Identification and functional analysis of interaction partners of the apoptosis inhibitor DIAP1 in *Drosophila*

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To my father
Mome Ocu

1. Introduction

1.1 Apoptosis during the life of an organism

1.1.1 General introduction

Evolution sculptures living organisms with the constant pressure of natural selection. Each organism develops lives and dies surrounded by a constantly changing environment. Adapting on the changes, successfully responding on the stimuli is the ultimate requirement for survival. Multicellular organisms developed during 1.5 billion years of eukaryotic history an astonishing number of different, highly specialized types of cells. Cells are forming tissues, which are then organized in organs, organs are connected in systems and all systems together make up an organism. The basis of this very old scholar systematization of the structure of a living organism forms an important biological principle. Diversity and differentiation are needed for sophisticated interactions with the environment but there is a strict hierarchy in place. What matters is the survival of an organism as a whole, even if individual cells have to be sacrificed.

The last two decades of biomedical research brought growing awareness that looking into intrinsic death pathways inside the organism could solve many open questions about regulation of the processes within living organisms. The death of a cell is a frequent event that plays a key role in development as well as in cellular defence. Replication, cell division and growth are main features of life but for the living organism cells that divide without control represent the biggest threat. Therefore, if effects of strong environmental stress (UV light, toxins, etc) or damage to the genetic material can not be neutralized, it is safer that such cells die than to loose control over their cell cycle and cell behaviour.

Death is also an inseparable part of morphogenetic processes and tissue homeostasis. Life span of epithelial cells in the intestine is only 48 hours (Grant et al., 2001) and all red blood cells are completely renewed in circulation every 90 days. Cells that have fulfilled their function are being gradually replaced in many organ systems.

Cells also die in large numbers during normal morphogenetic processes. Pluripotent progenitor cells usually proliferate at the beginning of development before they differentiate. At the onset of central nervous system formation, many more neuron and glial precursor cells are produced than are actually needed. Later, all neurons that did not establish a synapse are eliminated by cell death. In this case, the excessive number of neuronal progenitors allows maximal stringency of selection and “quality control”.

One of the most obvious and convincing examples of the importance of cell death for development is the formation of distal falangas in vertebrates. The developing finger bones are kept together by connective tissue. At a distinct time point the connective mesenchymal cells die and fingers are separated (Mori et al., 1995).

All these diverse cases of cell suicide are manifestations of a single conserved genetic program that is started when adequate conditions are met. The endogenous molecular mechanism that enables cells to die at a specific time point and without triggering of immune response is called apoptosis.

1.1.1.1 Characteristics of apoptotic cells

Apoptosis or programmed cell death is an irreversible process of self-destruction. Macrophages or even the neighbouring cells rapidly phagocytose the remaining “skeleton” of the former cell. There is no inflammation and no trace is left behind. That is the reason why apoptosis, although relatively early discovered in the work of Wyllie, Kerr and Currie (1972) remained for many years on the margin of interest of the mainstream biology. What the early research in this field mastered was histological description of the programmed cell death phenomenon.

Apoptotic cells have a very typical morphology. Main features are rounding up of the cells, condensation of the chromatin, blebbing of the membranes and formation of inclusion "apoptotic bodies". On the biochemical level apoptosis is marked by reduction of mitochondrial transmembrane potential, acidification of the cytoplasm, production of reactive oxygen species, proteolysis of selected protein substrates, and fragmentation of DNA into internucleosomal fragments (Willey et al., 1984). At any phase there is no release of cytoplasmic content into the intercellular space, what would be a hallmark of non-programmed necrotic cell death.

1.1.2 Molecular mechanisms of apoptosis

The importance of programmed cell death was comprehended only after several pathological conditions and human diseases have been studied. It became clear that apoptosis presents an important “safeguard” mechanism that neutralizes mistakes made in proliferation, cell cycle and cell division. In many cases cells are actually predetermined to die unless they receive a survival signal. Still, whether death of a cell is a “side effect” of the failure in house keeping functions or apoptosis exists as an independent mechanism remained for many years a controversial issue. The real breakthrough in understanding of the cell death phenomenon came with the discovery that programmed cell death is controlled by a set of genes. Pioneer work done by Robert Horvitz in the model system *Caenorhabditis elegans* gave evidence that apoptosis represents a conserved molecular mechanism controlled by a genetic pathway (Yuan and Horvitz, 1990). The nematode *C.elegans* is a hermaphrodite worm with a short life cycle and completely determined developmental program. The adult organism develops with exactly 1090 somatic cells, from which 131 undergo apoptosis.

Removal of excessive cells is a four-step process and each step is controlled by different genes: commitment phase, killing of the cell, engulfment of the corpses and phagocytosis of the corpses. A cystein protease encoded by the gene *ced-3* is the main player in execution of apoptosis in *C.elegans*. It belongs to the caspase family, a group of enzymes that proteolyticallly cleave peptide bonds after specific aspartate residues. CED3 is produced in inactive form and becomes processed in the commitment phase of the cell death program.

Activation of CED3 is genetically regulated by expression of the *ced-4* gene. Overexpression of *ced-4* triggers ectopic cell death but this is suppressed in *ced-3* mutant background suggesting that *ced-4* is genetically upstream or parallel. In vitro studies proved that CED4 oligomerizes, and that this property has critical importance for the process of caspase activation. Through CED4 oligomerization the attached CED3 procaspases are accumulated. Homophilic interactions create favourable conditions for the intermolecular proteolysis of caspase prodomains and active CED3 molecules are released. If the protein CED4 is mutated in a manner that it can not oligomerize, caspase activation is prevented.

Consequence of caspase action is the irreversible proteolysis of multiple targets, and therefore an additional control mechanism has been developed to minimize the possibility of accidental activation. The gene *ced-9* was identified as the main inhibitor of apoptosis in the nematode worm. Biochemical in vitro experiments showed that CED9 binds CED4 preventing it to activate CED3 (Seshagiri and Miller, 1997). CED9 also binds the EGL-1 apoptosis inducing gene product (Conradt and Horvitz, 1998). Egl-1 is epistatic to *ced-3*, *ced-4* and *ced-9*. Loss of function mutations in *egl-1* cause complete absence of developmental apoptosis. EGL-1 has a BH3 domain, which is a structural feature of many pro apoptotic proteins, identified in other model systems. Expression of *egl-1* in the cell represents the point of no return for the cell and execution of the apoptotic program begins (del Peso et al., 2000). There are indications that EGL-1 and related proteins represent the link between cell survival and commitment to cell death but all aspects of their function are yet to be discovered.

The molecular mechanism of the suicidal program established in *C.elegans* was reliable enough to be preserved as an “optimal” solution during evolution. Key regulatory points such as caspase activation or inhibition of apoptosis are fundamentally identical in all species, from the nematode worm to humans.

1.1.3 Control of Caspase activation: between death receptors and the BCl2 protein family

Genes closely related to *ced* genes of *C.elegans* have been found even in the mammalian genome. All core components of the apoptotic program are preserved but they are incorporated in the complex regulatory network of evolutionary younger species. More genes participate in the decision between life and death of a cell. In order to better respond to changes in the environment, molecular pathways are specialized for different types of signals. Death signals are often “delivered” through different receptors but can also be generated within the cell due to stress, mistakes in homeostasis etc. Thus, regardless of which upstream events occur during commitment phase they all result in caspase activation.

Caspases are synthesized as inactive zymogens. Catalytically active homodimers assemble after proteolytic removal of their prodomains. Active caspases start a proteolytic cascade and thus a wind range destruction that effectively kills the

cell. They can be classified in two groups, depending when and how processing takes place: initiator or apical caspases and effector, downstream caspases. Apical caspases have usually long prodomains, which contain protein-protein interaction domains while prodomains of downstream caspases are short. Interestingly, peptide sequences that are a cleavage target in the processing of the zymogen (e.g. QACXG) are identical to the target site of the activated enzyme. Therefore, only apical caspases need help of other proteins to remove their prodomains. Once they are active, caspases activate other caspases and the proteolytic cascade amplifies.

The mammalian counterpart of CED4, the protein that promotes caspase activation in *C.elegans*, is Apaf1. It mediates caspase activation as a response to the loss off mitochondrial membrane potential. Released cytochrome C binds to Apaf1 and induces a conformational change, which is followed by oligomerization. Bound pro-caspase 9 is processed and activates effector caspases (Cecconi et al., 1999).

In mammalian cells, the process of caspase activation is mainly controlled by the BCl-2 protein family members (Yang et al., 2000). Some members have anti apoptotic function and others are proapoptotic. The BCl-2 protein after whom the whole family got the name is a very potent protooncogene. Chromosomal translocations with duplication of *bcl-2* as well as changes in the regulatory sequence that cause increased expression are strongly correlated with occurrence of chronic lymphatic leukaemia. BCl-2, when overexpressed, prevents apoptosis of cells in culture that where exposed to a variety of death stimuli (Vaux et al., 1988). The molecular mechanism of action of BCL-2 family members remains unclear but they all influence the apoptosis program upstream of caspase activation.

Caspase activation is often triggered by death receptors as a response to the binding of different classes of ligands. The Tumor Necrosis Factor (TNF) Receptor superfamily comprises a large group of type I receptors that have cystein rich (CRD) regions within their extracellular domains. Members of the TNF subfamily have intracellular death domains (DD), a binding motif for the group of death adaptor proteins. When the receptor is activated by a ligand it changes conformation, adaptor proteins bind to the exposed death domain and via CARD or DED domains recruit apical caspases (Ahmad et al., 1997; Duan and Dixit, 1997). Activation of caspases through the death receptor signal is strong enough that it can not be inhibited by BCl-2 overexpression.

It remains unclear if there is a “threshold level” of caspase activity, when cell death cannot be prevented. What is known is that a certain level of caspase activity can be tolerated and neutralized within the cell. Therefore, nature developed the last barrier against unwanted apoptosis: Inhibitor of Apoptosis proteins (IAPs).

1.1.4 Inhibitors of Apoptosis, cellular caspase inhibitors: “IAPs”

The only so far isolated cellular proteins that are able to inhibit active caspases belong to the IAP family (Inhibitor of Apoptosis Protein). Functional and structural conservation between the species is proven in experiments where apoptosis in mammalian cell culture is prevented by expression of the baculoviral IAP (Manji et al., 1997). Main structural characteristic of all IAPs is the presence of up to three BIR domains (Baculovirus IAP Repeat). Additionally, many IAPs have a RING domain located near the C terminus. BIR was first identified as a viral protein that is able to inhibit protective suicidal response of the host cell (Crook et al., 1993; Birnbaum et al., 1994). It consists of ~70 amino acids and the regular distribution of Cys and His repeats suggests a Zn^{2+} ion binding structure (Hinds et al., 1999).

At least one BIR domain is required for anti apoptotic function of all IAP proteins. IAPs physically bind caspases and crystallization studies confirmed that the tertiary structure of BIR domains “fits” into the active site of caspases (Vucic et al., 1998). Although IAPs require a BIR domain to exhibit anti apoptotic function, not all proteins that contain BIR domain are involved in apoptosis. For example, the yeast protein Brp1 has a BIR domain but it plays a role in chromosome segregation and cytokinesis (Uren et al. 1999).

The human genome contains at least six genes that encode different IAPs. Their importance for cell survival is unclear because of functional redundancy. However, the NAIP encoding gene was first to be identified because it is depleted in patients with spinal muscular atrophy (SMA), a hereditary neurodegenerative disease (Rodrigues et al., 1996). Another human IAP, SURVIVIN is a reliable tumour marker because of its high expression levels in common cancers and its absence in the terminally differentiated tissues (Ambrosini et al., 1995).

It is shown that IAPs inhibit apoptosis by direct binding to caspases. Overexpression of IAPs in cell culture prevents apoptosis triggered by the activated form of caspases. XIAP1, c-IAP1 and c-IAP2 are shown to directly bind pro-caspase 9 and prevent apoptosis induced by release of cytochrome C from mitochondria (Srinivasula et al., 2001). At least in the mammalian system there are indications that IAPs regulate apoptosis by interacting with proteins associated with TNFR (tumour necrosis factor receptor). C-IAP2 is involved in a mechanism through which TNF induces NF-κB and thereby protects cell from apoptosis (Chu et al., 1997)

1.1.5 Apoptosis in *Drosophila melanogaster*

The fruit fly, *Drosophila melanogaster*, is a good model organism for the study of apoptosis because it offers powerful genetic tools for analysis. During *Drosophila* embryogenesis cell death is relatively widely spread and occurs in predictable patterns. It can be easily detected by acridin orange staining or by the TUNEL method that labels double stranded DNA breaks. Soon after germ band extension first apoptotic cells appear in the anterior region of the embryo. As development continues the number of dying cells rises and they can be seen in all tissues. The most prominent cell death activity occurs in the domain of nervous system formation. Although apoptosis in *Drosophila* follows a stereotyped pattern, there are small spatial and temporal differences. This proves that the cell death system has certain plasticity (Abrams et al., 1993) like it is the case in higher organisms.

Hid, *reaper, sickle* and *grim* genes are responsible for induction of almost complete embryonic apoptosis in *Drosophila*. They all reside within the same gene cluster on the left arm of chromosome 3 and a small deletion *Df(3L)H99* that removes the complete genomic fragment prevents cell death during development (White et al., 1994). Therefore, these genes are characterized as activators of cell death which are not involved in execution phase. This is based on the fact that full scale apoptosis can be induced in *Df(3L)H99* embryos by X rays radiation. Although Hid, Reaper and Grim proteins do not share a high homology it was found by Wing et al. (2001) that they have a 14 amino acids long conserved N terminus. Differences between single amino acid residues seem to determine functional aspects of *reaper hid* or *grim* killing (Lisi et al., 2000). A similar peptide sequence to the RHG “motif” is identified in the mammalian proapoptotic protein Smac/DIABLO (Silke et al., 2000) which is one

more example of conservation of the apoptotic pathway between the species. *Reaper*, *hid* and *grim* can induce apoptosis independently of each other but they often act synergistically. Their expression profiles combined, closely match pattern of TUNEL signal in WT embryo. It can be said though, that *rhg* genes are not the only apoptosis inducing factors in the fruit fly.

In the *Drosophila* genome seven genes encode functional capases. Three contain long prodomains and are therefore upstream caspases: *dcp-2/dredd* (Chen et al., 1998), *dronc* (Dorstyn et al., 1999) and *dream*. The remaining four have short prodomains and are considered to be downstream executioner caspases: *dcp-1* (Song et al., 1997), *drICE* (Fraser and Evan, 1997), *decay* (Dorstyn et al., 1999) and *daydream*. The mechanism of caspase activation in *Drosophila* is not well understood. The gene d*ark* is identified as a homologue of *ced-4/Apaf1.* It has been shown that DARK binds the apical caspases DREDD and DRONC and stimulates their activation but flies homozygously mutant for d*ark* are viable, although they have several physical abnormalities (Rodrigez et al., 1999). Caspase activation through TNF and Fas receptors cannot be excluded.

The DIAP1 protein, member of the IAP family, is the main inhibitor of caspases in *Drosophila*. It counteracts the RHG proteins but it also protects the cells form other so far unidentified caspase activators. This assumption is based on the strong apoptotic phenotype of DIAP1 loss off function/Df(3L)H99 double mutant situation. Although RHG are not expressed under these circumstances, lack of DIAP1 causes massive cell death (Wang et al., 1999). The *Drosophila* genome contains two IAP encoding genes resulting in the expression of DIAP1 and DIAP2, but only DIAP1 is proven to be required for cell survival. In higher organisms there are several IAPs with partially overlapping functions. This role of DIAP1 as a “single” inhibitor of apoptosis makes the fruit fly unique as a model system for studying the role of IAPs in keeping caspases in check.

1.1.5.1 Drosophila inhibitor of apoptosis, DIAP1

The DIAP1 protein, *Drosophila's* major inhibitor of apoptosis, encoded by the gene *thread* is of uttermost importance for the survival of all cells in the embryo. It was discovered as a negative regulator of apoptosis (Hay et al., 1995). Embryos homozygous for a *thread* null allele die at the beginning of germ band extension with practically all cells exhibiting the apoptotic phenotype. Heterozygous *thread* background strongly enhances killing triggered by Reaper and Grim (Hay et al., 1995, Lisi et al., 2000). The DIAP1 protein (438 amino acids) has two BIR domains and a C terminal RING domain. It is ubiquitously expressed and its turnover rate is very high. DIAP1 is able to bind caspases in both their active and inactive forms. Expression of proapoptotic genes in specific groups of cells during development disturbs the DIAP1/caspase interaction. The opposite is also true: DIAP1 when overexpressed prevents activation of caspases induced by RHG proteins (Goyal et al., 2000).

Wang et al. (1999) have described a mechanistic model for the control of apoptosis in Drosophila: the "double inhibition system": DIAP1 inhibits caspases and proapoptotic proteins inhibit DIAP1 function. On the structural level, current research data suggests that the interactions between caspases and DIAP1 and RHG-DIAP1 are competing with each other. Crystallization studies indicated that the thermodynamically most stable configuration of the RHG N terminus peptide fits into the caspase binding site of the BIR2 domain (Wu et al., 2001). It remains to be elucidated whether DIAP1 preferentially blocks activated caspases or in the first place inhibits the activation process itself.

Two possible models are proposed: a) Proapoptotic proteins bind DIAP1 and they work on the principle of anchor, so caspases are free to initiate the downstream cascade b) there is direct competition and proapoptotic proteins destabilize the DIAP1-caspase interaction. Several facts, however, indicate that these models do not give a complete explanation of regulation of apoptosis. Even if the peptide motif at the N-terminus is mutated Reaper and Grim still cause cell death although they do not bind DIAP1 (McCarthy et al., 1998). This argues that physical contact with the inhibitor might not always be necessary in order to promote caspase release.

1.1.5.2 Regulation of DIAP1 protein levels

Novel data published by several groups in the past two years, shifted the focus of interest in *Drosophila* programmed cell death from structural studies about caspase activation to the problem of stability of DIAP1. Yang et al. (2000) showed that DIAP1 is very unstable and undergoes autoubiqitination in vitro due to the presence of its E3 ubiquitin ligase domain. In the work of Yoo et al. (2002) it was shown that proapoptotic proteins Hid, Reaper and Grim induce apoptosis by reducing protein levels of DIAP1. At least part of Hid`s proapoptotic activity is exerted by increasing the autoubiquitination rate of DIAP1. Expression of Hid and Reaper leads to a decrease in protein concentration of DIAP1. In the case of Hid this effect is dependent on the activity of E3 ubiquitin ligase domain of the caspase inhibitor. Substantial evidence argues that Hid promotes cell death through two distinct mechanisms: it interacts via its N terminal domain with DIAP1 disrupting caspase inhibition, and in parallel promotes autoubiquitination and degradation of the inhibitor. Reaper and Grim, as authors suggested, also negatively influence DIAP1 protein levels but they work as general suppressors of translation. This could be sufficient to cause a decrease in concentration of the caspase inhibitor. Contrary to these results, Ryoo et al. (2002) demonstrated that Reaper can also promote DIAP1 ubiquitination but it requires help of the UbcD1 ubiquitin conjugating enzyme.

Despite of some discrepancies in the available data it clearly demonstrates that the protein level of DIAP1 plays an important role in regulation of apoptosis. Nevertheless, the ubiquitin ligase activity of DIAP1 can also have apoptosis inhibitory function as caspases are targeted for degradation by the proteasome as shown by Meier et al. (2000). The signalling events that are shifting the balance between DIAP1 auto degradation and caspase degradation remain to be defined.

1.2 Non apoptotic functions of DIAP1

The DIAP1 protein is required for the survival of nearly all cells in the *Drosophila* embryo but its function is not solely restricted to the inhibition of caspases. So far documented apoptosis unrelated roles of DIAP1 include spermatid individualization, induction of compensatory proliferation and regulation of cell migration during oogenesis.

1.2.1 DIAP1`s role in spermatid individualization and compensatory cell proliferation

The final step of spermatogenesis in *Drosophila* is the process of spermatid individualization. Haploid and mature spermatids that are part of the germline syncytium are separated and transformed into motile sperm. Not much is known about the molecular basis of these processes but there is evidence that limited caspase activity is necessary. Overexpression of DIAP1 in the germline tissue causes male sterility. This is one described example that genes involved in apoptosis might play a role in processes not related to death.

When apoptosis occurs as a part of the defensive mechanism after inflicted injury of the tissue, it is usually succeeded by proliferation of surrounding tissue. The genetically controlled total cell number of a tissue is rapidly recovered through compensatory cell divisions. Little is known about how tissue homeostasis is exactly regulated but latest evidence suggests that mitotic signals are sent directly from dying cells to the neighbouring tissue. Loss of DIAP1 protein in cells of the wing imaginal disc triggers the Jun kinase signalling pathway in parallel to caspase activation (Ryoo et al., 2004). The genes *wingless* and *decapentaplegic* (*dpp*) are transcribed in response to DIAP1 reduction. Both are secreted into the intercellular space as potent mitogens. The role of executioner caspases in this process is excluded, as coexpression of the viral inhibitor protein p35 does not reduce overgrowth of the surrounding tissue.

1.2.2 Epithelial cell migration

Oogenesis in *Drosophila melanogaster* is a complex developmental process that occurs within distinct structural units, the egg chambers. The germline is made of 15 nurse cells and a single layer of epithelial cells of somatic origin, the follicle cells, surrounds the growing oocyte. Nurse cells, like their name suggest, nourish the oocyte and stimulate its growth with loads of produced yolk, RNA and proteins. During egg maturation the follicle epithelium undergoes several morphological changes and different migration processes. After initial proliferation of the epithelial cells is finished they migrate to the posterior end of the egg chamber and form a columnar layer covering the oocyte. The remaining 30-40 cells flatten and stay on top of the nurse cells as a squamous epihelium. The first cell specification within the follicle epithelium occurs during early stages when two polar cells differentiate at the anterior and posterior ends of the chamber. During stage 9 the anterior polar cells recruit 6-8 epithelial cells in a process dependent on the JAK/STAT signalling pathway (Beccari et al., 2002). They form a rosette like epithelial patch, called border cells, which start migrating between the nurse cells towards the oocyte. Later, in the mature egg, border cells form the micropyle which functions as a sperm entry channel. At the time border cells arrive at the oocyte, follicle cells that are next to the nurse cells undergo cell shape changes (see Fig.1). This subpopulation of migrating follicle cells is called the centripetal cells. They are longer than their neighbouring epithelial cells and form protrusions to migrate inward until they completely cover the anterior of the oocyte. Together, border cells and centripetal cells are good examples of the inherent ability of epithelial cells to transform into invasive cells.

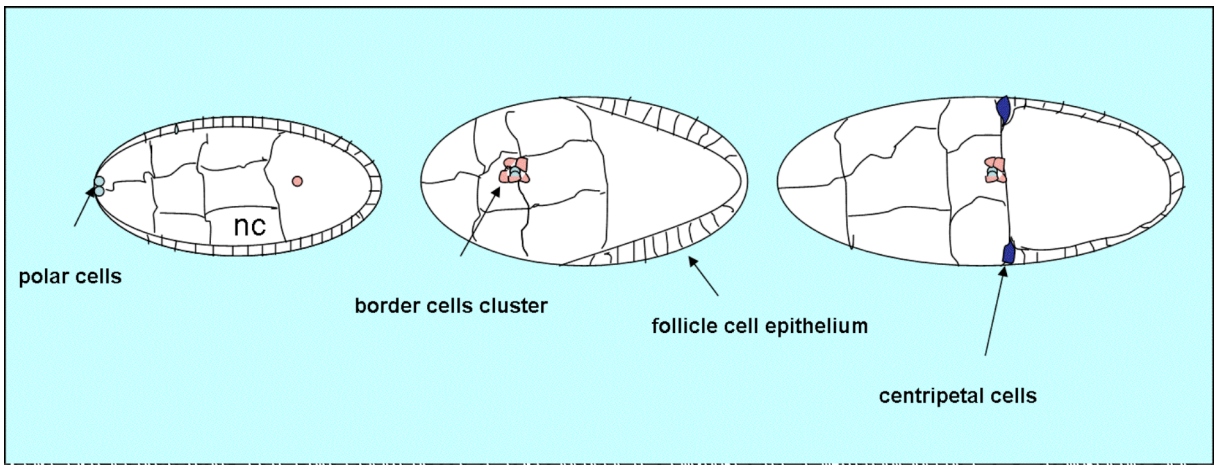


Figure 1: Model representing migratory processes during mid oogenesis
During midoogenesis the follicle cell epithelium undergoes several migratory processes. Initially, the epithelial sheat retracts to the posterior of the egg chamber; soon afterwards, in stage 9, border cells form and start migrating towards the ooycyte; Finally, centripetal cell migration occurs at stage 10, until anterior of the oocyte is completely covered.

Understanding of the underlying genetic mechanism which induces and controls this transition of epithelial into migratory cells might provide important clues about malignant tissue transformation. So far, it is known that adhesion plays a key role in facilitating movement. *D*E-Cadherin, a homophilic transmembrane adhesion protein is a component of the *zonula adherens* (ZA), a cell junction that maintains epithelial integrity. *D*E-Cadherin is encoded by the gene *shotgun* (*shg*). *Shg* is upregulated in migratory active regions of the follicle epithelium. Movement of border cells and centripetal cells depends on *D*E-Cadherin, as shown by Niewiadomska et al. (1999). Thus, the homophilic interaction between *D*E-Cadherin molecules might determine speed and direction of cell migration.

Several signalling pathways are identified as active during border cell migration. Recruitment and delamination steps are controlled independently of migration itself. The small GTPase Rac1 is established as the key player required for the continuing movement of the border cell cluster. Like other small GTPases, it cycles between a GTP and a GDP bound form. Experimental evidence suggests that Rac1 downstream effectors control actin cytoskeleton rearrangements (Aspenstrom et al., 1999). Overexpression of a dominant-negative form of Rac1 in the ovary causes inhibition of border cell migration (Murphy and Montell, 1996). In a genetic

suppressor screen for identification of proteins that rescue a Rac1 dependent phenotype, Geisbrecht and Montell (2004) identified DIAP1.
Interestingly DIAP1 is not required for cell survival during oogenesis, but border cells lacking DIAP1 fail to migrate. A model suggests a novel function of DIAP1 for cell migration, indicating that it might be involved in modulation of actin networks.

1.2.3 The JNK pathway as a stress response and cytoskeletal modulator

The Jun N terminal Kinase (JNK) pathway is an evolutionary conserved cascade of kinases that transcriptionally activate AP1 responsive genes (Li et al., 1996). The AP1 transcription factor consists of c-Jun and c-fos. C-Jun is phosphorylated by the Jun N terminal kinase, a member of the subgroup of Mitogen Activated Protein kinases (MAP-Kinase). Initially the JNK pathway was discovered as a part of the stress response in cells and part of a regulatory mechanism during cell proliferation. In mammalian cell culture, activation of Jun kinase triggers apoptosis (Xia et al., 1995). Contradictory results of in vivo experiments limited the importance of this finding by demonstrating that JNK signal transduction might result in proliferation as well. Therefore, it remains to be shown which control mechanisms regulate the decision between cell survival and cell death.

Recent data brought even more complexity into the field of cell biology showing an important role of JNK signalling in morphogenetic movements of epithelial sheets. In *Drosophila melanogaster* the JNK pathway controls the formation of actin stress fibres and lamellipodia (Royal et al., 2000; Wennerberg et al., 2002). During dorsal closure the two lateral sides of dorsal epithelia move towards the midline of the embryo until they completely cover the underlying amnioserosa. This complex morphogenetic movement depends on correct levels of JNK signalling. Mutation in one of the genes coding for the JNK pathway kinases results in defective dorsal closure, which can be seen as big dorsal holes in the embryonic cuticle. The *basket* gene encodes *Drosophila´s* Jun N terminal Kinase (JNK).

Several genes are activated in response to the JNK pathway, including numerous genes that regulate the cytoskeleton and cell adhesion. It is also

recognized that JNK might modulate the actin cytoskeleton in a transcription independent fashion via direct phosphorylation of paxilin (Otto et al., 2000).

Several proteins can activate the JNK pathway. It is documented that these activators determine specific functions of the pathway depending on the particular situation. For example, Frizzled mediated JNK signalling controls planar polarity in the eye (Weber et al., 2000) and differentiation of wing imaginal discs (Agnes et al., 1999). DTRAF mediated activation triggers apoptosis in the eye (Kaupilla et al., 2003) and Dreadlocks (DOCK) is involved in axon targeting processes (Hing et al., 1999). DTRAF activation pathway is particularly interesting because it transduces the signal from TNF receptors (see Figure 2).

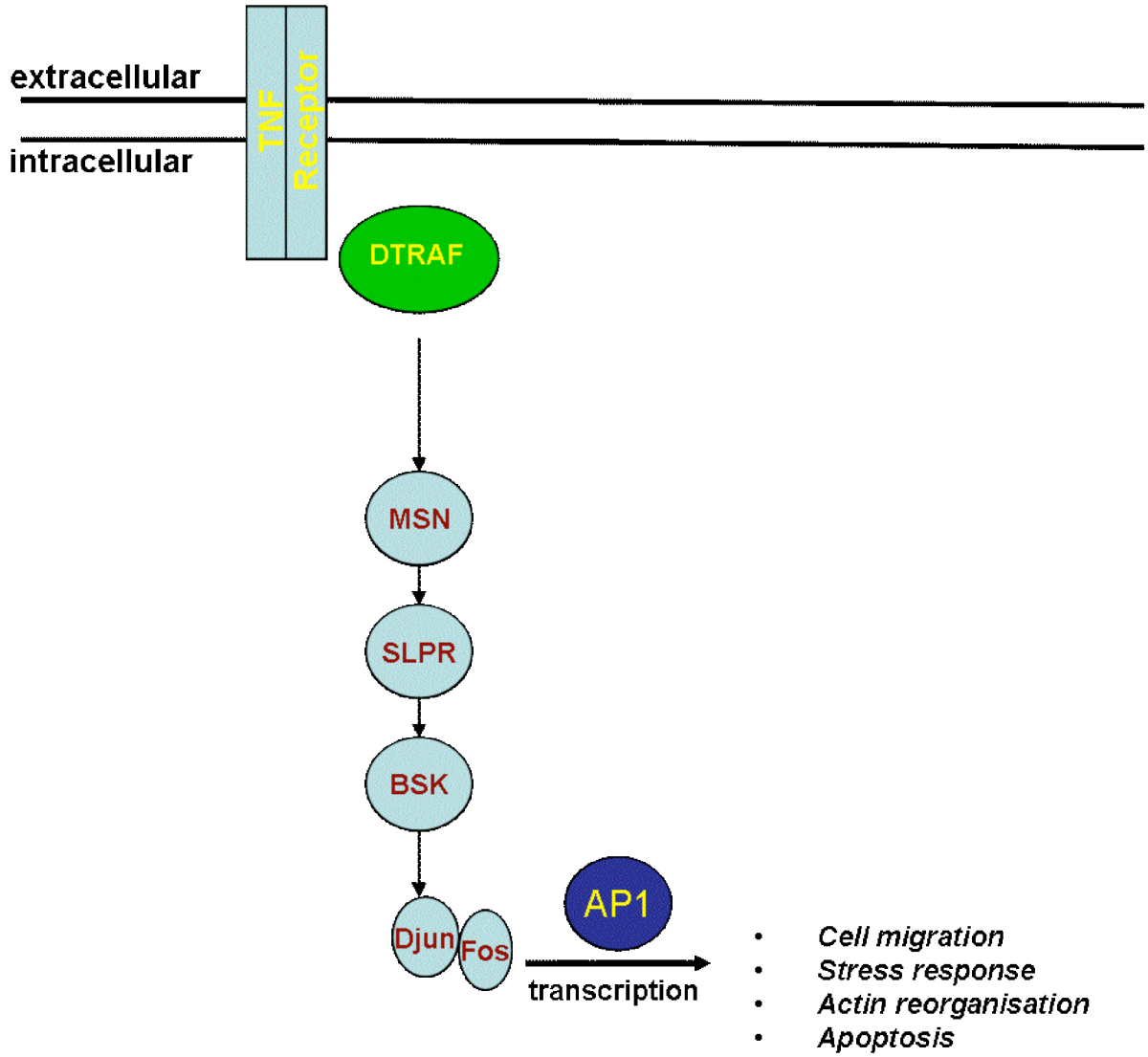


Figure 2: Model of the JNK signalling cascade
The Tumor Necrosis Factor (TNF) receptor becomes activated after TNF binds to its extracellular domain. The intracellular part of the receptor activates the JNK signalling cascade via the TNF receptor associated factor TRAF. The signal is transduced by an intracellular kinase cascade. In the model, the *Drosophila* homologues of the cascade proteins are named.

The main regulation of the JNK pathway signal is accomplished through a negative feedback loop. One of the first genes to be transcribed after c-Jun phosphorylation is *puckered*. It encodes a VH1-like phosphatase that can dephosphorylate Basket and by that turn it into an inactive state. *puckered* expression can thus be used as a reporter for JNK activation in vivo as well as in vitro. One of the most prominent characteristics of JNK signalling is, that it can not be observed as a variation of the two modes "on" and "off". The level of the signal determines the type of the resulting cellular response.

1.3 Aim of the Work

The Inhibitor of apoptosis DIAP1 protects all cells in the *Drosophila* embryo from cell death. It inhibits caspases and prevents their activation thereby blocking the start of a destructive proteolytic cascade. Although main players that control cell death during development of the fruit fly are already known, some important questions about regulation of apoptosis remain open. In this respect especially interesting is DIAP1`s function as a E3 ubiquitin ligase. It was shown that DIAP1 can autoubiquitinate and then undergoes degradation in the proteasome. Conversely DIAP1 can target caspases for degradation. It remains yet to be defined which cellular mechanisms regulate balance between the self destructing activity of the protein and its role in apoptosis inhibition. DIAP1 might also target proteins from other cellular pathways for degradation. The sub cellular localization of DIAP1 indicates that the protein has distinct functions in different tissues during development. DIAP1 is, until now, implicated in control of spermatid individualization, compensatory proliferation and most recently in regulation of cytoskeleton rearrangements.

In order to gain better insight into all functional aspects of DIAP1`s role during development, it is necessary to identify novel interaction partners. Aim of this work is identification and characterization of novel interaction partners of DIAP1 with the yeast two hybrid system. After putative novel binding partners are isolated, most interesting candidates will be selected for further studies. Experiments will include in vitro and in vivo binding assays to confirm the interaction. Finally, the aim is to find out the functional importance of the identified interactions. The best way to approach this problem is to analyse the phenotypes of available mutants. Additionally, the genetic interaction of DIAP1 and mutant candidate genes can be very informative. The tissue specific aspect of identified interactions will be addressed by overexpression of novel DIAP1 binding partners in a specific cellular environment.

2. Material and Methods

2.1 Material

2.1.1 Chemicals

All chemicals were, if not otherwise specified, manufactured according to *pro analysis* quality by the following companies: *Acros,*Geel, Belgium;*Baker*, Deventer, Netherland; *Biomol,* Hamburg; *Bio-Rad*, München; *Difco*, Detroit, USA; *Fluka*, Buchs, Switzeland; *Gibco/BRL* Life Technologies, Karlsruhe ; *Merck*, Darmstadt; Roth, Karlsruhe; *Serva*, Heidelberg; *Sigma-Aldrich*, Steinheim

All solutions were made with bi-distilled H_2O and autoclaved prior to use.

Enzymes required for molecular work were purchased from companies: *Boeringher/Roche Diagnostics* Mannheim; *MBI Fermentas*, St. Leon- Rot, *Promega*, Madison, USA.

2.1.2 Kit Systems

-Qiagen plasmid Midi Kit, *Qiagen*, Hilden

-Qiagen Plasmid Maxi Kit, *Qiagen*, Hilden

-In vitro Transcription Translation System, *Promega*, Madison, USA.

- Nucleobond gel extraction, PCR Purification, *Machery Nagel*, Düren

-Cell culture transfection, *Invitrogen*

-Ribomax large scale RNA production system, *Promega*/Madison USA.

2.1.3 General laboratory equipment

-electroporation: Gene Pulser II and Puls Controller Plus (*Bio Rad* Munich)

-UV spectral photometer: Gene Quant II (*Pharmacia Biotech*, Cambridge, UK)

-Sonificator: Labsonic U (*Braun Biotech*, Melsungen)

-SDS PAGE & Western Blotting : Miniprotean 3 (*Bio Rad*, Munich)

-Centrifuge: Heraeus biofuge fresco/pico (*Kendo lab products*, Langenselbold)

-PCR machine : MiniCycler (*MS Research*, Watertown)

-Micropipets puller: Sutter P-97 (*Science products*, Hofheim)

-Sonificator : Labsonic U (*Braun Biotech*, Melsungen)

2.1.4 Detection systems

X ray film development: film-Fuji Super RX, *Fuji*, Tokyo, Japan, developer- Tenetal Roentogen, *Tenetal*, Norderstedt, fixation- Tenetal Roentogen Superfix, *Tenetal*, Norderstadt

Fluorescence: confocal microscope Leica TCS NT, *Leica*, Heidelberg,

Light microscopy: Zeiss Axiophot2 , Zeiss, Oberkochen

Leica MZFLIII *Leica*, Heidelberg

2.1.5 Biological material

Yeast strains: AH109, Y190 *Clontech* , Palo Alto, USA.

Bacterial strains: Electrocompetent XL-1 Blue MRF

2.1.5.1 Plasmids

pBluescript KS+/SK+ -vector for in vitro transcription, sequencing, subcloning. (*Stratagene*, Heidelberg); ampicilin resistant.

pGBKT7-Yeast expression plasmid for expression of fusion proteins with DNA binding domain of Gal4 (*Clontech*, Heidelberg) kanamycin resistant.

pACT2-Yeast expression plasmid for production of fusion proteins with activating Gal4 domain (*Clontech* Heidelberg); ampicilin resistant.

pGEX4T1- For production of GST fusion proteins in *E.coli*, *Amersham Pharmacia Biotech*, Buckinghamshire, UK ampicillin resistance

pUAST- for expression of proteins in eukaryotic cells or generating transgenic lies; ampicillin resistance

2.1.5.2 Oligonucleotides: Primers for PCR

PGBKT7/DIAP1	5'CCGGAATTCATGGCATCTGTTGTAGCTG
	3'TGGGATCCTAAGAAAAATATACGCGC
PGBKT7/ΔRING	5'CCGGAATTCATGGCATCTGTTGTAGCTG
	3'CGCGGATCCGCACAACTTTTCCTCGGGT
PGBKT7/BIR1L1	5' CCGGAATTCATGGCATCTGTTGTAGCTG
	3'CGCGGATCCGGCGTATTCGGGATACTGGG
PGBKT7/L1BIR2L2	5'CCGGAATTCCGGCGCACTACCAACAATGTG
	3'CGCGGATCCGCACAACTTTTCCTCGGGT
DIAP1RNAi	5'TTAATACGACTCACTATAGGGAGAATGGCATCTGTTGTAGCTG
	3'TTAATACGACTCACTATAGGGAGAGGCGTATTCGGGATACTGGG
PUAST/DIAP1	5'ATAAGAATGCGGCCGCGGAGGAGCAGAAGCTGATCTCA
	3`CCGCTCGAGGGCGTATTCGGGATACTGGG
PUAST/ΔRING	5'ATAAGAATGCGGCCGCGGAGGAGCAGAAGCTGATCTCA
	3'CCGCTCGAGGCACAACTTTTCCTCGGGTATG
PUAST/LBIR1L	5'ATAAGAATGCGGCCGCGGAGGAGCAGAAGCTGATCTCA
	3`CCGCTCGAGTTAAGAAAAATATACGCGC

Tab.M1: Primers designed for cloning DIAP1 fragments into different expression vectors

2.1.5.3 Fly Stocks

Stocks	Remarks	Reference
1. Chromosome		
FM7	Balancer-Chr.	Lindsley und Zimm, 1992
FM7blue^{ftz}	Balancer-Chr. with P(*ftz-lacZ*)-Insertion	Y.Hiromi
2. Chromosome		
CyO	Balancer-Chr.	Lindsley und Zimm, 1992
3. Chromosome		
TM3	Balancer-Chr.	Lindsley und Zimm, 1992
TM6B	Balancer-Chr.	Lindsley und Zimm, 1992

Tab.M2: Balancer chromosomes used in this work

stocks	Description
th^{109}	null allele of DIAP1/*thread*
ral^{89}	hypomorphic *ral* allele
ral^{70}	null allele of *ral*
vha^{55}	point mutation of ß-subunit of the vacuolar H^+-ATPase / vha55
bsk^{1}	hypomorphic allele *basket*/JNK
hep^{1}	hypomorph allele of *hemipterous*/JNK Kinase

Tab.M3: Fly stocks carrying mutations that were used for this work

2.1.5.4 UAS transgenes and Gal 4 effector lines

Stocks	Description
UAS::Diap 1	full length DIAP1 transgene
UAS::Ral^{G20V}	Constitutive active Ral
UAS::Ral^{S25N}	Dominant negative Ral
EP(364)Dtraf	Gal 4 responsive dtraf gene
en::Gal4	*EngrailedGal4*
Gmr:: GAL4	Eye specific Gal4 driver line
Slbo::Gal4	*slow border cells Gal4* drives expression in border and centripetal cells
mat67::Gal4	maternal Gal4 driver line
puc::LacZ	LacZ reporter element insertion in *puckered* gene

Tab.M4: UAS transgenes and Gal4 effector lines used in overexpression experiments

2.1.5.5 Antibodies

Epitope	Organism	Dilution	Raised by
DIAP1	mouse	1:200	B.Hey
*D*E-Cadherin	rat	1:20	H. Oda
GFP	rabbit	1:100	BD Bioscience
Armadillo	rabbit	1:200	T.Kessler
Ral	rat	1:200	T.Kessler
Activated dRICE	rabbit	1:1000	Soon Ji Yoo
Myc	mouse	1:250	*SantaCruz Biotechnologies*

Tab.M5: Overview of primary antibodies

Secondary antibody	...Dilution	Raised by
Donkey-α-Mouse Cy2, Cy3,Cy5	1:200	Dianova
Donkey-α-Rat Cy2, Cy3,Cy5	1:200	Dianova
Donkey-α-Rabbit Cy2, Cy3,Cy5	1:200	Dianova
Goat-α-mouse HRP	1:10000	Dianova
Goat-α-Rat HRP	1:10000	Dianova

Tab.M6: Overview of secondary antibodies

2.2 Methods

2.2.1 Molecular methods

Standard protocols taken from Maniatis, were used for basic molecular manipulations of genetic material. Therefore, details will not be described for use of restriction enzymes, 5` dephosphorylation of DNA, ligation, agarose gel electrophoresis and phenol chlorophorm precipitation of DNA.

2.2.2 Transformation of electrocompetent cells

- Electroporation of electrocompetent cells was performed in Gene Pulser, Biorad. Pulses magnitude V=1,8kV
- Concentration of plasmid adjust to up to 100ng/μl by dilution in dH_2O
- thaw 50μl of Xl-1 electrocompetent cells on ice and add 1μl of DNA
- Transfer mixture into the electroporation cuvette (*Biorad*) and proceed with pulse transformation
- resuspend bacteria in LB growth medium and incubate 60 min at 37°C
- Plate bacteria on selective surface (for mini preps) or in selective medium (for midi prep) and let them grow overnight

Solutions:

LB medium: 1% Bactotrypton, 0,5 % Bactoyeast, 1% NaCl

LB Agar plate: 6,3gr Agar/300ml LB

For selection, always use antibiotic determined by the vector resistance e.g. ampicillin, kanamycin, chloramphenicol etc.

2.2.3 Isolation of DNA

Small scale DNA isolation (“Mini Prep”)

To screen a plate for a presence of a colony that contains a wanted plasmid a mini prep is performed, a small scale isolation of the DNA. Single colonies are propagated O.N at 37 in 3ml LB medium with selective antibiotic.

Plasmid extraction:

Spin down overnight culture for 1min at 13000 rpm

- Discard supernatant and resuspend pellet in 300μl S1
- Add 300 μl S2 lysis buffer, gently invert reaction tube several times and leave on ice for 5min
- Add 300 μl S3 neutralization buffer, invert several times and incubate 5 min on ice
- Centrifuge 12min at 4°C at 13000 rpm
- Take supernatant in the new reaction tube and add 700 μl isopropanol
- Centrifuge 30 min at 4 °C,13000 rpm
- Wash pellet 5min with ice cold 70 % ethanol and centrifuge for 5min at 13000 rpm
- Dry the pellet on air and resuspend in 30 μl dH2O

S1: 50mM Tris-HCl pH 8.0; 10mM EDTA; 100mg/ml RNaseA

S2: 200mM NaOH; 1% SDS

S3: 3.0M Kaliumacetat pH 5.5

For quantitative isolation of highly concentrated DNA (μg/μl) transformed cells were grown in the 30ml LB medium with antibiotic O.N. In medium scale DNA isolation, so called "midi prep" procedure, DNA was extracted by *Qiagen midi kit*.

2.2.4 Polymerase Chain Reaction PCR

Different DNA fragments, needed in the process of cloning were amplified by standardized method of PCR (Mullis and Faloona, 1987). Method is established on the basis of successive cycles of DNA replication. Both ends of amplified fragment are defined by two oligonucleotide, primers, that are complementary to 5`and 3`-terminal sequences of the wanted product. PCR reaction is performed in a thermal cycler. Necessary ingredient in the PCR mixture is a thermo stable polymerase, a DNA replicating enzyme that is stable at temperatures up to 95°. In successive steps of denaturation, annealing and elongation two chains of DNA are separated, designed nucleotides bind to the template and the polymerase replicates the sequence downstream of the primers. Thereby the amount of amplified product with each new cycle exponentially grows. Protocol was optimized each time based on the specificities of particular case (annealing temperature, time of elongation etc.).

PCR needs mixture of all necessary components to be prepared before reaction starts. Total volume per single amplification reaction was 50µl.

1 µl template DNA (10ng)
0,5µl primer 5` (25mMconc)
0,5µl primer 3' (25mMconc)
6µl $MgCl_2$
5µl 10xreact. Buffer
2µl dNTP mixture (25mM per nucleotide)
1µl Taq polymerase
34µl dH_2O

Standard parameters that were programmed to the thermal cycler are summarized in the table7:

	Duration	Temp	Functional meaning of the step
	5min	95°	Starting denaturation of the DNA
	30sec	95°	Denaturation step within the cycles
35x	30sec	55°*	Annealing of the primers to the template
	60sec	72°	Elongation-Polymerase synthesises novel DNA chain
	7min	72°	Final elongation
	∝	4°	End of the reaction

.

Tab.M7: Standart PCR programm
*Annealing temperature needs to be optimal for the set of the primers

2.2.5 Cloning of PCR fragments into plasmids

In this work several plasmid constructs were produced by inserting PCR products in vectors of choice (see table M8). Cloning procedure is a three step process and includes: Separate digestion of PCR fragment and vector with suitable restriction enzymes, ligation reaction during which fragment is incorporated into plasmid with the help of T4-DNA ligase and finally selection of the right clone by transformation in *E.coli*.

Successful ligation depends mostly on the concentration ratio between insert and vector. Excess of insert in general raises the probability that complementary ends will meet. Kinetics of reaction is influenced by the size of the molecules involved. Therefore following formula can be used to determine exact amount of insert and vector that should be in a ligation mixture:

ng (vector)x size of insert (kb)/ size of vector (kb)X 3= 1:1 insert /vector

Ligation reactions were performed at 16° overnight. In principle, volume of the reaction never exceeded 25µl.

2.2.6 Generated constructs

Vector	Insert (cDNA)	5' res	3'res
PGBKT7	DIAP1	EcoRI	BamH1
PGBKT7	ΔRING	EcoRI	BamH1
PGBKT7	BIR+L1	EcoRI	BamH1
PGBKT/	L1+BIR2+L2	EcoRI	BamH1
PACT2	GRIM	BamH1	Xho1
PACT2	REAPER	BamH1	Xho1
PGEX4T1	DIAP1	EcoRI	Sal1
PGEX4T1	ΔRING	EcoRI	Sal1
PGEX4T1	BIR+L1	EcoRI	Sal1
PUAST	DIAP1	NotI	Xho1
PUAST	BIR+L1	NotI	Xho1
PUAST	RAL	NotI	Xho1
pBluescript	Vha55	EcoR1	Xho1

Tab.M8: Constructs obtained by cloning ; Domain organization of DIAP1 protein is given in the supplementary data.

2.2.7 Amplification of the cDNA library

Drosophila cDNA library of 0-24h old embryos was amplified by standard methods and the vector pACT2 containing the cDNA was stored as a glycerol stock at -70°. Amplification of the library first required determination of the titre of the stock. Three dilutions 1:100 1:1000 1:10 000 of the stock in LB medium were plated on agar plates O.N at 37°C. Concentration that gave confluent growth of single colonies (1:1000) was used for full scale amplification:

- Bacterial stock diluted in LB 1:1000
- 300µl of the suspension was plated on the each of the 30 big agar-ampicillin plates
- incubation 48 hours at 37°
- Scrap the cells in a 1:5 glycerol/LB suspension
- Isolation of the plasmid DNA with Maxi prep , Qiagen

2.2.8 Transformation of plasmid DNA into *Saccharomyces cerevisiae*

Introduction of the yeast expression vectors pACT2 and pGBKT7 into the AH109 strain was performed by the Lithium Acetate (LiAC) precipitation.

- Inoculate an overnight culture of YPDA with a colony of AH109 strain
- Dilute the culture up to 0,2 OD and let it grow exponentially until it reaches 0,6-0,9 OD
- Spin 30 ml of the culture 5 min at 5000 rpm
- Wash the pellet with 20 ml dH_2O and spin for 5 min at 5000rpm
- Wash the pellet with 20 ml 0,1M LiAc and spin down 5min 5000rpm
- Resuspend the pellet in 1ml 0,1M LiAc
- Incubate 15min at 30 °
- Make a transformation mixture: 150 µl transformation competent yeast + 2µg HSP herring sperm protein + 2µg plasmid DNA + 750µl PEG7LiAc/TE solution
- Briefly vortex and incubate under shaking 30min at 30°
- Heatshock 20min in the water bath at 42°
  cool down samples on ice
- Spin down for 1 min at 7000 rpm
- Resuspend the pellet in 1ml YPDA and incubate 1h at 30°
- Centrifuge for 30 sec at 7000 rpm and discard supernatant
- Resuspend the pellet in 100µl dH2O
- Plate out the on the selective SD agar plate and grow 5-7 days

Solutions:

YPDA medium: 20 g/l Difco Peptone, 10g/l Yeast Extract, 300mg/l adenine sulfat
SD medium: 6,7 g Difco Yeast Notrogen Base without ammino acids dilute in 850 ml dH^2O
After autoclaving add 100ml DO medium and 50 ml 40 % glucose + according to selection needed add 10 ml 100x Leucin, 10 ml 100x Histidin and/or 10 ml 100x Tryptophan
SD agar: 15g agar/ 1L SD medium
10x Dropout solution: 300 mg/l L- Alanine, 1500 mg/l L-Valine, 200 mg/l L- Arginin HCl, 300 mg/l L- Lysin, 200 mg/l L- Methionin, 500 mg/l L-Phenyilalanin, 2000 mg/l L- Threonin, 300 mg/l L- Tyrosin, 200 mg/l L- Uracil

All solutions were autoclaved prior to use

2.2.9 Preparation of genomic DNA from *Saccharomyces cerevisiae*

Before isolation of the yeast DNA it is usually necessary to amplify the colony O.N at 30 ° in 1, 5 ml of appropriate medium.

- Spin the culture 5min at 13000 rpm
- Discard supernatant and add 200µl Yeast lysis solution
- Add 200 µl phenol/chlorophorm (1:1) mixture and 300 µl glass beads (425-600µm; Sigma #G-8772)
- Strongly vortex for 2min
- Centrifuge 5 min at 13 000 rpm
- transfer supernatant to a new reaction tube and add 0,3 vol. NaCl and 2,5 vol. Ethanol
- Leave to precipitate for min 2h at -20 °
- Centrifuge 30 min at 4 °
- Wash the pellet in ice cold 70 % ethanol
- Leave to dry and resuspend the pellet in 30 µl dH2O

2.2.10 β-galactosidase test

When “bait” and “prey” protein interact, a functional Gal4 transcription factor is formed and can drive expression of genes under control of the upstream activating sequence (UAS). The genome of AH109 contains four genes which are expressed under control of UAS sequence. Therefore test for β-galactosidase activity is additional way to confirm existence of the binding of “bait” and “prey”. The colony size should be 1-2mm in diameter before transferring them to a filter.

-Prepare Z-buffer/X-gal solution (2ml Z buffer, 5,4 µl Mercaptoethanol, 33,4 µl x-gal)

-Pre-soak sterile Whatman paper with Z-buffer/ X-gal solution (5 ml for 150 ml plates) and place it into a Petri dish

-Place nitrocellulose membrane over the agar plate and leave it for few moments until it completely attaches to the surface

-Lift the membrane, and transfer it to the liquid nitrogen for 30 sec

-Place the membrane, with colonies face up in the Petri dish with Whatman paper and reaction buffer

-Incubate min 6h at 30 °

-Colonies containing “bait” and “pray” proteins that interact will turn blue.

2.3 Propagation and transformation of Schneider S2 cells

The *Drosophila* cell culture line S2, Schneider cells, is propagated in complete medium containing fetal bovine serum as well as penicillin and streptomycin. Basic nutrient solution is provided by the company Invitrogen. In order to transform cells, the culture has to be in the exponential phase of growth ($1x10^6$ cells/ml). Plasmid DNA is administrated to the cells by $CaCl_2$ precipitation method. All solutions were a part of kit in vitro expression system from *Invitrogen.*

2.3.1 Antibody staining of Schneider cells

For convenient fixation and staining of S2 cells, they should be immobilized in six well chambers (Inivtrogen), prepared with poly-Lysin solution 1:10 with dH_2O as a substrate.

-In each well, pipette 300 µl of poly-lysine solution and leave for 30 min in the sterile hub.

-Remove the liquid and leave chambers to dry.

-Add 50µl of S2 cells diluted in 200µl complete medium

-Incubate O.N. at 25 °

-Remove the liquid and add 300µl fixation solution (4% Formaldehyd in PBS)

-Incubate 15min at RT

-Wash the cells 3x10min in PBS (Pipette carefully down the walls of the chambers to avoid detachment of the cells)

-Block 30 min in 5%NHS in PBT

-Add primary antibody in 5% NHS in PBT

-Incubate 1h at RT

-Wash 3x15 min in PBT

-Add secondary (Cy2, Cy3, C y5) antibody + 5 %NHS in PBT

-Incubate 1h at RT (cover with aluminium folia to prevent bleaching)

-Wash 3x15 min

-Remove plastic chamber walls from the slides

-Imbed in Moviol+ DABCO

Solutions:

PBS 1x : 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 1l H_2O; pH adjust to 7,4 and autoclave

PBT 1x : 1xPBS mit 0.1% Triton X-100

2.4 Immunocytochemistry

2.4.1 Cuticle preparation

Collect embryos of the wanted genotype after 24 h of egg laying and store them for additional 48h at 25° to make sure that all embryos developed cuticle. Collect the embryos wash them with dH_2O and dechorionate for 3-4 min using Na-Hypochlorite (Bleach). Wash them again with dH_2O and put them on a slide in one drop of Hoechst solution: lactic acid (1:1).

2.4.2 Fixation of embryos

For visualising the localization of proteins of interest in the embryo whole mount antibody stainings are performed. Preservation of protein localization that matches "in vivo" status is achieved by different fixation procedures. Depending on the nature of the protein of interest (i.e. membrane or cytoplasmic protein) different conditions and fixation solutions were used.
Stefanini fixation:

- Dechorionate embryos for 2-3 min in bleach
- Wash out the bleach with dH_2O
- Transfer the embryos into scintilation tubes that contain 4ml Stefanini solution+ 4ml Heptane
- Fixate embryos for 20-25 min at the shaker
- Remove the lower phase and add 4 ml methanol
- Strongly shake the bottle for 30 sec
- Collect the embryos that precipitated on the bottom and transfer them to eppi
- Wash embryos 3 times with Methanol
- Store embryos at -20 °

Solutions

Stefanini solution:1,1 ml 37% Formaldehyd + 1,5 ml 500mM PIPES+ 1,5 ml Picric acid+ 5,9 ml dH2O

Alternative fixative solutions can be 4% Formaldehyde, 8% Para-Formaldehyde

2.4.3 Immunofluorescence

-Wash embryos 3x20min with 1xPBT

-Block 1h at RT in 10%NHS+1xPBT

-Incubate with primary antibody O.N. at 4 ° +10% NHS

-Wash 4x15min with 1x PBT

-Incubate with fluorescently labelled secondary antibody for 2h at RT in 10%NHS+1xPBT

-Wash 4x15 min with 1xPBT

-Embed the embryos in Mowiol+DABCO

2.4.4 TUNEL assay

The TUNEL assay is a suitable method for positive identification of apoptotic cells by labelling DNA double strand breaks. TUNEL assay-kit from *Roche* was used in this work. Detection of apoptotic cells in S2 cell culture was performed according to the following protocol:

-Fix the S2 cells in 4% PFA PBS for 15 min

-Incubate 2x 10 min with PTX (1xPBS+ 0,3% TritonX-100)

-Incubate in CTX for 30 min at 65 °

-Wash the cells 2x10 min with TUNEL dilution buffer

-Incubate in TUNEL labelling buffer for 30 min at 37°

-Prepare TUNEL reaction mix (TUNEL enzyme : tunnel labelling mix = 1:10)

-Incubate in TUNEL reaction mix for 3h at 37°

-Wash the cells several times in PTX and mount in Mowiol (add very little DABCO)

2.4.5 Fixation and immunofluorescence of ovaries

Female flies not older than a week were put overnight on apple juice flasks and were additionally fed with yeast to enhance growth of the ovaries.
Since the structure of the ovaries is significantly more fragile than embryos, fixation conditions are milder. At the beginning, ovaries have to be extracted from the flies and immediately transferred to 1XPBS solution. Separation of the single egg chambers can be achieved by pipetting up and down. Afterwards immediately proceed with fixation:

- Add 110 µl 37 % Formaldehyde (4% fin. conc.) + 890 µl PBS and incubate on the shaker 10-15 min at RT
- Wash 4x10 min in 1xPBS
- Block ovaries for min 3h at RT in 5% NHS+ 5% Triton x-100 containing PBS
- Add primary antibody in 1%NHS PBS solution and incubate O.N. at 4°
- wash 4x15 min in PBT
- Add secondary antibody in 1%NHS PBT solution and incubate 1h at R
- Wash 4x15 min with 1xPBT
- Transfer on the slides in Mowiol+DABCO

2.5 Protein biochemistry

2.5.1 Isolation of protein extract from S2 cells

Coimmunoprecipitation is the method of choice to verify protein interaction *in vivo*. For this purpose, protein extract was made from S2 cells. Cells from 3 ml of culture in the exponential phase of growth were pooled and precipitated by centrifugation (1 min at 13 0000 rpm). After supernatant was discarded, pellet was resuspended in 150 µl of CHAPS IP buffer. CHAPS buffer creates relatively mild conditions for extraction which should favour maintenance of native structure of protein complexes. Cells were crashed with a micro syringe (*Hamilton*, Microliter syringe). Probes were incubated on ice for 20 min and then centrifuged for 20 min (13000 rpm at 4°). Supernatants were transferred to new reaction tubes. Concentration of isolated protein was determined with Bradford reaction (see below).

CHAPS IP buffer:
0,2% CHAPS
10mM Dithiotreitol (DTT)
100 mM HEPES
200mM NaCl
20% Sucrose

To prevent protein degradation during experimental procedure, protease inhibitors were added in 1:500 dilution from original stock solutions: Aprotinin (stock 10 mg/ml), Pepstatin (1mg/ml), Leupeptin (0.5 mg/ml) and Pefabloc (1mg/ml)

2.5.2 Measuring protein concentration by Bradford reaction

Protein concentration of extracts was determined using the commercially available reagent Coomassie Blue-G250 (*Roti-Quant*, Carl Roth GmbH, Karlsruhe). This colour substance has the property to absorb different wave lengths depending on its ionisation status in solution. Binding to the protein causes transition of the substance from cationic to anionic form and this change can be detecting with absorbance coefficient at λ= 595 nm.

- Dilute 2μl of the probe in 800 μl dH2O; same for 2 μl buffer control sample
- Add 200 µl 5x Color reagens
- Invert reaction tube several times and incubate for 2 min
- With the control sample set "null" absorbance value
- Determine absorbance of the sample at λ= 595 nm

Acquired value of absorbance was transformed into concentration of protein in solution (µg/µl) in relation to a BSA protein standard.

2.5.3 SDS PAGE Electrophoresis and Western Blotting

Presence of a protein of interest in the total protein extract can be shown by the western blot method. Proteins are denatured by SDS and afterwards separated in respect to size by SDS PAGE (Laemmli, 1972) electrophoresis on polyacrylamide gels. Complete protein content from the SDS gel is transferred on the nitrocellulose membrane.

Western Blot short protocol:

Assemble blot in a tray with Western transfer buffer. When sandwich is assembled transfer protein for 1 hour at 100V at 4°. Afterwards, disassemble sandwich, and check for the proteins on the nitrocellulose membrane by adding PonceauS staining solution for 1 min. Block the membrane 1h in blocking solution (TBST+3% skin milk powder+1%BSA). Dilute 1st antibody in desired concentration in blocking solution and

incubate the membrane O.N. at 4°. Wash the membrane 3-4 times 15 min in TBST. Incubate the membrane with the HRP conjugated secondary antibody. Wash the membrane 4x15 min in TBST. Prepare ECL reagent and incubate the membrane for 1 min. Wrap membrane and expose to X-ray film.

2.5.4 RNA interference in cultured S2 cells

RNA interference leads to knock down of gene function by targeting the RNA of a gene of interest for degradation (Fire et al., 1989). In cultured cells, addition of double stranded RNA to the culture is sufficient to reduce a targeted gene. Double stranded RNA was produced targeting DIAP1 RNA. Therefore, the DIAP1 cDNA was used for producing a DNA transcript by a standard PCR method and PCR primers with 5`elongated T7 transcription start sites. This PCR product was then used for in vitro transcription by T7RNA polymerase, following the manual of the in vitro RNA transcription kit (*Boehringer*, Mannheim). 15ug of the in vitro transcribed RNA were added to $1x10^6$ S2 cells in S2 RNAi incubation medium. This RNAi mix was incubated for 60min at room temperature. Afterwards 2ml of S2 Cell Growth Medium was added and the cells incubated at 25°C for 3 days until success of RNAi was determined.

S2 cell growth medium: FBS 10% Penicillin 50U/ml, Streptomycin 50ug/ml added to Schneiders`s Drosophila medium (*Invitrogen*).

S2 RNAi incubation medium: 2mM Glutamine added to DES Serum-free expression medium (Invitrogen).

2.5.5 Co-immuno precipitation

For confirming protein-protein interaction by coimmunoprecipitation, protein extracts from Drosophila S2 cells.were treated like follows:

- Prepare protein extract
- Leave on ice for 15 min
- Centrifuge 20 min at 13 000 rpm at 4°
- Transfer supernatant in the new reaction tube
- Determine concentration of the isolated protein with Bradford reaction
- Add 5 µl of the antibody to 0,5-1 mg of protein extract
- Incubate 2h at 4°

- Add 30 µl of protein A or protein G beads depending in which animal was antibody raised
- Incubate in over the top rotator O.N at 4 °
- Spin down 1 min at 13000 rpm and remove the supernatant. Keep aliquot of supernatant for testing
- Wash the beads 4x10 min in CHAPS IP buffer
- Add 2XSDS buffer and boil the samples

Samples further undergo SDS PAGE followed by Western Blotting procedures

2.5.6 Production of GST fusion proteins

Complete coding region of a cDNA or fragments of interest can be cloned 3`downstream of the glutathione-S-transferase (GST) coding sequence in pGEX expression vectors. For this work only pGEX4T1 was used. The plasmid can then be transformed to especially designed bacterial strains, like BL21pLysS, that allow high level expression of the fusion protein after addition of 1mM final concentration IPTG. Induction was performed for 3-4 hours at 28°.

Pellet was resuspended in 4ml 1xPBS containing proteinase inhibitors (apoprotin, pefabloc, pepstatin, leupeptin) and cells were sonificated for 60 sec (80 Hz). Triton X-100 was added to the extract up to a final concentration of 0,01%. Extract was rock over end incubated for 30min at RT and centrifuged 20min at 4°C to pellet bacterial debris. Supernatants were separated from the pellet and frozen at -20°C.

2.5.6.1 Purification of GST fusion proteins

GST fused proteins can be purified from the rest of the extracted proteins by coupling to Glutathione sepharose 4A beads. The GST part of the fusion protein strongly interacts with the substrate Glutathione and stays attached to the beads.

-Wash 30 µl of beads 2x5 min with 1xPBS

-Add 100µl protein extract and incubate 30-45 min at RT

-Wash the beads 4x10 min with PBS

-Add 2XSDS and boil the samples

Confirm binding of GST fusion protein by SDS PAGE electrophoresis and Comassie blue staining.

2.5 Genetic Methods

2.5.1 The Gal 4/UAS system

Brand and Perimmon (1993) developed an elegant and reliable genetic system for time and place controlled expression of any gene of interest. Activator, or driver, lines express the yeast transcription factor Gal4 under control of enhancers whose time and pattern of expression during development is well known (*engrailed*, *wingless, paired* etc.).

Effectors lines contain a P-element insertion carrying upstream activated sequence (UAS) 5` of the gene of interest. After crossing of activator and effector line, Gal4 is expressed and can bind to the UAS sequence upstream of the gene of interest to drive its expression.

2.5.2 The Flp/FRT system

Xu and Rubin developed the Flp/FRT system in 1993 (Figure 3). This system is based on the induction of mitotic recombination with the help of the yeast enzyme Flipase (Flp). Flipase mediates chromosomal exchange between two sister chromosomes at so call Flipase Recognition Target (FRT) sequences. One chromosome contains a wild type allele of a gene and the other one a mutant allele of the same gene. When mitotic recombination occurs, segregation of the chromosomes into daughter cells gives rise to one cell homozygous wild type, while the other is homozygous mutant.

In the *Drosophila* system any given, i.e. lethal, mutation can be meiotically recombined with available FRT chromosomes. This allows in later crosses to combine the mutant FRT chromosome with chromosomes carrying a transgene encoding for the Flipase. The Flipase can be conditionally expressed for example by a heat shock inducible promotor. Heatshock at 37°C for 1h then leads to induction of mitotic recombination and appearance of clonal populations of cells either wildtype or mutant for the given mutation. The wildtype chromosome is usually marked by a cell autonomous marker that allows to distinguish between wildtype and mutant cells. In

this work the marker is GFP under control of a ubiquitous promotor. In the homozygously mutant cells, GFP is missing.

For this work the following FRT chromosomes were used:

1st chromosome *ral*^{89} FRT101 /FM7 and *ral*^{70} FRT101/FM7

FRT101 was obtained from H.A.J. Müller

3rd chromosome w/w ;;*th*^{109} FRT2A/TM3 *ftz::lacZ*.

FRT2A was obtained from the Bloomington stock center, stock number BL-1997.

The chromosmes were crossed to males carrying a heat shock inducible Flp transgene on the 2nd or 1st chromosome, respectively. These males were either carrying *ubi*^{GFP} FRT101 or *ubi*^{GFP} FRT2A. The latter stocks were obtained from Bloomington stock center, stock number BL-5153 and BL-5825.

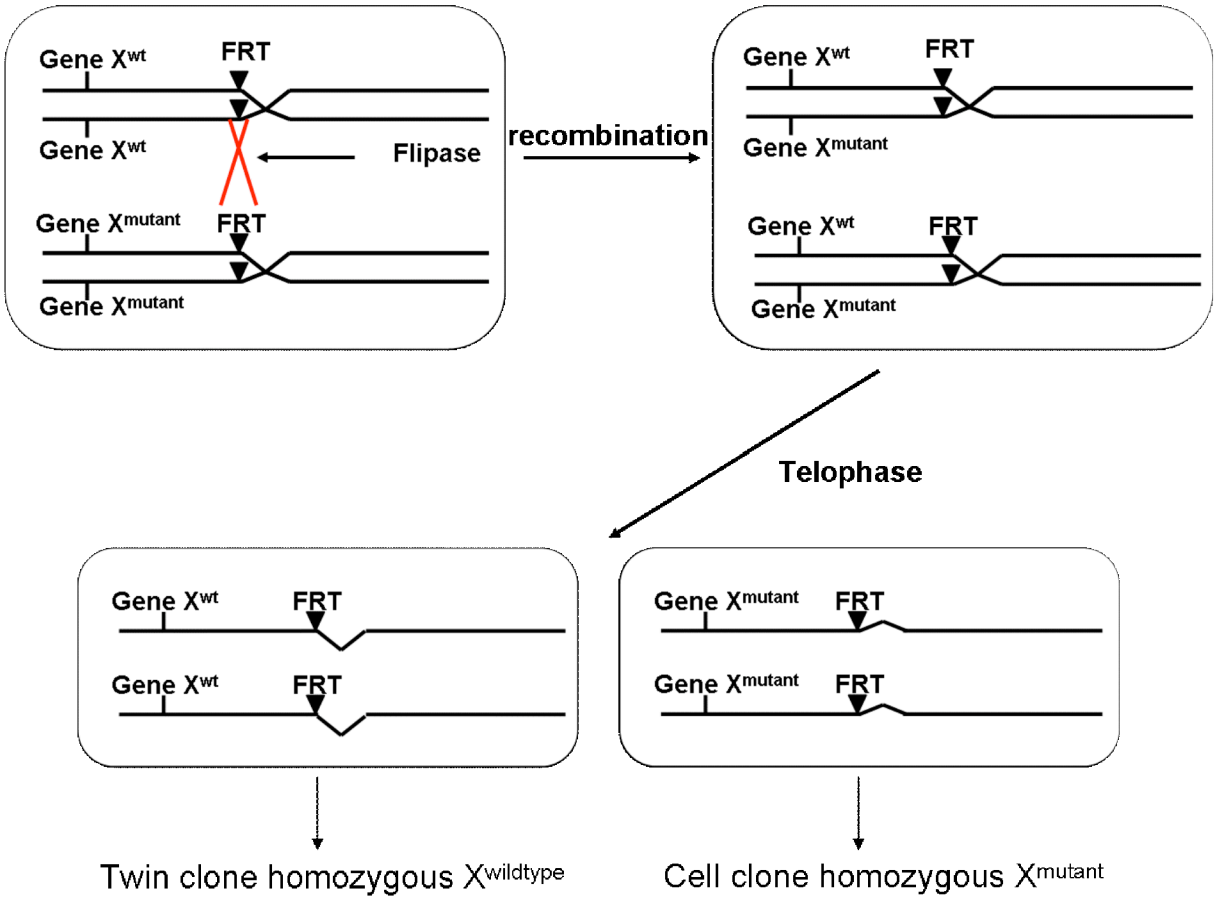


Figure 3: Schematic representation of the FRT/Flp system after Xu and Rubin, 1993
When Flipase is expressed after heat shock, it mediates mitotic recombination between two homologue chromosomes carrying FRT sequences. Wildtype and mutant alleles of gene X are exchanged by that event. When sister chromatides are separated, daughter cells obtain either two wildtype or two mutant alleles of gene X. Both daughter cells give rise to a clonal population of cells. The chromosome carrying the wildtype allele of X is usually carrying a cell autonomous marker like GFP^{ubi} as well (not shown in scheme). Wildtype cells can be identified by expression of GFP, while mutant cells lack GFP.

2.6 Preparation of *Drosophila* eyes for scanning electron microscopy

To test modification of the rough eye phenotype caused by expression of GMR::*reaper,* facette eyes of *Drosophila* were prepared for scanning electron microscopy (SEM). Therefore, eyes of adult F_1 females of the following crosses were prepared: *GMR::Gal4*/*GMR::Gal4;GMR::rpr*/TM3 x w^{1118}/Y , G*MR::Gal4*/*GMR::Gal4; GMR::rpr*/TM3 x w/w;*UAS::Ral*^{G20V}*; UAS::Ral*^{G20V} , *GMR::Gal4*/*GMR::Gal4 GMR::rpr*/TM3 x *UAS::DIAP1*/Y.
Only females were used that did not carry the balancer chromosome TM3.
As a control experiment *UAS::Ral*^{G20V} was expressed by crossing to *GMR::Gal4* alone.
Heads were separated from the thorax with the razor blade and then further cut in two. Eyes are dehydrated through consequent 5 min incubation steps in ethanol of rising concentration: 30 %, 50%, 70 %, 96% and absolute alcohol. Eyes were washed two times in dry acetone and incubated for 30min at 4° in 1:1 mixture of acetone and TMS (tetramethylsilane; Sigma). They were then incubated in a 1:2 mixture of acetone:TMS for 30 min. Pure TMS was added and left it open to dry overnight. Probes were sputtered with gold and used for scanning EM.

3. RESULTS

3.1 Differential localisation of DIAP1

The *Drosophila* Inhibitor of Apoptosis DIAP1 interacts with caspases and prevents their activation. Upon expression of proapoptotic genes or as a consequence of proapoptotic signals, caspases are being released and start proteolytic degradation of structural components in the cell. Thus, cytoplasmic presence of the DIAP1 protein is functionally important in all cells of the embryo.

Interestingly, immunofluorescence analysis shows that DIAP1 is differentially localized during embryonic development (Fig.4). In the early embryo a simple monolayer epithelium is formed. At this developmental stage DIAP1 appears associated with the plasma membrane (Fig.4A). In later stages of embryogenesis a high concentration of DIAP1 protein is present in the neuroblasts (Fig.4B). Neuroblasts are stem cells that undergo a series of cell divisions to form the central nervous system. A function of DIAP1 during central nervous system development other than cell death inhibition is not described. Thus, the exact functional requirement for DIAP1 accumulation in the neuroblast nuclei is not known. However, the overexpression of a full length DIAP1 transgene leads to accumulation of DIAP in the nucleus of almost all cells (Fig.4C).

The DIAP1 protein contains neither a predicted membrane anchor nor a nuclear localisation signal. The presence of DIAP1 in different cellular compartments implicates that other proteins control its subcellular localization. Increased concentration of a protein in a certain compartment is also a strong indication that the protein has some specific role in the local environment. As DIAP1 is crucial for cell death inhibition during embryogenesis possible apoptosis independent functions of DIAP1 might still be undiscovered.

The DIAP1 protein consists of two N-terminal BIR domains and a C-terminal RING domain. The BIR domains interact with caspases and proapoptotic proteins. The E3 ubiquitin ligase activity of the RING domain, depending on the cellular context, promotes autoubiquitination of DIAP1 or targets caspases for degradation. It can not be excluded that DIAP1`s E3 ubiquitin ligase recognizes additional proteins as targets for degradation and thereby influences multiple cellular pathways.

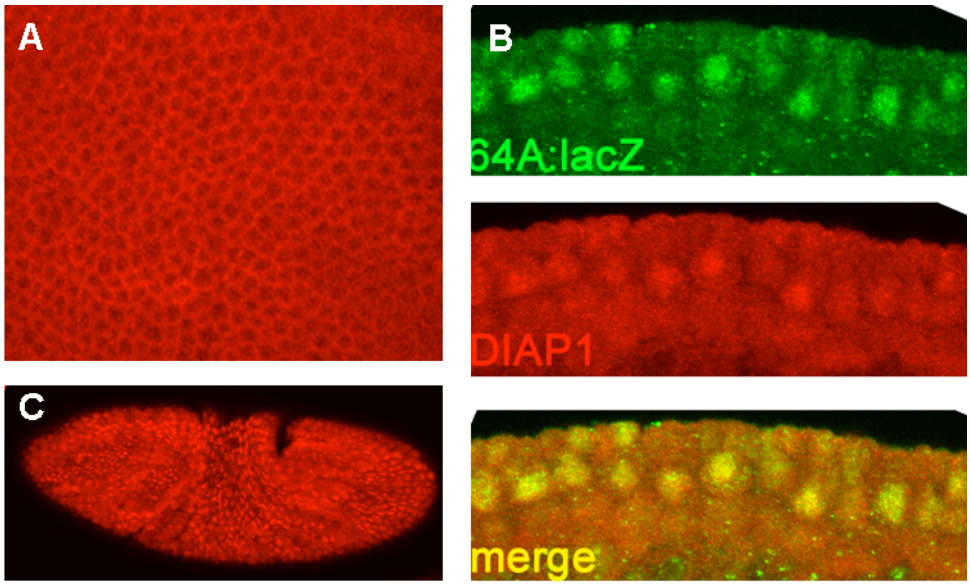


Figure 4: DIAP1 is differentially localized during embryogenesis
A) DIAP1 protein localization (red) during cellular blastoderm stage revealed by mouse anti DIAP1 antibody (red), surface view of the cellular blastoderm **B**) In stage 9 of development DIAP1 (red) is localized to the nuclei of neuroblasts (NB). NB are marked by lacZ transgene expression in the enhancer trap line 64A (green) **C**) Overexpression of *UAS::Myc-DIAP1* using a strong maternal Gal4 driver causes translocation of the protein to the nucleus in a stage 8 embryo, as revealed by anti Myc antibody (red).

One straightforward approach to address the question how regulation of DIAP1 is mediated is to identify interacting proteins. Characterization of novel binding partners is the best way to determine which molecular mechanisms are dependent on the caspase inhibitor.

3.2 A yeast two hybrid screen for novel interaction partners of DIAP1

To identify new DIAP1 interacting proteins, the yeast-two-hybrid has been chosen as a screening method (Song and Fields, 1989). This system for detection of protein-protein interactions is based on the capability of the yeast strain *Saccharomyces cerevisiae* to produce proteins from other species. The genetically modified yeast strain AH109 contains a promotor sequence in front of four reporter genes that can be activated by the Gal4 transcription factor. The Gal4 protein contains a DNA binding domain and a transactivation domain. Binding of functional Gal4 to the promoter leads to expression of the reporter genes.

The yeast two hybrid method takes advantage of the fact that DNA binding domain and activation domain of Gal4 can be encoded by two separate plasmids: pGBKT7 and pACT2. The cDNA of a "bait" protein of interest can be inserted into the vector containing the DNA binding domain. The other vector contains the transactivation domain and randomly inserted clones from an embryonic cDNA library of 0-12 h old *Drosophila* embryos (Clontech, Palo Alto, USA). Prior to the screen the cDNA library was amplified, to obtain sufficient amount of DNA for yeast transformations (see material and methods). The yeast two hybrid method requires transformation of both plasmids, pGBKT7 which contains the "bait" cDNA, and pACT2 which contains cDNA of a putative candidate, into the same cell. Interaction of "bait" and "prey" leads to assembly of functional Gal4 and activation of reporter genes as specific selection markers (Fig.5).

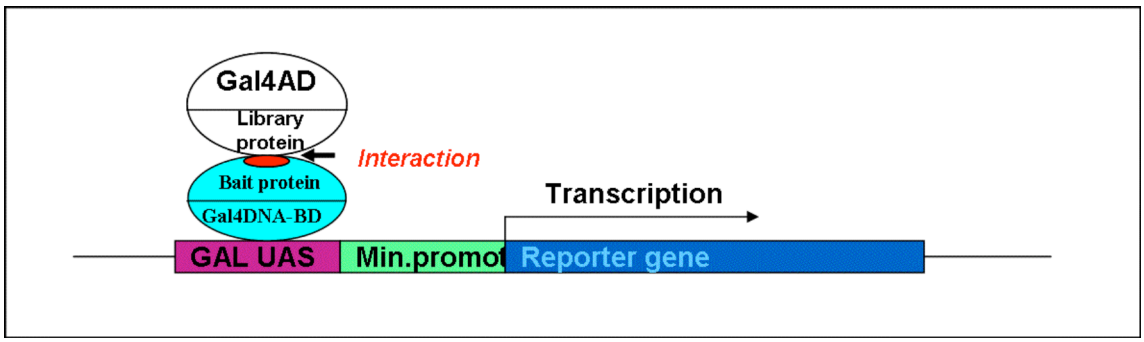


Figure 5: Schematic representation of the Yeast two hybrid method.
Protein interactions in the yeast two hybrid lead to formation of a functional transcription factor. Gal4 binds UAS sequences in front of reporter genes and drives their expression. AD: activation domain, BD: DNA binding domain

3.3 Screen with the full length DIAP1

Several versions of the DIAP1 coding sequence were cloned in frame into the pGBKT7 vector (Fig.6). In the initial screen full length DIAP1 was used as "bait". Constructs containing separate BIR domains were produced in order to map the region of interaction with putative candidates.

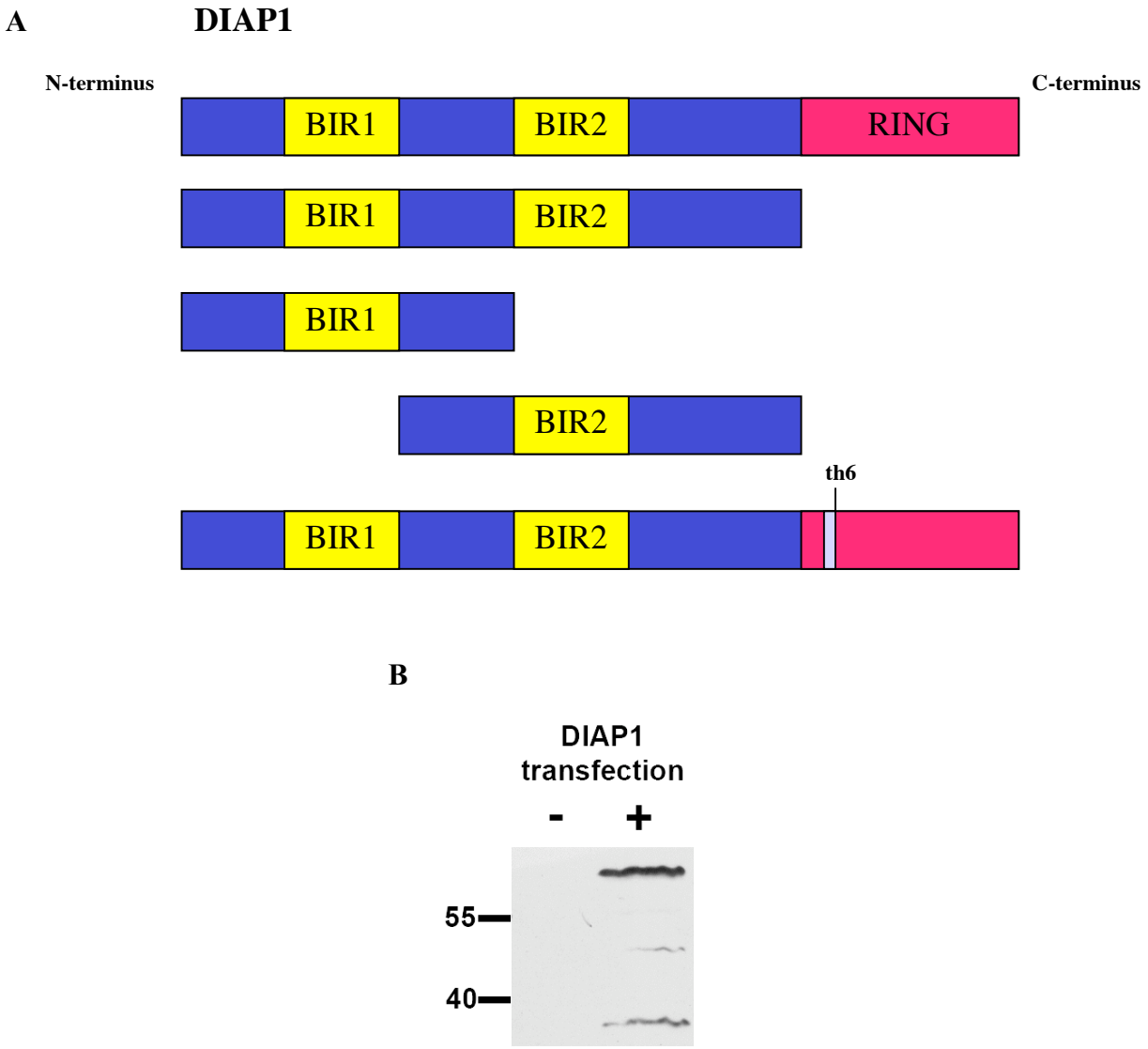


Figure 6: Schematic representation of Yeast two hybrid constructs.
A) The depicted different DIAP1 fragments were used as bait in the yeast two hybrid screen. Borders of the constructs are: BIR1L1 (1-225 amino acids), L1BIR2L2 (111-391) and ΔRING (1-391). Fragments were cloned in frame in front of the Gal4 DNA binding domain encoded by the PGBKT7 plasmid **B**) Success of bait transfection (+) was confirmed by western blot using anti myc antibody in comparison to untransfected yeast (-). Blot shows expression of full length DIAP1 fused to Gal4 BD.

Expression of the protein in the host strain was verified by western blotting against an N terminal Myc-epitope (Fig.6B). Ten large scale co transformations of pGBKT7/DIAP1 and pACT2/xcDNA were performed with an average efficiency of 285 000 colonies, and 2.85 million tested clones. Since the library should contain ~3 million of independent cDNA clones, the screen covered nearly all of them. However, yield of the potential candidates was lower than expected. Only two potential candidates gave positive interaction test after retransformation. One “prey” encoded for the gene RpS27A, the other for the gene RpL40.

Both candidate genes encode for proteins that are structural components of the ribosomes and contain an N terminal stretch of amino acids identical to ubiquitin. The function of these proteins is not well known. Some evidence points to their possible involvement in biogenesis of the ribosome and control of translation (Wittmann et al., 1989). Available mutant fly stocks that include point mutations show a minute phenotype. Homozygous mutants are embryonic lethal because of deficiencies in proteins synthesis.

Presence of ubiquitin in these proteins would make them interesting interaction partners with respect to DIAP1`s E3-ubiquitin ligase function. Additionally, a connection of DIAP1 with the translational machinery would be interesting as the proapoptotic proteins Reaper and Grim negatively regulate general translation via an unknown mechanism (Holley et al., 2002). However, several reasons argued against further research. Ribosomal proteins are frequent false positives in the yeast-two-hybrid system because of their abundance in the cell. Additional difficulty to analyse the mutants arises from their “housekeeping” function in the ribosome. It would be hard to distinguish if mutant cells develop abnormally because regulation of the cell death program is affected or because general translation is disturbed.

3.4 Screen with DIAP1-ΔRING protein

The screen with full length DIAP1 appeared to be problematic because of low yield. As western blotting confirmed that DIAP1 was produced in the yeast, it was concluded that intrinsic properties of the protein might have prevented a successful screen. The most likely explanation was that the E3 ubiquitin ligase domain negatively affected stability of interaction. Enzymatic active proteins tend to be “difficult” baits, because they can disturb formation of a functional transcription factor.

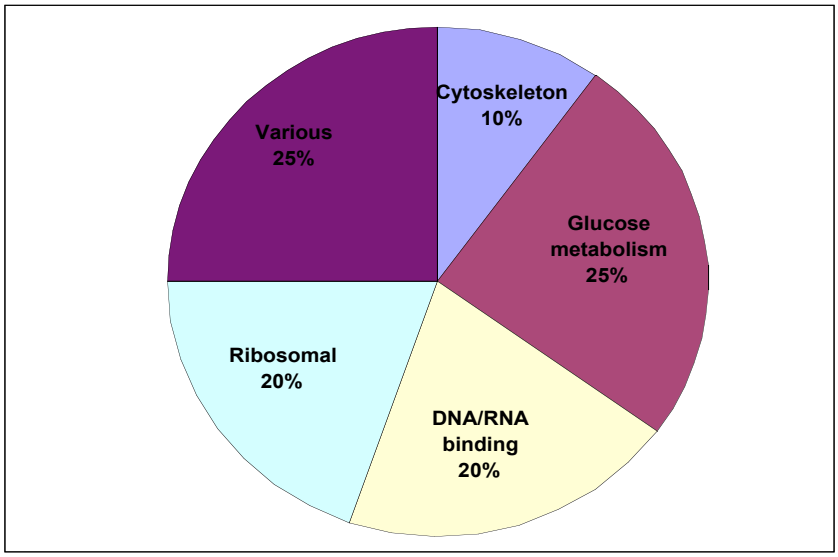
To circumvent putative problems with the E3-ligase domain, an alternative bait construct lacking the RING domain was developed (see supplementary data).

Eight transformations were performed with DIAP1-ΔRING as a "bait", yielding an average efficiency of 327 000 double transformants. In total, 2.61 million clones were screened. From 44 initially isolated colonies 29 turned positive after retransformation.

All cDNAs of the retrieved candidates were sequenced and 22 were confirmed to originate from coding regions of *Drosophila melanogaster* annotated genes.
Identification of the candidate sequence was done using the BLAST search program. The search engine is available online at the Berkley *Drosophila* Genome project (BDGP). It compares a given sequence with all sequences in the *Drosophila* genome and provides a list of identified genes or partial homologies. All up to date information about the annotated *Drosophila melanogaster* genome is assembled in Fly Base (flybase.org) under supervision of the *Drosophila* Research Center, Indiana. The data base includes additional information about available mutants, description of known protein functions based on references, or predictions of gene function based on structural homology. Known genes are mentioned by name while predicted genes, only identified by sequencing, are classified by a "CG" followed by a running number.
A summary of the putative novel interaction partners of DIAP1 is demonstrated in table 1.

Table1: Summary of yeast two hybrid results
A) Schematic representation of screen results indicated as percent of found clones. **B**) List of all candidate genes

A



B Candidate Genes	predicted function	protein domains
Further Characterized		
CG2849 Ral	Ras signalling pathway	Ras like GTPase
CG17369(Vha55)	H^+ transport / ATPase	ß-subunit of H^+ATP synthase
Cytoskeleton Proteins		
CG4027 (Act5C)	structural cytoskeletal	actin like ATPase domain
CG11605 (jbug/moa)	actin binding	calponin homology
DNA/RNA Binding		
CG2261 (CstF-50)	mRNA cleavage	WD repeat
CG3658 (cdc45L)	DNA replication	CDC45 like protein
CG7101	transcription factor	8x Zinc finger C2H2 type
CG10194	unknown / DNA repair	Nucleoside triphosphate pyrophorylase
Glucose metabolism		
CG9238 (PP1 binding protein)	glycogen metabolism regulator	
CG7070 (PyK)	pruvate kinase /glycolysis	Pyruvate kinase family
CG2103 (Pgant6)	N-acetylgalactosamintransferase	glycosyl transferase
CG14476	glucosidase II	Glycosyl hydrolase
CG10960	glucose transporter	general substrate transporters
Ribosomal Proteins		
CG15442 (RpS27A)	ribosomal component	L15 family
CG2960 (Rpl40)	ribosomal component	L40 family
CG5920 (sop)	ribosomal component	ribosomal proteinS5 dsRNA binding domain
CG3661 (RpL17A)	ribosomal component	L14b/L23 family
Various		
CG1188 (Rpn2)	proteasome endopeptidase	ARM repeats (see ref)
CG18641	lipase	esterase/lipase thioesterase active site
Cyp309a2	hormone synthesis	cytochrome P450 like
CG3420	unknown	unknown
CG13043 (RetininC)	unknown	unknown

3.5 Assessment of potential significance of isolated genes

The putative DIAP1 interaction partners identified in the screen belong to structurally very diverse protein families. Functionally they can be classified into five groups: general metabolic enzymes, cytoskeleton proteins, signalling molecules and proteins of unknown function (Table1A).

The yeast two hybrid method is well known as an efficient screening technique with sometimes a high background of false positives. From the aspect of this project the usual methodology of comparing expression profiles of the “bait” and “prey” proteins in the *Drosophila* embryo could not provide conclusions sufficient for elimination of the candidates. DIAP1 is ubiquitously expressed in almost every cell, so in situ hybridisations would have only descriptive value. For the same reason raising of antibodies against candidates was not an option. Therefore, filtering of potential candidates had to be performed based on theoretical relevance. The author of this work is aware of limitations of such an approach and of the danger that relevant candidates could be labelled as “background”. A short summary of main features that are known about some of the putative interaction partners are given in the next chapter. Although they have not been further tested in the course of this project because of time and practical constrains, it is possible that some of them are indeed interaction partners of DIAP1.

3.5.1 Potential candidates that have not been studied further

3.5.1.1 Cytoskeletal components

Components of the cytoskeleton are potentially interesting interaction partners of DIAP1 in respect of its newly discovered non apoptotic functions. Geisbrecht at al. (2004) demonstrated by coimmunoprecipitation that DIAP1 forms a protein complex with Rac1 GTPase and profillin, proteins that maintain a pool of monomeric actin in the cell. Additionally, DIAP1 has probably an F-actin stabilizing function under certain circumstances.

The gene *actin5C* was found to be an interaction partner of DIAP1. It encodes one of two cytoplasmic actins in the *Drosophila* genome. It is ubiquitously expressed throughout embryonic development. Interestingly, the peak of expression corresponds to the stages of maximal cell shape changes (Tseng et al., 2004).

Another candidate related to the cytoskeleton was the *jitterbug* gene. *Jitterbug* encodes a very large filamin protein (2963 amino acids) with three calponin homology domains and numerous filamin repeats. Functionally it is predicted to cross-link actin filaments into networks.

3.5.1.2 Candidates involved in glucose metabolism

Four isolated putative candidates are members of the sugar metabolism: Pyruvate kinase (PyK), CG14476, CG9238 and CG10960. CG10960 is predicted to be a glucose transporter and CG9238 is a homologue of the protein phosphatase 1 binding protein, one of the regulators of glycogen catabolism. PyK is the first enzyme in the citric acid cycle and CG1447 belongs structurally to the family of glycosyl hydrolases. All these putative candidates were eliminated based on the assumption that they are so abundantly expressed as “house keeping” components in the cell that they are likely to be false positives in the screen.

3.5.1.3 RPN2 (CG1188)

The endopeptidase encoded by this gene is a component of the 19S regulatory subunit of the proteasome. It contains an amino acid repeat motif of unknown function. The only protein that shares homology with RPN2 is located to APC1, a subunit of the large cyclosome complex that controls turnover of cyclins. Without RPN2 some natural substrates fail to be degraded in the proteasome (Kajava et al., 2003). The human homologue PSMD1 is shown to interact with TRAP-2, a protein related to tumor necrosis factor (TNF) receptors. Beaudenon et al. (1999) tested stress response of cells mutant for RPN2. UV light exposition triggered significantly higher level of cell death in culture of RPN2 mutant cells compared to the wild type. RNAi experiments targeting RPN2 also increased the number of apoptotic cells by 54%.

E3 ubiquitin ligase activity of DIAP1 links the apoptotic mechanism with proteasomal degradation. Biochemical data about the mechanism how proteins tagged with ubiquitin are loaded into the proteasome, how recognition of substrates takes place and how the whole process is regulated remain obscure. It would be interesting to know if DIAP1 influences specificity of the proteasome by binding to RPN2. Potential interaction of these two proteins could further explain regulation of apoptosis, and

provide valuable data in the field of protein degradation. A point mutation in the RPN2 gene causes embryonic lethality in the homozygous state, but no excessive apoptosis was detected (data not shown)

3.5.1.4 Genes involved in control of transmission of genetic information

In the screen several candidates were identified with predicted function in replication, transcription or translation. Some of them could perhaps mediate nuclear localization of DIAP1.

The gene *CG7101* is highly conserved between the species. The protein has a predicted Zink finger C2H2 type domain. It has therefore some properties of a transcription factor but there is not enough data for a definite classification.

CDC45L (*CG3658*) has a DNA binding domain and is localized to the nucleus. Based on the sequence similarity with homologues from *C.elegans*, *Mus musculus* and *Homo sapiens* it is required in the process of DNA replication.

The gene Cst-F (*CG2261*) encodes a protein that has structural homology with proteins involved in mRNA processing. EMBL data base predicts it as a component of RNA cleavage stimulating complex.

String of pearls (sop) or *CG5920* encodes a protein that is a structural component of the small subunit of the ribosome but also has a dsRNA binding domain. Therefore, it could be involved in regulation of translation. There are 8 mutants available. Loss of function mutations cause defects in development of bristles (macrocheata). Homozygous mutant females are sterile (Cramton and Laski, 1999).

3.5.2 Further investigated candidates: Ral GTPase and Vacuolar H^+-ATPase

Although several candidates have the potential to be analysed in more detail, the present work focused mainly on characterization of DIAP1`s interaction with Ral, a member of the Ras like small GTPase protein family. The yeast two hybrid result gave a first indication of a connection between the caspase inhibitor and signalling

processes in the cell. Experimental work presented below confirmed DIAP-Ral binding and gave insight into functional implications of this molecular interaction. Before data about Ral is presented, results of experiments performed with the vacuolar H^+-ATPase gene *vha55* are shown in the next chapter. *Vha55* was an interesting interaction partner because of the antiapoptotic function of its mammalian homologue.

3.5.2.1 Vha55 Vacuolar H^+-ATPase

In the screen a "prey" plasmid was recovered that contained a 650bp long cDNA fragment of *CG17369.* Product of this gene is the β subunit of the vacuolar H^+-ATPase. The enzyme as whole is a large complex, consisting of a catalytic and a regulatory domain. It resides in intracellular membranes and is important for the function of many organelles (Golgi, endosomes, lysosomes etc.). The vacuolar H^+-ATPase is using ATP for proton transport into intracellular compartments, therefore it maintains an acidic environment in the lysosomes. In some highly specialized tissues such as kidney or bone marrow, the vacuolar H^+-ATPase is localized to the apical plasma membrane.

Precise function of the β-subunit that appeared in the screen as DIAP1 partner remains yet to be elucidated. Several authors (Ohba et al., 1996; Akifusa et al., 1998) show that the ß-subunit in other organisms regulates enzyme activity without specifying the mechanism of regulation. Davies et al. (1996) molecularly characterized *vha55* encoding for the β-subunit of vacuolar H^+-ATPase in *Drosophila*. They analysed point mutations and P element excision of this gene. Their observations indicated that the ATPase is especially important in tissues whose function requires a strong proton gradient such as Malpigian tubules, intestine etc. Interestingly, the point mutation, when in homozygous state leads to embryonic lethality while excision of the gene shows a larval lethal phenotype. When there is no zygotic expression of v*ha55,* the maternal component is sufficient to maintain the function of the enzyme until larva. Presence of the altered β-subunit, on the other hand, causes earlier formation of a malfunctioning enzyme and results in embryonic lethality.

The decision to follow up the lead of the yeast two hybrid result in the case of *vha55* was based largely on exiting results from the studies of its mammalian homologues and their implications for cell survival.

3.5.2.2 Inhibition of Vacuolar H^+-ATPase in mammalian cells leads to apoptosis

Numerous studies of the v*ha55* homologue in mammalian cell culture demonstrated importance of the vacuolar H^+-ATPase for cell death. In these studies the pharmacological drug BafilomycinA1 (BafA1) was used that inhibits the enzyme by binding specifically to the β-subunit. The substance is an important component in cancer research because of its ability to induce apoptosis in malignant tissues.

BafA1 is also used in studies of neurodegenerative diseases such as Huntington Chorea and Parkinson disease (Soriano et al., 2003). These neurological disorders are characterized on the histological level by the presence of protein aggregates in the cytoplasm. After inhibition of the vacuolar H^+-ATPase, clearance of miss folded proteins is prevented and they accumulate in the cell.

How the functional cross–talk between vacuolar H^+-ATPase and the apoptotic program works is not known, but some data indicates that the connection is specific and controlled. Ishisaki et al. (1999) were comparing the effects of BafA1 and ammonium sulphate, a potent agent that abolishes acidic environment in the lysosomes. Addition of ammonium sulphate leads to necrosis while BafA1 treated cells show apoptosis. This argues against the possibility that apoptosis occurs simply because a constitutive part of cellular homeostasis, the low pH in the lysosomes, is affected and suggests that function of the vacuolar H^+-ATPase is not restricted to maintenance of the low pH.

When activated, human T lymphocytes are exposed to apoptotic stimuli targeting mitochondria (staurosporin), they enter early commitment phase of apoptosis in a caspase independent fashion (Dumont et al., 2001). Before a conserved apoptosis mechanism involving BCL2 family members is initiated, CathepsinD is released from the lysosomes into the cytosol (Bidere et al., 2003). Destabilization of lysosomal integrity is only limited and molecules are translocated in a size selective manner. It remains to be seen if the vacuolar H^+-ATPase in any way participates in the mechanism that controls release of lysosomal contents.

Multiple studies showed that BafA1 blocks the fusion of secondary endosomes with the lysosomes (van Deurs et al., 1996; Palokangas et al., 1997). This interaction is a critical step in the process of autophagy, an evolutionary very old form of cell death by which a cell “eats itself”. Fragments of the cell body, organelles, and structural components are being engulfed by membranes and transferred to the

lysosomes where they are digested. Autophagy is often induced by starvation and sometimes considered as an example of a “low cost” way of committing suicide (Yamamoto et al., 1998).

3.5.2.3 BafilomycinA1 triggers apoptosis in Schneider S2 cell culture

A potential interaction between the vacuolar H^+-ATPase and DIAP1 could link cell survival with integrity of the lysosomes. To investigate this possibility it was important to know how cells respond to functional deficiency of the *vha55* gene product: Does inhibition of the regulatory β-subunit trigger programmed cell death in *Drosophila* cells? To answer this question an experiment was set up to test the effects of BafA1 in the *Drosophila* Schneider (S2) cell culture.

Schneider cells are a cell line derived from neuroblasts. Under normal growth conditions up to 5% of cells in the culture undergo apoptosis at any time. S2 cells were treated for 24h with BafA1 at two different concentrations (0.25µM and 0.5µM). The effect of BafA1 on viability was quantified by a trypan blue test. Trypan blue is a dye that easily penetrates cell corpses but stays outside if the plasma membrane is intact.

The result of a quantitative analysis presented as a diagram (Fig.4) confirmed lethality of BafA1 for S2 cells but this could have been due to necrosis, autophagy or necrosis. Apoptosis can be positively identified by TUNEL assay, a reaction that labels double stranded DNA breaks with a fluorescent dye. As it is obvious on confocal images (Fig.7B), many more TUNEL positive cells (green) are present in the culture treated with BafA1 than in the DMSO treated control (Fig.7A). Notably cell culture density also appears lower in the culture exposed to BafA1 with some cells having pycnotic nuclei (transmission channels in Fig.7).

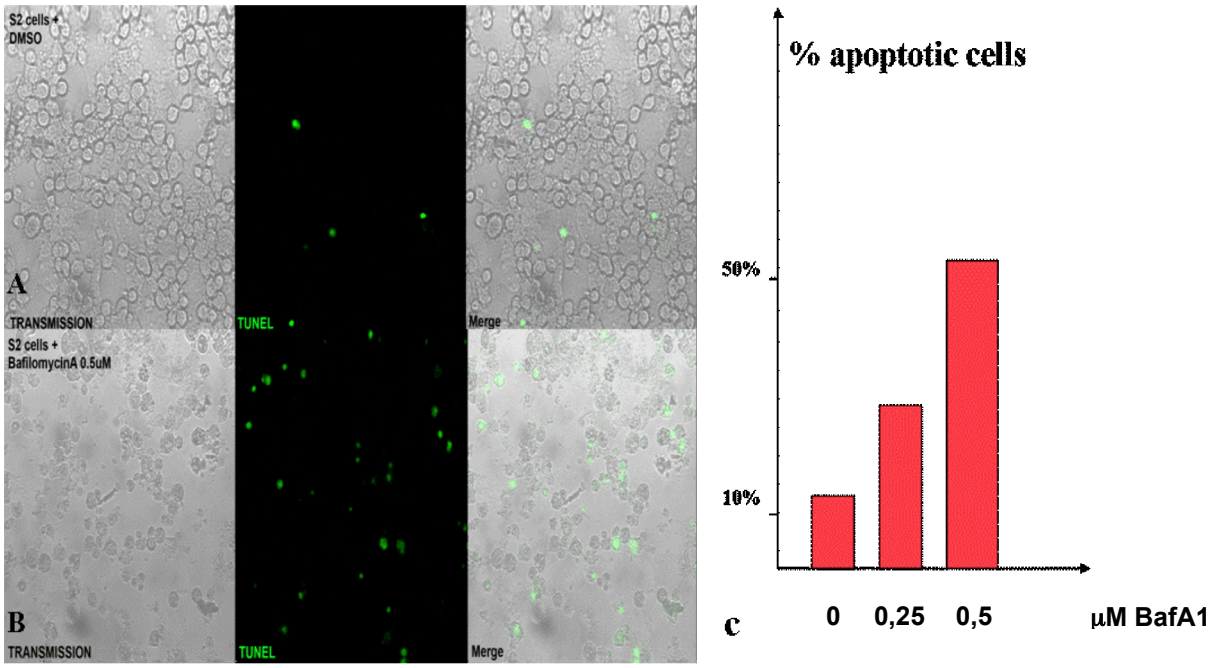


Figure 7: BafilomycinA1 triggers apoptosis in S2 cell culture

A) S2-cell culture treated with DMSO contains up to 5% apoptotic cells (TUNEL staining green) **B**) Number of apoptotic cells increases to over 50 % within 48h after addition of 0.5µM BafA1 **C**) The diagram shows quantification of the cell death rate at raising concentrations of the vacuolar-H^+-ATPase inhibitor BafA1.

It can be concluded that Bafilomycin A1 causes apoptosis in *Drosophila* S2 cells. To check which kind of effect, if any, treatment with the ATPase inhibitor leaves on distribution of DIAP1 protein in the cell, antibody staining against DIAP1 was performed. Confocal images demonstrated that staining pattern did not change significantly but the signal got weaker in the BafA1 exposed cells (data not shown).

3.5.2.4 Caspases are activated in Bafilomycin A1 treated cells

The results above confirmed the hypothesis that BafA1 induces apoptosis in *Drosophila* cells. What the experiment did not tell is whether the cell death mechanism triggered by the drug is identical to the one induced by expression of proapoptotic genes.

To address this problem, two experiments were performed. On one hand S2 cells treated with BafA1 were stained with the live dye Lysotracker to test integrity of lysosomes under these conditions. On the other hand activation of caspases was followed in western blot experiments. In untreated S2 cells Lysotracker is seen in many small vesicles throughout the cell (Fig.8A). When BafA1 is added, lysosomal

distribution within the cell is altered (Fig.8B). Lysotracker is found in 2-3 big spots within the cell under this condition (arrow in Fig.8B). This supports the idea that integrity of the lysosomes might be affected by BafA1 treatment.

To test whether BafA1 treatment leads to activation of caspases, cell lysates of BafA1 treated cells were probed for activation of the executioner caspase DRICE in western blotting experiments. To test a possible involvement of cathepsins in caspase activation, cells were additionally treated with the cathepsin inhibitor E64 and a combination of BafA1 and E64 (Fig.8C). Compared to Mock treated control cells, DRICE was activated in response to BafA1 treatment (arrowheads in Fig.8C). Caspase activation is almost reverted to basal level when E64 is present in BafA1 treated cells. Interestingly presence of E64 alone suppresses background activation of DRICE in DMSO treated cells.

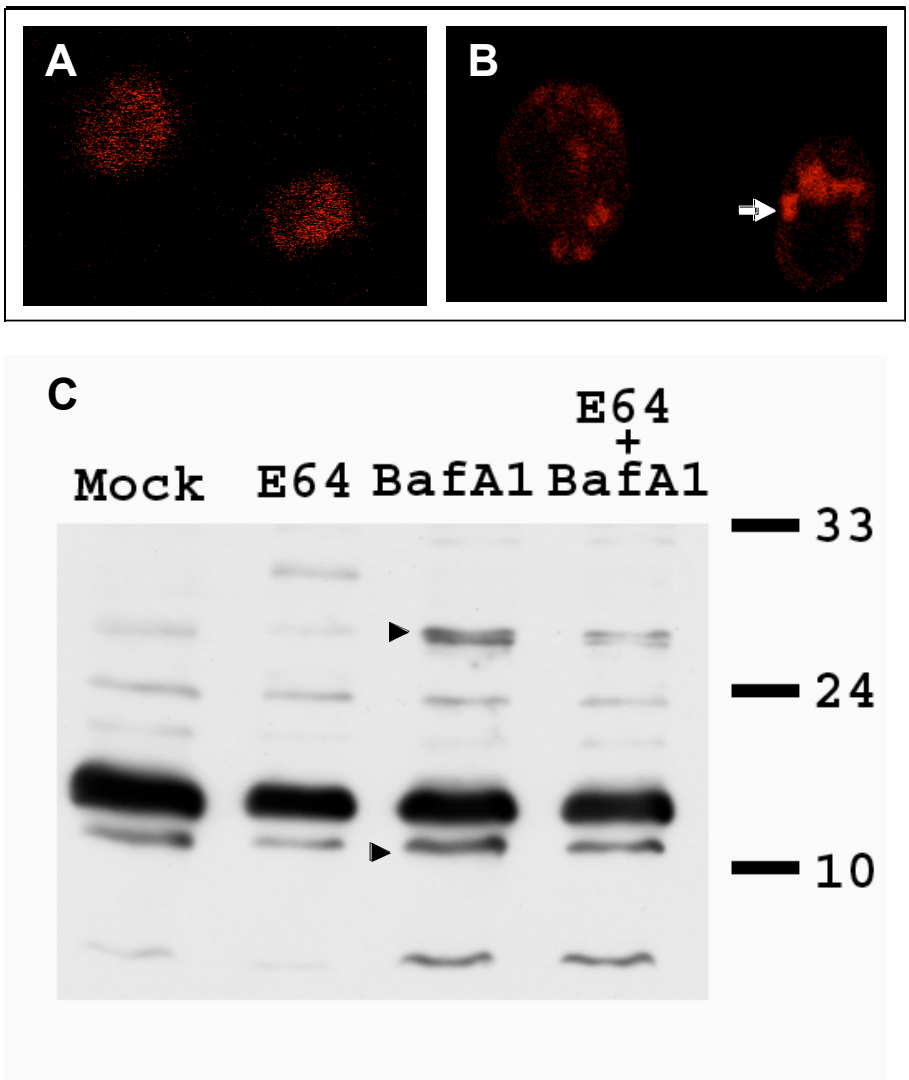


Figure 8: BafA1 affects subcellular lysosome distribution and leads to DRICE activation

A) S2 cells treated with DMSO were incubated with the live dye Lysotracker (red) to visualize lysosomes. **B)** S2 cells treated with BafA1 and Lysotracker (red). **C**) Western blot using anti activated DRICE antibody. S2 cells were treated with the indicated chemicals (Mock=DMSO see text for details). Same amounts of S2 cell proteins were separated by SDS-Page and subsequently blotted to nitrocellulose membrane. BafA1 induces DRICE activation as measured by appearance of separated large and small subunit (arrowheads in **C**). For details about caspase activation see introduction.

It can be concluded that reduced *vha55* function achieved by BafA1 treatment probably leads to release of cathepsins from lysosomes that subsequently stimulate caspase activation. So far nothing was known about the role of cathepsins in regulation of *Drosophila* apoptosis. Therefore the presented data provides an interesting starting point for further analysis of the vacuolar H^+-ATPase. Of crucial importance remains the confirmation of direct binding to DIAP1 by biochemical methods in vitro or in vivo.

3.5.3 The small GTPase Ral

3.5.3.1 Introduction

From the theoretical implications, the potentially most interesting candidate found in the screen was the Ral GTPase, known also as Rala (Fly Base). The interacting fragment that bound to DIAP1 was a 60 amino acids long C terminal part of the protein. Ral is a member of the Ras like family of small GTPases. In the cell it is present in two forms: an inactive GDP bound and an active GTP bound form (Fig.9). Two protein families control cyclical exchange of GTP and GDP.
Guanine nucleotide exchange factors (**GEFs**) facilitate release of GDP and binding of GTP while GTPase activating proteins (**GAPs**) trigger GTP hydrolysis.

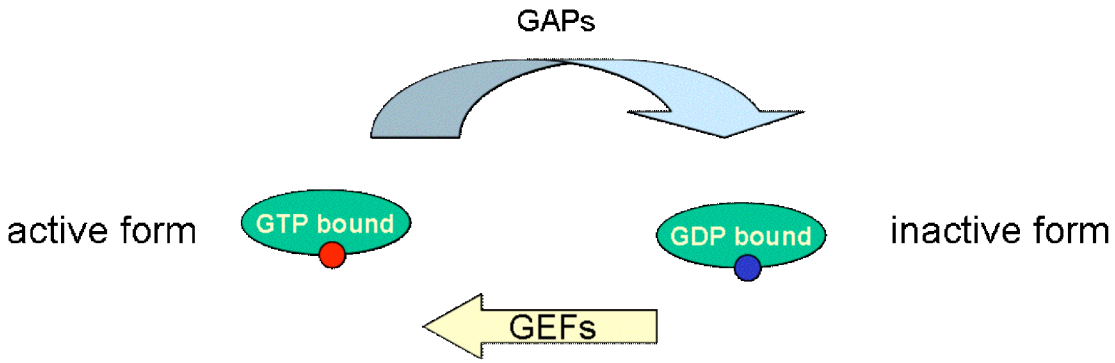


Figure 9: Schematic representation of the activation cycle of small GTPases
Small GTPases cycle between active and inactive states; GTPase specific GEF`s exchange GDP by GTP and promote the active form. The active GTP bound state allows binding of effector proteins and transduction of signals in a cascade. GTP hydrolysis is enhanced by GAP proteins that render the inactive state of the small GTPase and thus turn off the signalling cascade.

In the mammalian system Ral acts downstream of Ras (Feig et al., 1996). Several Ral GEFs have Ras interaction domains. It has been shown that human Ral is required for cell proliferation and morphologic changes during oncogenic transformation triggered by Ras (Urano et al., 1996). Ral has also been implicated in mammalian cells in control of receptor mediated endocytosis, induction of filopodia, activation of Src kinase and phospholipaseD, reorganization of the cytoskeleton etc (Frankel et al., 1999; Ladeda et al., 2001). Recent studies demonstrated that Ral is also a part of the exocyst complex (Moskalenko, 2002).

The function of Ral in *Drosophila melanogaster* is so far only fragmentary described. Ral can be activated independently of Ras (Park, 2001). By

overexpression of dominant negative and constitutive active forms of Ral, several groups demonstrated its importance for dorsal closure (see Introduction 1.1.3). In S2 cells, activated Ral prevents appearance of phosphorylated forms of Basket, the *Drosophila* Jun N terminal Kinase (Sawamoto et al., 1999). Camonis,J (unpublished) showed a genetic interaction between Ral and components of the JNK pathway with the same theoretical implication: Ral negatively regulates the JNK pathway.

3.5.3.2 Confirmation of Ral and DIAP1 binding

3.5.3.2.1 Mapping of protein interaction in the Yeast two hybrid system

Ral was identified as a potential binding partner of DIAP1 lacking the RING domain. Since shorter “bait” constructs were cloned covering sequences of different BIR domains, a simple mapping experiment was possible. Only the “bait” protein containing BIR1 interacted with Ral in the yeast (Fig.10) while no binding was detected in a parallel assay with BIR2. The experiment was also performed with the other candidate genes *actin5C*, *jitterbug* and *RPN2* with the same outcome.

The majority of experimental data so far referred to a crucial role of the BIR2 domain in inhibition of caspases and regulation of cell death. These experiments indicated that the BIR1 domain of DIAP1 might have high functional importance as well.

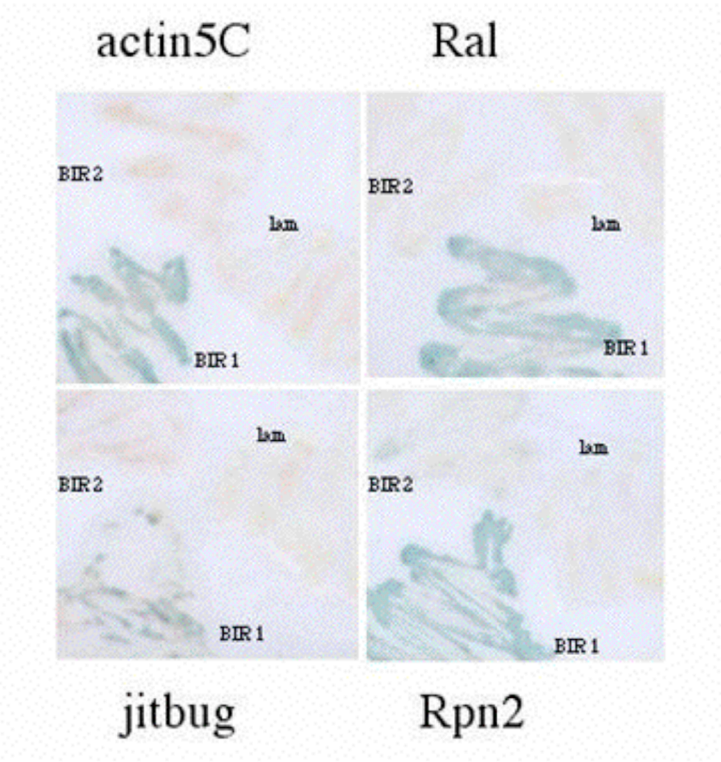


Figure 10: Ral binds to the BIR1 domain of DIAP1
Yeast two hybrid constructs containing either the BIR1 or the BIR2 domain of DIAP1 were used to confirm interaction of potential candidates. Interaction was tested with the 4 potential candidates *actin5C*, *ral*, *jittbug* and *rpn2*. The extracellular protein LamininA was used as a standard negative control. Positive interaction was tested by ß-galactosidase test resulting in blue color.of the colonies. All candidates interacted with the BIR1 domain of DIAP1 but neither with the BIR2 domain nor LamininA.

3.5.3.2.2 Analysis of Ral and DIAP1 interaction *in vitro*

Protein interactions detected in the yeast two hybrid screen need to be verified because of relative high frequency of false positive. “Bait” and “prey” proteins are produced as fusion proteins with the Gal4 transcription factor which can theoretically affect specificity of the binding. To confirm that Ral and DIAP1 bind to each other, the interaction was tested in vitro by GST pull down. Indeed, Ral binds to the BIR1 domain of DIAP1 as was shown in our lab (Kessler,T personal communication) Binding depends on activation of Ral as only activated Ral^{G20V} bound DIAP1 but not inactivated Ral^{S25N}.

To ensure that interaction indeed occurs in vivo, further studies were planned to show colocalization and binding of Ral and DIAP1. Therefore a *Drosophila* specific Ral antibody was produced. Purified GST-Ral protein was used for immunization of a rat to obtain rat-anti Ral polyclonal antiserum.

3.5.3.2.3 Ral and DIAP1 partially co localize in S2 cells

Both DIAP1 and Ral are expressed in S2 cells. To test for subcellular localization of both proteins, S2 cells were double stained with anti Ral antiserum and anti DIAP1 antibodiy. The two proteins are distributed in the cytoplasm in a punctuated fashion (Fig.11A-C). Signals of both antibodies partially overlap, arguing for colocalization of Ral and DIAP1 in specific situations (Fig.11A arrow). Nevertheless, Ral and DIAP1 often do not co-localize (Fig.11B+C)

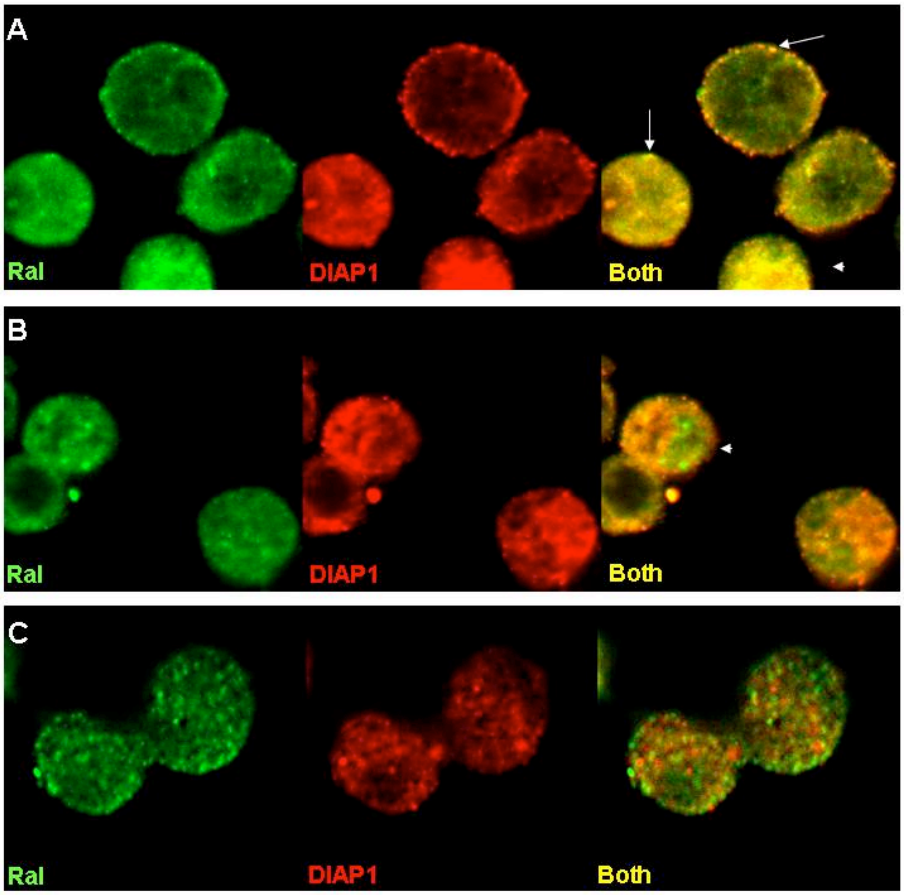


Figure 11: Partial co localization of Ral and DIAP1
Confocal images of S2 cells stained with anti Ral (green) and anti DIAP1 (red) antibodies with merged images to the right; Pictures were made from three focal planes: **A)** in the cell bases **B**) in the plane of the nucleus and **C**) from the top. Both proteins are distributed in a punctuated fashion throughout the cell and partially co-localize (arrows in **A**). Mostly they appear separated within the cell (Fig.11B arrowhead)

The staining pattern in S2 cells proved that both DIAP1 and Ral are abundantly present but just partially co-localize. This indicates that interaction of DIAP1 and Ral occurs under particular yet to be defined circumstances. This finding is further supported by the fact that coimmunoprecipitation of Ral and DIAP1 in a complex could not be obtained throughout this work.

3.5.4 Ral stabilizes DIAP1 protein levels

The binding of Ral to DIAP1 was shown in our lab by several experiments in vitro. The next step in characterization of this direct interaction was an attempt to find out its functional importance. Several theoretical models were possible, based on the structural and functional features of the two proteins. DIAP1 could be functionally upstream of Ral. In this case DIAP1`s E3 ubiquitin ligase could for example target Ral for degradation and thereby regulate Ral protein levels. That would implicate that DIAP1 down regulates the Ral signalling pathway. On the other hand the inhibitor of apoptosis could act functionally downstream in an effector pathway of the small GTPase Ral. If Ral was upstream of DIAP1 it could regulate DIAP1 function or affect its protein stability. It is known that DIAP1 undergoes autoubiquitination under certain circumstances (Yoo et al., 2002) and that Hid can promote this process. Ral could perhaps do the same and trigger apoptosis, or it could stabilize DIAP1 and thereby protect cells from apoptosis.

3.5.4.1 Loss of function Ral mutants cause decrease of DIAP1 protein levels in the follicle epithelium

To elucidate which of the given hypothesis was true, the constitutive active form of Ral (Ral^{G20V}) was overexpressed in the *Drosophila* embryo under control of the segment polarity gene *engrailed*. Increased concentration of active Ral in the stripes of *engrailed* expression should cause local stabilization of DIAP1 protein levels. Unfortunately DIAP1 is present in a high concentration in almost every cell of the embryo. Therefore, it was not possible to observe profound DIAP1 stabilization in this system (data not shown). As embryos mutant for Ral have early developmental defects, DIAP1 stability in the absence of Ral could not be analyzed (our group unpublished). Thus, definite proof that Ral is required for maintenance of DIAP1 protein levels could be obtained only by “loss of function” analysis.

The model system *Drosophila* offers a method to circumvent the problems of homozygous lethal mutations. Formation of the follicle cell epithelium at the beginning of oogenesis is accompanied by a series of mitotic divisions (see Introduction for details). The FRT/Flp system (see Material and Methods) can be used to induce mitotic recombination during development of the follicle epithelium to create local populations of cells with different genotypes. Cells that become

homozygously mutant for an allele of interest give rise to a clonal patch of follicle cells with the same genotype. Mutant clones can be identified by lack of anti GFP staining which is a marker for wild type chromosomes in this technique.

The FRT/Flp method was used to create cells homozygously mutant for the *Ral*^{70} "null" allele (Fig.12). When these ovaries were stained with anti-DIAP1 antibody it became visible that DIAP1 signal disappears in the cells that lack Ral. Notably, not all mutant cells exhibit the same DIAP1 concentration after removal of Ral. That indicates that degradation of DIAP1 takes some time to be completed. This confirms the model that puts Ral upstream of DIAP1. It can be concluded that Ral is required for stabilization of DIAP1 protein levels in vivo.

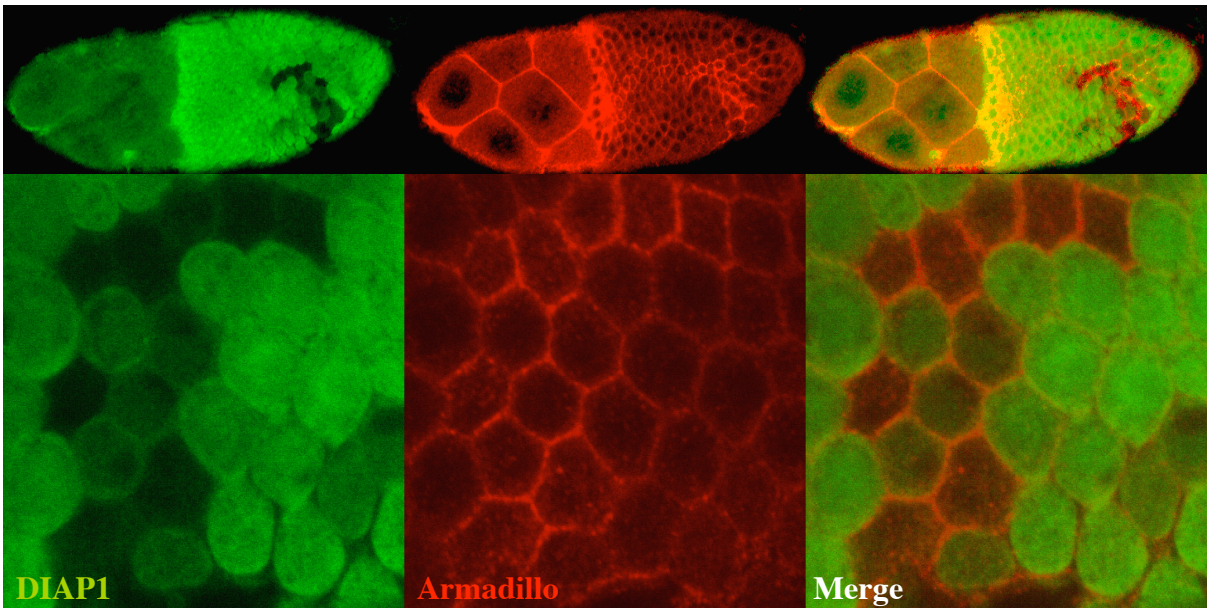


Figure 12: Ral stabilizes DIAP1 protein levels in vivo
A stage 10 egg chamber containing a follicle cell clone mutant for the null allele *ral*^{70}. Staining with an antibody against DIAP1 (green) shows reduced DIAP1 protein levels in the region of the clone. Cell outlines are marked by anti Armadillo staining (red). In the cells lacking DIAP1 Arm seems to accumulate at cell junctions. Merged pictures are shown to the right.

The functional connection between Ral and DIAP1 in vivo is supported by in vitro biochemical evidence (Kessler,T personal communication). A cell free assay confirmed that Ral stabilizes DIAP1 in a dose dependent manner. DIAP1 is strongly polyubiqitinated after 30 min in this system, giving rise to a characteristical laddering pattern on the western blot. If GST-Ral is added to this system, appearance of ubiquitinated DIAP1 forms is reduced. Addition of GST alone does not have any effect on DIAP1 protein stability.

At this point it can be concluded that Ral physically interacts with DIAP1 and that this interaction is required for the stabilization of DIAP1 protein levels *in vivo*.

3.5.4.2 Ral stabilization of DIAP1 suppresses Reaper induced apoptosis in the eye

DIAP1 is crucial for cell survival of most *Drosophila* tissues (Hay et al., 1995). A widely used model system for testing functional interactions of players in apoptosis in vivo is the *Drosophila* compound eye. When proapoptotic genes are overexpressed in the eye under control of the GMR promoter, massive apoptosis occurs resulting in much smaller and rough eyes. Two types of screens can be performed to search for genes that modify this phenotype. In both cases the gene dose of putative candidates is modulated. In the modifier screen using chromosomal deficiencies, “loss of function” of the potential candidate alters the phenotype, while a modifier screen using EP lines of candidate genes detects “gain of function” effects of gene overexpression with the help of the Gal4/UAS system (Brand and Perrimon, 1993).

In a modifier screen with deficiencies to find genes that modulate Reaper induced cell death in the eye, Kuranaga et al. (2002) obtained a strong rescue with the deficiency *Df(2L)sc19-8*. Subsequently the genomic region was narrowed down to the single gene *dtraf*. DTRAF is the adaptor protein which activates the Jun kinase signalling pathway in response to activation of the TNF receptor (Tumor Necrosis Factor). Rescue of the Reaper induced rough eye phenotype by reducing DTRAF function implied that the JNK pathway is required for induction of apoptosis. It was demonstrated in vitro that DIAP1 targets DTRAF for proteasomal degradation. When Reaper is expressed, the levels of DIAP1 are reduced and consequently DTRAF levels are stabilized. This leads to activation of downstream Jun kinases and triggers apoptosis. These experiments showed that a stable level of DIAP1 is necessary for inhibition of JNK induced cell death.

Analysis of the in vivo function of Ral in follicle cells showed that the GTPase is required for stabilization of DIAP1 protein levels in this particular tissue (see previous chapter). To further test whether the stabilizing effect represents a general mechanism in other tissues, constitutive active Ral^{G20V} was coexpressed with GMR::*reaper* in the eye. When Reaper is ectopically expressed, DIAP1 levels

decrease and massive apoptosis causes a small, rough eye phenotype (Fig.13A). The proapoptotic effect of Reaper is partially inhibited due to stabilization of DIAP1 in the presence of active Ral (Fig.13B) resulting in a slightly bigger eye. However, Ral maintains only existing DIAP1 levels and therefore the rescue is not as complete as in the case of DIAP1 overexpression. DIAP1 overexpression in GMR::*reaper* background completely prevents cell death as it blocks caspases and inhibits the JNK pathway at the same time. The resulting eye has almost wild type morphology (Fig.13C).

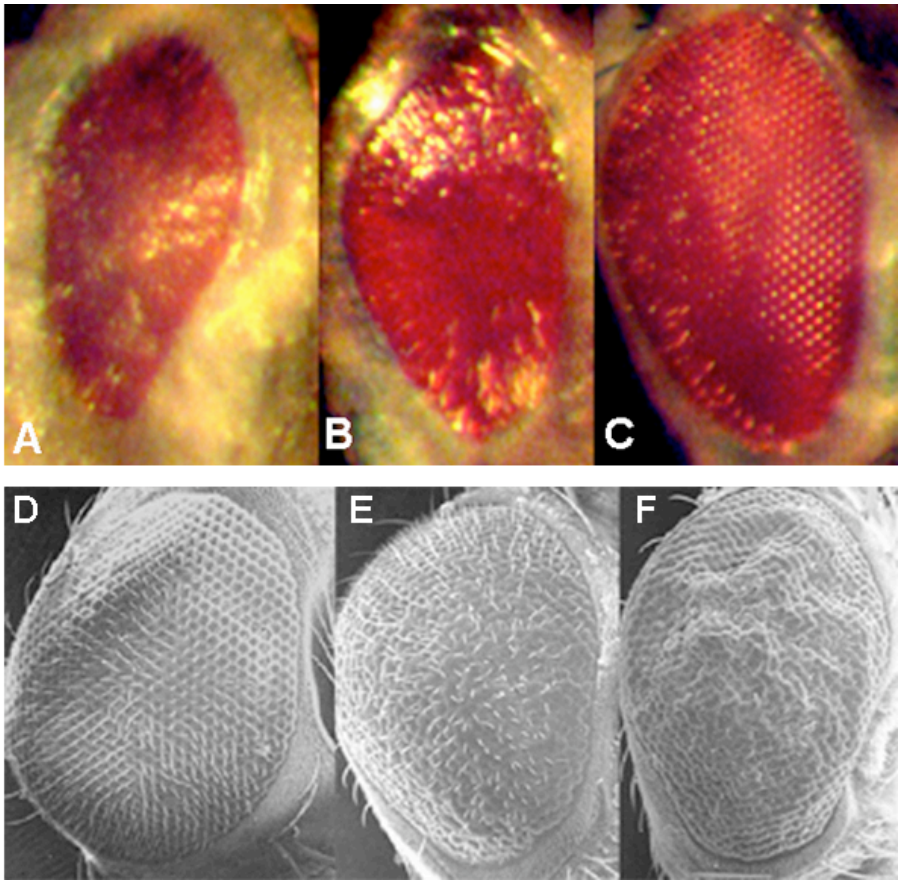


Figure 13 Activated Ral^{G20V} suppresses Reaper induced cell death in the eye
A-C Color pictures of Drosophila compound eyes with the following phenotypes: **A**) UAS::*reaper* expressed in the eye under control of the GMR::*Gal 4* promoter induces apoptosis and reduction in organ size **B**) Constitutive active form of Ral (Ral^{G20V}) partially suppresses GMR::*reaper* induced phenotype **C**) Expression of UAS::*DIAP1* completely rescues the phenotype of GMR::*reaper*. **D-F** SEM images of eyes with the following phenotypes **D**) GMR::*Gal4* expressed in WT eye at 29 °C causes mild changes in morphology **E**) Overexpression of dominant negative Ral^{S25N} causes rough eye phenotype and partial loss of bristles **F**) Expression of constitutive active Ral^{G20V} shows the strongest phenotype, hexagonal pattern is disturbed, ommatidia are fused, bristle loss is substantial.

To exclude the possibility that Ral rescues *reaper* induced cell death as a result of a GTPase autonomous pathway, Ral was overexpressed in the wild type eye. Both, dominant negative and constitutive active forms of Ral caused a mild rough eye phenotype (Fig.13 E+F). Increased levels of Ral, especially in its active form, lead to

disruption of ommatidial hexagonal pattern and accompanying bristles were missing. Interestingly, data from other research groups indicates that Ral might play a role in regulation of planar cell polarity in the eye (Mlodzik, M, unpublished) resulting in a similar phenotype.

Therefore it can be concluded that rescue of Reaper induced cell death is due to Ral stabilizing the function of DIAP1 and not caused by activation of an alternative apoptosis inhibitory pathway. Ral regulation of DIAP1 protein levels seems to be a general cellular mechanism rather than a tissue specific event restricted to the follicle epithelium.

3.5.5 Ral is required for morphogenetic movements of epithelia during oogenesis

3.5.5.1 Reduced level of Ral causes defects in centripetal cell migration

Previous experiments gave substantial insight into functional implications of the Ral-DIAP1 interaction. Rescue of Reaper induced cell death in the eye demonstrated that Ral has the potential to reduce apoptosis by stabilizing DIAP1. Nevertheless, this just proved that stabilization of DIAP1 through Ral was possible but it did not show when and where in development that happens. So, there was still the need to place this interaction into a broader biological context.

To find developmental processes that depend on a functional Ral-DIAP1 interaction, the phenotype of Ral mutants was evaluated. Embryos homozygous for the *ral*^{70} “null” allele die early during embryogenesis but embryos homozygous for the r*al*^{89} hypomorphic allele survive and develop into adult flies. The latter allele does not carry any mutations in the coding region of the gene. A fragment of upstream regulatory sequence is missing which causes reduction in gene expression (Camonis,J personal communication). As a result of the hypomorphic mutation males that are homozygous for *ral*^{89} exhibit a loss of thorax bristles, and the females are sterile.

Sterility of *ral*^{89} homozygous females indicated a role of Ral during ovary development. In order to find out what specific defect leads to sterility, homozygous

ral^{89} mutant egg chambers were analyzed in detail. Prior to characterization of the mutant phenotype extensive study was performed on wild type egg chambers.

3.5.5.2 *D*E-Cadherin is strongly upregulated in centripetal cells of wild type ovaries

Development of the wild type *Drosophila* egg chamber is a good example of coordinated morphogenetic movements. The follicle epithelium, a cell monolayer of somatic origin, undergoes continuous migratory processes during oogenesis in close contact to the underlying germ line (see Introduction for details). The most prominent migratory events in mid oogenesis are border cell migration and centripetal cell invagination. It is known that movement of these epithelial sheets depends on the cell adhesion molecule *D*E-Cadherin. In the absence of *D*E-Cadherin (in *shg* mutant follicle cell clones) migration stops (Niewiadaomska et al., 1999). To observe migratory features of the follicle cell epithelium in wild type chambers a monoclonal anti *D*E-Cadherin antibody was used. Additionally, ovaries were stained with anti Armadillo (Arm) antibody. Strong up regulation of *D*E-Cadherin can be observed in the border cell cluster and in the domain of centripetal cell migration (Fig.14 A+B). First cell shape changes in the region of centripetal cells coincide with arrival of the border cells to the anterior of the oocyte. On the subcellular level, *D*E-Cadherin redistributes along lateral membranes (arrows in Fig.14A magnification). Centripetal cells then extend, form protrusions and begin inward migration (Fig.14B magnification). The movement continues until the oocyte is completely separated from the nurse cells.

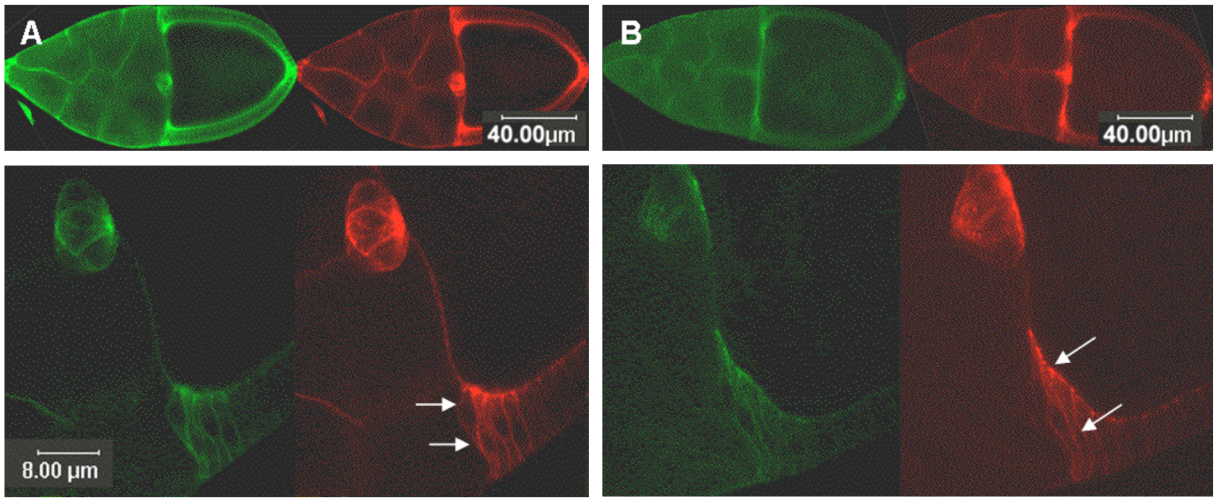


Figure 14 Wildtype egg chambers exhibit a series of genetically controlled migration processes
Confocal images of egg chambers stained with anti Armadillo (green) and anti *D*E-Cadherin (red) antibodies **A**) Stage 10a egg chamber, Arrow in the magnification points to cell shape changes of centripetal cells and *D*E-Cadherin accumulation at lateral membrane sites **B**) Stage 10b egg chamber, centripetal cells are elongated and cells migrate inwards. Arrows point to *D*E-Cadherin accumulation in the leading edge cells.

3.5.5.3 Reduced level of Ral causes migratory defects during oogenesis

Since reduced levels of Ral cause sterility in females, it was interesting to see if migration processes are affected in mutant egg chambers. Ovaries of females homozygous for r*al*^{89} *were* dissected and stained with anti *D*E-Cadherin antibody. Several types of aberrant morphogenetic movements of the follicle epithelium were observed (Fig.15). The most prominent defect was the absence of strong accumulation of *D*E-Cadherin in the migratory cells during mid oogenesis. Although, according to the size of the oocyte and the position of the border cells, the chamber reached the late developmental stage 10, centripetal cells did not elongate (Fig.15A). A high magnification of the centripetal cell domain revealed that the level of *D*E-Cadherin is not increased and the protein remains localized to the ZA (Fig.15C).
Border cells in the wild type chambers begin to migrate at the beginning of stage 9, when the oocyte enters a phase of intensive growth. At this stage the follicle cells surrounding the oocyte undergo a transition from cuboidal to columnar shape. Interestingly, a subset of r*al*^{89} mutant chambers with a very small oocyte and a follicle epithelium that did not retract to the posterior site of the egg chamber had border cell clusters well advanced in migration between nurse cells (Fig.15B). At this

developmental stage the border cell cluster is about to delaminate from the anterior tip during wild type oogenesis.

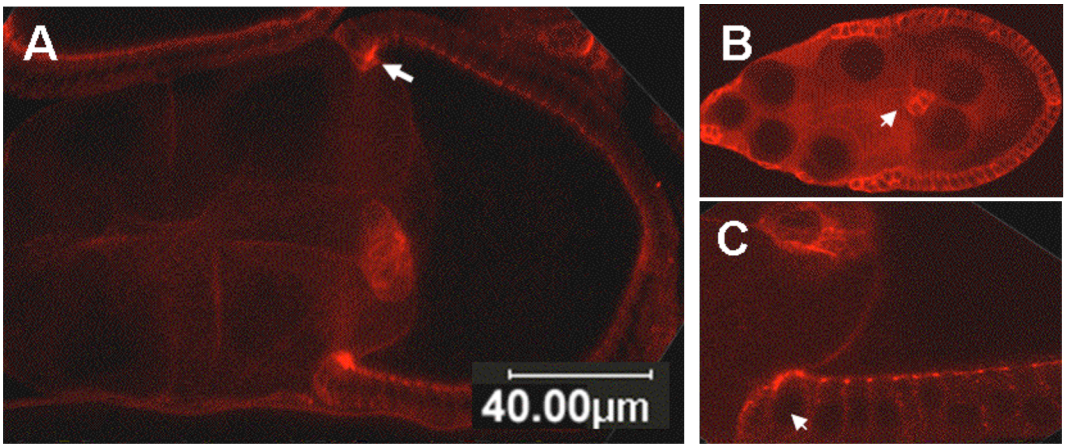


Figure 15 Egg chambers with reduced levels of Ral show migration defects

Confocal images of antibody stainings against *D*E-Cadherin (red in **A** and **C**) and spectrin (red in **B**). **A**) Size of the chamber indicates stage 10 of development but centripetal cells are not invaginating **B**) Small egg chamber, early stage 8; border cells reside in the middle of the chamber (arrow) **C**) Magnification of the centripetal cells shows no significant accumulation of *D*E-Cadherin in ral^{89} mutant egg chambers.

3.5.5.4 Reduced *ral* dose affects centripetal cell migration

The data shown in the previous chapter suggested that Ral is required during oogenesis for the regulation of morphogenetic movements of the follicle epithelium rather than in the germ line. This is supported by the fact that flies that have wild type amount of Ral in the follicle epithelium but carry two alleles of ral^{89} in the germ line are fertile (data not shown). Embryos that have reduced maternal component of the *ral* gene show strong defects during cellularization. Phenotypes of ral^{89} homozygous egg chambers indicate that the Ral protein level in the follicle epithelium is of crucial importance for regulation of proper migratory movements. To test this model chambers heterozygous for the Ral “null” allele ral^{70} could be susceptible to defects in oogenesis as well.

In order to confirm the dose requirement for Ral in migratory processes females heterozygous for the null allele *ral*^{70} were analysed. r*al*^{70}/Fm7 females were crossed with wild type males to eliminate potential effects of the Fm7 balancer chromosome.

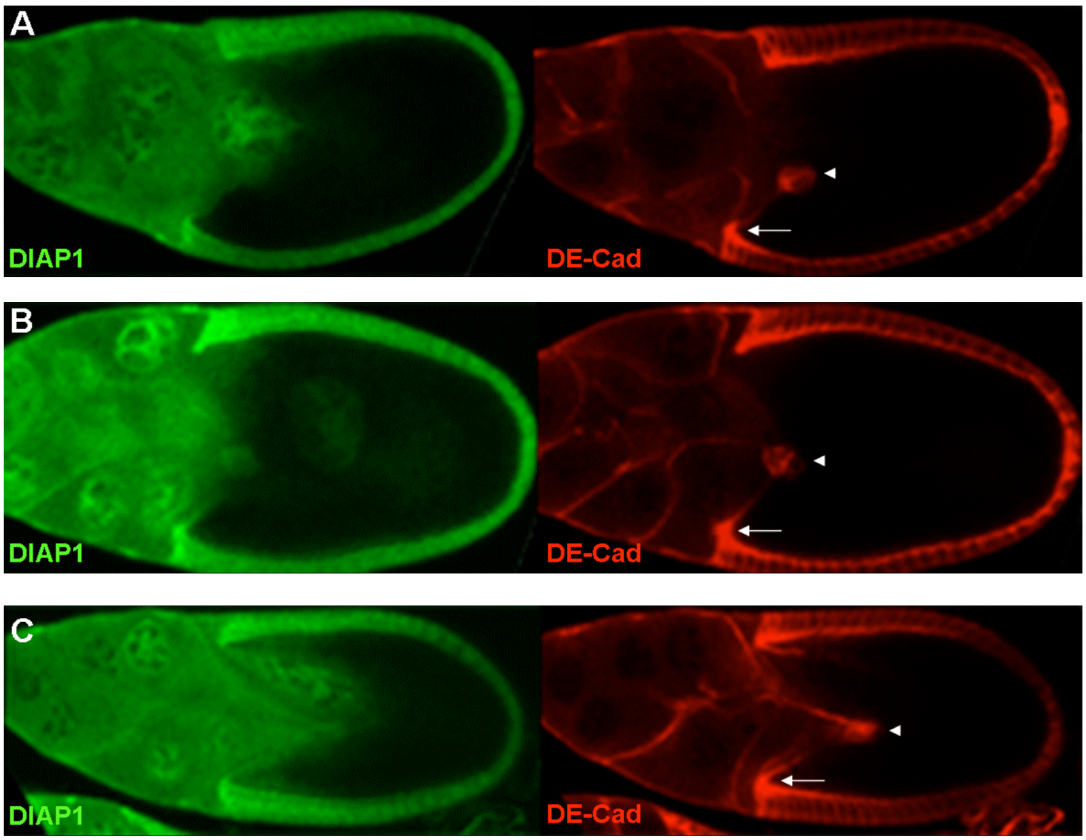


Figure 16: Reduced Ral function causes oogenesis defects

Examples of mutant egg chambers heterozygous for the null allele r*al*^{70} stained with anti-DIAP1 (green) and anti *D*E-Cadherin (red) antibodies; **A-C** Three egg chambers that reflect the phenotype of the *ral*^{70} mutation. Border cells remain in the tip of the nurse cells (arrowheads); centripetal cells do not elongate and migration does not take place (Arrows).

Ovaries of heterozygous *ral*^{70} females were dissected and stained for *D*E-Cadherin and DIAP1 (Fig.16A-C). Centripetal cell elongation did not take place in ~20% of late stage 10 egg chambers. *D*E-Cadherin appeared upregulated in centripetal cells but it was hard to conclude if the protein level corresponds to the wild type situation. In the absence of centripetal migration the oocyte and the nurse cells remained connected.

It was concluded that half of the dose of the *ral* gene is not sufficient to maintain its function during morphogenetic movements in the follicle epithelium. As a component of a signalling cascade Ral activates downstream targets. It cycles between active and inactive state and the cellular amount of the GTP bound form is a

result of upstream signalling events. If there is not enough Ral produced in the first place, transduction of signals to the Ral effector pathways might be occasionally interrupted. This could explain the aberrant morphogenesis observed in heterozygous *ral*^{70} mutants.

3.5.5.5 Complete loss of Ral in follicle cells causes changes in adhesion

Characterization of Ral mutant phenotypes revealed that centripetal cell migration depends on Ral signalling. In the experiments above both the germ line cells as well as the follicle cell epithelium were mutant for Ral. Thus, the specific cellular defect within the follicle cells in response to reduced Ral signalling had to be elucidated. Characterization of cellular changes that develop in the complete absence of Ral was supposed to give more conclusive evidence. Therefore, large r*al*^{70} mutant follicle cell clones were induced and changes in cell adhesion and modulation of the actin cytoskeleton analysed. For the latter experiment, fluorescent Phalloidin was used to stain F-Actin. In *ral* mutant cells the level of *D*E-Cadherin was lower than in the twin clones which carry two wild type copies of *ral* (Fig.17). It was observed that *D*E-Cadherin was still localized to the ZA as judged by its hexagonal staining pattern. A transversal view of the follicle epithelium revealed that in some cells the ZA localization of *D*E-Cadherin disappeared, leading to the formation of a multi layered tissue (Fig.17 arrows in 17B). These findings argue for an involvement of Ral in the maintenance of cell polarity but that is yet to be confirmed by analysis of specific cell polarity markers. Additionally, follicle cells mutant for Ral had a disorganized cytoskeleton visualized by phalloidin.

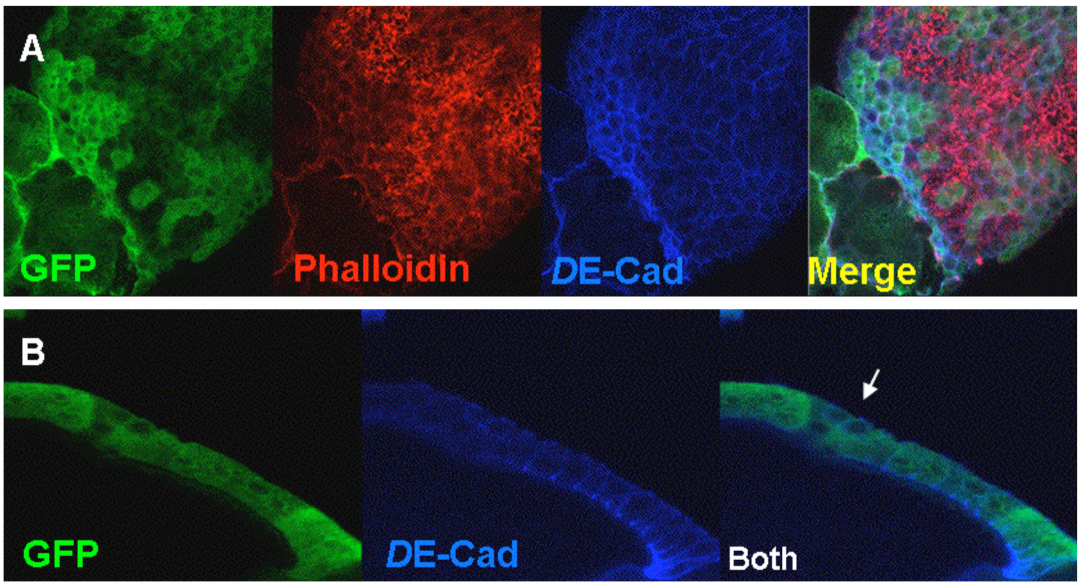


Figure 17 Large clones of *ral*^{70} mutant cells have severe cytoskeleton defects and lower level of *D*E-Cadherin

A) Surface view on a large r*al*^{70} mutant cell clone; Mutant cells are marked by lack of GFP expression (green). *D*E-Cadherin staining (blue) is reduced in the mutant cells and actin fibres are disorganized (red phalloidin) **B**) Side view on the follicle epithelium containing *ral* mutant cells marked by lack of GFP (green); *D*E-Cadherin signal (blue) is missing in some cells and a multilayered tissue forms (arrow)

Phenotype of the *ral*^{70} mutant follicle cell clones implied that the protein is required for control of adhesion mediated by *D*E-Cadherin within the epithelium. It remains to be investigated whether the observed cytoskeleton defect occurs due to loss of Ral or whether it represents a secondary effect of cell shape changes and a loss of cell polarity.

3.5.5.6 Overexpression of Ral^{G20V} in the centripetal cells accelerates cell migration

Migratory processes during mid oogenesis, border cell migration and centripetal cell migration have many similarities. One of the most important characteristic that they have in common is expression of the gene *Slow border cells (slbo)*. *Slbo* encodes a bZIP transcription factor (Montell et al., 1992) which drives expression of many genes important for migration such as the FGF-receptor *breathless* and *shotgun* encoding for *D*E-Cadherin. Expression of *slbo* is required for

border cell migration from stage 9 on and in the region of centripetal cells from stage 10a.

To investigate any effects on the cell migration in stages 9 and 10 that arise from an increased concentration of activated Ral, the Gal4/UAS system was used. With this method, it is possible to overexpress any gene in a strictly controlled fashion, at any particular time point of development and in a specified group of cells. In this case, a driver line was obtained which produces the yeast transcription factor Gal4 under control of the *slbo* promoter. This driver was crossed to flies carrying *UAS::Ral*^{G20V}.

As shown in previous experiments, low levels of Ral in the follicle epithelium negatively influence morphogenetic movements. Conversely, overexpression of constitutive active Ral^{G20V} led to enhanced migration in the centripetal cell region. In comparison to the wild type chamber in the same developmental stage, centripetal cells that overexpress Ral appear to be in more advanced phase of migration. (Fig.18). This phenotype further supports the hypothesis that Ral positively regulates transition of epithelial into migratory cells.

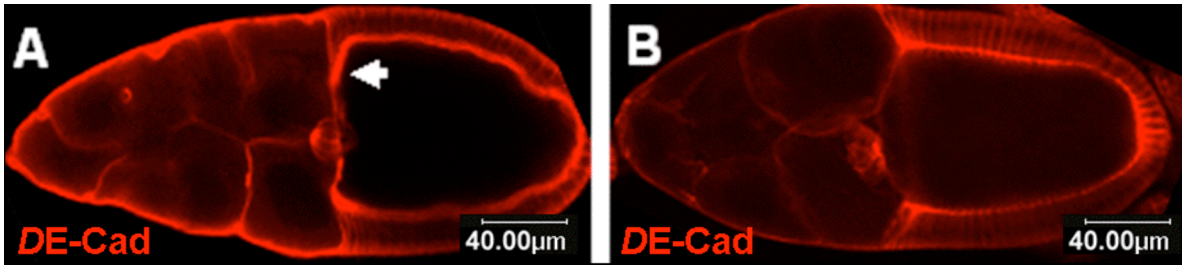


Figure 18 Expression of dominant mutant Ral causes accellerated centripetal cell movement
A) Egg chamber resulting from the cross Ral^{G20V}xslboGal4 stained with *D*E-Cadherin (red) is in more advanced phase of centripetal cell migration than comparable WT chamber **B)**

3.5.6 DIAP1 is required for morphogenetic movements in the ovary

As it was shown in the previous chapters, centripetal cell migration is sensitive to changes in functional Ral levels. Reduced amount of Ral, as in the case of *ral*^{89} homozygous egg chambers, causes inhibition of cell shape changes and movement in the centripetal domain. The phenotype of *ral*^{70} mutant follicle cells suggests that these defects might occur due to changes in cell adhesion as *D*E-Cadherin seems to be affected by the absence of Ral.

On the other hand, Ral stabilizes DIAP1 protein levels. The turnover rate of DIAP1 is increased in a *ral* mutant background due to enhanced autoubiquitination. Therefore an important question to answer was: Is DIAP1 involved in the control of morphogenetic movements in the follicle epithelium? The follicle cell epithelium is the only tissue in *Drosophila* in which DIAP1 is dispensable for cell survival. Thus, the developing egg chamber provides an excellent model system to study non-apoptotic functions of DIAP1. Experiments can be performed in a DIAP1 mutant background without a problem of imminent cell death in the tissue. Therefore, it was decided to investigate the potential role of DIAP1 as a downstream effector of Ral in the regulation of centripetal cell migration.

3.5.6.1 DIAP1 is abundantly present during oogenesis

DIAP1 is ubiquitously expressed, but is also differentially distributed during embryonic development. To reveal where DIAP1 is localized during oogenesis, double staining of wild type egg chambers was performed with anti DIAP1 and anti *D*E-Cadherin antibodies. Confocal imaging showed that DIAP1 is strongly accumulated in the nuclei of centripetal cells. Especially, a strong DIAP1 signal was seen in the cells that form the centripetal cell leading edge (Fig.19). In these cells DIAP1 is sequestrated to the nucleus but some protein appears also to be associated with the apical membrane (Fig.19 arrow). Notably, DIAP1 is present in high concentration in the germline as well, where it localizes to the oocyte nucleus and to the nuclei of the nurse cells.

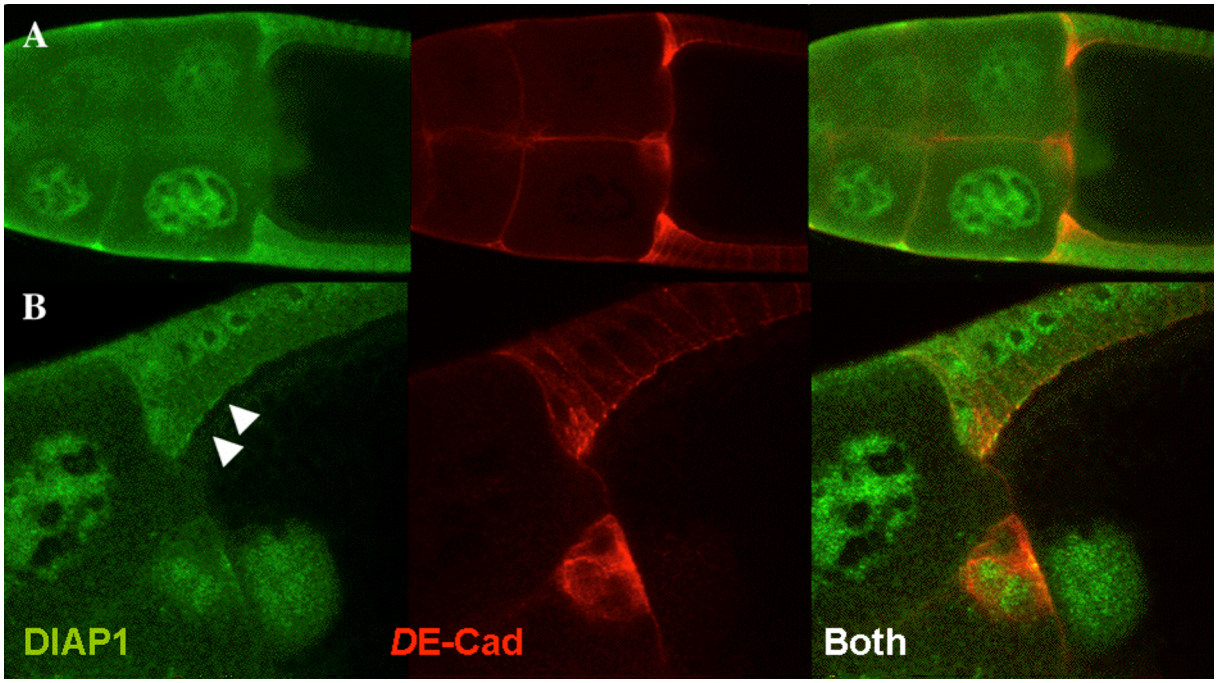


Figure 19: DIAP1 is abundantly present in all cells in the developing egg chamber

A) Confocal image of a wildtype egg chamber stage 10a; DIAP1 protein (in green) is present in the germline and in the follicle epithelium **B**) Magnified image of centripetal cells; DIAP1 locates to the nucleus and to the apical membrane (arrowheads); In red: *D*E-Cadherin.

3.5.6.2 Mutations in DIAP1 cause defects in oogenesis

The DIAP1 protein is abundant in the follicle cell epithelium, as it was shown by antibody staining in the wild type ovary. Migrating centripetal cells exhibit elevated levels of DIAP1 in their nuclei and the caspase inhibitor is associated with the apical membrane. Since the protein is strongly upregulated in the centripetal cells it was expected that it is functionally required in this tissue. To test whether DIAP1 levels influence migration of the centripetal cells, ovaries that have only half of the DIAP1 gene dose were dissected.

Quantitative analysis of the ovaries from heterozygous th^{109}/+ females showed that 34% of stage 10 chambers had an abnormal phenotype. Characteristics of the defect chambers were very similar to the ones seen in the case of reduced Ral function. Judged by the oocyte size and the flattened follicle epithelium the egg chamber represents stage 10b of development but no centripetal migration occurred (Fig.20). In comparison to the same stage wild type chamber it is obvious that centripetal cells did not elongate to the same extent. High magnification of the centripetal cell domain shows that *D*E-Cadherin is localized to the ZA and is not distributed along the lateral membranes.

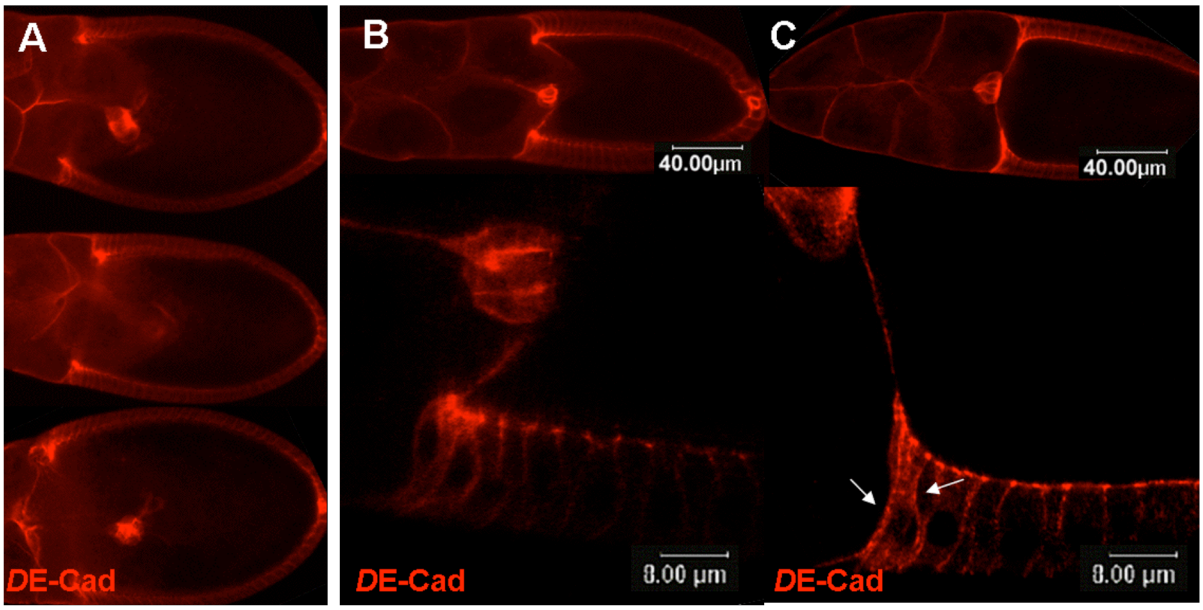


Figure 20: Centripetal cell migration is inhibited as a result of reduced DIAP1 expression

A) Examples of mutant egg chambers heterozygous for th^{109} stained with anti *D*E-Cadherin (red) **B**) th^{109}/+ stage 10b ;*D*E-Cadherin (red) remains localized to the ZA in **C**) WT stage 10 b; Centripetal cells elongated, lateral distribution of *D*E-Cadherin (red) marked by arrows.

The result indicates that stable levels of DIAP1 are as well required for proper regulation of migratory processes during mid oogenesis. At the subcellular level changes were detected in *D*E-Cadherin distribution similar to the Ral mutant situation. This could mean that Ral is needed to maintain DIAP1 protein levels in migratory cells and DIAP1 then regulates their adhesions properties. To confirm this model the phenotype of follicle cells completely lacking DIAP1 needed to be described.

3.5.6.3 th^{109} mutant follicle cells show low levels of *D*E-Cadherin

Since the DIAP1 null allel th^{109} is homozygous lethal, effects of DIAP1 removal in the follicle cells were analysed by inducing homozygous mutant cell clones. Ovaries were stained with three antibodies: anti-GFP to detected mutant cell clones, anti DIAP1 to verify that DIAP1 protein was absent and anti-*D*E-Cadherin. Follicle cells homozygous for th^{109} exhibit reduced *D*E-Cadherin levels in a cell autonomous fashion (Fig.21). *D*E-Cadherin is a component of the ZA and therefore an apical surface view of the follicle epithelium gives a hexagonal so called “honey comb”

staining pattern. Induced clones, marked by absence of GFP and DIAP1 signal, show nearly complete loss of *D*E-Cadherin from the membrane.

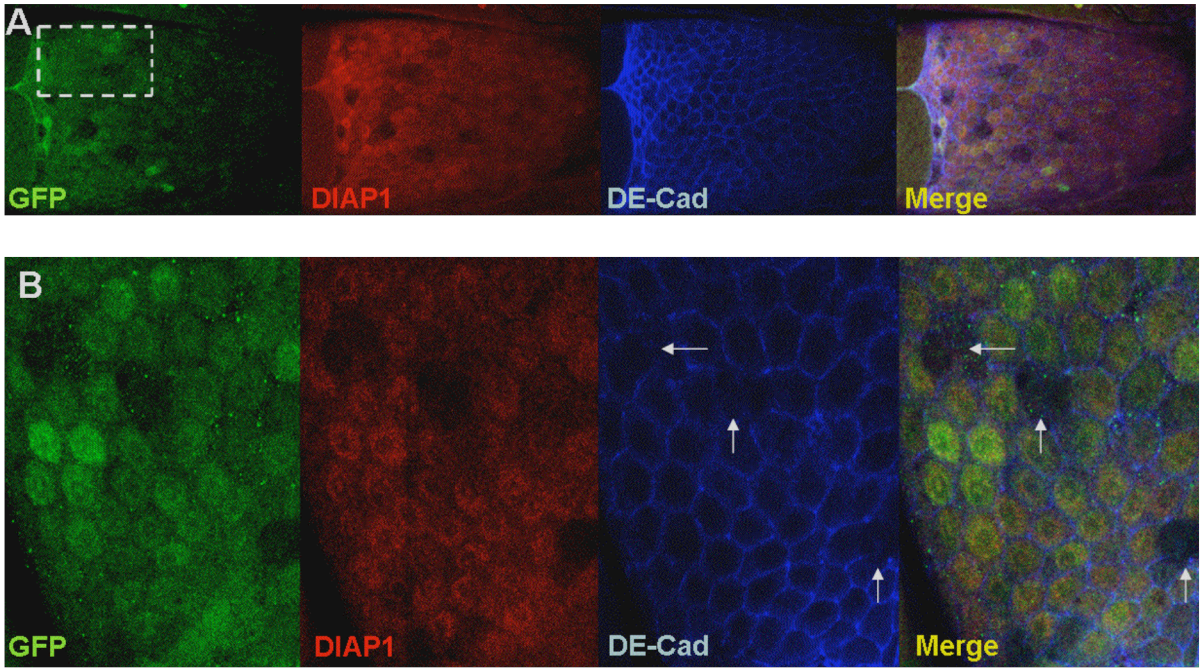


Figure 21: *th*^{109} mutant follicle cell clones exhibit loss of *D*E-Cadherin

Clonal cells homozygous mutant for the *thread* null allele *th*^{109}, are marked by absence of GFP staining (green). Absence of DIAP protein in the mutant cells is shown with anti DIAP1 staining (red). *D*E-Cadherin is seen in blue. **A**) Surface view on follicle epithelium; relatively small clones are induced in several regions of epithelium **B**) Close up of one area containing clones; Cells that are missing DIAP1 protein (red) show irregular *D*E-Cadherin signal (blue). *D*E-Cadherin is not found at the membrane contact sites of cells lacking DIAP1 with wildtype cells as marked by the arrows in ***B*.** As a result the hexagonal pattern characteristic for this adhesion molecule is clearly missing in the clones (arrows).

To confirm a function of DIAP1 during follicle cell migration, large clones in the region of the centripetal cell formation would be the most informative. Unfortunately, all induced *th*^{109} mutant clones were small and numerous, which can also be seen in the previous figure. No detected clone was bigger than 3-4 cells, and it was impossible to cover the complete centripetal domain.

Nevertheless, analysis of smaller DIAP1 mutant clones in the region of interest was sufficient to support the hypothesis that DIAP1 regulates *D*E-Cadherin in centripetal cells. While *D*E-Cadherin is strongly upregulated in the centripetal cells of wild type egg chambers, a single-cell clone mutant for DIAP1 shows strongly reduced *D*E-Cadherin in a leading edge cell (Fig.22B).

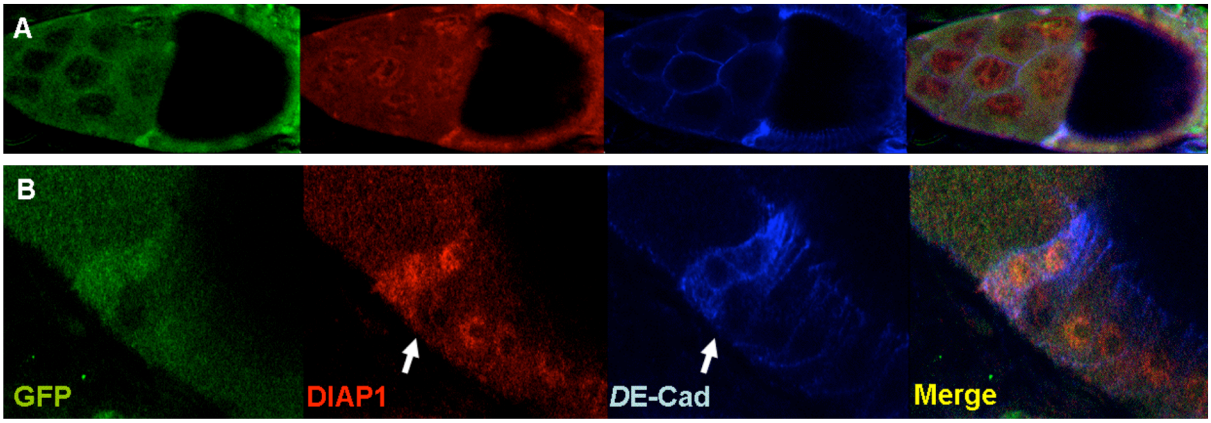


Figure 22: Upregulation of *D*E-Cadherin is missing in centripetal cell clones of DIAP1

A) Egg chamber in the stage 10a; follicle epithelium containing th^{109} mutant clones. **B**) Higher magnification of the region of centripetal cell migration which contains a one-cell clone (arrow). Shape of the mutant cells differs from elongated neighbouring cells on the left as well as from the epithelial cells on the right and *D*E-Cadherin signal (blue) is almost completely absent.

Thus, follicle cell clones mutant for DIAP1 confirmed the causal relationship between the protein level of the caspase inhibitor and *D*E-Cadherin. This proofs that DIAP1 is required for regulation of cell adhesion in the follicle epithelium and thereby probably influences transition from epithelial into migratory cells.

3.5.7 DIAP1 controls follicle cell migration through inhibition of the JNK pathway

The results, presented so far indicate that stable protein levels of DIAP1, maintained by Ral are of critical importance for the morphogenetic movements in the *Drosophila* follicle epithelium. As it was previously described, one of the proteins that are a target of DIAP1 ubiquitin ligase is DTRAF, an upstream activator of Jun kinase cascade.

Additionally to JNK dependent apoptosis, the Jun kinase pathway participates in many different types of cellular responses (see Introduction). Its well documented role in cytoskeletal rearrangements makes it a good candidate to be responsible for at least part of the changes in the follicle epithelium that occur when DIAP1 protein level is reduced. By targeting DTRAF for degradation, DIAP1 acts as a general inhibitor of the JNK pathway because it prevents transduction of the signal generated by active TNF receptor (Kuranaga et al., 2002). Stable protein levels of DIAP1 in the follicle epithelium, would then constantly suppress the amplitude of JNK signalling. When DIAP1 is gone or reduced, levels of DTRAF protein could be stabilized and

TNF receptor mediated signalling is transmitted without interruption. It has indeed been shown that balanced levels of activity of the JNK pathway are crucial during oogenesis (Dobens et al., 2001). Either too high or insufficient levels of JNK signalling cause defects in oogenesis. Based on these findings it was postulated that loss of DIAP1 leads to ectopic JNK activation and thereby negatively influences centripetal cell migration. If this hypothesis was true, mutations affecting components of Jun kinase should improve the phenotype of DIAP1 mutants.

3.5.7.1 Silencing the Jun kinase basket modifies the *thread* mutant phenotype

In line with the obtained results, the model was postulated that excessive activity of JNK signalling pathway in response to low DIAP1 levels causes defects in centripetal cell migration. To test this model it was necessary to characterize the effects that mutations in components of the JNK pathway have on centripetal cell migration.

First the phenotype of *basket (bsk)* mutants was examined. *Bsk* encodes the Drosophila Jun N terminal Kinase and *bsk* mutants have reduced levels of JNK signalling. Point mutations in the *bsk* gene are homozygous embryonic lethal. To test whether there was a dose dependent requirement for components of the JNK cascade for centripetal cell migration, ovaries of females heterozygous for *bsk* mutant alleles were dissected. Antibody staining was performed like in the previous cases using *D*E-Cadherin and Armadillo antibodies. Quantitative analysis of all stage 10 egg chambers showed that reduction of *bsk* gene dose causes an aberrant phenotype in 25% of the egg chambers. (Fig.23B)

If the observed defects in *thread* mutant egg chambers originate from increased activity of JNK, the *thread* phenotype should be suppressed when components of the JNK signalling pathway are mutated. Ovaries were dissected from trans-heterozygous females that carry both th^{109} and bsk^{1} mutations. The quantitative analysis of the resulting phenotype is summarized in the histogram (Fig.23A). The number of egg chambers that do not successfully complete centripetal cell migration due to reduced levels of DIAP1 is decreased if one copy of bsk^{1} is introduced. This argues in favour of the formulated hypothesis that DIAP1 and Bsk are in the same genetic pathway. bsk^{1}/+ ovaries contain 25% of chambers with defect centripetal cell

migration while *th*^{109}/+ ovaries contain 34%. In combination, both alleles compensate each other and the number of eggs with irregular centripetal cell migration falls to 16%. In relative terms that is improvement of the DIAP1 mutant phenotype by 30-40%.

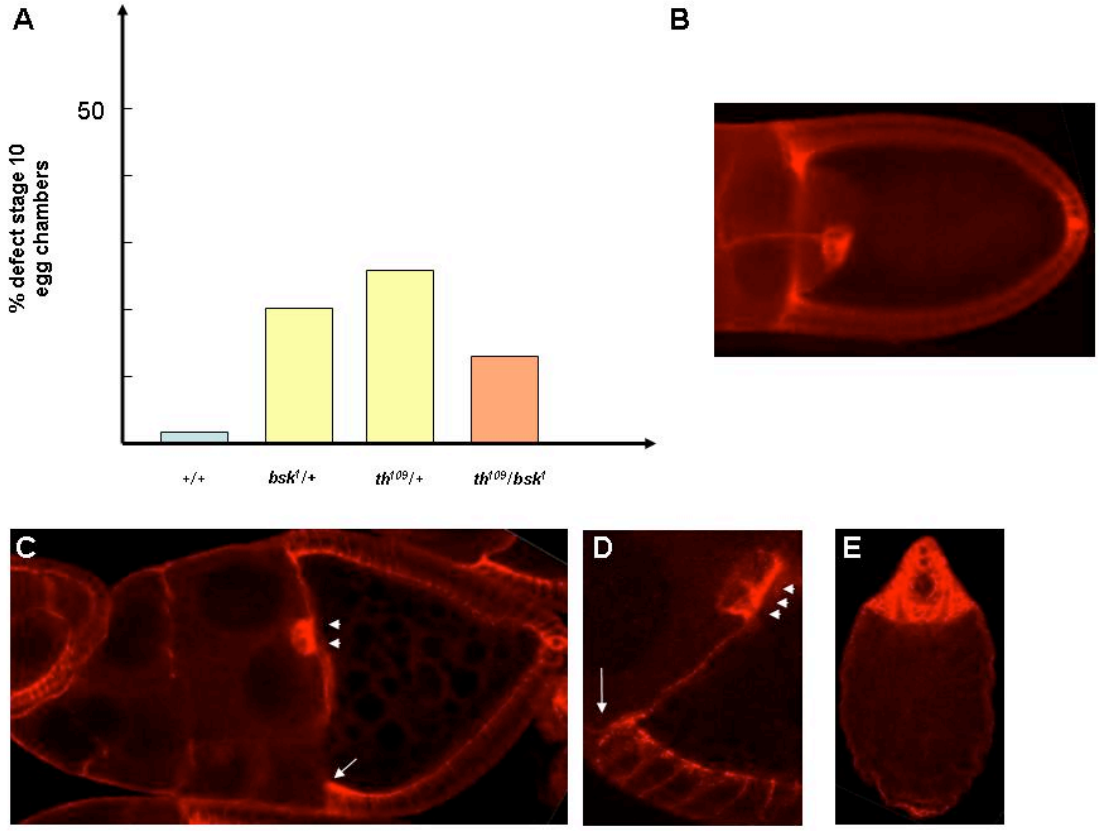


Figure 23 DIAP1 and the Jun-N-terminal Kinase build a signalling pathway during oogenesis

A) Histogram shows frequencies of abnormal stage 10 egg chambers in three different genotypes (n=100 for each type). **B**) Confocal image of stage 10 egg chamber heterozygous for *bsk*^{1}, stained with anti *D*E-Cadherin (red in **B**-**E**). No invagination of the centripetal cells is visible **C**) stage 10 egg chamber resulting from the cross UAS::*dtraf* x *slbo*Gal4 have reduced centripetal cell migration. **D**) There is no accumulation of *D*E-Cadherin at lateral sites of the leading edge cells. **E**) Example of the terminal phenotype resulting from *dtraf* overexpression. Arrowheads in **C** and **D** point to border cell cluster.

To further investigate the importance of right JNK signalling levels for the regulation of follicle cell migration, the JNK activator DTRAF (UAS::*dtraf*) was expressed under control of the *slbo* promotor in the developing egg chamber. As a result, centripetal cell migration was inhibited and cell shape changes were suppressed (Fig.23C). Due to aberrant centripetal cell migration a cup like egg chamber was formed with short dorsal appendages (Fig.23E). This is a phenotype characteristic for mutations that ectopically activate the JNK signalling cascade

(Suzanne et al., 2002). Thus, it was concluded that JNK signalling has to be silenced by DIAP1 to allow proper centripetal cell movements.

3.5.7.2 *DIAP1* and *Ral* modify *puckered* expression in the ovary

Activation of the JNK pathway as a consequence of reduced DIAP1 levels was demonstrated in the previous chapter. Nevertheless an attempt was made to find independent confirmation of this result to establish a firm connection between Ral, DIAP1, the JNK pathway and morphogenetic movements during oogenesis.

Activation of the JNK pathway can be measured by a lacZ insertion in the *puckered* gene (*puc::lacZ*) (Martin-Blanco et al., 1998). *Puc* belongs to the group of target genes that are immediately transcribed after formation of active AP1 transcription factor. A reduction of DIAP1 gene dose in th^{109} mutant background could lead to widening of the *puc* expression domain. The enhancer trap *puc::LacZ* exhibits a strong β-galactosidase signal in the border cell cluster of wild type ovaries (Fig.24A) but not in centripetal cells. In contrast to that, the Jun kinase pathway is activated also in the centripetal cells which have a lower DIAP1 level, as judged by elevated lacZ staining (Fig.24B). While wild type centripetal cells are elongated, centripetal cells with reduced DIAP1 level were shorter and round.

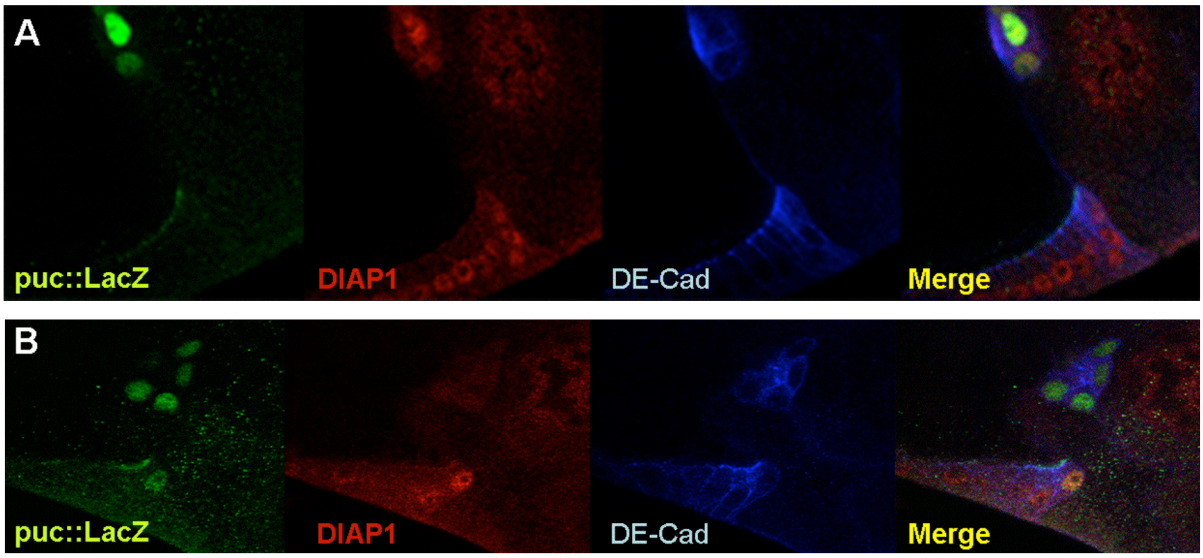


Figure 24: DIAP1 negatively regulates JNK activity in the centripetal cells

The gene *puckered* (*puc*) is expressed in response to Jun N terminal Kinase pathway activation and can be measured by LacZ expression. **A)** In the wildtype ovary, *puc::LacZ* is strongly expressed in the border cell cluster (green) while no *puc::LacZ* expression is seen in the leading edge cells of the inward migrating epithelium. **B**) In contrast to that, *puc*::*LacZ* expression becomes strongly upregulated in the nuclei of the leading edge cells in a th^{109} heterozygous genetic background that reduces DIAP1 gene expression by 50%. Expression of *puc*::*LacZ* in the border cells is not affected by this.

This result is in line with the expectation that Ral/DIAP1 are upstream of DTRAF1 and JNK signalling in the follicle cells. It confirms that DIAP1 levels affect amplitude of JNK signalling in follicle epithelium thereby influencing cell shape changes during centripetal cell formation.

3.5.7.3 DIAP1 and DTRAF overexpression influences border cell migration

In this work comprehensive evidence was presented that established a role for DIAP1 in regulation of centripetal cell formation. Results from loss of function studies, clonal analysis and genetic interaction tests strongly suggested that the newly described Ral-DIAP1-JNK pathway plays an important role in control of cell adhesion in the centripetal domain of the follicle epithelium. It is possible, though that this pathway also influences other migratory processes in the ovary. This assumption is supported by the finding that overexpression of DIAP1 frequently causes miss placement of border cells (Fig.25C). A similar phenotype is created with low penetrance by overexpression of DTRAF (Fig.25B).

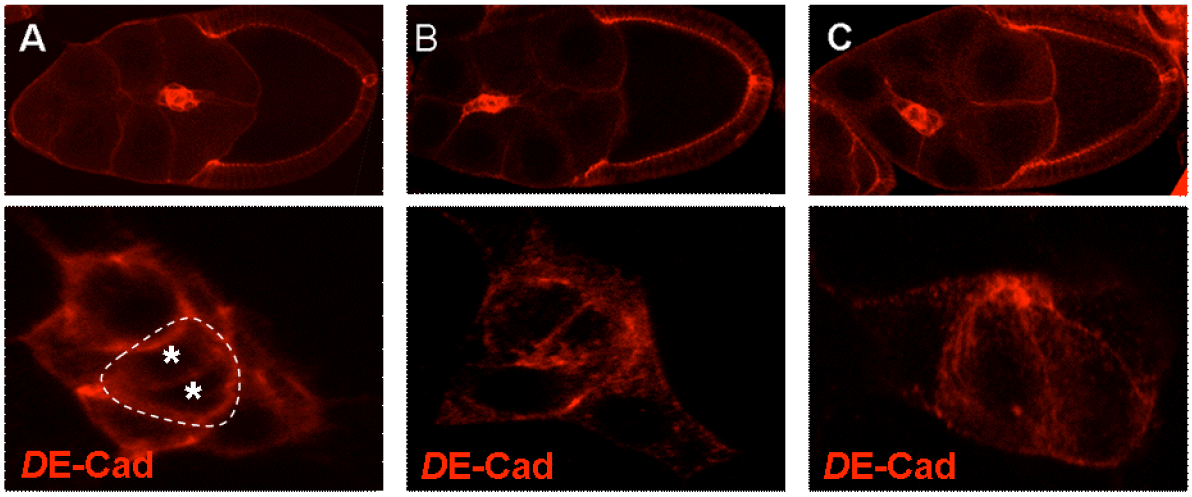


Figure 25: Overexpression of DIAP1 and DTRAF affects organization of the border cell cluster

A-**C** Egg chambers stained with *D*E-Cadherin antibody in red.
A) Wildtype egg chamber stained with *D*E-Cadherin antibody. Magnification of the border cell cluster reveals a highly organized pattern of *D*E-Cadherin distribution that form a “rosette” like structure. Polar cells can be easily discriminated within the cluster (asterisk in **A**). **B**) Egg chamber overexpressing DTRAF under control of the *slbo* promoter. The *UAS::DTRAF*x*slbo::Gal4* derived border cell cluster looses its epithelial organization and no discrimination of polar cells is possible. **C**) Egg chamber derived from the cross *UAS::DIAP1*x*slboGal4*; Morphology of the border cell cluster is as well affected and polar cells can not be discriminated.

High magnification of the border cell cluster reveals changes in the morphology. In the wildtype, border cells migrate in a organized cluster that resembles a rosette (Fig.25A). Polar cells are surrounded by 4-6 recruited follicle cells. When DIAP1 or DTRAF were overexpressed, organization of the border cell cluster was disrupted and *D*E-Cadherin is not correctly distributed (Fig.25B and C).

Further studies are needed to elucidate which role DIAP1 and the JNK pathway play in organization and migration of the border cell cluster. Antibodies against cell polarity markers like Crumbs, aPKC, Bazooka and others could show if epithelial structure of the border cell cluster is preserved under conditions that alter JNK signalling.

4. DISCUSSION

Genes control and propagate life but they can also cause its destruction. Apoptosis represents a cell intrinsic program of cell suicide that is genetically regulated. It can be seen as a normal part of morphogenesis and tissue homeostasis. Despite evolutionary divergence among species, the core molecular mechanism of apoptosis is preserved in almost all animals. In *Drosophila melanogaster* the DIAP1 protein, encoded by the *thread* gene, is required for survival of all cells in the embryo. DIAP1 binds caspases and inhibits their activation. The proapoptotic proteins Hid, Reaper and Grim can start the cellular apoptotic machinery through two different mechanisms. They directly bind DIAP1 thereby releasing caspases or they negatively influence protein levels of the inhibitor in the cell (Yoo et al., 2002; Ryoo et al., 2002; Wang et al., 1999).

Because of its requirement for cell survival it is difficult to investigate details of DIAP1 function in vivo. Nearly all loss of function mutations of *thread* cause massive apoptosis early in development. Removal of DIAP1 causes cell death even if no known proapoptotic genes are expressed. There is still no satisfactory explanation for activation of caspases in this case. In all other investigated model systems, apoptosis occurs only if there is some positive regulation of caspase activation. Initial cleavage of the caspase prodomains needs to be promoted by a CED4 like mechanism (see Introduction). In *Drosophila*, DIAP1 negatively regulates caspase activation, but why caspases are active if no proapoptotic proteins are present is an open question.

Parallel to the crucial role in keeping cells alive, DIAP1 might have additional functions in the embryo. Antibody staining against the protein in the embryo shows that it is differentially localized within different tissues at specific time points. In the early blastoderm stage it associates with membranes, additional to cytoplasmic localization. Later DIAP1 is upregulated in the neuroblasts and localizes to the nuclei. Nuclear localization can as well be observed in the follicle cells of the developing egg chamber. These properties can hardly be explained with the current model of DIAP1 function. Nothing is known about what DIAP1 might do in different cellular compartments or how it gets there.

It is likely that putative DIAP1 interacting proteins recruit the protein to distinct regions of the cell. While membrane association of DIAP1 seems to be unique for this IAP family member, nuclear localization and even chromatin association has

been reported for other BIR domain containing proteins. For example the *C.elegans* BIR1 protein is necessary for cell division (Fraser et al., 1999). The human BIR protein Survivin, a marker for tumour progression has a nuclear localization signal as well (Moon et al., 2003). Most interestingly, the human cIAP1 and cIAP2 proteins contain cryptic nuclear export signals. Translocation of both proteins is regulated by the TNF-Receptor associated protein TRAF2 (Vischioni et al., 2004). Yet a clear nuclear localisation signal in cIAP1 and cIAP2 is missing and what mediates cIAP`s nuclear import remains to be discovered. However, the author showed that forced translocation of cIAP1 to the nucleus does not impair its function as an apoptosis inhibitor. This suggests that IAPs might play an antiapoptotic role within the nucleus which could be the case for DIAP1 as well.

It is possible that a similar mechanism leads to the observed localization of DIAP1 to the nuclei of both neuroblasts and follicle cells. Unfortunately, no good candidate was identified in the yeast two hybrid screen that was recognized as a potential shuttle protein that would drive DIAP1`s nuclear localization.

4.1 Yeast two hybrid screen

As a possible approach to elucidate functional aspects of DIAP1 in the cell, it is helpful to know which other proteins interact with DIAP1. The aim of this project was to identify and characterize novel binding partners of the apoptosis inhibitor by conducting a yeast-two-hybrid screen with DIAP1 as bait.

Screening with full length DIAP1 lead to identification of two new binding partners: RpS27A and RpL40. Both proteins are structural components of the ribosome. Interestingly, the DIAP1 binding proapoptotic proteins Reaper and Grim have been shown to negatively regulate general translation of proteins (Yoo et al., 2002; Holley et al., 2002). The mechanism how Reaper and Grim achieve inhibition of translation is not known. Therefore, identification of the ribosomal proteins as DIAP1 interacting proteins could connect the apoptosis inhibitor and its proapoptotic binding partners with the core machinery of translation. Whether DIAP1 really binds to ribosomes and eventually regulates their function via interaction with RpL40 and RpS27A has yet to be elucidated.

Identification of only two binding partners of full length DIAP1 was considered to be a very low yield. It was assumed that intrinsic properties of the DIAP1 protein might be responsible for the low efficiency of the screen. The E3 ubiquitin ligase activity in particular might negatively influence homophilic protein-protein interactions. A recently published interaction map of all *Drosophila* proteins based on a bidirectional yeast two hybrid screen did not reveal any binding partners of full length DIAP1 as well (Giot et al., 2003). Any of the published interactions of full length DIAP1 with Hid, Reaper and Grim or caspases were also not confirmed.

To circumvent the problem with the DIAP1 full length protein, a “bait” construct without the RING domain was generated. After removal of the 48 amino acids from the C terminus, a novel screen yielded 29 putative candidates. Nevertheless, the second screen did not reach saturation as well. The yeast two hybrid screen is considered to be saturated when a high rate of multiple hits is reached. In this case all recovered candidates were isolated only once. Time constrains and the fact that from 29 sequenced plasmids 22 were encoding a fragment of a predicted gene, lead to the decision to proceed with the characterization of the candidate interacting proteins. Isolation of sequences that did not belong to any annotated gene in the Fly base can be explained by contamination of the cDNA library. RNA extracts that are used for creation of the cDNA library often contain additional amounts of tRNAs, rRNA and mitochondrial sequences (Johnson and Wodarz, 2003.)

The potential importance of candidate proteins had to be evaluated. Therefore, the list of interesting candidates was narrowed down after a thorough analysis of available data and published studies. Thus, it can not be excluded that interaction partners of DIAP1 have been overseen. The putative DIAP1 interacting proteins vacuolar H^+-ATPase and Ral were considered to be most interesting for further studies.

4.2 Regulatory subunit of Vacuolar ATPase- *vha 55*

House keeping genes encoding enzymes or abundant structural components in the cell tend to show up as false positives in yeast two hybrid screens. Nevertheless, the identification of the ß-regulatory subunit of the vacuolar H^+-ATPase as a potential novel interaction partner of DIAP1 protein was not discarded as insignificant. Studies of its close mammalian homologue indicated that the proton pump is functionally connected with apoptosis (Nishihara et al., 1995). The vacuolar H^+-ATPase is located to the membrane of lysosomes and is important for their integrity (Hishita et al., 2001). These organelles contain several classes of proteases in a highly acidic environment. Lysosomal proteases digest proteins that are delivered through fusion with secondary endosomes. Interestingly, leaking of proteases from the lysosomes into the cytoplasm induces caspase activation and apoptosis (Ishisaki et al., 1999). The involvement of the cathepsin class of lysosomal proteases during apoptosis induction is intriguing. Cathepsins are detected in the cytoplasmic fraction of cultured cell lines prior to the onset of apoptosis (Michallet et al., 2004) and they can activate executioner caspases in vitro (Ishisaki et al., 1999). Thus, lysosomal integrity is crucial for cell survival in mammalian cells.

The importance of the H^+-ATPase for cell survival in *Drosophila* was tested in cell culture. Inhibition of the H^+-ATPase β-subunit by the pharmacological drug Bafilomycin A1 (BafA1) induced a high rate of apoptosis in S2 cell culture. BafA1 induced apoptosis is accompanied by structural changes of the lysosomes detected by the live dye Lysotracker. Unfortunately, a release of cathepsins into the cytoplasm of apoptotic cells was not confirmed due to lack of *Drosophila* specific antibodies. Nevertheless, a specific antibody against activated DRICE confirmed activation of executioner caspases after apoptosis induction. Interestingly, a cathepsin inhibitor blocked DRICE activation. Thus, the *Drosophila* vacuolar H^+-ATPase responds to functional inhibition in a similar way like its mammalian counterpart.

A physical interaction between the ß-subunit of the vacuolar H^+-ATPase and DIAP1 still needs to be confirmed by independent in vitro experiments. However, a functional DIAP1/H^+-ATPase complex might be needed to maintain lysosomal integrity and control cathepsin release to the cytoplasm. This model could be tested in S2 cells depleted for DIAP1 using RNAi. Removal of DIAP1 would directly induce apoptosis by activation of caspases and probably mask DIAP1`s function for integrity

of the lysosomes. Cell permeable caspase inhibitors (e.g. z-VAD-FMK) can postpone the onset of cell death and therefore can be a useful tool to test apoptosis unrelated functions of DIAP1 in cell culture. Lysosomal integrity of DIAP1 RNAi treated cells could then be followed by probing the cytoplasmic fractions for the presence of cathepsins with *Drosophila* specific antibodies.

4.3 The small GTPase Ral, a member of the Ras family with antiapoptotic function

Identification of Ral as a putative binding partner of DIAP1 is certainly the most interesting result of the yeast two hybrid screen. Small GTPases are involved in diverse signal transduction cascades regulating growth and the cell cycle. Activity status cycles between an active, GTP bound, and an inactive, GDP bound state. Activation of GTPases is tightly regulated by specific GTPase activating proteins, (GAPs), and guanine nucleotide exchange factors (GEFs). Therefore this protein class can be observed like sensitive molecular switches.

In contrast to the well-studied Ras protein, which is known as one of the most potent proto oncogenes (Senger et al., 1988), the function of Ral is less well understood. Apart from several Ral specific GEFs and GAPs identified in different model systems, the signalling pathways that are controlled by Ral effector proteins are still not dissected. In the mammalian system Ral is in some cases downstream of Ras (Urano et al., 1996). Overexpression of a dominant negative form of Ral prevents oncogenic transformation caused by mutated *k-ras* (Feig et al., 1996, Reuther and Der, 2000). Additionally, experimental evidence points to involvement of Ral in activation of phospholipase D (Frankel et al., 1999), Src kinase (Goi et al., 1999) and receptor mediated endocytosis (Nakashima et al., 2000). Furthermore presence of activated Ral is a sufficient signal to promote expression of NF-κB and cyclinD1, two important signalling molecules for cell survival (Henry et al., 2000). Probably most interesting in context of this work is, that Ral is binding the exocyst complex (Moskalenko et al., 2002) and mediates basolateral membrane transport for example of E-Cadherin (Shipitsin and Feig, 2004).

Function of Ral in *Drosophila melanogaster* is even less understood. There are indications of at least one pathway of Ral activation independent of Ras (Mirey et al., 2003). Sawamoto and others (1999) could show that overexpression of dominant

mutant forms of Ral (Ral^{G20V} and Ral^{S25N}) during development causes various defects. Depending on the Gal4 driver line, they generated defects in dorsal closure during late embryogenesis or caused lack of bristles on the thorax of the adult fly.Thus, intensive cell shape changes and actin remodelling characterize processes influenced by Ral.

Interestingly, mutations silencing the Jun N terminal Kinase (JNK) pathway suppress the Ral^{S25N} induced phenotype (Sawamoto et al., 1999). On the other hand expression of constitutively active Ral^{G20V} prevents activation of the Jun N terminal kinase, Basket, itself. Therefore it was concluded that Ral is a negative regulator of the JNK pathway and modulates cell shape changes by an unknown mechanism.

The presented work defines a novel function of Ral as an important regulator of cell adhesion during follicle cell migration. The interaction of Ral and DIAP1 builds an upstream regulatory pathway of JNK signalling that defines protein levels and distribution of *D*E-Cadherin. In vivo observations and genetic evidence lead to a model that describes a new, cell death unrelated function, of the caspase inhibitor DIAP1.

4.4 Stability of DIAP1 is required for oogenesis and maintained by Ral

Interaction between DIAP1 and Ral was initially detected in the yeast two hybrid screen with DIAP1 lacking the RING domain. Specificity of the interaction was tested in the same system and revealed that Ral binds to the BIR1 domain located in the N terminus of DIAP1. Biochemical studies verified the DIAP1-Ral interaction *in vitro* (Kessler,T. personal communication). Interestingly, the interaction was dependent on the activation of Ral as Ral^{G20V} bound to DIAP1 while Ral^{S25N} did not. This suggests that DIAP1 is an effector molecule of Ral. Further studies indicated that Ral positively influences DIAP1 protein stability. Conversely there was no evidence that Ral itself might be targeted for degradation by DIAP1 (Kessler,T personal communication).

The significance of these findings needed to be confirmed in living cells under physiological conditions. Overexpression of Ral^{G20V} in the embryo leads to a

slight stabilization of DIAP1 protein levels. Probably, stabilization is not prominent because of DIAP1`s abundant high expression in all cells. Ral mutant embryos were not examined with regard to DIAP1 protein levels as reduced levels of Ral lead to very early developmental defects (Kessler,T personal communication).

Therefore, the stabilization of DIAP1 by Ral was investigated by clonal analysis in the follicle epithelium. Cells lacking Ral show a clear decrease of DIAP1 levels. The discovery that Ral is required for stabilizing DIAP1 protein levels has broad implications. Partial suppression of Reaper induced cell death by Ral^{G20V} argues for Ral as a DIAP1 stabilizing factor in a more general cellular context. Of course, stabilization of DIAP1 impacts on its function as a caspase inhibitor and its anti apoptotic role. The only known proteins that have similar properties are the proapoptotic proteins Hid, Reaper and Grim. In contrast to Ral, these proteins negatively influence DIAP1 protein levels and by that promote apoptosis. It would be interesting for further studies to test how balance between proapoptotic and anti apoptotic inputs on DIAP1 are regulated. Especially important would be the upstream activators of Ral that cause DIAP1 stabilization and by that provide a cell survival signal.

4.5 Role of Ral and DIAP1 in morphogenetic movements during oogenesis

Oogenesis in *Drosophila* represents a complex developmental process. A fertile egg is produced as a result of tightly regulated interactions between the germ line and the surrounding follicle epithelium of somatic origin. One of the most interesting aspects of these morphogenetic processes is the movement of the follicle cells. This simple epithelial monolayer has the intrinsic property to undergo cell shape changes and to migrate. Understanding of the cellular pathways that control these transitions could perhaps help to explain the behaviour of epithelial tumours. How do polarized, tightly connected and fully differentiated epithelial cells migrate? Or, perhaps even more crucial is the question, how do they migrate and retain their epithelial features? Several signalling pathways have so far been implicated in regulation of different phases of follicle cell movements. Members of the Rho GTPase family (Rac, Cdc42 etc) were characterized as important modulators of cell

shape changes on the level of cytoskeleton rearrangements. In this work, evidence is presented that interaction of the Ras like GTPase Ral and DIAP1 might be equally important for the control of changes in adhesion during centripetal cell formation.

The phenotype of egg chambers that had only half of the DIAP1 or Ral gene dose indicated that the protein levels of the two proteins influence migratory properties of centripetal cells. Heterozygous mutants provided some valuable information about changes in the centripetal morphology and migratory behaviour when DIAP1 or Ral levels are reduced. However, definite conclusions could not been made due to limitations of the experimental model. Namely, heterozygous egg chambers carry mutations both in the follicle cell epithelium and in the germ line cells. Therefore, it can not be excluded that the observed phenotype was partially caused by reduction of DIAP1 and Ral function in the nurse cells or the oocyte. The migratory processes in the follicle epithelium critically depend on the expression and distribution of *D*E-Cadherin, not only in the migrating cells but also in the underlying cells that provide surface for adhesion (e.g. border cells move between nurse cells). Clonal analysis was necessary to exclude influence by the mutant germline and to characterize subcellular effects that occur in the absence of Ral or DIAP1.

The loss of DIAP1 in ral^{70} mutant follicle cell clones is often accompanied by reduction of *D*E-Cadherin levels at the plasma membrane. Since *D*E-Cadherin promotes formation of a stable F-actin belt at the apical side of epithelial cells, loss of *D*E-Cadherin can result in a disorganized actin cytoskeleton as seen in large ral^{70} mutant cell clones. Thus, it is possible that Ral and DIAP1 have an organizing function for the actin cytoskeleton because they control localization of *D*E-Cadherin to the lateral sides of centripetal cells. Results of th^{109} mutant follicle cell analysis supported the idea that the phenotype detected in th^{109} heterozygous females originates from decreased *D*E-Cadherin levels. Complete removal of DIAP1 from centripetal cells leads to a sharp decrease of membrane bound *D*E-Cadherin protein. This effect is cell autonomous because neighbouring wild type twin cells had a normal *D*E-Cadherin distribution except on the side facing the mutant cell clone. It remains to be further investigated at which level *D*E-Cadherin is influenced, on transcriptional, translational or during post-translational modification.

Interestingly, the attempt to induce large clones of DIAP1 mutant cells was unsuccessful. Mutant clonal patches were never larger than 3-4 cells. This implies that DIAP1 is required cell autonomously for promoting the cell cycle and thus

needed for proliferation of cells, or for inhibition of cell death. This could be tested in early developmental stages of oogenesis where egg chambers undego extensive proliferation. Early induced mitotic recombination could then cause termination of egg chamber development due to insufficient number of follicle cells. As described by Ryoo et al. (2004) DIAP1 controls expression and secretion of wingless and dpp in the wing imaginal disc. These molecules are mitogens and stimulate division of the neighbouring cells. The whole process is caspase independent and considered to be an effect of JNK activation. Ectopic expression of *wingless* and *dpp* in DIAP1 mutant clones could for example be detected by antibodies against these signalling molecules. Analysing BrdU incorporation in the cells that surround th^{109} mutant clones could reveal whether proliferation is indeed induced. The fact, that ral^{70} mutant cell clones grow bigger than clones mutant for DIAP1 probably reflects inherent differences in DIAP1 protein levels between the two types of clones. In Ral mutant cell clones DIAP1 protein is destabilized and undergoes autoubiquitination at a fast rate. On the contrary, follicle cell clones mutant for th^{109} do not produce DIAP1 protein at all. Thus, ral^{70} mutant cells might still contain low levels of DIAP1, sufficient for sustained cell growth and cell division.

In some cases Ral mutant cells form a multilayered epithelium, which points to defects in maintenance of epithelial polarity or cell adhesion (Tanentzapf et al., 2000). Nevertheless, it cannot be excluded that Ral influences epithelial polarity and adhesion through an additional effector pathway parallel to DIAP1. However, effects of ectopic expression of Ral or DIAP1 in the domain of centripetal cells suggest the existence of additional mechanisms that regulate cell migration. Contrary to the loss of function mutant phenotypes, overexpression of constitutive active Ral^{G20V} in the centripetal cells stimulates their migration. The transition from columnar to cuboidal epithelium in late oogenesis is also inhibited. According to the established model that Ral stabilizes DIAP1, overexpression of DIAP1 should have similar effects. However this is not the case. Despite of high ectopic levels of DIAP1 in egg chambers, centripetal cell migration is inhibited, redistribution of *D*E-Cadherin resembles the situation in th^{109} heterozygotes and border cell migration is affected. These apparent discrepancies in the results can be due to cross talk of DIAP1 and Ral overexpression with other molecular pathways.

Experiments in the human epithelial MDCK cell line proved correlation between expression of activated Ral and the rate of delivery of E-Cadherin to the basolateral

membrane (Shipitsin and Feig.2004). A function for *Drosophila* Ral in the exocyst complex has not been described yet but eventually Ral`s function as a part of the exocyst complex is independent of DIAP1. However, expression of Ral^{G20V} could stimulate *D*E-Cadherin membrane delivery via the exocyst complex and thus promote centripetal cell migration in addition to a DIAP1 dependent pathway. The DIAP1 dependent activation of JNK signalling as described in this work would synergistically enhance the defects and could explain the strong phenotype by Ral^{G20V} overexpression. Upregulation of *D*E-Cadherin in response to stabilized DIAP1, coupled with a high rate of membrane targeting would then result in increased migratory behaviour of centripetal cells.

When DIAP1 is overexpressed, Ral independent cellular mechanisms might respond, as DIAP1 is also a member of the Rac1 effector pathway (Geisbrecht and Montell, 2004). DIAP1's function in the Rac1 complex depends on its caspase binding properties rather than its RING domain. The idea that caspases are required is supported by the finding that a dominant negative version of the apical caspase DRONC can rescue border cell migration to a similar extend as DIAP1 overexpression. Intriguingly the DIAP1/Rac1/caspase pathway does not impair centripetal cell movements arguing for at least two different complexes regulating cell migration in context of DIAP1.

DIAP1 protein levels, maintained by Ral, regulate adhesive properties of the centripetal cells. In this work evidence is presented, that activation of the JNK signalling pathway is at least part of the cellular response triggered in the absence of DIAP1. Genetic evidence showed that DIAP1 and components of the JNK signalling pathway belong to the same genetic pathway in the follicle epithelium. It was previously shown that DIAP1 targets DTRAF for degradation, one of the upstream activators of the JNK pathway. Dobens et al. (2002) showed, that the level of JNK signalling that is required for proper oogenesis differs depending on the stage and region of the follicle epithelium. Results presented in this work strongly argue that JNK signalling has to be reduced during centripetal cell formation migration. This reduction is maintained through high levels of DIAP1 protein in the centripetal cells (see model in Fig.22).

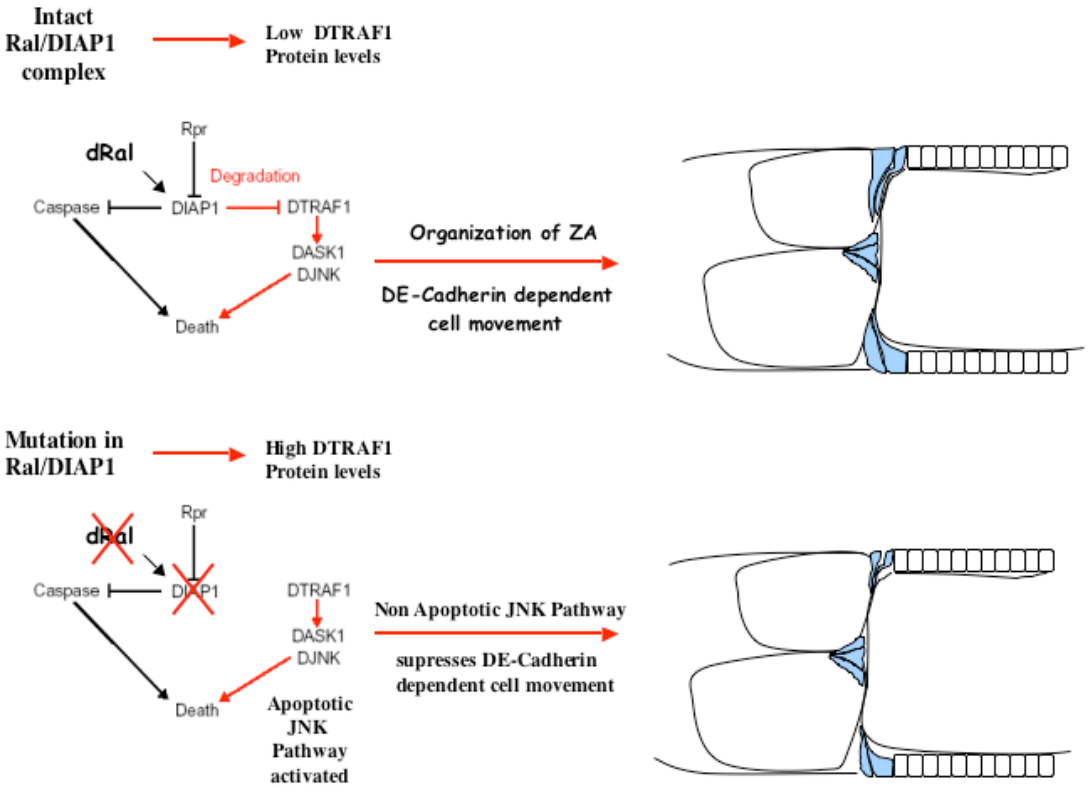


Figure 22: Schematic model how the Ral/DIAP1 complex regulates migration

Ral and DIAP1 downregulate DTRAF/JNK in centripetal cells and modulate migration. DIAP1 modulates JNK signal by constant targeting of DTRAF for degradation. When either Ral or DIAP1 are mutated, JNK is activated and centripetal cell migration is inhibited.

Ral becomes activated by a yet to be identified signal and stabilizes DIAP1. Interaction of Ral and DIAP1 probably leads to degradation of DTRAF, an upstream regulator of JNK pathway activity. Reduction of JNK signalling is precondition for the redistribution and upregulation of *D*E-Cadherin resulting in a positive migration stimulus.

It needs to be said that DIAP1 function in the ovary is almost certainly not restricted to the role in centripetal cell formation through inhibition of JNK signalling. This idea is based on the finding that DIAP1 is localized to the nucleus of centripetal cells. A function for this nuclear localization is not described yet but analysis of transgenes expressing mutated versions of DIAP1 could reveal domains necessary for its nuclear localization. To distinguish the mutated from the endogenous DIAP1, a tag like GFP could be inserted into the transgene. A further

approach to define DIAP1`s nuclear function could be a mutant replacement strategy. The method established by K. White`s lab (2004) combines depletion of the endogenous DIAP1 by RNAi in combination with ectopic expression of mutant DIAP1 forms. Such study could provide additional explanations about DIAP1`s functional domains and the nuclear function in particular.

The novel function of the caspase inhibitor in control of the morphogenetic movements in the follicle epithelium is an example of how different signalling pathways are integrated in larger protein networks. DIAP1 appears to be influenced by both Ral and Rac1 signals. In contrast to Rac1, which causes actin fibre remodelling and movement via profillin, Ral seems to influence actin organization indirectly via DIAP1 stabilization. It can be assumed that these two small GTPases and their downstream target DIAP1 represent a system in equilibrium depending on the cellular context.

The inhibitory effect of DIAP1 on Jun kinase signalling is not only significant for the regulation of migratory processes. A TNF induced signal can trigger expression of NF-κB, a factor required for cell proliferation (Majumdar et al., 2002). This and other yet to be discovered DIAP1 functions might be a part of the answer why the caspase inhibitor is so important in all developmental stages and in nearly every cell.

5. Summary

Drosophila melanogaster Inhibitor of apoptosis, DIAP1, is required for the survival of nearly all cells in the embryo during development. DIAP1 has two BIR domains and RING domain with E3 ubiquitin ligase activity. Its main role in the cells is to block caspases and prevent activation of the proteolytic cascade that leads to apoptosis. Additionally, DIAP1 differentially localizes within specific tissues in different developmental stages, which implicates possible other, apoptosis unrelated, functions. The yeast two hybrid screen performed in this work had the purpose to identify novel interaction partners of DIAP1. Among isolated candidates are members of diverse protein families: cytoskeleton components, signalling molecules, enzymes of general metabolism, proteins involved in the regulation of protein synthesis and degradation etc.

The vacuolar H^+-ATPase proved to be a potentially interesting candidate because of its functional importance in preserving integrity of the lysosomal compartment. In this work it has been shown that inhibition of v-H^+-ATPase causes apoptosis in *Drosophila* cell culture. This process is caspase dependent and is probably triggered by cathepsin release into the cytoplasm. These data provide a first evidence of a possible role that lysosomes have in the control of apoptosis in *Drosophila*. Identification of the Ral GTPase as a novel binding partner of DIAP1, and its characterization, helped to describe a function of the apoptosis inhibitor in regulation of migration processes during oogenesis. Evidence is presented that Ral partially co localizes with DIAP1 and that Ral is required for stabilization of DIAP1 protein levels. This mechanism influences cell survival as well as morphogenesis. Functional analysis of Ral and DIAP1 mutant cells strongly suggest that these proteins regulate changes in adhesion which are required for induction of movement in the follicle epithelium. It is shown that stable DIAP1 protein levels, maintained by Ral, are needed for regulation of *D*E-Cadherin, an adhesion molecule that is known to be of crucial importance for the cell migration during oogenesis. Genetic interactions and activity of reporter genes indicate that DIAP1 inhibits the JNK signalling pathway in the centripetal cells and thereby directly affects cell shape changes. It can be concluded that DIAP1, although necessary for inhibition of apoptosis, plays an additional important role in control of morphogenetic movements during development.

6. Literature

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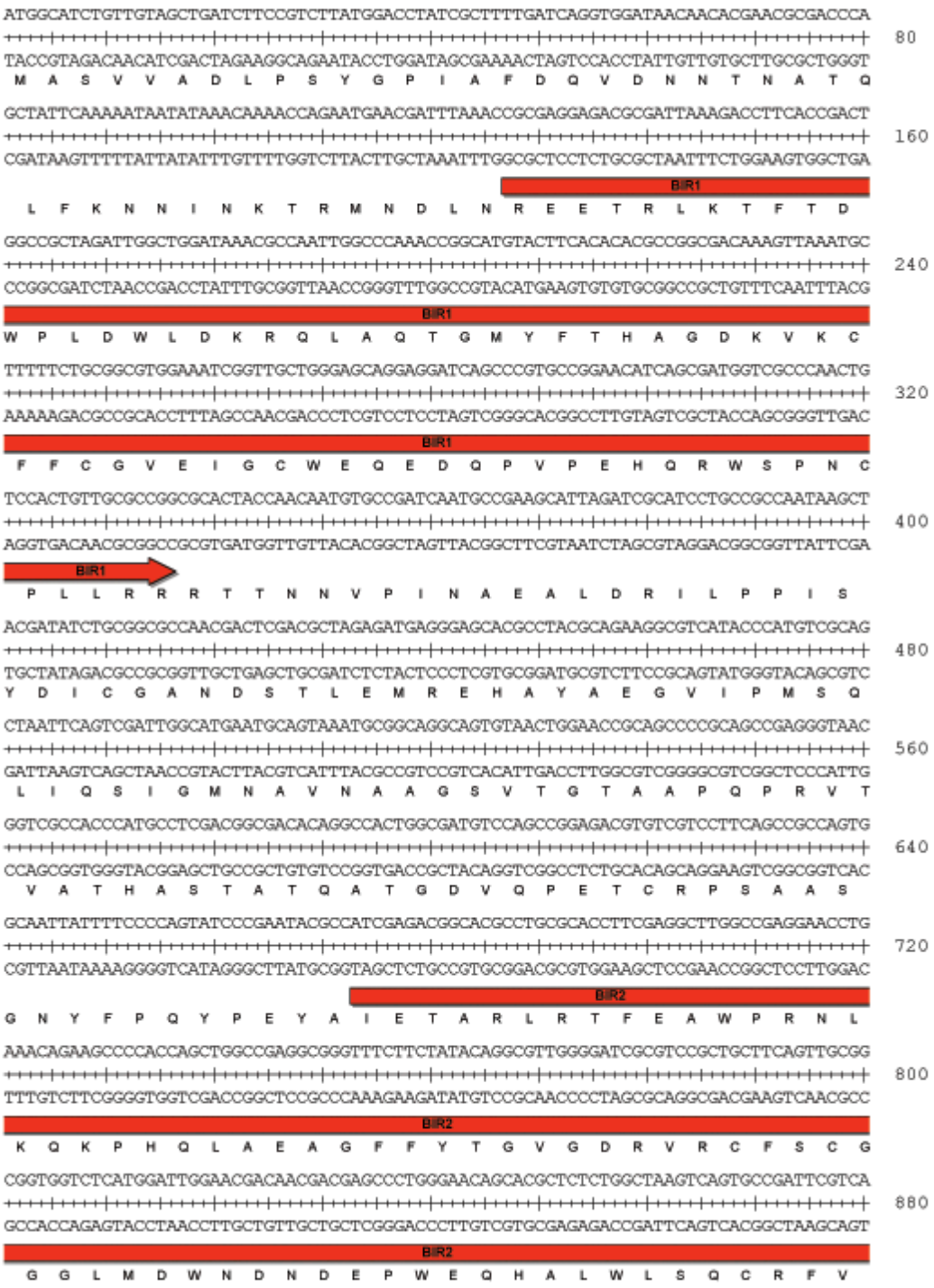
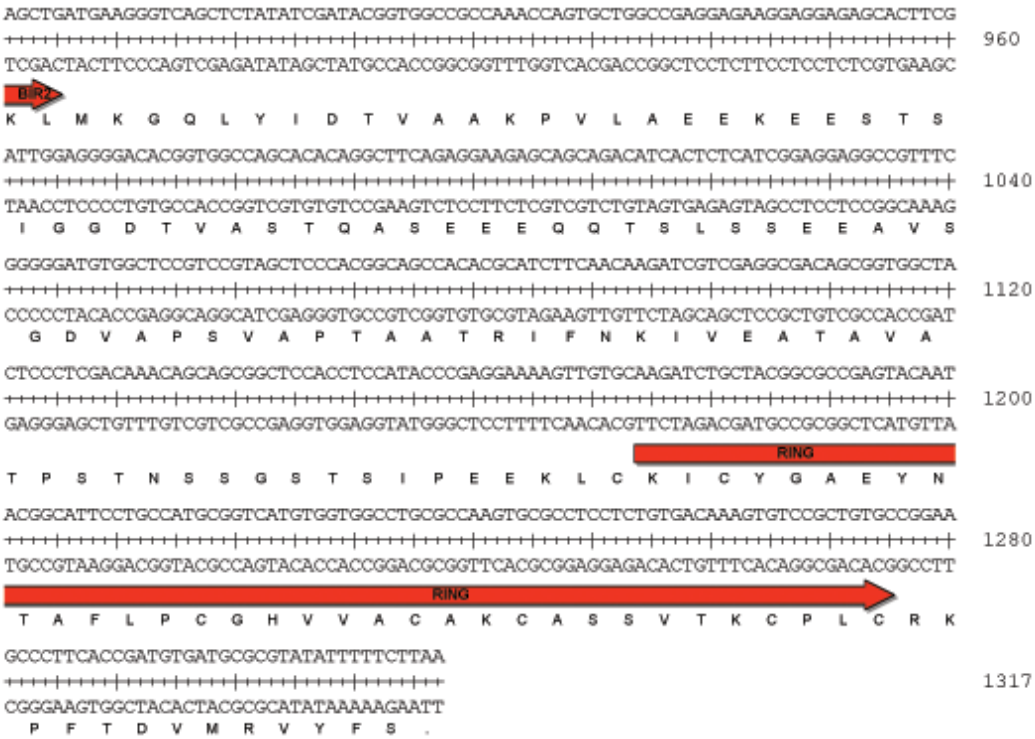
7. Supplementary Data

Schematic representation of the DIAP1 open reading frame and protein sequence
Regions of the BIR domains and the RING domain are marked as red arrows

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960
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BIR2
K L M K G Q L Y I D T V A A K P V L A E E K E E S T S
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1040
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I G G D T V A S T Q A S E E E Q Q T S L S S E E A V S
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1120
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G D V A P S V A P T A A T R I F N K I V E A T A V A
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1200
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RING
T P S T N S S G S T S I P E E K L C K I C Y G A E Y N
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1280
TGCCGTAAGGACGGTACGCCAGTACACCACCGGACGCGGTTCACGCGGAGGAGACACTGTTTCACAGGCGACACGGCCTT
RING
T A F L P C G H V V A C A K C A S S V T K C P L C R K
GCCCTTCACCGATGTGATGCGCGTATATTTTTCTTAA
1317
CGGGAAGTGGCTACACTACGCGCATATAAAAAGAATT
P F T D V M R V Y F S .

Sequences of clones which were isolated in the yeast two hybrid screen. Oligonucleotide sequence marked in red is the linker that connects the GAL4 AD with the sequence of the clone.

CG10960

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ANTTAAAGTCNCNGTTANTGCAAGTCTTTTGGCGGTT-3`

Rala GTPase (Rala)

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Vha55 CG17369

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Actin5C CG4027

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GCANNGAGTACANACGAGTCCGGCCCCTCCATTGGGCACC-3`

CG5920 Sop

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CstF-50 (CG2261)

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Pyk CG7070

5`CTTTTANCAANTTTNNTTTTAANAGACCCCNCCCGGCTTACCCATACGATGTTCCAGATTACGCT
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CG7101

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CG10194

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Jbug CG30092

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CG18641

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RPN2 CG11888

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CG4230

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CDC45L (CG3658)

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Cyp309 a2 (CG18559)

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CG9238

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Pgant6 CG2103

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RPL17 CG3661

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CG13043

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CG14476

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RpS27A (CG5271)

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RpL40 CG(2960)

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ABBREAVIATIONS

A	Adenine
Amp	Ampicillin
Bp	base pares
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C	Cytosine
°C	Celsius
DABCO	1,4-Diazabicyclo(2.2.2)octane
DMSO	Dimethylsulfoxid
Dpp	Decapentaplegic
DTT	Dithiothreitol
dNTP	Deoxyribonucleotide phosphates
dsRNA	Double stranded RNA
eg	lat exempli gratia,. For example
eng	engrailed
EtOH	Ethanol
Fig	Figure
G	Guanine
g	gramm
HRP	Horse Radish Peroxidase
LB-Medium	Luria Bertani Broth-Medium
M	Mole
m	Milli 10^{-3}
μ	Micro 10^{-6}
Min	Minute
mRNA	Messenger RNA
n	Nano 10^{-9}
OD	Optical density
O.N	Over night
PCR	Polymerase chain reaction
rpm	Rounds per minute
RT	Room temperature
T	Thymine
Taq	Termophilus aquatiqus
U	Unit
UV	Ultra violet
V	Volume
ZA	Zonula Adherens

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig und ohne unzulässige Hilfe angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und in Stellen der Arbeit - einschließlich Tabellen und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Fall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät zur Prüfung vorgelegen hat; dass sie noch nicht veröffentlicht worden ist, sowie dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der geltenden Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von PD. Dr. H. Arno J. Müller betreut worden

Düsseldorf, im März 2005

Special thanks

This work was done in the group of H. Arno J. Müller. I want to thank him for the opportunity he gave me, support and good advices. Through discussions we had and critics he gave me I learned the basis of scientific thinking. I am very grateful that he believed in me.

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My father Dusan I thank for the love, attention and all the times that he told me I should get better. Hvala tata. To my mother Ziza: I hope you are somewhere above proud of your girl. My brother Marko I thank for his bright spirit and great jokes that made me happier person.

I would like to thank all co workers at the Institute for the nice atmosphere and readiness to help in each situation. Especially, I would like to thank Sirin Otte and Wibke Meyer for their friendship and hilarious chatting sessions. Nadine Muschalik and Manuel Brauer I thank for their help to solve technical difficulties during the screen. Many thanks, to Diane Adam Egger, Annika Raupach and Nanette Fischer.

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