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# New insights into signaling pathways controlling pluripotency and differentiation in human induced pluripotent stem cells

Dissertation

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Presented by
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# Neue Erkenntnisse über Signalwege zur Steuerung der Pluripotenz und Differenzierung in humanen induziert pluripotenten Stammzellen

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# Summary

Stem cells are a population of undifferentiated cells which are defined by their ability to differentiate into various cell types (potency) and continuous proliferation (self-renewal). There are different sources of stem cells based on their potency. Pluripotent stem cells (PSCs) include embryonic stem cells (ESCs) derived from the inner cell mass of blastocyst and induced pluripotent stem cells (iPSCs) reprogrammed from somatic cells. Human iPSCs (hiPSCs) are widely studied in recent years owing to their different applications in developmental biology and regenerative medicine. They have broad potentials especially in human disease modeling *in vitro* by starting with reprogramming cells from patients to hiPSCs containing the disease-causing mutation(s) followed by differentiating to disease-relevant cell types. In order to realize the potential of hiPSCs in clinical applications, it is crucial to first address fundamental questions regarding their molecular nature of pluripotency and the underlying intracellular signaling pathways which maintain the characteristics of these cells. This doctoral thesis explored the molecular mechanisms involved in the transition from pluripotency to differentiation with the focus on bFGF signaling in hiPSCs. Our results revealed the prominent role of RAS-MAPK pathway as a downstream target of bFGF in maintaining pluripotency in hiPSCs as compared to other pathways, such as PI3K/AKT and JNK, which remain unchanged during differentiation. Interestingly, p38MAPK and JAK/STAT3 pathways were activated upon differentiation. Further analysis of RAS isoforms, showed that NRAS is the link between bFGF receptor and MAPK pathway leading to hiPSCs pluripotency. It has been shown that mutations in genes of RAS-MAPK pathway can lead to a group of developmental disorders called RASopathies, such as Noonan syndrome (NS). One of the mutated genes in NS patients in RAS-MAPK pathway is RAF1. Patients with $RAF1^{S257L}$ point mutation frequently display pathological hypertrophic cardiomyopathy (HCM), however, the underlying molecular mechanism is poorly understood. Thus, we generated iPSCs from a patient carrying $RAF1^{S257L}$ mutant and differentiated them to cardiomyocytes. Interestingly, patient-derived cardiomyocytes recapitulated the HCM phenotype, such as cell size enlargement, expression of fetal genes, as well as an increased sarcomere protein synthesis and myosin heavy chain beta to alpha switch with an abnormal sarcomere structure, increased calcium transient and cardiac contractility. Signaling analysis also confirmed a higher MAPK activity in mutant cardiomyocytes. These findings indicate that an increased RAS-MAPK signaling pathway in $RAF1^{S257L}$ cardiomyocytes may regulate the observed HCM phenotype. Fragile X syndrome (FXS) represents another developmental disorder that is based on the loss of *FMR1* gene which produce fragile X mental retardation protein (FMRP). FMRP plays a critical role in chromatin regulation, RNA binding, mRNA transport, and translation in many cell types, including several types of stem cells. However, the underlying mechanisms including the cellular FMRP protein networks remain elusive. This thesis has explored novel FMRP interacting proteins and their interaction networks in multiple cellular processes, suggesting that FMRP is central in several biological processes in various cell types.

# Zusammenfassung

Stammzellen sind undifferenzierte Zellen, die kontinuierlich proliferieren (Selbsterneuerung) und in verschiedene Zelltypen differenzieren können (Omni- bzw. Pluripotenz). Innerhalb der Stammzellen unterscheidet man basierend auf ihrer Differenzierungspotenz verschiedene Subklassen. Pluripotente Stammzellen (PSCs) umfassen embryonale Stammzellen (ESCs), gewonnen aus der inneren Zellmasse von Blastozysten, sowieinduzierte pluripotente Stammzellen (iPSCs), die aus reprogrammierten somatischen Zellen generiert werden können. Humane iPSCs (hiPSCs) wurden in den letzten Jahren eingehend bezüglich verschiedener Anwendungsmöglichkeiten in der Entwicklungsbiologie und der regenerativen Medizin untersucht. hiPSCs zeigen ein besonders großes Potential im Modellieren humaner Erkrankungen *in vitro*. Durch die initiale Reprogrammierung patienteneigener Zellen zu Stammzellen, welche die krankheitsrelevante Mutation enthalten, können Differenzierungen in krankheitsrelevante Zelltypen durchgeführt und diese untersucht werden. Um das Potential von hiPSCs in klinischen Anwendungen zu untersuchen, ist es von größter Bedeutung zunächst die molekularen Mechanismen der Pluripotenz sowie die zugrundeliegenden intrazellulären Signalwege zu verstehen. Diese Dissertation untersucht den molekularen Mechanismus des Übergangs von Pluripotenz zur Differenzierung mit dem Fokus auf die bFGF induzierte Signaltransduktion in hiPSCs. Die gewonnenen Befunde sprechen für eine dominante Rolle des RAS-MAPK Signalwegs in der bFGF-induzierten Aufrechterhaltung der Pluripotenz. Interessanterweise zeigten auch andere untersuchte Signalwege, wie p38MAPK und JAK/STAT3, eine differenzierungsassoziierte Aktivierung. Eine Analyse der RAS-Isoformen lässt vermuten, dass NRAS die Verbindung zwischen dem bFGF-Rezeptor und dem MAPK-Signalweg darstellt und dadurch die Pluripotenz der hiPSCs aufrechterhält. Des Weiteren konnte gezeigt werden, dass Mutationen in Genen des RAS-MAPK Signalweges zu Entwicklungsstörungen (wie z.B. dem Noonan Syndrom) führen, welche kollektiv als RASopathien beschrieben werden. Eines der mutierten Noonan-assoziierten Gene ist RAF1. Individuen mit einer $RAF1^{S257L}$-Punktmutation entwickeln mitunter häufig eine pathologische hypertrophe Kardiomyopathie (HCM), deren zugrundeliegender molekularer Mechanismus kaum verstanden ist. Wir generierten daher Kardiomyozyten aus patientenspezifischen iPSCs mit einer $RAF1^{S257L}$-Mutation. Diese Kardiomyozyten spiegeln den Phänotyp der HCM wieder, gezeichnet durch Charakteristika wie insbesondere Zellvergrößerung, Expression fetaler Gene, Steigerung der sarkomeren Proteinsynthese, abnormale Sarkomerstruktur, Wechsel der schweren Myosin-Ketten von beta nach alpha, gesteigerte Kalziumtransienten, und erhöhte kardiale Kontraktilität. Analysen der Signaltransduktion bestätigten eine höhere MAPK-Aktivität in mutanten Kardiomyozyten. Diese Ergebnisse sprechen dafür, dass die gesteigerte RAS-MAPK-Signalwegintensität in $RAF^{S257L}$-Kardiomyozyten verantwortlich ist für den beobachteten HCM-Phänotyp. Das Fragile-X-Syndrom (FXS) ist eine weitere Entwicklungsstörung, die auf dem Verlust des *FMR1* Genproduktes FMRP (fragile mental retardation protein) basiert. FMRP spielt hierbei eine entscheidende Rolle in zentralen Vorgängen in verschiedensten Zelltypen, insbesondere Chromatin-Regulation, RNA-Bindung und -Stabilität, mRNA Transport, und Translation. Zugrundeliegende Mechanismen und das zelluläre FMRP Proteinnetzwerks sind jedoch größtenteils unverstanden. In dieser Arbeit wurden daher zahlreiche neue FMRP-bindende Proteine beschrieben und deren Interaktionsnetzwerk in verschiedenen zellulären Prozessen untersucht. Basierend auf den gewonnenen Daten lässt sich FMRP als zentrales Regulationsprotein in verschiedensten biologischen Prozessen unterschiedlicher Zelltypen einglie

# Abbreviations

| | |
|---|---|
| aNSC | adult neural stem cell |
| ASC | adult stem cell |
| ATF-2 | cyclic AMP-dependent transcription factor 2 |
| bFGF | basic fibroblast growth factor |
| BMP | bone morphogenetic protein |
| CFC | cardio-facio-cutaneous syndrome |
| CM | conditioned medium |
| CM-AVM | capillary malformation– arteriovenous malformation syndrome |
| CR | conserved region |
| CS | costello syndrome |
| DAG | diacylglycerol |
| ECC | embryonic carcinoma cell |
| eNSC | embryonic neural stem cell |
| EpiSC | mouse epiblast stem cell |
| ERK | extracellular regulated kinase |
| ESC | embryonic stem cell |
| FGF | fibroblast growth factor |
| FGFR | fibroblast growth factor receptor |
| FMRP | fragile X mental retardation protein |
| FRS2α | fibroblast growth factor receptor substrate 2α |
| FXS | fragile X syndrome |
| GAB1 | GRB2-associated-binding protein 1 |
| GAP | GTPase-activating protein |
| GDP | guanosine diphosphate |
| GEF | guanine nucleotide exchange factor |
| GoF | gain-of-function |
| gp130 | glycoprotein 130 |
| GPCR | G-protein coupled receptor |
| GRB2 | growth factor receptor bound protein 2 |
| GSC | germ line stem cell |
| GTP | guanosine triphosphate |
| GTPases | guanosine triphosphatase |
| HCM | hypertrophic cardiomyopathy |
| hEGC | human embryonic germ cell |
| hESC | human embryonic stem cell |
| HRAS | harvey rat sarcoma |
| HS | heparin/heparan sulfate |
| HSPG | heparin/heparan sulfate proteoglycan |
| ICM | inner cell mass |
| IGF-I | insulin-like growth factor-I |
| iMEF | irradiated mouse embryonic fibroblast |
| Ins(1,4,5)P3 | inositol-1,4,5 trisphosphate |
| IP3 | inositol trisphosphate |
| iPSC | induced pluripotent stem cell |
| JAK | janus kinase |
| JNK/SPAK | c-Jun N-terminal kinase/ stress activated protein kinase |
| KH0 | K homology 0 domain |
| KLF4 | kruppel-like factor 4 |
| KRAS | kirsten rat sarcoma |
| KSR | knockout serum replacement |

| | |
|---|---|
| LATS | large tumor suppressor kinase |
| LIF | leukemia inhibitory factor |
| MAPK | mitogen-activated protein kinase |
| MEK | MAP/ERK kinase |
| mESC | mouse embryonic stem cell |
| mRNA | messenger RNA |
| MST | mammalian sterile 20-like kinase |
| mTORC | mammalian target of rapamycin |
| MYBPC3 | cardiac myosin-binding protein C |
| MYH7 | muscle β-myosin heavy chain |
| NES | nuclear export signal |
| NF1 | neurofibromatosis type 1 |
| NF1-like | neurofibromatosis type 1-like syndrome |
| NFAT | nuclear factor of activated T cell |
| NFκB | nuclear factor kappa B |
| NLS | nuclear localization signal |
| NoLS | nucleolar localization signal |
| NRAS | neuroblastoma rat sarcoma |
| NS | noonan syndrome |
| NSML | noonan syndrome with multiple lentigines |
| OCT3/4 | octamer-binding transcription factor 3/4 |
| P-bodies | processing bodies |
| PDK1 | phosphoinositide-dependent kinase 1 |
| PH | pleckstrin homology |
| PI3K | phosphatidylinositol-3-kinase |
| PIP2 | phosphoinositide (4,5) bisphosphate |
| PIP3 | phosphoinositide (3,4,5) trisphosphate |
| PKB | protein kinase B |
| PKC | protein kinase C |
| PLCγ | phospholipase Cγ |
| PSC | pluripotent stem cell |
| PTB | phosphotyrosine binding |
| RALBP1 | RALA binding protein 1 |
| RALGDS | RAL guanine nucleotide dissociation stimulator |
| RAS | rat sarcoma |
| RASSF | RAS-association domain family |
| RBD | RAS binding domain |
| RBP | RNA binding protein |
| RHO | RAS homolog |
| RNP | cytoplasmic ribonucleoprotein |
| RTK | receptor tyrosine kinases |
| SGs | stress granules |
| SH2 | Src homology 2 |
| SHP2 | Src homology region 2-containing protein tyrosine phosphatase 2 |
| SOS | son of sevenless |
| SOX2 | (sex determining region Y)-box 2, |
| STAT3 | signal transducer and activator of transcription 3 |
| TGF-β | transforming growth factor-β |
| TNNT2 | cardiac muscle troponin T |
| Tud | tudor domain |
| UTR | untranslated region |

# Amino acid abbreviations

| | |
|---|---|
| Arg (R) | arginine |
| Ala (A) | alanine |
| Asn (N) | asparagine |
| Asp (D) | aspartic Acid |
| Glu (E) | glutamic Acid |
| Gly (G) | glycine |
| Leu (L) | leucine |
| Lys (K) | lysine |
| Phe (F) | phenylalanine |
| Pro (P) | proline |
| Ser (S) | serine |
| Thr (T) | threonine |
| Tyr (Y) | tyrosine |
| Val (V) | valine |

*Dedicated to my husband Balal*

# Chapter 1
## General introduction

# 1.1 Stem cells

## 1.1.1 Background

Stem cells are biological and special cells, found in almost all multicellular organisms, which are defined by two main properties; they have the ability to continuously proliferate (self-renewal) and differentiate into different cell lineages (potency) (Kolios and Moodley, 2013). There are two main categories of stem cells including embryonic stem cells (ESCs) and adult stem cells (ASCs).

The discovery of ESCs represented a major advance in cellular biology and medicine (Keller, 2005). Research on ESCs began when the very first stem cells, the embryonic carcinoma cells (ECCs), were established in the early 1970s (Friedrich *et al.*, 1983; Kleinsmith and Pierce, 1964). Later in 1981, Evans and Kaufman were able to successfully cultivate cell lines from inner cell mass (ICM) of mouse blastocysts called mouse ESCs (mESCs). They established culture conditions for growing pluripotent mESCs *in vitro* (Evans and Kaufman, 1981). In 1998, Thomson *et al.*, derived human ESCs (hESCs) from the ICM of normal human blastocysts donated by couples undergoing treatment for infertility. Cells were cultured for many passages as long as retaining their high levels of telomerase activity, maintaining normal karyotypes and expressing markers specific for typical hECCs (Thomson *et al.*, 1998). At the same time, Shamblott and his colleagues derived human embryonic germ cells (hEGCs) from the gonadal ridge from a five to nine week aborted fetus. hEGCs were cultured *in vitro* for approximately 20 passages, and maintained normal karyotypes (Shamblott *et al.*, 1998). Since then, techniques for deriving and culturing hESCs are being developed and refined.

## 1.1.2 Stem cells characteristics

Two hallmark features of stem cells are self-renewal and potency. Self-renewal is defined as the capacity of stem cells to divide symmetrically or asymmetrically and generate daughter stem cells with the exact developmental potential (Fig. 1). Under special conditions and signaling, a stem cell is able to exit self-renewal and start to differentiate into any cells from germ layers (Fig. 1) (Romito and Cobellis, 2016). This process is crucial for stem cells to maintain their pool after injury, expand their numbers during development and to retain in adult tissues. Defects in self-renewal can cause cancer, premature aging and developmental disease (He *et al.*, 2009).

Potency is referred to the ability of differentiation into any specialized and mature cell types (Fig. 1). Depending on the differentiation potential, various levels of potency are described, totipotent, pluripotent, multipotent, oligopotent and unipotent (Hima Bindu and Srilatha, 2011). Totipotent stem cells can differentiate into embryonic and extraembryonic cell types (Weissman, 2000). Pluripotent cells can differentiate into any cells that are derived from three germ layers (ectoderm, mesoderm and endoderm) and they are the descendants of totipotent cells. Multipotent stem cells can differentiate into a number of cells with a closely related family of cells, like adult stem cells such as hematopoietic stem cells (Zuk *et al.*, 2002). Oligopotent stem cells can differentiate into only a few cells, like lymphoid or myeloid stem cells. Unipotent cells can produce only one cell type of their own as spermatogonial stem cells, but these cells have the property of self-renewal, which distinguishes them from non-stem cells (Hima Bindu and Srilatha, 2011).

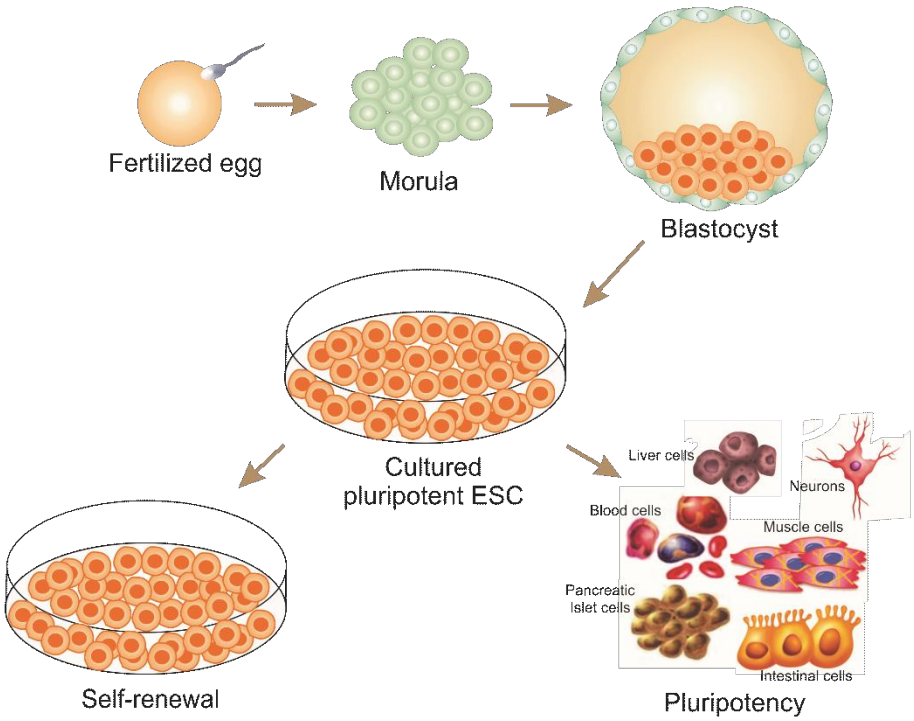



**Figure 1: Embryonic stem cells characteristics.**
Fertilization occurs when the sperm successfully enters and fuses with an egg and makes a zygote. The zygote divides through mitosis until the cell number reaches around sixteen and the solid sphere of cells is called a morula with totipotent cells. Then cells differentiate into outer layer and inner cell mass which is referred as blastocyst with pluripotent cells. ESCs are derived from the inner cell mass of blastocyst. They are able to go through numerous cycles of cell division while maintaining the undifferentiated state which is called self-renewal and upon stimulation they can differentiate into any specialized and mature cell types including blood cells, neurons, liver cells, muscle cells, pancreatic islet cells and intestinal cells.

### 1.1.3 Induced pluripotent stem cells (iPSCs)

ESCs were widely studied in recent years owing to their different applications. However, some complications have hindered their utilization such as difficulties in their derivation, immunological responses and ethical issues considering using them (Zhang *et al.*, 2011). The emergence of iPSCs has presented a breakthrough as an alternative for ESCs. iPSCs can be generated by a number of approaches like somatic cell nuclear transfer (Wilmut *et al.*, 1997; Tachibana *et al.*, 2013) and cell fusion (Tada *et al.*, 2001; Cowan *et al.*, 2005) from somatic cells. However, both methods were limited by low efficiency and ethical issues. Till 2006 and following 2007, Takahashi and his group were able to successfully induce pluripotency from mouse embryonic/adult and human adult fibroblasts by introducing four transcriptional factors OCT3/4, SOX2, c-MYC and KLF4 *via* ectopic expression using retroviral vectors (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007). At the same time, an American group also obtained iPSCs from human somatic cells with OCT4, SOX2, NANOG and LIN28 by using lentiviral vectors (Shi *et al.*, 2017). Using pluripotent cells reprogrammed from somatic cells solved efficiency, ethical and immunological issues. After Takahashi and Yamanaka innovation, many groups used these pioneering studies to generate pluripotent cells from somatic cells by using the same or other sources of cells with different inducing agents and vectors for delivery.

There are some differences between iPSCs and ESCs in gene expression signatures and epigenetic modifications (Chin *et al.*, 2009; Guenther *et al.*, 2010); however they share the same capacity of unlimited self-renewal and differentiation to any somatic cells and also they are similar in stemness marker expression, morphology and growth properties (Wang *et al.*, 2018).

### 1.1.4 Pluripotent stem cells applications

Over the last years, main progresses and discoveries in stem cell research were achieved by studying pluripotent stem cells including ESCs and iPSCs. The importance of ESCs can be divided to basic and biomedical research. In basic research, these cells represent the best model to study differentiation, function and the development of human tissues; they also provide different and early human cells that were previously almost inaccessible (Xu *et al.*, 2002). In biomedical research, ESCs are a powerful system to identify gene targets for new drugs and test the toxicity or teratogenicity of them and to study human genetic disease (Romito and Cobellis, 2016). They are promising tools for cell-based therapies in degenerative diseases (Kaji and Leiden, 2001) and transplantation of ESC-derived cells can replace cells which are damaged by various diseases such as Parkinson's disease, cardiac infarcts, juvenile-onset diabetes mellitus and leukemia (Amit *et al.*, 2000).

iPSCs have broad applications like ESCs especially in regenerative medicine. Soon after the development of the technology, hiPSCs were used to generate human disease *in vitro* models and used for drug screening for efficacy and potential toxicities. The advantage of hiPSCs in disease modeling include their human origin, expandability, accessibility, ability to differentiate into almost any cell types, no ethical concerns and the potential to develop personalized medicine using patient-specific iPSCs. Disease modeling by hiPSCs starts with reprogramming cells from patients to iPSCs containing the disease-causing mutation. These cells are then differentiated into disease-relevant cell types. The resultant cells are used to investigate the potential mechanisms involved in disease (Shi *et al.*, 2017).

So far, many diseases have been studied using a single disease-relevant cell type derived from iPSCs, such as RASopathies, Shwachman-Bodian-Diamond syndrome, adenosine deaminase deficiency-related severe combined immunodeficiency, Parkinson disease and Gaucher disease type III, they can be a perfect model for biological and pharmacological studies and drug screening (Gunaseeli *et al.*, 2010; Lee and Studer, 2010; Park *et al.*, 2008). The application of iPSCs as an *in vitro* model has been reviewed in different disease like neurogenetic disorders (Chamberlain *et al.*, 2008), iPS-derived cardiomyocytes in arrhythmic diseases (Tanaka *et al.*, 2009; Yokoo *et al.*, 2009) and they can also be a target in toxicology studies (Heng *et al.*, 2009).

iPSCs also showed a promising result in clinical study. In 2015, hiPSC-derived retinal pigment epithelial cells were used to treat macular degeneration (Kimbrel and Lanza, 2015), and it was reported that the treatment was successful to improve the patient's vision (Scudellari, 2016).

## 1.2 Signaling networks in embryonic stem cells

Pluripotency is a transient state during development that exits only for a short window of embryogenesis, but it can be recaptured *in vitro* by deriving ESCs or reprogramming somatic cells.

Shortly after the onset of embryogenesis, the cells within the embryo that are totipotent undergo differentiation and become either progenitor cells that later will form extra-embryonic tissues (the placenta and fetal extra-embryonic membranes) or become pluripotent cells which will form three germ layers from that all the tissues are generated.

Pluripotency consists of two distinct molecular states which is different according to the species (Kalkan *et al.*, 2017). Naïve pluripotency is the ground state of cells from the pre-implantation mESCs and primed pluripotency is the property of post-implantation mouse epiblast stem cells (EpiSCs) (Tesar *et al.*, 2007) and ESCs derived from ICM of a human embryo. EpiSCs and hESCs share many commonalities including a flat colony morphology, poor single-cell survival and dependent on activin A and fibroblast growth factor 2 (FGF2/bFGF), which proposed that human ESCs are also in a primed state (Li and Belmonte, 2017). Pluripotency is regulated by a highly interconnected gene regulatory network that is linked to a set of core pluripotency transcription factors (Fig. 2), the three most important ones are OCT4, SOX2 and NANOG (Li and Belmonte, 2017). This process is regulated by interaction between these genes and more aspects of regulation that include extrinsic factors and intrinsic signaling, chromatin regulators and regulatory RNAs (Fig. 2) (Pera and Tam, 2010; Li and Belmonte, 2017; Zhao and Jin, 2017).

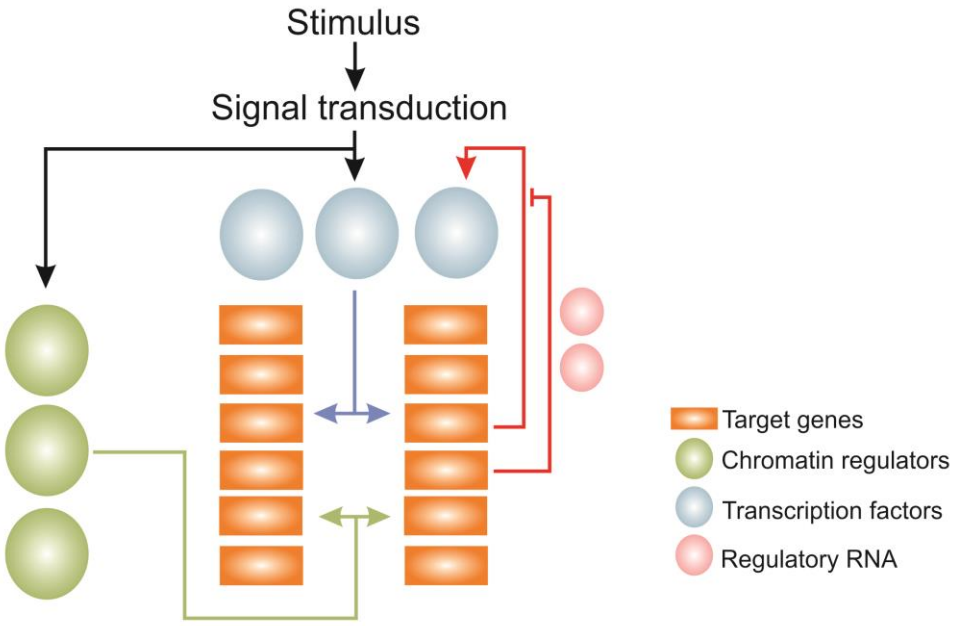



**Figure 2: Pluripotency regulation.**
The core of ESCs regulatory circuitry composed of many genes. The regulation of this gene program is a product of regulation by specific, chromatin-modifying enzymes from one hand, on the other side there are transcription factors which lead to transcription or silencing the target genes. There are also regulatory RNAs like miRNA which are a product of these program that can function at posttranscriptional levels and inhibit or activate some transcription factors. And on top of this highly interconnected network there are stimulus and signaling pathways which can positively or negatively regulate pluripotency.

### 1.2.1 The core pluripotency genes

Octamer-binding transcription factor 4 (OCT4; also known as POU5F1) which in human is coded by *POU5F1* gene, is involved in self-renewal and pluripotency *in vivo* and *in vitro* of stem cells and is expressed in ESCs and primordial germ cells (Scholer *et al.*, 1989; Nichols *et al.*, 1998). SRY (sex determining region Y)-box 2, also known as SOX2, is a transcription factor required for pluripotent epiblast formation and is a key regulator for OCT4 expression (Avilion *et al.*, 2003; Masui *et al.*, 2007). Loss of OCT4 and SOX2 expression will lead to trophectoderm differentiation, while the overexpression promotes mesendoderm and ectoderm differentiation (Niwa *et al.*, 2000; Thomson *et al.*, 2011). NANOG is the third important transcription factor in the core of pluripotency factors due to its role in the acquisition of pluripotency in the ICM (Mitsui *et al.*, 2003). OCT4, SOX2 and NANOG co-occupy hundreds of potential downstream targets promoters, including their own and make interconnected regulatory loops (Li and Belmonte, 2017) and pluripotency of ESCs and iPSCs is safeguarded by these three most important transcription circuitry.

### 1.2.2 Pluripotency-supporting signals

Among various signaling pathways, seven main signaling pathways have been reported to be involved in embryonic development (Brivanlou and Darnell, 2002), including JAK/STAT, NOTCH, NFκB, MAPK, PI3K/AKT, Wnt and TGF-β signaling (Dreesen and Brivanlou, 2007). The major signaling pathways modulating hESCs identity are described below.

**MAPK pathway**

MAPKs are Ser/Thr protein kinases which convert extracellular stimuli into a wide range of cellular responses. These Kinases regulate cell proliferation, differentiation, motility and survival in mammals. They comprise the ERK1/2, JNK, p38 and ERK5. Each group composed of a set of three evolutionarily conserved kinases; a MAPK, a MAPK kinase (MAPKK/MAP2K), and a MAPKK kinase (MAPKKK/MAP3K). The MAP3Ks are Ser/Thr protein kinases which are activated through phosphorylation or interaction with RAS/RHO family in response to extracellular stimuli. MAP3K activation leads to the activation and phosphorylation of a MAPKK, which then stimulates MAPK activity through dual phosphorylation on Thr and Tyr (Cargnello and Roux, 2011; Yoon and Seger, 2006).

One of the central components in the transmission network is the ERK cascade including RAF (MAP3K), MEK (MAP2K) and ERK (MAPK) (Yoon and Seger, 2006). RAF proteins are Ser/Thr kinases with three RAF isoforms CRAF (RAF1), ARAF and BRAF in mammalian. All of them contain an N-terminal RBD and C-terminal Ser/Thr kinase domain. Binding the N-terminal RBD of RAF kinase to RAS-GTP, brings RAF to the plasma membrane (Lavoie and Therrien, 2015), which leads to downstream signaling activation. RAF kinases phosphorylate S218/S222 and S222/S226 of MEK1/MEK2, respectively and phosphorylated and activated MEK1/2, phosphorylates position T202/Y204 of ERK1 and T185/Y187 of ERK2. ERK1/2 are the final kinases of this cascade which are triggered by RAS-GTP bound. ERK1/2, despite RAF and MEK kinase which have a limited number of substrate, possess approximately 200 cytoplasmic and nuclear targets (Yoon and Seger, 2006).

There have been conflicting reports on the role of MAPK/ERK signaling in hESCs but this pathway is generally considered to support hESCs self-renewal (Armstrong *et al.*, 2006; Li *et al.*, 2007), however a pro-differentiation role is also reported (Singh *et al.*, 2012).

**JNKs/SAPKs**

JNKs are cycloheximide-activated MAP2K regulate cell proliferation, embryonic development, inflammation, cytokine production, metabolism and apoptosis (Raman *et al.*, 2007), whether activation of JNK pathway leads to which process is based on the stimuli and the cell type (Fresno Vara *et al.*, 2004; Hennessy *et al.*, 2005). This signaling pathway can be activated by different stimuli such as growth factors (Vanhaesebroeck *et al.*, 2010), cytokines (Staal, 1987) and stress factors (Alessi *et al.*, 1996). JNKs also called SAPKs, have been first discovered in a c-Jun binding assay with extracts from UV-stimulated HeLa cells (Hibi *et al.*, 1993). In mammalian, three genes *JNK1*, *2*, *3* encoded the family of three isoforms (JNK1-3). *JNK1* and *JNK2* are widely expressed while *JNK3* is mainly confined to brain, heart and testis. JNKs have a docking domain in C-terminal and a glutamate/aspartate domain in their N-terminal which enable them to interact with upstream MAPKs and downstream targets (Haeusgen *et al.*, 2011). The main MAP2Ks upstream of JNK are MEKK4 and MEKK7, which phosphorylate JNK on the TPY motif within the activation loop with the preference of MEKK4 for tyrosine and of MEKK7 for threonine (Kishimoto *et al.*, 2003; Tournier *et al.*, 2001). Whereas MEKK7 is a specific activator of JNK, MKK4 can also phosphorylate p38 MAPK (Raman *et al.*, 2007). Monophosphorylation of JNKs on the threonine residue by MEKK7 is sufficient for enhancing its activity, while the additional phosphorylation of the tyrosine residue by MKK4 ensures optimal activation (Tournier *et al.*, 2001). After being phosphorylated and activated, JNKs phosphorylate some transcription factors such as c-JUN, ATF-2, p53, ELK-1and NFAT (Raman *et al.*, 2007). JNK signaling regulates apoptosis by phosphorylation of pro-apoptotic proteins (Dhanasekaran and Reddy, 2008). This pathway is also responsive to mitochondrial signals (Sehgal and Ram, 2013).

**P38MAPKs**

P38MAPKs are activated by a wide range of environmental stresses and inflammatory cytokines, and less by serum and growth factors. In mammals, there are four p38 MAPKs: α, β, γ and δ. p38α is the best characterized among all isoforms and is expressed in many cell types (Cuenda and Rousseau, 2007). Together with JNK family, p38 MAPKS are also known as SAPKs. The activation occurs *via* dual phosphorylation by MKK3 and MKK6 in the Thr–Gly–Tyr motif, in the activation loop (Kyriakis and Avruch, 2001). Upon activation, the phosphorylated p38 MAPK undergoes conformational changes which enhances access to substrate and increases enzymatic activity (Bellon *et al.*, 1999; Canagarajah *et al.*, 1997). p38 MAPK has both cytoplasmic and nuclear targets. In the cytoplasm, it phosphorylates other kinases like MNK1/2, while in the nucleus it can activate a large range of transcription factors such as ATF2, ELK-1, p53 and STAT1, which are involved in DNA damage response, apoptosis, inflammation, developmental processes and cellular proliferation (Cuadrado and Nebreda, 2010).

### PI3K/AKT pathway

PI3K/AKT is an intracellular signaling pathway that is crucial for metabolism, proliferation, cell survival and growth in response to extracellular signals (Hennessy *et al.*, 2005) which is mediated through phosphorylation of a wide range of downstream substrates. Key proteins involved in this signaling pathway are PI3K and AKT. PI3K constitutes a large family of lipid and Ser/Thr kinases, characterized by their ability to phosphorylate phosphatidylinositol and phosphoinositides (Hennessy *et al.*, 2005). Based on lipid substrate and sequence homology they are divided into three groups: class I, II and III. Upstream regulators of PI3K, mainly consist, RAS proteins, receptor tyrosine kinases and G protein-coupled receptors (Vanhaesebroeck *et al.*, 2010).

AKT or protein kinase B (PKB) is the human homologue of the viral oncogene *v-AKT* which is known to be responsible for a certain type of leukemia in mice (Fresno Vara *et al.*, 2004; Staal, 1987). There are three known AKT isoforms derived from distinct genes including AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ, which are closely related to each other with up to 80% homology in amino acid sequences. Each isoform has a pleckstrin homology (PH) domain which interacts with 3′-phosphoinositides and is contributing to recruitment of AKT to the plasma membrane (Fresno Vara *et al.*, 2004; Hennessy *et al.*, 2005). The subsequent kinase domain contains a threonine residue (T308) which is required for AKT activation after phosphorylation. Following the kinase domain, there is a hydrophobic C-terminal tail containing a second regulatory phosphorylation site (S473 in AKT1) (Fresno Vara *et al.*, 2004). Recruitment of AKT to the membrane results in a conformational change that exposes these two crucial amino acids, T308 is phosphorylated by constitutively active phosphoinositide-dependent kinase 1 (PDK1), whereas phosphorylation in S473 is mediated by PDK2 (Alessi *et al.*, 1996; Blume-Jensen and Hunter, 2001). Different potential PDK2s have been identified, including the mTOR rictor complex, integrin-linked kinase (ILK), PKCβII and even AKT itself (Kawakami *et al.*, 2004; Lynch *et al.*, 1999; Sarbassov *et al.*, 2005). Phosphorylation at T308 and S473 happens in response to extracellular stimuli and growth factors and it is essential for maximal AKT activation (Alessi *et al.*, 1996).

### JAK/STAT pathway

JAK/STAT pathway transduces a multitude of signals for development and homeostasis in animals, from humans to flies. Its activation stimulates cell proliferation, differentiation, cell migration and apoptosis (Rawlings *et al.*, 2004). JAKs are a family of intracellular tyrosine kinases which bind to the cytoplasmic regions of receptors (Blume-Jensen and Hunter, 2001). Upon ligand binding to the receptor, the receptor-associated JAKs are activated and in turn phosphorylate tyrosine residues in the cytoplasmic domain of the receptor. The phosphorylation provides a docking site for proteins with Src homology 2 (SH2) domains, one important class is STAT family (Lynch *et al.*, 1999). STAT proteins were first identified as cytoplasmic transcription factors, which were translocated to the nucleus upon JAK-mediated phosphorylation and dimerization (Kawakami *et al.*, 2004). In the nucleus, activated STAT can bind to consensus DNA-recognition motifs resulting in transcriptional activation (Sarbassov *et al.*, 2005). The mammalian STAT family comprises STAT1, 2, 3, 4, 5a, 5b and 6 (Cuenda and Rousseau, 2007), which an overall general structure that is organized into functional modular domains.

JAK/STAT pathway plays a central role in maintaining pluripotent state of mESCs (Smith *et al.*, 1988; Williams *et al.*, 1988). During *in vitro* culture, LIF binds to the heterodimeric cytokine receptor complex composed of LIF receptor b and gp130, resulting in the phosphorylation of gp130-associated JAK kinases and STAT3. pSTAT3 acts as a key factor, translocate to the nucleus and regulate the expression of downstream target genes which lead to maintenance of pluripotency in mESCs (Hirai *et al.*, 2011; Yoshida *et al.*, 1994). Despite the importance of LIF in mESCs culture, LIF does not support the pluripotency of hESCs when they are cultured in the absence of feeder cells and STAT1, 3 and 5 are not phosphorylated (Noggle *et al.*, 2005; Sato *et al.*, 2004). Consistent with these results, even addition of LIF to hESCs culture is not sufficient to maintain pluripotency. This contradiction between mouse and human ESC suggests that this pathway has different role in pluripotency in the two species.

## 1.3 bFGF in pluripotency

The family of fibroblast growth factor (FGF) consists of secreted signaling proteins (secreted FGF) that signal through receptor tyrosine kinases (RTK) and intracellular non-signaling proteins (intracellular FGF/iFGF) which are cofactors for voltage gated sodium channels. Secreted FGFs are widely expressed in almost all tissues and play essential roles in early stage of embryonic development, during organogenesis and in adults they serve as homeostatic factors which are important for metabolism, regeneration and tissue maintenance (Ornitz and Itoh, 2015). This group functions as autocrine or paracrine factors which are called canonical FGFs and endocrine factors. Canonical FGFs control cell proliferation, differentiation and survival and are tightly bound to heparin/heparan sulfate (HS) proteoglycans (HSPGs) that serves as cofactors to regulate the affinity and specificity for FGFR signaling (Fig. 3). Endocrine FGFs paly an essential role in regulating bile acid, carbohydrate, phosphate and lipid metabolism in addition to canonical FGFs functions and they require αKlotho, βKlotho or KLPH for receptor binding (Ornitz and Itoh, 2015).

The mammalian FGF family contains 5 subfamilies of canonical FGFs, one subfamily of endocrine FGF and one subfamily of iFGF, with 22 genes, 18 of which signal through FGF tyrosine kinase receptors. Subfamily of canonical FGFs are divided into FGF1, 4, 7, 8, 9 (Beenken and Mohammadi, 2009; Eswarakumar *et al.*, 2005; Ornitz and Itoh, 2001), from which FGF1 subfamily is of our interest.

The FGF1 subfamily consists of FGF1 and FGF2. These two growth factors lack secretory signal peptides but are readily exported by direct translocation across the cell membrane from cells (Prudovsky *et al.*, 2013). FGF2 which is also called basic FGF (bFGF), is a ubiquitously expressed FGF in various developmental stages. bFGF is a prototype member of the FGF family that is encoded by a single copy gene that produces one low (18-kDa) and four high (22-, 22.5-, 24-, and 34-kDa) molecular mass isoforms (Arnaud *et al.*, 1999; Delrieu, 2000). LMM bFGF signals through FGFRs (FGFR1-4) in a paracrine or autocrine manner with the highest affinity toward FGFR1 and 2 (Ibrahimi *et al.*, 2004a; Ibrahimi *et al.*, 2004b). bFGF signals by activating a smaller family of cell surface receptors called FGFRs.

FGFRs belong to the family of receptor tyrosine kinases of near 800 amino acids that are single-pass transmembrane receptors with three extracellular immunoglobulin-like domains (I, II, and III),

a transmembrane domain and two intracellular tyrosine kinase domains. Upon binding of the ligand to the extracellular domain of the receptor, the signal transduction will initiate which finally results in gene expression (Fig. 3) (Dailey *et al.*, 2005). The FGFR family consists of four genes encoding various FGFR isoforms by alternative splicing including FGFR1-4 (Dailey *et al.*, 2005; Eswarakumar *et al.*, 2005).

Signaling via FGFR, mediated through FGF binding, leads to receptor dimerization and activation of intrinsic tyrosine kinase and cause phosphorylation of multiple tyrosine residues on the receptors. These phosphorylation sites serve as docking sites for the recruitment of SH2 or PTB (phosphotyrosine binding) domains of signaling proteins (Eswarakumar *et al.*, 2005; Ornitz and Itoh, 2001). The best understood signal transduction activated by FGFs are RAS-MAPK including ERK1/2, JNK and p38, PI3K/AKT, JAK/STAT and phospholipase C (PLCγ) (Fig. 3) (Ornitz and Itoh, 2001; Dailey *et al.*, 2005). Phosphorylation of FRS2α activates RAS-MAPK and PI3K/AKT pathways. Activated FRS2α binds to GRB2 (growth factor receptor-bound 2) and tyrosine phosphatase SHP2 (Eswarakumar *et al.*, 2005; Beenken and Mohammadi, 2009). GRB2 activates RAS-MAPK through SOS and PI3K/AKT through GAB1 recruitment to the signaling complex (Fig. 3) (Beenken and Mohammadi, 2009; Prudovsky *et al.*, 2013).

Among the 22 FGF ligands, it is widely accepted that hESCs require exogenous bFGF to sustain self-renewal and the capacity to differentiate into a large number of somatic cell types (Xu *et al.*, 2001). bFGF maintains pluripotency either directly under feeder-free conditions supplemented with activin A (Zhao and Jin, 2017) or indirectly by stimulating irradiated mouse embryonic fibroblasts (iMEFs) to secrete activin A and other growth factors and cytokines necessary for hESCs pluripotency (Greber, 2011; Greber *et al.*, 2007; Levenstein *et al.*, 2006). Therefore, among many growth factors and cytokines which maintain pluripotency of hESCs and hiPSCs, bFGF was selected for monitoring downstream signaling pathways. All FGFR (1-4) are expressed in hESCs with specific pattern, with FGFR1 being the most abundant species and other receptors showing lower expression in the following order: FGFR1 > FGFR3 > FGFR4 > FGFR2 (Dvorak *et al.*, 2005).

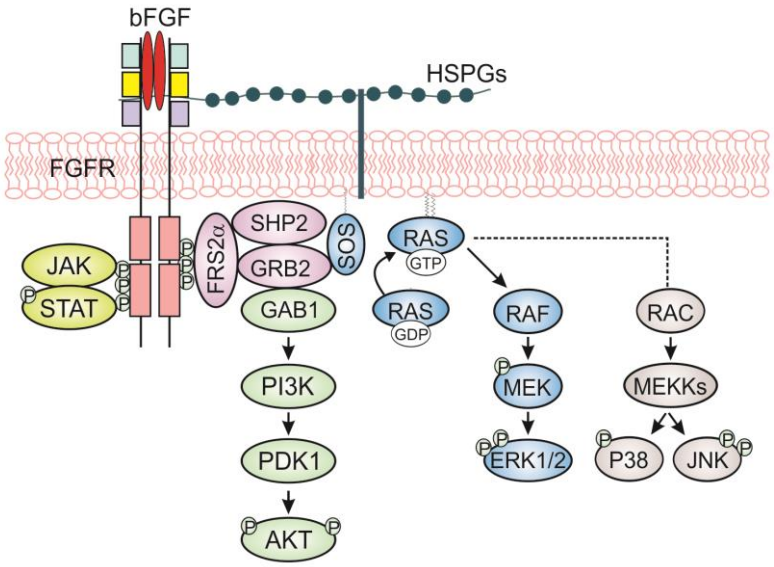



**Figure 3: Schematic view of signaling pathways downstream of bFGF.**
The FGF-FGFR signaling pathway is regulated at different levels. HSPGs act both as co-receptors and modulators of ligand bioavailability. Binding of bFGF to FGFR with HSPG induces the formation of ternary complex bFGF-FGFR-HSPG complex which activates FGFR intracellular tyrosine kinase domain by phosphorylation of specific tyrosine residues. Four main downstream pathways are MAPKs, JNK and p38 MAKPs, PI3K/AKT and JAK/STAT. The RAS-MAPK pathway: The major FGFR kinase substrate, FRS2$\alpha$ is phosphorylated by the activated FGFR kinase. Phosphorylated FRS2$\alpha$ recruits the adaptor protein GRB2, which then recruits the guanine nucleotide exchange factor SOS. The recruited SOS activates the RAS GTPase, which then activates the MAPK pathway. The PI3K/AKT pathway: The recruited GRB2 also recruits the adaptor protein GAB1, which then activates the enzyme PI3K, which then phosphorylates the enzyme AKT. The JAK/STAT pathway: FGFR kinase also activates JAK/STAT. This activated signaling pathway mostly regulates gene expression in the nucleus. bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; HSPG, heparan sulfate proteoglycans; FRS2α, fibroblast growth factor receptor substrate 2α; GRB2; growth factor receptor bound protein 2; SHP2: Src homology region 2-containing protein tyrosine phosphatase 2; SOS, son of sevenless; GAB1, GRB2-associated-binding protein 1; PI3K, phosphoinositide 3-kinase; PDK1, 3-phosphoinositide dependent protein kinase; JAK, Janus kinase; STAT, signal transducer and activator of transcription; MEK, MAP/ERK kinase; ERK, extracellular regulated kinase; JNK, c-Jun N-terminal kinases.

## 1.4 RAS superfamily at a glance

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). 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). RAS superfamily act as molecular switches cycling between a GTP-bound (active) and a GDP-bound (inactive) states (Wittinghofer and Vetter, 2011) and based on the sequence, structure and functional similarities they are divided into five major families: RAS, RHO, RAB, ARF and RAN (Wennerberg *et al.*, 2005). This superfamily plays a major role in signal transduction and transduces the signals from receptors at the membrane which regulate a variety of cellular processes. The RAS GTPases are involved in regulation of gene expression, cell proliferation, survival and differentiation. The RHO GTPases regulate actin organization and cytoskeleton. RAB and ARF GTPases play a role in vesicular trafficking, regulating endocytosis and

secretory pathways. RAN is involved in nuclear–cytoplasmic transport and mitotic spindle organization (Vigil *et al.*, 2010).

### 1.4.1 RAS family GTPases

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 (Nakhaei-Rad *et al.*, 2018). The most characterized RAS proteins are HRAS, KRAS and NRAS which 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). These three canonical RAS are highly conserved across different species and play significant roles in various cellular processes, including proliferation, differentiation, cell growth and cell death (Castellano and Santos, 2011).

### 1.4.2 RAS Effectors and signaling pathways

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. Activation of different transmembrane receptors, including receptor tyrosine kinases, 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 (Nakhaei-Rad *et al.*, 2018). 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 (Castellano and Downward, 2010; Cox and Der, 2003; Rajalingam *et al.*, 2007). More than 60 effectors reported for the RAS family proteins can activate about 49 pathways (Nakhaei-Rad *et al.*, 2018).

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 (Fig. 4) (Desideri *et al.*, 2015). 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 (Baljuls *et al.*, 2012; Jin *et al.*, 2006; Karbowniczek *et al.*, 2006; Mott and Owen, 2015; Self *et al.*, 2001; Wellbrock *et al.*, 2004).

The second best-characterized RAS effector family, PI3K (class I PI3K), phosphorylates phosphoinositide (4,5) bisphosphate (PIP2) and generates the second messenger phosphoinositide (3,4,5) trisphosphate (PIP3) that recruits the wide range of protein effectors through their 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 (Fig. 4) (Vanhaesebroeck *et al.*, 2001). HRAS1, NRAS, KRAS4B, ERAS, RRAS, and RAP1A activate PI3Ks (Nakhaei-Rad *et al.*, 2018).

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 (Fig. 4) (Ferro and Trabalzini, 2010).

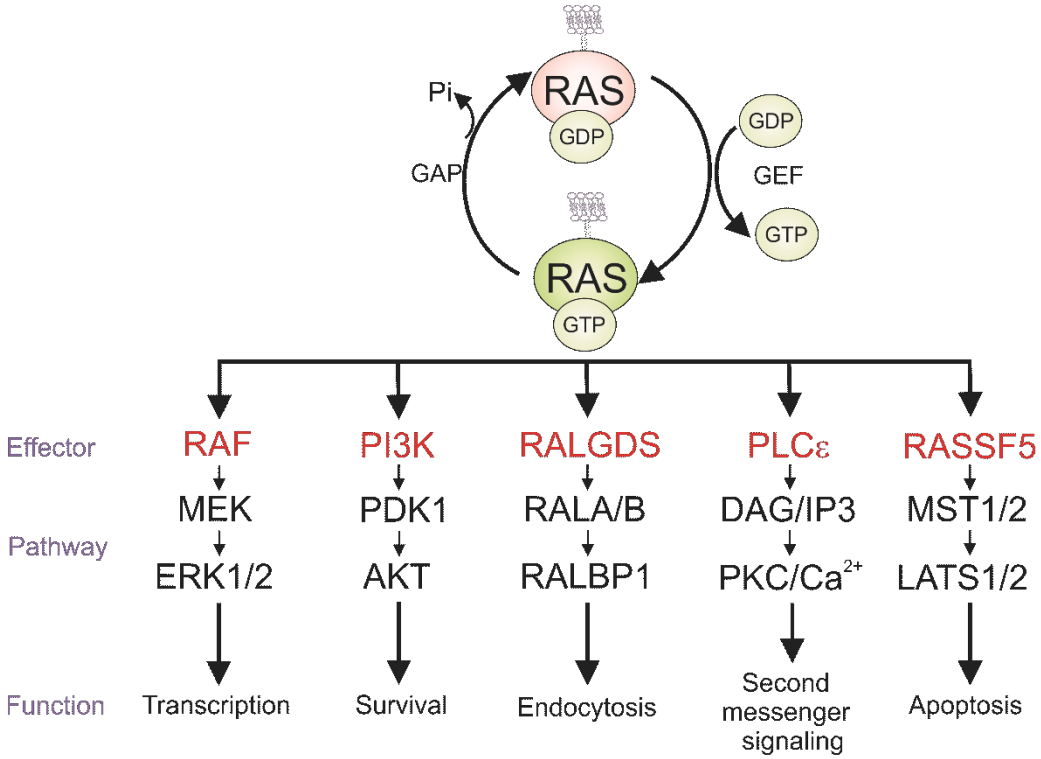



**Figure 4: Schematic representation of the RAS-GDP/GTP cycle and downstream signaling pathways.**
RAS proteins cycle between GDP and GTP form by two main regulatory proteins GEFs and GAPs. They only can transduce signal transduction when they are in a GTP form and bounded to the membrane by posttranslational modifications. Effector proteins of RAS-GTP are shown in red color and the downstream targets are in black. RAS, rat sarcoma; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; RALGDS, guanine nucleotide dissociation stimulator; RALBP1, RALA binding protein 1; PLCε, Phospholipase C ε; DAG, diacylglycerol; IP3, inositol trisphosphate; PKC, protein kinase C; RASSF5, RAS-association domain family; MST, mammalian sterile 20-like kinase; LATS, large tumor suppressor kinase.

## 1.5 RAS dysfunction 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, contribute to robust gain-of-function (GoF) effects and to various types of cancers as well as leukemia and lymphoma tumors (Simanshu *et al.*, 2017).

### 1.5.1 RASopathies

The RASopathies are a clinically defined group of developmental genetic disorders caused by germline mutations in genes that encode components or regulators of the RAS-MAPK pathway. RASopathies include noonan syndrome (genes encoding KRAS4B, NRAS, RRAS1/3, RIT1, SOS1, SOS2, RASGAP1M, CRAF, CBL), cardio-facio-cutaneous syndrome (KRAS4B, BRAF, ERK1/2), costello syndrome (HRAS1, HRAS2), neurofibromatosis type 1 (neurofibromin), legius syndrome (SPRED1), noonan syndrome with multiple lentigines or LEOPARD (BRAF, CRAF, SHP2), and capillary malformation/arteriovenous malformation syndrome (p120RASGAP) (Fig. 5) (Aoki *et al.*, 2016; Cao *et al.*, 2017; Flex *et al.*, 2014; Higgins *et al.*, 2017; Korf *et al.*, 2015; Lissewski *et al.*, 2015; Pantaleoni *et al.*, 2017; Rauen, 2013; Simanshu *et al.*, 2017; Tidyman and Rauen, 2016). Each RASopathy exhibit a unique phenotype but due to common underlying RAS-MAPK pathway dysregulation, they show various overlapping phenotypic features such as craniofacial dysmorphology, cardiac malformations, cutaneous, musculoskeletal, and ocular abnormalities, neurocognitive impairment, hypotonia and an increased cancer risk (Aoki *et al.*, 2016; Cave *et al.*, 2016; Gelb *et al.*, 2015; Lissewski *et al.*, 2015; Mainberger *et al.*, 2016; Simanshu *et al.*, 2017). Neurocognitive deficits and cardiac anomalies, particularly hypertrophic cardiomyopathy that is not necessarily present at birth and has no causal treatment, are among the health issues, which are most critical for life quality and expectancy in RASopathies (Wilkinson *et al.*, 2012).

### 1.5.2 Noonan syndrome (NS)

NS is a relatively common autosomal dominant developmental disorder that affects approximately 1 in 1,000–2,000 newborns. The principal features include congenital heart defects and hypertrophic cardiomyopathy, postnatally reduced growth, variable cognitive deficit, skeletal and hematologic anomalies an increased risk of developing cancer. This disorder is characterized by gain-of-function mutations in genes encoding components of the RAS-MAPK signaling pathway, such as PTPN11, SOS1, RAF1, KRAS, NRAS, SHOC2 and CBL (Fig. 5) (Tartaglia *et al.*, 2011). All of these genes harbor heterozygous germline mutations. The most common protein associated with NS is SHP2, encoded by *PTP11* gene, which accounts for approximately 50% of all cases (Tartaglia *et al.*, 2001). The second-most-common cause of NS is SOS1 missense mutations, accounting for approximately 15% of all cases (Roberts *et al.*, 2007; Tartaglia *et al.*, 2007). KRAS and NRAS mutations are a rare cause of NS and have been found in a very small number of individuals, respectively (Cirstea *et al.*, 2010; Schubbert *et al.*, 2006).

One of the mutated genes in RAS signaling is RAF1 as a downstream signal transducer. RAF1 mutations can be divided into three groups that affected three regions in the protein. The first group of mutations (70% of total RAF1 defects) occur in the N-terminal consensus 14-3-3 recognition sequence or adjacent residues. The second cluster (15% of RAF1 lesions) includes mutations which affect the activation region of the kinase domain (Asp486 and Thr491). The third group (15% of RAF1 mutations) occur at the two adjacent residues (Ser612 and Leu613) located at the C-terminal. These panel of RAF1 mutations differentially disrupt protein function and intracellular signaling (Tartaglia et al., 2011). Phenotype analysis of NS patients with RAF1 mutations (75% of cases) exhibit Hypertrophic cardiomyopathy (HCM). HCM is probably the major cause of unexpected death in patients with NS (Wilkinson *et al.*, 2012) and is characterized by an increase in left ventricular

wall thickness. Its pathophysiology is poorly understood, but there is evidence from *in vivo* models that aside from MAPK pathway also other RAS-dependent effector pathways are involved, and that pharmacological inhibition may prevent the myocardial changes (Dhandapany *et al.*, 2011; Marin *et al.*, 2011).

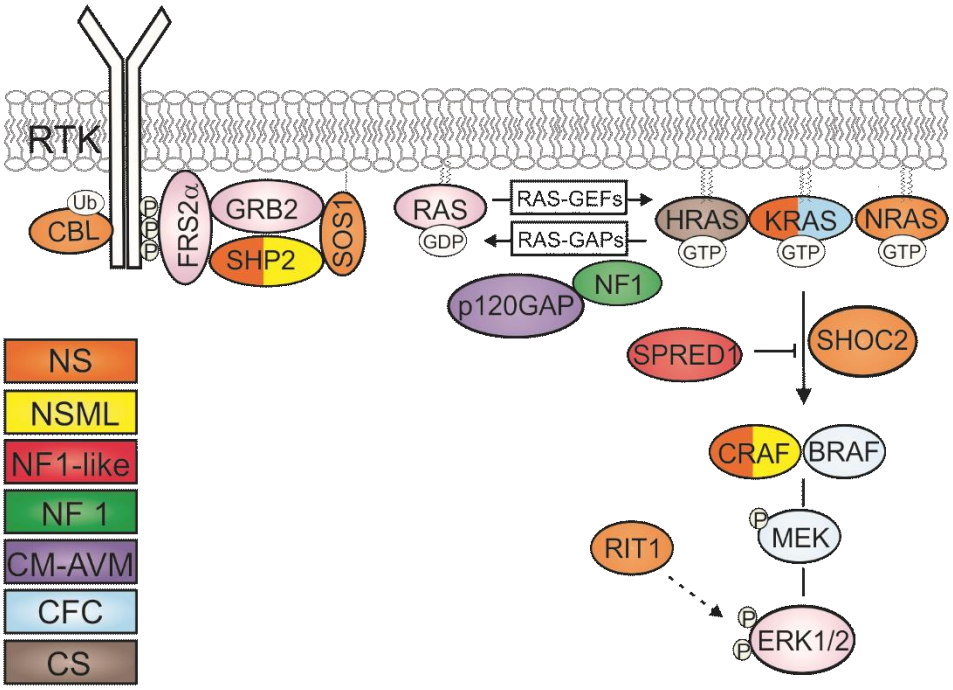



**Figure 5: Schematic view of mutated genes of RASophaties in RAS-MAPK pathway.**
RASopathies are a group of developmental disorders with overlying clinical features and characterized by germline mutations in genes that encodes for proteins involved in this pathway (illustrated in color code). This group of developmental disorders includes the following disorders: noonan syndrome (NS), noonan syndrome with multiple lentigines (NSML), NF1-like syndrome (NF1-like), neurofibromatosis type 1 (NF1), capillary malformation– arteriovenous malformation syndrome (CM-AVM), cardio-facio-cutaneous syndrome (CFC), and costello syndrome (CS). NF1, neurofibromin 1; SPRED1, sprouty-related, EVH1 domain containing protein.

### 1.5.3 Hypertrophic cardiomyopathy (HCM)

HCM is the most common inherited form of heart failures affecting up to 0.2% of the population, which is manifested as thickening of the left ventricular wall, contractile dysfunction and potentially fatal arrhythmias. The molecular events that lead to clinical phenotype of HCM is still unclear but mutations in more than 20 genes have been identified which elucidate the genetic basis of HCM. Most of these genes encode sarcomeric proteins including myosin 7 (also known as cardiac muscle β-myosin heavy chain; MYH7), cardiac myosin-binding protein C (MYBPC3) and cardiac muscle troponin T (TNNT2) (Frey *et al.*, 2011). As it has been mentioned earlier, NS patients according to the type of gene mutation, show different cardiac disease, such as HCM. In addition to the mutation in sarcomeric proteins, other stimuli can lead to cardiac hypertrophy that are divided into biomechanical and stretch-sensitive mechanisms which are associated with the release of growth

factors, cytokines, hormones and chemokines. These ligands are sensed by cardiac myocytes through different membrane-bound receptors such as RTK, GPCRs and gp130-linked receptors. These signaling directly leads to hypertrophic growth by changing gene expression, increasing protein translation and decreasing the rates of protein degradation. The important mediators of cardiac hypertrophy from proteins to signaling pathways are MAPK, NFAT, insulin-like growth factor-I (IGF-I), PI3K/AKT and mTOR (Heineke and Molkentin, 2006).

GPCRs are activated by angiotensin II, endothelin-1 and catecholamines, leads to generation of DAG which function as an intracellular ligand for PKC and its activation. PKC activation cause the production of inositol-1,4,5 trisphosphate which upon accumulation leads to the mobilization of internal $Ca^{2+}$ by directly binding to the Ins(1,4,5)P3 receptor located in the endoplasmic reticulum or the nuclear envelope. $Ca^{2+}$ storage mediates hypertrophic signaling through calcineurin–NFAT activation or calmodulin dependent kinase (CaMK)–HDAC inactivation (Wilkins and Molkentin, 2004).

In cardiac myocytes, MAPK signaling is initiated by different ligands such as IGF-1, TGF-β and cardiotrophin-1 binding to RTKs, GPCRs and gp130-linked receptors. Activates MAPKs including ERK, JNK and p38 phosphorylate multiple intracellular targets, including numerous transcription factors that induce the reprogramming of cardiac gene expression (Heineke and Molkentin, 2006). MEK/ERK signaling can induce cardiac hypertrophy by enhancing the transcriptional activity of NFAT (Sanna *et al.*, 2005).

## 1.6 Fragile X mental retardation protein (FMRP)

Genetic deficiency of the fragile X mental retardation protein (FMRP; also known as FRAXA, MGC87458, POF, POF1) results in the most common inherited form of intellectual disability, fragile X syndrome (FXS; also known as Escalante's syndrome or Martin–Bell syndrome) (Maurin *et al.*, 2014). It results from expansion of a CGG nucleotide repeat in the 5' untranslated region (UTR) of *FMR1* (Verkerk *et al.*, 1991) and the protein is ubiquitously expressed in different human cell types. During early embryonic development (0–14 days), FMRP is ubiquitously expressed similar to adult tissues. In the stage of day 15-19 of development, FMRP shows a specific pattern of expression, mainly in tissues from ectodermal lineage, such as brain, hair follicles, sensory cells and adrenal medulla (Bardoni *et al.*, 2001).

FMRP consists of an N-terminal domain containing two tudor (Tud) domains and one K homology 0 (KH0) domain, a central region containing two KH1 and KH2 domains, and a C-terminal domain containing a phosphorylation site (Bartley *et al.*, 2014) and an arginine-glycine-glycine (RGG) region (Fig. 6) (Myrick *et al.*, 2015). FMRP displays a nuclear localization signal (NLS), a nuclear export signal (NES) and two nucleolar localization signals (NoLSs) (Fig. 6) (Bardoni *et al.*, 1997; Feng *et al.*, 1997; Kim *et al.*, 2009; Taha *et al.*, 2014; Tamanini *et al.*, 1999), consequently localizing to different subcellular compartments in the cytosol and nucleus (Taha *et al.*, 2014). The N-terminus of FMRP harbors different protein binding characteristics due to various subdomains. Two conserved Tud1/2 domains (also called N-terminal domain of FMRP 1 and 2 or NDF1 and NDF2) (Ramos *et al.*, 2006; Taha *et al.*, 2014) are part of the royal family of proteins that also includes Agenet, MBT, PWWP, and chromo domains (Maurer-Stroh *et al.*, 2003). FMRP and Tud1/2 have

been shown to selectively associate with trimethyl-lysine peptides derived from histones H3K9 and H4K20 (Adams-Cioaba *et al.*, 2010; Ramos *et al.*, 2006) together with chromatin (Alpatov *et al.*, 2014). The N-terminus of FMRP has been proposed to be a platform for multiple protein-protein interactions (Ramos *et al.*, 2006) (Ramos *et al.*, 2006). A recent structure of the flexible $FMRP^{N\text{-}term}$ has revealed that this domain resembles a KH domain (Hu *et al.*, 2015) that is directly linked to the tandem KH domains of $FMRP^{central}$. KH domains are typical RNA and single strand DNA binding modules, which have been first described for the heterogeneous nuclear RNA-binding protein (hnRNP-)K (Nicastro *et al.*, 2015; Varelas *et al.*, 2008). $FMRP^{C\text{-}term}$ may apply for $FMRP^{N\text{-}term}$, i.e. its interactions may not all be direct protein-protein interactions but rather mediated via RNAs.

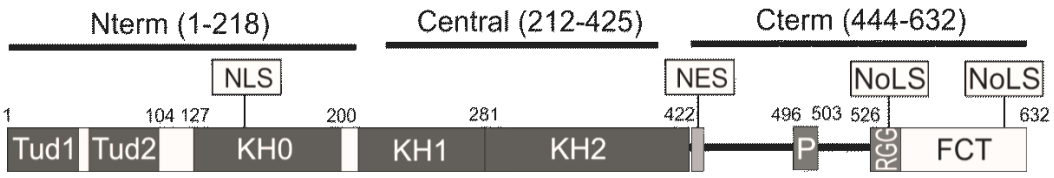



**Figure 6: Schematic diagram highlighting major domains and motifs of FMRP.**
FCT, FMRP C-terminus; KH0, KH1 and KH2, tandem K homology domain (first described for hnRNP K protein); NES, nuclear export signal; NLS, nuclear localization signal; NoLS, nucleolar localization signal; RGG, arginine-glycine-glycine region; P, phosphorylation sites; Tud1 and Tud2, tandem Tudor domains.

### 1.6.1 FMRP functions

FMRP has been described previously to be involved in different biological functions. The most prominent function of FMRP is regulation of translation. The mechanisms of translational regulation by FMRP are not entirely clear, although mounting evidence suggests that FMRP suppresses translation of its target mRNAs *via* association with either stalled, untranslating polyribosomes or microRNA (miRNAs) (Wang *et al.*, 2012a; Chen and Joseph, 2015; Irwin *et al.*, 2000; Kenny *et al.*, 2014). This can leads to the formation of cytoplasmic ribonucleoprotein (RNP) granules, which control the expression, repression, or decay of specific mRNAs (Alberti *et al.*, 2017). There are different types of cytoplasmic RNA granules, eukaryotic RNA processing bodies (P-bodies) and stress granules (SGs), which transport, store or degrade mRNAs, thereby indirectly regulating protein synthesis (Sfakianos *et al.*, 2016; Alberti *et al.*, 2017; Chyung *et al.*, 2018; El Fatimy *et al.*, 2016). There is an increasing evidence that such RNP granules are associated with several age-related neurodegenerative diseases (Maziuk *et al.*, 2017).

FMRP not only acts as an RNA binding protein (RBP) and local translational regulator for synaptic transmission (Ascano *et al.*, 2012; Brown *et al.*, 2001; Darnell and Klann, 2013; Darnell *et al.*, 2011; Fernandez *et al.*, 2013; Sakano *et al.*, 2017; Santoro *et al.*, 2012), but is also involved in the control of calcium channels (Ferron *et al.*, 2014), actin cytoskeletal dynamics (Billuart and Chelly, 2003; Nolze *et al.*, 2013; Schenck *et al.*, 2003), chromatin dynamics (Alpatov *et al.*, 2014), DNA damage response (DDR) (Alpatov *et al.*, 2014; Liu *et al.*, 2012), and replication stress response (Zhang *et al.*, 2014). These cellular functions presume physical properties for FMRP, which are

required not only for the recognition and localization of messenger RNA (mRNA) targets but also for direct association with a multitude of proteins and protein complexes (Pasciuto and Bagni, 2014a; Taha *et al.*, 2014).

### 1.6.2 FMRP and stem cells

As it mentioned above, stem cells are regulated by a complex mechanism to maintain their unique characteristics. Understanding stem cell regulation is critical to unlocking their therapeutic potential. Conversely, stem cells also give the opportunity to explore mechanisms of development, as well as developmental disorders, such as FXS. FMRP play significant roles in several types of stem cells, including GSCs, eNSCs, aNSCs, ESCs and iPSCs (Callan and Zarnescu, 2011). So understanding the role of FMRP in stem cell regulation is important for two reasons, first the post-translational function of FMRP likely plays a role in stem cell regulation and second, these cells provide a promising model to novel mechanisms and test potential treatments for FXS.

*FMRP and ESCs/iPSCs*, Eiges and his colleagues analyzed hESCs derived from a preimplantation FXS embryo to investigate the early events of *FMR1* gene inactivation. They showed that in undifferentiated FXS-ESCs with full expansion of CGG repeats, *FMR1* was expressed with acetylated promoter but in differentiated cells FMR1 gene was methylated. They showed for the first time that differentiation will trigged *FMR1* inactivation (Eiges *et al.*, 2007). Later, Telias et al. showed that during hESCs neural differentiation, FMRP expression had a steady upregulation, while FXS-hESCs could not upregulate FMRP during differentiation and exhibited aberrant expression of several neurogenesis markers. Although FXS-hESCs could differentiate to functional neurons, they had reduced synaptic connections (Telias *et al.*, 2013). FXS-hESCs are a prominent model for investigating the disease mechanism of FXS in human. The development of iPSCs technology made a revolution in human development and diseases. Despite intense interest, very few FXS-iPSC studies have been published. Urbach *et al.* reprogrammed fibroblasts from three FXS individuals to iPSCs and found that the *FMR1* gene remained transcriptionally silent and the promoter was methylated in all FXS-iPSCs. These data showed that there is a difference between hESCs and hiPSCs in FXS modeling and reprogramming has little effect on the silenced *FMR1* gene (Urbach *et al.*, 2010). Moreover, Sheridan et al. analyzed differentiation ability of FXS-hiPSCs to neurons and showed that FXS-iPSC differentiated neurons exhibited shorter neurites and fewer neurons, but more glia and also confirmed the lack of FMR1 gene reactivation in hiPSCs (Sheridan *et al.*, 2011). These studies prove how hPSCs have given us the opportunities to study FXS and related disorders in human systems and to investigate questions that cannot be answered using rodent models.

## Aims and objectives

The development of human embryonic stem cells opens new windows for basic research and regenerative medicine due to their two remarkable properties, self-renewal and pluripotency. A key goal in stem cell research is to identify the factors, which keeps human pluripotent stem cells (hPSCs) undifferentiated *in vitro* and differentiating later to mature functional derivatives. However, obtaining a clear and detailed view of how signaling pathways maintain pluripotency *in vitro* has been difficult to achieve due to some limiting factors including; disparate culture conditions, tools for evaluation of signal transduction pathways, their crosstalk and feedback loops. Basic fibroblast growth factor (bFGF) was the first factor found to be crucial for the maintenance of hPSCs *in vitro*. It promotes hPSCs self-renewal and pluripotency in two ways by directly activating RAS-MAPK and RAS-PI3K pathways and by indirectly stimulating autocrine effects. This thesis aimed at exploring and expanding in-depth the molecular mechanism of pluripotency with the focus of bFGF downstream signaling. Our data revealed that MAPK pathway appears to be the prime signaling pathway downstream of bFGF for maintaining pluripotency in hiPSCs (chapter II). Obtained knowledge about molecular properties and regulation of RAS GTPases is compiled in chapter III.

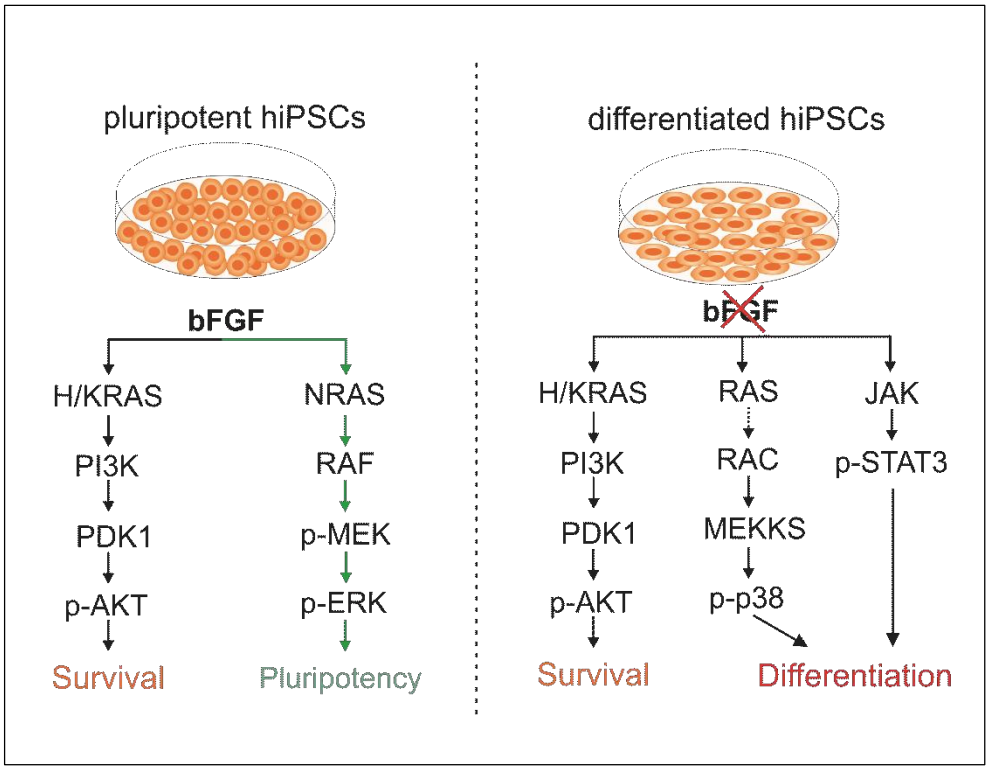
In addition to the role of RAS-MAPK pathway in maintaining pluripotency, dysregulation of this pathway causes a class of developmental syndromes called RASopathies. Noonan syndrome patients with $RAF1^{S257L}$ point mutation are frequently associated with pathological hypertrophic cardiomyopathy (HCM). Understanding the molecular mechanism of HCM, induced by $RAF1^{S257L}$, was another objective of this thesis. Therefore, patient-specific iPSCs, carried $RAF1^{S257L}$, were differentiated to cardiac myocytes and investigated the mechanism involved in HCM in details (chapter IV).

Another developmental disorder that can be investigated by the technology of hiPSCs is fragile X syndrome (FXS), with mutation in *FMR1* gene. The protein, FMRP, plays a critical role in chromatin regulation, RNA binding, mRNA transport, and translation and interestingly plays important regulatory roles in several types of stem cells. The underlying mechanisms, including the cellular FMRP protein networks, which has remained elusive, was another goal of this thesis. We explored multitudes of novel FMRP interacting proteins and described numerous novel FMRP interactors and networks, which are involved in diverse subcellular processes (chapter V).

# Chapter II

## bFGF-mediated pluripotency maintenance in human induced pluripotent stem cells is associated with NRAS-MAPK signaling

**bFGF maintains pluripotency in hiPSCs *via* NRAS-MAPK signaling pathway**

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# bFGF-mediated pluripotency maintenance in human induced pluripotent stem cells is associated with NRAS-MAPK signaling

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## Abstract

**Background:** Human pluripotent stem cells (PSCs) open new windows for basic research and regenerative medicine due to their remarkable properties, i.e. their ability to self-renew indefinitely and being pluripotent. There are different, conflicting data related to the role of basic fibroblast growth factor (bFGF) in intracellular signal transduction and the regulation of pluripotency of PSCs. Here, we investigated the effect of bFGF and its downstream pathways in pluripotent vs. differentiated human induced (hi) PSCs.

**Methods:** bFGF downstream signaling pathways were investigated in long-term culture of hiPSCs from pluripotent to differentiated state (withdrawing bFGF) using immunoblotting, immunocytochemistry and qPCR. Subcellular distribution of signaling components were investigated by simple fractionation and immunoblotting upon bFGF stimulation. Finally, RAS activity and RAS isoforms were studied using RAS assays both after short- and long-term culture in response to bFGF stimulation.

**Results:** Our results revealed that hiPSCs were differentiated into the ectoderm lineage upon withdrawing bFGF as an essential pluripotency mediator. Pluripotency markers OCT4, SOX2 and NANOG were downregulated, following a drastic decrease in MAPK pathway activity levels. Notably, a remarkable increase in phosphorylation levels of p38 and JAK/STAT3 was observed in differentiated hiPSCs, while the PI3K/AKT and JNK pathways remained active during differentiation. Our data further indicate that among the RAS paralogs, NRAS predominantly activates the MAPK pathway in hiPSCs.

**Conclusion:** Collectively, the MAPK pathway appears to be the prime signaling pathway downstream of bFGF for maintaining pluripotency in hiPSCs and among the MAPK pathways, the activity of NRAS-RAF-MEK-ERK is decreased during differentiation, whereas p38 is activated and JNK remains constant.

**Keywords:** bFGF, Differentiation, Induced pluripotent stem cells, MAPK, RAS, PI3K, Pluripotency

## Background

Embryonic stem cells (ESCs) are derived from the inner cell mass of human blastocyst [1], and represent promising tools in tissue engineering and cell therapy [2, 3]. What makes these pluripotent cells so valuable in developmental biology and regenerative medicine is their ability to differentiate into cell-types of different lineages both in vivo and in vitro. In order to realize the potential of ESCs in clinical applications, it is crucial to address fundamental questions regarding their molecular nature of pluripotency and the underlying intracellular signaling pathways which maintain the characteristics of these cells.

Various signaling pathways, including basic fibroblast growth factor (bFGF/FGF2), TGF-β/activin, WNT, EGFR family, insulin/IGF, PDGF, neurotrophin, integrin and NOTCH, participate in maintaining pluripotency in hESCs [4–12]. Among the 22 FGF ligands, it is widely accepted that hESCs require exogenous bFGF to sustain self-renewal and the capacity to differentiate into a large

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number of somatic cell types [13]. bFGF maintains pluripotency either directly under feeder-free conditions supplemented with activin A [14] or indirectly by stimulating irradiated mouse embryonic fibroblasts (iMEFs) to secrete activin A and other growth factors and cytokines necessary for hESCs pluripotency [15–17]. Therefore, among many growth factors and cytokines in maintaining pluripotency of hESCs and hiPSCs, bFGF was selected for monitoring downstream signaling pathways.

The stimulation of the bFGF receptors result in activation of various signaling pathways, including MAPK, PI3K/AKT and JAK/STAT [18]. The former two pathways are activated via RAS proteins, which control essential cellular processes, such as proliferation, differentiation, apoptosis, adhesion and migration and thus embryogenesis and normal development [19–21]. However, not much is known about the role of RAS proteins in regulating pluripotency or differentiation of hPSCs, especially hiPSCs. It has been shown that RAS proteins regulate the transition from naïve to primed ESCs in mice [22] and RAS nullyzygosity reduces the proliferation of mouse (m) ESCs and prohibits their differentiation [23].

In this study, we explored and expanded the molecular mechanism involved in the transition from pluripotency to differentiation with the focus on bFGF signaling in hiPSCs. We showed that, pluripotency markers were downregulated in hiPSCs following bFGF withdrawal and differentiated toward the ectoderm lineage. The MAPK pathway activity was significantly decreased, but interestingly no relevant changes were found in the activation of AKT or its downstream targets such as S6 kinase (S6K), FOXO-1, and JNK pathway. Investigating other signaling pathways downstream of bFGF revealed the activation of JAK/STAT3 and p38 as a result of differentiation. Moreover, we identified NRAS among the RAS paralogs as the likely link between bFGF receptor and the MAPK pathway that maintains hiPSCs undifferentiated.

## Methods

### Cell culture

Two clones of hiPSCs were generated by electroporating human foreskin fibroblasts (HFF, purchased from ATCC) with non-integrating episomal reprogramming vectors obtained from Addgene (pCE-hSK #41814, pCE-hOct3/4 #41813, pCE-hUL #41855, pCE-mp53DD #41856, pCXB-EBNA1 #41857) as previously described [24]. After 3–4 weeks, emerging hiPSC colonies were manually dissected under microscopic control and plated individually on mitotically inactivated (30 Gy gamma irradiation) iMEFs. Established clones were maintained as colonies on feeder layers in medium comprised of Dulbecco's Modified Eagle's Medium/Ham's F12 + GlutaMAX (DMEM/F12) (ThermoFisher, 31,331–028) supplemented with 20% knockout serum replacement (KSR) (ThermoFisher, 10,828,028), 1% non-essential amino acids (NEAA) (ThermoFisher, 11,140,035), 0.1 mM 2-mercaptoethanol (Millipore, ES-007.E), 25 ng/mL bFGF (Peprotech, 100-18B) and 50 units of penicillin/streptomycin (Genaxxon Bioscience, M3140.0100). The medium was changed every second day and colonies were passaged once per week using 0.4% (*w/v*) collagenase IV (ThermoFisher, 17,104,019). To initiate feeder-free cultures, almost confluent colony cultures were dissociated using Accutase (ThermoFisher, A1110501) and seeded onto Geltrex coated dishes (ThermoFisher, A14132–02, 1:400) at a seeding density of $2 \times 10^5$ cells/cm$^2$ with iMEF conditioned medium (CM, see below) supplemented with 100 ng/mL of bFGF and 10 μM Y27632 ROCK inhibitor (Selleckchem, S1049). After approximately 24 h, medium was exchanged with CM plus 100 ng/mL bFGF but without Y27632. Medium was replaced daily and confluent hiPSC monolayers were passaged every 3–4 days in the same manner using Accutase with seeding densities of $5 \times 10^4$ cells/cm$^2$. The CM was prepared as previously described [13]. Briefly, iMEFs were seeded at the density of $6 \times 10^4$ cells/cm$^2$ on precoated dishes with 1% gelatin (Sigma, G9391). One day after seeding, iMEFs were washed with PBS without calcium and magnesium (ThermoFisher, 10,010–015) and the medium was exchanged with DMEM/F12, 15% KSR, 1% NEAA, 0.1 mM 2-mercaptoethanol and 5 ng/mL bFGF. CM was replaced daily and collected for 7 days, filtered and aliquoted. HeLa and HFF cells were cultured in DMEM (ThermoFisher, 11,965,092), whereas NT2 cells were cultured in McCoy's media (ThermoFisher, 16,600,082), all supplemented with 10% FBS (ThermoFisher, 10,270–106) and 50 units of penicillin/streptomycin. Cell pellets from astrocytes were a gift from Dr. Boris Görg from the Heinrich-Heine University Düsseldorf.

### Long-term stimulation

In order to investigate the effect of bFGF and its downstream pathways on hiPSCs, cells were cultured under four different conditions; 100 ng/ml bFGF (CM-100) as the standard condition that was added to the medium each day freshly, 5 ng/ml bFGF (CM-5) as a lower concentration of bFGF, the conditioned medium without any bFGF (CM-0) and non-conditioned medium (non-CM) (as negative control) that contains only DMEM/F12, 15% KSR, 1% NEAA and 0.1 mM β-mercaptoethanol. For preparing CM-0, iMEFs were supplemented with DMEM/F12, 15% KSR, 1% NEAA, 0.1 mM β-mercaptoethanol without any bFGF and the CM-0 was collected daily for 7 days. Undifferentiated hiPSCs were seeded on Geltrex coated dishes with a density of $5 \times 10^4$ cells/cm$^2$ with CM-100 supplemented with 10 μM Y27632. The day after, cells were washed with PBS$^{-/-}$ and treated with CM-100, CM-5, CM-0 and non-CM and were kept in culture for 6 days. The medium was changed every day and at day 3, all cells were passaged by Accutase and seeded with the specific medium with the addition of

10 μM Y27632. Cells were harvested at the end of day 6 for RNA and protein level analysis.

#### Short-term stimulation

hiPSCs were seeded at a density of $5 \times 10^4$ cells/cm$^2$ and cultured until they reached 70–80% confluency and then washed two times with $PBS^{-/-}$ and incubated overnight (12 h) in DMEM/F12 deprived of bFGF and KSR. Cells were stimulated with 100 ng/ml bFGF for 15, 30, 60 and 120 min before preparation of cell lysates.

#### Reverse transcriptase polymerase chain reaction

The total RNA was extracted by the RNeasy Plus kit (Qiagen, Germany) according to the manufacturer's protocol. The quantity of the isolated RNA samples was analyzed by Nanodrop spectrophotometer. DNA-free™ DNA removal kit (Ambion, Life Technologies) was used to get rid of any possible contamination with genomic DNA. Complementary DNA (cDNA) was synthesized from DNase-treated RNA using ImProm-IITM reverse transcription system (Promega, Germany) and real-time PCR was performed using SYBR Green reagent (Life Technologies). *GAPDH* was used as an internal control. The $2^{-\Delta ct}$ method was used for estimating the relative mRNA expression levels. Primer sequences are listed in Additional file 1: Table S1.

#### Immunoblotting

Cell lysates were prepared using lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 1% Igepal CA-630, 10% glycerol, 20 mM β-glycerolphosphate, 1 mM $Na_3VO_4$, EDTA-free protease inhibitor (Roche Applied Science)), and protein concentrations were measured by Bradford assay (Bio-Rad). Equal amount of total cell lysates (t-p38/p-p38; STAT3/p-STAT3; t-JNK/p-JNK; t-RSK/p-RSK1: 40 μg; the remaining proteins: 10 μg) were loaded on SDS-PAGE. After electrophoresis, proteins were transferred into nitrocellulose membrane and blocked for one hour in 5% nonfat dry milk (Merck)/TBST (Tris-buffered saline, 0.05% Tween 20). Then membranes were probed with primary antibody at 4 °C overnight and later stained for one hour at room temperature with both horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5.000 dilution) and florescence secondary antibodies (1:10.000 dilution). Signals were visualized using ECL (enhanced chemiluminescence) reagent (GE Healthcare) and the Odyssey Fc Imaging System (LI-CORE Biosciences) respectively. The following antibodies were applied for immunoblotting: mouse anti-γ-tubulin (Sigma-Aldrich, T5326); mouse anti-OCT4 (Santa Cruz, sc-5279); rabbit anti-SOX2 (Invitrogen, PA1–16968); goat anti-NANOG (R&D systems, AF1997); mouse anti-SSEA4 (Millipore, MAB4304); mouse anti-α-SMA (DAKO, M0851); rabbit anti-GFAP (DAKO, Z0334); rabbit anti-MEK1/2 (#9126), rabbit anti-ERK1/2 (#9102), rabbit anti-RSK (#9355), rabbit anti-AKT (#9272), rabbit anti-p-MEK1/2 (S217/S221, #9154), rabbit anti-p-ERK1/2 (T202/T204, #9106), rabbit anti-p-p90RSK (T573, #9346), rabbit anti-p-AKT (S473, # 4060 and T308, #2965), rabbit anti-FOXO1 (#2880), rabbit anti-p-FOXO1 (S256, #9461), rabbit anti-S6 kinase (#2708), rabbit anti-p-p70 S6 kinase (T389, #9205), rabbit anti-p38 (#8690), rabbit anti-p-p38 (T180/Y182, #9211), rabbit anti-JNK (#9252), rabbit anti-p-JNK (T183/Y185, #9251), mouse anti-STAT3 (#9139S) and rabbit anti-p-STAT3 (Y705, #9145S) all from Cell Signaling.

#### Flow cytometry

For flow cytometric analysis, single-cell suspensions were obtained with Accutase and cells were washed with ice-cold $PBS^{-/-}$. Cells were fixed in 4% paraformaldehyde (PFA; Merck) for 10 min on ice and permeabilized with 90% ice-cold methanol for 15 min followed by a blocking step with 1.5% BSA and 2.5% goat or donkey serum diluted in PBS for 1 h at 4 °C. Cells were stained with primary antibodies overnight at 4 °C. Secondary antibodies, Alexa Fluor 488-conjugated goat anti-mouse IgG (A11029) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A21206) from Invitrogen, were used at a dilution of 1:2000 for one hour at room temperature. Samples were analyzed with FACS Canto II (BD Pharmingen) and FlowJo Software (Treestar, Ashland, OR).

#### Cell fractionation

Simple fractionation was performed as previously described [25]. Briefly, cells were washed with ice-cold PBS. 1 ml lysis buffer (without Igepal CA-630) was added to cells following centrifugation for 10 s, at 12000 rpm and 4 °C. The supernatant was removed and the pellet was resuspended in 900 μl ice-cold lysis buffer with 0.1% NP40 and was kept on ice for 2 min. 300 μl was taken as total cell lysate (TCL) and 100 μl of 4x Laemmli buffer was added to it. The rest of the supernatant was centrifuged and 300 μl was taken as cytosolic fraction (Cyt) following adding 100 μl of 4x Laemmli buffer. The remaining pellet was resuspended in 1 ml ice-cold lysis buffer with 0.1% NP40 and centrifuged again. The pellet was resuspended in 380 μl 1x Laemmli buffer and kept as nuclear fraction (Nuc). TCL and Nuc were sonicated at level 2 for 5 s and all fractions were boiled for 10 min at 95 °C. 20 μl from each fraction was loaded on SDS-PAGE. GAPDH (Cell signaling, #2118) and $Na^+/K^+$ ATPase (Sigma, A276) were used as cytosolic markers and histone H3 (Cell Signaling, #9715) and lamin B1 (Abcam, 16,048) were subjected as nuclear markers.

#### Immunostaining

Cells were seeded on Geltrex-coated coverslips with the density of $5 \times 10^4$ cells/cm$^2$. Cells were washed with PBS containing magnesium/calcium two times and fixed with

4% PFA for 20 min at room temperature. To permeabilize cell membranes, cells were incubated in 0.25% Triton X-100/PBS for 5 min. Blocking was performed by using 3% BSA in PBS for one hour, room temperature. Incubation with primary antibodies was performed overnight at 4 °C following three times washing steps with PBS and then incubation with secondary antibodies Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 488-conjugated donkey anti-rabbit IgG, Alexa Fluor 488-conjugated donkey anti-goat IgG (A11003) all from Invitrogen, used at a dilution of 1:500 for two hours at room temperature. Slides were washed three times and then stained with 4′,6-diamidino-2 phenylindole (DAPI) (Life Technologies) for five minutes and washed again for two times. ProLong Gold antifade was applied to mount coverslips. Confocal images were obtained using a LSM 510-Meta microscope (Zeiss, Jena, Germany).

### Pull-down assay

The RAS-binding domains (RBD) of effector proteins, including CRAF-RBD (a.a. 51–131) and PI3K-RBD (a.a. 127–314) were constructed as GST-fusions in pGEX-4 T and transformed in *Escherichia coli*. GST-fused proteins were obtained from total bacterial lysates. Glutathione Agarose 4B beads (Protino®) were coated with GST-fused CRAF-RBD and PI3K-RBD and GTP-bound RAS proteins were pulled down from total cell lysates and were probed by western blot. RAS paralogs were detected by validated antibodies: mouse anti-pan-RAS (Millipore, #05–516), mouse anti-KRAS (Sigma, WH0003845M1), mouse anti-NRAS (Santa Cruz, sc-31) and rabbit anti-HRAS (Santa Cruz, sc-520).

### Statistical analysis

All assays were carried out in three independent experiments in duplicates and triplicates. Data were analyzed by one-way analysis of variance (ANOVA). Differences in treatment levels were further evaluated for significance with Tukey post hoc comparisons. A level of $P < 0.05$ was considered significant. Statistical analysis was performed using SPSS software (SPSS v.20). Values are expressed as mean ± SD.

## Results

### Undifferentiated state of hiPSCs

To ensure stable conditions, hiPSCs were expanded on Geltrex-coated dishes with conditioned medium (CM) from iMEF supplemented with 100 ng/ml bFGF (see methods). We first analyzed the expression of pluripotency and differentiation markers at the mRNA and protein levels. Confocal imaging of hiPSCs revealed the presence of OCT4, SOX2, NANOG and SSEA4 and the absence of differentiation markers α-SMA (mesoderm) and GFAP (ectoderm) [26] in hiPSCs (Fig. 1a). Accordingly, flow cytometry data showed that more than 98% of the cells were positive for stemness makers, including OCT4, SOX2 and SSEA4 (Fig. 1b). Early and spontaneous lineage specific markers of mesoderm (*BRACHYURY*), ectoderm (*PAX6*) and endoderm (*AFP*) [26] were greatly absent at the mRNA level, while pluripotency genes were expressed (Fig. 1c). Western blot analysis verified the presence of OCT4, SOX2 and NANOG in hiPSCs and NT2 cells (pluripotent embryonal carcinoma cells, as a positive control), as well as the absence of α-SMA and GFAP (Fig. 1d). HeLa cells were used as negative control for all proteins and HFF cells and astrocytes were used as positive controls for α-SMA and GFAP, respectively (Fig. 1d). These data clearly confirmed that hiPSCs were undifferentiated for at least 15 passages under culture conditions using CM-100.

### bFGF maintains undifferentiated state of hiPSCs

In order to investigate the effect of bFGF on hiPSCs, feeder-free culture conditions were used to eliminate indirect effects of fibroblasts on hiPSCs. hiPSCs were cultured for 6 days under four different medium conditions containing various bFGF concentrations, i.e. CM-100, CM-5, CM-0 and non-CM used as a negative control. As indicated in Fig. 2a and Additional file 1: Figure S1, withdrawal of bFGF disrupted the compact morphology of hiPSCs, which spreaded out at day 6. These morphological changes are often correlated with the loss of pluripotency [1]. Therefore, we assessed the pluripotent state of the cells by determining OCT4, SOX2 and NANOG expression at mRNA and protein levels. qPCR data revealed a significant reduction in *POU5F1*, *SOX2* and *NANOG* expression 6 days after withdrawing bFGF (CM-0 and non-CM) as compared to CM-100 (Fig. 2b). Consistent with the mRNA expression data, the amount of OCT4, SOX2 and NANOG proteins were drastically and significantly reduced in the absence of bFGF (Figs. 2c, d). Interestingly, loss of pluripotency markers under CM-0 and non-CM at day 6 (Figs. 2b, c) were followed by the expression of GFAP, but not α-SMA, as differentiation marker (Fig. 2c). Moreover, confocal imaging also confirmed the reduction in OCT4 expression and differentiation toward the ectoderm and not mesoderm lineage in hiPSCs (Figs. 2e-g). Thus, bFGF is essential for maintaining pluripotency in hiPSCs and removing it from the culture medium leads to cell differentiation towards the ectoderm lineage.

### MAPK pathway is required for maintaining hiPSCs in an undifferentiated state

Conflicting studies have shown that MAPK pathway can positively and negatively regulate hESCs pluripotency [7, 27]. Thus, we first analyzed the activation status of this pathway. As shown in Fig. 3a (first lane from left), the MAPK pathway was highly active in undifferentiated hiPSCs (CM-100), as detected by immunoblotting of phosphorylated (p-) MEK and

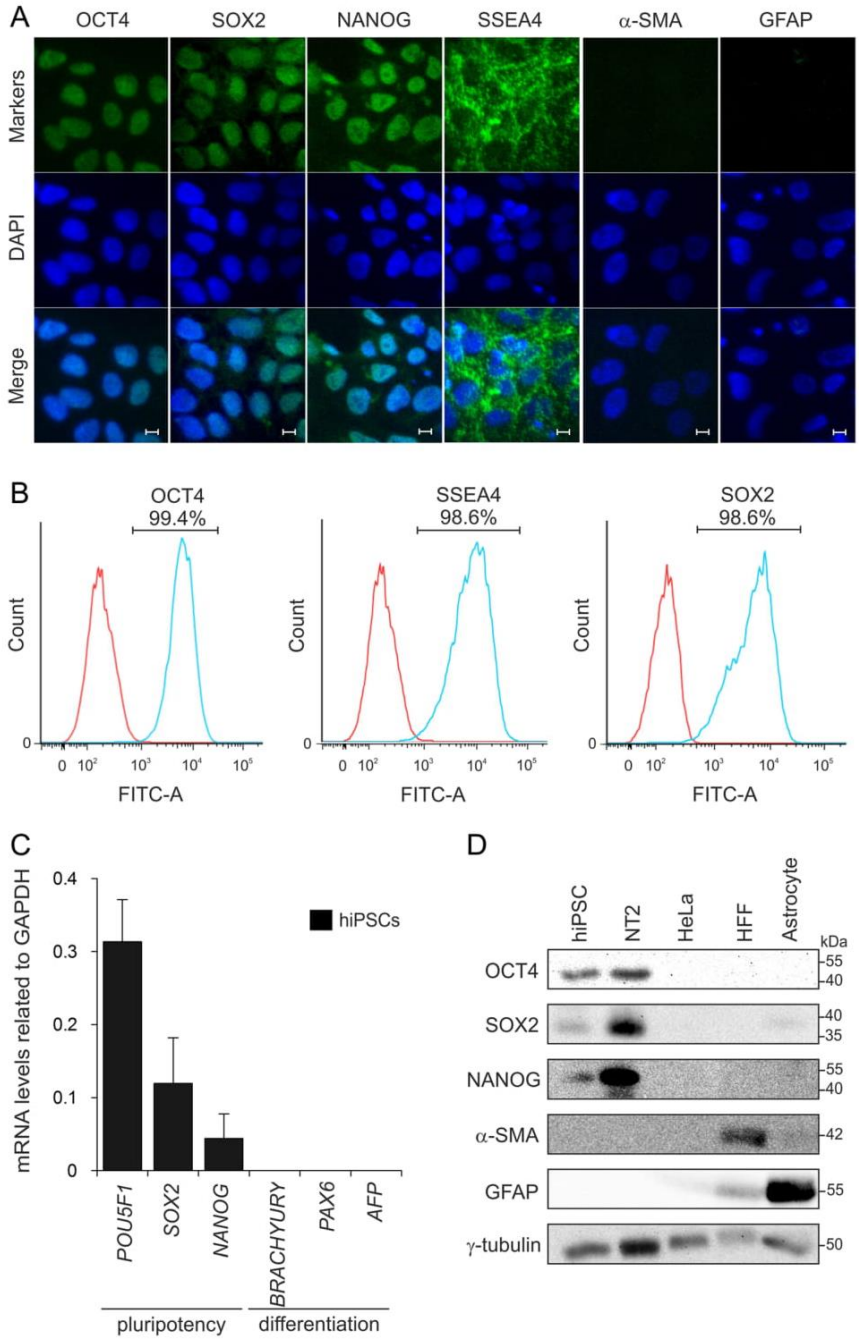


**Fig. 1** Long-term maintenance of undifferentiated hiPSCs. **a** Confocal imaging showed the expression of pluripotency markers (OCT4, SOX2, NANOG and SSEA4) and the absence of differentiation markers GFAP and α-SMA as ectodermal and mesodermal markers, respectively. Cell nuclei were stained with DAPI (blue). Scale bars, 10 μm. **b** Flow cytometry confirmed expression of OCT4, SSEA4 and SOX2 in hiPSCs with more than 98% of positive cells. **c** qPCR analysis for undifferentiated stem cell markers (*POU5F1*, *SOX2* and *NANOG*) and early commitment to differentiation markers (*BRACHYURY*, *PAX6* and *AFP*). *GAPDH* was used as an internal control. **d** Immunoblot analysis showing the specificity of antibodies and expression of markers. HeLa cells were used as negative control, HFF and astrocytes were used as positive control for α-SMA and GFAP, respectively

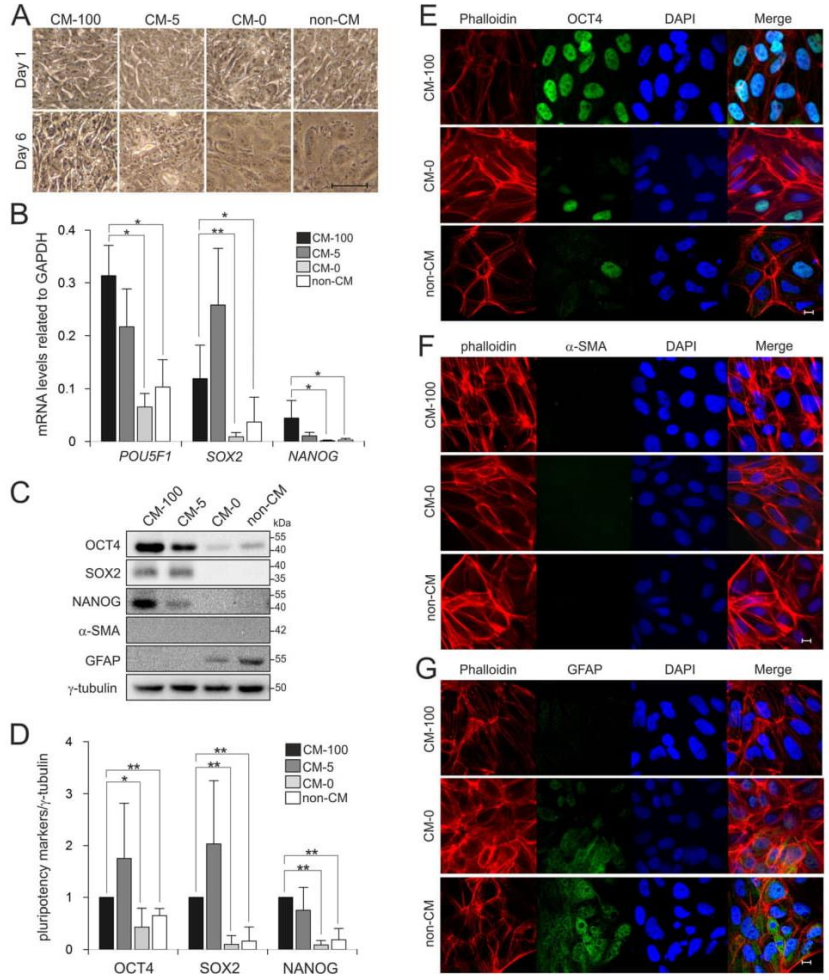


**Fig. 2** The critical role of bFGF for maintaining hiPSC pluripotency. **a** Phase contrast images of hiPSCs cultured under four different conditions, CM-100, CM-5, CM-0 and non-CM for 6 days. Undifferentiated hiPSCs formed compact colonies (CM-100), while without bFGF supplementation hiPSCs spread and flattened at day 6 (CM-0 and non-CM). Scale bar, 50 μm. **b** qPCR analysis showed the downregulation of pluripotency markers *POU5F1*, *SOX2* and *NANOG* in cells cultured in CM-0 and non-CM in comparison to control group (CM-100). All expression values were normalized to *GAPDH*. Results from three separate experiments, each carried out in triplicate, are shown as mean ± SD (ANOVA; $*p < 0.05$ and $**p < 0.01$). **c** Western blot analysis of pluripotency and differentation markers under four different conditions showed the downregulation of stemness markers and upregulation of GFAP. γ-tubulin served as loading control. **d** The graph represents densitometric analysis of three independent experiments, each carried out in duplicates. All values were normalized to γ-tubulin and relative to CM-100. Data are shown as mean ± SD (ANOVA; $*p < 0.05$ and $**p < 0.01$). hiPSCs were immunostained for OCT4 (**e**), α-SMA (**f**) and GFAP (**g**) under three different culture conditions; CM-100, CM-0 and non-CM. Cell nuclei were stained with DAPI (blue) and F-actin was stained with phalloidin (red). Scale bar, 10 μm

ERK1/2 proteins. This pathway is activated by bFGF [7] and given that bFGF is essential for maintaining undifferentiated state of hiPSCs (Figs. 2b, c), we next analyzed the MAPK pathway activity in pluripotent vs. differentiated hiPSCs. Our data showed that following withdrawal of bFGF (CM-0 and non-CM as compared to CM-100) the activity of MAPK pathway was reduced by 10- and 2-fold, as seen by decreased levels of p-MEK and p-ERK1/2, respectively (Figs. 3a, b). Thus, these data indicate that MAPK pathway activity downstream of bFGF is required to keep hiPSCs in a pluripotent state.

### PI3K/AKT pathway remains unchanged during hiPSCs differentiation

Another main signaling pathway downstream of bFGF is PI3K/AKT [18], which promotes activin A and Smad2/3-mediated self-renewal of hPSCs [27] and is essential for hiPSCs survival [28]. Therefore, we examined the

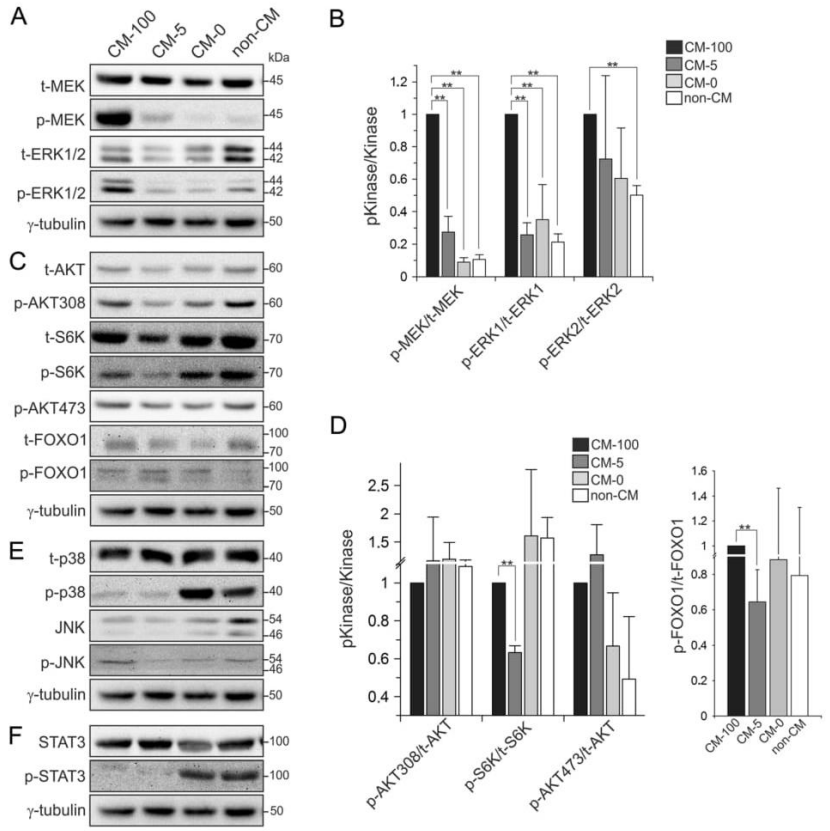


**Fig. 3** Signaling pathways downstream of bFGF in undifferentiated and differentiated hiPSCs. **a** Immunoblot analysis of the component of the MAPK pathway, including p-MEK and p-ERK1/2 in hiPSCs in undifferentiated (CM-100) and differentiated hiPSCs (CM-0 and non-CM). Total amounts of MEK and ERK1/2 as well as γ-tubulin served as loading controls. **b** The graph represents densitometric analysis of three independent experiments, each carried out in duplicates. All values were normalized to γ-tubulin and relative to CM-100. Data are shown as mean ± SD (ANOVA; $^{**}p < 0.01$). **c** Immunoblot of the phosphorylated signaling proteins downstream of the PI3K-AKT and mTORC2 axis in undifferentiated (CM-100) and differentiated hiPSCs (CM-0 and non-CM). Total amounts of AKT, S6K, FOXO1 as well as γ-tubulin served as loading controls. **d** The graph represents densitometric analysis of three independent experiments, each carried out in duplicates. All values were normalized to γ-tubulin and relative to CM-100. Data are shown as mean ± SD (ANOVA; $^{**}p < 0.01$). **e** Immunoblot of other MAPK pathways indulding p-p38 and p-JNK. Total amounts of p38, JNK and γ-tubulin served as loading controls. **f** Immunoblot analysis of p-STAT3 in undifferentiated (CM-100) and differentiated hiPSCs (CM-0 and non-CM). Total amounts of STAT3 and γ-tubulin served as loading controls

signaling activity of bFGF in undifferentiated and differentiated hiPSCs towards AKT and their downstream components S6K and FOXO1. Our data showed that AKT was activated in undifferentiated hiPSCs (CM-100) via both PI3K-PDK1-AKT and mTORC2-AKT pathways as monitored by p-AKT$^{T308}$ and p-AKT$^{S473}$ levels, respectively (Fig. 3c). The downstream components of these respective pathways were also phosphorylated (p-S6K$^{T389}$ and p-FOXO1$^{S256}$; Fig. 3c, first lane from left), presumably resulting in S6K activation and FOXO1 inhibition. However, we did not detect significant changes in the activity of the PI3K-PDK1 and mTORC2 pathways during bFGF withdrawal induced differentiation of hiPSC cells (Fig. 3c, d). Thus, PI3K-AKT signaling pathways are most probably involved in the control of cell survival rather than the maintenance of hiPSCs pluripotency.

### p38 and STAT3 activation during hiPSCs differentiation

In the next step, we monitored the activation state of p38, JNK and STAT3, respectively, as further candidate pathways downstream of FGF [18]. We found that p38 and STAT3 were not fully active in undifferentiated hiPSCs (CM-100) (Figs. 3e, f). Interestingly, however, following withdrawal of bFGF, the levels of phosphorylated and activated p-Thr180/Tyr182 p38 and p-Tyr705 STAT3, were clearly increased (Figs. 3e, f). Analysis of the JNK pathway showed no changes during differentiation (Fig. 3e). Taken together, p38 and STAT3, but not

JNK, are activated upon bFGF withdrawal and may play a role in hiPSCs differentiation.

### Dominant cytoplasmic localization of p-ERK

Our data indicate that the MAPK signaling is a prominent pathway downstream of bFGF, which maintains hiPSCs pluripotency and the activity will be decreased upon hiPSCs differentiation. Activation and nuclear translocation of MAPKs is necessary to initiate transcriptional programmes controlling cellular responses [29]. Thus, first we analyzed the nucleocytoplasmic distribution of these kinases in undifferentiated hiPSCs by subcellular fractionation. Total cell lysates (TCL) as well as cytosolic (Cyt) and nuclear (Nuc) fractions were analyzed for purity using antibodies directed against specific marker proteins (Fig. 4a). GAPDH and $Na^+/K^+$ ATPase were consistently found in the cytosol and histone H3 and lamin B1 in the nuclear fraction, confirming the purity of each fraction. Remarkably, both p-MEK/MEK and p-ERK1/2/ERK1/2 showed a predominantly cytosolic localization in undifferentiated hiPSCs (Fig. 4b, first lane from left). Subcellular fractionation was performed using another clone of hiPSCs and NT2 cells and comparable results were obtained (Additional file 1: Figure S3). Moreover, cells were starved for 12 h and then stimulated with 100 ng/ml bFGF for 15 min to observe any changes in p-ERK1/2 distribution upon stimulation. Interestingly both p-MEK/MEK and p-ERK1/2/ERK1/2 showed cytosolic localization even after bFGF stimulation, rather than nuclear localization (Fig. 4b). Consistent with this unexpected result, we investigated the phosphorylation of RSK, a cytosolic target of p-ERK1/2 that previously has been reported in hESCs [30, 31]. Our data revealed phosphorylation of RSK (at position T573) during hiPSCs differentiation (CM-0 or non-CM as compared to CM-100) (Figs. 4c, d), which is not consistent with predominant cytosolic localization of p-ERK1/2 (Fig. 4b).

### NRAS as an upstream regulator of undifferentiated hiPSCs

Small GTP-binding proteins of the RAS family transduce extracellular signals and activate a multitude of pathways via activation of effector proteins [32]. RAF kinases and PI3Ks are well-studied effectors of RAS family members which in turn activate MAPK and AKT, respectively [32]. As our data indicate that MAPK pathway is involved in maintaining pluripotency and PI3K/AKT likely involved in the survival of hiPSCs, we further investigated the RAS paralogs specificity downstream of bFGF and upstream of these pathways. First, the expression profile of three RAS paralogs (H/N/K) was investigated at both mRNA and protein levels in undifferentiated vs. differentiated hiPSCs. All four RAS genes were expressed and upregulated (3-fold for *HRAS* and *KRAS4B* and approximately 2-fold for *NRAS* and *KRAS4A*) upon differentiation (CM-0 and non-CM) (Fig. 5a). Interestingly, the amount of RAS proteins in differentiated hiPSCs (CM-0 and non-CM) was also increased 4-fold as compared to undifferentiated cells (CM-100) (Fig. 5b, c). To gain insights into the level of RAS activity (GTP-bound), total cell lysates of undifferentiated (CM-100) and differentiated (CM-0 and non-CM) hiPSCs were prepared and pull-down assays were performed with CRAF-RBD as RAS effector protein. As indicated in Fig. 5d, RAS activity was drastically reduced upon

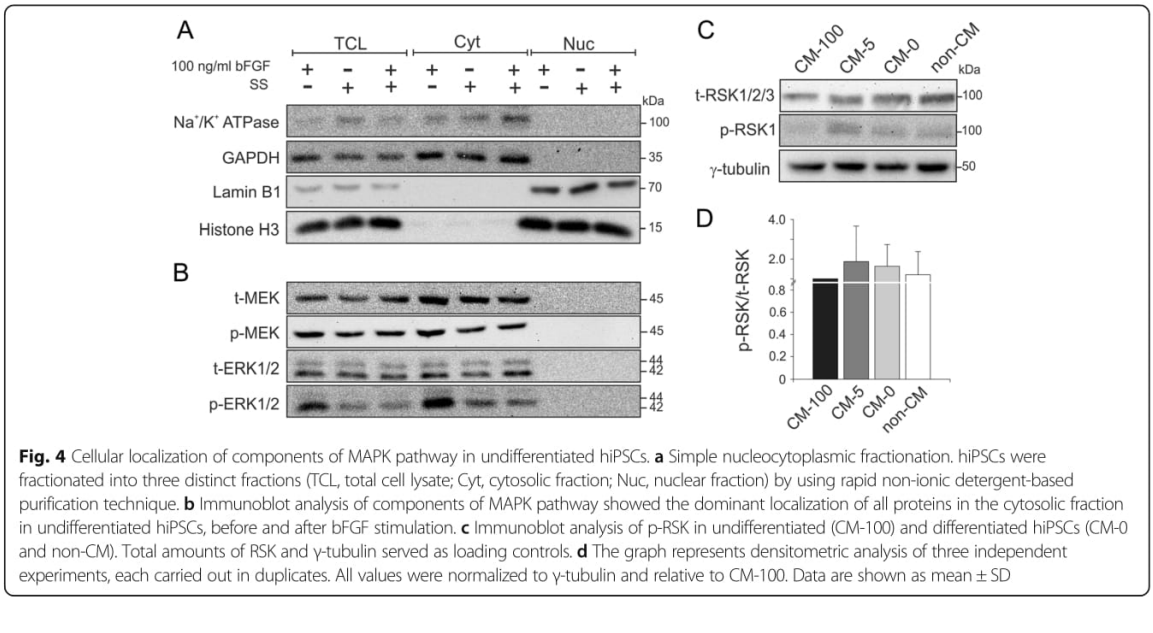


**Fig. 4** Cellular localization of components of MAPK pathway in undifferentiated hiPSCs. **a** Simple nucleocytoplasmic fractionation. hiPSCs were fractionated into three distinct fractions (TCL, total cell lysate; Cyt, cytosolic fraction; Nuc, nuclear fraction) by using rapid non-ionic detergent-based purification technique. **b** Immunoblot analysis of components of MAPK pathway showed the dominant localization of all proteins in the cytosolic fraction in undifferentiated hiPSCs, before and after bFGF stimulation. **c** Immunoblot analysis of p-RSK in undifferentiated (CM-100) and differentiated hiPSCs (CM-0 and non-CM). Total amounts of RSK and γ-tubulin served as loading controls. **d** The graph represents densitometric analysis of three independent experiments, each carried out in duplicates. All values were normalized to γ-tubulin and relative to CM-100. Data are shown as mean ± SD

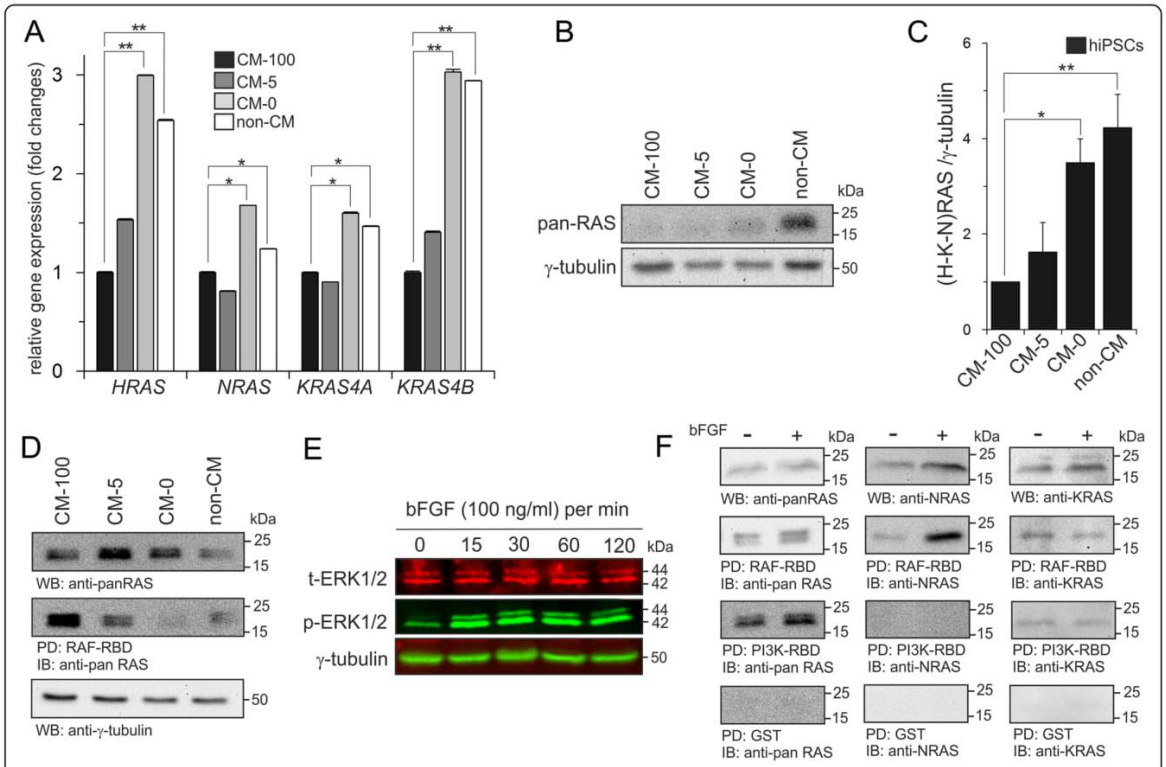


**Fig. 5** RAS paralogs display different expression and activation patterns in undifferentiated vs. differentiated hiPSCs. **a** qPCR analysis of RAS-related genes in undifferentiated vs. differentiated hiPSCs. Results from three separate experiments, each carried out in triplicate, are shown as mean ± SD (ANOVA; $*p < 0.05$ and $**p < 0.01$). **b** Immunoblot analysis of RAS protein expression in undifferentiated (CM-100) and differentiated hiPSCs (CM-0 and non-CM). **c** The graph represents densitometric analysis of three independent experiments, each carried out in duplicates. All values were normalized to γ-tubulin and relative to CM-100. Data are shown as mean ± SD (ANOVA; $**p < 0.01$). **d** CRAF-RBD-derived pull-down of GTP-bound RAS in differentiated (CM-0 and non-CM) and undifferentiated (CM-100) hiPSCs were analyzed by immunoblotting of pan-RAS. γ-tubulin was used as loding control. **e** Immunoblot analysis of p-ERK1/2 showed that transient bFGF stimulation induces phosphorylation of ERK1/2 in KSR-starved hiPSCs after 15 min. The amounts of ERK1/2 and γ-tubulin were used as loading controls. **f** RAS paralogs were subjected to pull-down analysis in undifferentiated hiPSCs in the presence and absence of bFGF using two GST-fused RAS effectors, CRAF-RBD and PI3Kα-RBD. RAS-GTP pull down assays were followed by western blot analysis using pan-RAS antibody and RAS paralog specific antibodies. Immunoblots of total cell lysates were served as loading controls to detect pan-RAS, NRAS and KRAS. GST and pan-RAS were used as negative and loading controls, respectivley

differentiation (CM-0 and non-CM) in comparison to the undifferentiated state (CM-100) that was in consistent with the reduction in MAPK pathway activity (Fig. 3a).

To examine the levels of active (GTP-bound) RAS paralogs, undifferentiated hiPSCs were serum starved for 12 h and re-stimulated with 100 ng/ml bFGF for different time points. Based on the phosphorylation levels of p-ERK1/2 (Fig. 5e), stimulation with bFGF for 15 min was chosen. For pull-down analysis, two major RAS effector proteins were employed; CRAF-RBD and PI3K-RBD which were used as GST-fusion proteins. As indicated in Fig. 5f, RAS activity was increased upon bFGF stimulation leading to a stronger RAF activation as compared to PI3K. To further investigate the activity of each of the three canonical RAS proteins, we used paralog-specific antibodies (Additional file1: Figure S4) [33]. Interestingly, we found that NRAS is the main RAS paralog which is activated upon bFGF stimulation (Fig. 5f). It preferentially and most strongly bound to CRAF as compared to PI3K for which no detectable binding was observed (Fig. 5f). KRAS bound to CRAF showed no elevated activity upon bFGF stimulation (Fig. 5f). The expression level of HRAS protein was very low in hiPSCs and was not detectable (data are not shown). Altogether, these data suggest that NRAS acts as a main RAS paralog that links bFGF signaling to the MAPK pathway.

## Discussion

This study provides novel molecular insight into the regulation of pluripotency maintenance of hiPSCs. Our findings indicate that among the signaling pathways downstream of bFGF, the MAPK pathway plays a critical role in maintaining pluripotency, whereas strong activation of p38 and

JAK/STAT3 signaling is linked to differentiation of hiPSCs. In contrast, no relevant changes occurred in the activation of AKT or JNK pathways from pluripotent hiPSCs towards differentiated cells. Moreover, we identified NRAS among the RAS paralogs as the likely link between bFGF receptor and the MAPK pathway that maintains hiPSCs pluripontency (Fig. 6).

Our data clearly suggest that bFGF transmits signals to promote pluripotency and suppress differentiation activities in hiPSCs (Fig. 6). We showed that bFGF withdrawal from the culture, markedly decreased OCT4, SOX2 and NANOG expression at the mRNA and protein levels, which was followed by the expression of GFAP and differentiation towards the ectoderm lineage. Consistent with our finding in hiPSCs, it was reported that bFGF also maintains hESCs in an undifferentiated state [11, 34].

Different studies suggest pleiotropic effects of bFGF activating different pathways in hESCs either directly or indirectly by inducing paracrine signaling via iMEFs in coculture [35]. For investigating the mechanistic effects of bFGF, we compared the signaling pathways in undifferentiated vs. differentiated hiPSCs obtained via bFGF withdrawal. FGF has been reported to activate multiple downstream signaling pathways, including MAPKs (ERK, JNK and p38), PI3K and JAK/STAT [36]. Our study demonstrates the activation of MEK-ERK1/2 pathway in undifferentiated hiPSCs and a remarkable decrease in the p-MEK and p-ERK1/2 levels by withdrawing bFGF which induces their differentiation. Previously, Li and colleagues have shown that inhibiting FGF signaling induces hESC differentiation into primitive endoderm and trophectoderm [7]. However, Singh et al. have reported a pro-differentiation role of MAPK pathway in hESCs [27]. These conflicting reports could be due to different culture conditions, cell lines or even pathway dose-dependency. Our data argue against a pro-differentiation role of the MAPK pathway. We used in this study a system for culturing hiPSCs with iMEF-CM that was supplemented with 100 ng/ml bFGF which was different from Li et al. and Singh et al. [7, 27]. Under these conditions we are able to dissect direct and paracrine iMEF-mediated influences of bFGF without the risk of confounding effects based on sample contamination with feeder cells. Our data clearly showed that MAPK pathway positively regulates hiPSC pluripotency. Culturing cells with CM-0 and non-CM, which led to differentiation toward ectoderm, was in correlation with a significant decrease in MAPK pathway activity between all groups (CM-100, CM-5, CM-0 and non-CM). As previously described, bFGF activates ERK1/2 at high concentrations (about 100 ng/ml) in ESCs [27]. Here, we observed no obvious differences between high dose (CM-100) and low dose (CM-5) of bFGF in maintaining the undifferentiated status of hiPSCs. Thus, the downregulation of ERK precedes the decrease of pluripotency markers and cannot be excluded under long-term culture (6 days) conditions. These observations suggest that homeostasis of MAPK signaling is a dose-dependent consequence of bFGF without direct impact on the expression level of pluripotency-associated transcription factors.

PI3K/AKT activation by bFGF has also been shown to be important for the maintenance of the undifferentiated state of hESCs [37]. This pathway contributes to a variety of important cellular processes including nutrient uptake, anabolic reactions, proliferation and survival [38]. Proliferation and survival can be controlled by mTORC1 mediated activation of S6K and mTORC2 mediated inhibition of FOXO-1, respectively [39, 40]. Armstrong and colleagues have shown that PI3K/AKT is important for maintaining pluripotency in hES-NCL1 cells and the key components of this pathway, such as p-PDK1, p-PTEN, p-AKT$^{308}$ and p-AKT$^{473}$ are downregulated during differentiation to embryoid bodies [30]. Li and coworkers have shown that PI3K/AKT pathway, downstream of bFGF, is highly active in hESCs, such as H1 and H9 cells, which supports hESC self-renewal and pluripotency [7]. Other studies have implicated the survival and anti-apoptotic role of PI3K/AKT in hESCs and hiPSCs [27, 28, 41]. In our study, two axes of AKT activation were investigated, PI3K-PDK1-AKT-S6K and mTORC2-AKT-FOXO1 as downstream pathways of bFGF, which is different from previous reports that just

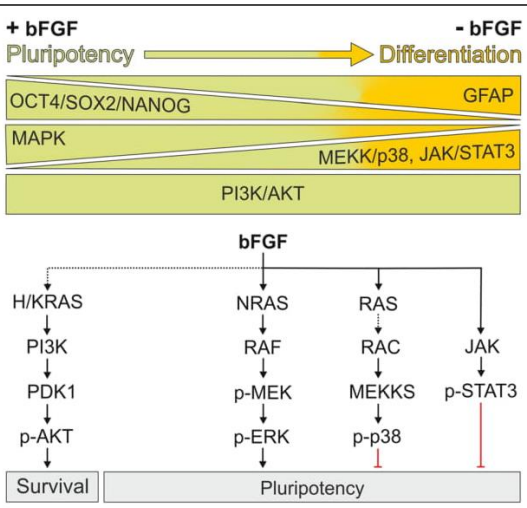


**Fig. 6** Proposed model for bFGF signaling in pluripotent (green) vs. differentiated (orange) hiPSCs (for details see discussion). bFGF, basic fibroblast growth factor; OCT4, octamer-binding transcription factor 4; SOX2, sex determining region Y-box 2; GFAP, glial fibrillary acidic protein; RAS, rat sarcoma; PI3K, phosphoinositide 3-kinase; PDK1, 3-phosphoinositidedependent protein kinase; MEK, MAP/ERK kinase; ERK, extracellular signal-regulated kinase; JAK, Janus kinase; STAT3, signal transducer and activator of transcription 3

showed the importance of PI3K/AKT in maintenance of pluripotency and not as a target of bFGF signaling [27, 28, 41]. Our results showed that there was no change in the activation level of these two pathways following hiPSCs differentiation (CM-0 and non-CM). This suggests that AKT-S6K and AKT-FOXO1 signaling remain unaffected in the presence and absence of bFGF during a long-term culture (6 days) which may be due to the presence of KSR in iMEF-CM. KSR contains high levels of insulin that can activate AKT pathways [27]. This rather suggests that PI3K/AKT is not critical for maintaining the undifferentiated state of hiPSCs and most probably plays an anti-apoptotic role required for survival of hiPSCs rather than their pluripotency.

In addition to MAPK and PI3K/AKT pathways, we also analyzed other signaling pathways, including p38 MAPK and JNK (c-Jun N-terminal kinase), both can be activated by FGF signaling [35]. p38 activation has been observed in response to a variety of extracellular stresses and mitogenic stimuli which lead to different cell-specific responses, including inflammation, cell death, senescence, survival, cell growth and differentiation [42]. So far, little is known about the role of p38 in pluripotency of hESCs. Neganova and colleagues demonstrated an increased activity of p38 MAPK during the early stage of reprogramming of human fibroblasts to hiPSCs and the importance of this pathway for obtaining fully reprogrammed cells [43]. Moreover, hESCs and hiPSCs are in a high-methionine metabolic state which decreases upon differentiation. In this regard, it has been shown that methionine deprivation triggering the activation of p53-p38 signaling leads to NANOG downregulation and differentiation into all three germ layers [44]. We showed in this study, for the first time, an increase in p38 MAPK activity during hiPSCs differentiation under bFGF starvation (CM-0). It can be proposed that p38 is inhibited as a downstream target of bFGF in undifferentiated hiPSCs.

Findings from *Drosophila* studies and some human cancers indicate that JNK might be a regulator of stem cells and cancer stem cells. Brill et al. observed a significantly elevated JNK activity in undifferentiated hESCs, which if blocked by JNK inhibitors under feeder-free conditions in the presence of CM, leaded to decreased OCT4 expression and differentiation [45]. A possible contribution of JNK signaling to the maintenance and/or self-renewal of hESCs was additionally confirmed in a different hESC line, Harvard's HUES-7. In response to BMP-induced differentiation, a transient elevation of c-Jun phosphorylation was observed, which indicates both the competence of the basal JNK pathway to maintain the stemness of the hESCs and a possible involvement of JNK activation in the initiation of hESC differentiation [46]. In our study, we observed the constant activation of JNK during hiPSCs differentiation in response to bFGF starvation. Thus, JNK pathway may be involved in other cellular responses rather than maintaining pluripotency or inducing differentiation.

mESCs can be maintained in vitro by adding leukemia inhibitory factor (LIF) to the medium and its withdrawal rapidly leads to differentiation [47, 48]. LIF activates Janus kinases (JAKs) which subsequently phosphorylate STAT3. Activated STAT3 translocates into the nucleus and activates transcription of target genes [49, 50]. Interestingly, LIF/STAT3 signaling fails to support self-renewal of hESCs and is nonresponsive to LIF/STAT3 [51]. Since LIF is not the only cytokine that activates JAK/STAT3 pathway, we analyzed the activity of this pathway downstream of bFGF. Similar to Humphrey and coworkers, who have shown that STAT3 phosphorylation was not detectable in undifferentiated hESCs [52], we also could not observe phosphorylation of STAT3 in undifferentiated hiPSCs. Interestingly, upon differentiation (CM-0 and non-CM), JAK/STAT3 pathway was activated in hiPSCs. It can be postulated that unlike mESCs, hiPSCs do not require STAT3 activity for the maintenance of their pluripotency but rather for their differentiation.

It has been shown that activated ERKs in hESC are translocated from the cytoplasm to the nucleus where they phosphorylate and activate nuclear transcription factors and effectors, such as ELK1 and MYC [30]. These downstream targets of MAPK pathway are downregulated during differentiation [30]. We performed subcellular fractionation of undifferentiated hiPSCs to analyze the cellular distribution of MAPK pathway components. Both p-MEK and p-ERK1/2 were located in the cytoplasm, even after stimulation with bFGF for 15 min. Therefore, we analyzed the activity levels of RSK as one possible cytosolic target of p-ERK1/2 in undifferentiated and differentiated hiPSCs. There was an increase in phosphorylation level of RSK in CM-0 and non-CM compared to CM-100 which was in conflict with the loss of ERK1/2 activity during differentiation of hiPSCs. Thus, these data suggest that p-ERK1/2 in hiPSCs most probably signal through further (critical) cytosolic targets other than RSK.

We demonstrated the critical role of MAPK pathway downstream of bFGF in maintaining pluripotency in hiPSCs. For further analysis of this pathway, we analyzed the expression of canonical RAS isoforms in undifferentiated vs. differentiated hiPSCs. Interestingly we found that in contrast to the decreased level of MAPK pathway activity in differentiated hiPSCs, the levels of RAS mRNA and protein were both upregulated upon differentiation. To elucidate the activity level of RAS (GTP-bound), pull down assays were performed with CRAF-RBD as an effector for RAS proteins. RAS activity was drastically reduced in hiPSCs treated with CM-0 and non-CM (differentiated cells) compared to undifferentiated cells (CM-100), consistent with the decrease of MAPK pathway activity levels. These findings suggest that RAS-RAF is upstream of MEK/ERK and its activity will be

decreased upon differentiation in hiPSCs. Furthermore, we analyzed main RAS paralogs, i.e. H-, K- and NRAS. Interaction analyses with two RAS effectors (RAF and PI3K) showed that among the RAS paralogs, NRAS preferentially interacts with RAF in the presence of bFGF and activates the MAPK pathway while no interaction was observed with PI3K independent of the bFGF stimulation status. KRAS interacts physically with RAF and PI3K but showed no preference for either of the effectors upon bFGF starvation or stimulation. Lastly, HRAS was the most difficult RAS to analyze. Although the mRNA levels of HRAS were higher than NRAS in hiPSCs (data not shown), at the protein level, HRAS was not detectable (data not shown).

Signaling pathways regulating ESC fate differ between mESCs and hESCs, and our study provides another aspect of this difference in signal transduction. Recently, Altshuler and colleagues have shown that all three RAS paralogs regulate the transition from naïve to primed state in mESCs and HRAS, KRAS and NRAS display a similar pattern of activation and overlapping roles in mESC differentiation [22]. However, we clearly observed the difference between the patterns of these paralogs in regulating the downstream pathways, which are involved in maintaining pluripotency. Further studies are needed to investigate the role of these RAS proteins in hiPSC differentiation which will provide a better understanding of pluripotency states and early human embryonic development.

## Conclusion

In conclusion, our study suggests that among the downstream pathways of bFGF, MAPK pathway plays a prominent role in keeping hiPSCs in a pluripotent state, while two axes of AKT pathway (PI3K-PDK1-AKT-S6K and mTORC2-AKT-FOXO1) remain unchanged during differentiation that propose a survival role of this pathway rather than maintaining pluripotency which is different from previous reports. Among other pathways, p38 and JAK/STAT3 were activated upon bFGF withdrawal and hiPSCs differentiation, and JNK, like AKT pathway, remain unchanged. Characterizing the MAPK pathway in more detail revealed that among RAS isoforms, NRAS is the link between bFGF receptor and MAPK pathway leads to hiPSCs pluripotency.

## Additional file

**Additional file 1: Table S1.** Primer sequences (5′ to 3′) for qPCR using the SYBR Green system obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/). **Figure S1.** Morphological changes of hiPSCs during 6 day culture of bFGF starvation. **Figure S2.** Quantitative western blot analysis of cell signaling in hiPSCs with different culture conditions. **Figure S3.** Subcellular distribution of ERK1/2. **Figure S4.** Specification and validation of RAS antibodies. (DOCX 2260 kb)

### Abbreviations

bFGF: Basic fibroblast growth factor; CM: Conditioned medium; ESC: Embryonic stem cell; GFAP: Glial fibrillary acidic protein; hESC: Human embryonic stem cell; HFF: Human foreskin fibroblast; hiPSC: Human induced pluripotent stem cell; iMEF: irradiated murine embryonic fibroblast; JAK/STAT: Janus kinase/signal transducers and activators of transcription; JNK: c-Jun N-terminal kinases; KSR: Knockout serum replacement; MAPK: Mitogen-activated protein kinase; mESC: Mouse embryonic stem cell; NEAA: Non-essential amino acid; non-CM: Non-conditioned medium; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; α-SMA: Alpha smooth muscle actin

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### Availability of data and materials

These are part of the Additional file 1 (see page 33).

### Authors' contributions

FH and MRA conceived and coordinated the study. FH and MRA designed the study and wrote the paper. FH, JD, SNR, AL, RP and MRA designed, performed and analyzed the experiments. JD, GK, IK and MZ reprogrammed human fibroblasts to iPSCs. All authors reviewed the results and approved the final version of the manuscript.

### Ethics approval and consent to participate

There is no need for an ethical approval and consent to participate. This study is purely cell-based. Studies iPSCs were generated from a HFF-1 cell line that was bought from ATCC.

### Consent for publication

All authors have read the manuscript and approved of the final version. Otherwise "Not applicable".

### Competing interests

The authors declare that they have no competing interests.

## Publisher's Note



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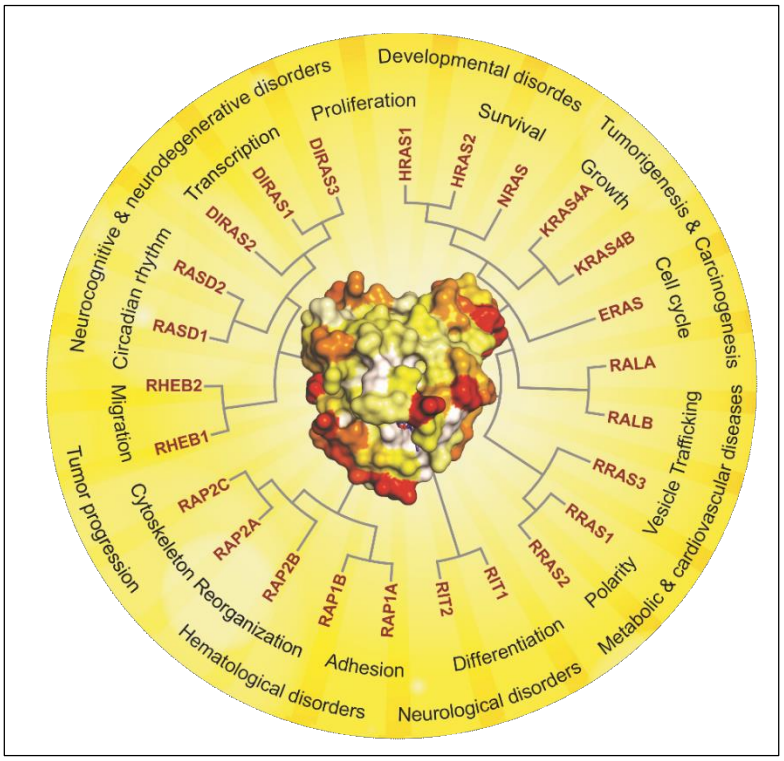
# Chapter III

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

**Features and differences of RAS family proteins regarding their structure, function, regulation, and involvement in diseases**

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REVIEW ARTICLE



# 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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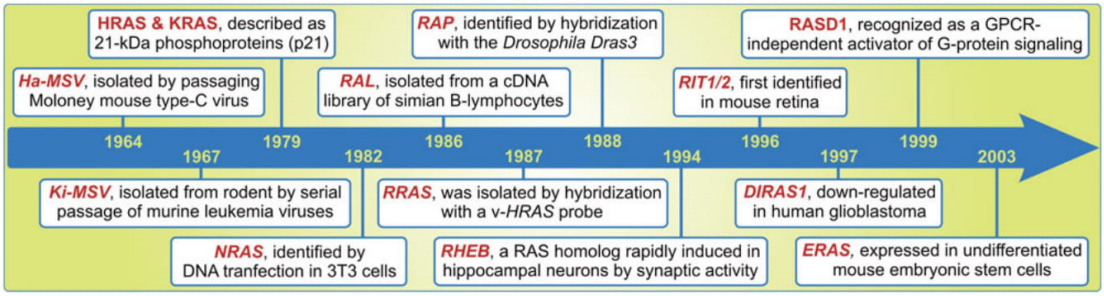


Figure 1. Historical timeline of the discovery of various members of the RAS family.

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

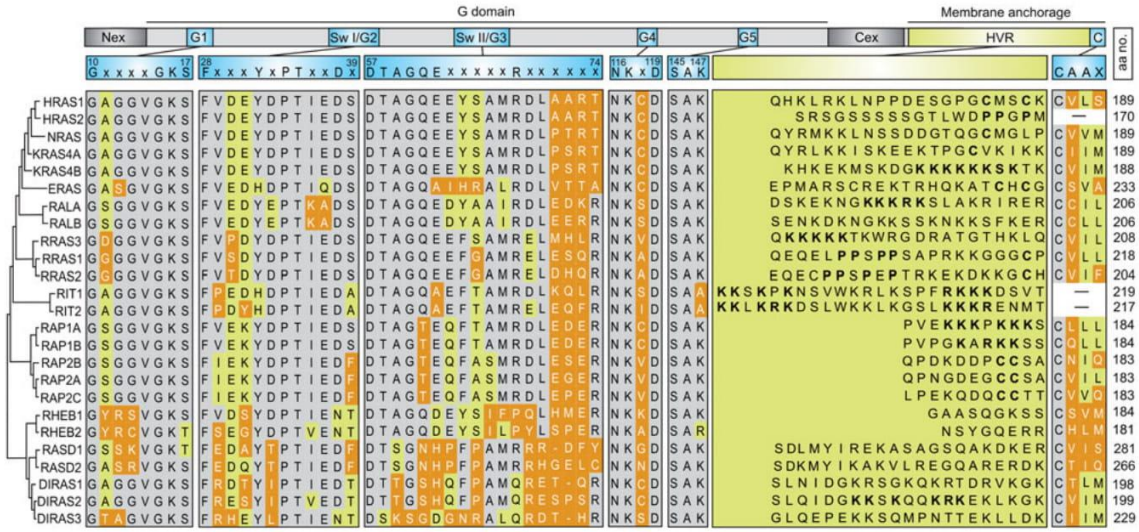


Figure 2. Evolutionary conservation of RAS family members. Signature motifs of 25 RAS-related proteins are presented according to their phylogenetic categorization. These proteins consist of a G domain with the five conserved motifs and a variable C-terminal membrane anchorage region, divided in hypervariable 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, | | JNK, MAP4K4, PARG1, TNIK, RPIP9, MINK, | Ger, Cm, Plam, P |
| RAP2B | | Platelet, neutrophils | Thrombin, convulxin | PDZGEF1 | | PLCε | 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

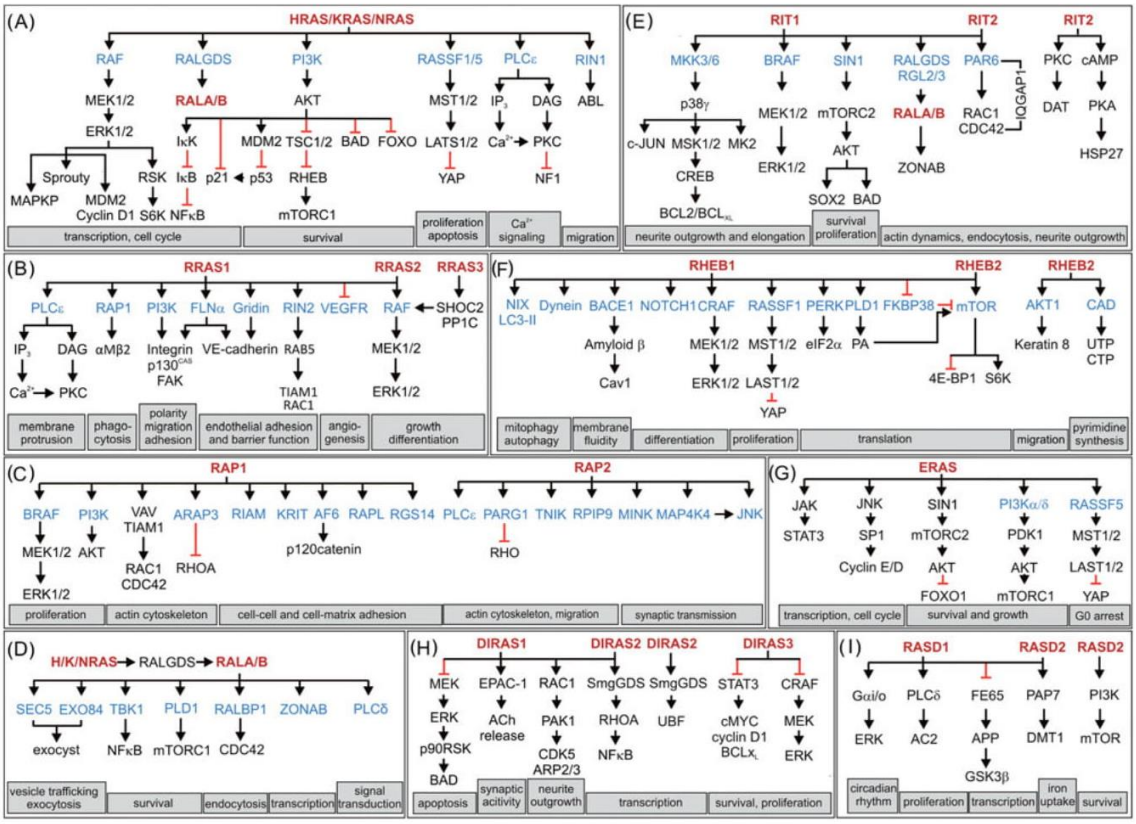


Figure 3. Signal transduction pathways downstream of the RAS family proteins. Signaling schemes are divided in different paralog in red colors (A–I). Reviewed effectors are shown in blue. Other downstream interacting proteins are shown in black. Black arrows indicate activating events and red lines inhibiting 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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The authors declare no conflict of interest.

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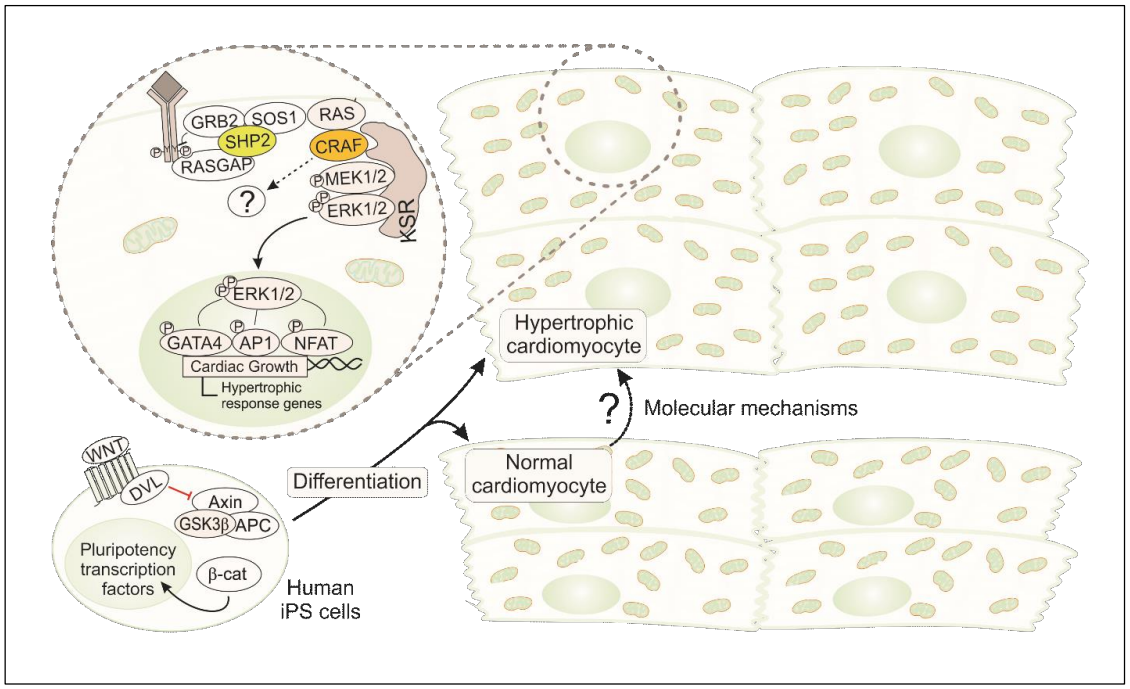
# Chapter IV

## Molecular mechanisms underlying hypertrophic cardiomyopathy caused by RAF1 missense mutation in Noonan patient

**Patient-derived cardiac myocytes (carried RAF1$^{S257L}$) recapitulated the HCM phenotype with increased RAS-MAPK signaling pathway**

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|---|---|
| Status: | In preparation |
| Impact Factor: | - |
| Own Proportion to this work: | 20%<br>iPSC culture (wt and RAF1 mutant), Differentiation to cardiomyocytes, Flow cytometry for pluripotency and cardiac markers, Cell cycle analysis, Beating rates, Figure preparation, Writing the manuscript |

## Molecular mechanisms underlying hypertrophic cardiomyopathy caused by RAF1 missense mutation in Noonan patient

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### Background

Hypertrophic cardiomyopathy (HCM) was reported as the first familial cardiomyopathy, is manifested as increase in left ventricular wall thickness and is the most common cause of sudden death in young people. HCM usually results from mutations in genes encodings structural components of sarcomere and signaling function. Dysregulation of sarcomeric proteins and signaling upregulate the fetal gene program, change the calcium transient rate, force generation and energy consumption. Altogether, these result in the increasement of cardiomyocytes size, fibrosis and pathological HCM.

The germline mutations of RAS-MAPK signaling components result in a set of developmental disorders collectively called RASopathies, which are characterized by craniofacial dysmorphology, delayed growth, neurocognitive impairment, cardiac abnormalities and an increased cancer risk. A number of cardiac defects are listed in RASopathy with various prevalence in different disorders. Among them, the mild to-severe HCM is the main complication in patient affected by RASopathy.

More than 30 years, RAS and its signaling components are studies as oncogenes. HCM is diagnosed in 80 % of LEOPARD individuals (PTPN11 and RAF1 mutations), 65% of Castello syndrome (HRAS mutation), 40% in Cardio-Facio-Cataneous syndrome (BRAF mutation) and 20% in Noonan Syndrome (NS) (RAF1>PTPN11>RIT1 mutations). Although, in general the frequency of HCM in NS syndrome is low (20%), notably, more than 90% of NS with RAF1 point mutation are associated with pathological HCM. RAF kinases, ARAF, BRAF and CRAF, share three conserved regions CR1-3. CR1 contains RAS binding domain (61-192aa) and CR2 (251-266) provides a regulatory phosphorylation site that acts as a docking site for 14-3-3 binding. Kinase domain are clustered in CR3 (333-625). Razzaque and Pandit reported, 80% NS individuals with S257L mutation

in the CR2 exhibit the severe HCM with disorganized muscle bundles. However, the molecular mechanism of HCM, induced by $RAF1^{S257L}$ is not fully understood. To investigate the mechanism underlying the RAF1 induced HCM, we need the human disease model of cardiomyocytes which endogenously express $RAF1^{S257L}$.

## Material and methods

### Generation and cultivation of human iPSCs

Dermal fibroblasts were obtained with the institutional ethics approval and under informed consent of the parents from a female who was diagnosed with rapidly progressive HCM and a S257L-mutation in exon 7 of RAF1. Human foreskin fibroblasts were purchased from ATCC and served as healthy controls. Fibroblasts were cultured in DMEM high glucose supplemented with 10% fetal calf serum, 1% non-essential amino acids, and 2 mM L-glutamine (all Thermo Scientific). Human iPSCs were generated using a cocktail of non-integrating episomal reprogramming vectors obtained from Addgene (pCE-hSK #41814, pCE-hOct3/4 #41813, pCE-hUL #41855, pCE-mp53DD #41856, pCXB-EBNA1 #41857) as previously published. In brief, $1x10^5$ human fibroblasts were mixed with 1 µg of each plasmid in a total amount of 100 µL resuspension buffer R (Thermo Scientific). After electroporation using the Neon Transfection system (Thermo Scientific) with two 20 ms pulses of 1650 V, fibroblasts were plated in fibroblast medium onto a Geltrex matrix coated 6-well culture dish. After 24 h, medium was exchanged and cells were maintained further on in DMEM/F12 + GlutaMAX supplemented with 1% N2 supplement, 2% B27 supplement, 1% non-essential amino acids and 100 ng/mL bFGF. After 3-4 weeks, emerging iPSC colonies were manually dissected under microscopic control and plated individually on mitotically inactivated (gamma-irradiation at 30 Gγ) murine embryonic fibroblast feeder layers (iMEFs). Established human iPSCs were then cultivated as colonies on iMEFs in iPSC-medium (DMEM/F12 + GlutaMAX supplemented with 20% (v/v) knockout serum replacement, 0.1 mM 2-mercaptoethanol (Sigma), 1% (v/v) non-essential amino acid stock and 25 ng/ml FGF-2 (Peprotech) or as feeder-free monolayers in Geltrex coated cell culture flasks in iMEF conditioned iPSC-medium incl. 100 ng/mL FGF-2 (CM+/100) and passaged every 3-4 days.

### Reverse transcriptase polymerase chain reaction

Cells were disrupted by TRIzol™ reagent (Ambion, Life Technologies, Germany) and total RNA was extracted *via* RNeasy plus kit (Qiagen, Germany) according to the manufacturer's protocol. Possible genomic DNA contaminations were removed using the DNA-free™ DNA Removal Kit (Ambion, Life Technologies, Germany). DNase-treated RNA was transcribed into complementary DNA (cDNA) using the ImProm-II™ reverse transcription system (Promega, Germany). Quantitative real-time reverse transcriptase polymerase chain reaction (qPCR) was performed using TaqMan probes or SYBR Green reagent (Life Technologies, Germany. The $2^{-\Delta\Delta Ct}$ method was employed for estimating the relative mRNA expression levels and $2^{-\Delta Ct}$ for mRNA levels. Among 6 different HKG that we tested HPRT1 showed the minimal variation among different cell lines and therefore, was used for normalization.

### Flow cytometry

For flow cytometric analysis, to prepare the single-cell suspensions of hiPSC-CBs, they were washed with phosphate buffer saline and incubated with Versene (EDTA-Solution, Gibco #15040066) for 10 min in Eppi-Thermomixer at 37°C. Continuously, TrypLE (10x) was added and incubate for additional 10 min at 37°C and 1200 rpm until the cell aggregates have disappeared. Cells were fixed in 4% paraformaldehyde (PFA; Merck) for 10 min on ice and permeabilized with 90% ice-cold methanol for 20 min followed by a blocking step with 1.5% BSA and 2.5% goat or donkey serum diluted in PBS for 1 h at 4°C. Cells were stained with primary antibodies included OCT3/4 (1:1000; Santa crus, # sc-5279), cardiac troponin t (1:200, Invitrogen, # MA5-12960) and Myosin light chain 2 V (1:100, Synaptic Systems, # 310111) overnight at 4°C. Secondary antibodies (Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated donkey anti-rabbit IgG) were from Life Technologies and used at a dilution of 1:2000 for one hour at room temperature. Samples were collected with FACScanto (BD PharMingen) and analyzed with FlowJo Software (Treestar, Ashland, OR).

**Immunoblotting**

To extract the total protein, hiPSC-CBs were washed with phosphate buffer saline and lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM MgCl2, 1% TritonX 100, 10% glycerol, 20 mM beta-glycerolphosphate, 1 mM Ortho-Na3VO4, EDTA-free protease inhibitor (Roche, Germany)) and to disturb the cell aggregate the sonicator with 70% power were used Sonicate for 40 second and they kept in rotor in 4ºC for 30 min. Protein concentrations were determined with Bradford assay (Bio-Rad). Equal amounts of cell lysates (10-50 µg), were subjected to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the proteins were transferred to a nitrocellulose membrane by electroblotting and probed with primary antibodies overnight at 4˚C. All antibodies from Santa Cruz were diluted 1:200 in 5% non-fat milk (Merck, Germany)/TBST (Tris-buffered saline, 0.05% Tween 20), remaining antibodies were diluted 1:1000. The following antibodies were applied for immunoblotting: mouse γ-tubulin (WB:1:2000, Sigma-Aldrich, # T5326),rabbit phospho-MEK1/2 (WB:1:1000, S217/S221, # 9154), rabbit phospho-ERK1/2 (WB:1:1000, T202/T204, # 9106), rabbit phospho-Akt (S473, WB:1:1000# 4060 and T308, #2965), phospho-YAP (WB 1:1000; Ser 127; Cell Signaling, #4911), YAP (WB 1:1000; Cell Signaling, # 4912), JNK (WB 1:1000; Cell Signaling, # 9252), phospho-JNK (WB 1:1000; Thr 183/Tyr185; Cell Signaling, # 9251), S6K (WB 1:1000; Cell Signaling, # 2708), phospho-S6K (WB 1:1000; Thr389; Cell Signaling, # 9205), phospho-p38 (WB 1:1000; Thr180/Tyr182; Cell Signaling, #9211) and p38 (WB 1:1000; Cell Signaling, # 8690), OCT3/4 (WB: 1:1000; Santa crus, #sc-5279), alpha-actinin (WB 1:1000, Sigma, # A7811), cardiac troponin t (WB:1000, Invitrogen, #MA5-12960), Myosin light chain 2 V (Synaptic Systems, # 310111), ATP2A2/SERCA2 (WB 1:1000; Cell Signaling, # 4388), RAF1 (WB 1:1000; abcam, #AB181115), phospho-RAF1 (WB 1:1000; S259, abcam, #ab173539). Membranes were stained with horse radish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution). Signals were visualized using ECL (enhanced chemiluminescence) reagent (GE Healthcare).

**Immunocytochemistry**

Immunostaining was performed as described previously (Nakhaei-Rad et al., 2015). Briefly, cells were washed twice with ice-cold PBS containing magnesium/calcium and fixed with 4%

Formaldehyde (Merck) for 20 min at room temperature. To permeabilize cell membranes, cells were incubated in 0.25% Triton X-100/PBS for 5 min. Blocking was done with 3% bovine serum albumin (BSA, Merck) and 2% goat serum diluted in PBS containing 0.25% Triton X-100 for 1 h at room temperature. Incubation with primary antibodies was performed overnight. Cells were washed 3-times for 10 min with PBS and incubated with secondary antibodies 2 h at room temperature. Slides were washed 3-times and the ProLong® Gold antifade mountant (4',6-diamidino-2-phenylindole) (Life Technologies) was applied to mount the coverslips. Primary antibodies included OCT3/4 (1:1000; Santa crus, #sc-5279), TRA-1-60 (1:100, abcam), SSEA4 (1:70, Hybridoma Bank), alpha-actinin (1:200, Sigma, # A7811), cardiac troponin t (1:200, Invitrogen, #MA5-12960), RAF1 (1:250; abcam, #AB181115). Secondary antibodies included Alexa488-conjugated goat anti-rabbit IgG (Invitrogen, #A11034), Alexa546-conjugated goat anti-mouse IgG (Invitrogen, #A11003), Alexa633-conjugated goat anti-rabbit IgG (#A4671), and Alexa488-conjugated goat anti-mouse IgG (Invitrogen, #A11029) (all from Life Technologies). Confocal images were obtained using a LSM 510-Meta microscope (Zeiss, Jena, Germany).

### Transmission electron microscopy (TEM)

Small biopsies were fixed with 6% glutaraldehyde/0.4 M phosphate buffered saline (PBS) and were processed with a Leica EM TP tissue processor with 1%-osmium-tetroxide and embedded in resin. For electron microscopy, ultrathin sections were contrasted with 3% lead citrate trihydrate with a Leica EM AC20 (Ultrastain kit II) and were examined using a Zeiss EM 109 transmission electron microscope equipped with a Slowscan-2K-CCD-digital camera (2K-wide-angle Sharp:eye).

## Results

### $RAF1^{S257L}$ causes left ventricular hypertrophy, fetal arrhythmia and perivascular fibrosis

The meanwhile 18-year old female patient is the sixth of six children of healthy, unrelated parents. Pregnancy was complicated by polyhydramnion and fetal arrhythmias (Figs. 1A and 1B). The patient was born at 36-weeks gestation by caesarean section without complications. Medial epicanthus, low-set ears, deep hair line, right sided ptosis, and lateralized mamillas were noticed at birth. Immediately after birth, the ECG revealed chaotic atrial arrhythmia and ventricular arrhythmias, necessitating treatment with atenolol, while the echocardiogram displayed a biventricular hypertrophic cardiomyopathy (HCM) (Figs. 1C and D). Septal hypertrophy inclined within the next years and resulted in obstruction of the left ventricular outflow tract. A cardiac catheter examination at age 5 1/2 years demonstrated a left-ventricular intracavitary pressure gradient of 70 mmHg, prompting transaortic septal myectomy. Since then, HCM is non-progressive, not limiting daily-life activities. At the age of 8 years, she suffered one afebrile seizure. At this time, a short stature (height 114 cm, 3 cm < third percentile) was noticed. Neither lentigines nor other cutaneous symptoms were visible. Now, Noonan syndrome was suspected because of her peculiar face (Fig. 1A) in conjunction with short stature and HCM. At last follow-up, at the age of 16 years and a height of 146 cm (5 cm < third percentile), her cardiac status was stable. She is still on beta-blocker, although Holter ECGs at age 15 and 16 years displayed nor arrhythmias. Cardiac MRI at this age disclosed a mild to moderate hypertrophy of the left ventricle with a septal wall thickness

during diastole of 15 mm and left-ventricular posterior wall thickness of 14 mm, being above the normal range in adults (Fig. 1D).

Compared to a healthy individual, histological analysis of a left ventricular endomyocardial biopsy performed at age 5 ½ years showed a cardiomyopathy with increased nuclear diameter and myofibrillar disarray in H&E and Desmin stained sections (Figs. 1 F/F` and G/G`). Fibrosis was pronounced perivascular (Masson Trichrome, MT) (Fig. 1 H/H`) and the endothelial cell layer of the myocardial vessels was enlarged demonstrated by immunohistochemistry against smooth muscle actin (SMA) (Fig. 1 I/I`).

**Reprogramming of the somatic cells towards hiPSCs**

To investigate the mechanism of HCM, we need the source of pluripotent and proliferative cells to produce large amount of the cells and later differentiate them to human cardiomyocytes. Fibroblasts were obtained from dermal biopsies of NS patients heterozygotes for *RAF1S257L* (Fig. 2A). The reprogramming of fibroblast to hiPSC was performed with cocktail of non-integrating episomal vectors harboring the transcription factors of *OCT3/4*, *SOX2*, *L-MYC*, *TRP53* and *EBNA-1* as previously published. The hiPSC-RAF1 colonies stained positive for pluripotent markers of Alkaline phosphatase, OCT4, SOX2, NANOG, TRA1-60 and SSEA4. Differentiation assay confirmed that they were able to differentiate to all three germ layer, ectoderm, mesoderm and endoderm (Fig. 2).

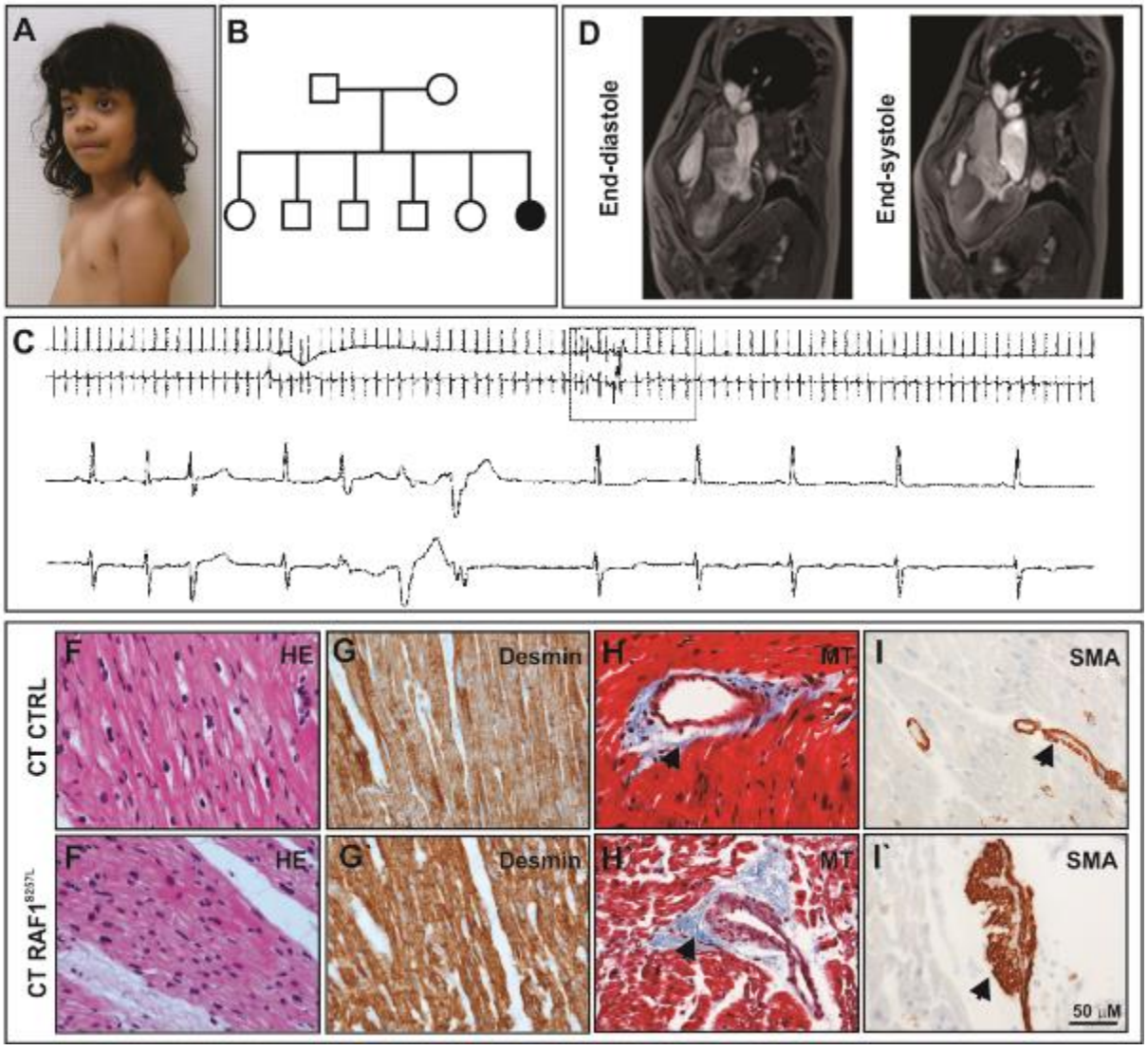


**Figure 1**. **Clinical manifestation of NS with RAF1 c.770C>T mutation.**
A) A girl with clinical diagnosis of Noonan syndrome was recognized with right-sided ptosis, down-slanted angles of the mouth, and lateralized mamillas. B) Schematic family pedigree of Noonan patient. C) ECG displaying chaotic supraventricular arrhythmia. 2-Channel Holter monitoring demonstrating a ventricular arrhythmia (triplet). D) Representative cardiac MRI in longitudinal axis during diastole (left) and systole (right) at age 16 years depicting moderate cardiac hypertrophy. F-I) Microscopy analysis of cardiac tissue (CT). F/F`) HE staining shows moderate variation of cardiomyocyte size and nuclei. G/G`) Desmin represent the microfilament disarray. H/H`) Masson trichrome staining (I/I`) shows perivascular fibrosis, smooth muscle actin staining (SMA) demonstrates endothelial thickening.

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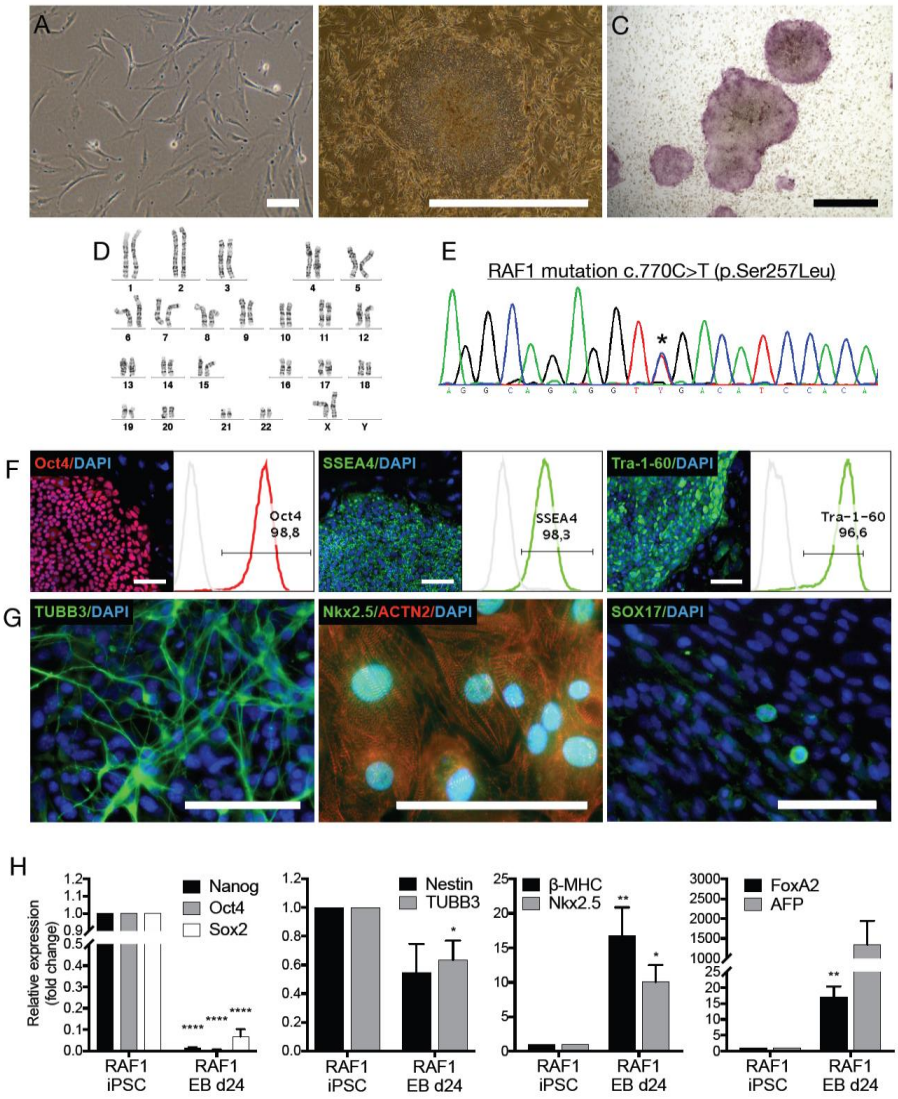


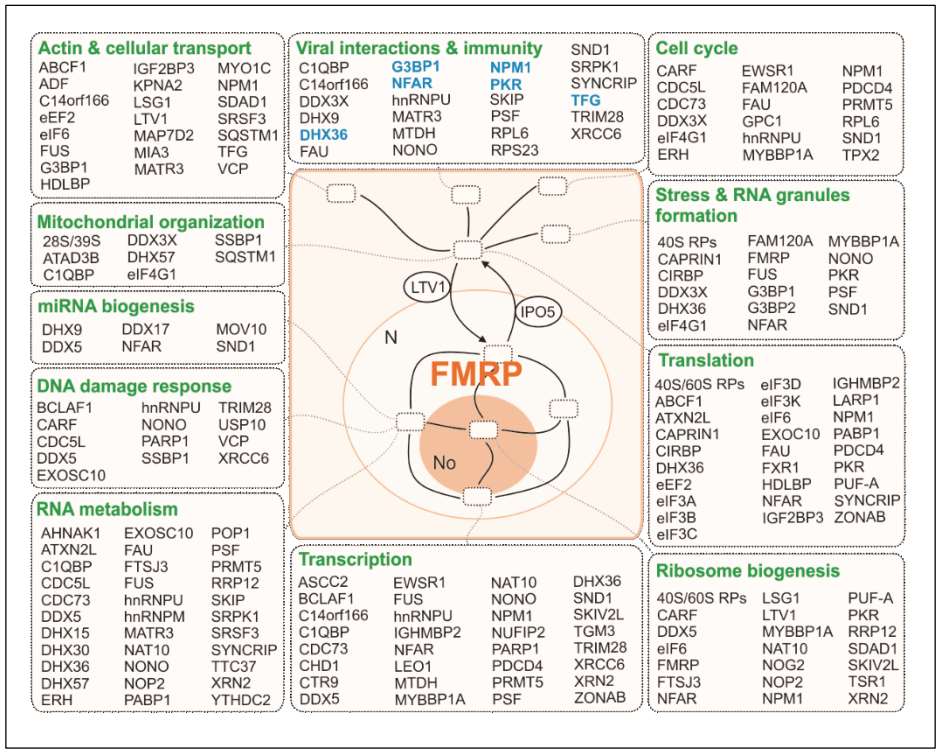

**Figure 2. Characterization of RAF1S257L-iPSCs.**

Human RAF1-iPSCs reveal a normal karyotype, express pluripotency markers and differentiate into ectodermal, endodermal and mesodermal derivatives *in vitro*. A) RAF-1 Patient derived from dermal fibroblasts. B) Typical RAF1-iPSC colony on mitotically inactivated murine feeder cells. C) Colonies stain positive for alkaline phosphatase activity. D) In passage 8 after reprogramming, iPSCs show a normal diploid karyotype. E) Sanger sequencing confirmed the heterozygous RAF1 S257L mutation in iPSCs (asterisk). F) Expression of pluripotency markers Oct4, SSEA4 and Tra-1-60 by immunofluorescence staining and flow cytometry. Gray histograms represent isotype controls. G) Trilineage differentiation of patient iPSCs. Expression of endodermal (TUBB3), mesodermal (Nkx2.5 and sarcomeric alpha actinin) and endodermal (Sox17) markers was detected. F) Relative gene expression of pluripotency (Nanog, Oct4, Sox2) and differentiation markers (Nestin, TUBB3, beta myosin heavy chain, Nkx2.5, FoxA2, alpha fetoprotein) of differentiated embryoid bodies on day 24 of differentiation relative to undifferentiated iPSCs normalized by beta actin expression. Bar graphs represent mean of three independent samples +/- SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, unpaired t test. Scale bars: A, F, G: 100 µm, B, C: 1000 µm.

# Chapter V

## Uncovering multiple protein interaction networks linked to fragile X mental retardation protein

### Physical and functional protein and RNA interaction networks of FMRP suggest its participation in different fundamental cellular processes

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|---|---|
| Status: | Submitted to Cell Communication and Signaling |
| Impact Factor: | 5.324 (2017) |
| Own Proportion to this work: | 40%<br>Cell culture (different cell lines), Immunobloting of FMRP, G3BP, Confocal microscopy, Figure preparation (Figs. 1 & 4), Documenting literatures and writing the manuscript |

# Uncovering multiple protein interaction networks linked to fragile X mental retardation protein

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**Abstract**

The fragile X mental retardation protein (FMRP) plays a critical role in RNA binding, mRNA transport, and mRNA translation in neuronal and nonneuronal tissues. However, the underlying molecular mechanisms, including the cellular FMRP protein networks, remain elusive. Here, we employed affinity pull-down and quantitative LC-MS/MS analyses with FMRP and identified known and novel candidate FMRP-binding proteins and protein complexes. FMRP interacted with 180 proteins, 28 of which interacted with the N-terminus of FMRP. Interaction with the C-terminus of FMRP was observed for 104 proteins, and 48 proteins interacted with both termini. This FMRP interactome comprised known FMRP-binding proteins, including the ribosomal proteins FXR1, NUFIP2, and Caprin-1, and numerous novel FMRP candidate interacting proteins that localize to different subcellular compartments, including CARF, LARP1, LEO1, NOG2, G3BP1, NONO, NPM1, SKIP, SND1, SQSTM1, and TRIM28. Our data considerably expand the protein and RNA interaction networks of FMRP and thereby suggest that, in addition to its known functions, FMRP participates in transcription, RNA metabolism, ribonucleoprotein granule formation, translation, DNA damage response, chromatin dynamics, cell cycle regulation, ribosome biogenesis, miRNA biogenesis, and mitochondrial organization. Thus, it is appropriate to appreciate and analyze the common roles of FMRP and novel candidate interacting proteins throughout the body, beyond the CNS.

**Key words:** FMRP, fragile X mental retardation protein, protein interaction network, RNA binding, signal transduction, ribosomes, gene ontology

**Introduction**

Genetic deficiency of the fragile X mental retardation protein (FMRP; also known as FRAXA, MGC87458, POF, and POF1) results in the most common inherited form of intellectual disability, fragile X syndrome (FXS; also known as Escalante's syndrome or Martin–Bell syndrome) [1]. FMRP is a well-studied RNA-binding protein (RBP) that regulates local translation [2-8] and is involved in the control of calcium channels [9], actin cytoskeletal dynamics [10-12], chromatin dynamics [13], DNA damage response (DDR) [13, 14], and replication stress response [15]. These cellular functions presume physical properties for FMRP, which are required for both the recognition and localization of messenger RNA (mRNA) targets and direct association with a multitude of proteins and protein complexes [16, 17]. In view of so many different, seemingly fundamental functions, it is appropriate to appreciate common roles of FMRP throughout the body, beyond the brain and spinal cord.

Despite its ubiquitous expression, the function and expression of FMRP remain understudied in nonneuronal tissues. FMRP plays critical roles in germline development during oogenesis [18], spermatogenesis [19], regulating heart rate during development [20], endothelial cell proliferation and angiogenesis [21], stem cell maintenance and differentiation [22], and tumor progression. FMRP controls downregulation of E-cadherin and upregulation of vimentin and is involved in different stages of aggressive breast cancer including the invasion of cancer cells into blood vessels and the spread of these cancer cells to other tissues [23]. FMRP is involved in relevant processes of melanoma progression. The reduction of FMRP in metastatic melanoma cell lines impinges on cell migration, invasion, and adhesion [24]. Enhanced FMRP expression in astrocytoma has been proposed to promote proliferation through the activation of MEK/ERK signaling [25].

The most prominent and studied function of FMRP is involved in translational regulation. The mechanisms of translational regulation by FMRP are not entirely clear, although mounting evidence suggests that FMRP suppresses the translation of its target mRNAs *via* association with either stalled nontranslating polyribosomes or microRNA (miRNAs) [26-29]. This can lead to the formation of cytoplasmic ribonucleoprotein (RNP) granules, which control the expression, repression, or decay of specific mRNAs [30]. There are two types of cytoplasmic RNA granules, eukaryotic RNA processing bodies (P-bodies) and stress granules (SGs), that transport, store or degrade mRNAs, thereby indirectly regulating protein synthesis [30-33]. There is increasing evidence suggesting that such RNP granules are associated with several age-related neurodegenerative diseases [34].

FMRP consists of an N-terminal domain comprising two tudor (Tud) domains and one K homology 0 (KH0) domain, a central region comprising two KH1 and KH2 domains, and a C-terminal domain comprising a phosphorylation site [35] and an arginine-glycine-glycine (RGG) region [36]. FMRP displays a nuclear localization signal (NLS), a nuclear export signal (NES) and two nucleolar localization signals (NoLSs) [17, 37-40], consequently localizing FMRP to different subcellular compartments in the cytosol and nucleus [17]. Nuclear FMRP has been suggested to regulate the

DDR and genomic stability as a chromatin-binding protein [13]. However, the interaction networks modulating these functions of FMRP remain unclear.

Taken together, we hypothesize that FMRP is involved in numerous cellular functions in various subcellular compartments. Therefore, in this study, we addressed novel FMRP-interacting proteins using a proteomic approach and described known and numerous novel and potential FMRP interactors and networks that are involved in diverse subcellular processes, both in neuronal and nonneuronal cells.

**Experimental section**

**Constructs and Proteins**

Amino-terminal (N-term; aa 1-218), central (aa 212-425) and carboxy-terminal (C-term; aa 444-632) fragments of human FMRP were amplified by standard PCR and cloned into pGEX-4T1-Ntev. *Escherichia coli* BL21 Rosetta was transformed with the respective FMRP plasmids. The expression and purification of the proteins was performed as previously described [41, 42]. pFLAG-CMV2 was used for G3BP1 constructs comprising full-length (FL), M1 (1-138), M2 (139-466), M3 (222-466) and M4 (338-466), as reported previously [43].

**Antibodies and other reagents**

The antibodies used in this study were as follows: anti-FMRP (ab17722) and anti-nucleophosmin (ab10530) (purchased from Abcam); anti-PKR (Sc-6282), anti-FMRP (F6072), and anti-G3BP (611126) (purchased from BD Transduction); and anti-γ-tubulin (T5326) (purchased from Sigma-Aldrich). The anti-GST antibody was made in our lab. 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Life Technologies, and sodium arsenite was purchased from Merck. Anti-FLAG (F3165) antibody and secondary antibodies (IgG) Alexa Fluor 488 and 564 were obtained from Molecular Probes (Oregon, USA). RNase treatment was performed using RNase A (Qiagen, Hilden, Germany) as described previously [17].

**Cell culture**

HEK293 (human embryonic kidney cells), HDF (human dermal fibroblasts), and cancer cell lines such as HepG2, HeLa, PANC1 and MCF7 were cultured in DMEM (ThermoFisher, 11965092), whereas NT2, BPH1, and SW480 cells were cultured in RPMI (ThermoFisher, 11875093); all media were supplemented with 10% FBS (ThermoFisher, 10270-106). Human iPSCs were cultured as previously described [44]; briefly, cells were cultured in irradiated mouse embryonic fibroblast (iMEF) conditioned medium supplemented with 100 ng/ml bFGF (Peprotech, 100-18B). HUVECs were cultured in Endothelial Cell Growth Medium (PromoCell) containing 100 U/ml penicillin and streptomycin (Genaxxon Bioscience, M3140.0100).

**Immunofluorescence microscopy**

Confocal imaging of HeLa cells was performed as described previously [17]. Images were obtained as single optical slides using an LSM510-Meta confocal microscope equipped with a 40x/1.3 immersion objective and excitation wavelengths of 364 nm, 488 nm, and 546 nm. Superresolution structured illumination microscopy (SR-SIM) was performed as described recently [45] using the ELYRA microscope (ZEISS, Jena, Germany) with an alpha Plan-Fluor 100x/1.45 M27 oil-immersion objective and immersion oil type 518F/30°C (ZEISS, Jena, Germany). Images were acquired using ZEN 2.0 software (ZEISS, Oberkochen, Germany).

**Transfection and pull-down assay**

GST pull-down experiments were conducted as described previously [17]. GST-FMRP$^{N\text{-term}}$, GST-FMRP$^{C\text{-term}}$, and GST as a negative control were immobilized on GSH agarose beads, subsequently mixed with HeLa total cell lysate and incubated for 1 h at 4°C to pull-down associating proteins and protein complexes. The beads from three independent experiments were washed four times, boiled in 1x SDS loading buffer for 5 min and separated on 10% tricine sodium dodecyl sulfate (SDS) polyacrylamide gels. Lanes of Coomassie Brilliant Blue (CBB)-stained SDS gels were cut in different sections, excluding the bands related to GST-FMRP$^{N\text{-term}}$, GST-FMRP$^{C\text{-term}}$, and the GST control. Gel sections were subjected to mass spectrometry. The pull-down of flag-tagged G3BP1 protein fragments that were overexpressed in HeLa cells was performed using purified GST-FMRP$^{N\text{-term}}$ and visualized by immunoblotting using anti-Flag and anti-GST antibodies. HeLa cells were transfected using TurboFect Transfection Reagent (Thermo Scientific) as previously described [17].

**Immunoprecipitation**

For coimmunoprecipitation, HeLa cells were lysed in immunoprecipitation buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40, 10 mM β-glycerophosphate, 0.5 mM $Na_3VO_4$, 10% glycerol, and EDTA-free

protease inhibitor). IP from total cell lysates was carried out for 2 h at 4°C with an anti-FMRP antibody (ab17722). The beads were washed 5 times with IP buffer lacking NP-40, and eluted proteins were heated in SDS-Laemmli buffer at 95°C and analyzed by immunoblotting.

**Immunoblotting**

Cell lysates were prepared using lysis buffer [50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 1% Igepal CA-630, 10% glycerol, 20 mM β-glycerophosphate, 1 mM $Na_3VO_4$, EDTA-free protease inhibitor (Roche Applied Science)], and protein concentrations were measured by Bradford assay (Bio-Rad). Equal amounts of total cell lysates (40 µg) were loaded on SDS-PAGE gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and blocked for one hour in 5% nonfat dry milk (Merck)/TBST (Tris-buffered saline, 0.05% Tween 20). Membranes were probed with primary antibodies at 4°C overnight and later stained for one hour at room temperature with both horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5.000 dilution) and fluorescent secondary antibodies (1:10.000 dilution) (DAKO, Germany). Signals were visualized using ECL (enhanced chemiluminescence) reagent (GE Healthcare) and the Odyssey Fc Imaging System (LI-COR Biosciences).

**Mass spectrometry and data analysis**

GST controls as well as N-term and C-term samples were cut into five and six gel pieces, respectively, excluding only the area of the GST fusion protein. Proteins in gel pieces were reduced by 10 mM dithiothreitol and alkylated with 55 mM iodoacetamide. Proteins were digested for 16 h at 37 °C with 0.1 µg trypsin (Serva, Heidelberg, Germany) in 100 mM ammonium hydrogen carbonate in water. Tryptic peptides were extracted twice with 1:1 (v/v) solution of acetonitrile and 0.1% trifluoroacetic acid, and after acetonitrile removal resuspended in 0.1% (v/v) trifluoroacetic acid and subjected to a liquid chromatography system (RSLC, Dionex/Thermo Scientific, Idstein, Germany) equipped with an Acclaim PepMap 100 C18 column (75 µm inner diameter, 50 cm length, 2 mm particle size from Dionex/Thermo Scientific, Idstein, Germany) coupled to an Orbitrap Elite mass spectrometer (Thermo Scientific, Bremen, Germany) essentially as described [46]. For protein and peptide identification and quantification, raw files were further processed using the MaxQuant software suite version 1.3.0.5 (Max Planck Institute of Biochemistry, Planegg, Germany). Database searches were carried out against the UniProt database (release 06.2013) using standard parameters. Label-free quantification was performed using the "match between runs" option with a time window of 2 min. Peptides and proteins were accepted at a false discovery rate of 1%, and proteins with quantitative information available for at least three analyzed samples were subjected to subsequent statistical analysis. Protein quantification was performed using the SAM algorithm [47] implemented in Perseus version 1.2.7.4 (Max Planck Institute of Biochemistry, Planegg, Germany) on log-transformed data (false discovery rate threshold: 0.01). Missing values were replaced by imputation (width: 0.3; downshift: 1.8).

**Gene Ontology analysis**

Gene Ontology (GO) terms for the biological processes, molecular functions, and cellular locations of FMRP-interacting proteins, including isoforms, paralogs or related proteins, were identified using the PANTHER database [48].

**Protein-protein interaction network**

A protein-protein interaction network for FMRP was generated with STRING 11 and visualized with Cytoscape 3.7.1.[49]

**Results**

**Ubiquitous expression of FMRP in human cells**

In a previous study, we observed large amounts of FMRP in different cell lines, i.e., COS7, HEK293, HeLa, MDCKII, MEF, and NIH3T3 [17]. To examine the existence of FMRP in various types of nonneuronal human cells, we investigated primary cells (stem and cancer stem cells) and different types of cancerous cells. Figure 1A clearly depicts that FMRP was expressed in many types of nonneuronal cells, consistent with findings in thrombocytes [50] and human embryonic stem cells [51]. This suggests that FMRP likely represents a protein with important and conserved functions across human tissues.

**Identification of novel FMRP-interacting proteins**

To identify proteins associated with different regions of FMRP in a proteomic approach, we used HeLa cells. This comprehensive study was based on several factors such as reproducible culture conditions, available subcellular fractionation and subcellular localization data [17], possible roles of

FMRP in cancer progression [23], and the fact that FMRP, as a multifunctional protein, exists in many types of nonneuronal cells (Fig. 1A). Thus, we conducted affinity pull-down experiments using total HeLa cell lysates and purified GST fusion protein fragments consisting of the N-terminal (N-term; aa 1-218), central (aa 212-425), and C-terminal (C-term; aa 444-632) domains of human FMRP.

Since most FMRP protein-protein interactions were exclusively achieved *via* the N- or C-termini and rarely *via* the central RNA-binding fragments [52-55], we excluded the latter from further analysis. Pull-down samples were separated on SDS gels, which were cut in different fractions, excluding the bands related to GST-FMRP$^{N\text{-}term}$, GST-FMRP$^{C\text{-}term}$, and the GST control (Fig. 1C, red boxes). The gel fractions were reduced, alkylated, and digested with trypsin. The resulting peptide mixtures were analyzed by mass spectrometry as described in the Materials and Methods.

All proteins interacting with GST-FMRP$^{N\text{-}term}$ and/or GST-FMRP$^{C\text{-}term}$ were detected and individually validated with a high degree of confidence based on the peptide sequences using specific databases and programs as described in the Materials and Methods. The criteria for considering proteins as significant interactors of FMRP included their presence in all three independent pull-down experiments, their absence in the GST pull-down controls, and *P*-values of $< 0.05$.

Collectively, we short-listed a set of 102 FMRP-interacting proteins, 22 of which were associated with FMRP$^{N\text{-}term}$, 67 were associated with FMRP$^{C\text{-}term}$, and 13 were found to bind to both termini (Tables 1-3). Table 4 summarizes 78 isoforms and paralogs, including the array of ribosomal proteins that were excluded from the major lists of binding partners. In our proteomic approach, we found nine previously reported FMRP interactors, i.e., ATXN2L, Caprin-1, DDX5, FMRP, FXRP, MOV10, NUFIP2, PABP1, and PARP1 [16, 29, 56-58]. Known FMRP-interacting partners, such as nucleolin and CYFIP [17], were excluded in this study as they were also present in the GST pull-down controls. Other FMRP-interacting proteins, including EIF4E [59], AGO1 [60] and β-catenin [61], were absent in our lists, which may be based on expression levels in different cell types and/or on experimental pull-down conditions.

A group of 13 proteins were found to be associated with both N-terminal and C-terminal fragments of FMRP (Table 3). One obvious explanation is that FMRP may interact with two proteins within the same protein complex. In this case, components of a protein complex that do not directly bind to FMRP will be pulled down. For these candidates, we calculated the values of the exponentially modified protein abundance index (emPAI) [62] by comparing the number of identified MS/MS spectra from the same protein in each of the multiple LC-MS/MS datasets. Therefore, the term 'abundance' was used to suggest one possible interacting domain. Based on the label-free quantification intensity value, we measured the abundance of each candidate in the FMRP$^{N\text{-}term}$ and FMRP$^{C\text{-}term}$ pull-down experiments (Fig. 1E). As exemplified for C16orf166, C1QBP, and PABP1, some FMRP-interacting proteins were clearly more capable of interacting with one FMRP terminus than the other, while other interacting partners (*e.g.*, Caprin-1 and NUFIP2) were found at nearly the same protein levels in both FMRP$^{N\text{-}term}$ and FMRP$^{C\text{-}term}$ pull-down experiments. In that case, the arrows were used to mark slightly higher protein levels. This differentiation method was prominent for some proteins, *e.g.*, the highly abundant protein G3BP1, which was shown by immunoblotting analysis to interact specifically and directly only with FMRP$^{N\text{-}term}$ (Fig. 1D). Another critical aspect is the oligomerization properties of FMRP through its N-terminus [40]. In this scenario, GST-FMRP$^{N\text{-}term}$ binds endogenous, oligomeric FMRP and, in this way, proteins interacting with its C-terminus.

Interestingly, the vast majority of interactions involve the C-terminus of FMRP (Table 2) and not its N-terminus, as we and others previously proposed [17, 53]. A striking characteristic of FMRP$^{C\text{-}term}$ is the presence of unstructured regions, including arginine-glycine-glycine-rich (RGG) motifs, phosphorylation sites, a nuclear export signal (NES), and two newly identified NoLSs [17, 63]. It has also been reported that many FMRP binding proteins contain RGG motifs [64], which may interact not only with proteins but also with RNAs [64-66]. A large number of the identified FMRP interaction partners were RNA-binding proteins, processing and/or transporting proteins, including components of the translation machinery, different helicases and transcription factors (Tables 1-4; highlighted in bold). Thus, we tested three different FMRP-interacting proteins such as nucleophosmin-1 (NPM1), protein kinase R (PKR), and RAS GTPase activating protein SH3 domain-binding protein 1 (G3BP1) for their RNA-

dependency on interacting with purified FMRP in the presence and absence of RNase A. As shown in Figure 1D, FMRP interacts with endogenous FMRP and G3BP1 *via* its N-terminal region and with NPM1 and PKR *via* its C-terminal region. None of these interactors bind to the central region of FMRP. RNase A treatment of the lysates for 45 min at 4°C did not affect these interactions. In contrast, G3BP1 binding to $FMRP^{N\text{-}term}$ appears to be facilitated.

The N-terminus of FMRP harbors different protein binding characteristics due to various subdomains (Fig. 1B). Two conserved Tud1/2 domains (also called the N-terminal domain of FMRP 1 and 2 or NDF1 and NDF2, respectively)[17, 53] are within the "Royal Family" of proteins, which includes Agenet, MBT, PWWP, and chromo domains [67]. FMRP and Tud1/2 have been shown to selectively associate with trimethyl-lysine peptides derived from histones H3K9 and H4K20 [53, 68] together with chromatin [13]. The N-terminus of FMRP has been proposed to be a platform for multiple protein-protein interactions [53]. However, we detected only a relatively small number of binding proteins for $FMRP^{N\text{-}term}$ compared to $FMRP^{C\text{-}term}$. A recent structure of the flexible $FMRP^{N\ term}$ has revealed that this domain resembles a K homology (KH) domain [69] that is directly linked to the tandem KH domains of $FMRP^{central}$ (Fig. 1B). KH domains are typically RNA and single-stranded DNA-binding modules; these molecules were first described for the heterogeneous nuclear RNA-binding protein (hnRNP-) K [70, 71]. A similar scenario as discussed above for $FMRP^{C\text{-}term}$ may apply for $FMRP^{N\text{-}term}$, i.e., its interactions may not all be direct protein-protein interactions but rather mediated *via* RNAs. G3BP1 belongs to the evolutionarily conserved hnRNP family. It is involved in an array of biological activities, ranging from cell-cycle regulation to mRNA metabolism and stress granule assembly [43, 72]; however, G3BP1 binds to $FMRP^{N\text{-}term}$ in an RNA-independent manner (Fig. 1D).

**FMRP is associated with multiple cellular processes**

FMRP has been described previously to be involved in different biological functions, *e.g.*, RNA transport, protein translation, actin cytoskeleton remodeling, and SG formation [1, 6, 8, 13, 26, 27, 73-80]. Most of these functions have been linked to the ability of FMRP to control the translation of numerous different mRNAs [81], potentially explaining why FMRP is expressed in several tissues and cell lines, including iPSCs (Fig. 1A). FMRP has been previously suggested to play a role in the maintenance and differentiation of iPSCs [22]. The FMRP-interacting proteins identified in this study were classified into three ontologies: biological process, molecular function, and cellular component (Fig. 2). The vast majority of these proteins are involved in binding nucleic acids, especially mRNA, rRNA, and miRNA (Tables 1-4; bold), and thereby participate in transcription, RNA metabolism, SG formation, and translation (Fig. 3). These functions imply an intracellular shuttling of FMRP into/between different subcellular compartments of the cell. FMRP has been previously described to be predominantly cytoplasmic [82]. However, in recent years, it has become increasingly evident that FMRP translocates into the nucleus due to sequence motifs responsible for its nuclear import and export as well as nucleolar localization [13, 15, 17, 39, 68, 83-88]. Notably, LTV1 and IPO5, which were found in our proteomic analysis, may be involved in nucleocytoplasmic shuttling of FMRP.

**Exemplified functional analysis of FMRP interactions**

To obtain specific functional insights into FMRP interactions, we performed immunoprecipitation of endogenous FMRP from HeLa cell lysates. Consistent with Figure 3, we again observed NONO and G3BP1 as interacting proteins in the immunoblot analysis (Fig. 4A). These two FMRP interactors have been previously described as components of neuronal cytoplasmic RNP granules [89]. A different type of cytoplasmic RNA granules represents stress granules (SGs), which have been shown to contain FMRP [90] and G3BP1 [43]. However, a direct interaction between FMRP and G3BP1 has not yet been reported. Thus, we pulled down different G3BP1 fragments (Fig. 4B) that were overexpressed in HeLa cells using purified GST-$FMRP^{N\text{-}term}$. As presented in Figure 4C, G3BP1 full-length and M3, but not M1, M2, or M4, bound to $FMRP^{N\text{-}term}$, suggesting that the proline-rich region (PXXP) of G3BP1, which is located between the acidic region and the RRM/RGG (Fig. 4B), may play a central role in the direct interaction with the N-terminal region of FMRP. The acidic region of G3BP1has been shown to have an inhibitory effect on G3BP1/2 protein interactions [43, 91]; therefore, we assume that G3BP1 has a similar effect when it is expressed without the NTF-like domain. Consequently, it may counteract the binding of G3BP1 M2 to $FMRP^{N\text{-}term}$ but not full-length G3BP1.

To investigate the functional relevance of this interaction, we next analyzed the relative levels of FMRP and G3BP1 as well as their localization in different cell lines treated with sodium arsenite, an oxidative stress agent. The expression levels of FMRP and particularly G3BP1 increased in cells under stress conditions, strikingly, in human dermal fibroblasts (Fig. 4D), suggesting that they have critical functions under stress conditions. This prompted us to examine their subcellular distributions in the same cell lines in the presence and absence of sodium arsenite. Confocal and superresolution microscopy imaging revealed that both FMRP and G3BP1 are recruited to and accumulated in SGs (Fig. 4E), presumably in large protein complexes, including Caprin-1 [92].

**Discussion**

In the following section, we discuss selected functional relationships of newly identified proteins that potentially interact with FMRP in selected cellular processes (Fig. 3 and Tables 1-4).

**Transcription**

Eukaryotic gene transcription is evolutionarily highly conserved between budding yeast and humans. This is based on deep structural and functional homologies among promoter factors, regulatory proteins, and RNA polymerases [93]. The latter are divided into three different enzyme systems: RNA polymerase I (RNAP I) synthesizes ribosomal RNA (rRNA); RNAP II synthesizes mRNAs and different types of noncoding RNA (ncRNA); and RNAP III synthesizes tRNA and some types of ncRNA. The fact that more than 400 different mRNAs are associated with FMRP [3, 23, 94] strongly indicates that FMRP may be involved in diverse processes, including mRNA synthesis by RNAP II, processing by the spliceosome, and transport. Interestingly, FMRP interacted with several RNAP II-associated factors, including C14orf166, CTR9, CDC73, and LEO1 (Fig. 3A). These proteins, together with PAF1 and RTF1, belong to the highly conserved and broadly utilized PAF1 complex (PAF1C), which regulates a variety of processes, such as transcription-coupled histone modifications; initiation, elongation and termination of transcription by RNAP II; and RNA processing [95]. Another possible role of FMRP in transcription and chromatin remodeling may be cell reprogramming and differentiation due to its interaction with key elements, such as its interaction with PSF, NONO and hnRNP-U, which together with RNAP II, p-TEFb, NWASP, and nuclear actin are key elements in cell reprogramming and differentiation [96]. P-TEFb also regulates transcription termination by promoting chromatin recruitment and activating FMRP-associating XRN2, which is a cotranscriptional RNA processing enzyme [97].

Various FMRP-associated proteins modulate NFκB-, Rb-, and p53-controlled transcription (Fig. 3A). SKIP potentiates the activity of important transcription factors, including the vitamin D receptor, CBF1, SMAD2/3, and MYOD, and synergizes with SKI in overcoming pRb-mediated cell cycle arrest [98]. BCLAF1, which also binds FXR1P [99], controls p53 expression in a PKCδ-dependent manner [100]. MYBBP1A has been reported to enhance p53 tetramerization and acetylation in response to nucleolar disruption [101]. NAT10 regulates p53 activation through p53 acetylation and MDM2 ubiquitination [102]. In contrast, EWSR1 induces acute myeloid leukemia by inhibiting the p53/p21 pathway [103]. The interaction of the nuclear corepressor TRIM28 with MDM2 contributes to p53 inactivation [104, 105]. ASCC2 and ASCC3 are components of the ASC-1 complex that stimulate transactivation by NFκB, SRF, and AP1 through direct binding to SRF, c-JUN, p50, and p65 [106]. PRMT5 dimethylates the p65 subunit to activate NFκB [107], whereas the tumor suppressor PDCD4 inhibits NFκB-dependent transcription, *e.g.,* in human glioblastoma cells by direct interaction with p65 [108]. XRN2 interacts with the NFκB-repressing factor and regulates transcription elongation [109]. MYBBP1A appears to be an NFκB corepressor of transcription by competing with p300 [110]. In this context, TGM3, a candidate tumor suppressor [111], appears to interfere with the NFκB signaling pathway and promote proliferation [112].

The FMRP-associated proteins AGR2, ATAD3A, DHX15, and RPL17 were identified in a proteome-wide analysis of hepatocellular carcinoma as binding partners of AGR2 [113]. Their FMRP-associated roles in transcriptional control remain to be investigated. Thus, FMRP appears to contribute to RNAP II-associated synchronization of biosynthesis, processing, transport, stability, and translational control of mRNAs through protein- and RNA-binding.

**RNA metabolism**

The majority of FMRP-interacting proteins are RBPs and are involved in RNA metabolism, which

refers to any event in the life cycle of RNA molecules, including their synthesis, folding/unfolding, splicing, modification, processing, nuclear export, transport, storage, translation activation or inhibition, and degradation. RNA helicases are RBPs, which represent a large family of proteins that play central roles in almost all biological processes in living cells [114]. The majority of FMRP-associated proteins are involved in RNA metabolism and include RNA helicases and splicing factors.

FMRP-interacting RNA helicases comprise DDX5, DHX9, and CHD1 (which are involved in the DNA damage response; see above) as well as DDX1, DDX3X, DHX15, DDX17, DHX36, IGHMBP2, MOV10, HLP/SKI2, YTHDC2, and YBX3 (Fig. 3B). DDX1 has been found together with the FMRP-associating proteins PSF, Caprin-1, and PQBP1 in a protein complex that interacts in an RNA-dependent manner [115]. PQBP1 has been shown to colocalize with FMRP to stress granules (SGs) [115]. DDX3X has been shown to stimulate the translation of a subset of mRNAs with long and structured 5′ UTRs, such as RAC1 [116]. On the other hand, DDX3X promotes cell migration and spreading by physically interacting with PABP1 and Caprin-1 [117], two other FMRP-associated proteins.

The FMRP-associated proteins AGR2, ATAD3A, DHX15, and RPL17 were identified in a proteome-wide analysis of hepatocellular carcinoma as binding partners of AGR2 [113]. A common function of FMRP and DHX36 is binding to RNA G-quadruplexes, which are stable secondary structures that play key roles in RNA metabolism [118]. In this way, DHX36 regulates p53 pre-mRNA 3'-end processing following UV-induced DNA damage [119], while CDC73 targets and destabilizes p53 mRNA [120]. IGHMBP2 was suggested to be a component of the translational machinery by physically associating with tRNAs [121]. Among other functions, DDX1, DDX3X, DHX9, and MOV10 are involved in nuclear RNA export [114]. Similar to FMRP, SKIP localizes both in the nucleolus and cytoplasm and may be involved in ribosome biogenesis and translation [122]. The functional relation of the RNA helicase YTHDC2 to FMRP remains unclear.

The removal of introns from pre-mRNA transcripts is a critical intermediate step in the expression of protein-coding genes. This process takes place in the nucleoplasm and is catalyzed by a large and dynamic small nuclear RNP (snRNP) complex called the spliceosome [123, 124]. Several FMRP-interacting proteins are well-studied splicing factors, such as ERH, NONO, PRMT5, PSF, SKIP, SRSF3, and SRPK1 (Fig. 3B). PSF and NONO form heterodimers and participate in various aspects of RNA metabolism, including transcription, pre-mRNA splicing, 3′ polyadenylation of mRNA, and nuclear retention of mRNA [125, 126]. The splicing of mRNA requires a group of essential factors known as SR proteins, which undergo cytoplasmic-nuclear shuttling upon phosphorylation by SRPK1 [125, 126]. Interestingly, the AKT-SRPK1-SR axis constitutes a major pathway in transducing EGF signaling to regulate alternative splicing in the nucleus. SRPK1-mediated phosphorylation of SRSF1 has been shown to regulate alternative splicing of RAC1B [127], which is a hyperactive form of RAC1 [128]. Moreover, ERH appears to regulate SRPK1-mediated phosphorylation of the lamin B receptor and SR proteins [129]. ERH is also associated with RNA processing complexes. It binds to the spliceosomal Sm complex and is required for splicing various mRNAs, including the mitotic motor protein CENP-E [130] and the DDR protein ATM [131]. PSF interacts with snRNA components of the spliceosome, is a component of the 3′ polyadenylation complexes SF-A, and binds together with MATR3 and NONO to hyperedited RNA [126]. SKIP counteracts p53-induced apoptosis by recruiting the 3' splice site recognition factor U2AF65 to the p21 pre-mRNA [132]. The methylation of arginines, *e.g.*, by PRMT5, enhances interactions with the Tudor domains of the splicing factors SMN and SPF30 [133]. SRSF3 links pre-mRNA processing to mRNA export by recruiting the nuclear export factor NXF1 [134]. The FMRP-interacting proteins C14orf166 and DDX1 form a complex with HSPC117 and FAM98B, underlay a nucleocytoplasmic shuttling in response to transcriptional activity, and may play a role in nuclear and cytoplasmic RNA fate [135]. Notably, C14orf166 interacts with RNAP II, modulates nuclear RNA metabolism, participates in RNA splicing, and is present in cytoplasmic RNA granules involved in localized translation [135].

**RNA granules**

Cytoplasmic RNP granules in germ cells (polar and germinal granules), somatic cells (SGs and processing bodies), and neurons (neuronal granules) have emerged as important players in the posttranscriptional regulation of gene

expression. RNA granules contain various ribosomal subunits, translation factors, decay enzymes, helicases, scaffold proteins, and RNA-binding proteins, and they control the localization, stability, and translation of their RNA cargo (Fig. 3C) [136, 137]. A very recent report has demonstrated a central role of FMRP in granule biology by monitoring the transport and fusion of RNA granules throughout neuronal processes [33]. Most interestingly, RNA granules, isolated and purified from mouse brain homogenates [33], contain more than 20 FMRP-interacting proteins along with most 40S and 60S ribosomal proteins, as detected in HeLa cells (Table 4). Three FMRP-associated proteins, G3BP1, NONO, and PSF, have been detected in complex with RBPMS in neuronal cytoplasmic RNA granules [89]. FMRP immunoprecipitation revealed that G3BP1 and NONO coimmunoprecipitated with FMRP in HeLa total cell lysates (Fig. 4A), suggesting their critical interrelated roles in the formation, integrity, and/or transport of cytoplasmic RNP granules. This and the fact that FMRP exists in almost every cell type (Fig. 1A) strongly indicate the existence of such RNA granules as the regulatory machinery for local translation in the cell.

The activation of stress response pathways often promotes the formation of stress granules (SGs) throughout the cytoplasm of stressed cells [138]. SGs are dynamic aggregates of untranslated mRNAs that are sorted between decay, storage, or polysome assembly [138, 139]. SGs also contain many signaling proteins [139]. The association of FMRP with the translation machinery and polysomes in SGs has been described frequently in several laboratories [5, 90, 140-143]. Thus, it was not surprising that FMRP itself and many FMRP-interacting proteins have been shown to localize in SGs [144-151], the vast majority of which are RBPs (Tables 1-4). Inhibition of translation initiation, achieved by the phosphorylation of eIF2α or by blocking assembly of the eIF4F complex (see below), results in the formation of SGs [139, 152]. NFAR, a double-stranded RNA-binding nuclear protein that is, similar to eIF2α, a PKR substrate [153], undergoes a heterodimeric complex with NF45 and thereby modulates RNA granule assembly and disassembly [154]. NFAR was identified in this proteomic study as one of many Caprin-1- and G3BP1-associated proteins, which we also found to be associated with FMRP [154]. SGs regulate double-stranded RNA-dependent PKR activation through a complex containing G3BP1 and Caprin-1 [155], and probably also FMRP. It has been reported that PKR recruitment to SGs requires the PXXP region of G3BP1 [156], which appears to be essential for the interaction with FMRP (Fig. 4 B-C). The role of FMRP in SG formation has been discussed in several studies [90, 140, 157], but the molecular mechanism of FMRP function awaits further investigation.

**Translation**

Translational control has an impact on many cellular and developmental processes, and most steps of translation are subjected to specific regulation. The role of FMRP as a regulator of local translation has been best investigated for neurons [4, 29]. FMRP transports coding and noncoding RNAs to the synapse and participates in local protein synthesis in dendrites. Thus, FMRP potentially influences signaling pathways involved in spine morphogenesis [4, 80, 158]. Increasing evidence suggests that FMRP interferes with the translation of its target mRNAs in two different ways: suppression of translational initiation and translocation and/or activation of miRNA pathways [1, 75-77, 158, 159].

In neurons, the vast majority of FMRP is associated with both its target mRNAs [5] and stalled, nontranslating polyribosomes [143, 160]. This process appears to be regulated by FMRP phosphorylation [35, 161]. The list of FMRP-interacting proteins that are involved in translation includes almost all ribosomal proteins, initiation factors eEF2, eIF3A, eIF4G1, and eIF6, eIF4-interacting PDCD4, ribosome-associated helicases IGHMBP2 and YBX3, and stress granule proteins ATXN2L, Caprin-1, SYNCRIP and VGL (Fig. 3D; Tables 1-4). eIF4G plays a key functional role in the initiation of cap-dependent translation by acting as an adapter to nucleate the assembly of the heterotrimeric eIF4F complex [162]. The latter consists of eIF4G, eIF4E, and eIF4A. Together with poly(A)-binding protein and eIF3, eIF4F subsequently triggers the recruitment of the 43S ribosomal preinitiation complex to the messenger RNA template [152]. PDCD4 suppresses cap-dependent translation initiation. PDCD4 tightly binds eIF4A in its inactive conformation and blocks its incorporation into the eIF4F complex, which consists of eIF4A, eIF4E, and eIF4G1/eIF4G3 [163], that then recruits the 40S ribosomal subunit to start translation initiation [164]. Analogous to PDCD4, LARP1 is also an FMRP-interacting protein and

directly binds the cap and region adjacent to the 5'-TOP motif of TOP mRNAs, thereby effectively impeding access of eIF4E to the cap and preventing eIF4F assembly [165]. Interestingly, IGF2BP3 in complex with the ribonuclease XRN2 (two FMRP-interacting proteins) destabilizes eIF4E-BP2, a negative regulator of eIF4E [166]. Caprin-1 binds G3PB1 and induces phosphorylation of eIF2α most likely through the activation of PKR, the inhibition of translation and the formation of cytoplasmic SG [167].

Several other FMRP-associated proteins act on translation at different levels and in different ways. The multi-KH protein VGL associates with free and membrane-bound ribosomes and is generally necessary for the localization of mRNAs to actively translating ribosomes [168]. IGHMBP2 is a component of the translational machinery, which physically associates with tRNAs [169]. Brain cytoplasmic RNA of 200 nucleotides (BC200 RNA) is a brain-specific, small noncoding RNA with a somato-dendritic distribution in primate neurons. SYNCRIP interacts specifically with BC200 RNA and may recruit it to mRNA transport complexes involved in the regulation of localized translation in dendrites [33, 170]. Remarkably, several other known translational regulators, including FMRP and PABP1, are components of the BC200 RNP complex [170]. FMRP promotes the translation of specific mRNAs in a complex with NAT1, eIF2 and PRRC2C [171]. In this context, we identified PRRC2C and NAT10, but not NAT1, as FMRP-interacting proteins (Fig. 3D). Another FMRP-associated protein is ABCF1, which influences the accuracy of initiation codon selection by binding eIF2 and efficiently initiates translation [172].

**Conclusion**

The data presented in this work considerably expand the physical and functional candidate protein and RNA interaction networks of FMRP and suggest its participation in various fundamental cellular processes throughout the body beyond the central and peripheral nervous systems. Accordingly, FMRP functions may start in the nucleolus following cytoplasmic–nuclear translocation, where it may be involved in the biogenesis of ribosomal subunits and most likely their nuclear export. FMRP may be part of the transcriptional factory by regulating gene expression *via* interaction and orchestration of RNA polymerase II, where it directly binds to a large set of mRNAs and transports them to sites of local translation. Upon any kind of cellular stress, FMRP accumulates at sites of stress responses and facilitates, for example, the stabilization of double-stranded RNA-binding and the activation of PKR, leading to the formation of stress granules. Moreover, our novel FMRP interactome analysis indicates that FMRP may play central roles in the DNA damage response, cell cycle regulation, intracellular transport and actin dynamics and that FMRP virally triggers innate immune responses. Of note, FMRP may also be involved in mitochondrial quality control and mitophagy, functions that are directly related to neurodegenerative and cognitive disorders, including FXS, Huntington's disease, Alzheimer's disease, Down syndrome, and progressive supranuclear palsy. Our work provides valuable insights and constitutes a useful starting point for future studies of the cellular functions of FMRP in both nonneuronal and neuronal cells.

**Abbreviations**
FMRP, fragile X mental retardation protein; FXS, fragile X syndrome; CNS, central nervous system; RBP, RNA binding protein; DDR, DNA damage response; RNP, ribonucleoprotein; SGs, stress granules; Tud, tudor domain; KH0, K homology 0 domain; NLS, localization signal; NES, nuclear export signal; NoLS, nucleolar localization signal; RGG, arginine-glycine-glycine-rich; NPM1, nucleophosmin; G3BP1, RAS GTPase activating protein SH3 domain-binding protein 1; PKR, protein kinase R; ncRNA, non-coding RNA; DSBs, DNA double-strand breaks; snRNP, small nuclear RNP; HDF, human dermal fibroblast; iPSC, induced pluripotent stem cells.

**Ethical Approval and Consent to participate**
There is no need for an ethical approval and consent to participate. This study is purely cell-based. The hiPSCs that were used in this study, were generated from a HFF-1 cell line that was bought from ATCC.

**Consent for publication**
All authors have read the manuscript and approved of the final version. Otherwise "Not applicable"

**Competing interests**
The authors declare that they have no competing interests.

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**Authors' contributions**
MRA conceived and coordinated the study. MST, FH and MRA designed the study and wrote the manuscript. MST, FH and RPP designed, performed and analyzed the experiments. MST, AS and KS performed the MS analysis, MST, SNR, BG and DH did the super-resolution imaging, and MF cloned various G3BP1 constructs. All authors reviewed the results and approved the final version of the manuscript.

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**Figure legends**

**Figure 1. FMRP protein interaction networks linked to the N- and C-terminal regions of FMRP.** (A) Immunoblot analysis depicting the expression pattern of FMRP in different human cell lines including epithelial HEK293 cells, benign prostatic hyperplasia epithelial cell line (BPH1), primary endothelial HUVECs, human dermal fibroblasts (HDF), human induced pluripotent stem cells (hiPSCs), embryonal carcinoma cells (NT2), and cancerous cell lines from different tissues (HeLa, HepG2, MCF7, PANC1 and SW480). (B) Schematic diagram highlighting major domains and motifs of FMRP. FCT, FMRP C-terminus; KH0, KH1 and KH2, tandem K homology domain (first described for hnRNP K protein); NES, nuclear export signal; NLS, nuclear localization signal; NoLS, nucleolar localization signal; RGG, arginine-glycine-glycine region; P, phosphorylation sites; Tud1 and Tud2, tandem Tudor domains. The FMRP fragments used in this study are the N-terminal (N-term), the central and the C-terminal (C-term) fragments. (C) Proteins from total HeLa cell lysates were affinity-purified using GST or GST-FMRP beads and analyzed by SDS-PAGE. Blue boxes indicate gel fragments excised for mass spectrometric (MS) analysis. Red boxes indicate GST, GST-FMRP$^{N\text{-}term}$, and GST-FMRP$^{C\text{-}term}$, which were excluded from MS analysis. Samples from three independent experiments were used for MS analysis. (D) Immunoblot analysis of newly identified FMRP-interacting proteins. GST pull-down experiments were conducted by mixing purified GST fusion proteins of FMRP$^{N\text{-}term}$, FMRP$^{central}$, and FMRP$^{C\text{-}term}$ as well as GST (negative control) immobilized on GSH agarose beads with total cell lysates (TCL) from HeLa cells in the presence (+) and in the absence (-) of RNase A. Proteins retained on the beads were resolved by CBB-stained SDS-PAGE (upper panel) and processed for Western blotting using monoclonal antibodies against GST and various FMRP-interacting proteins (lower panels). (E) Proteins identified to bind to both N- and C-terminal domains of FMRP (Table 3) revealed different abundances, which were determined based on the normalized values of the label-free quantification intensity values. The abundance is sorted from "high or 1" to "low or 0" based on color codes.

**Figure 2. Gene Ontology analysis of the identified FMRP-interacting proteins categorized according to biological process, molecular function, and subcellular localization.** (A) FMRP-associated partners were sorted into fifteen biological processes with a predominance of metabolic pathways (31% of all interactors). (B) From the molecular functions, nucleic acid- (RNA/DNA) binding proteins (35%) and ribosomal assembly factors (30%) are the major groups of FMRP interactors. (C) Cellular component classification revealed that FMRP is localized in different subcellular compartments, predominantly in the cytosol (35%).

**Figure 3. Functional interaction map for FMRP.** Interaction networks of FMRP-interacting proteins involved in transcription (A), RNA metabolism (B), RNA granules (C), and translation (D) were visualized by STRING. Black circle nodes indicate proteins identified in this study. Blue line edges indicate protein-protein interaction networks. The functions of the proteins highlighted in larger font were described in more detail in this study.

**Figure 4. FMRP physically interacts and colocalizes with G3BP1 in stress granules**. (A) Coimmunoprecipitation of G3BP1 and NONO with FMRP. Endogenous FMRP was immunoprecipitated (IP) from HeLa total cell lysates (TCL) using an anti-FMRP antibody. Immunoprecipitated endogenous proteins were probed with anti-G3BP and anti-NONO antibodies. γ-Tubulin was used as a negative control. (B) Domain organization of G3BP1 and the fragments used in this study. G3BP1 consists of four domains, an N-terminal nuclear transport factor 2-like domain (NTF2-like), an acidic region, a proline-rich region (PXXP), and a C-terminal region encompassing both an RNA recognition (RRM) and an arginine-glycine-glycine (RGG) domain. (C) Pull-down experiments were conducted by mixing GST-FMRP$^{N\text{-}term}$ immobilized on glutathione-agarose beads with HeLa cell lysates overexpressing Flag-tagged G3BP1 wild-type protein or deletion mutants. Proteins retained on the beads were resolved by SDS-PAGE and processed by Western blot analysis using anti-Flag and anti-GST antibodies to visualize G3BP1 and FMRP$^{N\text{-}term}$ proteins, respectively. (D) Increasing cellular FMRP and G3BP1 protein levels upon sodium arsenite treatment. (E) Colocalization of endogenous FMRP (red) and G3BP1 (green) in stress granules was visualized by structured illumination superresolution microscopy (SR-SIM) in HeLa cells or using an LSM510-Meta confocal microscope in

HEK293 and HDF cells treated with sodium arsenite as indicated. DNA was stained using DAPI (blue). Scale bar: 10 µm.

**Table 1.** Proteins interacting with the N-terminus of FMRP (FMRP$^{N\text{-}term}$).

| Protein name[b] | Function | MW kDa | p-value | Unique pep | Seq. coverage (%) | Acc. ID |
|---|---|---|---|---|---|---|
| Actin depolymerizing factor (ADF, Destrin) | Actin dynamics | 18.5 | 0.005 | 3 | 22.4 | P60981 |
| AHNAK nucleoprotein 2 (AHNAK2)* | Calcium signaling | 616.2 | 0.004 | 66 | 34 | Q8IVF2 |
| Anterior gradient protein 2 homolog (AGR2, AG2) | Differentiation | 20.0 | 0.016 | 3 | 20 | O95994 |
| ***Cold-inducible RNA-binding protein (CIRBP, A18hnRNP)*** | mRNA stabilization, translation, stress granules | 18.6 | 0.009 | 6 | 37.8 | Q14011 |
| **Collaborator of ARF (CARF, CDKN2AIP)** | DDR, cell growth | 61.1 | 0.004 | 7 | 21.9 | Q9NXV6 |
| ***DEAD Box Protein 5 (DDX5, p68)**** | Transcription, mRNA processing | 69.1 | 0.005 | 20 | 47.6 | P17844 |
| ***DEAH-Box protein 36 (DHX36, RHAU)**** | Transcription, mRNA processing, translation, stress granules | 114.7 | 0.003 | 9 | 11.4 | Q9H2U1 |
| Eukaryotic translation initiation factor 3 subunit K (eIF3K) | Translation | 25,043 | 0,020 | 7 | 38.5 | Q9UBQ5 |
| ***Ewing sarcoma breakpoint region 1 protein (EWSR1)*** | Transcription | 68.4 | 0.003 | 4 | 10.3 | Q01844 |
| ***Fragile X mental retardation protein (FMRP, FRAXA, POF1)*** | DDR, transcription, RNA processing, transport, translation, stress granules | 71.1 | 0.007 | 42 | 54 | Q06787 |
| ***Fused In Sarcoma (FUS, TLS)*** | mRNA splicing, transcription | 53.4 | 0.001 | 2 | 26.6 | P35637 |
| MAP7 domain-containing protein 2 (MAP7D2) | Microtubule cytoskeleton organization | 82.0 | 0.007 | 3 | 6.9 | Q96T17 |
| Melanoma inhibitory activity protein 3 (MIA3, TANGO) | Transport, ER-Golgi transport, exocytosis | 213.6 | 0.001 | 24 | 16.5 | Q5JRA6 |
| **Non-POU domain-containing octamer-binding protein (NONO)** | DDR, transcription, mRNA splicing, RNA granule, innate immune response | 54.2 | 0.007 | 9 | 27.8 | Q15233 |
| **Polypyrimidine tract-binding protein-associated splicing factor (SFPQ, PSF)*** | DDR, transcription, mRNA splicing, RNA granules, innate immune response | 76.1 | 0.002 | 2 | 19.6 | P23246 |
| Protein FAM98A | Lysosome localization, proliferation | 55.4 | 0.005 | 7 | 20.4 | Q8NCA5 |
| ***Protein LSM12 homolog (LSM12)*** | Posttranscriptional regulation, circadian clocks | 21.7 | 0.006 | 5 | 33.5 | Q3MHD2 |
| **Ski-interacting protein (SKIP, SNW1)** | mRNA processing, splicing | 61.5 | 0.002 | 4 | 9.6 | Q13573 |
| Transketolase (TKT) | Pentose phosphate pathway, growth regulation | 67.9 | 0.019 | 9 | 26.6 | P29401 |
| Tropomyosin-receptor kinase-fused gene protein (TFG) | Protein transport, secretory pathways | 43.4 | 0.011 | 12 | 41.5 | Q92734 |
| **40S ribosomal Protein S23 (RPS23)*** | Translation, stress granules | 15.8 | 0.003 | 8 | 49.7 | P62266 |
| **60S ribosomal protein L36 (RPL36)*** | Translation | 12.2 | 0.000 | 5 | 30.5 | Q9Y3U8 |

[a] Underlined proteins were previously described as direct or indirect interacting partners of FMRP; * Isoforms, paralogs or related proteins identified are listed in Table 4. Proteins highlighted in bold are RNA-binding proteins and proteins in *italic* are components of the stress granules. [b] Some proteins have more than one name (http://www.genecards.org), some alternative names or synonyms are therefore included in parentheses.

**Table 2.** Proteins interacting with the C-terminus of FMRP (FMRP$^{C\text{-}term}$).

| Protein name[b] | Function | MW kDa | p-value | Unique pep | Seq. coverage (%) | Acc. ID |
|---|---|---|---|---|---|---|
| Activating signal cointegrator 1 complex 2 subunit 2 (ASCC2)* | Transcription | 86.3 | 0.001 | 7 | 12.5 | Q9H1I8 |
| ATPase family AAA domain-containing protein 3A (ATAD3A)* | Cell growth, apoptosis | 71.3 | 0.010 | 8 | 13.7 | Q9NVI7 |
| **ATP-binding cassette subfamily F member 1 (ABCF1)** | Translation, transport | 96.0 | 0.002 | 10 | 12.8 | Q8NE71 |
| **Bcl-2-associated transcription factor 1 (BCLAF1, BTF)** | DDR, transcription, apoptosis | 106.1 | 0.000 | 7 | 10.4 | Q9NYF8 |
| **Cell division cycle 5-like protein (CDC5L)** | DDR, cell cycle, mRNA splicing, differentiation | 92.2 | 0.003 | 11 | 21.3 | Q99459 |
| **Cell division cycle protein 73 (CDC73, Parafibromin)** | Transcription, mRNA processing, apoptosis | 60.5 | 0.019 | 16 | 29.9 | Q6P1J9 |
| **Chromo-domain helicase DNA binding protein 1 (CHD1)** | Chromatin remodeling, DDR, transcription | 196.6 | 0.007 | 6 | 5.8 | O14646 |
| **Elongation factor 2 (eEF2)** | Translation | 95.3 | 0.020 | 27 | 43.1 | P13639 |
| **Eukaryotic translation initiation factor 4 gamma 1 (EIF4G1)*** | Translation, stress granules, mitochondrial organization, autophagy cell death, cell growth | 175.4 | 0.001 | 19 | 15.5 | Q04637 |
| Eukaryotic translation initiation factor 6 (eIF6, p27BBP)* | Ribosome biogenesis, transport, translation | 26.6 | 0.000 | 7 | 52.2 | P56537 |
| **Exosome component 10 (EXOSC10, RRP6)** | RNA Processing | 100.8 | 0.005 | 8 | 10.8 | Q01780 |
| Glypican-1 (GPC1) | Endosome localization, differentiation | 61.7 | 0.006 | 9 | 21 | P35052 |
| **Helicase-like protein (HLP, Ski2, SKIV2L)** | Transcription, ribosome biogenesis | 137.7 | 0.000 | 10 | 10.9 | Q15477 |
| **Immunoglobulin mu Binding Protein 2 (IGHMBP2, SMUBP2)** | Transcription, translation | 109.1 | 0.008 | 4 | 6.2 | P38935 |
| **Insulin-like growth factor 2 mRNA-binding protein 3 (IGF2BP3)** | mRNA transport, translation | 63.7 | 0.003 | 12 | 27.5 | O00425 |
| ***Karyopherin subunit α2 (KPNA2, SRP1-α)*** | Nuclear import | 57.8 | 0.006 | 7 | 22.1 | P52292 |
| Large subunit GTPase 1 homolog GTPase 1 (hLSG1) | Ribosome biogenesis, transport | 75.2 | 0.017 | 9 | 19.1 | Q9H089 |
| **Long-chain 3 Hydroxyacyl-CoA dehydrogenase (HADHA)** | RNA silencing, miRNA biogenesis | 83.0 | 0.002 | 24 | 42.5 | P40939 |
| **Matrin-3 (MATR3)** | Innate immune response | 94.6 | 0.006 | 5 | 8.9 | P43243 |
| **Metadherin (MTDH, AEG1, LYRIC)** | Transcription, NFκB pathway | 63.8 | 0.001 | 5 | 10.8 | Q86UE4 |
| **Mitochondrial ribosomal protein S28 (MRP-S28, S28mt)*** | Mitochondrial translation | 20.8 | 0.000 | 6 | 33.7 | Q9Y2Q9 |
| **Myb-binding protein 1A (MYBBP1A, p160)** | Transcription, stress response, cell cycle | 148.8 | 0.005 | 9 | 8.7 | Q9BQG0 |
| **N-acetyltransferase 10 (NAT10, ALP)** | RNA processing | 115.7 | 0.000 | 8 | 10.9 | Q9H0A0 |
| **Nuclear factors associated with dsRNA (NFAR, NF90, ILF3)** | Transcription, Translation, antiviral response | 95.3 | 0.001 | 9 | 11.8 | Q12906 |
| **Nucleolar GTP-binding protein 2 (GNL2)*** | Ribosome biogenesis | 83.6 | 0.001 | 6 | 10.8 | Q13823 |
| **Nucleolar protein 2 homolog (NOP2, NOL1, NSUN6)** | RNA processing, ribosome biogenesis | 89.2 | 0.001 | 7 | 10.9 | P46087 |
| **Nucleophosmin (NPM1, B23, Numartin)** | Ribosome assembly, biogenesis, mRNA stability, translation, transcription | 32.6 | 0.010 | 8 | 44.2 | P06748 |
| **Oxidative stress-associated Src activator (FAM120A, C9orf10)** | Oxidative stress | 121.8 | 0.000 | 10 | 12.4 | Q9NZB2 |
| **Poly [ADP-ribose] polymerase 1 (PARP1, ADPRT1)** | DDR, transcription, mitochondrial organization | 113.0 | 0.001 | 18 | 24.4 | P09874 |
| **Pre-rRNA-processing protein (TSR1)** | Ribosome biogenesis, RNA processing | 91.8 | 0.008 | 8 | 13.2 | Q2NL82 |
| **Programmed cell death protein 4 (PDCD4, H731)** | Transcription, cell cycle, apoptosis | 51.7 | 0.002 | 9 | 24.3 | Q53EL6 |
| **Protein arginine N-methyltransferase 5 (PRMT5, JBP1, SKB1)** | Transcription, spliceosome assembly | 72.6 | 0.010 | 9 | 20.6 | O14744 |
| **Protein FtsJ homolog 3 (FTSJ3, SB92)** | RNA processing, ribosome biogenesis | 96.6 | 0.000 | 6 | 9.7 | Q8IY81 |
| **Protein kinase RNA activated (PKR, EIF2AK2)** | Transcription, mRNA processing, translation | 62.1 | 0.019 | 11 | 21.6 | P19525 |
| Protein LTV1 homolog (LTV1) | RNA processing, 40S ribosome biogenesis | 54.8 | 0.005 | 5 | 11.4 | Q96GA3 |
| **Protein PRRC2C** | Translation, differentiation | 316.7 | 0.002 | 15 | 6.8 | Q9Y520 |
| **Pumiliohomolog 3 (PUFA)** | Translation | 73.5 | 0.013 | 8 | 16.5 | Q15397 |
| **Putative helicase MOV-10 (MOV10)** | Transcription, miRNA biogenesis, RNA interference, RNA granules | 113.6 | 0.005 | 5 | 7.3 | Q9HCE1 |
| **Ribonuclease P/MRP protein subunit (POP1)** | RNA processing | 114.6 | 0.002 | 9 | 11.8 | Q99575 |
| **RNA polymerase-associated protein (LEO1)** | Transcription, RNA metabolism | 75.4 | 0.001 | 6 | 10.5 | Q8WVC0 |
| RNA polymerase-associated protein CTR9 homologous (CTR9) | Transcription | 133.4 | 0.011 | 9 | 7.3 | Q6PD62 |
| **RRP12-like protein (RRP12)** | rRNA processing, ribosome biogenesis | 143.6 | 0.003 | 7 | 8.1 | Q5JTH9 |
| SDA1 domain-containing protein 1 (SDAD1, hSDA) | Ribosome biogenesis, transport, actin cytoskeleton organization | 79.8 | 0.013 | 3 | 4.7 | Q9NVU7 |
| Sequestosome-1 (SQSTM1, p62) | Mitophagy, stress response, differentiation | 47.7 | 0.011 | 8 | 31.8 | Q13501 |
| **Serine/Arginine-Rich protein specific Kinase 1 (SRPK1)** | RNA splicing, chromosome segregation | 74.3 | 0.000 | 4 | 9.4 | Q96SB4 |
| ***Serine/arginine-rich splicing factor 3 (SRSF3, SRP20)**** | RNA splicing, transport | 19.3 | 0.000 | 7 | 42.7 | P84103 |
| ***SERPINE1 mRNA Binding Protein 1 (SERBP1, PAIRBP1)*** | mRNA stability, apoptosis | 44.9 | 0.012 | 13 | 33.6 | Q8NC51 |
| **Single-stranded DNA-binding protein (SSBP1, SOSS)** | Replication, mitochondrion organization | 17.2 | 0.006 | 5 | 33.8 | Q04837 |
| **Staphylococcal nuclease domain-containing protein 1 (SND1, TDRD11)** | Transcription, RNA interference, stress granules | 101.9 | 0.010 | 11 | 18.7 | Q7KZF4 |
| ***Synaptotagmin binding cytoplasmic RNA interacting protein (SYNCRIP, hnRNPQ)**** | RNA processing, splicing, translation | 69.6 | 0.005 | 11 | 26.9 | O60506 |
| Targeting protein for Xklp2 (TPX2, DIL-2) | Microtubule organization, cell cycle, apoptosis | 85.6 | 0.020 | 12 | 20.2 | Q9ULW0 |
| **Tetratricopeptide repeat protein 37 (TTC37, Ski3)** | RNA processing | 175.4 | 0.000 | 16 | 13 | Q6PGP7 |
| **Transcription intermediary factor 1β (TRIM28, KAP1)** | DDR, transcription | 88.5 | 0.003 | 6 | 11.7 | Q13263 |
| Transglutaminase- 3 TGase-3 (TGM3, TGE) | Proliferation, migration, NF-κB pathway | 76.6 | 0.010 | 3 | 7.5 | Q08188 |
| **Ubiquitin carboxyl-terminal hydrolase 10 (USP10)** | DDR, autophagy | 87.1 | 0.003 | 6 | 9.8 | Q14694 |
| Unconventional Myosin-IC (MYO1C, MMIb) | Cytoskeletal rearrangements, motility | 121.6 | 0.012 | 5 | 7.1 | O00159 |
| **Valosin-containing protein (VCP)** | DDR, lysosome transport, autophagy | 89.3 | 0.019 | 5 | 9.4 | P55072 |
| **Vigilin (VGL, HDLBP)** | Lipid transport | 141.4 | 0.001 | 9 | 10.2 | Q00341 |
| **X-ray repair cross-complementing protein 6 (XRCC6, Ku70)*** | DDR, transcription, innate immune response | 69.8 | 0.001 | 38 | 61.7 | P12956 |
| **Y-box-binding protein 3 (YBX3, CSDA, ZONAB)** | Transcription, translation, RNA binding | 40.1 | 0.006 | 7 | 47 | P16989 |
| **YTH domain containing 2 (YTHDC2)** | RNA processing | 160.1 | 0.005 | 5 | 4.9 | Q9H6S0 |
| **Zinc finger CCCH-type antiviral protein 1 (ZC3HAV1, ZAP)** | Innate immune response | 101.4 | 0.003 | 5 | 8.7 | Q7Z2W4 |
| **Zinc finger protein 622 (ZNF622, ZPR9)** | ribosome biogenesis, apoptosis | 54.2 | 0.007 | 11 | 30 | Q969S3 |
| **40S ribosomal Protein S19 (RPS19)*** | Ribosome biogenesis, translation | 16.1 | 0.011 | 12 | 26 | P39019 |
| **40S ribosomal protein S30 (FAU)** | RNA processing, translation, apoptosis | 6.6 | 0.002 | 3 | 12.2 | P62861 |
| **5'-3' exoribonuclease 2 (XRN2)** | Transcription, RNA processing | 108.5 | 0.007 | 5 | 8 | Q9H0D6 |
| **60S ribosomal protein L6 (RPL6, TXREB1)*** | RNA processing, ribosome assembly, translation | 32.7 | 0.004 | 1 | 49.7 | Q02878 |

[a] Underlined proteins were previously described as direct or indirect interacting partners of FMRP; * Isoforms, paralogs or related proteins identified are listed in Table 4. Proteins highlighted in bold are RNA-binding proteins and proteins in *italic* are components of the stress granules. [b] Some proteins have more than one name (http://www.genecards.org), some alternative names or synonyms are therefore included in parentheses.

**Table 3.** Proteins (directly or indirectly) interacting with both $FMRP^{N\text{-term}}$ and $FMRP^{C\text{-term}}$

| Protein name[b] | Function | MW kDa | p-value | Unique pep. | Seq. coverage (%) | Acc. ID |
|---|---|---|---|---|---|---|
| Ataxin-2-like protein (ATXN2L) | RNA metabolism, processing, stress granules, p-body assembly | 113.3 | 0.006/ 0.004 | 13 | 16.2 | Q8WWM7 |
| Complement component 1 Q subcomponent-binding protein (C1QBP, HABP1) | Transcription, mRNA processing, ribosome assembly, mitochondrial translation apoptosis, innate immune response | 31.3 | 0.012/ 0.000 | 9 | 42.9 | Q07021 |
| *Cytoplasmic activation/proliferation-associated protein 1 (Caprin-1, GPIAP1, RNG105)* | Translation, differentiation | 78.3 | 0.000/ 0.000 | 17 | 34.1 | Q14444 |
| Enhancer of rudimentary homolog (ERH) | Cell cycle | 12.3 | 0.000/ 0.000 | 3 | 37.5 | P84090 |
| *Fragile X mental retardation syndrome-related protein 1 (FXR1, hFXR1P)* | RNA binding, translation, apoptosis, differentiation | 69.7 | 0.000/ 0.003 | 23 | 52.7 | P51114 |
| *Heterogeneous nuclear ribonucleoprotein U, hnRNPU (HNRNPU, SAF-A)** | Transcription, DDR, RNA processing, granules, mitotic spindle assembly | 90.5 | 0.000/ 0.000 | 8 | 14.4 | Q00839 |
| La-related protein 1 (LARP1)* | Translation, cell proliferation | 123.4 | 0.000/ 0.000 | 23 | 25.5 | Q6PKG0 |
| Nuclear fragile X mental retardation-interacting protein 2 (NUFIP2, 82-FIP) | RNA binding, transport, stress granules | 76.1 | 0.016/ 0.005 | 5 | 14.4 | Q7Z417 |
| Polyadenylate binding protein 1 (PABPC1, PAB1)* | RNA splicing, mRNA silencing, translation | 70.6 | 0.002/ 0.006 | 16 | 34.3 | P11940 |
| *RAS GTPase activating protein SH3 domain-binding protein 1 (G3BP1)** | Transport | 52.1 | 0.000/ 0.000 | 14 | 47.2 | Q13283 |
| RNA transcription, translation and transport factor protein (C14orf166, CGI-99, hCLE) | RNA metabolism, transport | 28.1 | 0.001/ 0.000 | 21 | 75.4 | Q9Y224 |
| Ubiquitin-associated protein 2-like (UBAP2L, NICE4)* | RNA binding | 114.5 | 0.000/ 0.001 | 8 | 12 | Q14157 |
| Uncharacterized protein C7orf50 | uncharacterized | 22.1 | 0.014/ 0.001 | 5 | 41.8 | Q9BRJ6 |

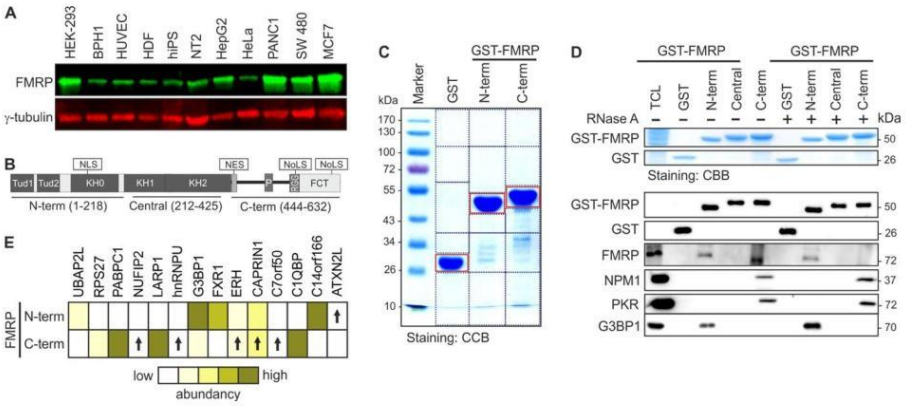
[a] Underlined proteins were previously described as direct or indirect interacting partners of FMRP; [a] Isoforms, paralogs or related proteins identified are listed in Table 4. Proteins highlighted in bold are RNA-binding proteins and proteins in *italic* are components of the stress granules. [b] Some proteins have more than one name (http://www.genecards.org), some alternative names or synonyms are therefore included in parentheses.

Table 4. Isoforms, paralogs, and related proteins of FMRP interacting proteins depicted in Tables 1-3.

| Interaction with $FMRP^{N\text{-}term}$ |
|---|
| **AHNAK1** (Q09666), **RPL13A** (P40429), **RPL18A** (Q02543), **RPS12** (P25398), **SF1** (Q15637), **UBAP2** (Q5T6F2) |
| **Interaction with $FMRP^{C\text{-}term}$** |
| ASCC3 (Q8N3C0), ATAD3B (Q5T9A4), **DHX9** (Q08211), **DHX15** (O43143), **DHX30** (Q7L2E3), **DHX57** (Q6P158), **eIF3A** (Q14152), **eIF3B** (P55884), **eIF3C** (Q99613), **eIF3D** (O15371), **GNL3** (Q9BVP2), **hnRNPM** (P52272), **hnRNPUL1** (Q9BUJ2), **LARP4** (Q71RC2), **MRPL12** (P52815), **MRPS2** (Q9Y399), **MRPS7** (Q9Y2R9), **NOG1** (Q9BZE4), **RPLP1** (P05386), **RPLP2** (P05387), **RPL4** (P36578), **RPL8** (P62917), **RPL9** (P32969), **RPL10** (P27635), **RPL10A** (P62906), **RPL13** (P26373), **RPL13A** (P40429), **RPL14** (P50914), **RPL19** (P84098), **RPL23A** (P62750), **RPL29** (P47914), **RPL35A** (P18077), **RPL37A** (P61513), **RPS5** (P46782), **RPS27L** (Q71UM5), **SRSF6** (Q13247), **XRCC5** (P13010) |
| **Interaction with both $FMRP^{N\text{-}term}$ and $FMRP^{C\text{-}term}$** |
| **DDX1** (Q92499), **DDX17** (Q92841), **DDX3X** (O00571), **DDX3Y** (O15523), **DHX36** (Q9H2U1), G3BP2 (Q9UN86), **PABP3** (Q3ZCS4), **RPL9** (P32969), **RPL10** (P27635), **RPL10A** (P62906), **RPL15** (P61313), **RPL17** (P18621), **RPL18** (Q07020), **RPL21** (P46778), **RPL23** (P62829), **RPL24** (P83731), **RPL26** (P61254), **RPL26L1** (P61254), **RPL27A** (P46776), **RPL28** (P46779), **RPL30** (P62888), **RPL38** (P63173), **RPS8** (P62241), **RPS9** (P46781), **RPS14** (P62263), **RPS16** (P62249), **RPS17** (P08708), **RPS17L** (P0CW22), **RPS18** (P62269), **RPS20** (P60866), **RPS21** (P63220), **RPS24** (P62847), **RPS26** (P62854), **RPS27** (P42677), **RPS28** (P62857) |

Gene names (accession numbers) of different isoforms, paralogs, and related proteins, including ribosomal proteins (RPs) associated with $FMRP^{N\text{-}term}$ and $FMRP^{C\text{-}term}$ are collected. Proteins highlighted in **bold** are RNA-binding proteins.

**Figure 1**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FMRP
γ-tubulin
B
NLS
NES
NoLS
NoLS
Tud1
Tud2
KH0
KH1
KH2
RGG
FCT
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Central (212-425)
C-term (444-632)
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PABPC1
NUFIP2
LARP1
hnRNPU
G3BP1
FXR1
ERH
CAPRIN1
C7orf50
C1QBP
C14orf166
ATXN2L
FMRP
N-term
C-term
low
high
abundancy
C
GST-FMRP
Marker
GST
N-term
C-term
kDa
170
130
100
72
55
43
34
26
10
Staining: CCB
D
GST-FMRP
GST-FMRP
TCL
GST
N-term
Central
C-term
GST
N-term
Central
C-term
RNase A
kDa
GST-FMRP
GST
50
26
Staining: CBB
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G3BP1
50
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72
37
72
70

**Figure 2**

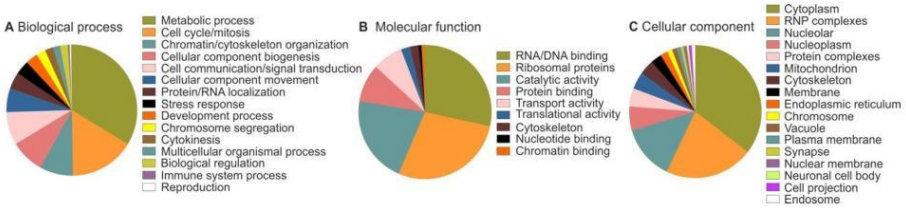
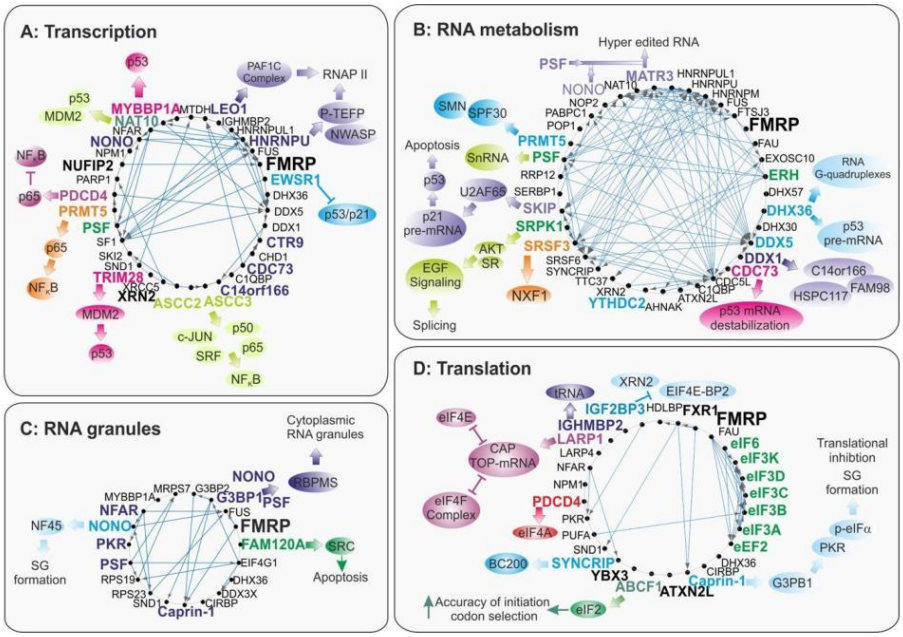

A Biological process
Metabolic process
Cell cycle/mitosis
Chromatin/cytoskeleton organization
Cellular component biogenesis
Cell communication/signal transduction
Cellular component movement
Protein/RNA localization
Stress response
Development process
Chromosome segregation
Cytokinesis
Multicellular organismal process
Biological regulation
Immune system process
Reproduction
B Molecular function
RNA/DNA binding
Ribosomal proteins
Catalytic activity
Protein binding
Transport activity
Translational activity
Cytoskeleton
Nucleotide binding
Chromatin binding
C Cellular component
Cytoplasm
RNP complexes
Nucleolar
Nucleoplasm
Protein complexes
Mitochondrion
Cytoskeleton
Membrane
Endoplasmic reticulum
Chromosome
Vacuole
Plasma membrane
Synapse
Nuclear membrane
Neuronal cell body
Cell projection
Endosome

**Figure 3**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RNAP II
p53
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MTDH
LEO1
NAT10
IGHMBP2
HNRNPUL1
P-TEFP
NWASP
NFAR
NONO
NPM1
HNRNPU
FUS
NF-κB
NUFIP2
FMRP
PARP1
EWSR1
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PDCD4
DHX36
PRMT5
DDX5
p53/p21
PSF
DDX1
SF1
CTR9
p65
SKI2
CHD1
SND1
CDC73
TRIM28
C1QBP
NF-κB
XRCC5
XRN2
C14orf166
ASCC2
ASCC3
MDM2
c-JUN
p50
p53
SRF
p65
NF-κB
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Hyper edited RNA
PSF
MATR3
HNRNPUL1
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NAT10
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SMN
SPF30
NOP2
FUS
PABPC1
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FMRP
POP1
Apoptosis
PRMT5
FAU
SnRNA
PSF
EXOSC10
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U2AF65
SERBP1
DHX57
SKIP
DHX36
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DHX30
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TTC37
CDC5L
NXF1
XRN2
C1QBP
HSPC117
FAM98
YTHDC2
AHNAK
ATXN2L
p53 mRNA destabilization
Splicing
C: RNA granules
Cytoplasmic RNA granules
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MYBBP1A
MRPS7
G3BP2
G3BP1
PSF
NFAR
FUS
NF45
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PKR
FAM120A
SRC
PSF
EIF4G1
SG formation
RPS19
DHX36
Apoptosis
RPS23
DDX3X
SND1
CIRBP
Caprin-1
D: Translation
tRNA
XRN2
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eIF4E
IGF2BP3
HDLBP
FXR1
FMRP
IGHMBP2
FAU
CAP
LARP1
eIF6
Translational inhibtion
TOP-mRNA
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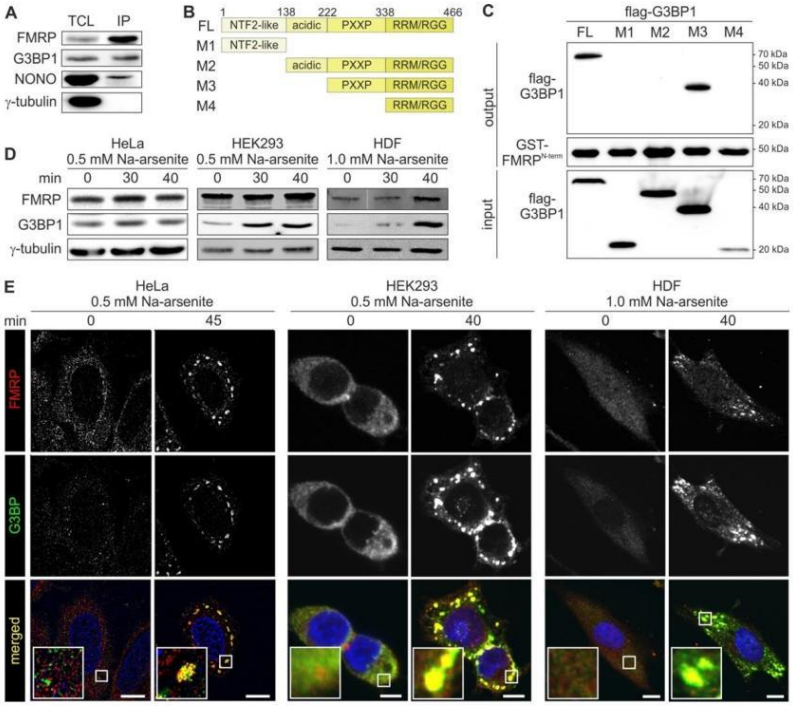
ABCF1
ATXN2L
Accuracy of initiation codon selection
eIF2

**Figure 4**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M3
M4
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FL
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M2
M3
M4
output
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input
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50 kDa
40 kDa
20 kDa
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HEK293
0.5 mM Na-arsenite
HDF
1.0 mM Na-arsenite
min
0
30
40
FMRP
G3BP1
γ-tubulin
E
HeLa
0.5 mM Na-arsenite
HEK293
0.5 mM Na-arsenite
HDF
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min
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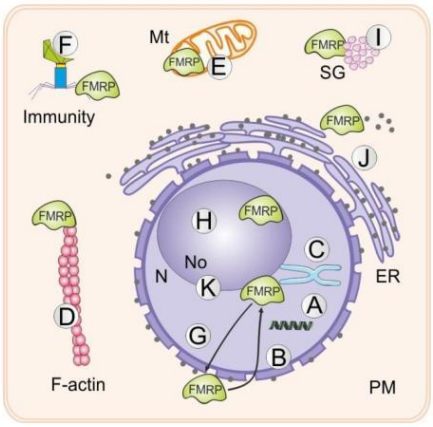
40
0
40
FMRP
G3BP
merged

**Table of Contents graphic**

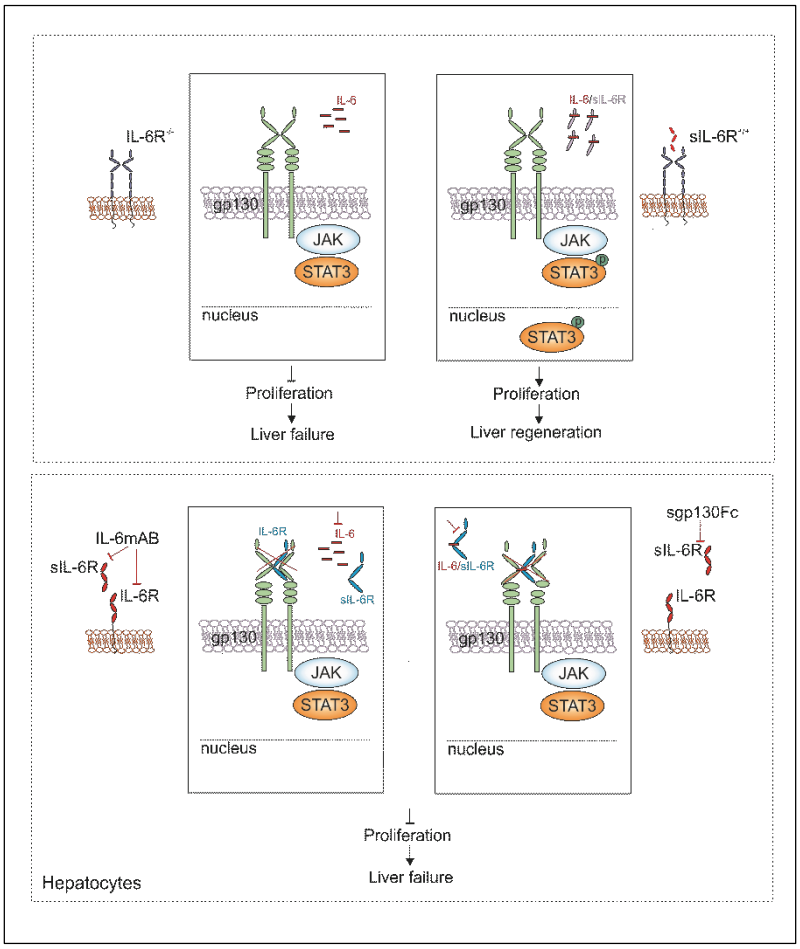



Identification of 180 FMRP interacting proteins in a proteomic approach strongly suggest a localization in diverse subcellular processes, including transcription (A), DNA damage response (B), cell cycle (C), actin dynamics and Intracellular transport (D), mitochondrial organization (E), innate immune response (F), RNA metabolism (G), ribosome biogenesis (H), RNA granules (I), translation (J), and miRNA biogenesis (K). ER, endoplasmic reticulum; Mt, mitochondrion; N, nucleus; No, nucleolus; PM, plasma membrane; SG, stress granules.

# Chapter VI

## IL-6 trans-signaling controls liver regeneration after partial hepatectomy

**IL-6 trans-signaling completely compensates for the loss of IL-6 classic signaling in liver regeneration after partial hepatectomy**




| | |
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| Status: | Published in Journal of Hepatology, 2019 May, 17 |
| Impact Factor: | 15.040 (2017) |
| Own Proportion to this work: | 10%<br>Immunobloting of STAT3, pSTAT3 and PCNA, Densitometry and statistical analysis, Figure preparation (Figs. 2, 4 & 6) |

HEPATOLOGY

HEPATOLOGY, VOL. 0, NO. 0, 2019

# IL-6 Trans-signaling Controls Liver Regeneration After Partial Hepatectomy

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Interleukin-6 (IL-6) is critically involved in liver regeneration after partial hepatectomy (PHX). Previous reports suggest that IL-6 trans-signaling through the soluble IL-6/IL-6R complex is involved in this process. However, the long-term contribution of IL-6 trans-signaling for liver regeneration after PHX is unknown. PHX-induced generation of the soluble IL-6R by ADAM (a disintegrin and metallo) proteases enables IL-6 trans-signaling, in which IL-6 forms an agonistic complex with the soluble IL-6 receptor (sIL-6R) to activate all cells expressing the signal-transducing receptor chain glycoprotein 130 (gp130). In contrast, without activation of ADAM proteases, IL-6 in complex with membrane-bound IL-6R and gp130 activates classic signaling. Here, we describe the generation of IL-6 trans-signaling mice, which exhibit boosted IL-6 trans-signaling and abrogated classic signaling by *genetic* conversion of all membrane-bound IL-6R into sIL-6R proteins phenocopying hyperactivation of ADAM-mediated shedding of IL-6R as single substrate. Importantly, although IL-6R deficient mice were strongly affected by PHX, survival and regeneration of IL-6 trans-signaling mice was indistinguishable from control mice, demonstrating that IL-6 trans-signaling fully compensates for disabled classic signaling in liver regeneration after PHX. Moreover, we monitored the long-term consequences of global IL-6 signaling inhibition versus IL-6 trans-signaling selective blockade after PHX by IL-6 monoclonal antibodies and soluble glycoprotein 130 as fragment crystallizable fusion, respectively. Both global IL-6 blockade and selective inhibition of IL-6 trans-signaling results in a strong decrease of overall survival after PHX, accompanied by decreased signal transducer and activator of transcription 3 phosphorylation and proliferation of hepatocytes. Mechanistically, IL-6 trans-signaling induces hepatocyte growth factor production by hepatic stellate cells. *Conclusion:* IL-6 trans-signaling, but not classic signaling, controls liver regeneration following PHX. (HEPATOLOGY 2019;0:1-17).

Synergistic action of hepatocyte growth factor (HGF) and interleukin 6 (IL-6) controls the early regenerative phase after partial hepatectomy (PHX) by promoting both mitosis and survival of hepatocytes.[1-5] IL-6 binds to the IL-6 receptor (IL-6R), followed by dimerization of glycoprotein 130 (gp130), leading to JAK/STAT, MAPK, and PI3K/AKT activation.[6] IL-6 or IL-6R alone have no affinity toward gp130. IL-6R expression is found on few cell types, including immune cells and hepatocytes, which are directly activated by IL-6 classic signaling.[7] Primarily ectodomain shedding by a disintegrin and metallo (ADAM) proteases generates the soluble IL-6R (sIL-6R).[8]

*Abbreviations: 3'UTR, 3'untranslated region; ADAM, a disintegrin and metallo; ELISA, enzyme-linked immunosorbent assay; Fc, fragment crystallizable; FRT, flippase recognition target; GFP, green fluorescent protein; gp130, glycoprotein 130; HGF, hepatocyte growth factor; HSCs, hepatic stellate cells; IL-6, interleukin 6; IL-6R, interleukin 6 receptor; mAb, monoclonal antibody; ns, not significant; PCNA, proliferating cell nuclear antigen; PHX, partial hepatectomy; sgp130, soluble gp130; sIL-6R, soluble IL-6 receptor; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; wt, wild type.*

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IL-6/sIL-6R complexes activate cells expressing only gp130, or superactivates cells expressing more gp130 than membrane-bound IL-6R.[9,10] Whereas global IL-6 signaling (classic and trans-signaling) is inhibited by neutralizing IL-6 or IL-6R antibodies, soluble gp130 (sgp130) selectively inhibits IL-6 trans-signaling.[11,12] Healthy individuals display serum levels of 250-400 ng/mL endogenous sgp130.[13] Recently, we have shown that endogenous serum levels of sgp130 are not sufficient to inhibit trans-signaling but might contribute to increase the serum half-life of IL-6.[7]

IL-6 trans-signaling has primarily pro-inflammatory functions and inhibition of IL-6 trans-signaling by application of sgp130 as fragment crystallizable (Fc) fusion protein (sgp130Fc) resulted in suppression of chronic inflammatory diseases in preclinical settings. Recently, sgp130Fc has entered phase 2 clinical trial.[13]

Although hepatocytes express relatively high levels of IL-6R, a modest increase in sIL-6R levels after PHX would enable local trans-signaling in the liver.[14] Therefore, heterologous application of the trans-signaling superagonist hyper-IL-6, which is a fusion protein of IL-6 and sIL-6R,[15] illuminates the maximal potential of trans-signaling *in vitro* and *in vivo*. Forced IL-6 trans-signaling by application of recombinant hyper-IL-6, but not classic signaling by IL-6, resulted in acceleration of liver regeneration after PHX.[16] Ectopic expression of IL-6 and sIL-6R in double transgenic mice, but not of IL-6 alone, leads to nodular hepatocellular hyperplasia.[17-19] Interestingly, IL-6/sIL-6R, but not IL-6 alone, cooperates with HGF to enhance hepatocyte proliferation, and short-time transient expression of sgp130Fc inhibits hepatocyte proliferation at early time points following PHX.[20] Even though these results suggest a role for IL-6 trans-signaling in liver regeneration, long-term experiments to determine the overall consequences of IL-6 trans-signaling during liver regeneration are lacking. Increased serum levels of sIL-6R are common events in pathophysiology (e.g., following PHX), suggesting a shift from classic toward trans-signaling.[14] Among others, ADAM proteases execute the stress-induced ectodomain shedding of IL-6R.[8] Analysis of ADAM activity using gene-deficient ADAM10/17 mice is hampered by the complexity of the ADAM10-induced and ADAM17-induced sheddome with more than 100 different protein substrates. Moreover, the phenotype of ADAM10 and ADAM17 deficient mice is dominated by shedding deficits of a few substrates, including epidermal growth factor receptor (EGFR) ligands, Notch, and tumor necrosis factor (TNF).[21] Therefore, consequences of abrogated shedding of the IL-6R could not be analyzed in ADAM10/17 deficient mice.[8]

Here, we generated and characterized IL-6 trans-signaling mice, which were genetically engineered to execute IL-6 trans-signaling and were indistinguishable from wild-type mice in liver regeneration after PHX.

# Materials and Methods

Floxed sIL-6R$^{fl/fl\text{-}NEO}$ mice were generated by ingenious targeting laboratory (www.genetargeting.com). The conditional targeting vector bearing the rearranged IL-6R exons 9 and 10 was electroporated into C57BL/6N embryonic stem cells. Targeted embryonic stem cells were microinjected into Balb/c blastocysts, and the resulting chimeras with a high percentage of black coat color were crossed to C57BL/6N mice to generate IL-6R$^{wt/fl\text{-}NEO}$. DNA from tail clippings was isolated using the

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DirectPCR-Tail kit with proteinase K (Peqlab, Erlangen, Germany) following the manufacturer's instructions for genomic PCR.

# Results

## IL-6 TRANS-SIGNALING SIL-6R$^{+/+}$ MICE HAVE DRASTICALLY INCREASED SIL-6R LEVELS

Here we generated transgenic soluble IL-6R$^{+/+}$ (sIL-6R$^{+/+}$) mice, a strategy to mimic ADAM10/17 hyperactivation for the single target protein IL-6R. sIL-6R$^{+/+}$ mice reflect a situation in which only trans-signaling is active, whereas classic signaling is abrogated. sIL-6R$^{+/+}$ mice exhibit amplified endogenous IL-6 trans-signaling due to an increased level of sIL-6R, which is not caused by ectodomain shedding but by Cre-mediated deletion of the genetic information coding for the transmembrane and intracellular domain of the IL-6R (Fig. 1A). In detail, exon 9 of the IL-6R gene codes for the transmembrane domain and the first part of the intracellular domain, and the last exon 10 codes for the second part of the intracellular domain followed by the 3′untranslated region (3′UTR). In the targeting vector, the 5,420-base pair (bp)-long intron 9 was deleted, resulting in functional fusion of the 100-bp and 223-bp-long coding regions of exon 9 and exon 10, respectively. Intron 9 was skipped because it would have complicated the construction of the targeting vector. The original IL-6R stop codon located in exon 10 was deleted, and a sequence coding for the 2A-peptide sequence followed by a KDEL-marked GFP was inserted. The 2A peptide from the foot-and-mouth disease virus is a self-processing sequence to achieve expression of at least two separate proteins from a single open reading frame.[22] The cleavage of the IL-6R and GFP is thought to happen in a co-translational process. The remaining 2A peptide fragment at the C-terminal end of IL-6R is then cleaved off by the protease furin. In addition, the endoplasmic reticulum retention signal KDEL[23] was fused at the C-terminal end of the green fluorescent protein (GFP). The E9-E10-GFP cassette was followed by the flippase recognition target (FRT)-neomycin-resistance-FRT cassette and the original 3′UTR of the IL-6R 3′UTR and ultimately flanked by loxP-sites.

In the founder transgenic mice, the FRT-neomycin-resistance-FRT cassette was deleted by crossing to Flp-recombinase expressing mice, referred to as homozygous sIL-6R$^{fl/fl}$ mice. We expected that the modified IL-6R protein in IL-6R$^{fl/fl}$ mice would be produced with comparable efficiency compared with the wild-type IL-6R protein and result in IL-6 classic and trans-signaling comparable to wild-type mice (Fig. 1B). Cre-mediated recombination in the germline resulted in homozygous IL-6R$^{+/+}$ mice carrying the deletion of the fused exons 9 and 10 on both alleles (Fig. 1A). As a consequence, the translated IL-6R in Cre-recombined sIL-6R$^{+/+}$ mice lack the trans-membrane and intracellular domains and will be directly secreted as soluble IL-6R (Fig. 1C, Supporting Fig. S1A,B). Due to the lack of membrane-bound IL-6R, sIL-6R$^{+/+}$ mice will selectively execute only trans-signaling. Introduction of the E9-E10-GFP cassette in sIL-6R$^{fl/fl}$ mice and deletion of the E9-E10-GFP cassette in sIL-6R$^{+/+}$ mice was confirmed by genomic PCR (Fig. 1D). Next, we quantified the *IL-6R* mRNA level in wild-type, sIL-6R$^{fl/fl}$, and sIL-6R$^{+/+}$ mice. Whereas the *IL-6R* mRNA level was comparable in wild-type and sIL-6R$^{fl/fl}$ mice, a significantly increased sIL-6R mRNA level was detected in sIL-6R$^{+/+}$ mice, suggesting that genetic rearrangement in sIL-6R$^{fl/fl}$ mice did not influence overall stability of the *IL-6R* mRNA but the deletion of most of the 3′UTR in sIL-6R$^{+/+}$ mice enhanced mRNA stability (Fig. 1E,F). Unfortunately, we were not able to detect GFP fluorescence in hepatocytes and immune cells from sIL-6R$^{fl/fl}$ mice, albeit western blotting of liver and spleen tissue clearly detected GFP proteins in sIL-6R$^{fl/fl}$ mice, but not in wild-type and sIL-6R$^{+/+}$ mice (Fig. 1G). Next, we performed immunohistochemistry of the IL-6R on liver samples from wild-type, IL-6R deficient, and sIL-6R$^{+/+}$ mice. We were able to detect IL-6R in cells of wild-type mice but not in IL-6R deficient mice, demonstrating that the staining was specific. Interestingly, staining for sIL-6R in sIL-6R$^{+/+}$ mice was also absent, indicating that all membrane-bound IL-6R was converted in sIL-6R in sIL-6R$^{+/+}$ mice and rapidly secreted (Fig. 1H). Moreover, staining of the IL-6R in wild-type mice suggests that IL-6R molecules were found primarily in intracellular compartments and not on the cell surface (Fig. 1H). Flow cytometry analysis of IL-6R on CD3$^{+}$ T cells and monocytes demonstrated that wild-type and sIL-6R$^{fl/fl}$ mice had comparable levels of IL-6R on the

cell surface, demonstrating that genetic rearrangement of IL-6R in sIL-6R$^{fl/fl}$ mice did not interfere with expression (Fig. 1I,J, Supporting Fig. S2). Importantly, IL-6R$^{-/-}$ and sIL-6R$^{+/+}$ mice lacked IL-6R cell surface expression, again supporting our conclusion that all membrane-bound IL-6R was converted into

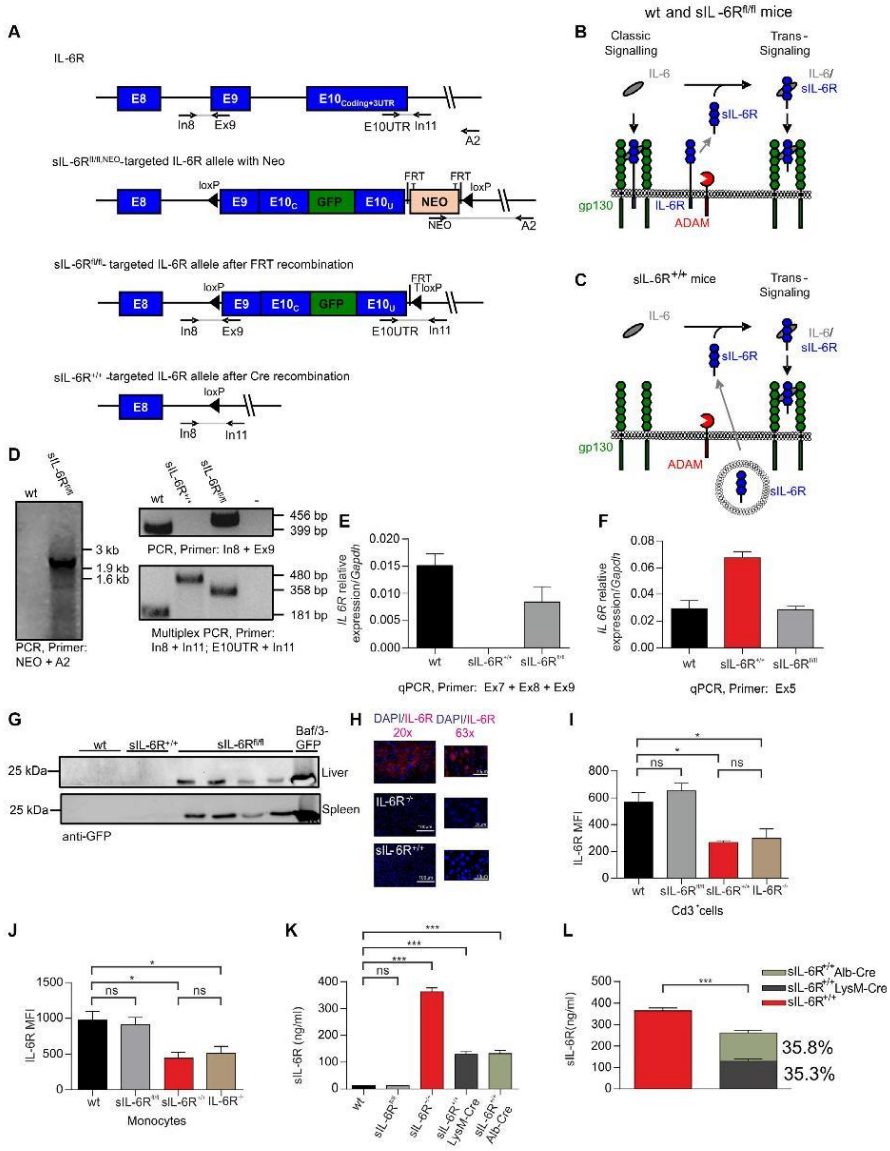

**FIG. 1.** Generation and characterization of soluble IL-6 trans-signaling mice (sIL-6R$^{+/+}$). (A) Schematic representation of targeting strategy for the generation of sIL-6R$^{fl/fl}$ and sIL-6R$^{+/+}$ mice. The arrows indicate the locations of primers used for genomic PCR (exon 8 to exon 10, blue). Exon 9 codes for the transmembrane domain of the IL-6R; exon 10 codes for the translated intracellular domain of the IL-6R (coding, C) and the 3′UTR (U). In sIL-6R$^{fl/fl}$ mice, exon 9 and exon 10 were fused. The natural stop codon of IL-6R was replaced by a 2A-GFP-KDEL coding sequence named GFP (green) followed by the 3′UTR of exon 10. After Cre-recombination, the E9-E10-GFP cassette is deleted, resulting in the generation of a mRNA that codes only for the soluble IL-6R. Schematic illustration of classic and/or trans-signaling in wild-type (wt)and sIL-6R$^{fl/fl}$ (B) and sIL-6R$^{+/+}$ mice (C). IL-6 (gray) in complex with membrane-bound IL-6R (blue) and gp130 (green) induce classic signaling. (B) In mice, sIL-6R (blue) is produced only by shedding of the membrane-bound IL-6R by ADAM proteases (red). (C) In sIL-6R$^{+/+}$ mice, sIL-6R is produced and secreted. IL-6 in complex with sIL-6R and gp130 induce trans-signaling. (D) PCR to characterize the genomic organization of the IL-6R locus in wt, sIL-6R$^{fl/fl}$, and sIL-6R$^{+/+}$ mice. Primer combinations and PCR products are indicated. (E,F) Quantification of IL-6R mRNA level in wt, sIL-6R$^{fl/fl}$, and sIL-6R$^{+/+}$ mice using the indicated primer combinations (n = 3). (G) Western blot against GFP on liver lysates from wt (n = 2), sIL-6R$^{fl/fl}$ (n = 4), and sIL-6R$^{+/+}$ (n = 2) mice. (H) Immunohistochemistry of IL-6R in liver samples from wt, IL-6R$^{-/-}$, and sIL-6R$^{+/+}$ mice (n = 2). (I,J) Expression of membrane-bound IL-6R was measured by flow cytometry from blood on two cell populations (CD3$^{+}$ cells [I] and monocytes [J]) from wt (black bar), sIL-6R$^{fl/fl}$ (gray bar), sIL-6R$^{+/+}$ (red bar), and IL-6R$^{-/-}$ (brown bar) mice. Results are presented as mean ± SEM of 4 animals/group. (K) The serum levels of sIL-6R in wt (black bar), sIL-6R$^{fl/fl}$ (gray bar), sIL-6R$^{+/+}$ (red bar), sIL-6R$^{+/+}$ LysM-Cre (dark gray), and sIL-6R$^{+/+}$ Alb-Cre (green bar) mice were determined by ELISA. Results are presented as mean ± SEM of at least 7 animals/group. (L) The amount of sIL-6R in sIL-6R$^{+/+}$ (red bar) was compared with the sum of sIL-6R in sIL-6R$^{+/+}$ LysM-Cre (dark gray) and sIL-6R$^{+/+}$ Alb-Cre (green bar) mice based on ELISA results.

sIL-6R after Cre-recombination in sIL-6R$^{+/+}$ mice (Fig. 1I,J, Supporting Fig. S2). Next, determination of the soluble IL-6R levels in the serum by enzyme-linked immunosorbent assay (ELISA) showed that wild-type and sIL-6R$^{fl/fl}$ mice had almost identical sIL-6R serum levels of approximately 11 ng/mL. Interestingly, sIL-6R$^{+/+}$ mice had about 33-fold increased sIL-6R levels of approximately 363 ng/mL (Fig. 1K). The increased sIL-6R level might at least to some degree also be caused by increased IL-6R mRNA levels in sIL-6R$^{+/+}$ mice. A previous report demonstrated that serum sIL-6R was originating almost completely from hepatocytes and neutrophils/macrophages, because Alb-Cre-recombined IL-6R$^{-/-}$ mice had 67.95% and LysMCre-recombined IL-6R$^{-/-}$ mice displayed 39.95% of the sIL-6R levels of wild-type mice (Supporting Table S1).[24] Here, we also crossed our sIL-6R$^{fl/fl}$ mice with the highly effective Alb-Cre and LysM-Cre lines (Supporting Fig. S3A,B).[25-28] Homozygous sIL-6R$^{+/+}$ LysM-Cre and sIL-6R$^{+/+}$Alb-Cre mice had about 129 ng/mL and 128 ng/mL sIL-6R in the serum, respectively (Fig. 1L). The sum of these sIL-6R serum levels was about 257 ng/mL, representing only about 70.8% of the sIL-6R levels detected in sIL-6R$^{+/+}$ mice (Fig. 1L, Supporting Table S1). These data suggest that although the native sIL-6R in wild-type mice were derived primarily from hepatocytes and monocytes/neutrophils, other cells also expressed appreciable amounts of IL-6R, even though they did not contribute to sIL-6R level in wild-type mice.

## SIL-6R IN SIL-6R$^{+/+}$ MICE MEDIATED IL-6 SIGNALS THROUGH TRANS-SIGNALING

Injection of recombinant IL-6 in wild-type mice showed that the colon, and to a lesser extent also the liver and lung, are targeted by IL-6 trans-signaling.[11] Our sIL-6R$^{+/+}$ mice have a 33-fold higher sIL-6R serum level compared with wild-type mice (Fig. 1K), suggesting that sIL-6R$^{+/+}$ mice efficiently and specifically execute trans-signaling. To test this, sIL-6R$^{+/+}$ mice were injected with 5 μg recombinant IL-6, and phosphorylation of signal transducer and activator of transcription 3 (pSTAT3) was analyzed in the colon, liver, and lung. IL-6 induced sustained STAT3 phosphorylation in these organs as demonstrated by immunohistochemical staining and western blotting compared with control mice (Fig. 2A-L, Supporting Fig. S4A-C). Moreover, mice were co-injected with 50 μg sgp130Fc or 250 μg neutralizing IL-6 monoclonal antibody (mAb) to block IL-6 signaling. Co-injection of IL-6 with sgp130Fc or IL-6 mAb significantly blocked pSTAT3 in colon, liver, and lung (Fig. 2A-L, Supporting Fig. S4A-C). To analyze whether the acute phase response differs between wild-type and sIL-6R$^{+/+}$ trans-signaling mice, we injected per mouse 5 μg IL-6 and analyzed the expression of the acute phase protein serum amyloid A2 (*Saa2*) by quantitative real-time PCR. As shown in Fig. 2M, induction of *Saa2* expression in wild-type and sIL-6R$^{+/+}$ trans-signaling mice was almost identical, suggesting

that at least in the liver, IL-6 trans-signaling can completely compensate for the loss of IL-6 classic signaling. Our results demonstrate that the sIL-6R in sIL-6R$^{+/+}$ mice is biologically active and transmits IL-6 signals through trans-signaling, thereby indicating that forced IL-6 trans-signaling can activate all cells of the organism. Thus, endogenous IL-6 trans-signaling is a very efficient cellular activation mode, which is specifically triggered during pathophysiological conditions.

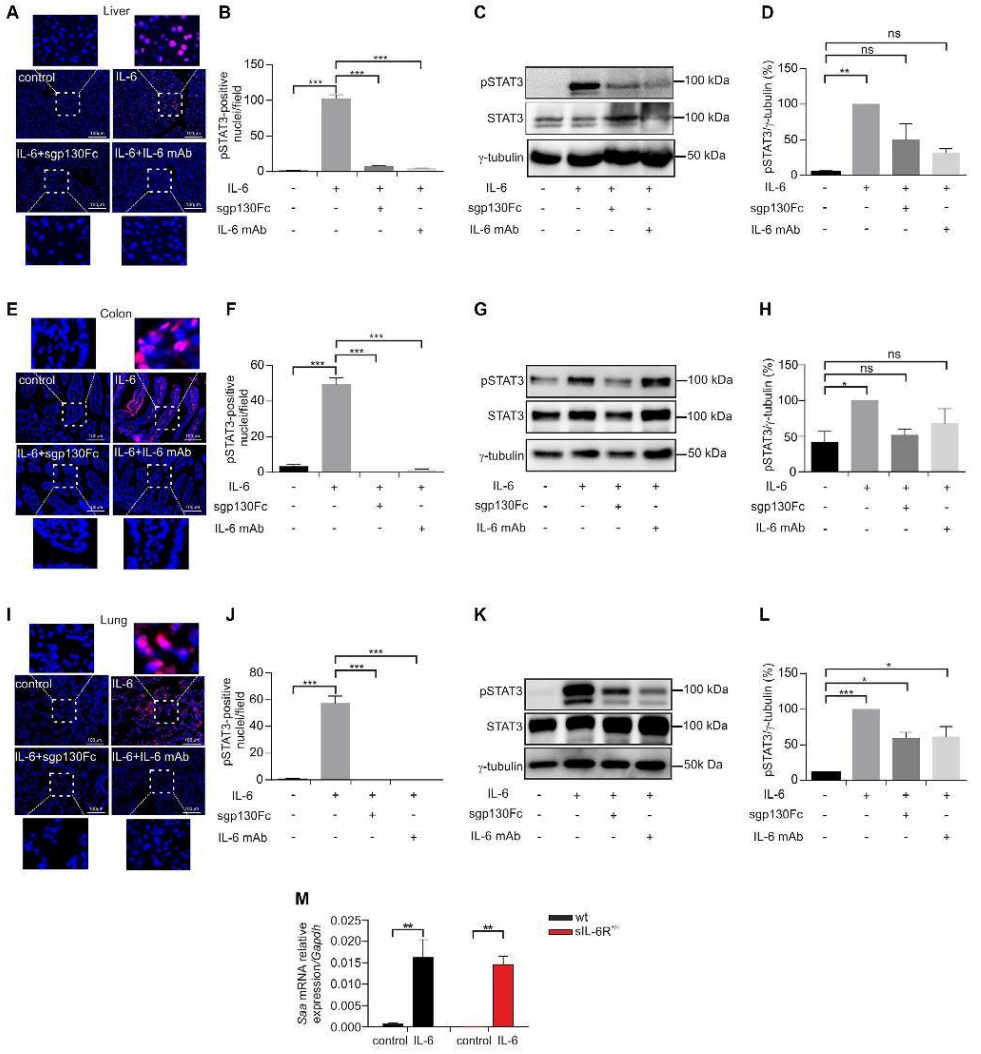

**FIG. 2.** Injection of IL-6 in sIL-6R$^{+/+}$ mice induced IL-6 trans-signaling. sIL-6R$^{+/+}$ mice were intraperitoneally injected with phosphate-buffered saline, IL-6, IL-6, and sgp130Fc or IL-6 and IL-6 mAb. Mice were sacrificed 90 minutes after injection (n = 3). (A) Sections of paraffin-embedded liver tissue were stained for pSTAT3. Higher magnification of each picture is indicated by dotted lines. (B) Quantification of (A) was performed from 10 visual fields per mouse (total of 30 visual fields). Results are presented as mean ± SEM of 3 animals/group. ***$P \leq 0.001$. (C) Liver lysates were prepared and stained for STAT3, pSTAT3, and γ-tubulin by western blotting. (D) Quantification of (C) of all samples (n = 3). Results are presented as mean ± SEM of 3 animals/group. (E) Sections of paraffin-embedded colon were stained for pSTAT3. Higher magnification of each picture is indicated by dotted lines. (F) Quantification of (E) was performed from 10 visual fields per mouse (total of 30 visual fields). Results are mean ± SEM of 3 animals/group. ***$P \leq 0.001$. (G) Colon lysates were prepared and stained for STAT3, pSTAT3, and γ-tubulin by western blotting. (H) Quantification of (G) of all samples. Results are presented as mean ± SEM of 3 animals/group. *$P \leq 0.05$. (I) Sections of paraffin-embedded lung tissue were stained for pSTAT3. Higher magnification of each picture is indicated by dotted lines. (J) Quantification of (I) was performed from 10 visual fields per mouse (total of 30 visual fields). Results are presented as mean ± SEM of 3 animals/group. ***$P \leq 0.001$. (K) Lung lysates were prepared and stained for STAT3, pSTAT3, and γ-tubulin by western blotting. (L) Quantification of (K) for all samples. Results are presented as mean ± SEM of 3 animals/group. *$P \leq 0.05$, ***$P \leq 0.001$. (M) Quantification of *SAA2* mRNA level in wt and sIL-6R$^{+/+}$ mice after injection of 5 μg IL-6 (n = 3).

## SIL-6R$^{+/+}$ MICE FULLY COMPENSATED DISABLED IL-6 CLASSIC SIGNALING BY IL-6 TRANS-SIGNALING DURING LIVER REGENERATION AFTER PHX

PHX was performed in wild-type, sIL-6R$^{fl/fl}$, sIL-6R$^{+/+}$, and IL-6R$^{-/-}$ mice to analyze whether IL-6 trans-signaling is sufficient for normal liver regeneration. Overall survival rate of wild-type and sIL-6R$^{fl/fl}$ mice 12 days after PHX was about 80%, whereas IL-6R deficient mice had an overall survival rate of only 20%. Survival of sIL-6R$^{+/+}$ trans-signaling mice after PHX was identical to wild-type mice and sIL-6R$^{fl/fl}$ mice (Fig. 3A). Moreover, large necrotic areas were only found in the liver of IL-6R$^{-/-}$ mice but not in wild-type, sIL-6R$^{fl/fl}$, and sIL-6R$^{+/+}$ mice (Fig. 3B). Consequently, the necrotic score was increased in IL-6R$^{-/-}$ mice in comparison to wild-type, sIL-6R$^{fl/fl}$, and sIL-6R$^{+/+}$ mice (Fig. 3C). There was, however, only a trend for increased alanine aminotransferase (ALT)/aspartate aminotransferase (AST) levels 12 and 24 hours following PHX in IL-6R$^{-/-}$ mice compared with wild-type, sIL-6R$^{fl/fl}$, and sIL-6R$^{+/+}$ mice (Supporting Fig. S5A,B). Liver weight to body weight ratio 12, 24, and 168 hours after PHX was not different between wild-type and sIL-6R$^{+/+}$ mice (Supporting Fig. S6A). Next, we analyzed sIL-6R levels following PHX in wild-type, IL-6R$^{-/-}$, and sIL-6R$^{+/+}$ mice. As reported previously[14] and supported by our data, sIL-6R level in wild-type mice increased 1.5 fold from 11 ng/mL to 15 ng/mL 24 hours after PHX (Fig. 3D), whereas no sIL-6R was detectable in IL-6R$^{-/-}$ mice at any time after PHX (Fig. 3E). Importantly, sIL-6R$^{+/+}$ mice also showed a 3.2-fold increase of sIL-6R from 372 ng/mL to 1,200 and 620 ng/mL 12 hours and 24 hours after PHX (Fig. 3F). As shown previously, the sIL-6R in sIL-6R$^{+/+}$ mice was not generated by ADAM-mediated shedding, but by direct secretion of sIL-6R. Therefore, we analyzed mRNA levels of *IL-6R* following PHX in wild-type and sIL-6R$^{+/+}$ mice in liver and spleen by quantitative real-time PCR. *IL-6R* mRNA levels were significantly increased in livers of wild-type mice and sIL-6R$^{+/+}$ mice by a factor of 6.6 and 5.5, respectively (Fig. 3G), suggesting that transcriptional activation of IL-6R is the main driving force of sIL-6R generation in sIL-6R$^{+/+}$ mice following PHX. Increased transcription likely contributes to increased sIL-6R level in wild-type mice following PHX; however, ectodomain shedding is the final trigger for sIL-6R generation. *IL-6R* mRNA levels were not increased in the spleen of wild-type mice and in sIL-6R$^{+/+}$ mice only after 24 hours (Fig. 3H). Because in wild-type mice the membrane-bound IL-6R is converted into sIL-6R by ectodomain shedding, production of sIL-6R will be delayed in comparison to direct secretion of sIL-6R in sIL-6R$^{+/+}$ mice, which might be the reason for the faster increase of the sIL-6R protein level in sIL-6R$^{+/+}$ mice compared with wild-type mice (Fig. 3D,F).

Next, we analyzed STAT3 phosphorylation in the liver of wild-type, IL-6R$^{-/-}$, and sIL-6R$^{+/+}$ 12 hours and 24 hours following PHX. STAT3 phosphorylation was comparable in wild-type and sIL-6R$^{+/+}$ mice as determined by immunohistochemistry (Fig. 4A). Importantly, IL-6R$^{-/-}$ mice had significantly reduced pSTAT3 levels in the liver 12 hours and 24 hours

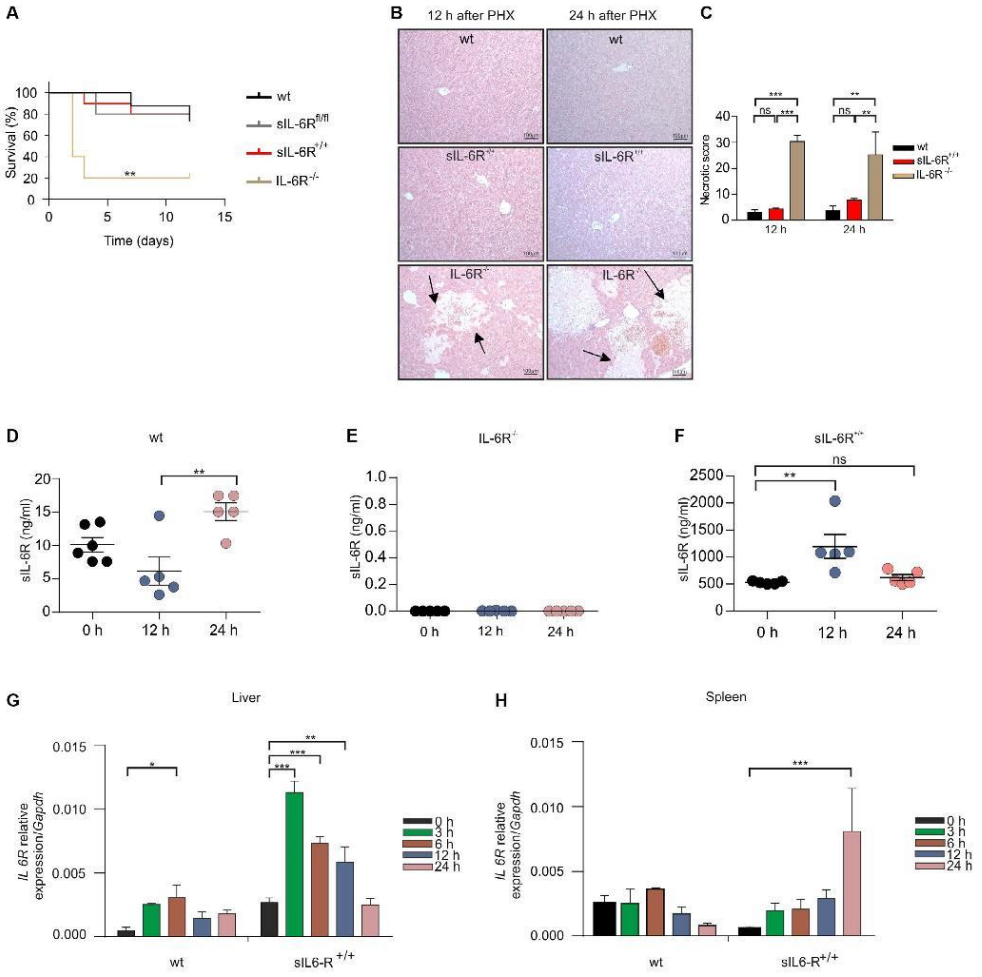


**FIG. 3.** Abrogated liver regeneration in IL-6R$^{-/-}$ but not in sIL-6R$^{+/+}$ mice after 70% PHX. (A) Mice were subjected to 70% PHX and survival was monitored for 12 days (wt [n = 10], IL-6R$^{-/-}$ [n = 10], sIL-6R$^{fl/fl}$ [n = 10], and sIL-6R$^{+/+}$ [n=10]). (B) Liver sections from wt, IL-6R$^{-/-}$, and sIL-6R$^{+/+}$ were stained with hematoxylin and eosin (H&E) (n = 3). Necrotic areas are marked with arrows. (C) Quantification of (B). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. (D-F) Zero, 12, and 24 hours after PHX, the sIL-6R serum level was determined in wt (D), IL-6R$^{-/-}$ (E), and sIL-6R$^{+/+}$ (F) mice by ELISA (n = 5). $^{**}P \leq 0.01$. (G) Total RNA was extracted from liver of wt and sIL-6R$^{+/+}$ mice 0, 3, 6, 12, and 24 hours after PHX, and mRNA levels of IL-6R were determined by quantitative real-time PCR (n = 5). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. (H) Total RNA was extracted from spleen of wt and sIL-6R$^{+/+}$ mice 0, 3, 6, 12, and 24 hours after PHX, and mRNA levels of IL-6R were determined by quantitative real-time PCR (n = 5). $^{***}P \leq 0.001$.

following PHX (Fig. 4A-D, Supporting Fig. S7). Proliferating cell nuclear antigen (PCNA) is required for DNA synthesis during replication and hepatocyte proliferation.[28] Immunohistochemical staining of PCNA in liver sections 24 hours after PHX revealed a reduction of about 76.4% of PCNA-positive cells in

IL-6R$^{-/-}$ mice compared with wild-type mice, whereas PCNA levels of wild-type and IL-6R$^{+/+}$ mice were comparable (Fig. 4E,F).

Taken together, our data demonstrate that IL-6 trans-signaling can fully compensate for the loss of classic signaling to ensure liver regeneration following PHX.

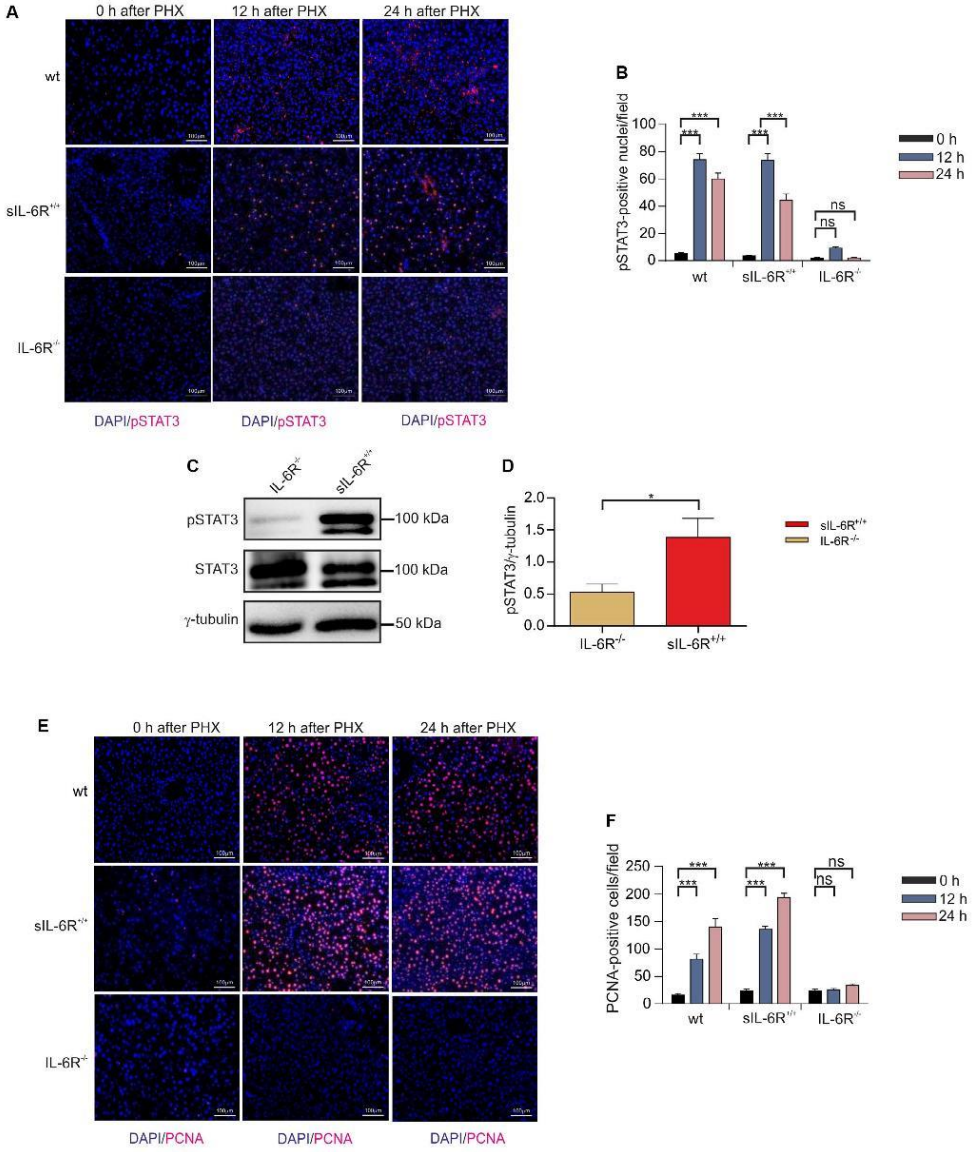

**FIG. 4.** Abrogated pSTAT3 and PCNA levels in IL-6R$^{-/-}$ but not in sIL-6R$^{+/+}$ mice after 70% PHX. (A) Sections of paraffin-embedded liver tissues from wt, IL-6R$^{-/-}$, and sIL-6R$^{+/+}$ were stained for pSTAT3 0, 12, and 24 hours after PHX. Representative examples are shown (n = 5 mice per group). (B) Quantification of (A) from 10 visual fields of every mouse (n = 5). ***$P \leq 0.001$. (C) Liver lysates from IL-6R$^{-/-}$ and sIL-6R$^{+/+}$ were prepared and stained for pSTAT3 in western blotting 12 hours after PHX. Representative examples are shown (n = 5 mice per group). (D) Quantification of (C) from western blots of 5 mice for each time point. *$P \leq 0.05$. (E) Sections of paraffin-embedded liver tissues from wt, IL-6R$^{-/-}$, and sIL-6R$^{+/+}$ were stained for PCNA 12 and 24 hours after PHX. Representative visual fields are shown (n = 5 mice per group). (F) Quantification of (E) from 10 visual fields of every mouse (n = 5). ***$P \leq 0.001$.

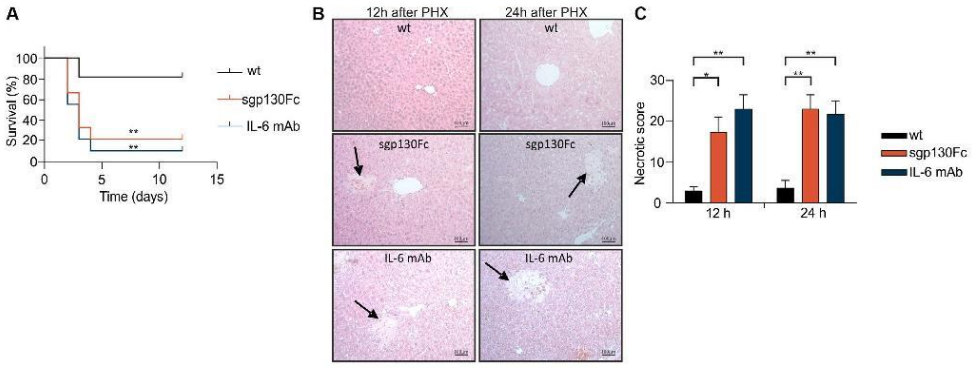


**FIG. 5.** Abrogated liver regeneration in mice treated with IL-6 mAb and sgp130Fc after 70% PHX. (A) Mice were subjected to 70% PHX and survival was monitored for 12 days (wt [n=10], wt+sgp130Fc [n = 10], and wt+IL-6 mAb [n = 10]). (B) Liver sections from wt, wt+sgp130Fc, and wt+IL-6 mAb mice were stained with H&E (n = 3). Necrotic areas are marked with arrows. (C) Quantification of (B). *$P \leq 0.05$, **$P \leq 0.01$.

## SELECTIVE INHIBITION OF IL-6 TRANS-SIGNALING PREVENTS LIVER REGENERATION FOLLOWING PHX

Discrimination between classic and trans-signaling is also possible by comparing the functional outcome of classic and trans-signaling blockade by IL-6 and/or IL-6R antibodies with the specific blockade of trans-signaling by sgp130Fc. Therefore, we injected a high dose of monoclonal antibodies directed against IL-6 (250 μg/mouse) and a low dose of sgp130Fc (50 μg/mouse) into wild-type mice, to independently characterize the contribution of classic and trans-signaling to liver regeneration. A total of 50 μg/mouse (1.25 mg/kg) sgp130Fc was chosen because previous studies showed selective inhibition of trans-signaling for this dosage, whereas lower amounts had no effect.[29,30] Abrogation of IL-6 classic and trans-signaling in wild-type mice treated with neutralizing IL-6 mAb (250 μg/mouse) every 2 days following PHX resulted in an overall survival of 10% after 12 days (Fig. 5A), which is in good agreement with the survival of IL-6R$^{-/-}$ mice after PHX (Fig. 3A). Importantly, selective inhibition of IL-6 trans-signaling by repetitive intraperitoneal injection of a low dose of 50 μg/mouse sgp130Fc (1.25 mg/kg) resulted in a comparably low survival rate as observed for mice treated with 250 μg/mouse IL-6 mAb (6.25 mg/kg) after PHX (Fig. 5A). In contrast, an even lower dose of 10 μg/mouse sgp130Fc (0.25 mg/kg) had no effect on survival (Supporting Fig. S8). Histological examination of the overall liver damage 12 hours and 24 hours after PHX showed larger necrotic areas in mice treated with IL-6 mAb and sgp130Fc (1.25 mg/kg) compared with control mice (Fig. 5B). Consequently, the necrotic score was increased in IL-6 mAb and sgp130Fc treated mice in comparison to control mice (Fig. 5C). There was, however, just a trend for increased ALT/AST level 12 hours and 24 hours after PHX in IL-6 mAb treated mice compared with wild-type and sgp130Fc treated mice (Supporting Fig. S5A,B). Liver weight

to body weight ratio 12 hours and 24 hours after PHX was not different between experimental groups (Supporting Fig. S6B). Due to the low survival rate of IL-6 mAb-treated and sgp130Fc-treated mice liver weight to body weight ratio at a later time point was not determined. Next, we analyzed STAT3 phosphorylation in the liver following PHX in mice treated with IL-6 mAb, sgp130Fc, or left untreated. STAT3 phosphorylation in the liver was decreased after global blockade of IL-6 signaling for 12 hours and 24 hours compared with control mice, as determined by immunohistochemistry (Fig. 6A,B) and western blotting (Fig. 6C-F, Supporting Fig. S9A,B). Importantly, selective inhibition of IL-6 trans-signaling also prevented phosphorylation of STAT3 in the liver 12 hours and 24 hours after PHX (Fig. 6A-F, Supporting Fig. S9A,B). Immunohistochemical staining of PCNA in liver sections 24 hours following PHX demonstrated a 94.1% reduction of PCNA-positive cells after global blockade of IL-6 signaling and a 85.4% reduction after selective inhibition of IL-6 trans-signaling compared with control mice (Fig. 6G,H). Taken together, our data showed that IL-6 trans-signaling is the main pathway controlling liver regeneration following PHX.

### IL-6 TRANS-SIGNALING INDUCES HGF EXPRESSION IN MICE FOLLOWING PHX AND IN HEPATIC STELLATE CELLS

HGF is important for induction of hepatocyte proliferation[26] and was up-regulated following PHX in wild-type mice in the liver at the mRNA level (Fig. 7A). Importantly, inhibition of classic and trans-signaling by IL-6 mAb or trans-signaling by sgp130Fc prevented up-regulation of *Hgf* on mRNA level in wild-type mice following PHX (Fig. 7A). Up-regulation of *Hgf* was, however, abrogated in IL-6R$^{-/-}$ mice (Fig. 7A,B), but not in sIL-6R$^{+/+}$ mice (Fig. 7A,B). Next, we determined HGF level in the liver lysate of mice following PHX. Whereas wild-type and sIL-6R$^{+/+}$ mice had significantly increased HGF-level 12 hours and 24 hours following PHX, the IL-6 mAb-treated, sgp130Fc-treated, and IL-6R$^{-/-}$ mice failed to show up-regulation of HGF (Fig. 7C). These data indicate that HGF up-regulation following PHX is dependent on IL-6 trans-signaling.

Hepatic stellate cells (HSCs) are a known source of HGF following PHX.[31] To answer the question of whether IL-6 classic and/or trans-signaling trigger expression of HGF, rat HSCs were analyzed. Interestingly, HSCs express *IL-6R* and *Gp130* on the mRNA level as determined by quantitative real-time PCR (Fig. 8A). Expression of IL-6R protein was verified by immunohistochemical staining (Fig. 8B), and again IL-6R was found primarily in intracellular compartments and only sparingly on the cell membrane. Despite the expression of IL-6R, only the stimulation of HSCs with hyper-IL-6 but not with IL-6 or sgp130Fc, resulted in increased of *Hgf* mRNA level (Fig. 8C). Even though increased HGF protein level found only in lysates of hyper-IL-6 stimulated HSCs (Fig. 8D), release of HGF was not stimulated by IL-6 trans-signaling in this *in vitro* setting (Fig. 8E). Our data show that IL-6 trans-signaling induced HGF production in HSCs, but suggested that in addition to IL-6 trans-signaling, additional factors might be needed to induce release of HGF from HSCs.

## Discussion

We present three major findings in this study that define the role of IL-6 signaling in the process of liver regeneration. First, our data suggest that IL-6 trans-signaling is the main mechanism of IL-6 action in liver regeneration following PHX in mice. Second, the trans-signaling sIL-6R$^{+/+}$ mice represent a genetic strategy to phenocopy substrate-selective hyperactivation of ectodomain shedding that is executed primarily by ADAM proteases.[8] Third, using our trans-signaling sIL-6R$^{+/+}$ mice, we independently demonstrate that IL-6 trans-signaling controls liver regeneration, and show that classic signaling is absolutely dispensable for this process in this setting. Exemplified on the basis of liver regeneration, the trans-signaling sIL-6R$^{+/+}$ mouse model is a valuable tool to study IL-6 trans-signaling under physiological and non-physiological conditions, including acute and chronic inflammation and cancer development. This strategy might also serve as a blueprint to study increased release of other ADAM substrates. Of note, it is not possible to study most substrates in ADAM overexpressing mice, because the phenotypes in these mice would be dominated by only a subset of dominating substrates, including EGFR ligands, NOTCH, and TNF. Our strategy also complements existing models, including mice with noncleavable ADAM-substrates,

as shown for L-selectin, which is an elegant way to analyze diminished ADAM activity for a selected substrate.[32]

The genetic conversion of a transmembrane protein into a soluble protein to phenocopy single-substrate ADAM activation has to be carefully

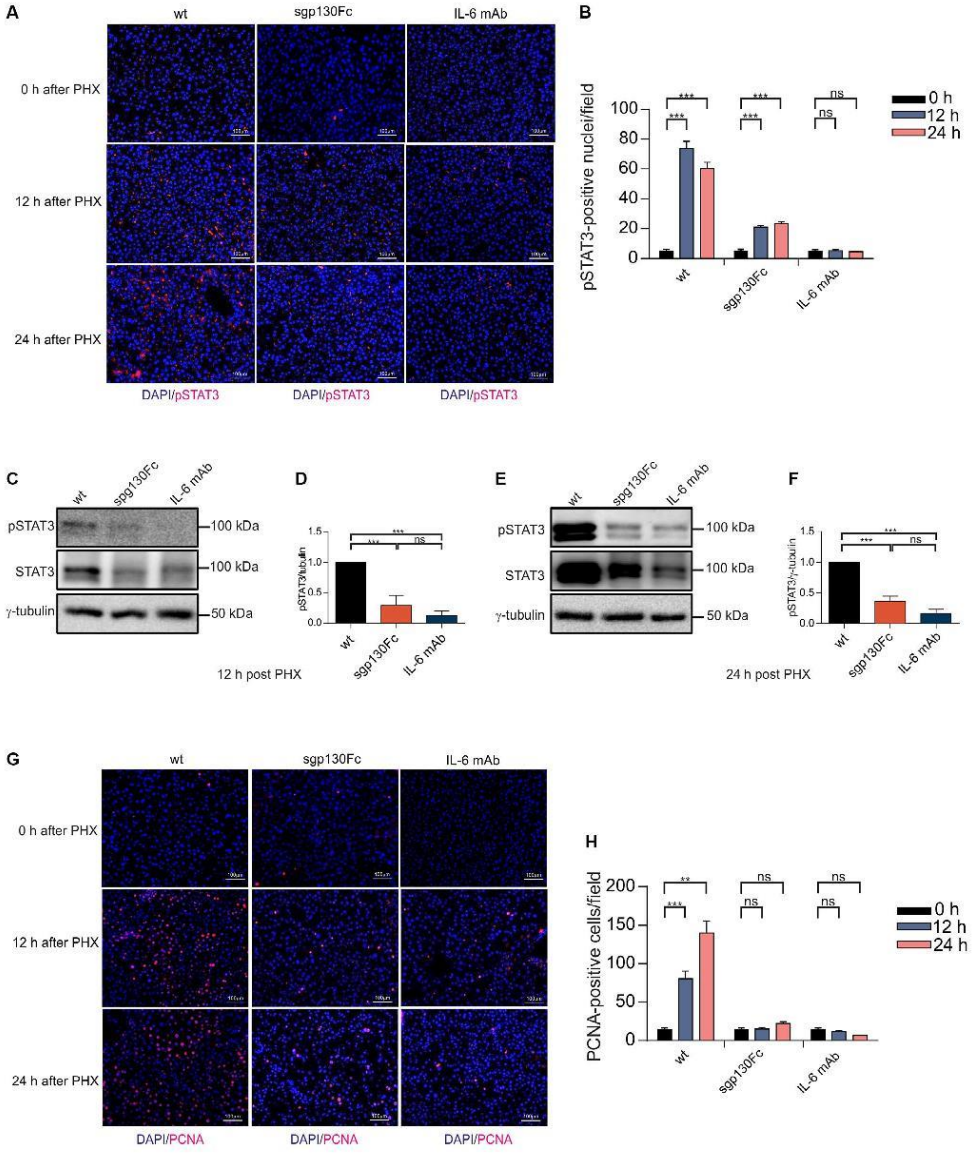

**FIG. 6.** Abrogated pSTAT3 and PCNA signals in wt mice treated with sgp130Fc and IL-6 mAb after 70% PHX. (A) Sections of paraffin-embedded liver tissues from wt, wt+sgp130Fc, and wt+IL-6 mAb mice were stained for pSTAT3 0, 12, and 24 hours after PHX. Representative examples are shown (n = 5 mice per group). (B) Quantification of (A) from 10 visual fields of every mouse (n = 5). ***$P \leq 0.001$. (C) Lysates from liver tissues from wt, wt+sgp130Fc, and wt+IL-6 mAb mice were prepared and stained for pSTAT3 in western blotting 12 hours after PHX. Representative examples are shown (n = 5 mice per group). (D) Quantification of (E) from western blots of 5 mice each. ***$P \leq 0.001$. (E) Lysates from liver tissues from wt, wt+sgp130Fc, and wt+IL-6 mAb mice were prepared and stained for pSTAT3 in western blotting 24 hours after PHX. Representative visual fields are shown (n = 5 mice per group). (F) Quantification of (C) from western blots of 5 mice each. ***$P \leq 0.001$. (G) Sections of paraffin-embedded liver tissues from wt, wt+sgp130Fc, and wt+IL-6 mAb mice were stained for PCNA 12 and 24 hours after PHX. Representative examples are shown (n = 5 mice per group). (H) Quantification of (G) from 10 visual fields of every mouse (n = 5). **$P \leq 0.01$, ***$P \leq 0.001$.

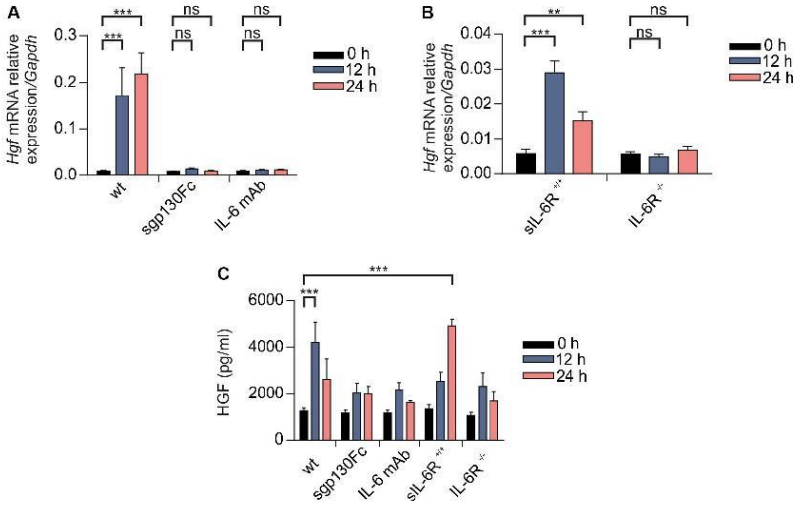


**FIG. 7.** Abrogated HGF expression in mice treated with sgp130Fc and IL-6 mAb after 70% PHX. (A) Total RNA was extracted from liver tissues of wt, wt+sgp130Fc, and wt+IL-6 mAb mice 0, 12, and 24 hours after PHX. Subsequently, the RNA levels of *Hgf* were determined by quantitative real-time PCR (n = 5). ***$P \leq 0.001$. (B) Total RNA was extracted from liver tissues of sIL-6R$^{+/+}$ and IL-6R$^{-/-}$ mice 0, 12, and 24 hours after PHX. Subsequently, the RNA levels of *Hgf* were determined by quantitative real-time PCR (n = 5). **$P \leq 0.01$, ***$P \leq 0.001$. (C) Total HGF protein levels in liver lysates of wt, wt+sgp130Fc, wt+IL-6 mAb, sIL-6R$^{+/+}$, and IL-6R$^{-/-}$ mice 0, 12, and 24 hours after PHX were determined by ELISA (n = 5). ***$P \leq 0.001$.

designed, because deletion of exons/introns might also interfere with the generation and stability of the respective mRNA. Importantly, genetic fusion of exon 9 and 10 and introduction of a 2A-GFP cassette did not influence endogenous IL-6R mRNA and protein production. However, partial deletion of the 3′UTR of the IL-6R by Cre-mediated recombination resulted in increased sIL-6R-level, suggesting that negative regulatory elements are located in the IL-6R 3′UTR. Moreover, in the case of IL-6R, the genetically engineered soluble IL-6R was terminated by a stop codon in the former intron 8, which becomes part of the sIL-6R mRNA after Cre-mediated recombination. As a consequence, the sIL-6R in IL-6R$^{+/+}$ mice contained the three additional C-terminal amino acids (Gly, Lys, and Arg) after the last original amino acid $Leu_{352}$, coded by the last codon of exon 8 and located close to the C-terminal end of the stalk region of the translated IL-6R. Based on structural evidence, it is highly unlikely that these three additional C-terminal amino acids will influence the function of the soluble IL-6R, as the IL-6 binding domains of the IL-6R are not altered.[33] Moreover, the naturally occurring and biologically active human sIL-6R, which is

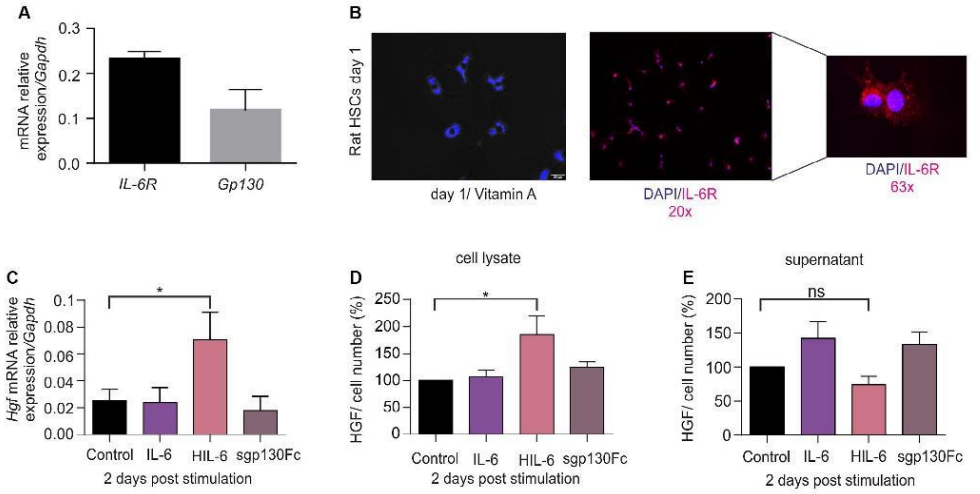


**FIG. 8.** IL-6 trans-signaling induces HGF expression in HSCs. (A) Total RNA was extracted from rat HSCs, and mRNA level of *IL-6R* and *Gp130* was determined by quantitative real-time PCR (n = 3). (B) Immunohistochemistry of IL-6R and 4′,6-diamidino-2-phenylindole in rat HSCs. (C) Rat HSCs were stimulated for 48 hours with IL-6 (100 ng/mL), hyper-IL-6 (100 ng/mL), or sgp130Fc (1 μg/mL). Total RNA was extracted from rat HSCs, and mRNA level of *Hgf* was determined by quantitative real-time PCR (n = 7). $^*P \leq 0.05$. (D) Total HGF protein in lysates of rat HSCs stimulated for 48 hours with IL-6, hyper-IL-6, or sgp130Fc was determined by ELISA (n = 4). $^*P \leq 0.05$. (E) Total HGF protein in cell culture supernatants of rat HSCs stimulated for 48 hours with IL-6, hyper-IL-6, or sgp130Fc was determined by ELISA (n = 3). $^*P \leq 0.05$. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole.

produced by differential splicing, contains 10 additional C-terminal amino acids (GSRRRGSCGL) that are not present in the membrane-bound IL-6R.[34] These additional amino acids showed no effect on the binding affinity of IL-6 to the alternatively spliced sIL-6R-protein.[35] In mice, the situation is much less complicated, because the murine IL-6R is not differentially spliced and the sIL-6R is only produced by ectodomain shedding.[36] Importantly, our experiments showed that the sIL-6R in trans-signaling sIL-6R$^{+/+}$ mice was biologically active, because injection of IL-6 efficiently induced trans-signaling in sIL-6R$^{+/+}$ mice.

In wild-type mice, sIL-6R serum levels are in the range of 6-15 ng and may rise 2-fold to 3-fold under stress conditions.[20,37,38] Previously, it was calculated that all circulating sIL-6R in wild-type mice is derived from neutrophils/macrophages and hepatocytes, suggesting that these cells are also the main producer of membrane-bound IL-6R.[24] However, here we show by using LysM- and Alb-Cre-recombined sIL-6R$^{+/+}$ mice that only about 70.8% of all IL-6R is produced by neutrophils/macrophages and hepatocytes, indicating that the remaining 29.2% must be expressed by other cell types (Fig. 1J). Even though neutrophils/macrophages and hepatocytes generate more than 90% of the naturally formed sIL-6R in the serum, our data indicate that they express proportionally less membrane-bound IL-6R. It might just be that neutrophils/macrophages and hepatocytes release proportionally higher amounts of sIL-6R compared with other cells that also express IL-6R. Neutrophils are exceptionally efficient sIL-6R releasers. They have a very short life span before they rapidly undergo apoptosis. Apoptosis, however, is an efficient trigger of ADAM17-mediated ectodomain shedding of the IL-6R.[36]

Many studies highlight the critical role of IL-6 in liver regeneration after PHX, with the consistent finding that IL-6 deficient mice show impaired liver regeneration based on reduced proliferation of hepatocytes accompanied by a high mortality rate of 40%-80% versus 10% in wild-type mice.[1,2,16,20,39]

After PHX, gut-derived factors including lipopolysaccharide activate liver-resident Kupffer cells to secrete IL-6 in a TNFα-dependent manner.[4] The parallel increase of sIL-6R after PHX[20] opens a functional window for IL-6 trans-signaling. Because hepatocytes express much more gp130 than IL-6R, the increased presence of IL-6 and sIL-6R will result in more gp130 activation and a stronger IL-6 signal. This is accompanied by the longer duration of the IL-6 signal when mediated through trans-signaling, because of the slower internalization of IL-6/sIL-6R complexes compared with IL-6/membrane-bound IL-6R complexes.[40] Our previous study using hydrodynamic injection of a sgp130Fc expression plasmid was designed to study the first 48 hours of liver regeneration after PHX.[20] Although it lacks a proper anti-IL-6 control, it already suggested a positive role for trans-signaling.[20] The data presented here suggest that blocking of IL-6 trans-signaling is as detrimental for liver regeneration after PHX as blocking of global IL-6 signaling. Nevertheless, all *in vivo* data with the trans-signaling inhibitor sgp130Fc have to be interpreted with caution, because we previously showed *in vitro* that high concentrations of sgp130Fc also cross-inhibit classic signaling at molar ratios for sIL-6R/IL-6 larger than 1, which are typically found in the serum of healthy and moderately inflamed mice.[41] Importantly, recent studies defined the minimal effective dose of sgp130Fc *in vivo*, clearly differentiating between classic and trans-signaling effects in a sepsis and a bacterial infection model.[29,30] Here we used 1.25 mg sgp130Fc/kg body weight, which is at the lower border of this minimal effective dose, to ensure inhibition of trans-signaling, but largely to exclude cross-inhibition of classic signaling.

Previously, failure of liver regeneration after PHX was shown in IL-6 antibody-treated[42] and IL-6 deficient mice.[1] Consistently IL-6R deficient mice were compromised in liver regeneration following PHX in our setting.[1] This is noteworthy, because in a murine skin wound-healing model, only IL-6 but not IL-6R deficient mice showed delayed healing,[24] which might be explained by the ability of the IL-6R to bind also to at least two other cytokines of the IL-6 family, CNTF and p28.[43-45] Therefore, our working hypothesis was that after PHX, IL-6R deficient mice might also behave like wild-type mice and not like IL-6 deficient mice, which also warrants the requirement to include treatment with IL-6R mAbs as additional control in PHX. Importantly, IL-6R deficient mice showed the same phenotype after PHX as IL-6 deficient mice, exhibiting reduced STAT3 phosphorylation and hepatocyte proliferation as determined by reduced expression of PCNA.

HGF is produced by HSCs and plays an important role in the induction of hepatocyte proliferation following PHX.[27] Although HSCs express *Gp130* and *IL-6R*, production of HGF was stimulated only through IL-6 trans-signaling. The reason for this phenomenon is unknown, but immunohistochemical staining of IL-6R in HSCs revealed that most IL-6R proteins are present in intracellular compartments, which might prevent efficient induction of classic signaling.

In summary, our data show that IL-6 trans-signaling is the main driver of liver regeneration following PHX. Although hepatocytes express membrane-bound IL-6R and are therefore also a target for classic signaling, the signal induced through the membrane-bound IL-6R on hepatocytes alone is not sufficient to induce a proliferative response. In wild-type mice, the combined injection of IL-6 plus sIL-6R, but not of IL-6 alone, accelerates liver regeneration after PHX.[16] Our study now provides functional proof that IL-6 trans-signaling has not only the potential to accelerate liver regeneration, but it is critically involved in normal liver regeneration, which might have implications for the use of anti-IL-6/IL-6R therapeutics in the clinic and for the forthcoming clinical evaluation of sgp130Fc.

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# Supporting Information

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# Chapter VII
## General discussion

Pluripotent stem cells possess two remarkable properties: pluripotency or the ability to give rise into all the tissues of adult body and immortality or indefinite self-renewal. The derivation of hESCs from human embryo (Thomson *et al.*, 1998) and the development of hiPSCs by reprogramming somatic cells (Takahashi *et al.*, 2007) are the main technological breakthroughs in biomedical research. Investigating the molecular basis of hESCs or hiPSCs as well as studying how pluripotency is maintained and how lineage commitment is regulated, are crucial not only for understanding the human embryogenesis and differentiation into different cell types but also for human disease modeling, drug discovery and stem cell therapy; these potentials are on the beginning to be identified (Pera and Tam, 2010). Therefore, it is noteworthy to search for the key signaling pathways that govern pluripotent state of hiPSCs. Moreover, studying the pathological mechanisms underlying human diseases plays an essential role in discovering novel therapeutic strategies. Disease modeling using primary cells from patients is useful for developing therapeutic strategies and aetiology of human diseases. However, there are some limitations such as lack of accessible source of primary cells from patients. hiPSCs are an attractive alternative since they can easily be reprogrammed from different cell types, such as skin fibroblasts and blood cells, from different patients. Due to properties of hiPSCs, self-renewal and potential to differentiate into nearly any cell type in the body, patient-specific-iPSCs can provide wide range of disease-relevant cells and various cell types that were previously not possible to access like neurons and cardiomyocytes (Shi *et al.*, 2017).

Small GTPases of RAS superfamily are central nodes of intracellular signaling which are involved in almost every aspect of cell biology. This family composed of different families (RAS, RHO, RAN, RAD, RAG, RAB and ARF) with specific expression, regulation and effector proteins which can activate different signaling pathways and exert their cellular function.

This doctoral thesis provided new insights into molecular mechanism of pluriotency in hiPSCs with the focus of bFGF downstream signaling (chapter II) continued by expanding the knowledge about molecular properties and regulation of RAS GTPases (chapter III). In chapter IV, the advantage of hiPSC is introduced by reprogramming dermal fibroblasts from a NS patients with HCM due to a RAF1 mutation. Specific hiPSCs were differentiated into cardiomyocytes and the mechanism involved in HCM was investigated in details. In chapter V, another developmental disorder, fragile X mental retardation, is introduced and the interaction networks of a large number of novel FMRP binding proteins are studied in details.

## 7.1 hiPSCs and pluripotency

This thesis provides novel molecular insight into the regulation of pluripotency maintenance of hiPSCs (chapter II). Our findings indicate that among the signaling pathways downstream of bFGF, the MAPK pathway plays a critical role in maintaining pluripotency, whereas strong activation of p38 and JAK/STAT3 signaling is linked to differentiation of hiPSCs. In contrast, no relevant changes occurred in the activation of AKT or JNK pathways from pluripotent hiPSCs towards differentiated cells. Moreover, we identified NRAS among the RAS paralogs as the likely link between bFGF receptor and the MAPK pathway that maintains hiPSCs pluripontency. Each signaling will be discussed in details below.

*MAPK signaling pathway and pluripotency* – Different studies suggest pleiotropic effects of bFGF activating different pathways in hESCs either directly or indirectly by inducing paracrine

signaling *via* iMEFs in coculture (Lanner and Rossant, 2010). For investigating the mechanistic effects of bFGF, we compared the signaling pathways in undifferentiated *vs.* differentiated hiPSCs obtained *via* bFGF withdrawal. FGF has been reported to activate multiple downstream signaling pathways, including MAPKs (ERK, JNK and p38), PI3K and JAK/STAT (Bottcher and Niehrs, 2005). Our study demonstrates the activation of MEK-ERK1/2 pathway in undifferentiated hiPSCs and a remarkable decrease in the p-MEK and p-ERK1/2 levels by withdrawing bFGF which induces their differentiation. Previously, Li and colleagues have shown that inhibiting FGF signaling induces hESC differentiation into primitive endoderm and trophectoderm (Li *et al.*, 2007). However, Singh *et al.* have reported a pro-differentiation role of MAPK pathway in hESCs (Singh *et al.*, 2012). These conflicting reports could be due to different culture conditions, cell lines or even pathway dose-dependency. Our data argue against a pro-differentiation role of the MAPK pathway. We used in this study a system for culturing hiPSCs with iMEF-CM that was supplemented with 100 ng/ml bFGF which was different from Li *et al.* and Singh *et al*. (Li *et al.*, 2007; Singh *et al.*, 2012). Under these conditions we are able to dissect direct and paracrine iMEF-mediated influences of bFGF without the risk of confounding effects based on sample contamination with feeder cells. Our data clearly showed that MAPK pathway positively regulates hiPSC pluripotency.

*PI3K/AKT signaling pathway and pluripotency* – PI3K/AKT activation by bFGF has also been shown to be important for the maintenance of the undifferentiated state of hESCs (Eiselleova *et al.*, 2009). This pathway contributes to a variety of important cellular processes including nutrient uptake, anabolic reactions, proliferation and survival (Yu and Cui, 2016). Proliferation and survival can be controlled by mTORC1 mediated activation of S6K and mTORC2 mediated inhibition of FOXO-1, respectively (Laplante and Sabatini, 2012; Chiang and Abraham, 2005). Armstrong and colleagues have shown that PI3K/AKT is important for maintaining pluripotency in hES-NCL1 cells and the key components of this pathway, such as p-PDK1, p-PTEN, p-AKT$^{308}$ and p-AKT$^{473}$ are downregulated during differentiation to embryoid bodies (Armstrong *et al.*, 2006). Li and coworkers have shown that PI3K/AKT pathway, downstream of bFGF, is highly active in hESCs, such as H1 and H9 cells, which supports hESC self-renewal and pluripotency (Li *et al.*, 2007). Other studies have implicated the survival and anti-apoptotic role of PI3K/AKT in hESCs and hiPSCs (Hossini *et al.*, 2016; Romorini *et al.*, 2016; Singh *et al.*, 2012). In our study, two axes of AKT activation were investigated, PI3K-PDK1-AKT-S6K and mTORC2-AKT-FOXO1 as downstream pathways of bFGF, which is different from previous reports that just showed the importance of PI3K/AKT in maintenance of pluripotency and not as a target of bFGF signaling (Hossini *et al.*, 2016; Romorini *et al.*, 2016; Singh *et al.*, 2012). Our results showed that there was no change in the activation level of these two pathways following hiPSCs differentiation. This suggests that AKT-S6K and AKT-FOXO1 signaling remain unaffected in the presence and absence of bFGF during a long-term culture which may be due to the presence of knockout serum replacement (KSR) in iMEF-CM. KSR contains high levels of insulin that can activate AKT pathways (Singh *et al.*, 2012). This rather suggests that PI3K/AKT is not critical for maintaining the undifferentiated state of hiPSCs and most probably plays an anti-apoptotic role required for survival of hiPSCs rather than their pluripotency.

*p38 MAPK and JNK signaling pathways and pluripotency* – In addition to MAPK and PI3K/AKT pathways, we also analyzed other signaling pathways, including p38 MAPK and JNK, both can be activated by FGF signaling (Lanner and Rossant, 2010). p38 activation has been observed

in response to a variety of extracellular stresses and mitogenic stimuli which lead to different cell-specific responses, including inflammation, cell death, senescence, survival, cell growth and differentiation (Zarubin and Han, 2005). So far, little is known about the role of p38 in pluripotency of hESCs. Neganova and colleagues demonstrated an increased activity of p38 MAPK during the early stage of reprogramming of human fibroblasts to hiPSCs and the importance of this pathway for obtaining fully reprogrammed cells (Neganova *et al.*, 2017). Moreover, hESCs and hiPSCs are in a high-methionine metabolic state which decreases upon differentiation. In this regard, it has been shown that methionine deprivation triggering the activation of p53-p38 signaling leads to NANOG downregulation and differentiation into all three germ layers (Shiraki *et al.*, 2014). We showed in this study, for the first time, an increase in p38 MAPK activity during hiPSCs differentiation under bFGF starvation. It can be proposed that p38 is inhibited as a downstream target of bFGF in undifferentiated hiPSCs. Findings from *Drosophila* studies and some human cancers indicate that JNK might be a regulator of stem cells and cancer stem cells. Brill *et al.* observed a significantly elevated JNK activity in undifferentiated hESCs, which if blocked by JNK inhibitors under feeder-free conditions in the presence of conditioned medium (CM), led to decreased OCT4 expression and differentiation (Brill *et al.*, 2009). A possible contribution of JNK signaling to the maintenance and/or self-renewal of hESCs was additionally confirmed in a different hESC line, Harvard's HUES-7. In response to BMP-induced differentiation, a transient elevation of c-Jun phosphorylation was observed, which indicates both the competence of the basal JNK pathway to maintain the stemness of the hESCs and a possible involvement of JNK activation in the initiation of hESC differentiation (Van Hoof *et al.*, 2009). In our study, we observed the constant activation of JNK during hiPSCs differentiation in response to bFGF starvation. Thus, JNK pathway may be involved in other cellular responses rather than maintaining pluripotency or inducing differentiation.

*JAK/STAT3 signaling pathway and pluripotency* – mESCs can be maintained *in vitro* by adding LIF to the medium and its withdrawal rapidly leads to differentiation (Smith *et al.*, 1988; Williams *et al.*, 1988). LIF activates JAKs which subsequently phosphorylate STAT3. Activated STAT3 translocates into the nucleus and activates transcription of target genes (Sasse *et al.*, 1997; Wang *et al.*, 2012b). Interestingly, LIF/STAT3 signaling fails to support self-renewal of hESCs and is nonresponsive to LIF/STAT3 (Daheron *et al.*, 2004). Since LIF is not the only cytokine that activates JAK/STAT3 pathway, we analyzed the activity of this pathway downstream of bFGF. Similar to Humphrey and coworkers, who have shown that STAT3 phosphorylation was not detectable in undifferentiated hESCs (Humphrey *et al.*, 2004), we also could not observe phosphorylation of STAT3 in undifferentiated hiPSCs. Interestingly, upon differentiation, JAK/STAT3 pathway was activated in hiPSCs. It can be postulated that unlike mESCs, hiPSCs do not require STAT3 activity for the maintenance of their pluripotency but rather for their differentiation.

*RAS paralogs and pluripotency* – We demonstrated the critical role of MAPK pathway downstream of bFGF in maintaining pluripotency in hiPSCs. For further analysis of this pathway, we analyzed the expression of canonical RAS isoforms in undifferentiated *vs.* differentiated hiPSCs. Interestingly we found that in contrast to the decreased level of MAPK pathway activity in differentiated hiPSCs, the levels of RAS mRNA and protein were both upregulated upon differentiation. To elucidate the activity level of RAS (GTP-bound), pull down assays were performed with CRAF-RBD as an effector for RAS proteins. RAS activity was drastically reduced

in differentiated hiPSCs compared to undifferentiated cells, consistent with the decrease of MAPK pathway activity levels. These findings suggest that RAS-RAF is upstream of MEK/ERK and its activity will be decreased upon differentiation in hiPSCs. Furthermore, we analyzed main RAS paralogs, i.e. H-, K- and NRAS. Interaction analyses with two RAS effectors (RAF and PI3K) showed that among the RAS paralogs, NRAS preferentially interacts with RAF in the presence of bFGF and activates the MAPK pathway while no interaction was observed with PI3K independent of the bFGF stimulation status. KRAS interacts physically with RAF and PI3K but showed no preference for either of the effectors upon bFGF starvation or stimulation.

## 7.2 hiPSCs and disease modeling

Noonan syndrome is an autosomal dominant disorder of RASopathies which is manifested by heart defects, facial dysmorphism, ectodermal abnormalities and mental retardation. One of the common molecular pathogenesis of these disorders are the mutations in genes of RAS-MAPK signaling pathway. The role of RAS-MAPK in HCM remains controversial. In 2007, mutation in RAF1 were identified in patients with NS and LEOPARD syndrome (Pandit *et al.*, 2007; Razzaque *et al.*, 2007). RAF1 is a Ser/Thr kinases which transmits signals from RAS proteins (from the cell surface) to the nucleus. Regulation of RAF1 is complex and involves protein–protein interactions, phosphorylation at multiple residues (inhibitory and activatory) and localization (Wellbrock *et al.*, 2004). RAF isoforms, RAF1, ARAF and BRAF share three conserved regions CR1, CR2 and CR3 (Mercer and Pritchard, 2003). BRAF mutations identified in CFC patients were located in CR1 and CR3 domains (Aoki *et al.*, 2008), meanwhile mutations in RAF1 reported in NS and LEOPARD patients mostly were clustered in CR2 domain and some mutations in CR3 domain. 80% of NS individuals with S257L mutation in the CR2 domain of RAF1, exhibit the severe HCM with disorganized muscle bundles and enhanced phosphorylation of ERK1/2 (Pandit *et al.*, 2007; Razzaque *et al.*, 2007).

Previously, Kobayashi and colleagues identified eight RAF1 mutations in 18 out of 119 patients with NS and related conditions without any mutations in other genes of RAS-MAPK pathway. The frequent phenotype of these patients was HCM and short stature. Four of these mutations were clustered in CR2 domain (p.S257L, p.S259F, p.P261A, and p.N262K) which has an inhibitory phosphorylation site (serine at position 259; S259). Among all mutations, S257L, was found in 11 patients. Moreover, they investigated the molecular mechanisms by which RAF1 mutants are activated. Mutations in CR2 domain including S257L, had impaired phosphorylation of S529, which will lead to non-efficient binding of RAF1 to 14-3-3 proteins, resulting in partial activation of ERK, suggesting that mutations in CR2 domain lead to dephosphorylation of S259, which is the primary pathogenic mechanism in the activation of RAF1 mutants as well as the downstream ERK (Kobayashi *et al.*, 2010). Later, Dhandapany *et al.* investigated the role of RAF1 signaling in HCM in neonatal and adult rat cardiomyocytes. Overexpression of wild-type and different mutations of RAF1 including S257L and L613V in adult rat cardiomyocytes, caused HCM by activating ERK and calcineurin pathways whereas similar RAF1 overexpression in neonatal rat cardiomyocytes only activated calcineurin signaling. These data suggested that RAF1 overexpression induced HCM *via* two pathways, ERK and calcineurin, which depends on the developmental stage of heart (Dhandapany *et al.*, 2011). In the current status of the art, our understanding about molecular mechanism of HCM is mainly based on animal models. Animal models will provide the scientist

valuable information about the whole organism; however, several remarkable differences exist between the mouse and human models. For instance, the resting beating rate of the mouse is 10-time more that human. In mouse, the myosin heavy chain 6 (faster isoform) is a dominantly expressed in ventricle and in human myosin heavy chain 7 (slower). The heart development, ion channels contribution and therefore electrical properties varies between human and mouse. One other hand, there are difficulties to obtain the human heart tissue samples. Regardless of this issue, since the adult cardiac myocytes represents the terminally differentiated cells, they are not surviving in long-term culture for further studies. Additionally, in the case of RASophaty, since the patients have the developmental disorders and exhibits the postnatal and neonatal HCM (below 2 years), the adult cardiac myocytes will not be helpful here. To circumvent these hurdles, the patient-specific human pluripotent stem cell-derived cardiomyocytes will be beneficial for overall understanding of human HCM. So, to investigate the mechanism underlying the RAF1 induced HCM, we generated ventricular hiPSC-cardiac bodies from patient carried $RAF1^{S257L}$ in 3D. Patient-derived cardiac myocytes recapitulated the HCM phenotype such as cell size enlargement, expression of fetal genes, and increased sarcomere protein synthesis and myosin heavy chain beta to alpha switch. $RAF1^{S257L}$ cardiac myocytes exhibited the abnormal sarcomere structure, increased calcium transient and cardiac contractility. CB-$RAF1^{S257L}$ illustrated a specific SERCA2 upregulation that may affect the calcium uptake via SR from cytoplasm. Signaling analysis also confirmed the higher MAPK activity in mutant CBs. These findings indicated an increased RAS-MAPK signaling pathway in $RAF1^{S257L}$ cardiac myocytes may regulate the observed HCM phenotype.

## 7.3 FMRP and multiple cellular process

FMRP has been described previously to be involved in different biological functions, *e.g.*, RNA transport, protein translation, actin cytoskeleton remodeling, and SG formation (Chen and Joseph, 2015; Sethna *et al.*, 2014; Bardoni *et al.*, 2006; Sidorov *et al.*, 2013; Kenny and Ceman, 2016; Kim and Ceman, 2012; Wang *et al.*, 2012a; Santoro *et al.*, 2012; Fernandez *et al.*, 2013; Maurin *et al.*, 2014; Zalfa *et al.*, 2006; Alpatov *et al.*, 2014; Garber *et al.*, 2006; Santos *et al.*, 2014). Most of these functions have been retained to the ability of FMRP to control translation of many different mRNAs (Pasciuto and Bagni, 2014b). This can explain why FMRP is expressed in several tissues and cell lines including iPSCs. FMRP has been previously suggested to play a role in maintenance and differentiation of iPSCs (Li and Zhao, 2014). Identified proteins in this study which interact with FMRP were classified into three ontologies, cellular component, molecular function, and biological process. The vast majority of these proteins are involved in binding of nucleic acids, especially mRNA, rRNA and miRNA and participate in RNA metabolism, ribosome biogenesis, RNA interference, mRNA processing and transport, actin dynamics, mitochondrial stability, SGs formation and translation. Other major functions include the regulation of the DNA damage response (DDR), transcription, cell cycle regulation, apoptosis, antiviral response, immunity, and proliferation. These functions imply an intracellular shuttling of FMRP into different subcellular compartments of the cell. FMRP has been previously described to be predominantly cytoplasmic (Tamanini *et al.*, 1997). In recent years, it has become increasingly evident that FMRP translocates into the nucleus due to sequence motifs responsible for its nuclear import and export as well as nucleolar localization (Taha *et al.*, 2014; Adams-Cioaba *et al.*, 2010; Dube *et al.*, 2000; Bardoni *et al.*, 2003; Lai *et al.*, 2006; Kim *et al.*, 2009; Dury *et al.*, 2013; Alpatov *et al.*, 2014; Zhang *et al.*, 2014; Okray *et al.*, 2015; Tan *et al.*, 2016)

The data presented in this work considerably expand the physical and functional protein and RNA interaction networks of FMRP and suggest its participation in various fundamental cellular processes throughout the body beyond the central and peripheral nervous system. Accordingly, FMRP functions may start in the nucleolus following cytoplasmatic–nuclear translocation, where it may be involved in DNA damage response, maintenance of genome stability, biogenesis of ribosomal subunits and most likely their nuclear export. FMRP may be part of the transcriptional factory by regulating gene expression *via* interaction and orchestration of RNA polymerase II, where it directly binds to a large set of mRNAs and transport them to sites of local translation. Upon any kind of cellular stress, FMRP accumulates at sites of stress responses and facilitates for example stabilization double-strand RNA-binding and activating PKR and as consequence, leading to the formation of stress granules. Moreover, our novel interactome indicates that FMRP plays a central role in mitochondrial quality control and mitophagy, functions that are directly related to neurodegenerative and cognitive disorders, including FXS, Huntington's disease, Alzheimer disease, Down syndrome, and progressive supranuclear palsy. Our work provides valuable insights and constitutes a useful starting point for future studies of the cellular functions of FMRP.

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# Curriculum Vitae

## Education

| | |
|---|---|
| Since 03.2016 | PhD candidate at the Institute of Biochemistry and Molecular Biology II, Heinrich-Heine University of Düsseldorf, Germany<br>Thesis title: “New insights into signaling pathways controlling pluripotency and differentiation in human induced pluripotent stem cells”<br>Supervisor: Prof. Reza Ahmadian |
| 2008-2011 | Master’s Degree in Cell and Molecular Biology<br>Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran.<br>Thesis title: "Investigating the cytotoxicity effects of cobalt (III)-salen and two coumarin compounds, umbelliprenin and 7-isopentenyloxycoumarin, *in vitro*"<br>Supervisors: Dr. Maryam M.Matin and Dr. Ahmad Reza Bahrami<br>GPA: 18.22 out of 20 |
| 2003-2007 | Bachelor’s Degree in Genetic<br>Faculty of Science, Isfahan University, Isfahan, Iran<br>GPA: 16.34 out of 20 |

## Skills

| | |
|---|---|
| **Cell Biology** | Cultivation of various eukaryotic cell lines including human and rat pluripotent cells (feeder-based and feeder-free), *in vitro* differentiation of iPS cells, transfection, transduction, fluorescence imaging including confocal laser scanning microscopy, Sub-cellular fractionation, flow cytometry, cell cycle analysis (PI, 7-AAD) |
| **Molecular Biology** | PCR (standard-PCR, RT-PCR, Quantitative real-time PCR), DNA recombination techniques and cloning, virus packaging |
| **Protein Biochemistry** | Gene expression in Escherichia coli, Characterization of proteins by using Western blotting, Immunoprecipitation and pull down |
| **Computer Skills** | Systems: Windows<br>Softwares: MS-Office, Corel Draw, EndNote |
| **Bioinformatics Skills** | Clone Manager, BioEdit, Image J, CLC main work Bench, Primer Premier, SnapGene, Prism, GraFit |

## Practical courses

| | |
|---|---|
| 2017 | Scientific Image Processing and Analysis |
| 2016 | Good Scientific Practice for Doctoral Researchers |

## Work Experience and Teaching

| | |
|---|---|
| 2013-2014 | Lab assistant<br>Dr. Bazrafshani Medical Genetic Lab, Kerman, Iran<br>Manager: Dr. Mohammad Reza Bazrafshani<br>Lab assistant in Molecular Biology Section |
| 2012-2014 | Lab assistant<br>Dr. Bazrafshani Medical Genetic Lab, Kerman, Iran<br>Manager: Dr. Mohammad Reza Bazrafshani<br>Lab assistant in Research and Development Section |
| 2011-2012 | Lab Assistant<br>International Centre for Science, High Technology and Environmental Sciences, Kerman, Iran<br>Assistant Professor: Dr. Malek Hossein Asadi |

2014-2015 English Teacher
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## Awards

2018 Travel award from 6th Annual GSCN Conference, September 19th-21th 2018, Heidelberg, Germany

2011 Ranked the "2nd Position" among M.Sc. students of Cell and Molecular Biology

## Conference attendance

poster 6th Annual GSCN Conference, September 19th-21th 2018, Heidelberg, Germany

Talk 12th BMFZ-Klausurtagung, April 19th-20th 2018, Bergisch-Gladbach, Germany

Trainee 32th Ernst Klenk Symposium in Molecular Medicine Precision oncology, December 8th-10th 2016, Cologne, Germany

Trainee Genome Editing for Gene and Cell Therapy, November 3th-4th 2016, Hannover, Germany

Talk Iranian Congress on Biology and Applications of Stem cells, April 27th-29th 2011, Mashhad, Iran

## Publications

### Research articles and reviews

2019 Fazel Modares N, Polz R, **Haghighi F**, Lamertz L, Behnke K, Zhuang Y, Kordes C, Häussinger D, Sorg UR, Pfeffer K, Floss DM, Moll JM, Piekorz RP, Ahmadian MR, Lang PA, Scheller J. " IL-6 trans-signaling controls liver regeneration after partial hepatectomy". *Journal of Hepatology*. 2019.

2018 **Haghighi F**, Dahlmann J, Nakhaei-Rad S, Lang A, Kutschka I, Zenker M, Kensah G, Piekorz RP, Ahmadian MR. "bFGF-mediated pluripotency maintenance in human induced pluripotent stem cells is associated with NRAS-MAPK signaling". *Cell communication and signaling*. 2018, 16(1): 96. PMID: 30518391.

2018 Youhannayeea M, Nakhaei-Rad S**, Haghighi F**, Klauke K, Janiak C, Ahmadian MR, Rabenalt R, Albers P, Getzlaf Z. "Physical characterization and uptake of iron oxide nanoparticles of different prostate cancer cells". *Journal of Magnetism and Magnetic Materials*. In Press**.**

2018 Nakhaei-Rad S, **Haghighi F**, Nouri P, Rezaei Adariani S, Lissy J, Kazemein Jasemi NS, Dvorsky R, Ahmadian MR. "Structural fingerprints, interactions, and signaling networks of RAS family proteins beyond RAS isoforms". *Critical Reviews in Biochemistry and Molecular Biology*. 2018, 53(2):130-156. PMID: 29457927

2015 Sadeghi B, **Haghighi F**, Pishbin M, Mirahmadi M. "MicroRNAs and Cancer: prespective on the discovery and function*". Journal of Cellular Immunotherapy.* 2015, 1(1):8-9.

2015 Falahati-pour S, Lotfi A, Ahmadian G, Baghizadeh A, Behroozi R, **Haghighi F**. "High Level Extracellular Secretion of Organophosphorous Hydrolase of Flavobacterium sp. in Escherichia coli BL21(DE3)pLysS". *Biotechnology and Applied Biochemistry.* 2015, PMID: 26331355

2014 **Haghighi F**, M. Matin M, Bahrami AR, Iranshahi M, Behnam Rassouli F, Haghighi talab A. "The cytotoxic activities of 7-isopentenyloxycoumarin on 5637 cells via induction of apoptosis and cell cycle arrest in G2/M stage". *DARU journal of pharmaceutical sciences*. 2014, 22(1):3. PMID: 24393601.

2014 Haghighitalab A, M. Matin M, Bahrami AR, Iranshahi M, **Haghighi F**, Porsa H. "Enhancement of cisplatin cytotoxicity in combination with herniarin *in vitro*". *Drug and Chemical Toxicology.* 2014, 37(2):156-62. PMID:24116377

2014 Haghighitalab A, M. Matin M, Bahrami AR, Iranshahi M, Saeinasab M, **Haghighi F**. "*In vitro* investigation of anticancer, cell-cycle-inhibitory, and apoptosis-inducing effects of diversin, a natural prenylated coumarin, on bladder carcinoma cells". *Z Naturforsch C*. 2014, 69(3-4):99-109. PMID: 24873030.

**In preparation**

Taha MS[§], **Haghighi F**[§], Stefanski A, Nakhaei-Rad S, Görg B, Fujii M, Häussinger D, Piekorz RP, Stühler K, Ahmadian MR. "Uncovering multiple protein interaction networks linked to fragile X mental retardation protein". [§]co-first author. Submitted in *Cell communication and signaling.*

Nakhaei-Rad S, Dahlmann J, Bucholzer M[§], **Haghighi F**[§], Schänzer A, Borchardt A, Kronenbitter AV, Scheller J, Piekorz RP, Reichert A, Krüger M, Schmitt J, Hahn A, Zenker M, Kensah G, Ahmadian MR. "Mechanisms underlying hypertrophic cardiomyopathy caused by RAF1 germline mutation in an individual with Noonan syndrome".[§]co-second author. In preparation.

## Languages

English **Advance**

2015 IELTS (International English Language Testing System)
Overall band score: 7 out of 9
Listening: 7.5, Reading: 7.5, Speaking: 6, Writing: 6

German **Basic (A2)**

Persian **Mother's tongue**

# Eidesstattliche Erklärung

Hiermit erkläre ich, Fereshteh Haghighi, an Eides statt, dass ich die hier vorgelegte Dissertation eigenständig und ohne unerlaubte Hilfe angefertigt habe. 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 keener anderen Institution eingereicht und es wurden bisher keine erfolglosen Promotionsversuche von mir unternommen.

Signed: Fereshteh Haghighi
Date: Düsseldorf, January 2019