *et al*. has recently demonstrated that ubiquitylation of KRAS at L147 impairs RAS-RASGAP interaction and facilitate RAS-CRAF association and MAPK signaling (64). Barceló *et al*. have shown that PKC-catalyzed phosphorylation of KRAS at S181 results in an increased interaction of KRAS with CRAF and PI3Kα (65). Several studies have previously shown that CRAF CR domain undergoes direct interaction with HRAS, which appears to be enhanced by the farnesyl moiety if using farnesylated RAS (15,66-71). A possible HRAS-CRAF CR domain interaction has been proposed to be, contrary to CRAF RB domain, outside of the switch regions of HRAS and thus independent of its nucleotide-bound state.

Another aspect related to very low affinity interactions involves a secondary RAS binding site, in addition to the RA/RB domain, in terms of a two-step, two-domain binding model. The two-domain model accommodates at least two different enhancer mechanisms. One is direct enhancement of a selective RAS-effector interaction required for effector activation, proposed for the interactions of yeast RAS2 with two sites in adenylyl cyclase (72), HRAS with RB and CR domains of CRAF (32), and HRAS with two RA domains of PLCε (73). The latter may involve a high-affinity, GTP-dependent binding of RA2 domain accompanied by low-affinity, GTP-independent binding of RA1 domain. Deletion of one of the RA domains inhibits HRAS-induced PLCε activation (73). Notably, AF6 also possesses two RA domains and RGS12/14 two RB domains, respectively (43). Such tandem arrangement of RA respective RB domains may enhance their affinity towards RAS, increase effector occupancy by additional endogenous events and thus the signaling output. An emerging concept, therefore, is the action of membrane binding CR domain that stabilizes RAS-CRAF RB domain interaction accompanied with S621 phosphorylation, and 14-3-3 binding that collectively facilitates RAF activation (67,68,74-77).

The formation of multiprotein complexes underlies a multistep assembly mechanism that follows a defined and probably short path from the cytoplasm, just underneath the membrane, to the membrane where membrane associated proteins, for example RAS proteins, are anchored. The first step, which has been designated as the piggyback mechanism (78), most likely increases local concentrations of protein components in a small volume and may drive cytoplasmic phase separations (79-81). The second step is site-specific association of assembled protein complex with membrane-associated components, such as RAS proteins, which in turn are connected to receptor and co-receptors (43,80,81). In this way, a machinery of signaling molecules is orchestrated before the ligand activates the receptor. This is fine-tuned and prepared for an efficient signal transduction. Of course, it remains to be figure out why some interactions are in nanomolar range (e.g. 20 nM) and some in micromolar range (e.g. 20 μM or more). Given that the latter

is involved in the initiation of multivalent macromolecular interactions, final complex formation come along after multivalent interactions have proceeded (82). This obviously increases significantly both the number of interacting complexes and overall binding affinity by orders of magnitude (43). The nanomolar affinity, however, may determine the selectivity for a sequential formation of two complexes. These interactions are often characterized by fast association and slow dissociation rates, indicating formation of stable complexes (83-85).

EXPERIMENTAL PROCEDURES

***Constructs*–Fragments of human genes encoding RAs of RASSF1 (accession number Q9NS23; amino acids or aa 194-288), RASSF2 (P50749; aa 176-264), RASSF3 (Q86WH2; aa 79-187), RASSF4 (Q9H2L5; aa 174-262), RASSF5 (Q8WWW0; aa 200–358), RASSF6 (Q6ZTQ3; aa 218-306), RASSF7 (Q02833; aa 6-89), RASSF8 (Q8NHQ8, aa 1-82), RASSF9 (O75901, aa 25-119), and RASSF10 (A6NK89; aa 4-133) as well as CRAF RB domain (P04049, aa 51–131) and TIAM1 RB domain (Q13009, aa 765-832) were cloned into pMal-c5X-His vector. Constructs for the expression of human HRAS, RRAS, RALA, RHEB1, RIT1, RAP2A and RAP1B isoforms were described previously (6).**

***Protein purification*–All RASSF and RAS proteins were expressed in *Escherichia coli* using the pMal-His and pGEX expression systems and purified by using Ni-NTA and glutathione based affinity chromatography as described previously (86). RAS-mGppNHp was prepared as described (86) .**

Fluorescence measurements–RAS-effector interaction was performed in 50 mM Tris/HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$ and 3 mM dithiothreitol at 25˚C using a Fluoromax 4 fluorimeter in polarization mode as described (86). Increasing amounts of MBP-tagged effector proteins (0.025–300 μM) titrated to 1 μM RAS-mGppNHp resulted in an increase of polarization. Equilibrium dissociation constants (K_d) were calculated by fitting the concentration dependent binding curve using a quadratic ligand binding equation.

Bioinformatics–Information about RB and RA domains were obtained either from annotations in UniProt database or in parallel using the program suite HAMMER [http://hmmer.org/]. Sequence alignments were performed with Bioedit program using the ClustalW algorithm (87). By using Chimera the sequence alignments was adjust with superimposed structures (24). An interaction matrix is based on intermolecular contacts in complex structures (21). A python code was written to match sequence alignments with complex structures (Table S7) and calculated intermolecular contacts were put in the form of interaction matrix. The intermolecular contacts were defined as pair residues with a distance 4.0 Å between effectors and RAS proteins in available complex structures in the protein data bank (http://www.pdb.org). Biopython modules (88) were also used to elucidate corresponding residues in all available complex structures. The structural representation were generated using Pymol viewer (89).

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AUTHORS´ CONTRIBUTIONS
M.R.A. conceived and coordinated the study; S.R.A., R.D., C.A.M.S. and M.R.A. designed and wrote the paper; S.R.A., N.S.K.J., and C.W. designed, performed, and analyzed the experiments; S.R.A. and R.D. and E.A. performed structural analysis; all authors reviewed the results and approved the final version of the manuscript.

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COMPETING INTERESTS
The authors declare no competing financial interest.

REFERENCES

1. Jaiswal, M., Dvorsky, R., Amin, E., Risse, S. L., Fansa, E. K., Zhang, S. C., Taha, M. S., Gauhar, A. R., Nakhaei-Rad, S., Kordes, C., Koessmeier, K. T., Cirstea, I. C., Olayioye, M. A., Haussinger, D., and Ahmadian, M. R. (2014) Functional cross-talk between ras and rho pathways: a Ras-specific GTPase-activating protein (p120RasGAP) competitively inhibits the RhoGAP activity of deleted in liver cancer (DLC) tumor suppressor by masking the catalytic arginine finger. *J Biol Chem* **289**, 6839-6849

2. Simanshu, D. K., Nissley, D. V., and McCormick, F. (2017) RAS proteins and their regulators in human disease. *Cell* **170**, 17-33

3. Gutierrez-Erlandsson, S., Herrero-Vidal, P., Fernandez-Alfara, M., Hernandez-Garcia, S., Gonzalo-Flores, S., Mudarra-Rubio, A., Fresno, M., and Cubelos, B. (2013) R-RAS2 overexpression in tumors of the human central nervous system. *Molecular cancer* **12**, 127

4. Karnoub, A. E., and Weinberg, R. A. (2008) Ras oncogenes: split personalities. *Nature reviews Molecular cell biology* **9**, 517

5. Herrmann, C. (2003) Ras–effector interactions: after one decade. *Current opinion in structural biology* **13**, 122-129

6. Nakhaei-Rad, S., Nakhaeizadeh, H., Götze, S., Kordes, C., Sawitza, I., Hoffmann, M. J., Franke, M., Schulz, W. A., Scheller, J., and Piekorz, R. P. (2016) The role of embryonic stem cell-expressed RAS (ERAS) in the maintenance of quiescent hepatic stellate cells. *Journal of Biological Chemistry*, jbc. M115. 700088

7. Castellano, E., and Downward, J. (2010) Role of RAS in the regulation of PI 3-kinase. in *Phosphoinositide 3-kinase in Health and Disease*, Springer. pp 143-169

8. Chan, J. J., Flatters, D., Rodrigues-Lima, F., Yan, J., Thalassinos, K., and Katan, M. (2013) Comparative analysis of interactions of RASSF1-10. *Advances in biological regulation* **53**, 190-201

9. Bunney, T. D., and Katan, M. (2011) PLC regulation: emerging pictures for molecular mechanisms. *Trends in biochemical sciences* **36**, 88-96

10. Ferro, E., and Trabalzini, L. (2010) RalGDS family members couple Ras to Ral signalling and that's not all. *Cellular signalling* **22**, 1804-1810

11. Rajalingam, K., Schreck, R., Rapp, U. R., and Albert, Š. (2007) Ras oncogenes and their downstream targets. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* **1773**, 1177-1195

12. Nussinov, R., Tsai, C.-J., Muratcioglu, S., Jang, H., Gursoy, A., and Keskin, O. (2015) Principles of K-Ras effector organization and the role of oncogenic K-Ras in cancer initiation through G1 cell cycle deregulation. *Expert review of proteomics* **12**, 669-682

13. Ahearn, I. M., Haigis, K., Bar-Sagi, D., and Philips, M. R. (2012) Regulating the regulator: post-translational modification of RAS. *Nature reviews Molecular cell biology* **13**, 39

14. Hennig, A., Markwart, R., Esparza-Franco, M. A., Ladds, G., and Rubio, I. (2015) Ras activation revisited: role of GEF and GAP systems. *Biological chemistry* **396**, 831-848

15. Fischer, A., Hekman, M., Kuhlmann, J., Rubio, I., Wiese, S., and Rapp, U. R. (2007) B-and C-RAF Display Essential Differences in Their Binding to Ras THE ISOTYPE-SPECIFIC N TERMINUS OF B-RAF FACILITATES RAS BINDING. *Journal of Biological Chemistry* **282**, 26503-26516

16. Mott, H. R., and Owen, D. (2015) Structures of Ras superfamily effector complexes: What have we learnt in two decades? *Critical reviews in biochemistry and molecular biology* **50**, 85-133

17. Vetter, I. R., and Wittinghofer, A. (2001) The guanine nucleotide-binding switch in three dimensions. *Science* **294**, 1299-1304

18. Filchtinski, D., Sharabi, O., Rüppel, A., Vetter, I. R., Herrmann, C., and Shifman, J. M. (2010) What makes Ras an efficient molecular switch: a computational, biophysical, and structural study of Ras-GDP interactions with mutants of Raf. *Journal of molecular biology* **399**, 422-435

19. Erijman, A., and M Shifman, J. (2016) RAS/effector interactions from structural and biophysical perspective. *Mini reviews in medicinal chemistry* **16**, 370-375

20. Nassar, N., Horn, G., Herrmann, C., Block, C., Janknecht, R., and Wittinghofer, A. (1996) Ras/Rap effector specificity determined by charge reversal. *Nature structural biology* **3**, 723

21. Nakhaeizadeh, H., Amin, E., Nakhaei-Rad, S., Dvorsky, R., and Ahmadian, M. R. (2016) The RAS-effector interface: isoform-specific differences in the effector binding regions. *PloS one* **11**, e0167145

22. Repasky, G. A., Chenette, E. J., and Der, C. J. (2004) Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis? *Trends in cell biology* **14**, 639-647

23. Wohlgemuth, S., Kiel, C., Krämer, A., Serrano, L., Wittinghofer, F., and Herrmann, C. (2005) Recognizing and defining true Ras binding domains I: biochemical analysis. *Journal of molecular biology* **348**, 741-758

24. Goddard, T. D., Huang, C. C., Meng, E. C., Pettersen, E. F., Couch, G. S., Morris, J. H., and Ferrin, T. E. (2018) UCSF ChimeraX: Meeting modern challenges in visualization and analysis. *Protein Science* **27**, 14-25

25. Nassar, N., Horn, G., Herrmann, C. A., Scherer, A., McCormick, F., and Wittinghofer, A. (1995) The 2.2 Å crystal structure of the Ras-binding domain of the serine/threonine kinase c-Raf1 in complex with RaplA and a GTP analogue. *Nature* **375**, 554-560

26. Bunney, T. D., Harris, R., Gandarillas, N. L., Josephs, M. B., Roe, S. M., Sorli, S. C., Paterson, H. F., Rodrigues-Lima, F., Esposito, D., and Ponting, C. P. (2006) Structural and mechanistic insights into ras association domains of phospholipase C epsilon. *Molecular cell* **21**, 495-507

27. Stieglitz, B., Bee, C., Schwarz, D., Yildiz, Ö., Moshnikova, A., Khokhlatchev, A., and Herrmann, C. (2008) Novel type of Ras effector interaction established between tumour suppressor NORE1A and Ras switch II. *The EMBO journal* **27**, 1995-2005

28. Pacold, M. E., Suire, S., Perisic, O., Lara-Gonzalez, S., Davis, C. T., Walker, E. H., Hawkins, P. T., Stephens, L., Eccleston, J. F., and Williams, R. L. (2000) Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase γ. *Cell* **103**, 931-944

29. Smith, M. J., Ottoni, E., Ishiyama, N., Goudreault, M., Haman, A., Meyer, C., Tucholska, M., Gasmi-Seabrook, G., Menezes, S., and Laister, R. C. (2017) Evolution of AF6-RAS association and its implications in mixed-lineage leukemia. *Nature communications* **8**, 1-13

30. Kiel, C., Wohlgemuth, S., Rousseau, F., Schymkowitz, J., Ferkinghoff-Borg, J., Wittinghofer, F., and Serrano, L. (2005) Recognizing and defining true Ras binding domains II: in silico prediction based on homology modelling and energy calculations. *Journal of molecular biology* **348**, 759-775

31. Donninger, H., Schmidt, M. L., Mezzanotte, J., Barnoud, T., and Clark, G. J. (2016) Ras signaling through RASSF proteins. in *Seminars in cell & developmental biology*, Elsevier

32. Rezaei Adariani, S., Buchholzer, M., Akbarzadeh, M., Nakhaei-Rad, S., Dvorsky, R., and Ahmadian, M. R. (2018) Structural snapshots of RAF kinase interactions. *Biochemical Society Transactions* **46**, 1393-1406

33. Haghighi, F., Dahlmann, J., Nakhaei-Rad, S., Lang, A., Kutschka, I., Zenker, M., Kensah, G., Piekorz, R. P., and Ahmadian, M. R. (2018) bFGF-mediated pluripotency maintenance in human induced pluripotent stem cells is associated with NRAS-MAPK signaling. *Cell Communication and Signaling* **16**, 96

34. Desideri, E., Cavallo, A. L., and Baccarini, M. (2015) Alike but different: RAF paralogs and their signaling outputs. *Cell* **161**, 967-970

35. Castellano, E., and Downward, J. (2011) RAS interaction with PI3K: more than just another effector pathway. *Genes & cancer* **2**, 261-274

36. Ross, E. M., and Wilkie, T. M. (2000) GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annual review of biochemistry* **69**, 795-827

37. Willard, F. S., Willard, M. D., Kimple, A. J., Soundararajan, M., Oestreich, E. A., Li, X., Sowa, N. A., Kimple, R. J., Doyle, D. A., and Der, C. J. (2009) Regulator of G-protein signaling 14 (RGS14) is a selective H-Ras effector. *PloS one* **4**

38. Malliri, A., and Collard, J. G. (2003) Role of Rho-family proteins in cell adhesion and cancer. *Current opinion in cell biology* **15**, 583-589

39. Rooney, C., White, G., Nazgiewicz, A., Woodcock, S. A., Anderson, K. I., Ballestrem, C., and Malliri, A. (2010) The Rac activator STEF (Tiam2) regulates cell migration by microtubule-mediated focal adhesion disassembly. *EMBO reports* **11**, 292-298

40. Yamauchi, J., Miyamoto, Y., Tanoue, A., Shooter, E. M., and Chan, J. R. (2005) Ras activation of a Rac1 exchange factor, Tiam1, mediates neurotrophin-3-induced Schwann cell migration. *Proceedings of the National Academy of Sciences* **102**, 14889-14894

41. Nakhaei-Rad, S., Haghighi, F., Nouri, P., Rezaei Adariani, S., Lissy, J., Kazemein Jasemi, N. S., Dvorsky, R., and Ahmadian, M. R. (2018) Structural fingerprints, interactions, and signaling networks of RAS family proteins beyond RAS isoforms. *Critical reviews in biochemistry and molecular biology* **53**, 130-156

42. Kiel, C., Foglierini, M., Kuemmerer, N., Beltrao, P., and Serrano, L. (2007) A genome-wide Ras-effector interaction network. *Journal of molecular biology* **370**, 1020-1032

43. Ibáñez Gaspar, V., Catozzi, S., Ternet, C., Luthert, P. J., and Kiel, C. (2020) Analysis of Ras-effector interaction competition in large intestine and colorectal cancer context. *Small GTPases*, 1-17

44. Ghai, R., Mobli, M., Norwood, S. J., Bugarcic, A., Teasdale, R. D., King, G. F., and Collins, B. M. (2011) Phox homology band 4.1/ezrin/radixin/moesin-like proteins function as molecular scaffolds that interact with cargo receptors and Ras GTPases. *Proceedings of the National Academy of Sciences* **108**, 7763-7768

45. Ghai, R., and Collins, B. (2011) PX-FERM proteins: A link between endosomal trafficking and signaling? *Small GTPases* **2**, 7763-7768

46. Amendola, C. R., Mahaffey, J. P., Parker, S. J., Ahearn, I. M., Chen, W.-C., Zhou, M., Court, H., Shi, J., Mendoza, S. L., and Morten, M. J. (2019) KRAS4A directly regulates hexokinase 1. *Nature* **576**, 482-486

47. Chowdhury, D., and Hell, J. W. (2020) How CBP/Shank3 Guards Rap and H-Ras. *Structure* **28**, 274-276

48. Cai, Q., Hosokawa, T., Zeng, M., Hayashi, Y., and Zhang, M. (2020) Shank3 Binds to and Stabilizes the Active Form of Rap1 and HRas GTPases via Its NTD-ANK Tandem with Distinct Mechanisms. *Structure* **28**, 290-300. e294

49. Miyan, J., Asif, M., Malik, S. A., Dubey, P., Singh, V., Singh, K., Mitra, K., Pandey, D., Haq, W., and Amita, H. (2019) Direct physical interaction of active Ras with mSIN1 regulates mTORC2 signaling. *BMC cancer* **19**, 1-16

50. Iwasa, H., Hossain, S., and Hata, Y. (2018) Tumor suppressor C-RASSF proteins. *Cellular and Molecular Life Sciences* **75**, 1773-1787

51. Gordon, M., and Baksh, S. (2011) RASSF1A: not a prototypical Ras effector. *Small GTPases* **2**, 5729-5740

52. van der Weyden, L., and Adams, D. J. (2007) The Ras-association domain family (RASSF) members and their role in human tumourigenesis. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* **1776**, 58-85

53. Thillaivillalan, D., Singh, S., Killoran, R. C., Singh, A., Xu, X., Shifman, J., and Smith, M. J. (2020) RASSF effectors couple diverse RAS subfamily GTPases to the Hippo pathway. *bioRxiv*

54. Bauer, B., Mirey, G., Vetter, I. R., García-Ranea, J. A., Valencia, A., Wittinghofer, A., Camonis, J. H., and Cool, R. H. (1999) Effector recognition by the small GTP-binding proteins Ras and Ral. *Journal of Biological Chemistry* **274**, 17763-17770

55. Nelson, N., and Clark, G. J. (2016) Rheb may complex with RASSF1A to coordinate Hippo and TOR signaling. *Oncotarget* **7**, 33821

56. Devanand, T., Venkatraman, P., and Vemparala, S. (2018) Phosphorylation promotes binding affinity of Rap-Raf complex by allosteric modulation of switch loop dynamics. *Scientific reports* **8**, 1-15

57. Yee, W. M., and Worley, P. F. (1997) Rheb interacts with Raf-1 kinase and may function to integrate growth factor-and protein kinase A-dependent signals. *Molecular and cellular biology* **17**, 921-933

58. Karbowniczek, M., Robertson, G. P., and Henske, E. P. (2006) Rheb inhibits C-raf activity and B-raf/C-raf heterodimerization. *Journal of Biological Chemistry* **281**, 25447-25456

59. Heard, J. J., Phung, I., Potes, M. I., and Tamanoi, F. (2018) An oncogenic mutant of RHEB, RHEB Y35N, exhibits an altered interaction with BRAF resulting in cancer transformation. *BMC cancer* **18**, 69

60. Yaoita, M., Niihori, T., Mizuno, S., Okamoto, N., Hayashi, S., Watanabe, A., Yokozawa, M., Suzumura, H., Nakahara, A., and Nakano, Y. (2016) Spectrum of mutations and genotype–phenotype analysis in Noonan syndrome patients with RIT1 mutations. *Human genetics* **135**, 209-222

61. Shao, H., Kadono-Okuda, K., Finlin, B. S., and Andres, D. A. (1999) Biochemical characterization of the Ras-related GTPases Rit and Rin. *Archives of biochemistry and biophysics* **371**, 207-219

62. Shi, G.-X., and Andres, D. A. (2005) Rit contributes to nerve growth factor-induced neuronal differentiation via activation of B-Raf-extracellular signal-regulated kinase and p38 mitogen-activated protein kinase cascades. *Molecular and cellular biology* **25**, 830-846

63. Fard, S. S., Kele, J., Vilar, M., Paratcha, G., and Ledda, F. (2010) Tiam1 as a signaling mediator of nerve growth factor-dependent neurite outgrowth. *PloS one* **5**

64. Thurman, R., Siraliev-Perez, E., and Campbell, S. L. (2017) RAS ubiquitylation modulates effector interactions. *Small GTPases*, 1-6

65. Barceló, C., Paco, N., Morell, M., Alvarez-Moya, B., Bota-Rabassedas, N., Jaumot, M., Vilardell, F., Capella, G., and Agell, N. (2014) Phosphorylation at Ser-181 of oncogenic KRAS is required for tumor growth. *Cancer research* **74**, 1190-1199

66. Thapar, R., Williams, J. G., and Campbell, S. L. (2004) NMR characterization of full-length farnesylated and non-farnesylated H-Ras and its implications for Raf activation. *Journal of molecular biology* **343**, 1391-1408

67. Li, Z.-L., Prakash, P., and Buck, M. (2018) A "tug of war" maintains a dynamic protein–membrane complex: molecular dynamics simulations of C-Raf RBD-CRD bound to K-Ras4B at an anionic membrane. *ACS central science* **4**, 298-305

68. Li, S., Jang, H., Zhang, J., and Nussinov, R. (2018) Raf-1 cysteine-rich domain increases the affinity of K-Ras/Raf at the membrane, promoting MAPK signaling. *Structure* **26**, 513-525. e512

69. Hu, C.-D., Kariya, K.-i., Tamada, M., Akasaka, K., Shirouzu, M., Yokoyama, S., and Kataoka, T. (1995) Cysteine-rich region of Raf-1 interacts with activator domain of post-translationally modified Ha-Ras. *Journal of Biological Chemistry* **270**, 30274-30277

70. Williams, J. G., Drugan, J. K., Yi, G.-S., Clark, G. J., Der, C. J., and Campbell, S. L. (2000) Elucidation of binding determinants and functional consequences of Ras/Raf-cysteine-rich domain interactions. *Journal of Biological Chemistry* **275**, 22172-22179

71. Ke, H., Matsumoto, S., Murashima, Y., Taniguchi-Tamura, H., Miyamoto, R., Yoshikawa, Y., Tsuda, C., Kumasaka, T., Mizohata, E., and Edamatsu, H. (2017) Structural basis for intramolecular interaction of post-translationally modified H-Ras• GTP prepared by protein ligation. *FEBS letters* **591**, 2470-2481

72. Shima, F., Okada, T., Kido, M., Sen, H., Tanaka, Y., Tamada, M., Hu, C.-D., Yamawaki-Kataoka, Y., Kariya, K.-i., and Kataoka, T. (2000) Association of yeast adenylyl cyclase with cyclase-associated protein CAP forms a second Ras-binding site which mediates its Ras-dependent activation. *Molecular and cellular biology* **20**, 26-33

73. Kelley, G. G., Reks, S. E., Ondrako, J. M., and Smrcka, A. V. (2001) Phospholipase Cϵ: a novel Ras effector. *The EMBO journal* **20**, 743-754

74. Travers, T., López, C. A., Van, Q. N., Neale, C., Tonelli, M., Stephen, A. G., and Gnanakaran, S. (2018) Molecular recognition of RAS/RAF complex at the membrane: Role of RAF cysteine-rich domain. *Scientific reports* **8**, 1-15

75. Lakshman, B., Messing, S., Schmid, E. M., Clogston, J. D., Gillette, W. K., Esposito, D., Kessing, B., Fletcher, D. A., Nissley, D. V., and McCormick, F. (2019) Quantitative biophysical analysis

defines key components modulating recruitment of the GTPase KRAS to the plasma membrane. *Journal of Biological Chemistry* **294**, 2193-2207

76. Park, E., Rawson, S., Li, K., Kim, B.-W., Ficarro, S. B., Gonzalez-Del Pino, G., Sharif, H., Marto, J. A., Jeon, H., and Eck, M. J. (2019) Architecture of autoinhibited and active BRAF–MEK1–14-3-3 complexes. *Nature* **575**, 545-550

77. Jang, H., Zhang, M., and Nussinov, R. (2020) The quaternary assembly of KRas4B with Raf-1 at the membrane. *Computational and Structural Biotechnology Journal*

78. Kholodenko, B. N., Hoek, J. B., and Westerhoff, H. V. (2000) Why cytoplasmic signalling proteins should be recruited to cell membranes. *Trends in cell biology* **10**, 173-178

79. Sukenik, S., Ren, P., and Gruebele, M. (2017) Weak protein–protein interactions in live cells are quantified by cell-volume modulation. *Proceedings of the National Academy of Sciences* **114**, 6776-6781

80. Case, L. B., Ditlev, J. A., and Rosen, M. K. (2019) Regulation of transmembrane signaling by phase separation. *Annual Review of Biophysics* **48**, 465-494

81. Bratek-Skicki, A., Pancsa, R., Meszaros, B., Van Lindt, J., and Tompa, P. (2020) A guide to regulation of the formation of biomolecular condensates. *The FEBS Journal*

82. Banani, S. F., Lee, H. O., Hyman, A. A., and Rosen, M. K. (2017) Biomolecular condensates: organizers of cellular biochemistry. *Nature reviews Molecular cell biology* **18**, 285-298

83. Kiel, C., Selzer, T., Shaul, Y., Schreiber, G., and Herrmann, C. (2004) Electrostatically optimized Ras-binding Ral guanine dissociation stimulator mutants increase the rate of association by stabilizing the encounter complex. *Proceedings of the National Academy of Sciences* **101**, 9223-9228

84. Kiel, C., Filchtinski, D., Spoerner, M., Schreiber, G., Kalbitzer, H. R., and Herrmann, C. (2009) Improved binding of Raf to Ras· GDP is correlated with biological activity. *Journal of Biological Chemistry* **284**, 31893-31902

85. Schreiber, G., Haran, G., and Zhou, H.-X. (2009) Fundamental aspects of protein− protein association kinetics. *Chemical reviews* **109**, 839-860

86. Gremer, L., Merbitz-Zahradnik, T., Dvorsky, R., Cirstea, I. C., Kratz, C. P., Zenker, M., Wittinghofer, A., and Ahmadian, M. R. (2011) Germline KRAS mutations cause aberrant biochemical and physical properties leading to developmental disorders. *Human mutation* **32**, 33-43

87. Hall, T. A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. in *Nucleic acids symposium series*, [London]: Information Retrieval Ltd., c1979-c2000.

88. Cock, P. J., Antao, T., Chang, J. T., Chapman, B. A., Cox, C. J., Dalke, A., Friedberg, I., Hamelryck, T., Kauff, F., and Wilczynski, B. (2009) Biopython: freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics* **25**, 1422-1423

89. DeLano, W. L. (2002) The PyMOL molecular graphics system. *http://www. pymol. org*

ABBREVIATIONS AND NOMENCLATURE

AF6, ALL1-fused gene from chromosome 6; CR domain, cysteine-rich domain; ERK, extracellular signal-regulated kinase; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GTP, guanosine triphosphate; GTPase, guanosine triphosphatase; HK1, hexokinase-1; HRAS, Harvey rat sarcoma; KRAS, Kristen rat sarcoma; MAPK, mitogen-activated protein kinase; MBP, maltose binding protein; MEK, MAPK/ERK kinase; NKIRAS, NF-kappa-B inhibitor-interacting RAS-like protein; NORE1, novel RAS effector; NRAS, neuroblastoma RAS; PDZGEF, PDZ domain-containing guanine nucleotide exchange factor; PI3K, phosphoinositidine 3-kinase; PKC, protein kinase C; PLCε, phospholipase C epsilon; RA domain, RAS association domain; RAF, rapidly accelerated fibrosarcoma; RALA, RAS-like protein A; RALGDS, Ral guanine nucleotide dissociation stimulator; RAP, RAS proximate; RAS, rat sarcoma; RASIP1, RAS-interacting protein 1; RASD, Dexamethasone-induced RAS-related; RASSF, RAS association domain family; RB domain, RAS binding domain; RERG, RAS-related and estrogen-regulated growth inhibitor; RERGL, RAS-related and estrogen-regulated growth inhibitor-like protein; RGL, Ral guanine nucleotide dissociation stimulator-like; RGS, regulator of G protein signaling; RHEB, RAS homologous enriched in brain; RHO, RAS homologous; RIN, RAS and RAB interactor; RIT, RAS-like protein expressed in many tissues; RRAS, RAS-related protein; SARAH domain, Salvador-RASSF-Hippo domain; SHANK, SH3 and multiple ankyrin repeat domain; SIN1, stress-activated protein kinase-interacting protein 1; SNX17, sorting nexin-17; TIAM, T-lymphoma invasion and metastasis protein.

FIGURE LEGENDS

Figure 1. Domain organization of effector proteins. Schematic representation of RASSF1-10 proteins, CRAF RB and TIAM1 RB domains. Different domains are highlighted, including RAS Association domain (RA) in red, RAS Binding domain (RB) in yellow, and other domains in blue. Based on their domain organization, the RASSF family proteins are divided in group 1 (RASSF1-6) and group 2 with N-terminal RA domains (RASSF7-10). Coomasssie brilliant blue stained SDS-gels show purified RAS proteins as well as the RA/RB domains purified as MBP fusion proteins.

Figure 2. Differential binding affinities for the RA/RB domain interactions with various RAS subfamily members. The interactions between 7 RAS subfamily members with 12 effector proteins (10 RA domains of the RASSF protein family and 2 RB domains of CRAF and TIAM1, respectively) were determined by titrating mGppNHp-bound, active forms of RAS proteins (1 µM, respectively) with increasing concentrations of the respective effector domains as MBP fusion proteins (Fig. S5 and S6). (A) Data of four representative experiments are shown for the interaction of RALA, RAP2A, RRAS1and RIT1 with RASSF1, 5, 7 and 9, respectively. (B) Evaluated K_d values (above the bars; Table S5) were divided in high affinity (0.1 – 5 µM; green), intermediate affinity (6 – 30µM; blue), low affinity (31 – 90 µM; red) and very low affinity (91-510 µM; black). No binding (n.b.) stands for K_d values higher than 500 µM.

Figure 3. Interaction matrix adapted for the structures of RAS complexes with effector domains. (A) Secondary structures of HRAS and RA/RB domains along with the interacting residues are illustrated. (B) Interaction matrix of RAS and effector proteins (boxed in red) is showed to demonstrate interaction residues in all available structures (see Table S6). It comprises the amino acid sequence alignments of the RAS proteins (lower left panel) and the effector domains (upper middle panel), respectively, extracted from the complete alignments in Fig. S2-S4. Each element corresponds to a possible interaction of RAS residues (row; HRAS numbering) and effector (column; CRAF and RASSF5 numbering, respectively). The number of actual contact sites between RAS and the effector domains (with distances of 4 Å or less) were calculated and are indicated with positive numbers for matrix elements. The structures of bolded proteins were used to generate the matrix. Underlined proteins were biochemically investigated in this study. (C) Extracted structures of HRAS (in orchid) and the RA/RB domains (in olive) from their surface complexes are presented. Key interaction hotspots with the same color codes are highlighted on the surface structures as well as in the interaction matrix and the secondary structures, respectively.

FIGURE 1

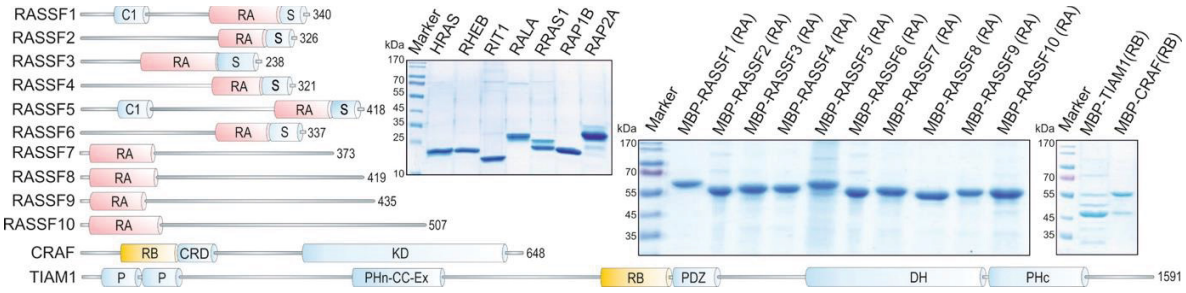

RASSF1
RASSF2
RASSF3
RASSF4
RASSF5
RASSF6
RASSF7
RASSF8
RASSF9
RASSF10
CRAF
TIAM1
C1
RA
S
RB
CRD
KD
P
PHn-CC-Ex
PDZ
DH
PHc
340
326
238
321
418
337
373
419
435
507
648
1591
kDa
Marker
HRAS
RHEB
RIT1
RALA
RRAS1
RAP1B
RAP2A
MBP-RASSF1 (RA)
MBP-RASSF2 (RA)
MBP-RASSF3 (RA)
MBP-RASSF4 (RA)
MBP-RASSF5 (RA)
MBP-RASSF6 (RA)
MBP-RASSF7 (RA)
MBP-RASSF8 (RA)
MBP-RASSF9 (RA)
MBP-RASSF10 (RA)
MBP-TIAM1(RB)
MBP-CRAF(RB)
170
70
55
45
35
25
10

FIGURE 2

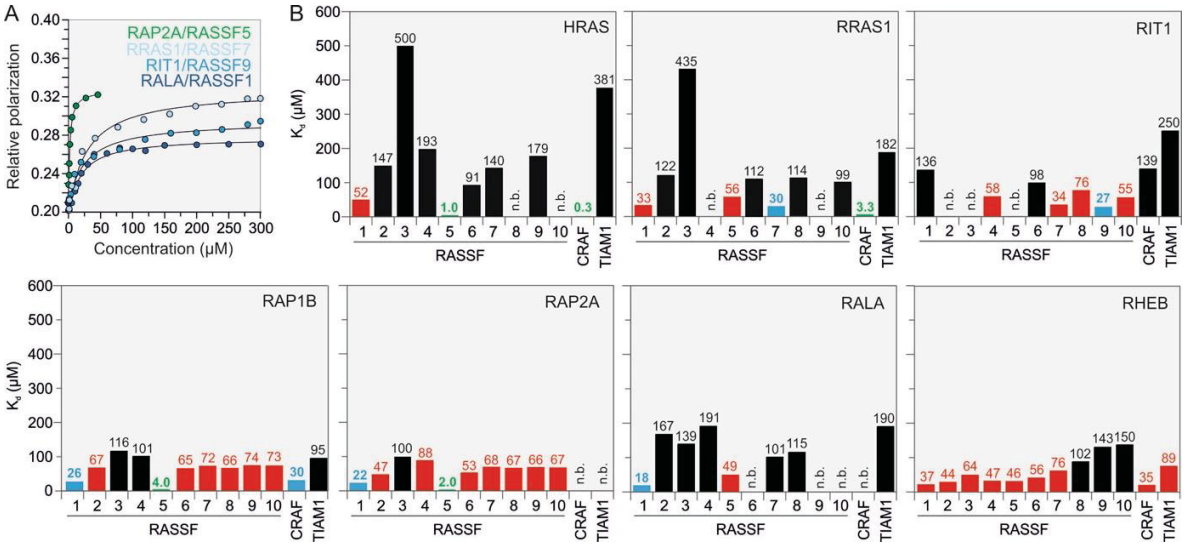
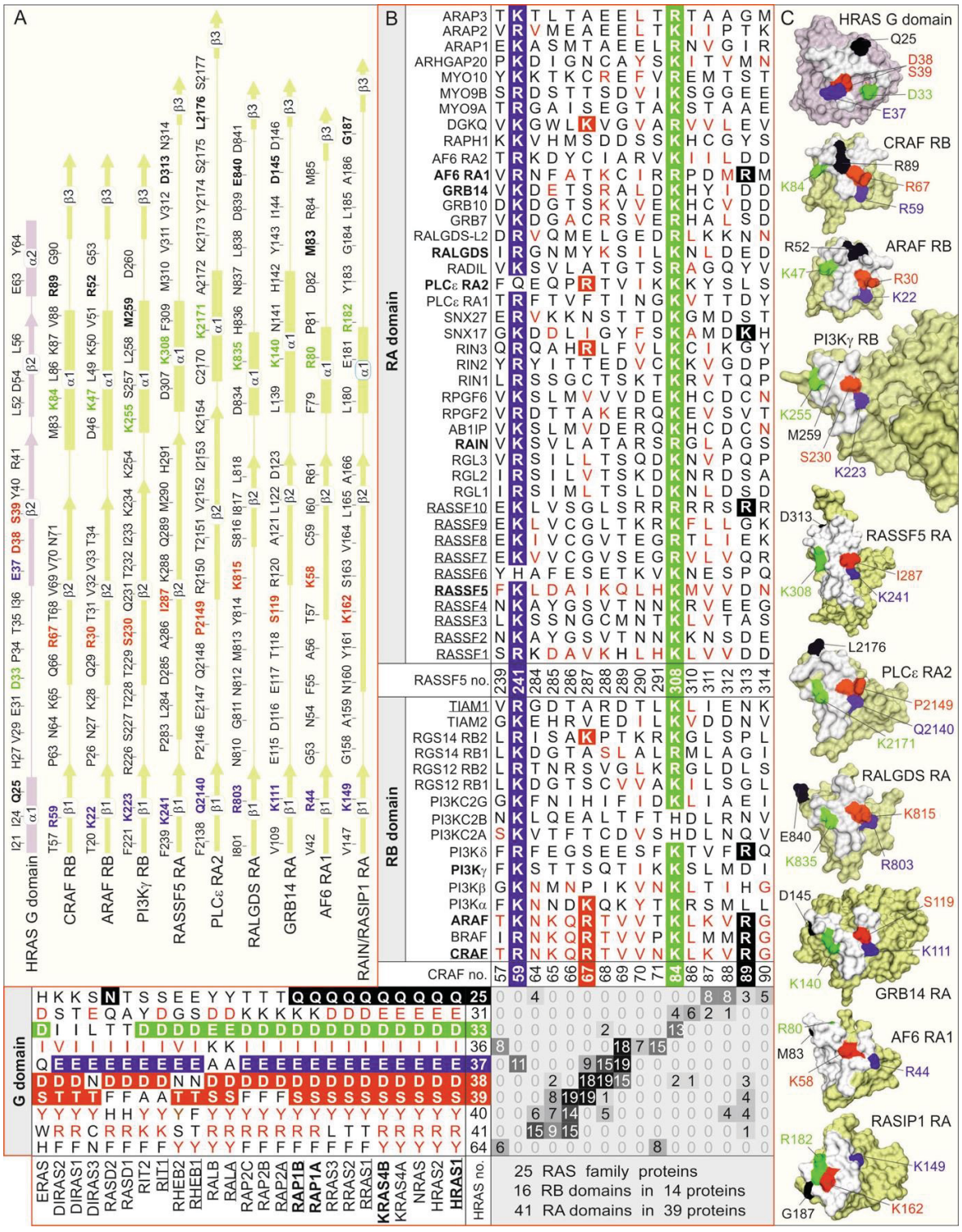

A
Relative polarization
Concentration (μM)
RAP2A/RASSF5
RRAS1/RASSF7
RIT1/RASSF9
RALA/RASSF1
B
HRAS
RRAS1
RIT1
RAP1B
RAP2A
RALA
RHEB
K_d (μM)
RASSF
CRAF
TIAM1
n.b.

FIGURE 3


A
HRAS G domain
CRAF RB
ARAF RB
PI3Kγ RB
RASSF5 RA
PLCε RA2
RALGDS RA
GRB14 RA
AF6 RA1
RAIN/RASIP1 RA
B
RA domain
RB domain
RASSF5 no.
CRAF no.
G domain
ERAS
DIRAS2
DIRAS1
DIRAS3
RASD2
RASD1
RIT2
RIT1
RHEB2
RHEB1
RALB
RALA
RAP2C
RAP2B
RAP2A
RAP1B
RAP1A
RRAS3
RRAS2
RRAS1
KRAS4B
KRAS4A
NRAS
HRAS2
HRAS1
HRAS no.
25 RAS family proteins
16 RB domains in 14 proteins
41 RA domains in 39 proteins
C
HRAS G domain
Q25
D38
S39
D33
E37
CRAF RB
R89
K84
R67
R59
ARAF RB
R52
K47
R30
K22
PI3Kγ RB
K255
M259
S230
K223
RASSF5 RA
D313
I287
K308
K241
L2176
PLCε RA2
P2149
Q2140
K2171
RALGDS RA
K815
E840
K835
R803
D145
S119
K111
K140
GRB14 RA
R80
AF6 RA1
M83
R44
K58
RASIP1 RA
R182
K149
G187
K162

Supplemental data

The binding selectivity of effectors for RAS proteins

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Table S1. Human proteins containing RAS association (RA) domain

No.	Entry	Protein name
1	Q9NS23	RASSF1, NORE2, PDA32
2	P50749	RASSF2, CENP34, RASFADIN
3	Q86WH2	RASSF3
4	Q9H2L5	RASSF4
5	Q8WWW0	RASSF5, RAPL, NORE1
6	Q6ZTQ3	RASSF6
7	Q02833	RASSF7, HRC1
8	Q8NHQ8	RASSF8, HOJ1
9	O75901	RASSF9, PCIP1, PAMCI
10	A6NK89	RASSF10
11	P55196	AF6, AFDN, MLLT4
12	Q12967	RALGDS, RALGEF, RGF, RGDS
13	O15211	RALGDSL2, RAB2L
14	Q9NZL6	RGL1
15	Q9BSI0	RGL2
16	Q3MIN7	RGL3
17	Q9Y4G8	PDZGEF1, RAPGEF2, RAGEF1
18	Q8TEU7	PDZGEF2, RAPGEF6, RAGEF2
19	Q9P212	PLCε1, PPLC, NPHS3
20	Q13671	RIN1, JC99
21	Q8WYP3	RIN2, JC265
22	Q8TB24	RIN3
23	Q5U651	RAIN, RASIP1
24	Q7Z5R6	RIAM, APBB1IP, PREL1, RARP1
25	Q96JH8	RADIL, RASIP2
26	Q14451	GRB7, B47
27	Q13322	GRB10, GRB-IR, Meg1, RSS
28	Q14449	GRB14
29	Q15036	SNX17
30	Q96L92	SNX27
31	Q70E73	RAPH1, PREL2
32	P52824	DGKQ
33	Q96P48	ARAP1
34	Q8WZ64	ARAP2
35	Q8WWN8	ARAP3
36	B2RTY4	MYO9A
37	Q13459	MYO9B
38	Q9HD67	MYO10

39	Q9P2F6	ARHGAP20

Table S2. Human proteins containing RAS-binding (RB) domain

No.	Entry	Protein name
1	P10398	ARAF, RAFA1, PKS
2	P15056	BRAF, NS7, p94
3	P04049	CRAF, CMD1NN, NS5
4	P42336	PI3Kα, p110α, CLAPO, CLOVE
5	P42338	PI3Kβ, p110β
6	P48736	PI3Kγ, ß110γ, PIK3
7	O00329	PI3Kδ, p110δ
8	O00443	PI3KC2A, PI3KC2α
9	O00750	PI3KC2B, PI3KC2β
10	O75747	PI3KC2G, PI3KC2γ
11	O14924	RGS12
12	O43566	RGS14
13	Q13009	TIAM1
14	Q8IVF5	TIAM2, STEF

Table S3. Proposed RAS effectors with no RA/RB domains

No.	Entry	Protein name	Reference
1	Q8IZJ4	RGL4	[1]
2	O95398	RAPGEF3, Epac1	[1, 2]
3	Q8WZA2	RAPGEF4, Epac2	[1, 3]
4	Q92565	RAPGEF5, Repac	[1]
5	O00522	KRIT1, Krit	[1, 4]
6	P19367	HK1	[5]
7	Q9BPZ7	SIN1, MAPKAP1	[6]
8	Q9BYB0	SHANK3	[7, 8]
9	Q9UPX8	SHANK2	[8]
10	Q8N9S9	SNX31	[9]
11	Q75LH2	FLJ10324	[10]

[1] Ibáñez Gaspar V, Catozzi S, Ternet C, Luthert PJ, Kiel C. Analysis of Ras-effector interaction competition in large intestine and colorectal cancer context. Small GTPases. 2020:1-17.

[2] Kiel C, Wohlgemuth S, Rousseau F, Schymkowitz J, Ferkinghoff-Borg J, Wittinghofer F, et al. Recognizing and defining true Ras binding domains II: in silico prediction based on homology modelling and energy calculations. Journal of molecular biology. 2005;348:759-75.

[3] Kiel C, Foglierini M, Kuemmerer N, Beltrao P, Serrano L. A genome-wide Ras-effector interaction network. Journal of molecular biology. 2007;370:1020-32.

[4] Wohlgemuth S, Kiel C, Krämer A, Serrano L, Wittinghofer F, Herrmann C. Recognizing and defining true Ras binding domains I: biochemical analysis. Journal of molecular biology. 2005;348:741-58.

[5] Amendola CR, Mahaffey JP, Parker SJ, Ahearn IM, Chen W-C, Zhou M, et al. KRAS4A directly regulates hexokinase 1. Nature. 2019;576:482-6.

[6] Schroder WA, Buck M, Cloonan N, Hancock JF, Suhrbier A, Sculley T, et al. Human Sin1 contains Ras-binding and pleckstrin homology domains and suppresses Ras signalling. Cellular signalling. 2007;19:1279-89.

[7] Chowdhury D, Hell JW. How CBP/Shank3 Guards Rap and H-Ras. Structure. 2020;28:274-6.

[8] Cai Q, Hosokawa T, Zeng M, Hayashi Y, Zhang M. Shank3 Binds to and Stabilizes the Active Form of Rap1 and HRas GTPases via Its NTD-ANK Tandem with Distinct Mechanisms. Structure. 2019.

[9] Ghai R, Bugarcic A, Liu H, Norwood SJ, Skeldal S, Coulson EJ, et al. Structural basis for endosomal trafficking of diverse transmembrane cargos by PX-FERM proteins. Proceedings of the National Academy of Sciences. 2013;110:E643-E52.

[10] The crystal structure of the RA domain of FLJ10324 (RADIL): http://www.rcsb.org/structure/3EC8.

Table S4. Human proteins containing RAS-related GTP-binding domain

No.	Entry	Protein name
1	P01112-1	HRAS1, p21HRAS
2	P01112-2	HRAS2, p19HRAS
3	P01111	NRAS
4	**P01116-1**	KRAS4A
5	**P01116-2**	KRAS4B, RASK2
6	Q7Z444	ERAS, KRAS2, HRASP
7	P11233	RALA
8	P11234	RALB
9	P10301	RRAS, RRAS1
10	P62070	RRAS2 TC21
11	O14807	RRAS3, MRAS
12	Q92963	RIT1, RIT, RIBB, ROC1
13	Q99578	RIT2, RIN, ROC2
14	P62834	RAP1A, KREV1
15	P61224	RAP1B
16	P61225	RAP2B
17	P10114	RAP2A
18	Q9Y3L5	RAP2C
19	Q15382	RHEB1
20	Q8TAI7	RHEB2
21	Q9Y272	RASD1, AGS1, DEXRAS1
22	Q96D21	RASD2, RHES, TEM2
23	O95057	DIRAS1, RIG, GBTS1
24	Q96HU8	DIRAS2
25	O95661	DIRAS3, ARHI, NOEY2, RHOI

Table S5. Dissociation constants determined for the RAS-effector interactions.

	HRAS	RRAS1	RAP1B	RAP2A	RALA	RHEB	RIT1
RASSF1	**52**	**33**	**26**	**22**	**18**	**37**	**136**
RASSF2	**147**	**122**	**67**	**47**	**167**	**44**	n.b.
RASSF3	**500**	**435**	**116**	**100**	**139**	**64**	n.b.
RASSF4	**193**	n.b.	**101**	**88**	**191**	**47**	**58**
RASSF5	**1.0**	**56**	**4.0**	**2.0**	**49**	**46**	n.b.
RASSF6	**91**	**112**	**65**	**53**	n.b.	**56**	**98**
RASSF7	**140**	**30**	**72**	**68**	**101**	**76**	**34**
RASSF8	n.b.	**114**	**66**	**67**	**115**	**102**	**76**
RASSF9	**179**	n.b.	**74**	**66**	n.b.	**143**	**27**
RASSF10	n.b.	**99**	**73**	**67**	n.b.	**150**	**55**
CRAF	**0.3**	**3.3**	**30**	n.b.	n.b.	**35**	**139**
TIAM1	**381**	**182**	**95**	n.b.	**190**	**89**	**250**

Dissociation constants (K_d values) were determined by evaluating the fluorescence polarization data (Figures S1, S2) shown in Figure 3 as bar charts. No binding (n.b.) stands for K_d values higher than 500 μM.

Table S6. Published structures of the RAS and Effector protein complexes.

Structures	PDB code	Res. (Å)	Ref.[a]
RB domains			
RAP1A-GTP-CRAF RB	1C1Y	2.2	[1]
RAP1A(E30D/K31E)-GppNHp-CRAF RB	1GUA	2.0	[2]
RAP1A(E30D/K31E)-GDP-CRAF RB(A85K/N71R)	3KUC	1.92	[3]
HRAS-GDP-CRAF-RB(A85K)	3KUD	2.15	[3]
HRAS-GppNHp-CRAF-RB	4G0N	2.45	[4]
HRAS(Q61L)-GppNHp-CRAF-RB	4G3X	3.25	[4]
KRAS-GppNHp-ARAF-RB	2MSE	NMR	[5]
HRAS(G12V)-GppNHp-PI3Kγ-RB(V223K/V326A)	1HE8	3.0	[6]
HRAS-GppNHp-Byr2-RB	1K8R	3.0	[7]
RA domains			
HRAS(D30E/E31K)-GppNHp-RASSF5-RA (L285M/K302D)	3DDC	1.8	[8]
HRAS(G12V)-GTP-GRAB14-RA/PH (K272A/E273A)	4K81	2.4	[9]
HRAS-GppNHp-RALGDS	1LFD	2.1	[10]
HRAS(G12V)-GTP-PLCε(Y2176L)	2C5L	1.9	[11]
HRAS-GppNHp-Afadin RA1	6AMB	2.5	[12]
RAP1B-GppNHp-Rasip1 RA	5KHO	2.78	[13]

[a] References are listed below.

References

[1] Nassar N, Horn G, Herrmann CA, Scherer A, McCormick F, Wittinghofer A. The 2.2 Å crystal structure of the Ras-binding domain of the serine/threonine kinase c-Raf1 in complex with RaplA and a GTP analogue. Nature. 1995;375:554.

[2] Nassar N, Horn G, Herrmann C, Block C, Janknecht R, Wittinghofer A. Ras/Rap effector specificity determined by charge reversal. Nat Struct Biol. 1996;3:723-9.

[3] Filchtinski D, Sharabi O, Rüppel A, Vetter IR, Herrmann C, Shifman JM. What Makes Ras an Efficient Molecular Switch: A Computational, Biophysical, and Structural Study of Ras-GDP Interactions with Mutants of Raf. Journal of Molecular Biology. 2010;399:422-35.

[4] Fetics Susan K, Guterres H, Kearney Bradley M, Buhrman G, Ma B, Nussinov R, et al. Allosteric Effects of the Oncogenic RasQ61L Mutant on Raf-RBD. Structure. 2015;23:505-16.

[5] Mazhab-Jafari MT, Marshall CB, Smith MJ, Gasmi-Seabrook GMC, Stathopulos PB, Inagaki F, et al. Oncogenic and RASopathy-associated K-RAS mutations relieve membrane-dependent occlusion of the effector-binding site. Proceedings of the National Academy of Sciences. 2015;112:6625-30.

[6] Pacold ME, Suire S, Perisic O, Lara-Gonzalez S, Davis CT, Walker EH, et al. Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma. Cell. 2000;103:931-43.

[7] Scheffzek K, Grunewald P, Wohlgemuth S, Kabsch W, Tu H, Wigler M, et al. The Ras-Byr2RBD complex: structural basis for Ras effector recognition in yeast. Structure. 2001;9:1043-50.

[8] Stieglitz B, Bee C, Schwarz D, Yildiz Ö, Moshnikova A, Khokhlatchev A, et al. Novel type of Ras effector interaction established between tumour suppressor NORE1A and Ras switch II. The EMBO journal. 2008;27:1995-2005.

[9] Qamra R, Hubbard SR. Structural Basis for the Interaction of the Adaptor Protein Grb14 with Activated Ras. PLoS One. 2013;8:e72473.

[10] Huang L, Hofer F, Martin GS, Kim SH. Structural basis for the interaction of Ras with RalGDS. Nat Struct Biol. 1998;5:422-6.

[11] Bunney TD, Harris R, Gandarillas NL, Josephs MB, Roe SM, Sorli SC, et al. Structural and Mechanistic Insights into Ras Association Domains of Phospholipase C Epsilon. Molecular Cell. 2006;21:495-507.

[12] Smith MJ, Ottoni E, Ishiyama N, Goudreault M, Haman A, Meyer C, et al. Evolution of AF6-RAS association and its implications in mixed-lineage leukemia. Nature communications. 2017;8:1099.

[13] Gingras AR, Puzon-McLaughlin W, Bobkov AA, Ginsberg MH. Structural basis of dimeric Rasip1 RA domain recognition of the Ras subfamily of GTP-binding proteins. Structure. 2016;24:2152-62.

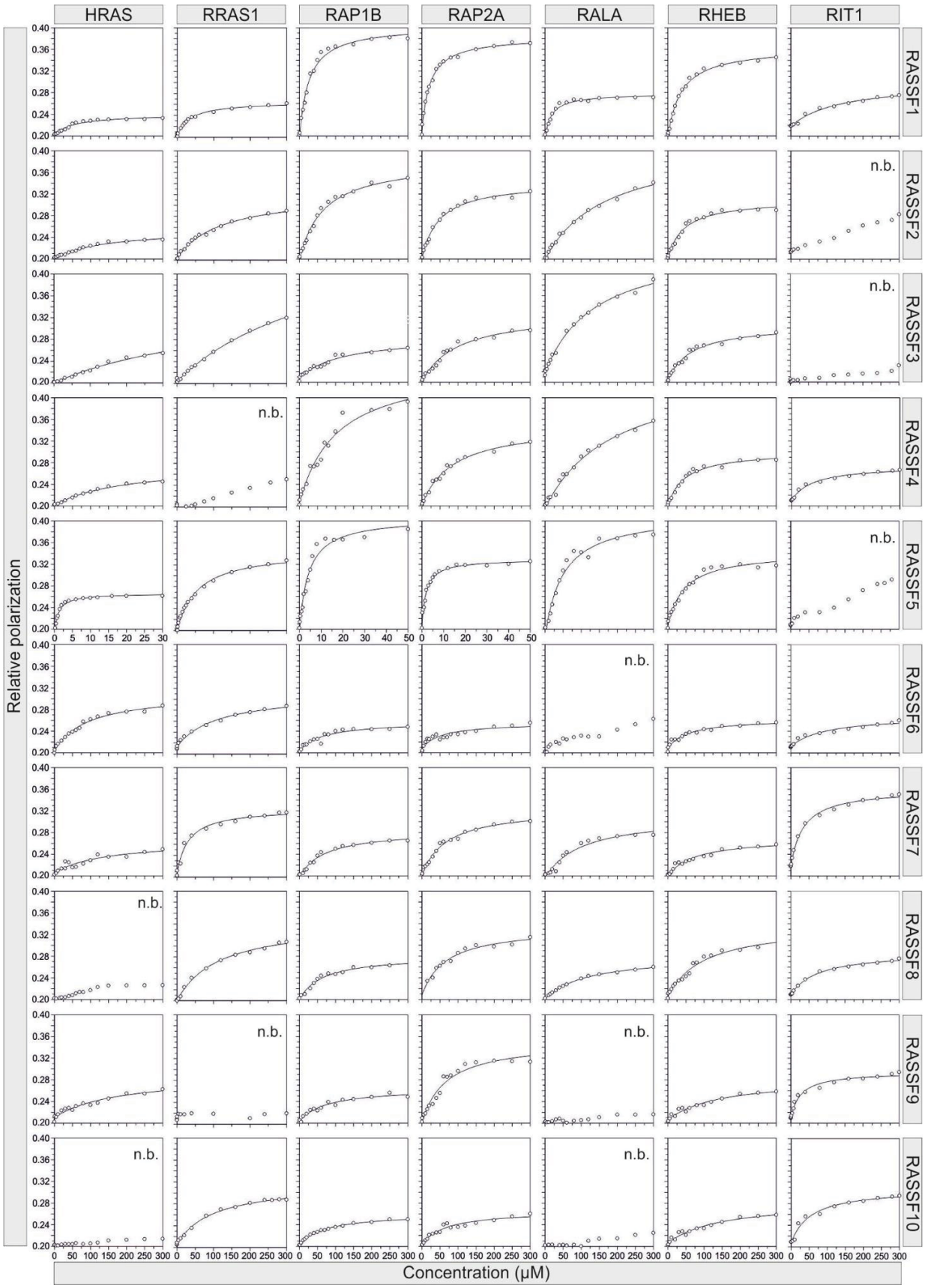
HRAS
RRAS1
RAP1B
RAP2A
RALA
RHEB
RIT1
RASSF1
RASSF2
RASSF3
RASSF4
RASSF5
RASSF6
RASSF7
RASSF8
RASSF9
RASSF10
n.b.
Relative polarization
Concentration (μM)

Figure S1. Fluorescence polarization measurements of RAS interactions with RASSF RA domains. Fluorescence polarization experiments were conducted to determine the dissociation constants (K_d) by titrating the active, mantGppNHp-bound form of RAS proteins (1 µM) with increasing concentrations of the respective effector domains. The X-axis represents the concentration of the effector domain as MBP fusion proteins in µM and Y-axis represents fluorescence polarization. The lines through the data points indicate that equilibrium K_d values have been determined for the respective measurements. The K_d values are summarized in Figure 3 and Table S5.

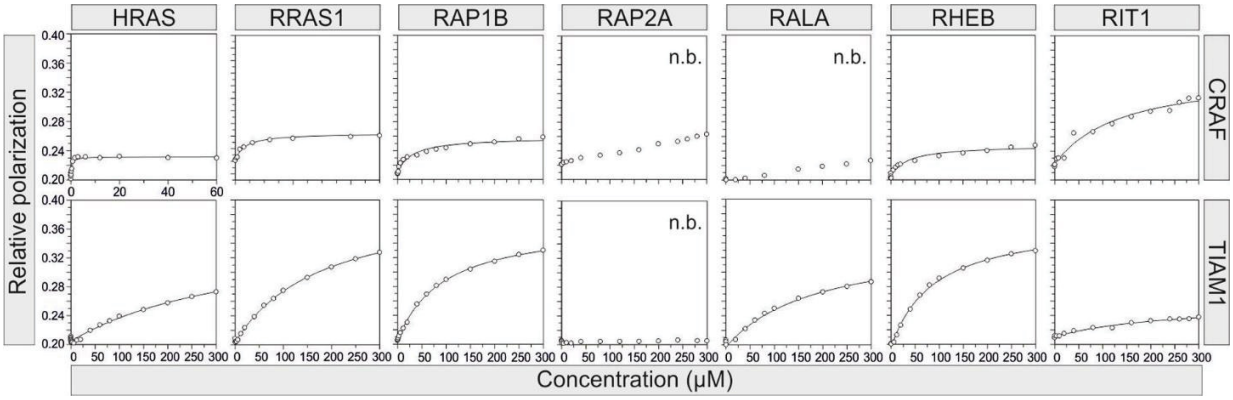

Figure S2. Fluorescence polarization measurements of RAS interactions with CRAF and TIAM1 RB domains. Fluorescence polarization experiments were conducted to determine the dissociation constants (K_d) by titrating the active, mantGppNHp-bound form of RAS proteins (1 μM) with increasing concentrations of the RB domains of CRAF and TIAM1. The X-axis represents the concentration of the effector domain as MBP fusion proteins in μM and Y-axis represents fluorescence polarization. The lines through the data points indicate that equilibrium K_d values have been determined for the respective measurements. The K_d values are summarized in Figure 3 and Table S5.

SIAS results: Seuqences identity of 9.2 % and similarity of 20.2 % for the RA domain of 41 proteins

Figure S3. Multiple sequence alignment of human RA domains. Amino acid sequences of 41 RA domains of 39 RA domain-containing proteins were aligned by using ClustalW and implemented in Bioedit with default multiple alignment parameters. Asterisks highlight RAS-binding amino acids of the respective effectors as indicated in red, green, magenta, blue, orange and purple. Underlined proteins were biochemically investigated in this study.

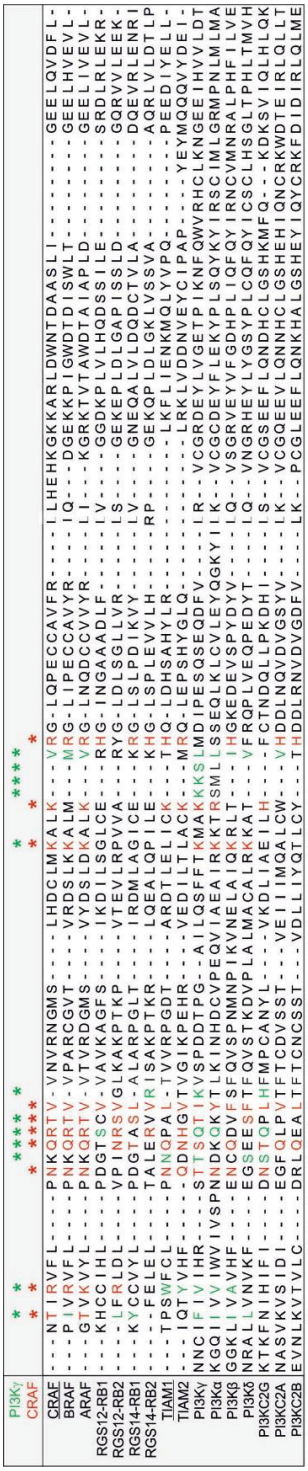

Figure S4. Multiple sequence alignment of human RB domains. Amino acid sequences of 16 RB domains of 14 RB domain-containing proteins were aligned by using ClustalW and implemented in Bioedit with default multiple alignment parameters. Asterisks highlight RAS-binding amino acids of the respective effectors as indicated in green and red. Underlined proteins were biochemically investigated in this study.

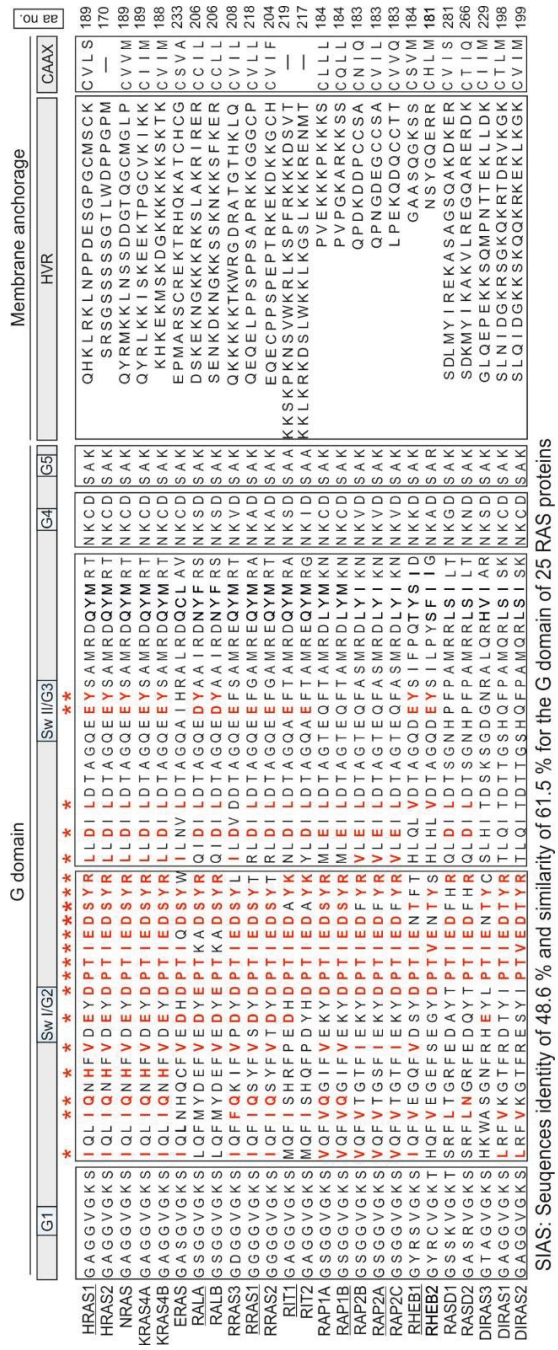

Figure S5. Multiple sequence alignment of human RAS protein family. Amino acid sequences of 25 RAS family proteins were aligned by using ClustalW implemented in Bioedit with default multiple alignment parameters. Asterisks highlight effector-binding amino acids as indicated in red. Conserved signatures of the RAS proteins critical for GDP/GTP binding, GTP hydrolysis and proteins interactions are represented as G1 (or P loop for phosphate binding and magnesium ion coordination), G2 (or switch I for magnesium ion coordination and γ-phosphate binding), G3 (or switch II for γ-phosphate binding containing the catalytic glutamine), G4 (major determinant of guanine base binding specificity) and G5 box (for guanine base binding). HVR (hypervariable region) and CAAX (C is cysteine, A is any aliphatic amino acid, and X is any amino

acid) are critical motifs for association with cell membrane. Underlined proteins were biochemically investigated in this study.

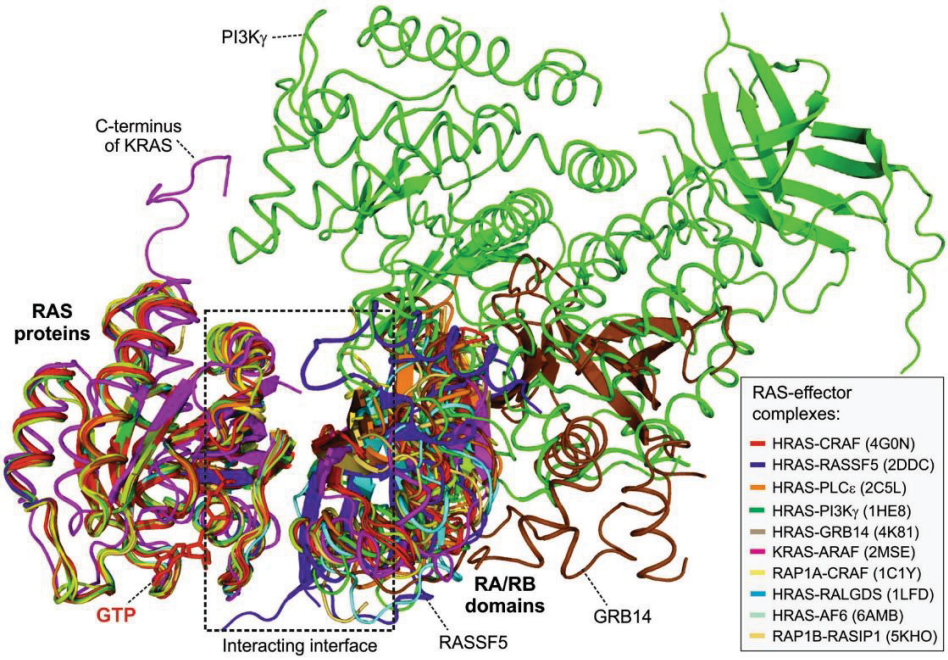

Figure S6. Superposition of all available RAS–effector complex structures. Ten structures of RAS-RA/RB domain complexes were overlaid in ribbon presentation. Additional properties outside the interaction interface (box) are indicated. For more details see Table S6.

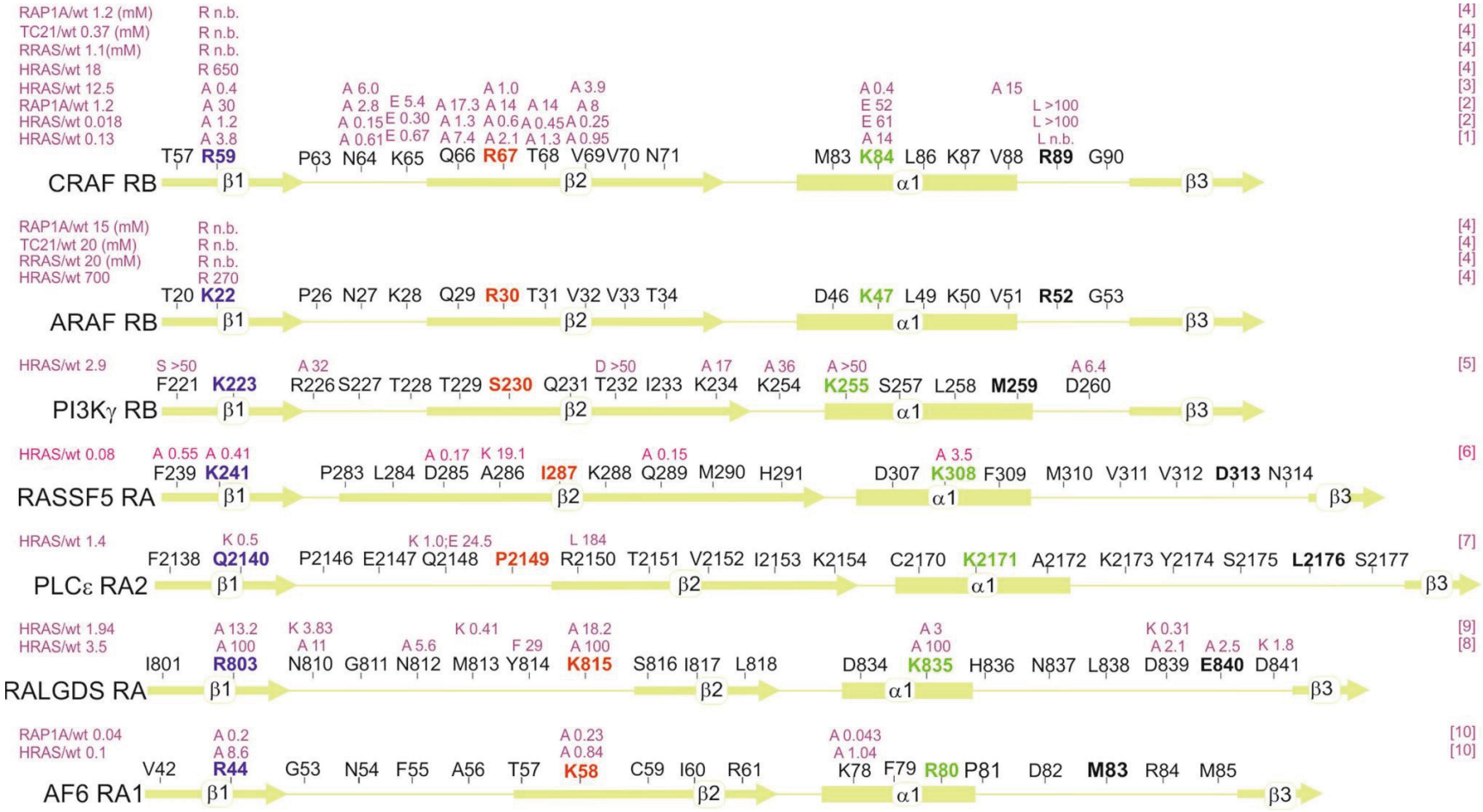
RAP1A/wt 1.2 (mM) R n.b. [4]
TC21/wt 0.37 (mM) R n.b. [4]
RRAS/wt 1.1(mM) R n.b. [4]
HRAS/wt 18 R 650 [4]
HRAS/wt 12.5 A 0.4 A 6.0 A 1.0 A 3.9 A 0.4 A 15 [3]
RAP1A/wt 1.2 A 30 A 2.8 E 5.4 A 17.3 A 14 A 14 A 8 E 52 L >100 [2]
HRAS/wt 0.018 A 1.2 A 0.15 E 0.30 A 1.3 A 0.6 A 0.45 A 0.25 E 61 L >100 [2]
HRAS/wt 0.13 A 3.8 A 0.61 E 0.67 A 7.4 A 2.1 A 1.3 A 0.95 A 14 L n.b. [1]
T57 R59 P63 N64 K65 Q66 R67 T68 V69 V70 N71 M83 K84 L86 K87 V88 R89 G90
CRAF RB β1 β2 α1 β3
RAP1A/wt 15 (mM) R n.b. [4]
TC21/wt 20 (mM) R n.b. [4]
RRAS/wt 20 (mM) R n.b. [4]
HRAS/wt 700 R 270 [4]
T20 K22 P26 N27 K28 Q29 R30 T31 V32 V33 T34 D46 K47 L49 K50 V51 R52 G53
ARAF RB β1 β2 α1 β3
HRAS/wt 2.9 S >50 A 32 D >50 A 17 A 36 A >50 A 6.4 [5]
F221 K223 R226 S227 T228 T229 S230 Q231 T232 I233 K234 K254 K255 S257 L258 M259 D260
PI3Kγ RB β1 β2 α1 β3
HRAS/wt 0.08 A 0.55 A 0.41 A 0.17 K 19.1 A 0.15 A 3.5 [6]
F239 K241 P283 L284 D285 A286 I287 K288 Q289 M290 H291 D307 K308 F309 M310 V311 V312 D313 N314
RASSF5 RA β1 β2 α1 β3
HRAS/wt 1.4 K 0.5 K 1.0;E 24.5 L 184 [7]
F2138 Q2140 P2146 E2147 Q2148 P2149 R2150 T2151 V2152 I2153 K2154 C2170 K2171 A2172 K2173 Y2174 S2175 L2176 S2177
PLCε RA2 β1 β2 α1 β3
HRAS/wt 1.94 A 13.2 K 3.83 K 0.41 A 18.2 A 3 K 0.31 [9]
HRAS/wt 3.5 A 100 A 11 A 5.6 F 29 A 100 A 100 A 2.1 A 2.5 K 1.8 [8]
I801 R803 N810 G811 N812 M813 Y814 K815 S816 I817 L818 D834 K835 H836 N837 L838 D839 E840 D841
RALGDS RA β1 β2 α1 β3
RAP1A/wt 0.04 A 0.2 A 0.23 A 0.043 [10]
HRAS/wt 0.1 A 8.6 A 0.84 A 1.04 [10]
V42 R44 G53 N54 F55 A56 T57 K58 C59 I60 R61 K78 F79 R80 P81 D82 M83 R84 M85
AF6 RA1 β1 β2 α1 β3

Figure S7. Dissociation constants (K_d) for the interaction of the RAS proteins with RB/RA effectors and their variants. Interacting amino acids and their corresponding variants along with determined K_d values are represented above the secondary structures of HRAS and the RA/RB domains, respectively. The numbers at the right side refer to the original studies listed below.

References

[1] Block C, Janknecht R, Herrmann C, Nassar N, Wittinghofer A. Quantitative structure-activity analysis correlating Ras/Raf interaction in vitro to Raf activation in vivo. Nature structural biology. 1996;3:244-51.

[2] Nassar N, Horn G, Herrmann C, Block C, Janknecht R, Wittinghofer A. Ras/Rap effector specificity determined by charge reversal. Nature structural biology. 1996;3:723.

[3] Rudolph MG, Linnemann T, Grünewald P, Wittinghofer A, Vetter IR, Herrmann C. Thermodynamics of Ras/effector and Cdc42/effector interactions probed by isothermal titration calorimetry. Journal of Biological Chemistry. 2001;276:23914-21.

[4] Weber CK, Slupsky JR, Herrmann C, Schuler M, Rapp UR, Block C. Mitogenic signaling of Ras is regulated by differential interaction with Raf isozymes. Oncogene. 2000;19:169-76.

[5] Pacold ME, Suire S, Perisic O, Lara-Gonzalez S, Davis CT, Walker EH, et al. Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase γ. Cell. 2000;103:931-44.

[6] Stieglitz B, Bee C, Schwarz D, Yildiz Ö, Moshnikova A, Khokhlatchev A, et al. Novel type of Ras effector interaction established between tumour suppressor NORE1A and Ras switch II. The EMBO journal. 2008;27:1995-2005.

[7] Bunney TD, Harris R, Gandarillas NL, Josephs MB, Roe SM, Sorli SC, et al. Structural and mechanistic insights into ras association domains of phospholipase C epsilon. Molecular cell. 2006;21:495-507.

[8] Vetter IR, Linnemann T, Wohlgemuth S, Geyer M, Kalbitzer HR, Herrmann C, et al. Structural and biochemical analysis of Ras-effector signaling via RalGDS. FEBS letters. 1999;451:175-80.

[9] Kiel C, Selzer T, Shaul Y, Schreiber G, Herrmann C. Electrostatically optimized Ras-binding Ral guanine dissociation stimulator mutants increase the rate of association by stabilizing the encounter complex. Proceedings of the National Academy of Sciences. 2004;101:9223-8.

[10] Kiel C, Wohlgemuth S, Rousseau F, Schymkowitz J, Ferkinghoff-Borg J, Wittinghofer F, et al. Recognizing and defining true Ras binding domains II: in silico prediction based on homology modelling and energy calculations. Journal of molecular biology. 2005;348:759-75.