The function of the *Drosophila* caspase inhibitor DIAP1 in the control of epithelial integrity and cell polarity

Inaugural Dissertation

zur

Erlangung des Doktorgrades der

Mathematisch-Naturwissenschaftlichen Fakultät der

Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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April 2006

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Prof. Dr. Elisabeth Knust
Tag der mündl. Prüfung
17.5.2006

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine Universität Düsseldorf

Für meine Familie

1. Introduction

Epithelia are tissues that build the surface of organs, confine the border between different body compartments or mediate the matter exchange between the outside environment and the lumen of the organism. Depending on their differentiation, epithelia can have very different functions like resorption (gut epithelium), secretion (glands) or protection (skin epidermis) (Rodriguez-Boulan und Nelson, 1989).

Two intrinsic properties are essential for the function and integrity of epithelia. On one hand, the plasma membrane and the associated cytoskeleton of epithelial cells are highly polarized and cellular components within the cells are localized asymmetrically. On the other hand, epithelial cells form tight cellular contacts among themselves and to the extracellular matrix (Alberts et al., 1994).

The plasma membrane of epithelial cells is divided into two functional domains, the apical and the baso-lateral plasma membrane. The apical plasma membrane faces the outside environment while the basal plasma membrane is in contact with the inside of the organism. Differential localization of proteins to either the apical or the baso-lateral plasma membrane is necessary for regulation of directional ion and matter transport across the epithelial cell sheet (Eaton and Simons, 1995; Drubin and Nelson, 1996). Several genes have been identified that are necessary for establishment of apico-basal cell polarity. Interestingly, the molecular mechanisms that are involved in this process are highly similar from evolutionary old organisms like *Caenorhabditis elegans* to man (Knust and Bossinger, 2002; Margolis and Borg, 2005). Therefore, genetically accessible model organisms like *Drosophila melanogaster* can reveal detailed insight into the general molecular mechanisms necessary for establishment of cell polarity in epithelia.

Membrane contact sites of epithelial cells can be distinguished by their function. At the basal plasma membrane, cell-matrix contact sites connect the cell to the components of the underlying basal lamina. At the lateral plasma membrane cell contacts can build a diffusion barrier or connect neighbouring cells to each other. In *Drosophila* three different types of cell-cell contacts can be distinguished. 1) Gap junctions physiologically connect cells in the epithelium (Bryant, 1997). 2) Septate junctions (SJ) block para-cellular solvent and ion exchange and build a trans-epithelial barrier (Skaer et al., 1987). SJ are probably a functional homologues of vertebrate tight

junctions although they do not contain similar proteins. 3) The adherens junctions that connect neighbouring cells to each other and are often organised as a belt like structure, the zonula adherens (ZA). Proteins that are involved in establishment of the ZA are highly homologous in invertebrates and vertebrates. Thus, analysis of the mechanisms that regulate the ZA in *Drosophila* can reveal principles how adhesive contacts are regulated in higher organisms.

1.1 Epithelia in *Drosophila melanogaster*

Epithelia in *Drosophila* are classified as primary and secondary epithelia (Tepass and Hartenstein, 1994). All primary epithelia are derivatives of the cellular blastoderm epithelium. Examples for primary epithelia are the epidermis, part of the gut epithelium as well as tracheae, malpigian tubules and salivary glands. Secondary epithelia derive from mesenchymal tissue or stem cells. Examples for secondary epithelia are the midgut and the follicle epithelium.

1.2 Cell adhesion in *Drosophila* primary epithelia

The *Drosophila* embryonic surface consists of the ectoderm cell layer with distinct apical and basolateral membrane domains, typical for epithelia. Cell adhesion in the ectodermal epithelium is mediated by the *Drosophila* homologue of the transmembrane protein E-Cadherin, *D*E-Cadherin (*D*E-Cad) that mediates Ca^{2+} dependent cell adhesion (Oda et al., 1996). The cytoplasmic tail of *D*E-Cad binds to Armadillo (Arm), the *Drosophila* homologue of ß-Catenin (Peifer and Wieschaus 1990). Arm binds to *Drosophila* α-Catenin (*D*αCat) by that linking *D*E-Cad to the actin cytoskeleton either directly, or via interaction with α–Actinin or vinculin. The protein complex formed by *D*E-Cad, Arm and *D*αCat is necessary to organize the actin cytoskeleton of epithelial cells into an apical adhesion belt. Zygotic mutants of either Arm or *D*E-Cad show relatively mild cell adhesion defects mostly affecting cells that undergo strong cell shape changes during development. Absence of stronger defects can be explained by the presence of maternally provided Arm and *D*E-Cad that can compensate for the zygotic loss of

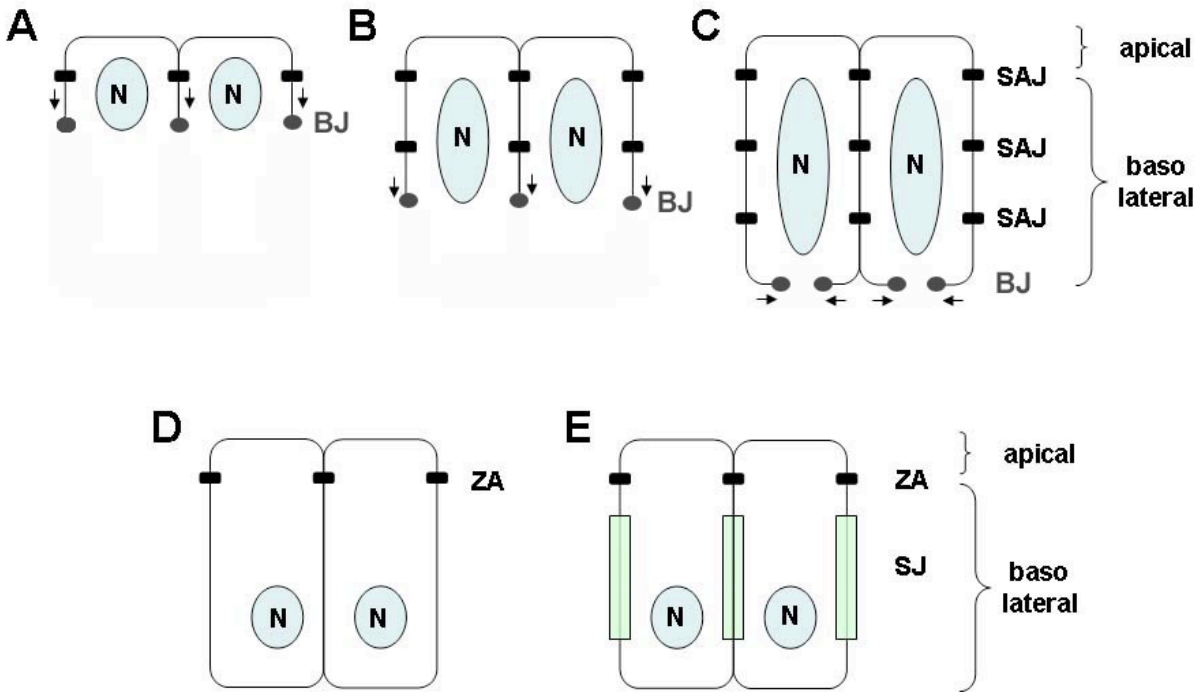


Figure 1: Establishment of cell adhesion in the *Drosophila melanogaster* embryo

A-D Cellularization in the *Drosophila* embryo. (A) After fertilization, the nuclei of the *Drosophila* embryo undergo 13 mitotic divisions without cytokinesis, forming the syncytial blastoderm. With onset of zygotic gene expression, cleavage occurs and new plasma membrane is inserted in between the nuclei. This process is called cellularization. Arrows depict the movement direction of the invaginating membrane, the cleavage furrow. (B) The in-growing plasma membrane separates the nuclei from each other. Spot like adherens junctions (SAJ) along the lateral plasma membrane form that contain the cell adhesion molecules *D*E-Cad, Arm and *D*αCat. (C) At the end of cellularization, the cleavage furrows widen and fuse with each other, forming the cellular blastoderm embryo. SAJ start to assemble into a single apical region, the zonula adherens (ZA). (D) After cellularization is completed, the embryo undergoes gastrulation. The ZA mediates cell adhesion in the gastrulating embryo and separates the apical and baso-lateral site of the cells. (E) By stage 14 of embryogenesis, septate junctions (SJ) appear that provide additional sites of close membrane contact in the epithelium. N: Nucleus; BJ: Basal Junctions; SAJ: Spot Adherens Junction; ZA: Zonula Adherens; SJ: Septate Junctions; Modified after Müller and Bossinger, 2003.

expression to some extent. A mutation affecting the *D*α-Cat locus has not been described so far, but embryos depleted for *D*αCat mRNA show a massive loss of cell adhesion similar to Arm and *D*E-Cad maternal and zygotic mutants (Magie et al., 2002). Thus, the complex of *D*E-Cad, Arm and *D*αCat is essential for integrity of the primary epithelium in the *Drosophila* embryo.

The *Drosophila* embryo develops as a syncytium until mitotic cycle 13, when newly synthesized membrane inserts between the nuclei, separating individual cells from each other. This process is called cellularization. *D*E-Cad, Arm and *D*αCat are already present during that phase (Fig. 1A-C). *D*E-Cad, Arm and *D*αCat accumulate in adhesive sides at the ingrowing plasma membrane, the so-called spot adherens

junctions (SAJ; Fig. 1B+1C). After cellularization, the embryo undergoes gastrulation accompanied by rapid morphological changes during mesoderm invagination and germband extension. During gastrulation, the epithelium further differentiates with *D*E-Cad, Arm and *D*αCat accumulating in the zonula adherens (ZA) at the apical site of the cell (Fig. 1D). *D*E-Cad, Arm and *D*αCat still localize to some extend to the lateral plasma membrane of the epithelium but are not organized into SAJ any longer. The ZA separates the apical from the basolateral plasma membrane domain ensuring differential localization of transmembrane proteins along the cell surface. From stage 14 on, additional sites of close membrane contact emerge at the basolateral plasma membrane, the septate junctions (SJ; Fig. 1E).

1.3 Cell polarity in *Drosophila* primary epithelia

Cell adhesion in primary *Drosophila* epithelia is mediated by *D*E-Cad, Arm and *D*αCat that are accumulated in the ZA as described in chapter 1.1. Clustering of *D*E-Cad, Arm and *D*αCat into the ZA at the end of cellularization requires expression of the genes *bazooka* (*baz*) and *crumbs* (*crb*). Mutants for either the *baz* or the *crb* gene show defects in assembly of the ZA and embryos die by stage 11 of development. Studies in the recent years showed that the cytoplasmic protein Bazooka (Baz) and the transmembrane protein Crumbs (Crb), in combination with many other proteins in a large complex, are necessary for establishment of apical basal cell polarity.

The cytoplasmic protein Baz is the *Drosophila* homologue of the partition defective 3 protein (PAR-3; Kuchinke et al., 1998). PAR-3 was first discovered to regulate asymmetric distribution of cell fate determinants in the *C.elegans* zygote in combination with other PAR proteins (Suzuki and Ohno, 2006). During cellularization in *Drosophila*, Baz localizes to the apical membrane of the embryo, were it is required for establishment of a functional ZA (Müller and Wieschaus, 1996; Cox et al, 1996; Harris and Peifer 2004). Baz localization itself is independent of adherens junctions and depends on an intact microtubule network and dynein (Hutterer et al., 2004; Harris and Peifer 2005). Baz directly interacts with *Drosophila* atypical Protein Kinase C (*D*aPKC) and the *Drosophila* homologue of PAR-6 (*Dm*PAR-6) via distinct protein domains

(Wodarz et al., 2000; Yamanaka et al., 2003). The interaction with Baz is necessary to restrict the localization of both *D*aPKC and *Dm*PAR-6 to the apical plasma membrane. This restriction is required for building of a continuous ZA as absence of Baz, *D*aPKC or *Dm*PAR-6 leads to defects in ZA maturation (Yamanaka et al., 2003; Betschinger et al., 2003; Hutterer et al., 2004; Harris and Peifer 2005). During cellularization, *D*aPKC and *Dm*PAR-6 rarely colocalize with Baz at the apical plasma membrane, and it hasn't been proven so far that direct binding to Baz is really necessary at this stage of development (Harris and Peifer 2005). However, the interaction of *D*aPKC, *Dm*PAR-6 and Baz is clearly required in later stages of development were they control the asymmetric cell division in the neuroblasts, progenitors of the *Drosophila* nervous system, (Wodarz et al., 2000).

Baz controls initial establishment of cell polarity as described above. After the initial polarization is achived, the Crb protein complex becomes crucial for maintenance of cell polarity in addition to Baz. The Crb protein complex consists of Crb, Stardust (Sdt) and the *Drosophila* Protein Associated with Tight Junctions (*D*PATJ) (Tepass et al., 1990; Bachmann et al., 2001; Hong et al., 2001; Pielage et al., 2003). *Crb* or *sdt* mutant embryos fail to establish an apical ZA, retain SAJ and show reduced cell adhesion (Grawe et al., 1996; Müller und Wieschaus, 1996; Tepass, 1996).

Crb is a transmembrane protein with a large extracellular and a small (37 amino acid) intracellular domain (Tepass et al., 1990). Mutation of the intracellular domain of Crb leads to a loss of function phenotype. Interestingly, a dominant phenotype can be obtained by overexpression of the cytoplasmic domain alone (Wodarz et al., 1993; Wodarz et al., 1995). The intracellular domain of Crb binds to the Spectrin cytoskeleton via *D*Moesin (Medina et al., 2002). It also mediates the interaction with Sdt via a PDZ (PSD95/discs large/zonula occludens) binding motif in the very C-terminus (Bachmann et al., 2001).

Sdt is a Membrane Associated Guanylate Kinase (MAGUK) that contains several protein-protein interaction domains. The PDZ domain of Sdt binds binds to Crb while one C-terminal L27 (Lin2/Lin7) domain interacts with *D*Lin7 and another L27 domain interacts with *D*PATJ (Roh et al., 2002; Bachmann et al., 2004; Kempkens, 2005). The binding partners of an SH3 domain and the function of the GUK domain are currently unknown. Interestingly, a conserved N-terminal region of Sdt interacts with *Dm*PAR-6

and this binding provides a direct link between the two apical protein complexes organized by Crb and Baz (Wang et al., 2004; Kempkens 2005).

In the mature ectoderm epithelium, the Crb complex co-localizes with Baz in the subapical region (SAR) adjacent apical to the ZA (Wodarz et al., 2000; Petronczki und Knoblich, 2001; Bachmann et al., 2001; Hong et al., 2001). At the apical plasma membrane, Crb and Baz act antagonistic to proteins that localize to the lateral plasma membrane, like the tumour suppressors Scribbled (Scrib), Discs large (Dlg) and Lethal Giant Larvae (Lgl) (Bilder and Perrimon, 2000; Bilder et al., 2001; Tanentzapf and Tepass, 2003). These proteins control localization of the ZA by restricting the size of the apical plasma membrane domain. Mutants for *scribble* and *dlg* show an expanded apical plasma membrane domain and a multilayered, rather than a single layered epithelium (Bilder and Perrimon, 2000). It is not clear so far whether Scrib, Dlg and Lgl form a large protein complex at the lateral plasma membrane. However, Scrib is necessary for localization of other lateral membrane determinants like Dlg or Lgl (Bilder et al., 2000). Lgl interacts with the secretory machinery of the cell by that regulating secretion of proteins and probably membrane insertion at the lateral plasma membrane itself (Arquier et al., 2001; Peng et al., 2000; Gangar et al., 2005). Interestingly, Lgl localization also depends on interaction with *D*aPKC and *Dm*PAR-6 as *D*aPKC phosphorylates Lgl to prevent its apical accumulation (Hutterer et al., 2004). However, the interplay between lateral proteins among each other and their interaction with the apical localized protein complexes is not very well understood.

1.4 Cell adhesion and polarity in the follicle epithelium

As described above, primary epithelial cells of *Drosophila* adhere to each other via the ZA. The localization of the ZA is controlled by the lateral Dlg/Scrib complex and Lgl as well as the apical Crb and Baz protein complexes. The mechanisms that control cell adhesion and polarity in the primary epithelium are also present in some secondary epithelia but additional factors are required.

A well characterized secondary epithelium of *Drosophila* is the follicle epithelium in the female germline. The follicle epithelium derives from somatic stem cells in the anterior tip of each ovariole. This region of the ovariole is called germarium and also

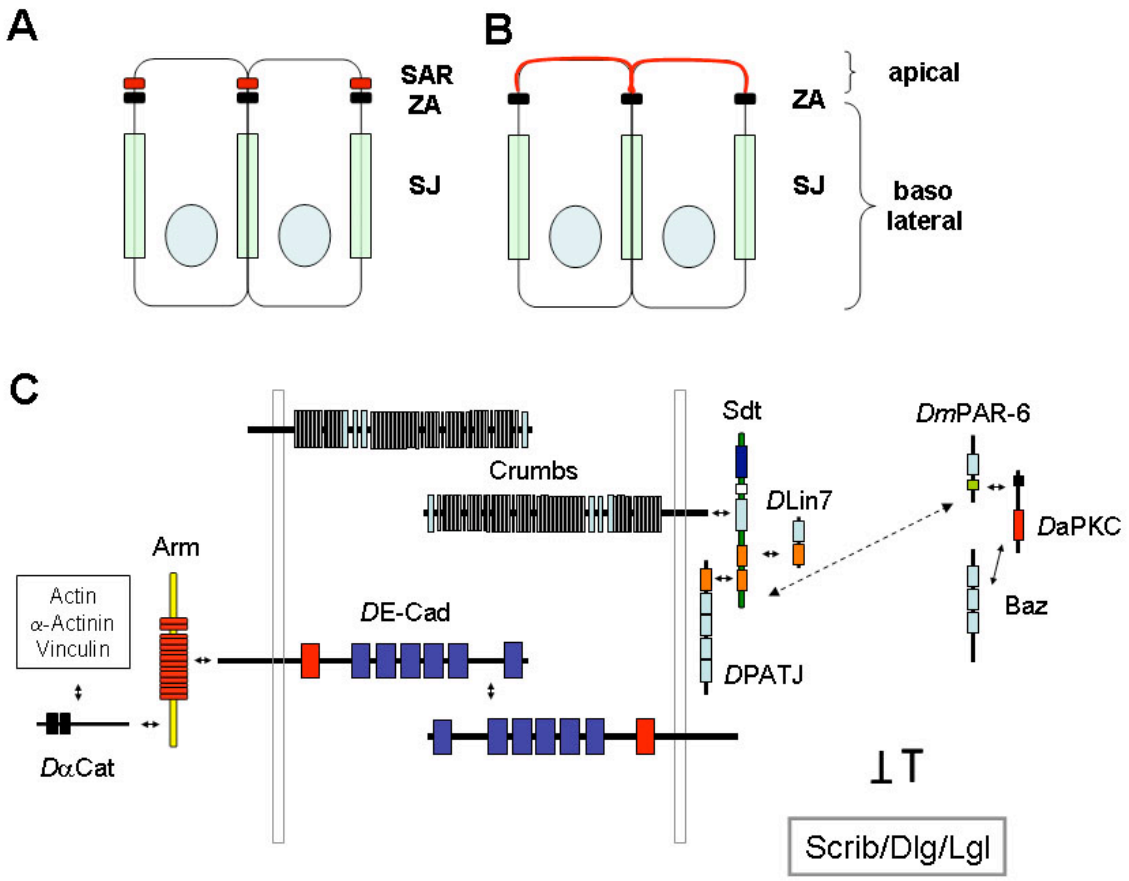


Figure 2: Large protein complexes regulate *Drosophila* cell polarity

(A) Proteins that define apical plasma membrane identity of primary *Drosophila* epithelia localize apical of the zonula adherens (ZA) to the Sub-Apical-Region (SAR). (B) In the follicle epithelium, the SAR seems to expand along the entire apical plasma membrane. The apical plasma membrane of follicle cells faces the oocyte while the basal site has contact with the basal lamina. (C) Proteins that mediate cell adhesion and cell polarity in *Drosophila* epithelia. The ZA contains *D*E-Cad, Arm and *D*αCat. The extracellular domain of the transmembrane protein *D*E-Cad contains 6 Cadherin repeats (dark blue boxes) and one LaminiG domain with unknown function (red box). The Cadherin repeat domains mediate homophilic interaction with Cadherin molecules on neighbouring cell in the presence of Ca^{2+}. The cytoplasmic tail of *D*E-Cad binds to the Arm-repeat region (red boxes) of *Drosophila* ß-Catenin, Armadillo (Arm). An N-terminal region adjacent to the Arm-repeats is necessary for binding of Arm to *D*α-Cat. *D*α-Cat in turn is associated with the actin-cytoskeleton either directly or via Vinculin or α–Actinin. Positioning of the ZA is under control of proteins that localize to the lateral or the apical plasma membrane. The apical proteins Bazooka (Baz), *Drosophila* atypical Protein Kinase C (*D*aPKC) and *Dm*PAR-6 form a protein complex that is necessary for initial clustering of the *D*E-Cad complex into the belt-like apical zonula adherens. The Baz complex is also required for localization of the transmembrane protein Crumbs (Crb) apical of the *D*E-Cad complex. The extracellular domain of Crb contains EGF- and Laminin-like domains with unknown function. The cytoplasmic domain of Crb binds to the PDZ domain (light blue boxes) of the MAGUK protein Stardust (Sdt) as well as *D*Moesin that provides a link to the spectrin cytoskeleton (not depicted). Sdt binds to *D*Lin7 and *D*PATJ via the L27 domain (orange boxes) and the N-terminus of Sdt binds to the PDZ domain of *Dm*PAR-6 linking the Crb protein complex to the Baz complex. The lateral group contains, among others, the proteins Dlg, Lgl and Scrib and restricts localization of the ZA and SAR by an unknown mechanism. Modified after Bilder, 2004.

contains the germ line stem cells. The follicle epithelium covers the germline cells and forms the egg chamber together with the underlying nurse cells and the oocyte. The egg chamber undergoes distinct stages of development to a mature egg (Fig. 3). The germ cells massively grow during stages 8-10 of development. The follicle cells stop proliferation by stage 7 and accumulate over the oocyte. These cells form a single layered columnar epithelium and are also called main body follicle cells. In contrast to the main body cells, the follicle cells associated with the nurse cell cluster are flattened and form a squamous epithelium.

In the mature follicle epithelium *D*E-Cad, Arm and *D*αCat accumulate in the ZA at the apical site of the cell (Fig. 2B). Additionally, *D*N-Cadherin (*D*N-Cad), another Cadherin expressed in follicle cells, localizes to the ZA as well. Arm function is essential for follicle cell adhesion as *arm* mutant cells show reduced adhesion and *D*E-Cad and *D*N-Cad are not accumulated in the ZA (Müller, 2000; Tanentzapf et al, 2000). In contrast to the reduced adhesion in *arm* mutants, follicle cells mutant for *D*E-Cad show only mild adhesion defects. This observation suggests that *D*E-Cad is not essential for cell adhesion in this secondary epithelium and it has been proposed that *D*N-Cad acts redundantly to *D*E-Cad (Tepass, 1999). However, involvement of *D*N-Cad in follicle cell adhesion has not been directly shown so far by loss of function mutations.

*D*E-Cad expression is not essential for cell adhesion in the follicle epithelium as described above. Interestingly *D*E-Cad is required for the movement of a subset migratory follicle cells, the border cells and the centripetal cells (Niewiadomska et al., 1999). Border cells delaminate from the follicle epithelium at stage 8 and migrate through the nurse cell cluster towards the oocyte. By stage 10, the border cells arrive at the oocyte and expand laterally. At this time point, another set of follicle cells start migrating centripetally towards the border cells. These centripetal cells undergo prominent cell shape changes. The apical plasma membrane domain of centripetal cells seems to shrink and the lateral plasma membrane appears to expand while the cells penetrate the germ cell cluster and move in between the nurse cells and the oocyte (Fig. 3D). Presence of *D*E-Cad in border and centripetal cells is necessary for migration as well as the accompanied cell shape changes.

Positioning of the ZA in follicle cells depends on the Crb and the Baz protein complexes (Tanentzapf et al., 2000; Bilder et al., 2003). Both the Crb and the Baz complexes localize to the entire apical plasma membrane of follicle cells and are not

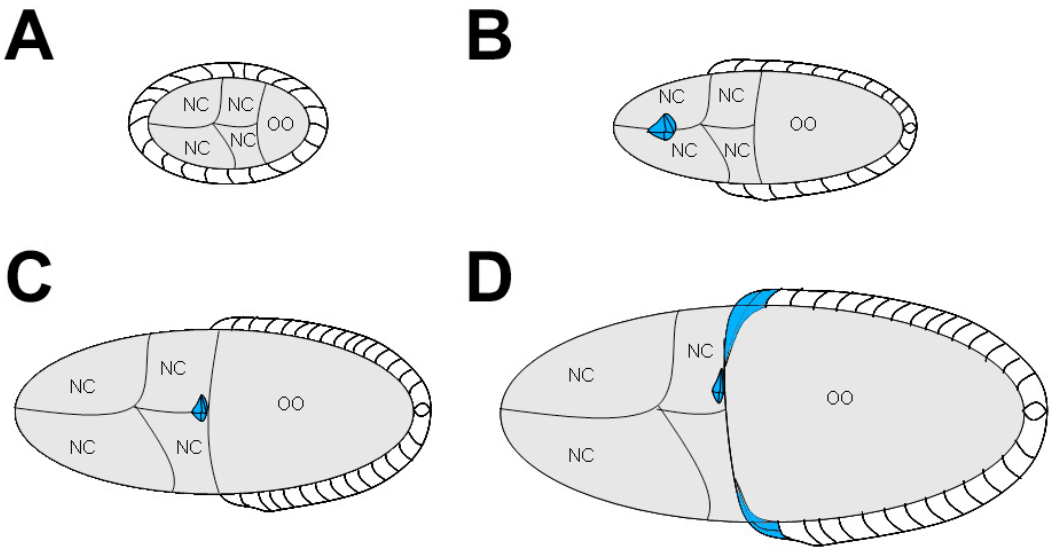


Figure 3: Development of the *Drosophila* egg chamber

A-D schematically depicts stages of egg chamber development in the *Drosophila* ovary. A single egg chambers contains the germ cells and an overlaying single cell sheet, the follicle epithelium. The germ cells consist of 15 nurse cells (NC) and one oocyte (OO) that are connected via cytoplasmic bridges. (A) Stage 5 egg chamber: the follicle epithelium forms a single cell layer that completely surrounds the germ cells. (B) Stage 8 egg chamber; the germ cells start to grow massively; the follicle epithelium above the NC starts to flatten and forms a squamous epithelium (not depicted in B-D); the follicle epithelium that lays above the oocyte remains columnar; the border cells (blue) delaminate and start migrating towards the oocyte. (C) Stage 10a egg chamber; border cells reach the oocyte; the columnar follicle epithelium is restricted to the region above the oocyte. (D) Stage 10b egg chamber; Centripetal cells (blue) start to penetrate between nurse cells and oocyte; centripetal cells flatten in direction of movement towards the border cells. In later development, nurse cells dump their content into the oocyte, the centripetal cells enclose the oocyte completely and nurse cells undergo programmed cell death.

restricted to a distinct sub apical region like in the embryo (Fig. 2B). The Crb complex is required for the initial polarization of the follicle cells. Large *crb* mutant clones that are induced early in follicle cell development show morphological abnormalities like multilayering of the epithelium. In contrast to this, *crb* mutant clones which are induced after maturation of the follicle epithelium show no obvious phenotype. Therefore, it has been suggested that Crb is dispensible for maintenance of cell polarity and ZA positioning in the follicle epithelium (Tanentzapf et al., 2000). Loss of *baz* function on the other hand severely affects establishment and maintenance of epithelial polarity and results in a stronger phenotype than *crb* mutation (Benton and StJohnson, 2003). Multilayering of the follicle epithelium is even more frequent in *sdt,baz* double mutant follicle cell clones, showing that Baz and Crb act redundantly to regulate cell polarity in the main body follicle cells (Bilder et al., 2003).

Baz is required for *D*E-Cad localization in the main body follicle cells and also in the border cells (Pinheiro and Montell, 2005). During border cell migration, Baz and *Dm*PAR-6 regulate membrane localization of *D*E-Cad which in turn is required for the movement as mentioned above. Interestingly, the Crb protein complex has been shown to localize asymmetrically in border cells, but the function of Crb in migration has not been addressed so far (Niewiadomska et al., 1999). A function of both the Crb and the Baz complex for movement of the centripetal cells at stage10 of oogenesis has not been analysed.

Baz and Crb regulate cell polarity in the follicle epithelium in interplay with several proteins. The cytokine receptor Domeless (Dome) for example has been shown to regulate expression or localization of Baz and Crb (Ghiglione et al., 2002; Pinheiro and Montell, 2005). Loss of *dome* during establishment of cell polarity results in a *crb* like phenotype (Ghiglione et al., 2002). Crb in turn regulates localization of Baz in the follicle epithelium in combination with the *Drosophila* homologues of the *C.elegans* PAR proteins PAR-1 and PAR5 (Benton and StJohnson, 2003). PAR-1 is a kinase that localizes to the lateral plasma membrane of follicle cells and phosphorylates Baz. Phosphorylated Baz can bind to 14-3-3, the homologue of PAR-5. Binding of Baz and 14-3-3 prevents assembly of the Baz complex at the lateral plasma membrane. Thus, PAR-1 and 14-3-3 are necessary to exclude Baz from the lateral plasma membrane compartment in main body follicle cells. It is not clear so far whether PAR-1 and 14-3-3 are effectors of the lateral Scrib/Dlg protein complex. However, mutants in either gene result in multilayering of the follicle epithelium very similarly to PAR-1 mutants. Direct interaction of Scrib or Dlg with PAR-1 and 14-3-3 has not been confirmed so far and it remains possible that these proteins act through different signalling pathways.

1.5 Terminal differentiation of epithelial cells undergoing apoptosis

The function of epithelia depends on tight intercellular contacts which are under control of cell polarity genes (chapter 1.1. and 1.2). Some developmental processes require negative regulation of both cell polarity and adhesion of epithelial cells. The prerequisite for neural crest cell migration in vertebrates or mesoderm migration in *Xenopus laevis* and *Drosophila melanogaster* for example is an epithelial to mesenchymal transition (Hay, 2005). Another developmental program that involves

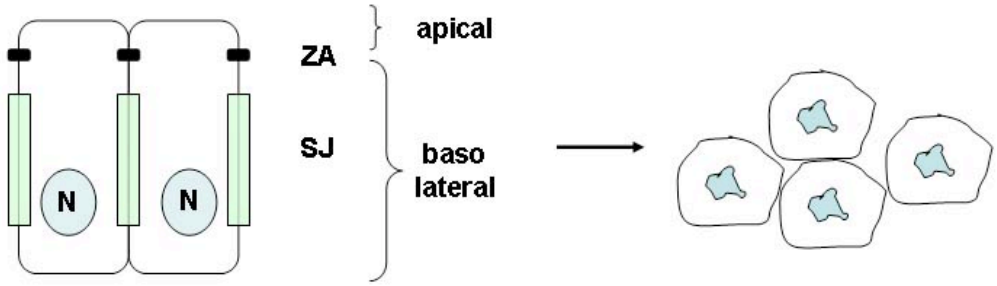


Figure 4: Terminal differentiation during apoptosis

From left to right: Apoptosis is executed upon activation of apoptotic enzymes, the caspases. Within cells of the highly polarized epithelium, caspases are regulated by Inhibitor of apoptosis proteins (IAP) and other upstream inhibitors of caspase activation. If negative regulation of caspases fails, an irreversible process of proteolytical degradation occurs. As a result cell polarity and adhesion is lost, accompanied by a series of morphological changes like cell shrinkage, fragmentation of the nucleus and blebbing of the plasma membrane.

negative regulation of cell polarity and adhesion of epithelial cells is the execution of programmed cell death, apoptosis. Apoptosis is a genetically controlled process that leads to removal of damaged or unwanted cells from the otherwise healthy tissue (Rodriguez et al. 1997).

Apoptosis is accompanied by typical morphological changes including cell shrinkage, membrane blebbing, and nuclear as well as DNA fragmentation. Finally, apoptotic cells are fragmented into membrane engulfed apoptotic bodies that are excluded from the tissue and cleared by macrophage uptake. The cellular mechanisms that lead to theses distinct morphological changes are mainly unknown.

Execution of apoptosis is mediated by apoptotic enzymes, the caspases that proteolytically cleave certain target proteins (Degterev et al., 2003). As proteolytic cleavage is irreversible, the process of caspase activation has to be tightly controlled. In *Drosophila*, expression of the cellular Inhibitor of Apoptosis Protein1 (DIAP1) is necessary to block caspases and thereby prevent cell death (Wang et al., 1999; Lisi et al., 2000). The function of IAPs in vertebrates is less well understood, as they act in a partially redundant manner (Vaux and Silke, 2005).

Many structural proteins are targets of caspase mediated cleavage and this cleavage might partially explain the cellular phenotype of apoptotic cells (Utz and Anderson, 2000). However, the functional importance of caspase dependent cleavage of structural proteins has not been demonstrated directly so far (Steinhusen et al., 2000; Steinhusen et al., 2001; Brancolini et al., 1997; Rao et al., 1996). Interestingly,

caspases regulate developmental processes different from apoptosis as well, including sperm cell differentiation (Arama et al., 2006; Arama et al., 2003; Huh et al., 2004), lymphocyte activation (Kabra et al., 2001; Chun et al., 2002) and extracellular signalling (Randle et al., 2001; Siegmund et al., 2001). As caspases have a highly specific substrate recognition, analysis of caspase dependent cleavage in apoptotic cells might provide insight into various other cellular processes that involve caspases, as cleavage events might be similar.

1.5.1. Caspases are proteolytic enzymes that lead to cell death

Apoptosis is mediated by cystein proteases that cleave their target polypeptides after an aspartate residue and are therefore called Caspases. Substrate specificity of caspases is unique, as no other known protease requires Asp for cleavage except GranzymeB (Thornberry et al., 1997). Caspases were first discovered to regulate developmental cell death in *Caenorhabditis elegans* (Ellis and Horvitz, 1986; Hengartner et al., 1992; Yuan et al., 1993). The *ced-3* gene encodes for the single *C.elegans* caspase and when CED-3 is absent, adult animals have more cells than their wildtype counterparts. Thus, the *C.elegans* caspase controls the invariant cell number in the adult worm by inducing cell death during development.

Caspases not only mediate cell death in *C.elegans* but in all multicellular organisms analyzed so far. In humans the *ced-3* homologous caspase is the Interleukin-1ß Converting Enzyme (ICE=caspase-1) one out of 11 caspases that were identified in mice and man (Lamkanfi et al., 2002). Caspases are potentially very dangerous molecules as they induce cell death when overexpressed in mammalian cells (Miura et al., 1993). Therefore, activation of caspases has to be a tightly regulated process to prevent unwanted cell death in the animal. The enzymatic steps that directly lead to caspase activation and the machinery that controls these actvation steps are very similar among the organisms.

Caspases are synthesized as inactive zymogens. The zymogen, or pro-caspase, contains a large and a small catalytic subunit as well as an N-terminal regulatory pro-domain. Caspases with long pro-domains are classified as initiator caspases. The long pro-domain of initiator caspases contains regulatory domains like a caspase-recruiting domain (CARD) or a death effector domain (DED).

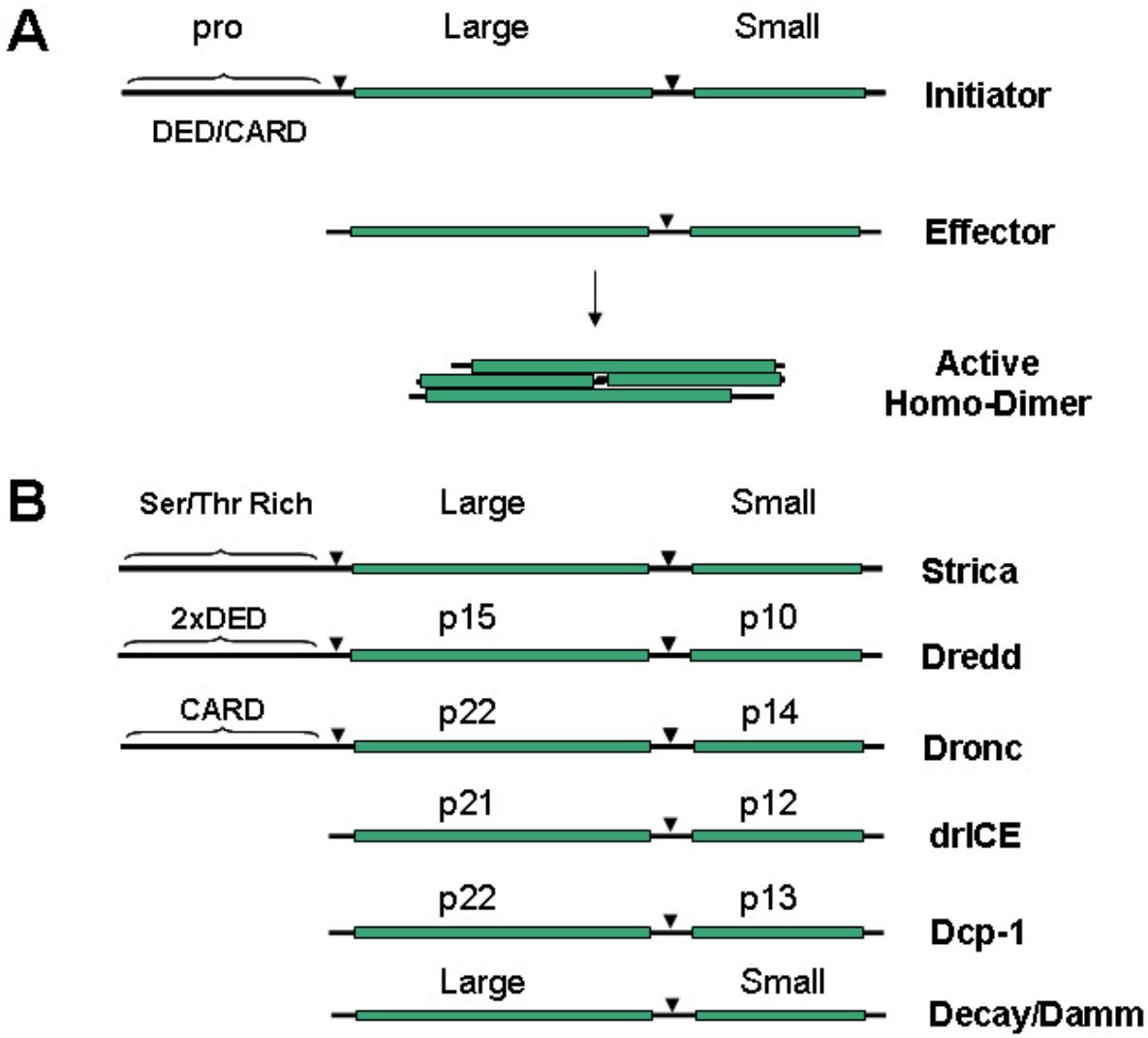


Figure 5: Activation of *Drosophila* caspases

Apoptosis is executed by caspases, cystein proteases that cleave target molecules at Asp residues in distinct tetra peptide motives. (A) Caspases are synthesized as zymogens that contain a large and a small catalytic domain. Caspases are characterized as initiator-caspases when they contain an N-terminal pro-domain with either a Death Effector Domain (DED) or a Caspase Recruiting Domain (CARD). DED or CARD are absent in executioner caspases and the pro-domain is short or absent. The zymogene becomes activated by cleavage at sites that resemble the caspase cleavage motive in target molecules (arrowheads (A) and (B)). Cleaved subunits interact with each other to form the active homo-dimeric caspase. (B) The *Drosophila* genome encodes for 7 caspases. The initiator caspases Dredd and Dronc contain either two DED or a CARD domain. A third caspase, Strica, contains an untypical Serin/Threonin rich pro-domain. drICE, DCP-1, Decay and Damm are executioner caspases. Modified after Kumar, 2000.

Both CARD and DED domains can be utilized to link upstream signalling events with activation of executioner caspases and subsequent cell death (Muzio et al., 1996; Juo et al., 1998; Varfolomeev et al., 1998). In contrast to initiator caspases, executioner caspases contain a short pro-domain without regulatory domain. Activation of both the initiator and the effector caspases involves proteolytic cleavage between the large and the small enzyme subunit as well as removal of the pro-domain. Cleavage of the

zymogene then leads to assembly of the active, homo-dimeric caspase enzyme (Fig. 5A).

The *Drosophila* genome encodes for the three caspases Dronc, Dcp-2 and Dream that contain long pro-domains and function as initiator caspases (Chen ate al., 1998; Dorstyn et al., 1999). The four *Drosophila* caspases Dcp-1, drICE, Decay and Daydream are executioner caspases with a short pro-domain (Fig. 5B; Song et al., 1997; Fraser and Evan, 1997; Dorstyn et al., 1999).

Active human caspases show individual substrate specificity as shown in vitro using a peptide library (Thornberry et al., 1997). Initiator caspases preferentially cleave a XEHD tetra peptide motif in the target molecule with X being an amino acid, with residue of large or intermediate size. In contrast to that, executioner caspases preferentially cleave tetra peptides similar to DEXD with X being a small amino acid like Histidin or Valin. Initiator and effector caspases not only differ in the length of the pro-domain but also show different substrate specificities. The consensus sequences XEHD and DEXD represent the most likely cleaved tetra peptide motifs in vitro and cleavage in vivo involves a similar spectrum of cleavage sites. Interestingly, caspases themselves contain conserved cleavage sites that lead to activation of one caspase by an array of other caspases. Thus, caspases are not only regulators of specific target molecules during apoptosis but also regulate activity status of other caspases (Degterev et al., 2003). The substrate specificity of *Drosophila* caspases does not significantly differ from vertebrate caspases (Thornberry et al., 1997; Hawkins et al., 2000; Song et al., 2000).

Similar to vertebrate caspases, activation of *Drosophila* caspases involves autocatalytic cleavage of tetra peptide motifs in the pro-caspase that resemble the consensus sequences in target molecules. In vertebrates, the pro-apoptotic protein Apaf-1 can trigger auto-activation of caspases in a structure called apoptosome by clustering of initiator-caspase-9 (Cain et al., 2000; Acehan et al., 2002). This clustering then leads to subsequent activation of effector caspases and cell death (Cecconi et al., 1999). Activation of caspase-9 in the apoptosome requires binding of Apaf-1 to Cytochrome C (CytoC), a protein that is localized to the mitochondria and becomes released to the cytoplasm prior to caspase activation. Release of CytoC from mitochondria is controlled by a set of proteins, the BCL-2 protein family, that resemble *C.elegans* CED-9 (Hengartner and Horvitz, 1994; Conradt and Horvitz, 1998; Vaux et al., 1988). In addition to the apoptosome, caspases can be activated by recruitment to

cell death receptors like the Tumor Necrosis Factor Receptor (TNFR). The TNF receptor exposes a death domain upon ligand binding that recruits CARD and DED domain containing adaptor molecules. These adaptor molecules themselves bind to initiator caspases and promote their activation (Ahmad et al., 1997; Duan and Dixit, 1997). The functions of the *Drosopila* BCL-like proteins and the TNF-Receptor homologue Wengen are not very well described in contrast to vertebrates (Claveria and Torres, 2003; Igaki et al., 2003; Kauppila et al., 2003).

In *Drosophila* the process that leads to activation of caspases is less known than in vertebrates. The fly homologue of Apaf1, Ark, binds and activates the initiator caspases Dronc and Dredd and induces cell death when overexpressed as an activated form in cultured cells (Zimmermann et al., 2002). Reduced levels of Dark in turn can suppress cell death induced by pro-apoptotic proteins in the *Drosophila* eye showing involvement of Ark in apoptosis regulation (Rodrigez et al., 1999). Interaction of Ark with *Drosophila* CytoC is reportedly important for caspase activation and results in male sterility of *ark* and *cytoC* mutants (Kanuka et al., 1999; Arama et al., 2003; Arama et al., 2006; Huh et al., 2004). As cellular levels of active caspases can depend on Ark and CytoC expression, both *ark* and *cytoc* are classified as pro-apoptotic genes. However, mutation of *ark* results in a weak embryonic phenotype comparing to mutation of other pro-apoptotic genes, namely *reaper (rpr)*, *grim* and *head involution defective* (*hid).* Absence of all three genes results in a complete lack of developmental cell death during *Drosophila* embryogenesis (Bergmann et al., 2003). The proteins Reaper, Hid and Grim promote cell death by direct interaction with cellular Inhibitor of Apoptosis Proteins, IAPs, the only know proteins that block apoptosis once caspases are activated (Zachariou et al., 2003).

1.5.2. Inhibitor of Apoptosis proteins directly interact with caspases

Apoptosis is induced upon distinct intrinsic or extrinsic signals, including DNA damage and infection with a pathogenic bacterium or virus. To avoid cell death of an infected host cell, the genome of some pathogens encodes inhibitory proteins that directly bind to caspases and by that inhibit apoptosis. These Inhibitor of Apoptosis molecules (IAP) act as pseudo substrates for caspases, blocking access of the active

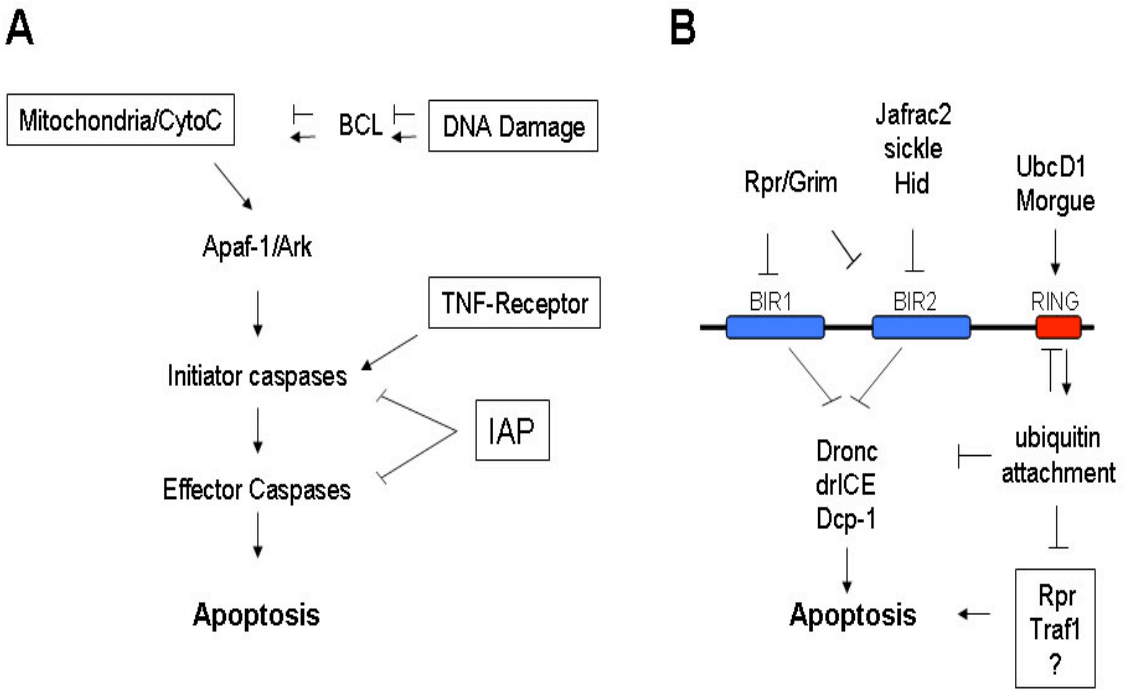


Figure 6: Regulation of Apoptosis in *Drosophila*

(A) The *Drosophila* Apaf-1 homologue Ark can activate initiator caspases by direct binding and assembly into a large protein complex, the apoptosome. Initiator caspases can activate effector caspases by direct cleavage. Activation of effector caspases leads to cleavage of cellular target molecules and apoptosis. Activation of Ark requires CytochromeC (CytoC) but unlike vertebrates *Drosophila* CytoC is not released to the cytoplasm. Activation of CytoC is mediated by *Drosophila* BCL homologues for example upon DNA damage. TNF receptors can activate Initiator caspases probably independent of the apoptosome but for *Drosophila* this has not been shown so far. Both Initiator-and effector caspases can be blocked by the *Drosophila* Inhibitor of apoptosis (DIAP1). (B) Regulation of caspases activation requires interaction of the Inhibitor of apoptosis DIAP1 with pro-and antiapoptotic proteins. The Baculoviral Repeat Regions BIR1 and BIR2 of DIAP1 inhibit caspases by direct binding. The proapptotic protein like Hid, Reaper, Grim, Sickle or Jarfrac2 can directly compete for binding and release the block of caspases. Thus, expression of pro-apoptotic proteins leads to cell death. DIAP1 contains a C-terminal RING domain with E3 ubiqitin ligase activity. The RING domain can target pro-apoptotic molecules like Reaper (Rpr), TRAF1 or Dronc for proteasomal degradation. Additionally, the proapoptotic proteins Hid and Reaper can trigger autocatalytic activity of the RING domain and lead to downregulation of DIAP1 protein levels. Reaper requires a specific E2 enzyme, UbcD1 for this. Morgue is an SCF like ubiquitin ligase that has been shown to regulate DIAP1 as well. Modified after Kornbluth and White, 2005; Vaux and Silke, 2005

centre to target proteins. The first discovered IAP was p35, a viral protein that inhibits host insect cell death upon infection (Clem et al., 1991). Subsequently, homologes genes in all multicellular organisms were identified that encode cellular IAPs with Baculoviral Inhibitor of Apoptosis Repeat (BIR) domains similar to p35 (Vaux and Silke, 2005). All cellular IAP proteins contain two or three BIR domains that facilitate binding to and inhibition of caspases. Additionally, cellular IAPs except NAIP contain a conserved C-terminal RING domain that can acts as an E3-Ubiquitin Ligase domain (Vaux and Silke, 2005). The function of the RING domain for apoptosis inhibition is not completely understood. On one hand, overexpression of the RING domain alone induces apoptosis

in the *Drosophila* eye and the RING domain negatively regulates the half-life of IAPs expressed in cultured cells (Harvey et al., 1997). These findings suggest that the RING domain might be necessary to target IAPs for degradation in an autocatalytic manner thereby acting as a pro-apoptotic regulatory domain. On the other hand, the RING domain is important to negatively regulate initiator caspases and other pro-apoptotic proteins acting as an anti-apoptotic domain (Lisi et al., 2000; Muro et al., 2002; Kuranaga et al., 2002). A model that partially explains these opposing functions of the IAP RING domain emerged from experiments using *Drosophila* as a model system.

Drosophila contains four genes encoding for the BIR containing proteins DIAP1, DIAP2, the survivin homologue deterin and dBruce (Hay, 2005). DIAP2 regulates the innate immune response (Gesellchen et al., 2005; Kleino et al., 2005). Additionally, DIAP2 can negatively regulate apoptosis as overexpression of DIAP2 counteracts Reaper, Hid and Grim (RHG) induced apoptosis in the *Drosophila* eye (Hay et al., 1995). Similarly, gain of function of dBruce can inhibit apoptosis (Vernooy et al., 2002). Deterin seems to be involved in regulation of apoptosis and cell cycle regulation (Wenzel et al., 2000; Jones et al., 2000; Gigoux et al., 2002).

In contrast to the other BIR containing proteins, DIAP1 is clearly necessary to prevent apoptosis in *Drosophila*. DIAP1 is encoded by the gene *thread* and several mutations of *thread* show its anti apoptotic requirement (Lisi et al., 2000). Embryos mutant for *thread* loss of function alleles exhibit massive apoptosis in early embryo development accompanied by strong activation of executioner caspases (Wang et al., 1999; Yoo et al., 2002). Inhibition of caspases by DIAP1 is mediated by direct binding of the initiator caspase Dronc and the effector caspases drICE and Dcp-1 to the BIR domains of DIAP1. RHG proteins can also directly bind to DIAP1 BIR domains via an N-terminal sequence that is conserved among the RHG proteins and the vertebrate pro-apoptotic proteins Smac/DIABLO and HtrA2/Omi (Chai et al., 2000; Wu et al., 2000; Srinivasula et al., 2001). Binding of RHG competes with the binding of DIAP1 to caspases, thus initiating caspases activation and cell death (Yan et al., 2004).

RHG proteins share the N-terminal sequence necessary for binding to DIAP1 but differ in their binding capacity to either the BIR1 or the BIR2 domain (Zachariou et al., 2003). The functional importance for differential binding is not clear but probably reflects mechanistic differences how RHG promote cell death. Analysis in the *Drosophila* eye identified three classes of *thread* alleles that act as enhancers or suppressors of RHG

provoked cell death (Lisi et al., 2000). These classes include loss of function mutations that enhance cell death induced by either *hid, reaper* or *grim*. Interestingly, the gain of function mutation th^{SL} affects the BIR1 domain of DIAP1, and enhances HID mediated apoptosis but suppresses Rpr and Grim mediated cell death. In contrary to that, the th^{6} and $th^{81.03}$ alleles enhance Rpr and Grim mediated apoptosis but suppress the effect of overexpressed Hid. Th^{6} and $th^{81.03}$ are point mutations within the RING domain, suggesting that Hid mediated apoptosis depends on a functional E3 ubiquitin ligase activity of DIAP1 while Reaper and Grim might act differently. Indeed, Hid expression can enhance E3 dependent auto degradation of DIAP1 and leads to reduced cellular DIAP1 levels, subsequent caspase activation and cell death (Yoo et al., 2002). In contrast to Hid, Reaper can negatively regulate DIAP1 in combination with the E3 ubiquitin ligase UbcD1 (Ryoo et al., 2002). Thus, RHG proteins can regulate caspase activity by direct competition for binding and by targeting DIAP1 for proteasomal degradation.

DIAP1 can target itself or the pro-apoptotic proteins Dronc, TRAF1 and Rpr for proteasomal degradation (Meier et al., 2000, Kuranaga et al. 2002). At present it is unclear which mechanism regulates the switch between auto-ubiquitination and ubiquitination of pro-apoptotic proteins but it could involve yet unknown DIAP1 binding proteins. These putative regulators of IAPs E3-ligase domain might act in other organisms in a similar way given the high homology of the IAP protein family. As mentioned above, the pro-apoptotic proteins Smac/DIABLO and HtrA2/Omi regulate caspase activation in vertebrates by binding to IAPs via an RHG-like N-terminal motif. Thus, analysis of *Drosophila* proteins that interact with DIAP1 can reveal useful insights to cell death pathways in general.

1.6 Aim of Work

Apoptosis in *Drosophila melanogaster* is negatively regulated by the main Inhibitor of apoptosis protein, DIAP1. When DIAP1 is missing, cell death occurs accompanied by distinct morphological changes that are typical for apoptotic cells in all multicellular organisms. The molecular mechanisms that lead to most of these hallmarks of apoptosis are not well understood so far. One striking morphological phenotype of apoptotic epithelial cells is the loss of cell adhesion during early stages of apoptosis. This can also be observed in *Drosophila* embryos mutant for DIAP1 and is accompanied by strong activation of apoptotic enzymes, the caspases (Wang et al., 1999).

One aim of this work is to identify a molecular mechanism that regulates the loss of cell adhesion in apoptotic cells of *Drosophila.* Therefore, DIAP1 mutant embryos will be analyzed for changes in localization and stability of cell adhesion molecules during apoptosis. The caspase dependent cleavage of one ZA component, Armadillo, will be analyzed in detail. The functional importance of Arm cleavage for down-regulation of cell adhesion during early stages of apoptosis will be assessed in vitro and in vivo.

The anti-apoptotic function of DIAP1 is based upon inhibition of caspases in the cytoplasm of epithelial cells. Recently, the work in our group revealed that DIAP1 is not only localized to the cytoplasm but also localizes to a distinct compartment in the epithelial plasma membrane (Gagic M., 2005). It has not been reported so far that caspases localize to this membrane compartment, suggesting a novel function of DIAP1 that is probably independent of caspase inhibition.

It is the aim of this work to identify the mechanism that recruits DIAP1 to the apical plasma membrane of follicle cells and to analyze the functional importance of DIAP1 during egg development. DIAP1s apical localization will be analysed with respect to known protein complexes that regulate cell polarity. The function of DIAP1 in the follicle epithelium will be determined by analysis of DIAP1 mutant follicle cell clones. In vitro assays will be used to characterize a novel function of DIAP1s E3 ubiquitin ligase for regulation of a protein complex that controls cell polarity. Additionally, an upstream signalling pathway will be analyzed that regulates the function of DIAP1 in the follicle epithelium.

2. Results

Regulation of apoptosis in all multicellular organisms requires the activation and inhibition of caspases, aspartate specific cystein proteases. The inhibition of caspases is mediated by the Inhibitor of Apoptosis protein (IAP) family that blocks cell death by direct binding to caspases or targeting them for proteasomal degradation. In *Drosophila* the main Inhibitor of Apoptosis Protein (DIAP1) is encoded by the gene *thread.* Expression of DIAP1 throughout the *Drosophila* life cycle is essential to prevent cell death in almost all tissues examined.

Caspases as well as IAPs are usually expressed in the cytoplasm of all cells, but in some cases a differential localization within the cell has been reported. For example caspases 8 as well as cIAP2 localize to the nucleus of cultured cells (Besnault-Mascard et al., 2005; Vischioni et al., 2004). Also DIAP1 is not solely a cytoplasmic protein but differential subcellular localization has been reported by a number of researche groups (Gagic, 2005; Yoo et al., 2002, Geisbrecht and Montell, 2004; Vucic et al., 1998; Yokokura et al., 2004). The functional importance of the differential localization of caspases and their inhibitors within the cell as well as the mechanism that regulates this is not clear.

The fruit fly *Drosophila melanogaster* provides excellent tools to study the mechanisms that control the cell death machinery and the first part of this thesis is focused on a novel function of DIAP1 in the follicle epithelium of the *Drosophila* ovary. As it will be shown, DIAP1 is differentially localized within this tissue and controls aspects of apical-basal cell polarity. The second part of this thesis is focused on the implication of DIAP1 in control of cell polarity and cell adhesion in the *Drosophila* embryo. Both parts of this study reveal the general importance of DIAP1 for integrity of and cell polarity in *Drosophila* epithelia.

2.1 DIAP1 is differentially localized within somatic tissue of the ovary

The follicle epithelium is a single layered epithelium that surrounds the developing germline cells (Fig. 7A). In stage 10 of egg development, the anterior follicle cells surround the nurse cells (NC) forming a squamous epithelium. The posterior follicle cells above the oocyte (O) form a columnar epithelium.

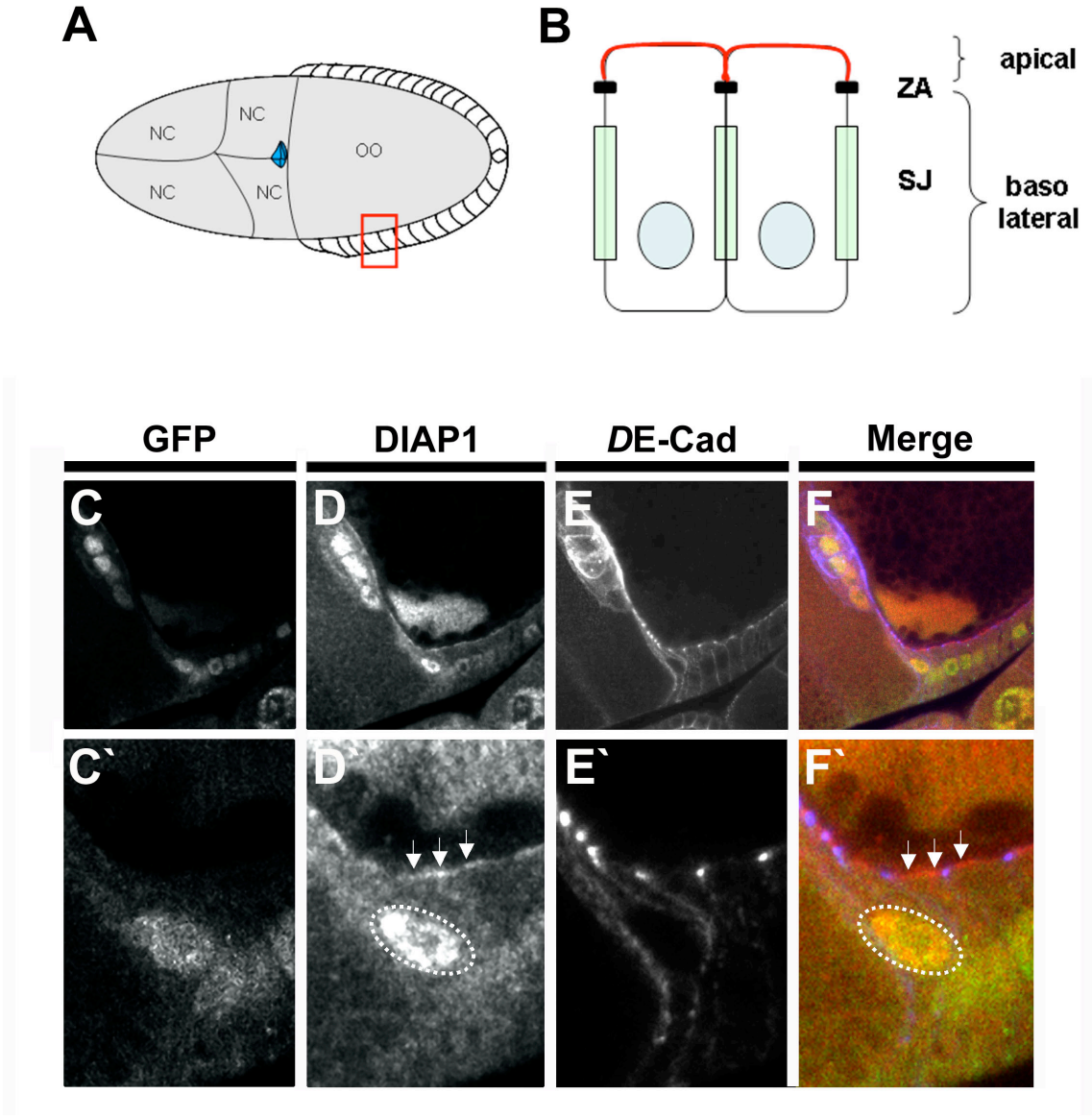


Figure 7: DIAP1 localizes to the apical membrane domain of follicle cells

(A) A schematic stage 10 egg chamber. Nurse cells (NC); Oocyte (OO); border cells indicated by blue colour. The epithelium above the oocyte is columnar. Red square: area of magnification in B. (B) Schematic representation of cell polarity and adhesion within follicle cells. Apical and basolateral membrane domains are indicated. The zonula adherens (ZA) separates both membrane domains. Septate junctions (SJ) are additional sites of close membrane contact. (C-F) Localization of DIAP1 in the columnar follicle epithelium of a stage 10 egg chamber stained for (C) nuclear GFP, (D) DIAP1 and (E) *D*E-Cad. (C`-E`). Higher magnification of C-E. (F+F`) show an overlay of C-E and C`-E` with GFP in green, DIAP1 in red and *D*E-Cad in blue. DIAP1 localizes to the cytoplasm, the nucleus and the apical membrane domain of follicle cells (Arrows in D` and F`). Centripetal cells show enhanced accumulation of nuclear DIAP1 (dotted circle in D` and F`). Here and below: Anterior is to the left.

The columnar epithelium consists of cells with distinct apical and a basolateral plasma membrane domains (Fig. 7B). The apical plasma membrane domain faces the oocyte while the basolateral domain is in contact with the basal lamina. The zonula

adherens (ZA) contains the Cadherin/Catenin system of cell adhesion molecules and separates the apical and basolateral membrane domains.

DIAP1 is localized to the cytoplasm and the nuclei of all follicle cells (Fig. 7). Nuclear localization of DIAP1 is enhanced in a subset of migrating epithelial cells, the centripetal cells and the border cells (Fig. 7D+D`). Additionally, DIAP1 is localized to the apical plasma membrane of the follicle cells facing the oocyte suggesting a novel function of the caspase inhibitor at this cellular compartment.

2.2. DIAP1 colocalizes with and binds to *D*PATJ in follicle cells

DIAP1 localizes to the apical plasma membrane in the follicle epithelium as shown above. To investigate the importance of DIAP1 enrichment at the apical plasma membrane, it was tested wether a functional connection between DIAP1 and other proteins that localize apically could be established.

Two large protein complexes, the Bazooka (Baz) and the Crumbs (Crb) complex localize to the apical plasma membrane and regulate establishment of cell polarity (Müller, 2000; see introduction for details). One component of the Crb complex that marks the apical plasma membrane of follicle cells is *D*PATJ (Tanentzapf et al., 2000). DIAP1 co-localizes with *D*PATJ at the apical plasma membrane of the follicle epithelium (Fig. 8). The apical determinants Baz and *Dm*PAR-6 are localized to the apical membrane in the same way as *D*PATJ and therefore also co localize with DIAP1 (see below). However, *D*PATJ and DIAP1 do not completely co-localize in the follicle epithelium as *D*PATJ localizes to a basal membrane area where the columnar epithelium meets the squamous epithelium while DIAP1 does not.

To test whether DIAP1 not only co localizes with but also binds to the Baz- or the Crb complex, GST-Pulldown experiments were performed using a GST-DIAP1 fusion protein and embryo or ovary tissue extract. The presence of putative interaction partners bound to GST-DIAP1 was tested by Western blotting using antibodies against *D*PATJ and Bazooka (Fig. 8E). Presence of DIAP1 was revealed by anti DIAP1 antiserum. In all experiments *D*PATJ was pulled down by DIAP1 but not to GST alone. In contrast to this, GST-DIAP1 never pulled down Baz nor did GST alone. This data suggests that DIAP1 and *D*PATJ directly or indirectly bind to each other in the protein extract, while DIAP1 does not physically interact with the Baz protein complex under the same conditions.

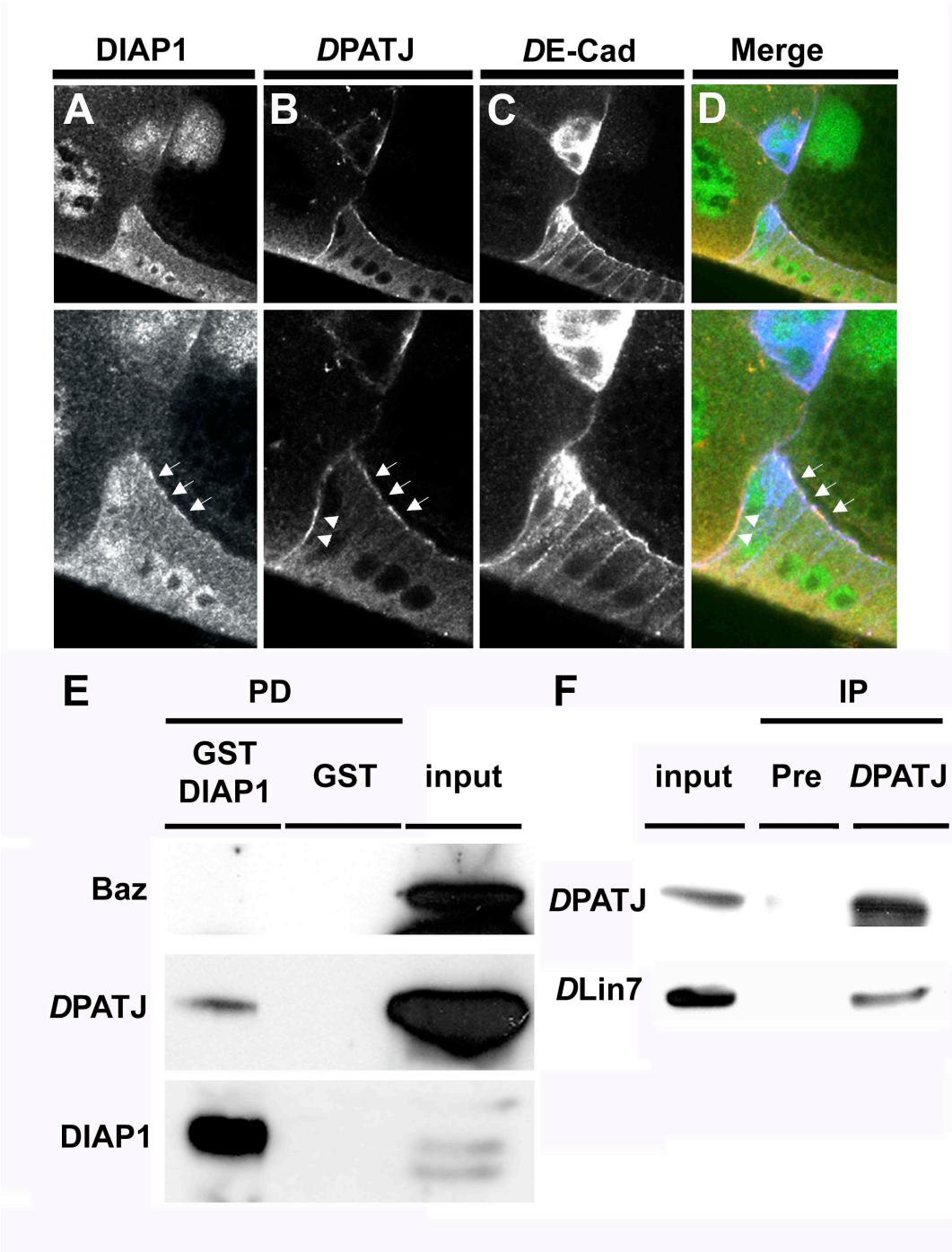


Figure 8: DIAP1 co localizes with and binds to the apical determinant *D*PATJ

A-D Localization of DIAP1 in a stage 10 egg chamber with respect to the apical marker protein *D*PATJ. Lower panel shows a higher magnification of the same egg chamber (A) Localization of DIAP1 (B) Localization of *D*PATJ (C) ZA marked by DE-Cad (D) Overlay of A-C with DIAP1 in green, *D*PATJ in red and *D*E-Cad in blue. Arrows in D mark co-localization of DIAP1 and *D*PATJ. Arrowheads in B mark additional localization of *D*PATJ at the lateral plasma membrane. (E) Western blot of a GST-Pulldown (PD) with GST or GST-DIAP1 using embryonic tissue extract. DIAP1 has the M_r of ~50 kDa (indicated DIAP1). Baz has a M_r of ~190 kDa (indicated Baz). *D*PATJ has the M_r of ~120 kDa (indicated *D*PATJ). *D*PATJ is pulled down by GST-DIAP1 but not by GST alone while Baz is neither pulled down by GST alone nor by GST-DIAP1. (F) Co-immunoprecipitation of *D*PATJ and *D*Lin7 using *D*PATJ antiserum; the precipitate was blotted for presence of *D*PATJ and *D*Lin7; *D*PATJ antiserum precipitates *D*PATJ and *D*Lin7 from ovary extract while control preimmune serum (Pre) does not.

The function of the Crb complex for establishment of cell polarity in the follicle epithelium has been clearly shown (Tanentzapf et al., 2000). However, the composition of the Crb complex in follicle cells has not been studied. In the embryo, Crb directly binds to the MAGUK protein Sdt, which in turn binds to *D*PATJ as well as *Drosophila* Lin7 (Bachmann et al., 2001; Bachmann et al., 2004; Kempkens, 2005). To test, whether a similar protein complex is present during egg chamber development *D*PATJ was precipitated from ovary extract and the precipitate was analyzed for presence of *D*Lin7 (Fig. 8E). Indeed, *D*Lin7 can be precipitated by *D*PATJ antiserum suggesting that *D*PATJ and *D*Lin7 form a stable protein complex in ovary extract. It is highly likely due to the published data that Sdt is found in this complex as well (Kempkens, 2005; Bachmann et al., 2004).

2.3 Apical localization of DIAP1 is altered in *sdt* mutant follicle cells

DIAP1 localizes to the apical membrane of follicle cells as shown above. This localization might be due to the interaction with *D*PATJ, as DIAP1 itself contains no reported membrane binding domain. Thus, the question arises whether DIAP1 still localizes to the apical membrane in *D*PATJ mutant cells. Unfortunately, null mutations affecting the *D*PATJ locus are not available (Bhat et al., 1999; Richard et al., 2005). However, *D*PATJ directly interacts with Stardust and this interaction recruits *D*PATJ to the apical membrane (Tanentzapf et al., 2000). If *D*PATJ localization was necessary to recruit DIAP1, the apical localization of DIAP1 should be missing in *sdt* mutant follicle cells. To test this hypothesis the FRT/FLP system was employed (Xu and Rubin, 1993). The FRT/FLP system allows the induction of mitotic recombination for example in somatic tissue like the follicle epithelium. As a result, clones of mutant cells are generated that are surrounded by wildtype tissue. To distinguish the cell populations from each other the wildtype chromosome contains a marker gene, like *lacZ* (ß-Gal) or nuclear localized Green Fluorescent Protein (GFP). All cells lacking this marker are homozygous mutant for the gene of interest.

sdt^{XP96} mutant cells show reduced membrane association of DIAP1 in comparison to wildtype cells (Fig. 9B). The overall cellular level of DIAP1 in the clonal area seems unchanged comparing to wildtype cells, suggesting that sdt^{XP96} affects

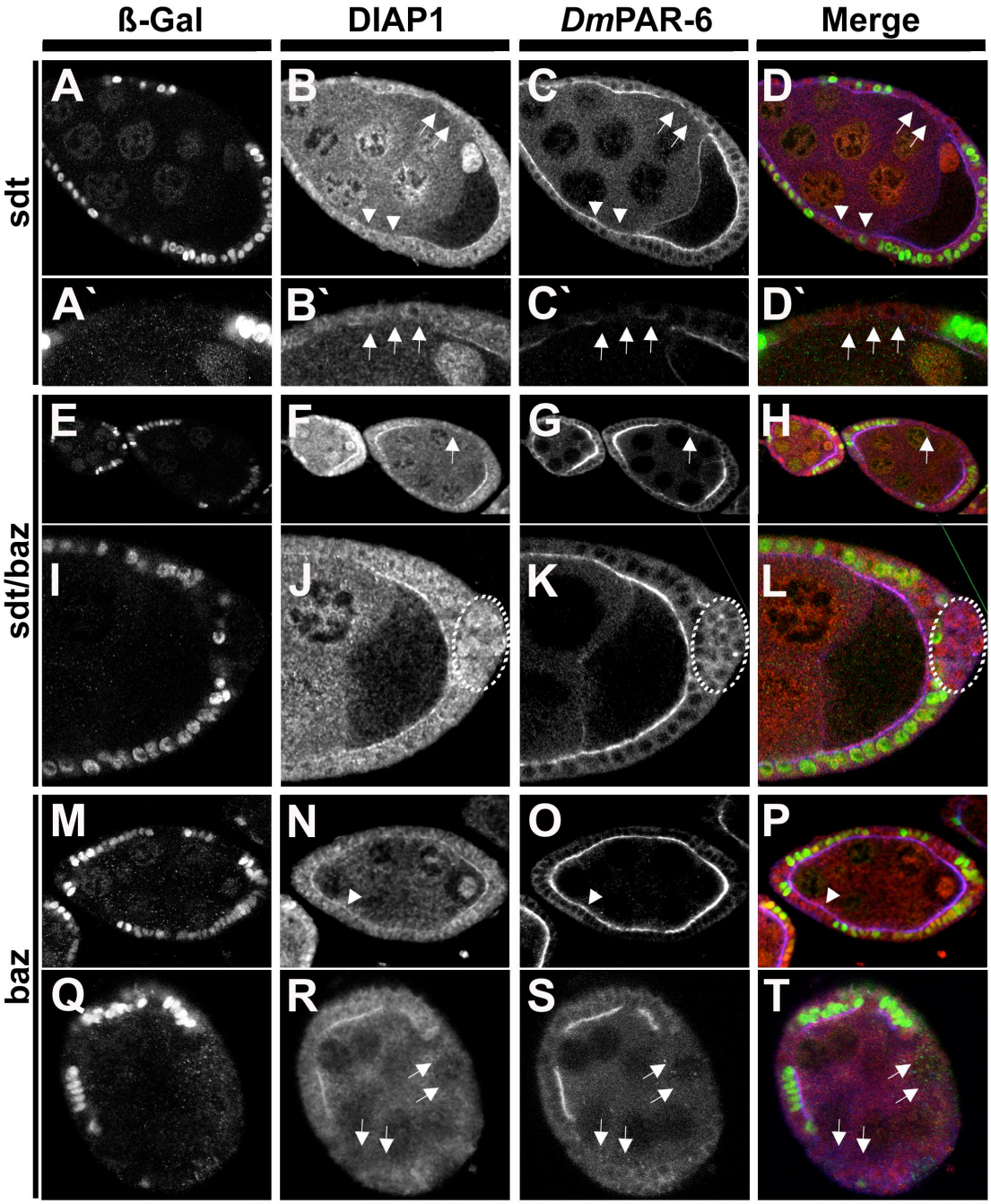


Figure 9: DIAP1 localization in *sdt* and *baz* mutant follicle cells

(A-D) Stage 6 egg chamber with two large sdt^{XP96} mutant clones (A`-D`) Higher magnification of the upper clonal area (A´+A`) *lacZ* expression (B+B`) DIAP1 localization and (C+C`) *Dm*PAR-6 localization (E-H). Stage 4 egg chamber with sdt^{XN05},baz^{YD97} double mutant clones (I-L) sdt^{XN05},baz^{YD97}double mutant clone at the posterior pole of a stage 8 egg chamber (E+I) *lacZ* expression (F+L) DIAP1 localization (G+K) *Dm*PAR-6 localization; Lack of apical DIAP1 and *Dm*PAR-6 in sdt^{XP96} and baz^{Xi106} and sdt^{XN05},baz^{YD97} mutant cells is indicated by arrows; Note that sdt^{XN05},baz^{YD97} double mutant epithelia become multilayered (dotted circle in J-L). (M-P) Stage 5 egg chamber with multiple baz^{Xi106} mutant clones (M+Q) *lacZ* expression (N+R) DIAP1 localization (O+S) *Dm*PAR-6 localization; Note that DIAP1 and *Dm*PAR-6 localize to the apical plasma membrane in small baz^{Xi106} mutant clones (arrowhead in N-P) but not in large baz^{Xi106} mutant clones (arrows in Q-T); Merged images with ß-Gal in green, DIAP1 in red and *Dm*PAR-6 in blue. Mutant cells are identified by lack of ß-Gal.

localization but not expression of DIAP1. *Dm*PAR-6 is localized to the apical membrane in wildtype cells where it colocalizes with DIAP1, but apical localization is missing in sdt^{XP96} mutant cells (Fig. 9C). Thus, DIAP1 and *Dm*PAR-6 localization in the follicle epithelium depends on Sdt and therefore a functional Crb complex.

Baz regulates establishment of cell polarity in the follicle epithelium in combination with Crb. Interestingly, DIAP1 localization at the apical membrane is not changed in small baz^{Xi106} mutant clones (Fig. 9N+9O) probably due to perdurance of the Baz protein (Cox et al., 1996). Similarly, localization of *Dm*PAR-6 is not altered in small baz^{Xi106} mutant clones. In large baz^{Xi106} mutant clones however, both DIAP1 and *Dm*PAR-6 do not localize to the apical plasma membrane (Fig. 9R and 9S), probably as the Crb protein itself is delocalized (Tanentzapf et al., 2000).

To test whether DIAP1 localization is affected when both the Crb and the Baz complex are missing, DIAP1 localization in sdt^{XN05},baz^{YD97} double mutant cells was analyzed. Similar to sdt^{XP96} single mutants, DIAP1 is not localized to the apical plasma membrane in sdt^{XN05},baz^{YD97}double mutant cells while the cellular level of DIAP1 is unchanged (Fig. 9F+J). *Dm*PAR-6 is not localized to the apical membrane in the same cells (Fig. 9G+K). Interestingly, the follicle epithelium becomes multilayered in small sdt^{XN05},baz^{YD97} mutant clones and DIAP1 and *Dm*PAR-6 are delocalized in these small clones (Fig. 9J+9K). The observation that the sdt^{XN05},baz^{YD97} double mutant phenotype is more severe than the phenotype of each single mutant shows that *sdt* and *baz* act redundantly to control cell polarity in the follicle epithelium. Together, these findings suggest that apical localization of DIAP1 in follicle cells is controlled by the Crb protein complex as well as the Baz protein complex.

2.4. Functional importance of DIAP1 in the follicle epithelium

2.4.1 DIAP1 mutations alter cell polarity in the follicle epithelium

As demonstrated in chapter 2.2, DIAP1 co localizes with and is pulled down by *D*PATJ. *D*PATJ interacts with the Crb complex that regulates cell polarity within the follicle epithelium. Also, DIAP1 localization depends on the Crb complex as shown in chapter 2.3. These findings raise the question whether DIAP1 itself might control aspects

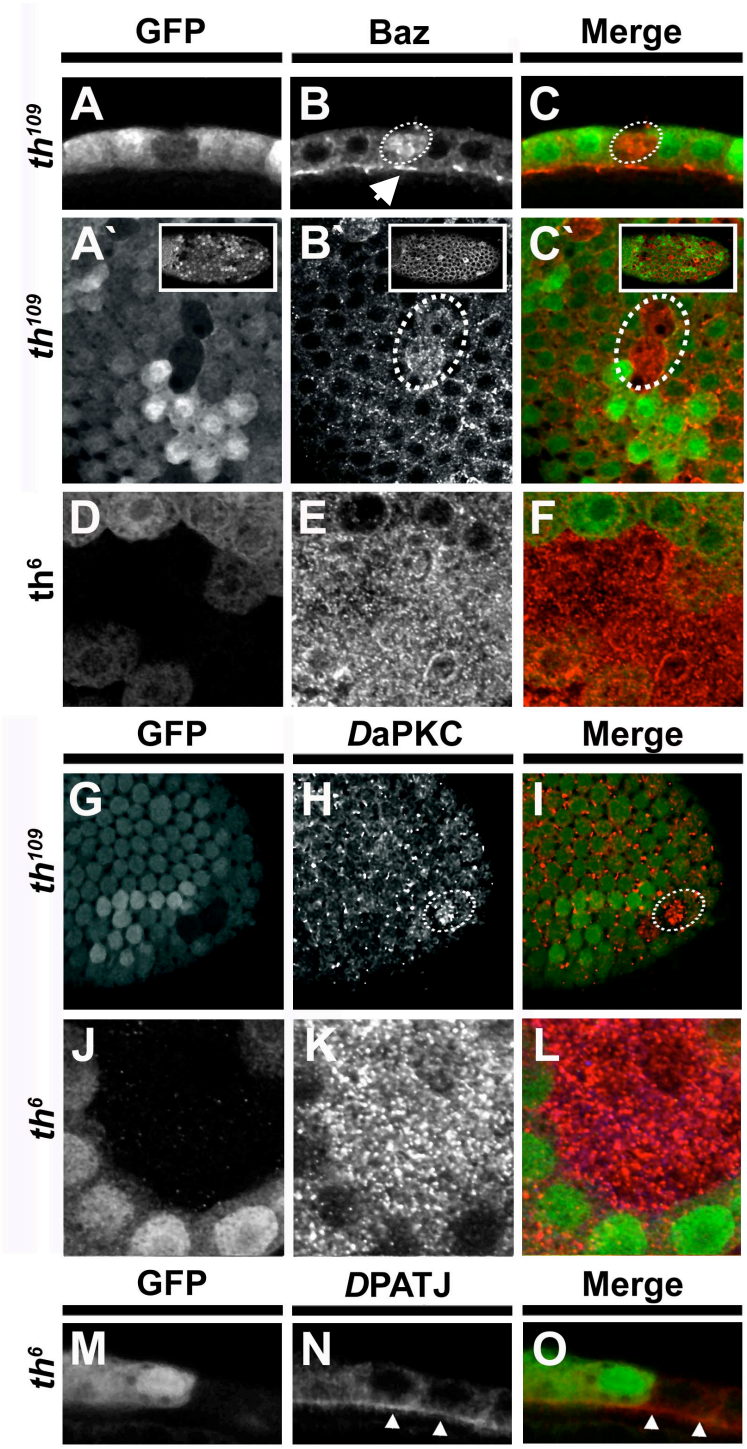


Figure 10:
Cytoplasmic accumulation of Baz and *D*aPKC in DIAP1 mutant follicle cells

(A-C and A´-C`) Stage 10 egg chamber containing cells mutant for the DIAP1 null allele $th^{109}$ (A+A`) GFP expression (B+B`) Baz localization (C+C`) overlay with GFP in green and Baz in red, mutant cells are marked by absence of GFP. Baz localizes to the apical membrane in wildtype and th^{109} homozygous cells (arrow in B); elevated cytoplasmic levels of Baz in th^{109} homozygous mutant cells are indicated by the dotted circle in B + B`. (D-F) Stage 10 egg chamber with a large th^{6} mutant clone stained for (D) GFP and (E) Baz; (F) Overlay of D and E with GFP in green and Baz in red. Note the cytoplasmic accumulation of Baz in th^{6} mutant cells. (G-I) Stage 10 egg chamber with a single th^{109} mutant clone stained for (G) GFP and (H) *D*aPKC. (I) Overlay with GFP in green and *D*aPKC in red; Note cytoplasmic accumulation of *D*aPKC in the th^{109} homozygous cell (dotted circle in H). (J-L) Stage 10 egg chamber with a th^{6} mutant clone stained for (J) GFP and (K) *D*aPKC (L) Overlay with GFP in green and *D*aPKC in red; Note accumulation of *D*aPKC in th^{6} homozygous mutant cells; (M-O) th^{6} mutant follicle cell clone stained for (M) GFP and (N) *D*PATJ; (O) Overlay with GFP in green and *D*APTJ in red; *D*PATJ localization is not altered in th^{6} mutant cells (arrowheads in N).

of cell polarity in the follicle epithelium. To answer this question, clonal analysis of DIAP1 mutant cells was performed using the FRT/FLP system and two alleles of the DIAP1 encoding gene *thread* (*th*).

The allele th^{109} is a point mutation that leads to a premature stop codon within the first BIR domain of DIAP1 and is a protein null mutation (Wang et al., 1999). The allele th^{6} is a point mutation within the C-terminal RING domain of DIAP1, that abolishes the E3 ubiquitin ligase function of DIAP1 (Lisi et al., 2000).

To test whether DIAP1 mutant cells show any alteration of cell polarity, DIAP1 mutant tissue was analyzed for localization of the apical determinants Baz, atypical Protein Kinase C (*D*aPKC) and *D*PATJ. In wildtype tissue both Baz and *D*aPKC are mainly associated with the apical plasma membrane domain with some residual protein localized to the cytoplasm. When DIAP1 is absent, Baz and *D*aPKC predominantly localize to the cytoplasm (Fig. 10B, 10B` and 10H). Additionally, staining intensity for Baz and *D*aPKC appears stronger when DIAP1 is absent indicating that protein levels of Baz and *D*aPKC might be elevated. Note that the size of th^{109} homozygous clones is much smaller than the corresponding wildtype clones (inset of Fig. 10C) which is not the case in th^{6} mutant clones. Interestingly, the cytoplasmic accumulation of Baz and *D*aPKC can also be observed in cells homozygous mutant for th^{6} (Fig. 10E +10K). However, the localization of *D*PATJ does not change in th^{6} mutant cells comparing to wildtype (Fig. 10M-O). This indicates that in wildtype cells accumulation of Baz and *D*aPKC in the cytoplasm is suppressed by the E3 ligase domain of DIAP1. The localization of *D*PATJ instead seems to be independent of DIAP1 domain.

2.4.2. DIAP1 induces ubiquitination of *D*aPKC

Protein levels of Baz and *D*aPKC are elevated or de-localized in follicle cells lacking DIAP1 as seen in Figure 10. This observation suggests a number of models how DIAP1 regulates the Baz protein complex. However, the fact that th^{6} mutant clones show Baz and *D*aPKC accumulation suggests that in wildtype cells the E3 ligase domain of DIAP1 controls protein levels of Baz and *D*aPKC by inducing their proteasomal degradation. To test this hypothesis, stability of the putative E3 target proteins was assessed in a cell free system under conditions that allow DIAP1 to function as an E3 ubiquitin ligase. Therefore, cell extract was incubated with buffer alone, buffer plus Ubiquitin or buffer and Ubiquitin and DIAP1. After the reaction, the proteins were separated by SDS-PAGE, transferred to nitrocellulose and *D*aPKC, Baz, *D*PATJ and *Dm*PAR-6 were detected by western blotting using specific antisera. Recombinant Ubiquitin (Ub) was added to the extract, as it appears to be rate limiting for the ubiquitination process. The *D*aPKC protein has the predicted molecular weight (M_r) of ~90kDa. Additionally, multiple high molecular weight forms of *D*aPKC are present in tissue extract and levels of these high M_r forms are increased upon addition

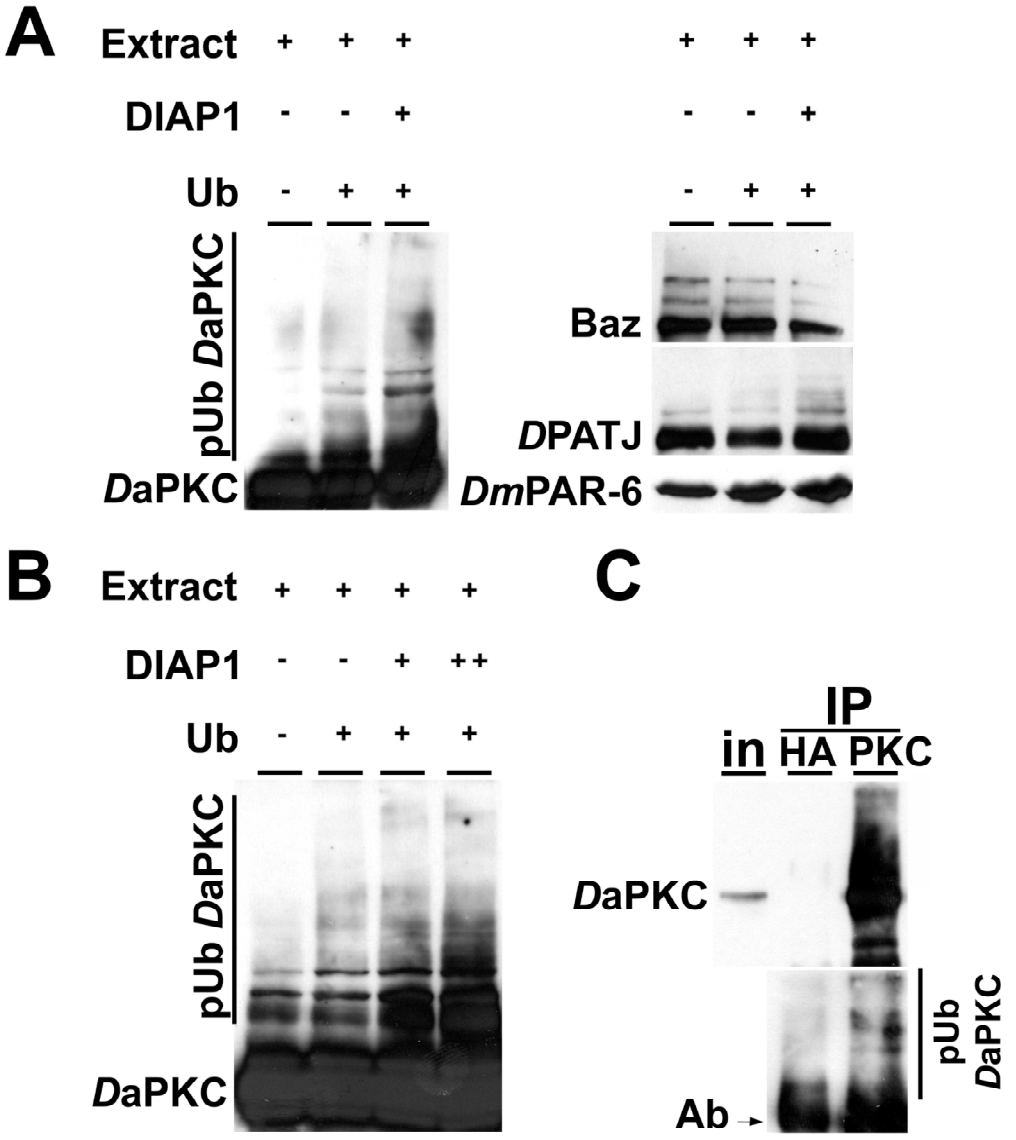


Figure 11: DIAP1 induces polyubiquitination of *D*aPKC but not Baz, *Dm*PAR-6 or *D*PATJ

(A) Protein levels of *D*aPKC, Baz, *Dm*PAR-6 and *D*PATJ in protein extract treated with buffer alone (-DIAP1; -Ub), recombinant Ubiquitin (-DIAP1; +Ub) and recombinant DIAP1 and Ub (+DIAP1;+Ub). *D*aPKC has the M_r of ~66 kDa. High M_r forms of *D*aPKC are elevated upon incubation with Ub and Ub plus DIAP1 comparing to buffer alone (indicated by pUbaPKC). Baz has the M_r ~160kDa and levels of high M_r forms of Baz are not changed in the same reaction. *D*PATJ has the M_r ~96kDa and a small increase of high M_r forms can be observed upon addition of Ub and DIAP1. *Dm*PAR-6 has the M_r ~38kDa and high M_r forms cannot be observed; (B) Addition of 1ug (++) of recombinant DIAP1 increases the levels of high M_r *D*aPKC stronger than 0.5ug (+) of DIAP1 comparing to addition of buffer or Ub alone; (C) Immunoprecipitation (IP) of full length *D*aPKC and western blotting against *D*aPKC (upper lane) and anti-Ubiquitin (lower lane) Upper lane: *D*aPKC and high M_r *D*aPKC forms are immunoprecipitated by *D*aPKC antiserum but not by the control anti HA-antiserum. Lower lane: The high molecular weight forms of *D*aPKC strongly cross-react with anti Ubiquitin antiserum (vertical bar in C), indicating that *D*aPKC is indeed heavily ubiquitinated in wildtype cell lysate (pUb*D*aPKC). Unspecific signals from rabbit antiserum (Ab) are indicated by the arrow in C.

of Ub to the extract (Fig. 11A). An anti-Ubiquitin antibody strongly binds to these high M_r *D*aPKC showing that the high M_r forms represent polyubiquitinated forms of *D*aPKC (Fig. 11C). Addition of recombinant DIAP1 to this system further enhances amount of high M_r forms of *D*aPKC (Fig. 11A). This enhanced ubiquitination of *D*aPKC occurs in a dose dependent manner suggesting that DIAP1 indeed induces polyubiquitination of *D*aPKC in the cell free system (Fig. 11B). In contrast to *D*aPKC, protein stability of the other tested candidates Baz, *D*PATJ and *Dm*PAR-6 was not significantly altered upon addition of DIAP1 (Fig. 11A). These results show that protein stability of *D*aPKC in tissue extract as well as in the follicle epithelium is under control of the E3 Ubiquitin ligase DIAP1.

2.5. DIAP1 is regulated by the small GTPase Ral

The experimental evidence presented in this work shows that DIAP1 is necessary to maintain the polarized distribution of the Baz protein complex in follicle cells. The Baz complex is crucial for establishment and maintenance of cell polarity as in *baz* mutants, cell polarity is lost and the follicle epithelium becomes multilayered. Thus DIAP1 seems to act in a regulatory pathway that controls development or maintenance of follicle cell polarity upstream of Baz. However, the question remains how DIAP1 activity itself is regulated. One candidate protein that might act upstream of DIAP1 is Ral, a Ras-like small GTPases (Mirey et al., 2003). Ral was found in a yeast two hybrid screen for novel interaction partners of DIAP1 to directly interact with BIR-1 domain of DIAP1 (Gagic, 2005).

In the follicle epithelium Ral controls wildtype protein levels of DIAP1 as follicle cells homozygous mutant for the *ral* null allele ral^{70} show less DIAP1 protein comparing to wildtype tissue (Fig. 12B; also Gagic, 2005). Thus, in the follicle epithelium Ral acts upstream of DIAP1. Interestingly, the protein level of DIAP1 in the follicle epithelium is specifically under control of Ral as lack Ras or of Rap1, a second Ras-like protein encoded by the *Drosophila* genome, does not alter DIAP1 protein level (Fig. 12E-12L).

The finding that Ral controls cellular levels of DIAP1 in the follicle epithelium suggests that Ral acts upstream of DIAP1 to control localization of Baz and *D*aPKC. In this case, ral^{70} mutant cells should show a cellular phenotype very similar to DIAP1

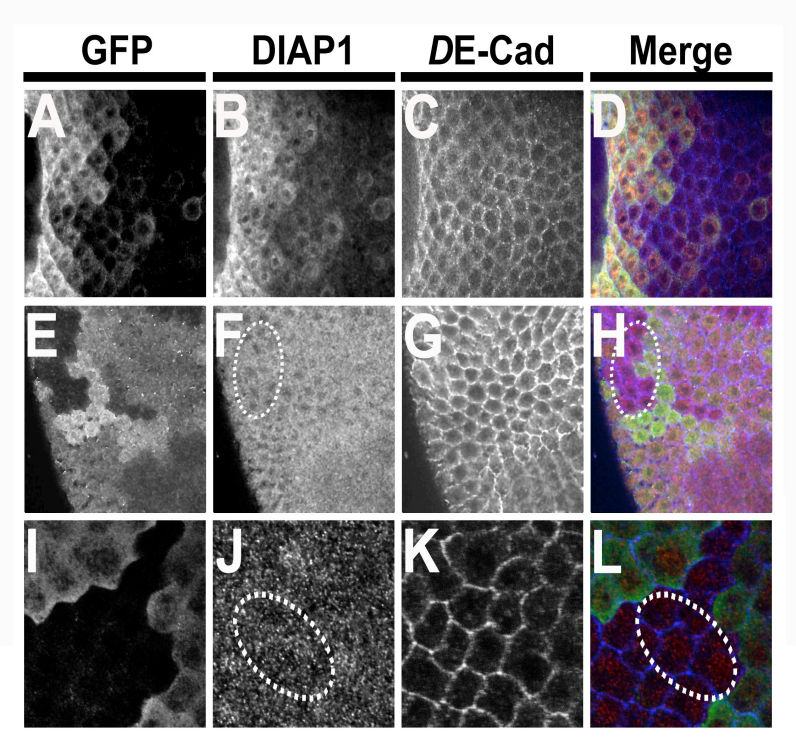


Figure 12:
DIAP1 protein levels in follicle cells mutant for the small GTPases Ral, Ras and Rap1

(A-D) Stage 10 egg chamber containing *ral*^{70} mutant follicle cells stained for (A) GFP, (B) DIAP1 and (C) DE-Cad; (D) Overlay with GFP in green, DIAP1 in red and *D*E-Cad in blue. *ral*^{70} mutant tissue contains less DIAP1 than wildtype tissue. (E-H) Stage 10 egg chamber with *rap1*^{B1} mutant clone stained for (E) GFP expression, (F) DIAP1 and (G) *D*E-Cad; (H) Overlay with GFP in green, DIAP1 in red and *D*E-Cad in blue. (I-J) Stage 10 egg chamber with *ras*$^{\Delta C40}$ mutant clone stained for (I) GFP, (J) DIAP1 and (K) *D*E-Cad; (L) Overlay with GFP in green, DIAP1 in red and *D*E-Cad in blue. The levels of DIAP1 are not altered in *rap1*^{B1} or *ras*$^{\Delta C40}$ mutant tissue comparing to wildtype cells.

mutant cells. Indeed, in *ral*^{70} mutant tissue the localization of Baz, *D*aPKC and *Dm*PAR-6 is altered comparing to wildtype cells. Baz is localized to the apical plasma membrane of *ral*^{70} mutant cells and is accumulated in small cytoplasmic dots (Fig. 13B). *D*aPKC is accumulated in the cytoplasm of *ral*^{70} mutant cells as well but to a lesser extend than in *th*^{6} mutant cells (Fig. 13E). The *Dm*PAR-6 protein does not localize to the apical membrane of *ral*^{70} mutant cells while *D*PATJ does (Fig. 13I+13J). Thus, both *ral*^{70} and *th*^{6} mutant cells show very similar changes in the distribution of cell polarity markers in the follicle epithelium. This supports the idea that DIAP1 is an effector of Ral in a novel signalling pathway that controls maintenance of certain aspects of cell polarity.

2.6 Activated Ral binds to DIAP1

Ral and DIAP1 control the differential localization the Baz protein complex in the mature follicle epithelium as shown in the previous chapter. Ral is necessary to sustain DIAP1 levels in the *Drosophila* follicle epithelium suggesting that DIAP1 is a downstream effector of Ral. Ral is a small GTPase that acts as a molecular switch by binding to downstream effector molecules only in its activated GTP bound state (Rosse et al., 2003). Thus, if DIAP1 is indeed an effector molecule of Ral, binding to DIAP1 should depend on activation status of Ral. To test whether the interaction with DIAP1

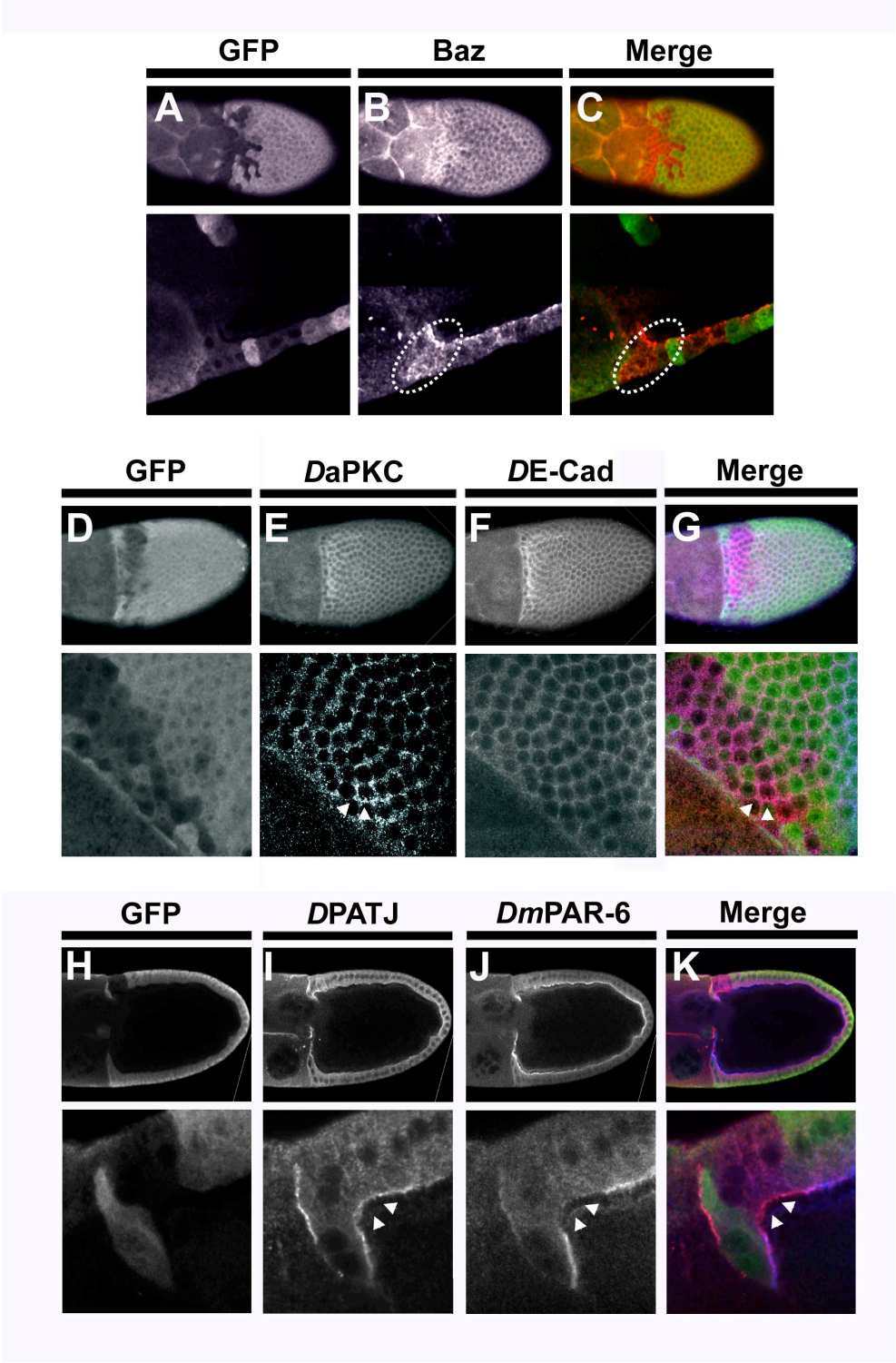


Figure 13: Cell polarity is altered in *ral* mutant follicle cells

(A-K) Localization of the apical determinants Baz, *D*aPKC, *D*PATJ and *Dm*PAR-6 in stage 10 egg chambers harbouring clones of *ral*^{70} mutant cells. (A-C) Stage 10 egg chamber stained for (A) GFP, and (B) Baz; (C) Overlay with GFP in green and Baz in red; Cytoplasmic Baz accumulation in *ral*^{70} mutant cells is marked by dotted circle in (B). (D-G) Stage 10 egg chamber stained for (D) GFP, (E) *D*aPKC and (F) *D*E-Cad); (G) Overlay with GFP in green *D*aPKC in red and *D*E-Cad in blue; Note that *D*aPKC is slightly elevated in *ral*^{70} mutant cells marked by arrowheads in (E). (H-K) Stage 10 egg chamber stained for (H) GFP, (I) *D*PATJ and (J) *Dm*PAR-6; (K) Overlay with GFP in green, *D*PATJ in red and *Dm*PAR-6 in blue; Note that *D*PATJ localizes at the apical membrane of *ral*^{70} mutant cells while *Dm*PAR-6 does not (arrowheads in I+K).

was dependent on activity status of the small GTPase Ral, GST-Pulldown experiments with recombinant proteins were performed. Ral was modified in a way that either rendered the protein constitutively GTP bound (active Ral^{V20}) or constitutively GDP bound (inactive Ral^{N25}). Interestingly, DIAP1 exclusively binds to active Ral^{V20}, while no interaction is observed with inactive Ral^{N25} or GST alone (Fig. 14A). Unmodified wildtype Ral also shows no binding to DIAP1 in the presence of GDP. This result supports the idea that DIAP1 is indeed an effector molecule of Ral.

To identify domains of DIAP1 important for the interaction with Ral, three known point mutations affecting the BIR1 domain were reconstituted by site directed mutagenesis. The mutant DIAP1 protein was then used for a GST-Pulldown experiment with active GST-Ral^{V20} (Fig. 14B) using wildtype DIAP1 as positive control. While the mutation $V^{85}M$ and $P^{105}S$ only slightly reduce binding, the amino acid exchange $P^{55}L$ strongly affects binding of DIAP1 to Ral (Fig. 14B). This indicates that interaction of Ral^{V20} with its downstream partner DIAP1 depends on the BIR1 domain of DIAP1 and is consistent with the results from the yeast two hybrid screen (Gagic, 2005).

The direct interaction between Ral and DIAP1 in vitro is clearly shown by the GST-Pulldown experiments. Whether this interaction occurs in vivo as well was tested by immunoprecipitation (IP) experiments using monoclonal DIAP1 antibody. In the input lysate a ~50kDa form of DIAP1 is present as well as additional 2 bands, probably modified versions of DIAP1 (Fig. 14D). All three protein species are precipitated by the monoclonal DIAP1 antibody but not by the control IgG. Note that one form of DIAP1 is enriched in the precipitate. Presence of Ral was assessed using a monoclonal antibody against human RalA that cross reacts with *Drosophila* Ral in the input lysate (Fig. 14D). Ral is precipitated from the lysate by the monoclonal anti DIAP1 antibody but not by the control antibody (Fig. 14D). These results lead to the conclusion that Ral and DIAP1 form a protein complex in the tissue extract. The functional importance of this complex might be to stabilize DIAP1 and by that to control cell polarity in the follicle epithelium.

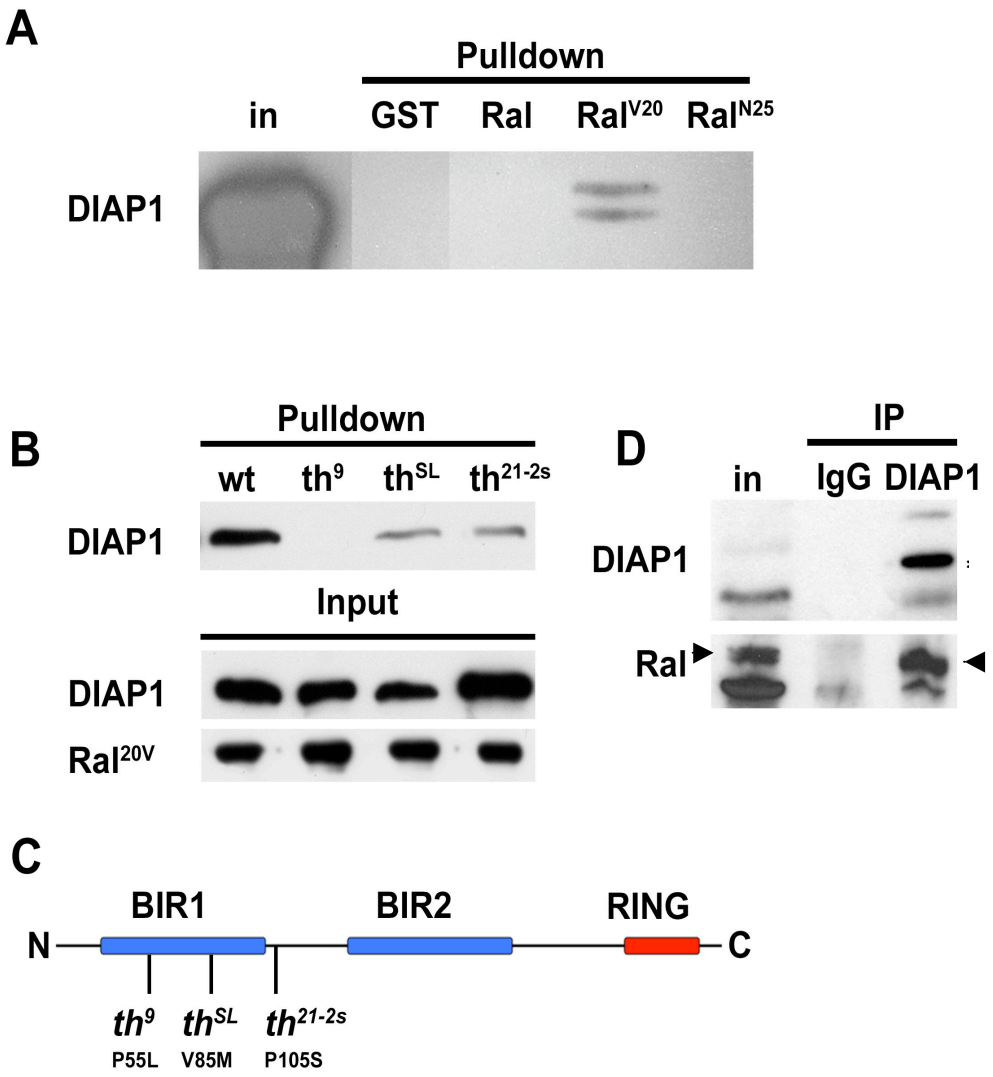


Figure 14: The small GTPase Ral binds to DIAP1

(A) GST-Pulldown of recombinant DIAP1 with GST and modified versions of GST-Ral. (A) Recombinant DIAP1 has the M_r of 50 kDa (indicated as DIAP1). DIAP1 binds active Ral (GST-Ral^{V20}) but not GST alone. Inactivate Ral (GST-Ral^{N25}) or Ral in presence of excessive GDP does not bind to DIAP1 as well.
(B) GST-Pulldown of unmodified and mutant DIAP1 with active Ral^{V20}; upper lane: wildtype DIAP1 binds active Ral while binding of the DIAP1 mutant *th*^{SL} and *th*$^{21-2s}$ is reduced. The DIAP1 mutant *th*^{9} shows even stronger reduction in binding to Ral; middle lane: input levels of wildtype and mutant DIAP1; lower lane: input levels of GST-Ral^{V20}. (C) Schematic overview of DIAP1 indicating the position and nature of the point mutations *th*^{9}, *th*^{SL} and *th*$^{21-2s}$. (D) Co-immunoprecipitation of Ral and DIAP1 out of embryo tissue extract. DIAP1 is present in the extract with the M_r of ~50 kDa. Two other forms of DIAP1 are present in the extract as well (M_r ~55kDa and 60kDa). In the precipitate, the 55 kDa form of DIAP1 is accumulated (star in D). Ral has the M_r of ~ 28kDa (arrow in D) and co-precipitates with DIAP1 (arrowhead in D). Neither DIAP1 nor Ral is precipitated by an unrelated control antibody (IgG).

2.7 DIAP1 is stabilized by small GTPases in vitro

2.7.1 DIAP1 is stabilized by Ral in a cell free system

Ral controls protein levels of DIAP1 in the follicle epithelium as shown in chapter 2.5. Interestingly, Ral directly binds to DIAP1 as shown in chapter 2.6. The latter finding suggests that direct binding by Ral might be important to control stability of cellular DIAP1 levels. A good method to test the influence of a candidate protein on stability of DIAP1 is a cell free system developed by Yoo and others in 2002.

DIAP1 becomes polyubiquitinated over time when it is subjected to the influence of tissue extract and recombinant Ubiquitin (Fig. 15). Reconstitution of the *th*^{6} mutation that contains a single amino acid exchange within the N-terminal RING domain of DIAP1 prevents ubiquitination of DIAP1 (Fig. 15A). This shows that DIAP1`s RING domain has E3 ubiquitin ligase activity and DIAP1 can act in an autocatalytic manner. Polyubiquitinated proteins are instable as they are targeted for degradation by the proteasome. Thus, reduced concentration of pUbDIAP1 can be interpreted as enhanced protein stability of DIAP1.

In vivo, cellular levels of DIAP1 depend on regulation of its E3 ligase function but which proteins are actually involved in regulation of DIAP1`s E3 ligase function is not known. Based on the results presented in chapter 2.5, Ral might be a good candidate to negatively regulate DIAP1 E3 ligase function in vivo. Thus, the influence of recombinant GST-Ral on stability of DIAP1 was tested in the cell free system. The influence of GST alone or GST-fusion proteins of Ras and Rap1, two other Ras like GTPase encoded by the *Drosophila* genome, was tested as well. Addition of GST-Ral strongly reduces autoubiquitination of DIAP1 in a dose dependent manner (Fig. 14A+14C) while GST alone has no stabilizing effect (Fig. 14B). Addition of GST-Ras to DIAP1 results also in a marked increase of DIAP1 stability in this assay (Fig. 14A). Again this stabilizing effect is clearly dependent on the amount of protein added (Fig. 14D). In contrast to this, GST-Rap1 does not stabilize DIAP1 in a similar concentration range as the other GTPases (Fig. 14E). These results suggest a specific function of Ras and Ral to stabilize DIAP1 depending on a functional E3 ubiquitin ligase activity of DIAP1. When the E3 ligase domain is made inactive, no ubiquitination occurs showing that DIAP1 acts autocatalytically in this system. Thus, both Ral and Ras seem to have a direct influence

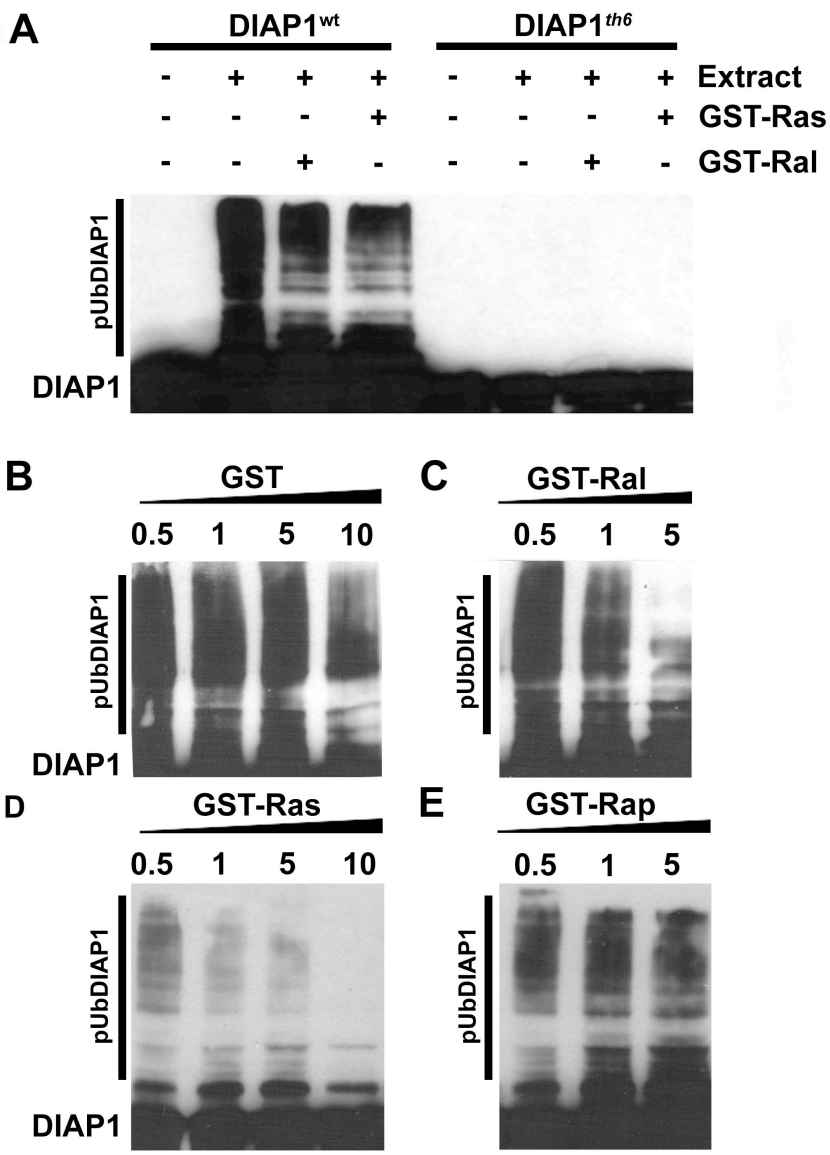


Figure 14:

Ral stabilizes DIAP1 in a cell free system

(A) Stabilization of DIAP1 by recombinant GTPases in a cell free system. Recombinant DIAP1 has the M_r of ~50 kDa as indicated (-extract). Polyubiquitinated, high M_r forms of DIAP1 (pUbDIAP1) accumulate upon addition of tissue extract (+Extract) but not when DIAP`s E3 ligase domain is rendered inactive (DIAP1*th*^{6}). Levels of high M_r forms of DIAP1 are reduced when 1µg Ral (+GST-Ral) or Ras (+GST-Ras) are present. Both Ras and Ral have no influence on DIAP1 *th*^{6}. Reduction of pUbDIAP1 is interpreted as stabilization of DIAP1. (B-E) Dose dependent stabilization of DIAP1 by Ras and Ral
(B) Stability of DIAP1 is not altered upon addition of 0.5µg-5µg of recombinant GST while addition of 10µg GST slightly reduces polyubiquitination of DIAP1; (C) Stability of DIAP1 is increased by addition of 0.5µg-5µg of GST-Ral; (D) Stability of DIAP1 is increased by addition of 0.5µg-10µg recombinant GST-Ras; (E) Stability of DIAP1 is not affected by 0.5µg-5µg GST-Rap1.

on DIAP1 autoubiquitination, rather than inhibition of a putative second E3 enzyme that regulates DIAP1 in trans.

2.7.2. Interaction of DIAP1 with Rac1

In the previous chapters it has been shown that the small GTPase Ral binds to DIAP1 and by that positively regulates DIAP1 protein levels in the follicle epithelium. This positive regulation is important for localization of proteins that control cell polarity. Another set of small GTP binding proteins that regulate certain aspects of cell polarity is

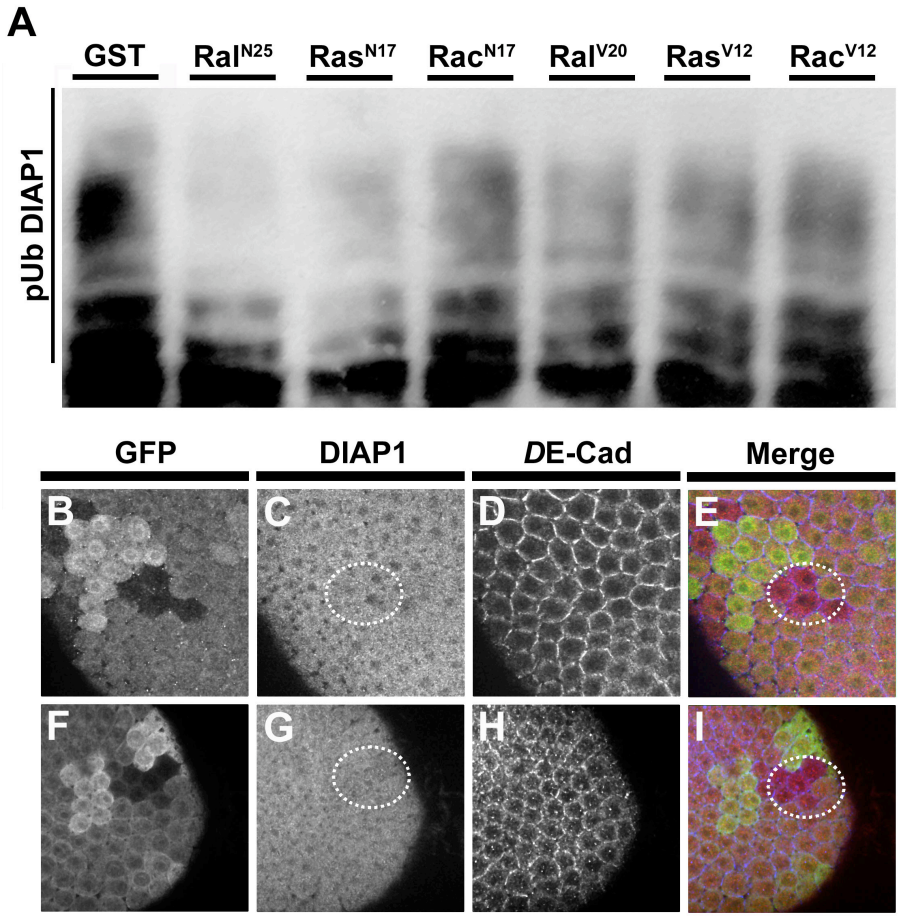


Figure 15: Rac1 stabilizes DIAP1 in the cell free system by but not in follicle cells

(A) DIAP1 stabilization by constitutive active and inactive small GTPases. DIAP1 is polyubiquitinated in presence of GST (pUbDIAP1). Addition of Ral^{N25} and Ras^{N17} strongly reduces polyubiquitination of DIAP1 while Ral^{V20} and Ras^{N17} have a weaker effect. Rac1^{V12} and Rac1^{N17} reduce polyubiquitination of DIAP1 to a lesser extend than Ral or Ras. (B-E) Stage 10 egg chambers with follicle cell clones homozygous mutant for *rac1^{J11},rac2$^{\Delta}$* and heterozygous mutant for *mtl$^{\Delta}$* stained for (B) GFP, (C) DIAP1 and (D) *D*E-Cad; (F-I) stage 10 egg chamber with a *mtl$^{\Delta}$* homozygous mutant clone stained for (F) GFP, (G) DIAP1 and (H) *D*E-Cad; (E+I) overlay with GFP in green, DIAP1 in red and *D*E-Cad in blue. DIAP1 levels are not significantly altered in *rac* or *mtl* mutant cells.

the Rho family of GTPases (Wennerberg and Der, 2004). Rac1 belongs to this Rho-like family of small GTPases. Recently, binding of Rac1 to DIAP1 has been described (Geisbrecht and Montell, 2004) and the question arises whether this binding might be necessary to regulate cell polarity similar to the binding to Ral. It is possible though that Rac1 has a similar stabilizing effect on DIAP1`s E3 ligase domain than small GTPases of the Ras-like family. This hypothesis is supported by the finding that overexpression of wildtype Rac1 stabilizes DIAP1 protein levels in border- and polar cells (Geisbrecht and Montell, 2004). The underlying molecular mechanism however is not clear so far and it was therefore tested whether Rac1 stabilizes DIAP1 by regulating its RING domain.

As DIAP1 is stabilized in a cell free system due to direct binding to the Ras like protein Ral (see above), the question was addressed whether a similar stabilization can be observed by binding to Rac1. Therefore, constitutively GTP bound (active Rac1 ^{V12}) and constitutively GDP bound (inactive Rac1 ^{N17}) versions of Rac1 were added to the cell free system and stability of DIAP1 was assessed by western blotting as described. Modified versions of Ral and Ras (active Ras^{V12} and inactive Ras^{N17}) were used as well. Addition of buffer or GST alone had no influence on appearance of polyubiquitinated DIAP1 (Fig. 15A). In contrast, addition of active or inactive versions of Ras, Ral and Rac1 strongly reduced the amount of polyubiquitinated DIAP1 independent of their activity status (Fig. 15A). Thus, Ras, Ral and Rac1 can negatively regulate the E3 ligase function of DIAP1 in the cell free system.

Ral binds to DIAP1 in its activated form while Rac1 has been shown to bind to a Profilin/DIAP1 complex independent of its activation status. In contrast to these findings both Ral and Rac1 negatively regulate DIAP1 protein levels independent on their activity status in vitro. This finding might be explained by a crosstalk between the Ras, Ral and the Rac1 signalling pathway in embryo extract. On the other hand it is possible that stabilization of DIAP1 occurs via a domain shared by Ras, Rac1 and Ral but that is not present in Rap1. Thus, it remained to be shown whether Rac1 stabilizes DIAP1 protein levels in vivo in a tissue dependent manner.

Based on the observation in the cell free system the question arises whether Rac1 is relevant for stable DIAP1 protein levels in the follicle epithelium similar to Ral. This question was addressed using the FRT/FLP system and mutant alleles of *rac1* and *rac2* and *mig-2-like* (*mtl)*, two additional *rac* genes that act redundantly to *rac1* (Hakeda-Suzuki et al., 2002). Loss of *mtl* alone does not alter DIAP1 protein levels in the mutant clone comparing to wildtype tissue (Fig. 15F-15I). Similarly, the loss of both *rac1* and *rac2* and heterozygosis for *mtl* does not change DIAP1 levels significantly (Fig. 15B-15F). Thus, DIAP1 protein levels in the follicle epithelium are not under major control of Rac1.

2.8 Requirement of Ral and DIAP1 for follicle cell morphogenesis

The wildtype main body cells form a columnar follicle epithelium surrounding the oocyte (see introduction). Crb and Baz are required for development of cell polarity in the follicle epithelium (Benton and St Johnston, 2003; Tanentzapf et al, 2000). The

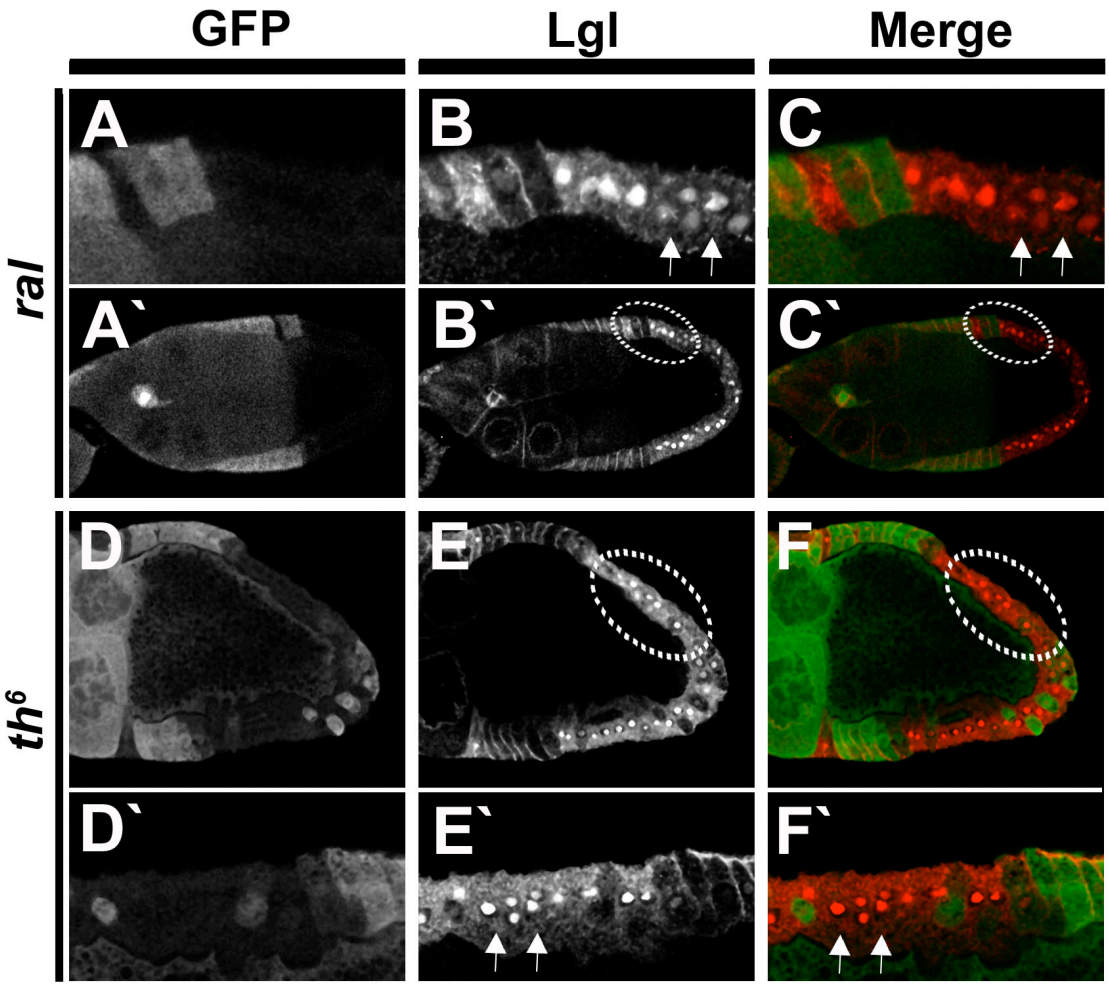


Figure 16: Multilayering in follicle epithelia lacking Ral or DIAP1

(A-C`) Multilayering of *ral*$^{70}$ mutant follicle epithelium; (A`-C`) stage 8 egg chamber with a large *ral*$^{70}$ mutant clone covering half of the posterior follicle epithelium stained for (A`) GFP and (B`) Lgl; dotted circle in (B`) marks the area magnified in (A-C). (C+C`) Overlay with GFP in green and Lgl in red. (D-F`) Multilayering of *th*^{6} mutant follicle epithelium; (D-F) Stage 10 egg chamber with a large *th*^{6} mutant clone covering the lateral sites of the egg chamber stained for (D) GFP and (E) Lgl; The dotted circle in (E) marks the area of higher magnification in (D`-F`). Note that Lgl does not localize to the lateral plasma membrane of *ral* and *th*^{6} mutant cells and the epithelium is multilayered (arrows in B,C+E`,F`).

function of Crb and Baz during establishment of cell polarity can be distinguished into two phases. The initial phase of polarization requires both Baz and Crb; follicle cells that loose Baz or Crb in this early phase grow into large clones with defective cell polarity. As a result of *baz* and *crb* mutation the normally single layered epithelium becomes multilayered (Tanentzapf et al, 2000; Benton and St.Johnson, 2003). Multilayering is often found in large *baz* single mutant clones but rarely in *crb* single mutants suggesting that Baz is more important than Crb for initial development of cell polarity in the epithelium (Tanentzapf et al, 2000). In phase two of follicle cell development, the function of either the Crb or the Baz complex for maintenance of cell polarity is

dispensable as small *sdt* or *baz* clones show no obvious mutant phenotype. However, small sdt^{XN05},baz^{YD97} double mutant clones that become induced after the initial polarization phase show morphological defects and multilayering of the epithelium suggesting that the Baz and the Crb complex act redundantly after the initial polarization phase (Fig. 9).

As both Ral and DIAP1 regulate localization of the Baz complex in the mature follicle epithelium (Fig. 10+13) the question arises whether morphological changes similar to *baz* mutant clones can be observed in *ral* and *thread* mutant follicle cells that are induced during establishment of cell polarity. To address the question whether Ral and DIAP1 are necessary for initial establishment of cell polarity in the follicle epithelium large ral^{70} and th^{6} mutant follicle cell clones were analyzed. The lateral plasma membrane of wildtype follicle cells is marked by the protein Lethal Giant Larvae (Lgl) and Lgl is also found in the cytoplasm and the nucleus (Fig. 16 and Dollar et al., 2005). Cytoplasmic and nuclear levels of Lgl are clearly enhanced in ral^{70} and th^{6} mutant follicle cells while lateral localization of Lgl disappears (Fig. 16B+16E`).

The nuclei of ral^{70} and th^{6} mutant cells accumulate Lgl and do not align in one plane of the follicle epithelium. Similar misalignment of the nuclei reveals multilayering of the epithelium in large *crb* and *baz* mutant follicle cell clones (Tanentzapf et al., 2000). Thus, presence of Ral and DIAP1 is necessary for the initial development of cell polarity in the follicle epithelium. When Ral and DIAP1 are absent during the establishment of cell polarity morphology of the epithelium is affected and the follicle cell sheet becomes multilayered.

2.9 DIAP1 is required for epithelial integrity in the *Drosophila* embryo

In the first part of this thesis a functional connection between the small GTPase Ral and the caspase inhibitor DIAP1 has been established. Both Ral and DIAP1 are necessary for maintenance of cell polarity within the *Drosophila* follicle epithelium and are therefore important for egg chamber development in oogenesis. Further on, evidence was presented that Ral acts through DIAP1. Thus, the question arises whether Ral and Diap1 influence cell polarity in other epithelia as well, for example the ectoderm epithelium in the embryo. The experimental outline to investigate in the role of Ral and DIAP1 in the embryo lead to observations shortly described in the following.

Loss of Ral function in the female germline leads to very small ovaries that do not develop mature egg chambers. This hypotrophy of *ral*^{70} mutant ovaries shows a novel and yet not described function of Ral in the germline and might reveal further insight into the cellular function of Ral in the ovary. Interestingly, removal of DIAP1 from the germline leads to a strong hypotrophy of the affected ovaries similar to the *ral*^{70} mutant ovaries. The phenotype is largely the same if DIAP1 is removed completely (*th*^{109} germline clones) or if DIAP1`s E3 ligase function is defective (*th*^{6} germline clones).

Germline cells mutant for a null allele of either Ral or DIAP1 do not produce mature egg chambers as described above and fertilization cannot take place. Therefore, embryos with a complete lack of maternally contributed Ral or DIAP1 cannot be analyzed. However, a hypomorphic *ral* allele (*ral*^{89}) leads to reduced Ral expression and allows egg development, fertilization and embryonic development until stage 5. The phenotype of these embryos is presented in chapter 2.9.1.

Removal of maternally provided DIAP1 prevents development of mature eggs while zygotic loss of DIAP1 leads to massive programmed cell death by stage 8 of development (Wang et al., 1999). Thus, it is not possible to describe a function of DIAP1 in the embryo prior to stage 8 using the DIAP1 alleles *th*^{109} and *th*^{6}. However, zygotic removal of DIAP1 leads to apoptosis accompanied by characteristic changes of cell polarity and cell adhesion in the ectoderm epithelium. Aspects of the cellular phenotype of DIAP1 mutant embryos with respect to cell polarity are described in chapter 2.9.3. Chapter 2.9.4 and following focus on the phenotype of DIAP1 mutant embryos with respect to cell-cell adhesion. Evidence for a molecular mechanism that leads to the changes in cell-cell adhesion during apoptosis is presented.

2.9.1. Maternal Ral controls early steps of *Drosophila* embryogenesis

In the follicle epithelium Ral regulates cell polarity as described in chapter 2.8. Embryos homozygous mutant for the *ral* null allele die in late embryonic stages. This might be because maternally provided Ral is sufficient for early development. To examine *ral* function during early embryogenesis the amount of maternally provided protein was reduced using the hypomorphic *ral*^{89} allele and the FRT/DFS system (Chou and Perrimon, 1992).

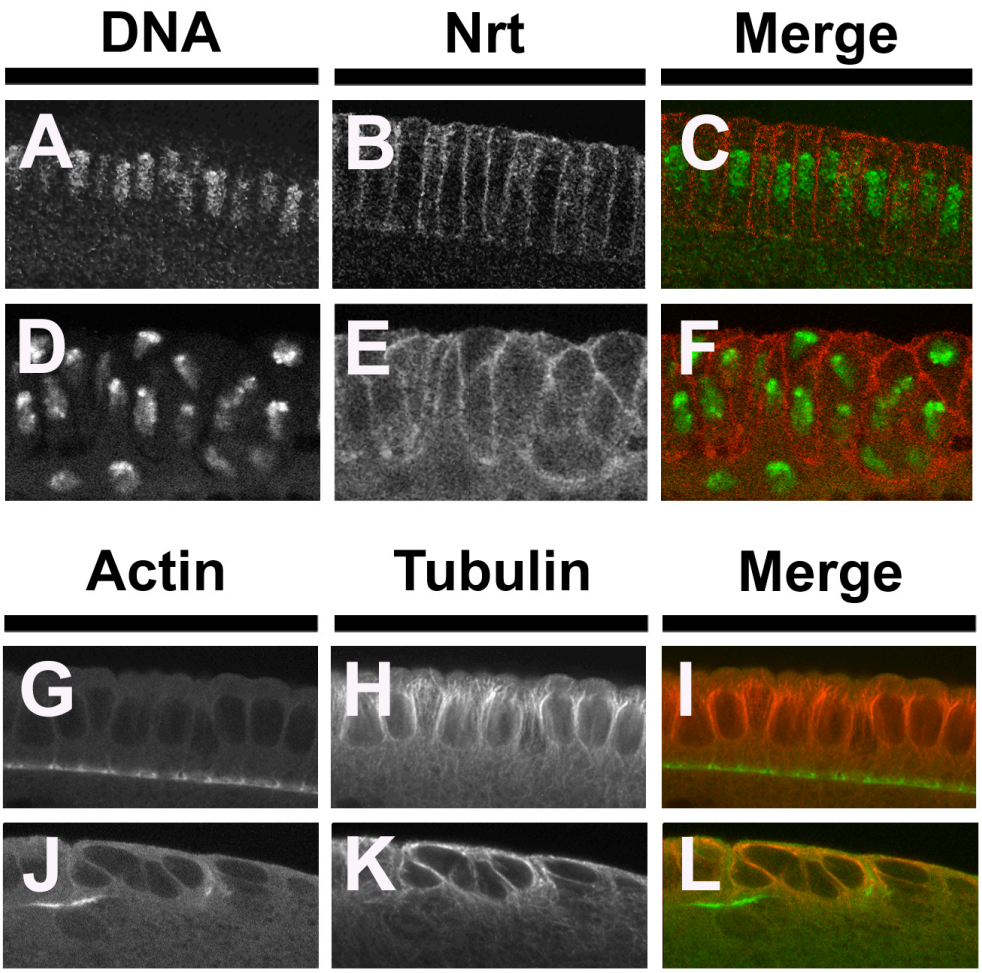


Figure 17: Phenotype of Ral germline clone embryos

(A-L) Cellular phenotype of stage 5 wildtype and *ral*^{89} maternal mutant embryos; (A-C) Wildtype embryo stained for (A) DNA and (B) Neurotactin (Nrt); (D-E) Similar staged embryo with reduced maternal Ral stained for (D) DNA and (E) Nrt. (C+F) Overlay with DNA in green and Nrt in red. (G-I) Wildtype embryo stained for (G) Actin and (H) Tubulin. (J-L) Embryo of a similar stage with reduced maternal Ral stained for (J) Actin and (K) Tubulin. (I+L) Overlay with Actin in green and Tubulin in red.

Wildtype *Drosophila* embryos develop as a syncitial blastoderm until mitotic cycle 13. In this developmental step the nuclei are aligned in a highly ordered fashion (Fig. 17A). When cellularization occurs, new membrane becomes inserted in between the nuclei. This new membrane is marked by the transmembrane protein Neurotactin (Nrt; Fig. 17B; Müller and Wieschaus, 1996). In *ral* germline clone embryos the nuclei are not properly aligned at the apical site of the embryo (Fig. 17D). Nrt is inserted into the in-growing plasma membrane showing a disordered membrane distribution (Fig. 17E). Some *ral*^{89} mutant cells contain more than one nucleus and some nuclei are not surrounded by membrane at all (Fig. 17F). In the wildtype embryo the tip of the in-growing membrane is marked by strong F-Actin accumulation (Fig. 17G). The microtubule network is highly organized within the cells with Tubulin accumulation at

both sides of the nuclei (Fig. 17H). In *ral*^{89} germline clone embryos, Actin is associated with the in-growing membrane randomly, with some cells having no F-Actin accumulation at all (Fig. 17J). Tubulin is distributed randomly within cells of Ral germline clone embryos (Fig. 17K). Thus, embryos with reduced amount of maternal Ral show severe defects in cellularization and do not form a properly polarized blastoderm epithelium.

2.9.2. Zygotic expression of DIAP1 sustains epithelial integrity

As shown in chapter 2.4.1, DIAP1 is necessary for localization of the apical protein markers Baz, *Dm*PAR-6 and *D*aPKC in the follicle epithelium. Baz and *Dm*PAR-6 also regulate apical-basal cell polarity during cellularization of the *Drosophila* embryo while the function of *D*aPKC is less clear (Müller and Wieschaus, 1996; Cox et al., 1996; Hutterer et al., 2004). DIAP1 is expressed during cellularization and the cellular DIAP1 pool consists of maternally contributed as well as zygotically expressed DIAP1. As DIAP1 might have a yet undescribed function during building of cell polarity, maternal DIAP1 was removed using the FRT/DFS system (Chou and Perrimon, 1996). However, removal of maternal DIAP1 protein leads to a block in egg development and thus prevents further investigation in the embryo as mentioned above. Therefore, embryos lacking zygotically expressed DIAP1 were analyzed for changes in cell polarity.

DIAP1 is the main inhibitor of apoptosis in the *Drosophila* embryo. Apoptosis is a form of programmed cell death executed by proteolytic enzymes, the caspases. DIAP1 binds to caspases in the cytoplasm and by that inhibits their activity. When inhibition by DIAP1 is blocked caspases become active and induce apoptosis by cleavage of certain target proteins within the cell. Apoptosis is a process that normally occurs during wildtype *Drosophila* development were some excessive cells are removed during embryogenesis. Theses dying cells can be visualized by staining against fragmented DNA or by an antibody that specifically recognizes the activated form of the effector caspase drICE (Fig 19A+B). When DIAP1 is missing like in *th*^{109} homozygous embryos apoptosis is induced and all cells in the embryo are positive for both TUNEL and active caspase (Fig. 19D+E). It is obvious that *th*^{109} homozygous embryos show a severe disruption of overall morphology including disruption of cell-cell adhesion in the ectoderm epithelium.

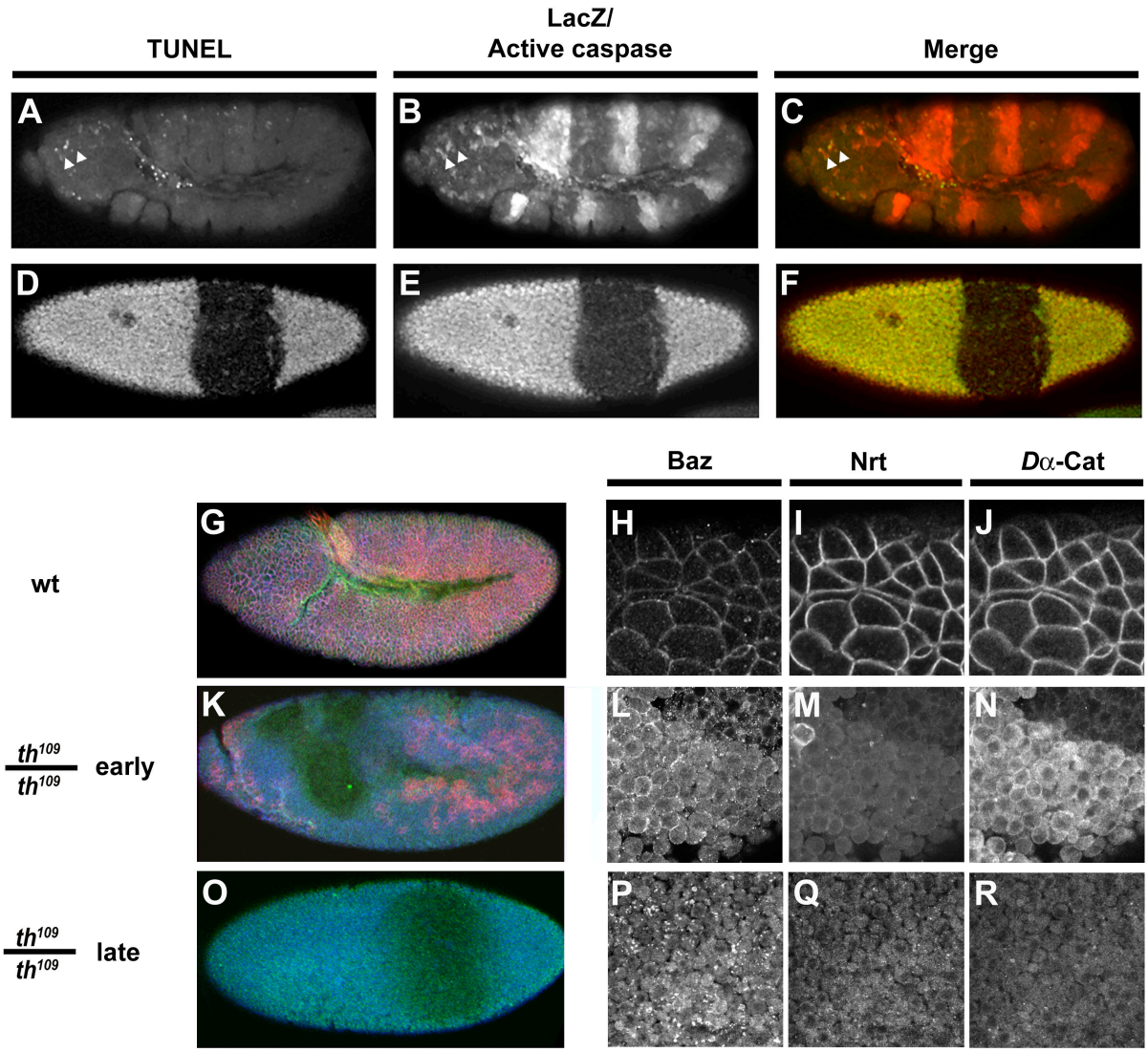


Figure 19: Zygotic expression of DIAP1 prevents apoptosis and maintains epithelial integrity

(A-F) Apoptosis in th^{109} heterozygous and homozygous embryos; (A-C) stage 9 th^{109} heterozygous embryo stained for (A) fragmented DNA (TUNEL), (B) expression of *ftz::lacZ* and activation of the executioner caspase drICE; arrowheads indicate apoptotic cells in the head region. (D-F) stage 9 th^{109} homozygous embryo stained for (D) fragmented DNA and (E) expression of *ftz::lacZ* and active drICE; Note that (E) shows active drICE only as th^{109} homozygous embryos do not express *lacZ*. (C+F) Overlay with TUNEL in green and *lacZ* and active drICE in red. (13 G-J) th^{109} heterozygous embryo stained for (H) Bazooka (Baz), (I) Neurotactin (Nrt) and (J) *D*α-Catenin (*D*α-Cat). (K-L) th^{109} homozygous embryo 45 min after cephalic furrow formation stained for (L) Baz, (M) Nrt and (N) *D*α-Cat. (O-R) th^{109} homozygous embryo 60 min after cephalic furrow formation stained for (P) Baz, (Q) Nrt and (R) *D*α-Cat.

Cell adhesion in the ectoderm epithelium is mediated by *D*E-Cadherin as well as *D*α-Catenin and Armadillo (see introduction for details). These three proteins form the adhesive complex that is organized in the ZA. Positioning of the ZA in the embryo is under control of the Crb/Sdt/*D*PATJ complex and the Baz/*Dm*PAR-6/*D*aPKC complex. Mutants of either of these complexes show delocalization of the ZA and impaired cell-cell adhesion. Thus, disruption of cell adhesion in th^{109} homozygous embryos could be a

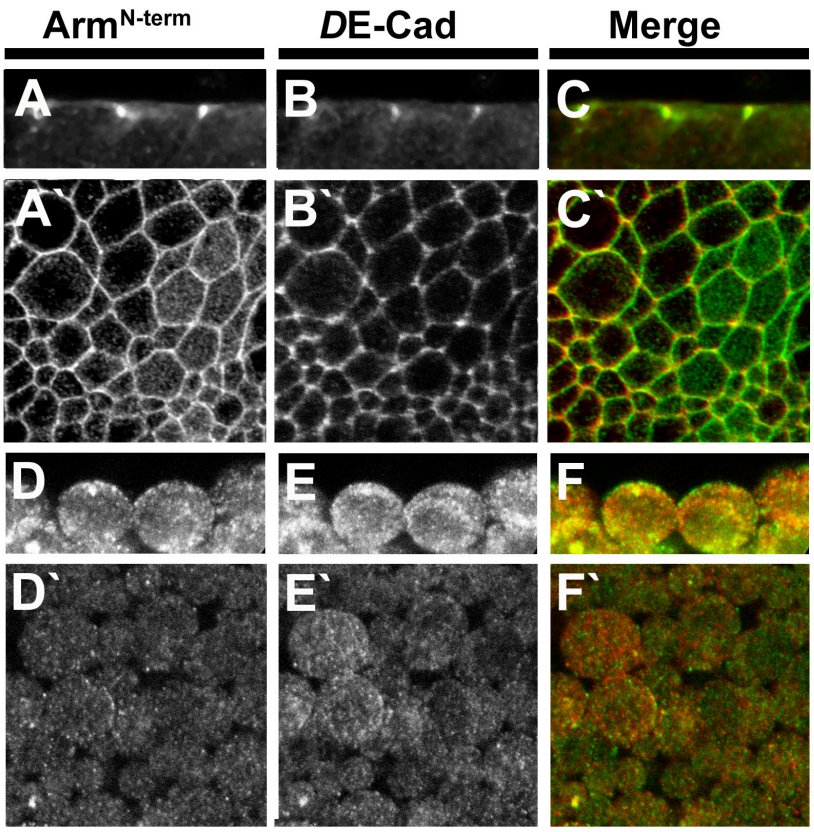
result of altered cell polarity and delocalization of the ZA. In wildtype embryos, Baz is localized at the subapical region that partially overlaps with the ZA (Fig. 19 H+J). The membrane protein Nrt marks the basolateral cell outlines (Fig. 19I). *th*^{109} homozygous embryos show altered overall morphology and beginning of disruption of cell adhesion ~45min after cephalic furrow formation (Fig. 19K and19L-N) comparing to wildtype embryos (Fig. 19 G). Baz remains membrane associated in some of the cells undergoing early phases of apoptosis while Nrt is absent in all dying cells (Fig. 19L+19M). *D*α-Cat is predominantly associated with the plasma membrane of *th*^{109} homozygous cells (Fig. 19N). Disruption of the epithelium becomes more severe with progression of apoptosis and ~60min after cephalic furrow formation all epithelial cells are rounded (Fig. 19O). Neither Baz nor Nrt can be found on the plasma membrane in the late phase of apoptosis indicating that expression of the caspases inhibitor DIAP1 is necessary for wildtype localization of both cell polarity markers (Fig. 19P+Q).

Similar to Baz and Nrt, the ZA associated molecule *D*α-Cat is not localized to the membrane of cells undergoing the late phase of apoptosis in stage 9 of embryogenesis suggesting that the ZA is severely disrupted in these embryos (Fig. 19R). This phenotype is different from mutants affecting cell polarity, for example Crb mutants, which show structurally intact spot adherens junctions that are not properly located at the apical site of the epithelium (Grawe et al., 1996).

2.9.3 The cell adhesion molecule Arm is degraded in DIAP1 mutant cells

DIAP1 mutant embryos show altered cell polarity and a lack of cell adhesion by stage 9 of development. To investigate in detail the molecular mechanism that leads to disruption of cell adhesion in DIAP1 mutant embryos, experiments were carried out focusing on the cell adhesion molecules *D*E-Cadherin (*D*E-Cad), *D*α-Catenin (*D*α-Cat) and Armadillo (Arm).

In wildtype or *th*^{109} heterozygous embryos, Arm and *D*E-Cad co-localize with *D*α-Cat forming an apical adhesion belt, the zonula adherens (Fig. 19+20A+B). In a surface view of the epithelium, the ZA of neighbouring cells form a highly ordered hexagonal pattern (Fig. 20A`+B`). In *th*^{109} homozygous mutant embryos of the same age, both Arm and *D*E-Cad rarely co-localize, indicating absence of a functional ZA (Fig. 20F+F`). The area of membrane contact between the epithelial cells is strongly reduced and the cells



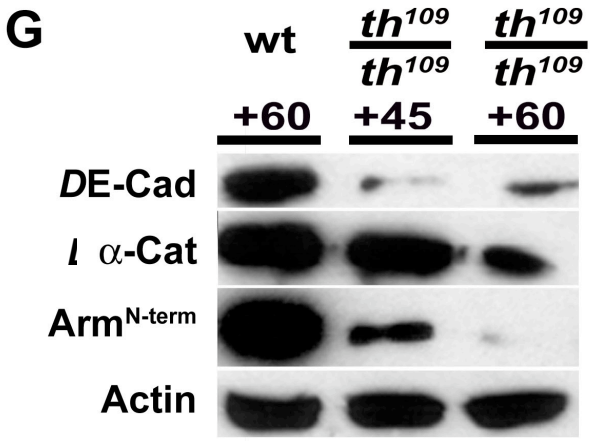


Figure 20:

Arm is degraded during apoptosis

(A-C`) *th*$^{109}$ heterozygous stage 9 embryo stained for (A+A`) Arm and (B+B`) *D*E-Cad; (A+B) confocal cross-section, (A`+B`) surface view. (D-F`) Same stage *th*^{109} homozygous embryo stained for (D+D`) Arm and (E+E`) *D*E-Cad; (D+E) confocal cross section and (D`+E`) surface view; (C+, C`, F and F`) Overlay with Arm in green and *D*E-Cad in red. (G) Protein levels of *D*E-Cad, *D*α-Cat, Arm and Actin in *th*^{109} heterozygous (indicated as wt) and *th*^{109} homozygous embryos 45min and 60min after cephalic furrow formation (indicated as +45 and +60).

are rounded. The cellular concentration of Arm seems to be reduced comparing to *D*E-Cad indicating that Arm might be degraded in *th*^{109} homozygous cells (Fig. 20D`+E`).

The latter finding suggests that reduced cell adhesion during apoptosis is due to altered protein stability of adhesion proteins. To test this hypothesis, protein levels of Arm as well as *D*E-Cad and *D*α-Cat in *th*^{109} homozygous and heterozygous embryos were compared by western blotting (Fig. 20G). Indeed, concentration of Arm is significantly reduced in *th*^{109} mutant embryos that undergo early steps of apoptosis comparing to the Arm protein level in *th*^{109} heterozygous embryos (Fig. 20G). Arm

concentration further decreases during the late phase of apoptosis. The latter finding shows that Arm is instable throughout progression of apoptosis. Contrary to Arm, *D*α-Cat concentration is similar to wildtype during early apoptosis and *D*α-Cat concentration during late apoptosis is only slightly reduced. *D*E-Cad concentration is markedly reduced with onset of apoptosis but does not change significantly over time, indicating that *D*E-Cad is stable throughout apoptosis. Concentration of Actin in th^{109} homozygous embryos was not altered comparing to heterozygous siblings therefore Actin was used as a loading control. These results indicate that reduced cell adhesion in th^{109} homozygous embryos is accompanied by down-regulation of the adhesion molecule Arm, while *D*E-Cad and *D*α-Cat protein levels are stable throughout apoptosis.

2.9.4 Arm is cleaved by the executioner caspase drICE

Decreased cell adhesion in th^{109} homozygous embryos is accompanied by strong activation of the executioner caspase drICE and gradual down-regulation of the cell adhesion protein Arm (Fig. 19+20). Thus, down-regulation of Arm protein levels could be due to enzymatic cleavage by drICE. Indeed Arm is cleaved by recombinant drICE but not by other executioner caspases (Keßler, 2002). Interestingly, Arm contains four putative cleavage motifs for drICE (Fig. 21A; see introduction for details). One of these motifs ($TQFD^{123}$) is conserved in human ß-Catenin and one other motif ($DQVD^{88}$) is present in DIAP1 itself (Steinhusen et al., 2002; Dizel et al., 2003). Additionally, there are two putative caspase cleavage sites with lower similarity to the DXXD cleavage motif ($NDED^{172}$ and $LPID^{755}$).

To identify the amino acid within Arm that is cleaved by drICE, the P_1 position of putative cleavage motifs were altered to alanin by site directed mutagenesis. Mutant Arm was then tested for cleavage by drICE in vitro (Fig. 21B). Full length Arm is stable upon addition of drICE when the $DQVD^{88}$ motif is altered by the mutation $D^{88}A$. In contrast to that, full length Arm is still susceptible to drICE cleavage when all other putative cleavage motifs are mutated ($D^{123}A$, $D^{170}A$, $D^{172}A$ and $D^{755}A$; Fig 21B). In the latter case Arm is cleaved by drICE leading to a stable cleavage product with the M_r of 90 kDa ($Arm^{\Delta N}$). This shows that activated drICE specifically cleaves the DQVD motif in the N-terminus of Arm.

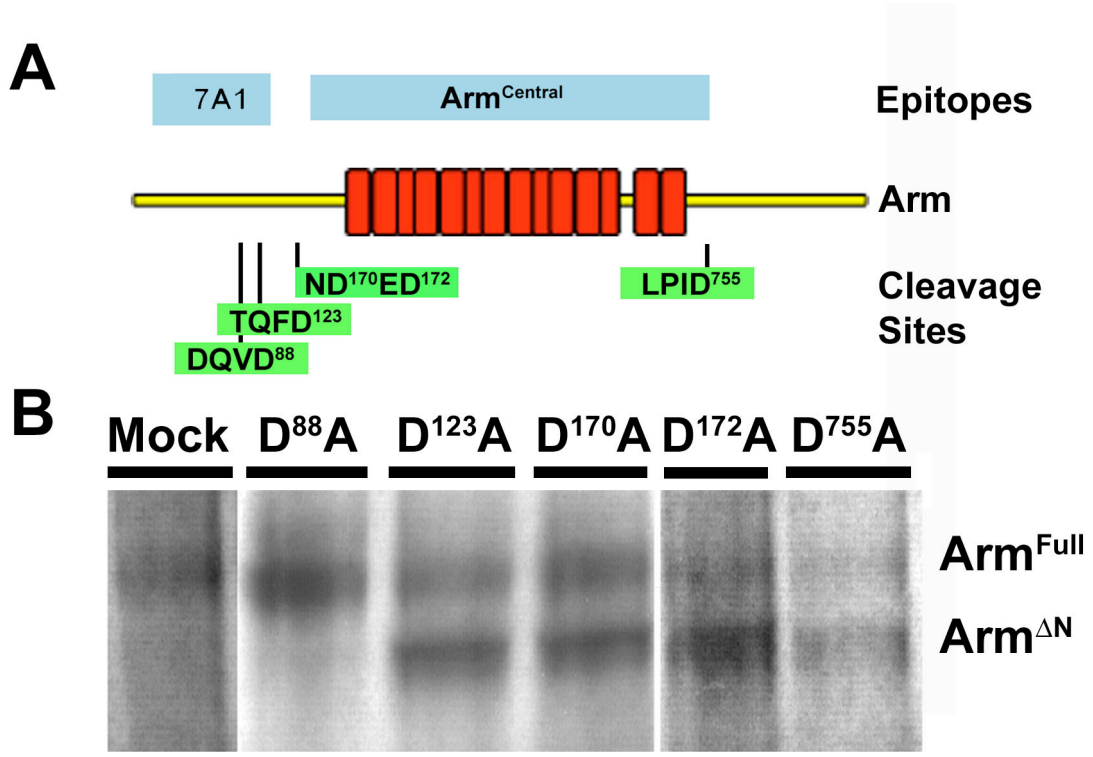


Figure 21: The N-terminus of Arm is cleaved by the executioner caspase drICE

(A) Schematic representation of Arm primary structure. Arm repeats are indicated as red boxes. Putative caspase cleavage sites are indicated in green boxes. Epitopes of the $Arm^{N\text{-}term}$ (7A1) and the $Arm^{Central}$ antibody are indicated by blue bars. (B) Cleavage of in vitro translated mutant forms of Arm incubated with buffer (Mock) or recombinant drICE (all other lanes); drICE cleavage produces a stable product of the Arm mutants $D^{123}A$, $D^{170}A$, $D^{172}A$ and $D^{755}A$. The cleavage product has the M_r of 90kDa and is marked as $Arm^{\Delta N}$; Full length Arm (Arm^{Full}, M_r 110 kDa) is reduced in these lanes. The mutation $D^{88}A$ prevents cleavage by drICE and full length Arm is stable.

Cleavage of Arm by drICE leads to the removal of an 88 amino acid N-terminal fragment. This fragment contains a big part of the region that includes the epitope recognized by the $Arm^{N\text{-}term}$ antibody (Fig. 21A) and cleavage might impaire detection of full length Arm in th^{109} homozygous embryos (Fig. 20G). Additionally, the stable C-terminal fragment that results from drICE cleavage can not be detected by the $Arm^{N\text{-}term}$ antibody in western blots. To circumvent this problem a novel antibody was produced using the central domain of Arm as an epitope ($Arm^{Central}$). This antibody specifically binds to full length Arm in extract of th^{109} heterozygous embryos and to Arm immunoprecipitated by the $Arm^{N\text{-}term}$ antibody (Fig. 22A+22B). Full length Arm is massively reduced in th^{109} homozygous embryos and an additional apoptotic form of Arm is present with the M_r of 90kDa ($Arm^{\Delta N}$; Fig. 22B). This apoptotic form of Arm corresponds to the drICE cleavage product obtained in vitro indicating that the Arm N-terminus is indeed cleaved off by drICE at the onset of apoptosis in th^{109} homozygous embryos.

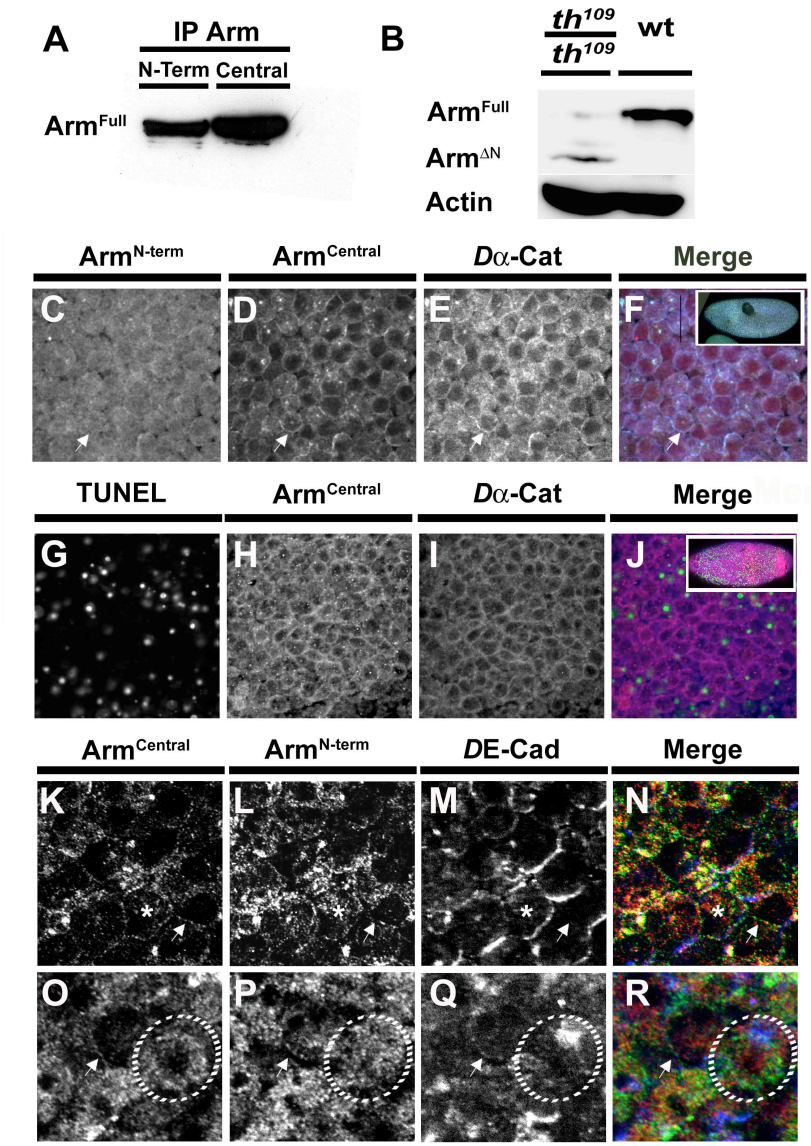


Figure 22:

A stable Arm cleavage product persists throughout apoptosis

(A) $Arm^{Central}$ antibody immunoprecipitates full length Arm; Full length Arm (M_r 110 kDa; indicated as Arm^{Full}) is immunoprecipitated (IP) by the $Arm^{N\text{-}term}$ and the $Arm^{Central}$ antibody. In this western blot Arm^{Full} is detected by the $Arm^{N\text{-}term}$ antibody. (B) Protein levels of Arm^{Full} and $Arm^{\Delta N}$ in th^{109} heterozygous (indicated as wt) and th^{109} homozygous embryos. The $Arm^{Central}$ antibody binds Arm^{Full} (M_r 110kDa) in wt embryos; Levels of Arm^{Full} are decreased in th^{109} homozygous embryos and an apoptotic form of Arm with Mr ~90kDa appears corresponding to $Arm^{\Delta N}$ lacking the N-terminal 88 amino acids. Actin was used as loading control. (C-F) Localization of $Arm^{\Delta N}$ in th^{109} homozygous embryos stained for (C) $Arm^{N\text{-}term}$, (D) $Arm^{Central}$ and (E) $D\alpha$-Cat; (F) Overlay with $Arm^{N\text{-}term}$ in green, $Arm^{Central}$ in red and $D\alpha$-Cat in blue. (G-J) th^{109} homozygous embryo in early stage 9 (inset in J) stained for (G) TUNEL, (H) $Arm^{Central}$ and (I) $D\alpha$-Cat; (J) Overlay with TUNEL in green, $Arm^{Central}$ in red and $D\alpha$-Cat in blue. (K-R) Localization of $Arm^{\Delta N}$ in comparison to Arm^{Full} and *D*E-Cad in early (K-N) and late (O-R) phase of apoptosis; (K-N) early apoptotic cells stained for (K) $Arm^{Central}$, (L) $Arm^{N\text{-}term}$ and (M) *D*E-Cad; (O-R) late apoptotic cells stained for (O) $Arm^{Central}$, (P) $Arm^{N\text{-}term}$ and (Q) *D*E-Cad; (N+R) overlay with $Arm^{Central}$ in green, $Arm^{N\text{-}term}$ in red and *D*E-Cad in blue; In early apoptotic cells $Arm^{\Delta N}$, Arm^{Full} and *D*E-Cad mostly colocalize at the plasma membrane (star in K-M) but co-localization is reduced throughout apoptosis (arrows in 16 K-Q). The dotted circle in Fig. 16 O-R marks a late apoptotic cell with no co-localization of $Arm^{\Delta N}$, Arm^{Full} and *D*E-Cad.

To determine the subcellular localization of the apoptotic Arm cleavage product, th^{109} homozygous embryos were immunolabeled using the $Arm^{Central}$, the $Arm^{N\text{-}term}$ and the $D\alpha$-Cat antibody (Fig. 22C-J). $Arm^{\Delta N}$ is localized to the plasma membrane of cells in the early phase of apoptosis while full length Arm is absent. In these cells $Arm^{\Delta N}$ colocalizes with $D\alpha$-Cat (Fig. 22F), suggesting that removal of the N-terminus does not alter the binding capacity of Arm to $D\alpha$-Cat significantly. Co localization persists to advanced stages of apoptosis where most cells are TUNEL positive. $Arm^{\Delta N}$ rarely co

localizes with *D*E-Cad throughout apoptosis. In early apoptotic cells, $Arm^{\Delta N}$ mostly localizes to membrane areas that are depleted of Arm^{Full} as well as *D*E-Cad (Fig. 22K-N). In some cases $Arm^{\Delta N}$ colocalizes with *D*E-Cad but not with Arm^{Full}. Co-localization of $Arm^{\Delta N}$, Arm^{Full} and *D*E-Cad can not be detected in late apoptosis (Fig. 22O-R). These experiments clearly show that Arm is cleaved upon caspase activation in th^{109} homozygous embryos. This cleavage of Arm by drICE can be followed throughout progression of apoptosis in vivo using the $Arm^{Central}$ antibody.

2.9.5 Expression of non-cleavable Arm in th^{109} homozygous embryos

As shown above, the N-terminus of Arm is cleaved by the effector caspase drICE at the onset of apoptosis. The resulting C-terminal fragment localizes to the plasma membrane but obviously, truncated Arm is not sufficient to sustain cell adhesion between apoptotic cells. This suggests that the presence of the Arm N-term is necessary for cell adhesion. The functional importance of this observation was tested by expressing non cleavable Arm^{D88A} in cells undergoing apoptosis using the GAL4/*UAS*-system (Brand and Perrimon, 1993). If the N-term was crucial for Arm function, late apoptotic cells expressing Arm^{D88A} should maintain cell adhesion. This hypothesis was tested by comparing cell adhesion in wildtype and th^{109} homozygous embryos to cell adhesion in th^{109} homozygous embryos expressing Arm^{D88A}.

Wildtype embryos show highly regular expression of patterning genes that define the borders of the prospective segments. A good marker for this developmental patterning during early embryogenesis in the wildtype embryo is the expression of the segment polarity gene *engrailed*. Engrailed defines the posterior border of each of the 14 parasegmental boundaries in the *Drosophila* embryo (Fig. 23A; Di Nardo et al., 1985). In contrast to wildtype embryos, th^{109} homozygous embryos show a complete block of morphogenesis by stage 9 of development and the regular pattern of *engrailed* expression is disrupted (Fig. 23B). The overall morphology and *engrailed* expression pattern of th^{109} homozygous embryos expressing Arm^{D88A} instead looks very similar to wildtype embryonic development (Fig. 23C). Interestingly, apoptosis in th^{109} homozygous embryos expressing Arm^{D88A} occurs with similar kinetics than in th^{109} embryos according to massive appearance of TUNEL positive cells in stage 9 (Fig. 23D). The Arm^{D88A} transgenic protein localizes to the plasma membrane of apoptotic

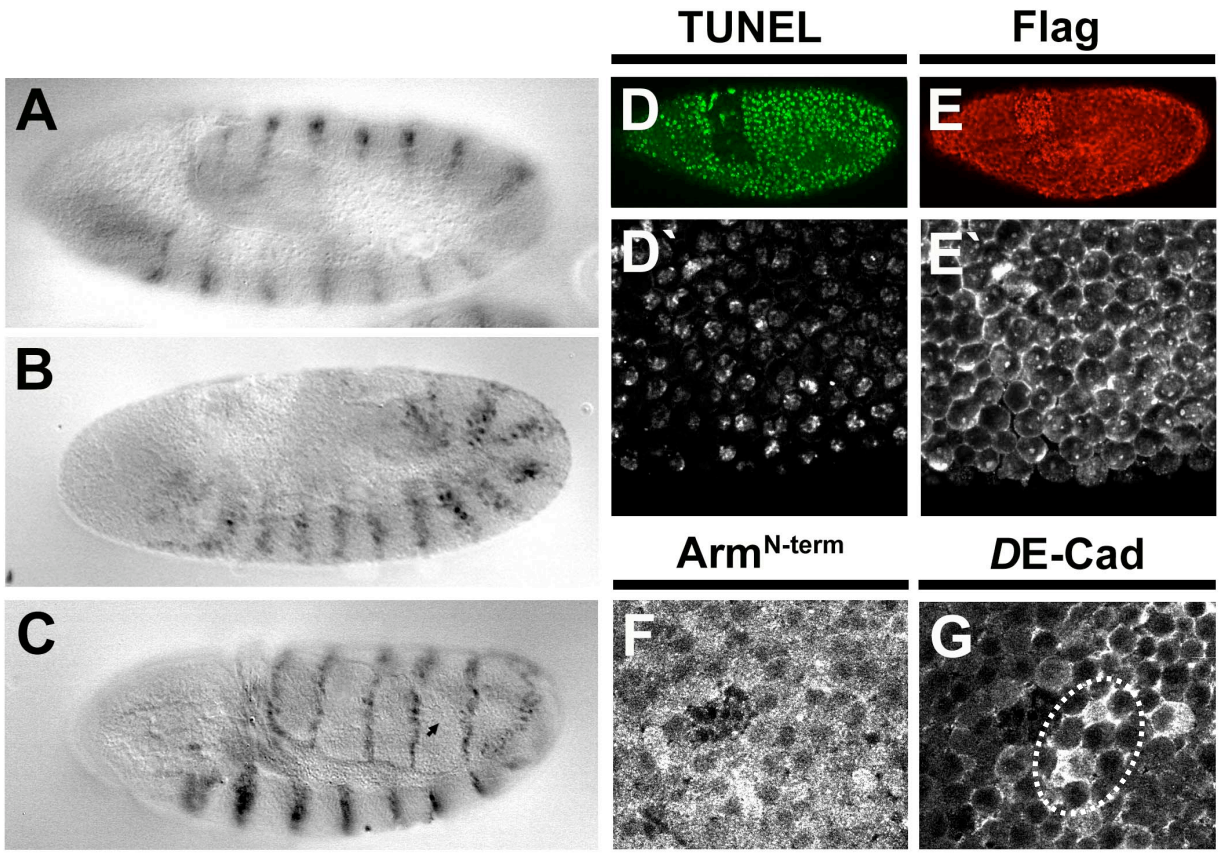


Figure 23: Cell adhesion in *th*^{109} homozygous embryos expressing Arm^{D88A}

(A-C) *engrailed* expression in (A) stage 9 *th*^{109} heterozygous (wt), (B) stage 9 *th*^{109} homozygous and (C) stage 9 *th*^{109} homozygous embryos expressing Arm^{D88A} under control of the maternal Gal4 driver *mat15::Gal4*; (D+E) *th*^{109} homozygous embryo expressing Arm^{D88A} stained for (D) TUNEL and (E) expression of the Arm^{D88A} transgene (Flag in E). (F+G) *th*^{109} homozygous embryo expressing Arm^{D88A} stained for (F) Arm and (G) *D*E-Cad; An area of enhanced localization of *D*E-Cad at the plasma membrane is indicated by the dotted circle in G.

cells while endogenous Arm does not (Fig. 23E). These results support the idea that the N-term of Arm is cleaved during onset of apoptosis in vivo and that this can be prevented when the caspase cleavage site is mutated.

Cleavage of Arm might then result in de-localization of *D*E-Cad during progression of apoptosis in *th*^{109} homozygous embryos. This hypothesis is supported by the finding that *D*E-Cad is localized to the plasma membrane of *th*^{109} homozygous cells that express Arm^{D88A} (Fig. 23G). In this particular case the effect of Arm^{D88A} expression is restricted to clusters of cells and *D*E-Cad is localized at the cell surface in such a cluster. In cells outside the indicated cluster *D*E-Cad is not localized at the cell surface similar to *th*^{109} homozygous embryos without Arm^{D88A} expression. This shows that the N-terminus of Arm is at least partially necessary for maintenance of epithelial integrity and localisation of *D*E-Cadherin at the plasma membrane.

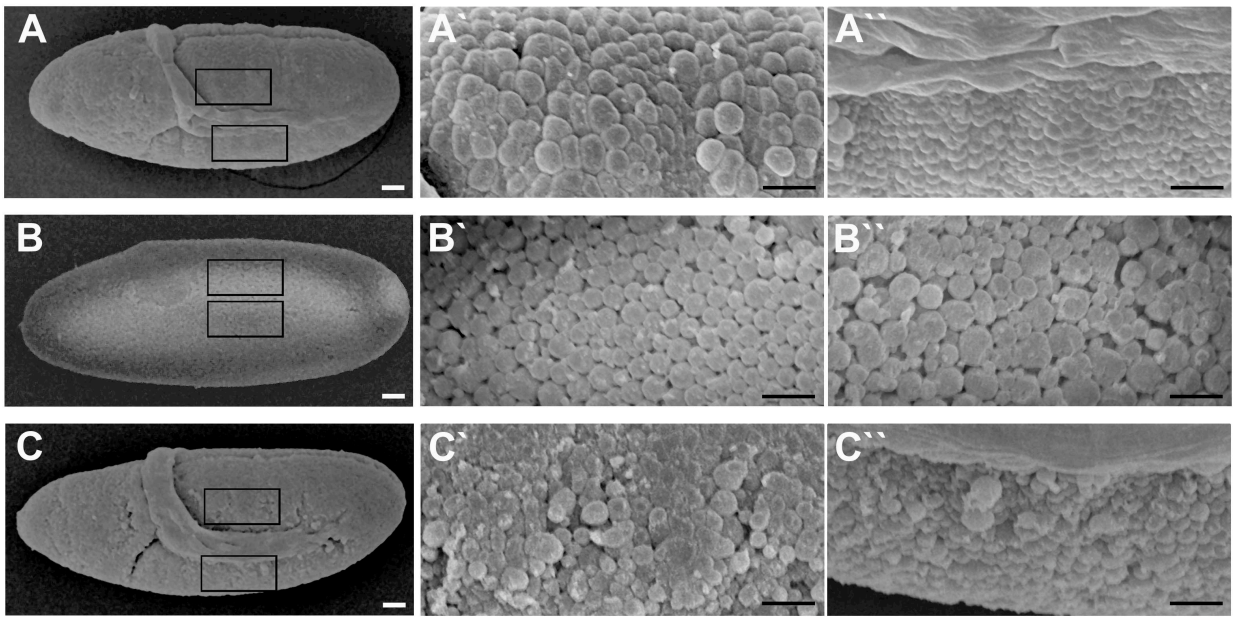


Figure 24: Epithelial integrity in *th*^{109} mutant embryo expressing ArmD^{88}A

(A-C) Scanning electron microscopy of (A) *th*^{109} heterozygous (wt), (B) *th*^{109} homozygous and (C) *th*^{109}homozygous embryos expressing Arm^{D88A}, magnification 250x, Scale bar: 20µm; upper boxes: area of higher magnification in A`-C` (1500x, scale bar: 10µm), lower boxes: area of higher magnification in A``-C``; (A`+A``) Wildtype epithelium; all cells adhere to each other forming a continuous cell sheet; (B`-B``) Integrity of epithelium in *th*^{109} homozygous embryos is impaired; cells are rounded and do not adhere to each other; (C`-C``) Integrity of epithelium in *th*^{109} homozygous embryos expressing Arm^{D88A} is widely normal as cells adhere to each other; only some areas show reduced cell adhesion.

Arm^{D88A} expression seems to be sufficient to maintain cell adhesion in apoptotic cells as shown above. Additionally, *th*^{109} homozygous mutant embryos continue morphogenesis until stage 9 only when Arm^{D88A} is present supporting the idea that cell adhesion in the epithelium is maintained. To further characterize maintenance of cell adhesion in *th*^{109} embryos expressing the Arm^{D88A} transgene, scanning electron microscopy was performed. In wildtype or *th*^{109} heterozygous embryos, cells tightly adhere to each other and form a highly organized tissue sheet (Fig. 24A-24A``). In all *th*$^{109}$ homozygous embryos the overall epithelial structure is severely disrupted 60min after onset of gastrulation. Indeed, all cells in *th*$^{109}$ homozygous embryos undergo apoptosis, the cells do not adhere to each other and have a rounded morphology (Fig. 24B-B``). At the same time point, a 42% (15/35) of *th*^{109} homozygous embryos expressing the Arm^{D88A} transgene maintain overall epithelial integrity. The epithelium of these embryos remains organized and only clusters of cells do not adhere to each other (Fig. 24C-C`). This result is a further indication that cleavage of Arm^{Full} at the onset of apoptosis is an essential step to down-regulate cell adhesion in apoptotic cells. When cleavage of Arm is prevented like in Arm^{D88A} expressing *th*^{109} homozygous embryos, cell adhesion is maintained and the overall morphology is similar to wildtype.

3. Discussion

Epithelia are built by highly polarized cells that are tightly connected with each other by cellular junctions. The function of epithelia crucially depends on proper establishment of apical-basal cell polarity and cell junctions. The unique tisse architecture enables the epithelium to function as a selective barrier, facilitates active transport, secretion or absorbtion depending on the differentiation of the cells. Therefore, genes that regulate cell polarity and cell adhesion are important for the function of many organs including kidney, gut or skin. Most important for cell adhesion in epithelia is the organization of the E-Cadherin/Catenin protein complex into an apical adhesive belt, the zonula adherens (ZA). The ZA separates the apical from the basolateral plasma membrane and therefore allows establishment of two functionally different membrane domains. Initially, positioning of the ZA is defined by large protein complexes that localize asymmetrically within the cell and provide positional information.

In *Drosophila* epithelia apical-basal cell polarity is organized by two large protein complexes that localize apical of the ZA to the subapical region, SAR. One of these SAR protein complexes is organized by the transmembrane protein Crb while the other requires the cytoplasmic protein Baz. An important function of Crb and Baz is to control establishment of intercellular contacts in epithelia by positioning of the cell adhesion molecules *D*E-Cad, Arm and *D*α-Cat to the ZA. These proteins accumulate at a functional ZA only when Crb and Baz are present showing that expression of both is crucial for epithelial integrity (Knust et al., 1993; Tepass et al., 1990; Grawe et al., 1996; Cox et al., 1996; Müller and Wieschaus, 1996). Crb and Baz counteract the activity of the Scrib/Dlg protein complex that localizes to the lateral plasma membrane. Scrib/Dlg localize to a region directly basal to the ZA, the apical lateral membrane (ALM). Similar to Baz and Crb, the ALM proteins are required for positioning of the ZA as they restrict the size of the apical plasma membrane by an unknown mechanism (Bilder et al., 2003).

Crb and Baz define the apical identity of the plasma membrane domain by providing positional information within the cell and by counteracting the activity of the ALM complex (Bilder et al., 2003; Tanentzapf and Tepass, 2003). The molecular mechanisms by which Crb and Baz act are just beginning to be understood. Direct binding of Crb and Baz to scaffolding proteins organize large protein complexes that

might be interconnected with each other. Effector proteins of this scaffolding complex include the kinase *D*aPKC and other classical effector proteins like phosphatases or small GTP binding proteins all of which can directly change the activity status of downstream proteins (von Stein et al., 2005; Hutterer et al., 2004, Chen et al., 2005). However, the regulatory interplay between the scaffolding proteins and the effector molecules during establishment of cell polarity is not very well described.

This work identifies the *Drosophila* inhibitor of apoptosis protein DIAP1 as a molecule that interacts with the Crumbs protein complex in follicle epithelial cells. The functional implication of this binding for cell polarity and its dependence on the small GTPase Ral is discussed in chapter 3.1-3.3. In the *Drosophila* embryo, the main function of DIAP1 is the inhibition of cell death. In DIAP1 mutant embryos, caspases become active and cleave the ZA associated molecule Arm. This cleavage likely results in the loss of cell adhesion observed in DIAP1 mutant embryos as discussed in chapter 3.4.

3.1 The Crb protein complex interacts with DIAP1

The Crb protein complex is an essential regulator of epithelial development in *Drososphila*. In absence of Crb, establishment of cell polarity fails and causes morphologically abnormal epithelial development. The most obvious morphological change in *crb* mutant tissue comparing to the wildtype epithelium is the appearance of multilayered regions (Tepass et al., 1990; Grawe et al., 1996; Tanentzapf et al.,, 2000). Thus, Crb controls the overall integrity and morphology of *Drosophila* epithelia. Although the molecular mechanism that regulates cell polarity downstream of Crb is not completely elucidated it is know to be dependent on the spectrin cytoskeleton and the cytoplasmic protein Sdt (Medina et al., 2002; Bachmann et al., 2001). This work shows that Sdt is required to localize DIAP1 to the apical plasma membrane. Additionally, evidence is presented that a mutation in the RING domain of DIAP1 results in a morphological phenotype very similar to Crb mutants. According to these results, one way how Crb might regulate cell polarity is by localizing DIAP1 to the apical plasma membrane or by inducing degradation of a yet unknown target molecule via the E3 ubiquitin ligase DIAP1.

Interestingly, DIAP1 induces polyubiquitination of *D*aPKC in vitro and in follicle cells as discussed below. *D*aPKC is a member of the Baz protein complex and can act as a negative regulator of the Crb complex in the embryo (Sotillos et al., 2004). Thus it is tempting to speculate that Crb inhibits this negative regulation by inducing *D*aPKC degradation via DIAP1. However, further experiments will be needed to show whether Crb is indeed necessary to induce *D*aPKC degradation by DIAP1 or whether localization of the E3 ligase DIAP1 at the apical plasma membrane is sufficient for this process. Further on, it would be interesting to analyze the function of other Crb complex proteins in *D*aPKC degradation namely *D*PATJ, *D*Lin7, *Dm*PAR-6 and Sdt.

Sdt is a membrane associated guanylate kinase that binds to *D*Lin7 and to *D*PATJ, a protein with four PDZ and an L27 domain (Bachmann et al., 2001; Bachmann et al., 2004; Kempkens, 2005). The interaction of Sdt with *D*Lin7 and *D*PATJ requires homophilic interaction of the L27 domains (see below). The vertebrate homologue of *D*PATJ is necessary for localization of the tight junction proteins ZO3 and Claudin and regulates cell polarity in cultured cells. In the *Drosophila* eye, *D*PATJ is required for localization of the Crb complex and as well regulates planar cell polarity (Richard et al., 2005; Dijane et al., 2005).

This work shows that *D*PATJ interacts with DIAP1, linking the cell polarity machinery to a key regulator of programmed cell death. The main function of DIAP1 is to inhibit caspases, and by that prevents onset of apoptosis in almost all *Drosophila* tissues (Wang et al., 1999; Lisi et al., 2000). Inhibition of caspases depends on two known protein interaction domains in DIAP1, the BIR1 and BIR2 repeat region. The structure of these BIR domains is highly conserved throughout evolution and they are functionally important for regulation of apoptosis in all IAP homologues (Hay, 2000). The binding specificity of BIR domains to other proteins is not analyzed in detail and it is yet to be tested whether the DIAP1-*D*PATJ interaction requires the BIR domains of DIAP1. *D*PATJ however contains five well characterized protein interaction domains, one L27- and four PDZ domains. DIAP1 does not contain an L27 domain that could mediate homophilic interaction with the L27 domain of *D*PATJ but DIAP1 might directly bind to one of its PDZ domains.

Generally, binding to PDZ domains requires a short amino acid stretch, the PDZ binding motif. Such a binding motif is usually located at the very C-terminus of a protein but can also be mimicked by an internal secondary structure (Sheng et al., 2001). The C-terminus of DIAP1 contains the three amino acids YFS that are similar to a novel

class III PDZ binding motif (X-W-C/S-COOH; Maximov et al., 1999) and could therefore mediate the binding to *D*PATJ. The C-terminal four amino acids that represent the putative PDZ binding motive in DIAP1 are conserved in the human proteins cIAP1, cIAP2 and XIAP (see Supplementary Fig. 1). Thus, it is possible that human IAP proteins could interact with the mammalian homologues of *D*PATJ or other binding partners of the vertebrate Crb complex. The requirement of the YFS motif for binding to PATJ could be tested in GST-Pulldown experiments using mutant IAP forms deleted for this motive. This experiment could also reveal whether binding of IAPs to PATJ is direct or indirect involving PATJ binding partners. The presence of the putative PDZ binding motive might be necessary to recruit IAPs to the apical plasma membrane of polarized cells.

In higher organisms, localization of either of the mammalian IAPs at the plasma membrane has not been reported so far. Most studies of mammalian IAP proteins were conducted in non-adherent cells and intended to show the involvement of IAPs in regulation of apoptosis instead of polarity. It is not clear whether IAPs are generally associated with the plasma membrane of polarized cells like for example MDCK cells. Immunofluorescence using commercially available IAP antibodies or transfection with tagged IAP-forms could therefore easily reveal the localization of IAPs in polarized vertebrate cells. Tagged IAP-forms could as well be used to test the interaction with vertebrate Patj and other components of vertebrate tight junctions in vivo. In *Drosophila*, expression of a transgenic DIAP1 version lacking the C-terminal four amino acids could reveal whether the YFS motif is indeed necessary for DIAP1s apical localization in follicle cells. The localization of this transgenic DIAP1 could easily be followed in vivo by addition of a tag like Green Fluorescent Protein (GFP).

Localization of DIAP1 in follicle cells depends on expression of a functional Crb complex as discussed above. DIAP1 might be linked to this complex via *D*PATJ and Sdt and this seems to be necessary to control establishment of cell polarity in the follicle epithelium. As shown before Sdt, *D*Lin7 and *D*PATJ form a protein complex via the interaction of L27 domains (Bachmann et al., 2004; Kempkens, 2005). This work shows that a similar protein complex might be present in the ovary as the *D*PATJ antibody immunoprecipitates *D*Lin7 from ovary extract. The functional importance of this interaction is not clear however as *D*Lin7 mutants have no obvious phenotype and a null mutation affecting *D*PATJ is not available (Bachmann et al., 2004). *D*Lin7 could link the Crb complex to the exocytosis machinery as mammalian Lin7/Veli interacts with Mint1

and MUNC18/sec1, proteins that are directly associated with Syntaxin and the exocyst complex (Butz et al., 1998; Biederer and Sudhof, 2000).

Similarly to *D*Lin7, the function of *D*PATJ for establishment of cell polarity in *Drosophila* is not clear as a null mutation of *D*PATJ is missing (Pielage et al., 2003). However, localization of *D*PATJ in follicle cells depends on expression of Crb as well as another unknown apical recruiting mechanism (Tanentzapf et al., 2000). This additional recruiting mechanism might be provided by the Baz protein complex and it has been suggested that *Dm*PAR-6 is a linker protein between the Crb and the Baz protein complex (Hurd et al., 2003). Existence of such a link in *Drosophila* follicle cells has not been shown yet, but could be tested for example by analysis of Crb or *D*PATJ localization in *Dm*PAR-*6* mutant cell clones. *Dm*PAR-6 mutant cells could show severe defects in cell polarity as function of both Crb and Baz mediated cell polarity might be impaired.

Evidence for interplay between Baz and Crb in the follicle epithelium is provided in this work by the finding that *Dm*PAR-6 localization is altered in *baz* as well as *sdt* mutant follicle cells as described. Baz controls cell polarity by the interaction with *D*aPKC, *Dm*PAR-6, cdc42 and Rac1 (Joberty et al., 2000). Localization of either protein depends on localization of the other members of the complex, explaining why *Dm*PAR-6 is delocalized in *baz* mutant cells. In *sdt* mutant cells an additional apical recruiting mechanism for *Dm*PAR-6 might be impaired as *D*PATJ does not localize apically. To show whether *Dm*PAR-6 localization really depends on *D*PATJ expression, one could generate transgenic flies carrying a *D*PATJ RNAi construct that allows conditional knock-down of *D*PATJ function in follicle cells. Another approach would be generation of a *D*PATJ null allele using targeted gene deletion (Rong et al., 2002). While the exact mechanism that recruits *Dm*PAR-6 to the apical membrane remains to be shown, control of *Dm*PAR-6 localization by Sdt and Baz might reflect an interaction of the Crb and Baz protein complexes via *Dm*PAR-6.

3.2 Localization of DIAP1 might involve posttranslational modification

DIAP1 not only localizes to the apical plasma membrane of follicle cells but to the nuclei as well. As DIAP1 contains no obvious nuclear import signal, translocation of DIAP1 to the nucleus might involve shuttling by unknown binding partners or

posttranslational modification (see below). One way to find other putative protein domains necessary for differential localization of DIAP1 could be to analyze subcellular localization of mutant DIAP1 proteins. Several point mutations affecting the *thread* gene have been identified, that lead to cell death in the embryo and were classified as DIAP1 null mutations (Lisi et al., 2000; Goyal et al., 2000). The molecular basis of how these null mutations lead to cell death has been shown only for a small subset of mutations that affect binding to pro apoptotic proteins and caspases (Zachariou et al., 2003). These findings suggest that modular binding of proteins to DIAP1 is necessary for its antiapoptotic function. However, in the follicle epithelium, DIAP1 is not necessary to protect from cell death as DIAP1 null mutant cells do not undergo apoptosis (Geisbrecht and Montell, 2005). Thus, follicle cell clones mutant for different *thread* alleles might reveal insight into the mechanisms that control subcellular localization of DIAP1 if they impair for example the binding to the factor that mediates nuclear transport. These experiments might as well explain a complementary localization of DIAP1 at the lateral membrane domain that has been shown recently, using a different DIAP1 antibody (Geisbrecht and Montell, 2004). While lateral localization of DIAP1 seems dispensable for the migration process studied in this paper, it is not known which protein recruits DIAP1 to the lateral membrane. Putative protein interaction domains within DIAP1 that mediate this localization could be revealed by the experiments mentioned above and could give further insight into the mechanisms that control differential localization of IAPs in general.

In follicle cells, DIAP1 localizes to the lateral as well as the apical plasma membrane as mentioned above. This finding suggests that two subpopulations of DIAP1 exist within epithelial tissues of *Drosophila*. This view is supported by the finding in this work, that tissue extracts from ovaries as well as embryos contain three DIAP1 forms. Only one of these forms accumulates in immunoprecipitates using the monoclonal DIAP1 antibody, probably reflecting a preference of the antibody for this DIAP1 form. How the putative forms of DIAP1 differ from each other is yet unknown, but regulation of DIAP1 localization might involve posttranslational modification like ubiquitination or phosphorylation.

The work of several groups conclusively showed that DIAP1 is a target for E3 ubiquitin ligases like UbcD1 and DIAP1 itself (Ryoo et al., 2002). Both DIAP1 and UbcD1 E3 ligases catalyze the addition of polyubiquitin chains to target protein, priming them for proteasomal degradation. In several cellular contexts, this ubiquitin dependent

process negatively regulates DIAP1 protein levels and thereby promotes onset apoptosis (Yoo et al., 2002; Ryoo et al., 2002). However, it cannot be ruled out that regulation of DIAP1 involves yet an unknown Ubiquitin dependent process that changes its activity status rather than its protein stability. One class of E3-ubiquitin ligases, the SCF complex, can trigger changes of the activity status of a target molecule by ubiquitination and regulate for example the progression of the cell cycle (Deshaies, 1999). This process can involve polyubiquitination as well as monoubiquitination. Evidence exists that IAP proteins can be regulated by monoubiquitination, for example the mammalian XIAP1 that is monoubiquitinated upon brain injury by an unknown mechanism (Lotocki et al., 2002). This monoubiquitination of XIAP is associated with changes in its cellular localization, while the cellular function if this localization is not known. Interestingly, DIAP1 can be regulated by a SCF protein, Morgue (Wing et al., 2002) and it remains to be shown whether localization of DIAP1 is regulated by monoubiquitination similar to XIAP.

Another posttranslational modification utilizes addition of a small ubiquitin related molecule, SUMO, to target molecules. SUMOylated molecules can show an altered cellular localization and activation status (for review see Hay, 2005; Gill, 2004). Caspase 8 for example is localized to the nucleus of cells in a SUMO dependent manner (Besnault-Mascard et al., 2005). SUMOylation of Caspase 8 is necessary for differentiation and maturation of different cell types, providing further evidence that caspases are involved in processes different from apoptosis (Wang and Lenardo, 2000). Interestingly, the primary structure of DIAP1 contains four putative SUMOylation sites although predicted with a relative low probability (http://www.abgent.com/-tool/sumoplot). The functional importance of SUMOylation for localization of DIAP1 within the cell remains to be shown.

Phosphorylation of conserved consensus sequences within the primary structure of a kinase target molecule is also a well know cellular strategy to alter localization of a protein. DIAP1 contains several putative phosphorylation sites for Serin/Threonin and Tyrosin kinases as predicted by the NetPhos program (http://www.cbs.dtu.dk/services/-NetPhosK/). Some of these phosphorylation sites for Protein Kinase A, B and C are predicted with a high probability (~0.8). These kinases have been implicated in regulation of exocytosis (PKA), as well as cell polarity (PKB and PKC) and might provide positional cues for DIAP1 localization within the cell. Putative phosphorylation of DIAP1 could be tested by immunoprecipitation and subsequent western blotting using

phospho-specific antibodies. However, identification of the kinase that phosphorylates DIAP1 would require direct testing of many kinases including Protein Kinase A, B and C. An interesting candidate kinase could be Par1, a protein that localizes to the lateral plasma membrane and controls cell polarity by phosphorylation of target molecules.

3.3 Functional importance of DIAP1 in the follicle epithelium

DIAP1 is required for proper development of the follicle epithelium as described in this work. Loss of E3 ligase function in the th^6 allele during early development of the follicle epithelium leads to multilayering and defective epithelial integrity similar to mutants affecting the classical polarity regulator Crb. As analysis of follicle cell clones shows, DIAP1 regulates cellular levels of Baz and *D*aPKC. Therefore it seems likely that the multilayering of th^6 mutant follicle epithelium is due to altered activity of Baz or *D*aPKC. Thus, both establishment of epithelial polarity and maintenance of asymmetric localization of the Baz complex in the mature epithelium might be under control of DIAP1s RING domain. These results indicate that regulation of cell polarity in the follicle epithelium requires degradation of one or several molecules that are targets of the E3 ligase DIAP1.

The C-terminus of almost all cellular IAP like proteins contains a RING domain as mentioned above. This RING domain provides E3 ubiquitin ligase activity and DIAP1 has been shown to induce proteasomal degradation of E3 target proteins (Yoo et al., 2002; Ryoo et al., 2002; Muro et al., 2002; Kuranaga et al., 2002). In presence of pro apoptotic proteins the RING domain can target IAPs for proteasomal degradation. This event leads to caspase activation and cell death in *Drosophila*. Additionally, the E3 ligase DIAP1s has been shown to negatively regulate cell death in cultured cells by targeting the proapoptotic proteins Rpr (Olson et al., 2003), Dronc (Wilson et al., 2002) and TRAF1 (Kuranaga et al., 2002) for proteasomal degradation. In the case of TRAF1, negative regulation by DIAP1 inhibits cell death in the adult fly but functional relevance of Rpr or Dronc degradation remains to be shown. One would predict that cellular levels of E3 target proteins rise, when DIAP1`s E3 ligase function is absent. This hypothesis has not been confirmed *in vivo* for any known E3 target of DIAP1 yet, probably due to induction of apoptosis in DIAP1 mutant cells.

This work identifies *D*aPKC as a novel target of DIAP1s E3 ligase function in vitro. *D*aPKC is a member of the Baz protein complex that is involved in regulation of

cell polarity of *Drosophila* epithelia. Interestingly, DIAP1 does not to regulate *Dm*PAR-6, Baz or *D*PATJ suggesting that DIAP1 specifically regulates *D*aPKC by ubiquitination. This observation supports the idea that DIAP1 is functionally downstream of the Crb protein complex and regulates the Baz protein complex by ubiquitination of *D*aPKC.

Drosophila aPKC has not been shown so far to be regulated by proteasome mediated degradation. However, a close mammalian homologue of *D*aPKC, PKCλ, is regulated by an E3 ligase complex containing the tumor suppressor van Hippel Landau protein (VHL; Okuda et al., 2001). The functional importance of this PKCλ degradation by VHL is not clear. Interestingly, the *Drosophila* homologue of VHL can act as an E3 ligase in cultured cells with similar substrate specificity than its mammalian homologue (Aso et al., 2000). In vivo, the dVHL protein regulates cell division and tracheal development (Adryan et al., 2000) a phenotype not described for *D*aPKC. Thus, it remains open whether dVHL is involved in regulation of *D*aPKC in other tissues. However, the VHL protein mediates substrate recognition of a SCF like Ubiquitin ligase complex via a protein interaction domain that is conserved in dVHL. It is possible that substrate specificity of SCF like ubiquitin ligase complexes is mediated by different E3 ligases that become assembled in a modular way in a cell type dependent manner. DIAP1 can genetically interact with a SCF like protein complex as DIAP1 is negatively regulated by the SCF E3 ligase Morgue. It would therefore be interesting to test whether DIAP1 is found in complex with dVHL by that mediating degradation of *D*aPKC.

Elevated cytoplasmic levels of *D*aPKC detected in th^6 mutant cells support the in vitro data and define DIAP1 as a protein responsible for negative regulation of *D*aPKC in the follicle epithelium. Similar to *D*aPKC, Baz is strongly accumulated in the cytoplasm of large th^6 mutant clones although Baz is not directly regulated by DIAP1 in the cell free system. As the cell free system is based on embryonic tissue extract, it cannot be ruled out that DIAP1 directly regulates Baz stability in other tissues like the follicle epithelium. Interestingly, PAR-2, a protein that contains a RING domain, has been described to regulate localization of the Baz homologue PAR-3 in the *C.elegans* zygote in response to phosphorylation by the *D*aPKC homologue PKC3 (Cheng et al., 1995; Boyd et al., 1996; Hao et al., 2006). No clear homologue of PAR-2 has been described in other organisms, leaving open, whether PAR-2 function is required in evolutionary younger organisms like *Drosophila* or whether other E3 ligases are employed for Baz regulation.

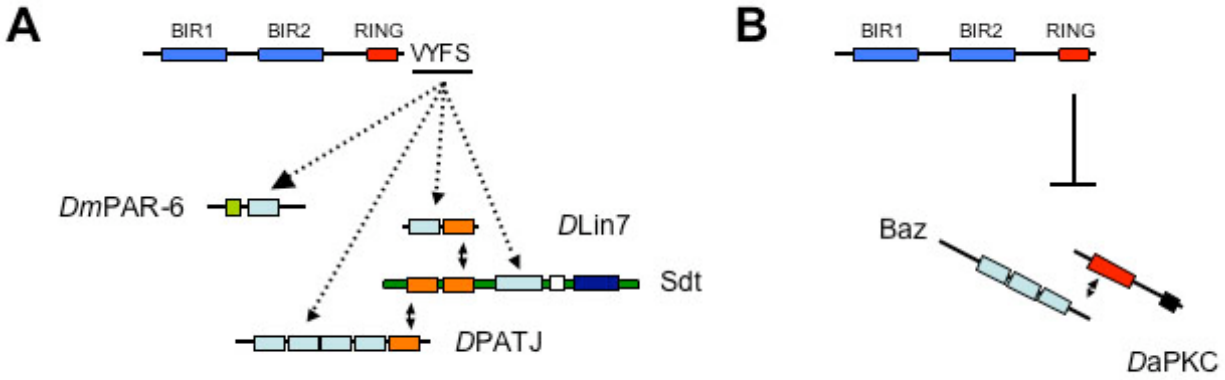


Figure 25: Interaction of DIAP1 with the Crb- and the Baz protein complex

(A) DIAP1 might interact with proteins that contain PDZ domains via a putative C-terminal PDZ binding motive (VYFS). As apical localization of DIAP1 in follicle cells depends on the Crb complex, binding might involve *D*PATJ, *D*Lin7, *Dm*PAR-6 or Sdt. (B) The RING domain of DIAP1 is necessary to repress the cytoplasmic accumulation of Baz and *D*aPKC in follicle cells probably in response to apical localization of DIAP1 by the Crb complex.

Interestingly, cytoplasmic Baz localization in the follicle epithelium seems to be controlled by *D*aPKC dependent phosphorylation and a Baz construct lacking a putative *D*aPKC phosphorylation site is accumulated in the cytoplasm (Fischer, personal communication). In line with that observation is the result that a putative kinase dead*D*aPKC form leads to elevated cytoplasmic Baz levels in follicle cells (Kim, unpublished). Thus, elevated cytoplasmic levels of Baz might therefore due to inactivation of *D*aPKC in DIAP1 mutant follicle cells. But how could elevated *D*aPKC levels in *thread*^{6} mutant cells lead to inactivation of *D*aPKC kinase function?

Full length *D*aPKC contains an N-terminal auto-inhibitory domain that is believed to block the kinase domain and this block can be overcome by phosphorylation by PDK1 (Le Good et al., 1998). It is possible that elevated levels of *D*aPKC saturate this activation mechanism, provoking a dominant effect on Baz protein levels. Alternatively, DIAP1 might primarily regulate regulate Baz localization in follicle cells rather than its stability. As this work shows, *Dm*PAR-6 is not localized at the apical plasma membrane of cells lacking DIAP1. This delocalization of *Dm*PAR-6 could also lead to accumulation of Baz in the cytoplasm as localization of Baz complex members is strongly interdependent. This hypothesis could be tested by analyzing Baz protein localization in *Dm*PAR-6 mutant follicle cells.

3.4 Ral is a potential regulator of cell polarity upstream of DIAP1

As described in this work, DIAP1 regulates establishment of cell polarity during follicle cell development. This novel function of DIAP1 is probably independent of its antiapoptotic function and likely requires upstream regulatory mechanisms that allow proper egg development. One candidate protein that might act upstream of DIAP1 in the follicle epithelium is the small GTPase Ral (this work and Gagic, 2005). Ral is a Ras-like small GTPase involved in many cellular processes like actin nucleation, negative regulation of the JNK signalling pathway and regulation of exocytosis (Sawamoto et al., 1999; de Ruiter et al., 2000; Moskalenko et al., 2002; Rosse et al., 2003; Shipitsin et al., 2004).

In general, small GTPases are molecular switches that trigger downstream events by binding to effector molecules. This work present evidence that Ral binds to the BIR1 domain of DIAP1 in an activation dependent manner suggesting that DIAP1 is an effector of Ral. This view is supported by the finding that in Ral mutant follicle cells, DIAP1 is absent, placing Ral upstream of DIAP1 in a regulatory mechanism.

The cellular phenotype of Ral mutant cells is very similar to DIAP1 mutant cells, as *D*aPKC and Baz are accumulated in the cytoplasm, *Dm*PAR-6 does not localize to the apical plasma membrane and *D*PATJ localization is not affected. Strikingly, absence of *ral* expression during establishment of cell polarity results in multilayering of the epithelium very similar to DIAP1 mutants as described in this work. These findings strongly support the idea that in wildtype cells Ral stabilizes DIAP1 protein levels and by that regulates localization of apical plasma membrane determinants.

Control of proteins associated with the apical plasma membrane is a novel function for Ral. Neither *Drosophila* nor vertebrate Ral have been reported to associate with the apical plasma membrane. Rather than that mammalian Ral has been implicated in regulation of the basolateral plasma membrane by targeting secretion via the exocyst complex (Shipitsin and Feigh, 2004). It is not know so far whether *Drosophila* Ral localizes to the apical or baso-lateral plasma membrane in follicle cells. Future experiments with Ral antiserum or tagged versions of Ral will therefore be required to reveal its subcellular localization. Interestingly, localization of the exocyst complex in *Drosophila* follicle cells seems not to be restricted to the lateral plasma membrane and apical localization cannot be excluded (Murthy et al., 2005). Ral binds to

exocyst complex members and regulates their assembly in vertebrates (Moskalenko et al., 2003). Therefore Ral act similarly in *Drosophila* and Ral could be localized to the apical plasma membrane domain in follicle cells. In contrast to Ral, the small GTPases Ras and Rap1 have been implicated in control of apical cell polarity in other tissues than the follicle epithelium (James et al., 2002; Asha et al., 1999). However, small Rap1 or Ras mutant clones do not show obvious alterations in DIAP1 protein levels suggesting that DIAP1 dependent regulation of cell polarity is specifically regulated by Ral.

As shown in this work, Ral regulates DIAP1 levels in follicle cells. One known cellular mechanism that regulates DIAP1 protein levels involves regulation of DIAP1 protein stability via its E3 ubiquitin ligase domain. The pro apoptotic protein Hid enhances autoubiquitination of DIAP1 (Yoo et al., 2002). Additionally, direct binding of Hid to the BIR2 domain of DIAP1 has been confirmed (Zachariou et al., 2003). Interestingly, addition of Ral stabilizes DIAP1 in the same assay, suggesting that Ral binding negatively regulates DIAP1´s E3 ubiquitin ligase function. This finding supports a model in which presence of Ral is required to stabilize DIAP1 protein levels. The negative regulation of DIAP1s E3 ligase domain might be missing in *ral* mutant follicle cells and explains why DIAP1 levels rapidly decrease.

Similar to Ral, Ras and Rac1 (but not Rap1) have a similar stabilizing effect on DIAP1 protein levels in the cell free system. It is not clear from this assay whether the stabilizing effect of Ras and Rac1 is direct or indirect as addition of the recombinant GTPase might influence the activity of other GTPases in the extract. This cross activation might then lead to the observation that all tested GTPases stabilize DIAP1 independent on their activity status. This hypothesis could be further tested by analyzing the effect of small GTPases on DIAP1 protein stability in absence of protein extract. One way to test specificity of the DIAP1 stabilizing effect of GTPases could be an assay that utilizes recombinant proteins only. Recombinant E1 and E2 proteins are commercially available and in combination with DIAP1 are sufficient to induce DIAP1 ubiquitination (Yoo et al., 2002). Addition of recombinant GTPases to this assay could resolve the question if DIAP1 protein levels are indeed regulated exclusively by Ral or by Ras and Rac1 as well. It remains to be determined though if the small GTPases that act as DIAP1 stabilizing proteins in vitro are regulators of DIAP1 levels in vivo. This work only considered the situation in the follicle epithelium and it is possible that DIAP1 stabilization depends on different small GTPases in other tissues.

3.5 Function of Ral/DIAP1 in the embryo

In the follicle epithelium DIAP1 is needed for localization of cell polarity marker proteins like Baz, *D*aPKC and *Dm*PAR-6. These proteins were first discovered to regulate establishment of cell polarity in the *Drosophila* embryo and both maternal and zygotic expression of Baz is required for proper embryogenesis (Müller and Wieschaus, 1996; Cox et al, 1996; Wodarz et al., 2000; Hutterer et al., 2004). Both DIAP1 and Ral are provided maternally to the embryo but an embryonic phenotype is inaccessible as null mutations for both genes abolish egg development and early morphogenesis respectively (shown in this work). Thus, the function of maternal Ral and DIAP1 for establishment of cell polarity in the embryo remains obscure. A widely used method to analyze the function of maternal mutations that prevent egg development is silencing target genes by RNA interference (RNAi; Bilder 2003). In the case of Ral, RNAi might result in an even stronger phenotype than observed in hypomorphic Ral germline clone embryos which show clear defects in cellularization. It is possible however, that this *ral* phenotype is a consequence of an earlier defect, for example during migration of the nuclei to the periphery of the syncytial blastoderm embryo (Campos-Ortega and Hartenstein, 1997). Thus, more careful analysis of maternal *ral* mutants will be necessary to describe its function for early morphogenesis and might reveal a functional importance of Ral for establishment of cell polarity. Similar to Ral RNAi, DIAP1 depletion could block embryogenesis before cell polarity is being established as maternal DIAP1 is sufficient to block apoptosis in zygotic DIAP1 mutants until stage 9 of development. Thus, removal of maternal and zygotic DIAP1 by RNAi might result in massive “cell” death in early stages of embryo development.

3.5.1 DIAP1 regulates tissue integrity in the ectoderm epithelium

Zygotic expression of DIAP1 is necessary to prevent apoptosis, an irreversible process of cell differentiation. In zygotic DIAP1 mutants the localization of Baz, the membrane protein Neurotactin and the ZA component *D*αCat is altered compared to wildtype. These findings indicate that caspase activation leads to loss of cell polarity and cell adhesion. With progression of apoptosis DIAP1 mutant embryos completely lack cell adhesion by stage 9 of embryogenesis as marked by rounding of all cells. The

lack of cell adhesion in DIAP1 mutants is reminiscent of embryos maternal and zygotic mutant for the ß-Catenin homologue Arm (Müller und Wieschaus, 1996; Cox et al., 1996). Arm links the transmembrane protein *D*E-Cad to *D*α-Cat and this binding is necessary for cell adhesion and building of a functional ZA. In Arm mutants, the interaction between *D*E-Cad and *D*α-Cat is impaired and therefore cell adhesion is reduced and cells round up (Cox et al., 1996; Orsulic and Peifer, 1996). In th^{109} homozygous embryos, levels of full length Arm are rapidly decreasing during progression of apoptosis while *D*E-Cad and *D*α-Cat are more stable. These finding suggests thatreduced cell adhesion in apoptotic cells is caused by reduced protein levels of full length Arm.

3.5.2 Functional importance of Arm cleavage

Caspase activation in culture cells is accompanied by cleavage of ß-Catenin and E-Cadherin (Brancolini et al., 1997; Steinhusen et al., 2000; Steinhusen et al., 2001). However, the functional importance of ß-catenin or E-Cad cleavage for their functional inactivation and down-regulation of cell adhesion has not been shown. In DIAP1 mutant embryos, levels of full length Arm are reduced upon caspase activation and the activated effector caspase drICE can cleave Arm in vitro (Kessler, 2002). Analysis of Arm`s primary structure revealed putative caspase cleavage sites in the N-terminus of Arm (shown in this work). In contrast to Arm both *DE*-Cad and *D*α-Cat do not contain bona fide caspase cleavage sites. Thus, reduced levels of full length Arm are likely to be a result of Arm cleavage while *D*E-Cad and *D*α-Cat are not cleaved by caspases and appear more stable. The N-terminus of Armadillo shares high homology with the ß-Catenin homologue of *Danio rerio*, *mus musculus* and human (Fig. 19). The 25 amino acid stretch located at amino acid 25-50 and a more C-terminal 20 amino acid stretch between aa 70 and 90 has the highest sequence homology. The conserved N-terminal aa stretch of mouse, fish and human ß-Catenin proteins contains the SYLD motif that can be cleaved by human caspase 3 but this motif is not conserved in *Drosophila.* The more C-terminal conserved region contains the DQVD caspase cleavage site found in *Drosophila* Arm and in direct vicinity the ADID motif that can be cleaved by human Caspase 3. Thus, cleavage of the ß-Catenin N-terminus seems to be evolutionary conserved, but its functional importance in other organisms remains to be elucidated.

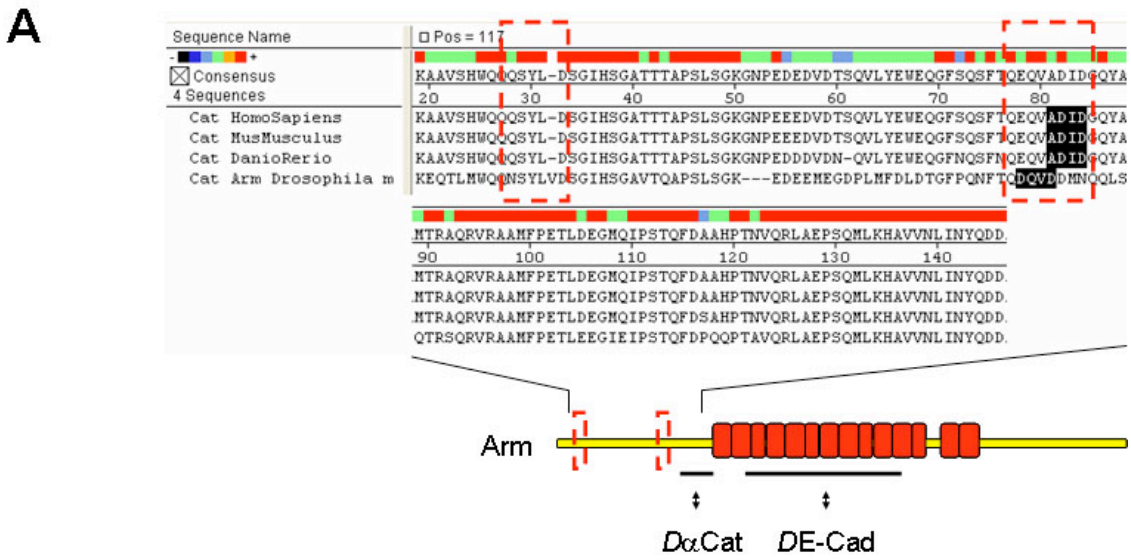


Figure 26: Arm/ß-Catenin contains caspase cleavage sites at conserved N-terminal regions

(A) Alignment of *Drosophila* Arm and the ß-Catenin of *Danio rerio*, *Mus musculus* and *Homo sapiens*; The N-terminus of Arm is cleaved by the caspase drICE at position 88 (DQVD highlighted in black); The DQVD motive itself is not conserved in ß-Catenins of higher organisms but the homologous region contains the caspase-3 cleavage site ADID (highlighted in black) at a similar position; The region of Arm and ß-Catenin that contains the caspase cleavage sites (red square to the right) lies N-terminal of the a-Cat binding domain. An N-terminal region of ß-Catenin (red square to the left) contains another caspase-3 cleavage site (SYLD) that is not conserved in *Drosophila* Arm.

Interestingly, during apoptosis all ß-Catenins are cleaved C-terminal of the GSK3 phosphorylation sites that are conserved between the species and necessary to target ß-Catenins for proteasomal degradation in the absence of Wnt/wingless signalling. One function of caspase cleavage could therefore be stabilization of ß-Catenin by removing the regulatory N-terminal domain. This hypothesis is supported by the finding that *Drosophila* full length Arm is strongly reduced during progression of apoptosis while the Arm cleavage product lacking the GSK3 phosphorylation site is stable (this work). $Arm^{\Delta N}$ does not to localize to the nucleus of apoptotic cells, making it unlikely that $Arm^{\Delta N}$ mediates enhanced transcription of wingless-responsive genes. Instead, antibody staining reveals that $Arm^{\Delta N}$ does not co-localize with *D*E-Cad in late apoptosis suggesting that binding is impaired by cleavage.

Several deletion variants of Arm have been analyzed for their binding capacity to either *D*E-Cad or *D*α-Cat. This analysis revealed that the minimal *D*E-Cad binding domain of Arm requires the C-terminus including all Arm repeats while the complete N-terminus is dispensable (Pei et al., 1996). The *D*α-Cat binding domain of Arm has been restricted to amino acids 87-139 as a construct lacking the amino acids 37-87

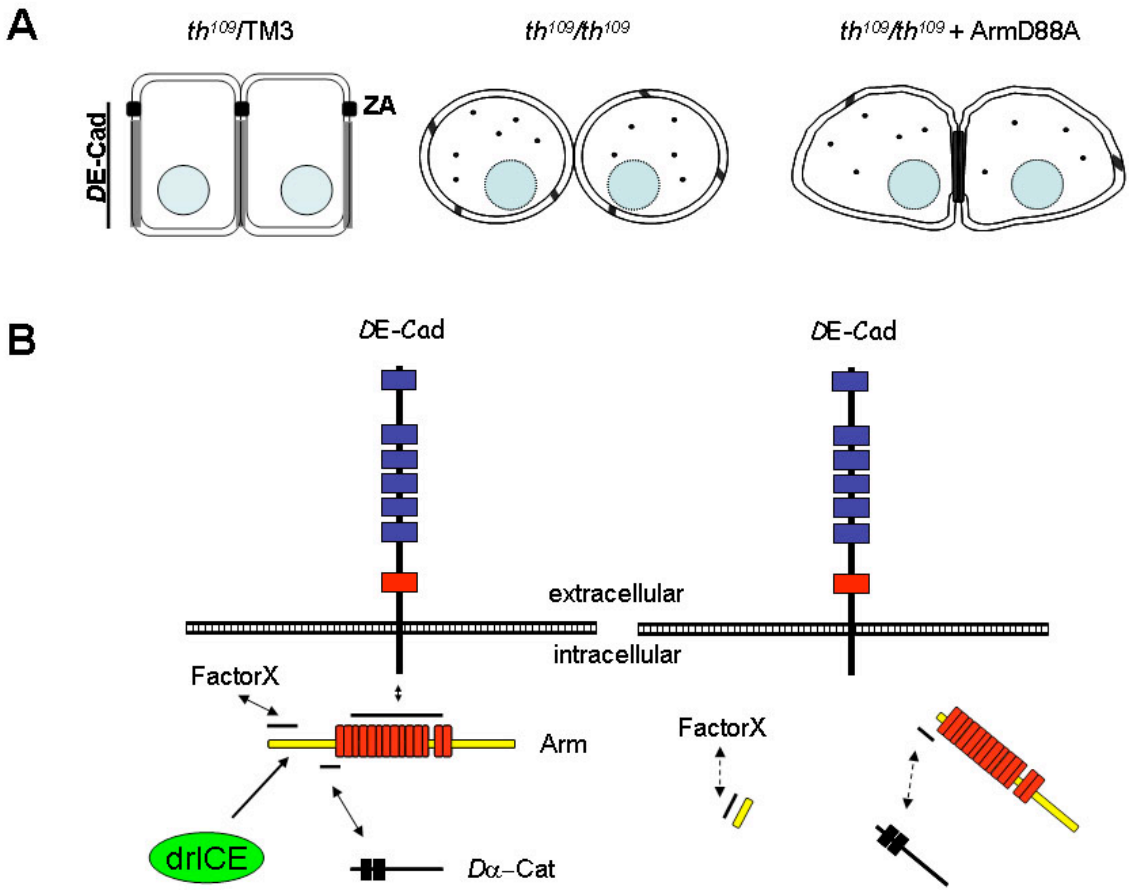


Figure 27: Schematic representation of cleavage events during apoptosis

(A) Left: *th*^{109} heterozygous or wildtype epithelial cells mainly adhere to each other via the zonula adherens (ZA) at the boundary between apical and basolateral plasma membrane. The lateral membrane adhere probably due to weak *D*E-Cad mediated cell adhesion at this membrane compartment. Middle: In *th*^{109} homozygous embryos the ZA is lost and cell adhesion is lost. *D*E-Cad is delocalized in these cells. Right: *th*^{109} homozygous embryos that express Arm^{D88A} retain *D*E-Cad localization in clusters at the plasma membrane in advanced stages of apoptosis. This seems to be sufficient to maintain cell adhesion in late stages of apoptosis.

(B) Progression of apoptosis from left to right: The central repeat region of Arm interacts with the cytoplasmic tail of *D*E-Cad in wildtype and early *th*^{109} homozygous epithelial cells and connects *D*E-Cad with the cytoskeleton via *D*αCat. When the caspase drICE becomes activated at the onset of apoptosis the N-terminus of Arm is cleaved. This cleavage either removes the Arm binding domain to an unknown regulator of cell adhesion (FactorX) or impairs binding of Arm to *D*E-Cad. Cleavage of Arm results in delocalization of *D*E-Cad, disassembly of the ZA and subsequently the loss of cell adhesion

localizes apical and retains *D*α-Cat binding capacity (ArmS10; Pei et al., 1996) while a construct lacking aa 101-139 retains *D*E-Cad but nor *D*α-Cat binding. According to this data, the Arm cleavage product (ArmCentral aa 88-end) should retain binding capability to both *D*E-Cad and *D*α-Cat. It cannot be excluded, though, that cleavage impairs binding of Arm$^{\Delta N}$ to *D*α-Cat as Arm$^{\Delta N}$ is missing aa 87. In addition to the interaction with *D*E-Cad and *D*α-Cat, recent data shows that the Arm C-term interacts with the exocyst complex Sec10 and by that might regulate *D*E-Cad exocytosis and ZA

stability (Langevin et al., 2005). The latter finding probably shows a cell type specific function of Arm as Arm is dispensable for cell adhesion in other tissues (Pacquelet et al., 2003). Human ß-Catenin binds to *D*E-Cad and *D*α-Cat only when the N-terminal region is present suggesting that the N-terminal region of ß-Catenin/Arm might have a cryptic functional domain that allows regulation of cell adhesion (Castano et al., 2002). This hypothesis is in line with the finding that full length Arm localizes to the plasma membrane of apoptotic cells only when the N-terminus is present like in non cleavable Arm^{D88A}. Localization of non cleavable Arm is sufficient to maintain *D*E-Cad at the plasma membrane of apoptotic cells and prolongs cell adhesion. Cleavage of Arm might therefore be a negative regulatory step to down-regulate adhesion in apoptotic cells and indicates a novel function for the N-terminal region of Arm. It will be interesting to test whether the N-terminal regulatory domain directly influences binding of Arm to *D*E-Cad or the exocyst complex. Alternatively, the very N-terminal domain of Arm could bind to a novel interaction partner that is involved in regulation of cell adhesion. To identify this putative Arm binding partner one could perform a yeast two hybrid screen with the Arm N-terminus as bait.

3.6 DIAP1 expression is crucial for integrity of *Drosophila* epithelia

The epithelium requires establishment and maintenance of distinct plasma membrane compartments and a functional ZA throughout development. The data presented in this work show that the *Drosophila* inhibitor of apoptosis DIAP1 regulates both aspects of epithelial development and is therefore generally required for the function of *Drosophila* epithelia. Interestingly, the function of DIAP1 for establishment of cell polarity and cell adhesion can be separated into the RING domain and the N-terminal domain including the BIR1 and BIR2 repeats.

It is clear from the presented data that establishment of cell polarity in the follicle epithelium depends on the E3 ligase domain in the C-terminus of DIAP1. When the E3 ligase function is abolished by a single point mutation in the RING domain, localization of the Baz protein complex is impaired. This delocalization of the Baz complex most likely affects downstream components and leads to aberrant development and multilayering of the follicle epithelium. This work shows also that the cellular DIAP1 level in the follicle epithelium is under control of the small GTPase Ral (also Gagic, 2005).

Ral is the only molecule described so far that binds to DIAP1 and positively regulates its protein stability by interfering with the E3-ubiquitin ligase domain. Ral has not been implicated in regulation of apical cell polarity until now, and future work will be required to identify regulatory mechanisms that trigger the Ral/DIAP1 interaction upstream of Ral.

Generally, little is known about mechanisms that lead to activation of small GTPases in *Drosophila.* It is tempting to speculate that the Ras signalling pathway acts upstream of Ral/DIAP1. Ras has been shown to interact with a guanine nucleotide exchange factor (GEF) that activates Ral in vertebrates (Wolthuis et al., 1999). One of these Ral GEFs is present in the *Drosophila* genome but conservation of a Ras/Ral signalling pathway could not be confirmed so far. Instead, it has suggested that Rap1 acts upstream Ral under conditions were both proteins are overexpressed (Mirey et al., 2003). Whether this interaction of Ral and Rap1 is present under physiological conditions has not been analyzed. Therefore it remains elusive whether Rap1, Ras or other proteins might activate Ral during follicle morphogenesis in the *Drosophila* ovary. Indeed, vertebrate Ral can be activated by multiple Ras independent proteins but the presence of either of these Ras independent activation mechanisms has not been confirmed in *Drosophila* (de Bruyn et al., 2000; Rebhun et al., 2000; Wang et al., 1999; Park et al, 2001).

Evidence presented in this work suggests that Ral and DIAP1 are required for follicle morphogenesis. It remains to be tested whether the interaction of Ral and DIAP1 is required for establishment of cell polarity in other tissues as well. In the embryo Ral has been implicated in regulation of cell sheet movements as misexpression of dominant mutant Ral results in dorsal closure defects. It has been suggested that Ral acts during dorsal closure by modulating the JNK signalling pathway (Sawamoto et al., 1999). Interestingly, zygotic th^6 mutant embryos show a weak dorsal closure defect and DIAP1 has been implicated in regulation of JNK signalling and cell sheet movement as well (Wang et al., 1999; Kuranaga et al., 2002; Gagic, 2005). These findings support the hypothesis that Ral and DIAP1 might act downstream of an extracellular signalling pathway that regulates JNK signalling and/or cell sheet movements in *Drosophila*. One candidate upstream protein might therefore be the Dpp receptor Thick Veins (Tkv) that regulates epithelial movements in the *Drosophila* embryo (Affolter et al., 1994). Tkv is also expressed in the follicle epithelium and interestingly interaction of DIAP1 with Tkv has been reported (Mantrova et al., 1999; Oeda et al., 1998). It would certainly be

interesting to test the functional importance of the putative Ral/DIAP1/Tkv interaction for epithelial cell sheet movements in vivo.

The RING domain of DIAP1 is required for dosal closure during late embryo development as mentioned above. This suggests that the RING domain is not essential for earlier development of the *Drosophila* embryo. In contrast to this, the BIR domains of DIAP1 are clearly required for development as they are necessary for maintenance of epithelial integrity during gastrulation as shown in this work. The BIR domains of DIAP1 bind to caspases and block their activation. When the blocking activity of the BIR domains is overcome, caspases are activated and apoptosis takes place. Execution of apoptosis is accompanied by distinct changes in cell morphology and a hallmark of apoptosis is the loss of cell adhesion between apoptotic cells. This loss of cell adhesion can be explained by cleavage of the cell adhesion molecule Arm by caspases as described in this work. When Arm cannot be cleaved, cell adhesion in apoptotic cells is enhanced and morphogenesis of the *Drosophila* embryo is prolonged.

Thus, DIAP1 controls integrity and development of *Drosophila* epithelia by negatively regulating caspases and by regulating cell polarity via its E3 ubiquitin ligase domain.

3.7 Summary

Cells in epithelial sheets are highly polarized and adhere tightly to each other. Both cell polarity and adhesion are crucial for the function of the tissue. In *Drosophila* epithelia, the formation of a functional epithelium depends on the Crumbs (Crb) and the Bazooka (Baz) protein complex. Crb and Baz act in concert with other proteins to regulate establishment of cell polarity. Proper establishment of cell polarity in turn is necessary for assembly of cell adhesion proteins in the zonula adherens (ZA). This work defines the *Drosophila* inhibitor of apoptosis, DIAP1, as a novel player in the cellular network which controls apico-basal polarity of epithelial cells and integrity of the epithelium.

In the *Drosophila* embryo, DIAP1 inhibits the action of caspases by that preventing apoptotic cell death. Evidence is presented that in the absence of DIAP1, caspases become activated and enzymatically cleave off the N-terminus of the cell adhesion molecule Armadillo (Arm). Cleavage of Arm can be prevented by mutagenesis of the caspases target site both in vitro and in vivo. Expression of non-cleavable Arm is sufficient to maintain cell adhesion in apoptotic epithelial cells. The data presented in this work strongly suggests that Arm cleavage is a major event during down-regulation of cell adhesion at the onset of apoptosis.

In the *Drosophila* follicle epithelium, DIAP1 functionally interacts with both the Crb and the Baz protein complexes as shown in this work. The results suggest that DIAP1 is recruited to the apical plasma membrane by direct binding to the Crb complex. Absence of functional DIAP1 leads to accumulation of *D*aPKC and Baz in the cytoplasm and evidence is presented that defines DIAP1 as a negative regulator of *D*aPKC protein levels in vitro. Additional evidence shows that morphogenesis of the DIAP1 mutant follicle epithelium is severely affected and that a similar phenotype can be observed in follicle cells lacking Ral. Ral is a small Ras like GTPase that stabilizes DIAP1 protein levels as confirmed both in vitro and in vivo. Ral binds to DIAP1 in an activation dependent manner.

This study provides multiple lines of evidence for a previously undescribed function of DIAP1 in regulation of cell polarity and epithelial integrity.

4. Material and Methods

4.1. Materials

4.1.1 Chemicals

All chemicals were obtained in *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 destilled H_2O and autoclaved prior to use.

Enzymes required for molecular work were purchased from:

Boeringher/Roche Diagnostics Mannheim; *MBI Fermentas*, St. Leon- Rot, *Promega*, Madison, USA

4.1.2 General laboratory equipment

Electroporation: Gene Pulser II and Puls Controller Plus (*Bio Rad* Munich);UV Spectrophotometer: Gene Quant II (*Pharmacia Biotech*, Cambridge, UK); Sonificator: Labsonic U (*Braun Biotech*, Melsungen); SDS PAGE & Western Blotting : Miniprotean 3 (*Bio Rad*, Munich); Centrifuge (*Heraus* biofuge fresco and pico); PCR machine (MS Research MiniCycler); Micropipets puller: Sutter P-97 (*Science products*, Hofheim); Sonificator : Labsonic U (*Braun Biotech*, Melsungen) ; X ray film development: film: Fuji Super RX, Fuji, Tokyo, Japan, developer: Tenetal Roentogen, Tenetal, Norderstedt, fixation: Tenetal Roentogen Superfix, Tenetal, Norderstadt; Confocal microscope: Leica TCS NT, Leica, Heidelberg, and Zeiss 510Meta, Zeiss Jena; Light microscopy: Zeiss Axiophot2, Zeiss Oberkochen, Scanning electron microscope: Leo Supra, Leo Electrone Microscopy Ltd., Cambridge; Pictures were handeled with Adobe Photoshop CS, Text and calculations were done with Microsoft Office 2003 Students Edition

4.2 Methods

4.2.1 Molecular methods

If not otherwise stated, standard methods were used for molecular cloning, transformation of bacteria, heterologous expression in bacteria and purification of these proteins like described in Molecular Cloning, A Laboratory manual, 2nd edition; Smabrook, Fritsch and Maniatis; Cold Spring Harbour Laboratory Press. Similarly, antibody purification, SDS-Page and subsequent Western blotting was performed according to standard protocols. Whenever kit-systems were used, protocols were followed according to the manufacturer instruction.

4.2.2 Kit Systems

Qiagen plasmid Midi Kit, Qiagen, Hilden; PCR Purification Nucleobond gel extraction; TNT In vitro Transcription Translation System, Promega, Madison, USA; QuickChange Site directed muatagenesis Kit, Stratagene, La Jolla; Actin Spin down kit, Cytoskelleton Inc, Denver, USA; AminoLink Plus, Stratagene LaJolla;

4.2.3 Polymerase Chain Reaction PCR

For molecular cloning, DNA fragments were amplified by PCR according to Mullis and Faloona, 1987. Annealing temperature and amplification time of a standard protocol indicated below were adjusted according to the needs of the particular experiment.The reaction volume was set to 50ul in all reactions. A standard PCR protocol was used with 36 cycles of denaturation, annealing and elongation:

4.2.4 Cloning of PCR fragments into plasmids

Purified PCR products were digested by restriction endonuclease enzymes for subsequent cloning into vectors. Restriction sites were usually inserted by the PCR primer pairs. The pBluescript KS+/SK+ vector (Stratagene, Heidelberg, Amp^R) was used for amplification of cDNA and vectors of the pGEX series **(**Amersham Pharmacia

Biotech, Buckinghamshire, UK Amp^R) were used for bacterial expression of GST-fusion proteins. The pUAST-vector (Brand and Perrimon 1993; Amp^R) was used for generation of transgenic fly lines. The electrocompetent bacterial strain XL-1 Blue MRF was used for propagation of plasmids, while BL21(pLysS) bacteria (Stratagene, LaJolla, USA) were used for protein expression.

Mutated Arm cDNA (see below) was cloned in frame to a modified pUAST vector carrying an N-terminal FLAG epitope using Not1 and Xba1 introduced with the primer pair 5` AAGGAAAAAAGC-GGCCGCCACCATGAGTTACATGCCAGCCCAGAAT3` and 5` GCTCTAGACTAACA-ATCGGTATCGTACCAGG3`. RalG20V (see below) was cloned from pGex4t-Ral to the pUAST-HA vector using gateway technology and the following primers: 5`: CACCATGAGCAAGAAGCCGACAG; 3`: AAGTAGG-GTACACTTAAGTC

4.2.5 Site directed mutagenesis

Site directed mutagenesis is a simple PCR based method that allows exchange of defined Nucleotides within cloning vectors. Usually a single or double nucleotide exchange leads to changes in the amino acid sequence when the DNA is expressed. These changes are inserted into the original construct during PCR by using mismatch primer pairs. Thus, this method can be used to answer a variety of experimental questions. In this work several constructs were produced using the QuickChange site directed mutagenesis kit (Stratagene, LaJolla, USA)

$Armadillo^{D88A}$ was obtained by site directed mutagenesis of the Armadillo cDNA E91 in the pBluescript KS+ vector using the primer

ArmD88A: 5` CAAGACCAAGTGGCT-GATATGAACCAG and its reverse complement changing aspartate 88 to alanin.

The other mutated forms of Arm were generated using the primers

ArmD123A coding: CCACCCAGTTTGCGCCCCAACAGCCG;

ArmD170A coding: CAAGCTGCTGAACGCGGAGGATCAGGTGG;

ArmD172A coding: TGCTGAACGATGAGGCGCAGGTGGTAGTAG; and

ArmD755A coding: CTCTACCAATAGCGTCGATGCAGGGTCTG.

E91 and the mutated Arm cDNA´s were used for in vitro caspase cleavage experiments. All mutations were confirmed by sequencing. ArmD^{88}A was as well used for generation of transgenic flys carrying *UAS::Arm^{D88A}*. To obtain dominant active and dominant inactive versions of the small GTPases Ras, Rap and Ral (provided by J.Camonis) as well as Rac1 (provided by J.Grosshans), site directed mutagenesis was performed using the cDNAs cloned to pGex-plasmids and the following mutagenesis primers (sense primer only):

RapG12V:GTCCTTGGAAGCGTCGGCGTGGGCA
RapS17N:CGGCGTGGGCAAAAACGCGCTGACAGTCC
RacG12V:GTCGTGGGCGACGTAGCCGTGGGAAAG
RacT17N:GGAGCCGTGGGAAAGAATTGCCTGCTGATCAGC
RasS17N:GGAGGCGTGGGCAAGAATGCGCTCACCATCCAG
RasG12V:GTCGTTGGAGCCGTAGGCGTGGGCAAG
RalG20:VATGGTGGGCAGTGTCGGCGTGGGAAAG
RalS25N: GGCGGCGTGGGAAAGAATGCCCTCACACTGCAG

DIAP1 mutants were reconstituted using DIAP1 cDNA in the pGex vector and the following mutagenesis primers (sense only):

DIAP1 *th^{9}*/P55L:CACCGACTGGCTGCTAGATTGGCTG
DIAP1 *th^{SL}*/V85M: CTTTTTCTGCGGCATGGAAATCGGTTG
DIAP1 *th^{21-2s}* : P105S:GCGATGGTCGTCCAACTGTCCACTG

4.2.6 Proteins

4.2.6.1 Protein extraction from bacterial cells

The expression vector pGEX allows simple production of GST-fusion proteins in bacteria after addition of 1mM final concentration IPTG for 3-4 hours at 28°C. The fusion protein can then be purified after resuspending the bacteria in 1xPBS containing proteinase inhibitors in 1:500 dilution (aprotinin, pefabloc, pepstatin, leupeptin), sonification for 60 sec (80 Hz) and addition of 0.01% Triton X-100. The extract was incubated for 30min at room temperature and centrifuged 20min at 4°C to pellet bacterial debris. The supernatant was then used for further analysis.

4.2.6.2 Purification of GST-fusion proteins

GST-fusion proteins can be purified using Glutathione Sepharose 4A beads (Amersham). GST strongly interacts with its substrate Glutathione covalently coupled to the beads. Thus, short time (30-60min) incubation of bacterial extract with these beads and 4 subsequent washing steps for 10min are sufficient to purify GST fusion proteins from the bacterial crude extract. Binding of the GST fusion protein can be revealed by SDS PAGE electrophoresis and subsequent Comassie blue staining (BioRad, Hercules, USA) of the gel or by western blotting using anti GST antibody.
It is possible to elute the GST fusion protein from the beads by incubation with 10-10-100mM Glutathione in PBS pH8 for 2hours-over night at 4°C. As well, the fusion partner alone can be eluted by incubation with either Thrombin or TEV protease (Invitrogen, USA) when the respective protease recognition sequence was inserted during cloning. In this work GST fusion proteins of several small GTPases were purified and eluted by 100mM Glutathione. For further experiments, protein concentrations were adjusted to similar levels, according to a BSA protein standard in SDS-PAGE. DIAP1 was cleaved from the GST fusion partner by addition of 1-10µl TEV-protease in Buffer A (50mM HEPES pH 7.5, 100mM NaCL, 1mM EDTA, 0,1%CHAPS, 10%Sucrose, 5mM DTT; Wang et al., 1999) and incubation for 1h at room temperature or over night at 4°C. The supernatant was used for further analysis after incubation with 10µl Ni-NTA (Qiagen, Germany) matrix to remove the TEV protease from the solution. Total removal of GST-fusion protein from the solution was monitored by western blotting using a DIAP1 antibody.

4.2.6.3 GST-Pulldown

The GST-Pulldown method allows verification of direct protein protein interactions (Maniatis). One protein is expressed as a GST-fusion protein in bacteria and subsequently bound to Glutathion Sepharose 4A beads as described above. Then, a prospective binding partner is added that either contains a different tag or that can easily be detected by a specific antibody. Both proteins are incubated for 2h at room temperature or over night at 4°C in 500µl of binding buffer, the binding partner is removed and the beads are washed 4-5 times with binding buffer. Presence of the GST-

fusion protein as well as of the prospective binding partner is then verified by Western blotting using anti GST antibody. In this work, binding of DIAP1 to several small GTPases is shown. Therefore, the GTPases were bacterially expressed as GST-fusion proteins. 1-2µg GST-fusion protein was bound to Glutathione Sepharose 4A and washed for 3 times in PBT. Afterwards the beads were washed once with Exchange Buffer (20mM TrisCl pH 7,5, 10mM EDTA, 5mM $MgCl_2$, 1mM DTT) and the GTPases were loaded with 90uM GDP or GTP in exchange buffer for 1 hour at 4°C. Then, the loaded GTPases were incubated with 0.5µg of TEV-purified DIAP1 protein in Buffer UR over night on 4°C. Beads were washed 5 times with buffer UR and western blotting was performed using anti GST or anti DIAP1 antibody. To determine binding of DIAP1 to *D*PATJ, GST-DIAP1 fusion protein was incubated with embryo or ovary protein extract using RIPA protein extraction buffer. GST-DIAP1 was coupled to Glutathione-Sepharose-4B, incubated with extract over night on 4°, washed 5 times with RIPA buffer and 1 time with buffer A. DIAP1 was eluted from the Sepharose using 2µl TEV protease. The supernatant was then seperated by SDS-PAGE, transferred to nitrocellulose and presence of *D*PATJ, Baz, and DIAP1 was confirmed using specific antisera (4.2.7.4)

4.2.6.4 Proteinextraction from *Drosophila* tissue

To isolate proteins of *Drosophila* tissue, embryos were collected, dechorionated and extracted manually in one of the following buffers:
CHAPS immunoprecipitation buffer: 0,2% CHAPS, 10mM Dithiotreitol (DTT), 100 mM HEPES, 200mM NaCl; RIPA immunoprecipitation buffer: 150 mM NaCl, 1% NP40, 0.5% Deoxycholate, 0.1% SDS, 50 mM Tris [pH = 8.0], 0.4 mM EDTA; Ubiquitination assay extraction buffer/Buffer EX: 20mM TrisCL pH 7,5, 100mM NaCl, 5mM ATP2, 5mM $MgCl_2$, 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) and the proteasome inhibitor Lactacystin (stock 0.5µg/ul). Protein concentration of extracts was determined using Coomassie Blue-G250 (Roti-Quant, Carl Roth GmbH, Karlsruhe) according to the manufacturer, detecting absorbance coefficient at λ= 600 nm. Acquired value of absorbance was transformed into concentration of protein in solution (µg/µl) in

relation to a BSA protein standard. Presence of a protein of interest in the total protein extract was controlled by SDS PAGE (Laemmli, 1972) and subsequent western blotting.

4.2.6.5 In vitro ubiquitination

DIAP1 has been shown to be regulated by ubiquitination and subsequent degradation by the proteasome and DIAP1 is quickly becoming ubiquitinated in presence of protein extract from 2-5h old embryos containing His-tagged ubiquitin (Biomol, USA). In this assay, addition of proapoptotic proteins as GST or HIS fusion proteins enhances ubiquitination and degradation of DIAP1 and thus renders it more instable. The method was employed to test the influence of several small GTPases as purified GST fusion proteins on the stability of DIAP1. Therefore, 0.5 µg of TEV-purified DIAP1 protein was incubated with 10µg of protein extracts of 2-5h old wildtype flies made with Buffer EX in the presence of Lactacystin (Biomol, USA), protease inhibitors and 2µg His-Ubiquitin. For analysis of dose dependent effects of the small GTPases Ras, Rap and Ral, 0.5µg, 1ug, 2.5µg and 5µg purified GST fusion proteins were added and adjusted to a final volume of 15µl with buffer UR (25mM TrisCL pH 7,4, 0,5mM DTT, 2mM ATP, 5mM $MgCl_2$). For experiments with activated and inactivated mutants of small GTPases, 1µg of each fusion protein was used. Incubation lasted for 30min at 37°C and was stopped by addition of 6xSDS sample buffer. The reaction was separated by SDS-PAGE and ubiquitinated DIAP1 forms were visualized by Western blotting using the monoclonal anti DIAP1 antibody. Alternatively, the reaction was probed for ubiquitination of *D*aPKC, Baz, *Dm*PAR-6 or *D*PATJ

4.2.6.6 Specification of caspase cleavage sites

^{35}S-labeled Arm protein was in vitro translated using the T7/T3 rabbit reticulocyte lysate system (Promega, Madison) according to the manufacturers instructions using the Arm cDNA E91 or mutated Arm versions respectively (see site directed mutagenesis). For experiments with the recombinant proteins His-tagged versions of drICE, DCP1 and Decay were induced in BL21pLysS bacteria and purified under native conditions as described (Wang et al., 1999). Plasmids were a generous gift of Bruce Hay. 2µg of purified caspase was used for cleavage of Arm. For all experiments in vitro

translated Lamin DMo protein was used as a positive indicator for active caspases in the extracts and in the purified recombinant caspase fractions as cleavage of LaminDMo protein by caspases has been described before. The plasmid encoding *Drosophila* LaminDMo was a kind gift of (Fischer,PA).

4.2.7 Immunocytochemistry

Immunocytochemistry uses the specific binding of antibodies to their antigens to detect these proteins in fixed cells or precipitate them out of cell lysates. These methods are widely used in this work.and this chapter gives an overview about the protocols for fixation of cells and immunprecipitation.

4.2.7.1 Fixation of embryos

Fixation using the heat/methanol protocol

Embryos were dechorionated using 20% Na Hypochlorid and shortly washed with dH_2O. Embryos were transfered to scintillation tubes containing boiling Triton-X-Salt-Solution (3 ml Triton X-100, 40 g NaCl ad 1l) and immediately cooled down with ice-cold Triton-X-Salt-Solution on ice. Salt solution is discarded, replaced by Heptan and Methanol and the vitellin membrane of the embryos is removed by strong shaking. Embryos are transferred to reaction tubes and washed two times with methanol, while storage at -20°C is possible in methanol.

Fixation using Formaldehyde:

Embryos are dechorionated as described above. Embryos are transferred to scintillation tubes containing the fixative and Heptane and fixed for 25 minutes under agitation. The lower phase is removed, Methanol is added and vitellin membranes are removed by strong shaking. After washing with methanol embryos can be stored at -20°C. Formaldehyde containing fixatives: Stefanini solution:1,1 ml 37% Formaldehyd; 1,5 ml 500mM PIPES; 1,5 ml Picric acid; 5,9 ml dH2O; alternatively fixative solutions can be used containing 4% Formaldehyde, or 4-8% Para-Formaldehyde in PBS.

For subsequent detection of F-Actin by Fluorescently labelled Phalloidin derivates a 37% Formaldehyde fixation method was used. Vitellin membrane was removed using 80% Ethanol instead of Methanol.

Fixation of ovaries:

Ovaries of 2-10 day old females were dissected under PBS and subsequently fixed in 4% Formaldehyde for 20min. Formaldehyde was removed by washing 3x15min with PBS. Ovaries have to be permeabilized well to allow antibody access to the antigen throughout the entire structure. Thus, permeabilization was performed for minimum 6h in PBS containing 5% NHS and 5% Triton X-100 at room temperature or over night at 4°C.

4.2.7.2 Immunofluorescence

Indirect immunfluorescence was used almost exclusively for detection of proteins in fixed embryos. Ovaries were treated in analogous way. Therefore, fixed embryos were washed 3x20min with PBT, unspecific binding sites saturated by blocking with 10%NHS in PBT for 1h at room temperature and then incubated with primary antibodies in PBT/ 10% NHS over night at 4 ° C. Embryos were washed 4 times 15min with PBT and incubated with fluorescently labelled secondary antibody in PBT/10%NHS for 2h at room temperature. After 4 washings steps with PBT for 15min each, embryos were embedded in Moviol containing DABCO to prevent bleaching of fluorescence during confocal microscopy. In some cases the presence of apoptotic cells was subsequently monitored using the TUNEL method (Boehringer). Therefore, embryos were incubated 2 times 15min with PTX (1xPBS+ 0,3% TritonX-100), 1 time for 15min in CTX (100mM NaCitrat, 0,3% Triton-X100), 1 time 30min in CTX at 65°C, 2 times 15min in TUNEL dilution buffer at room temperature and 1 time 30min at 37°C in TUNEL labelling buffer. The TUNEL reaction was then performed in TUNEL labeling mix containing 1:10 TUNEL enzyme for minimum 2h at 37°C. Afterwards embryos were washed 3 times in PTX and mounted in Mowiol with very little DABCO (1,4-Diazabicyclo(2.2.2)octane).
Ovaries were always treated with 1%NHS instead of 10% and primary antibodies were incubated in presence of 0,05%Triton-X100 instead of Tween.

4.2.7.3 Immunoprecipitation

The immunprecipitation method is widely used to show interaction of a protein complex under native conditions. Therefore, proteins were extracted from tissue as described above, but the crude extract was incubated on ice for 20-30min before 10min centrifugation at 4°C. 30-40µg protein was removed as input control. 500-1000µg of total protein was then incubated with 30µl of Protein-A or Protein-G beads to remove proteins that bind to the beads. The supernatant was incubated with 1µg of antibody and 30µl of Protein-A or Protein-G beads for 2h up to over night at 4°C under agitation. Beads were washed 4-5 times with buffer and then boiled in presence of SDS-sample buffer. Presence of precipitated proteins was detected by subsequent SDS-PAGE and Western blotting.

4.2.7.4 Antibodies

The following primary antibodies were used for immunofluorescence: mouse-anti-DIAP1 1:100 (B.Hay); rat-anti-*D*E-Cadherin 1:20 (H.Oda); mouse-anti-Armadillo7A1 1:10 (DSHB); affinity purified rabbit-anti-Armadillo^{Central} 1:200 (this work); rat-anti-*D*_Cat 1:50 (H.Oda); rabbit-anti-active drICE 1:200 (B.Hay); rabbit-anti-*D*PATJ 1:1000 (E.Knust); rabbit-anti-Bazooka 1:1000 (A.Wodarz); guinea pig-anti *Dm*PAR-6 1:1000 (A.Wodarz); rabbit-anti-Lgl 1:100 (J.Knoblich); mouse-anti-engrailed 1:10 (DSHB); mouse-anti-Neurotactin 1:10 (DSHB); rabbit-anti-nPKC_ C20 (SantaCruz Biotechnologies); mouse anti-Tubulin 1:500. F-Actin was stained using FITC-Phalloidin 1:100.

The following primary antibodies were used for Western-blotting: mouse-anti-DIAP1 1:1000; rabbit-anti-DIAP1 1:7000 (P.Meier); rat-anti-*D*E-Cadherin 1:10; mouse-anti-Armadillo7A1 1:10; affinity purified rabbit-anti-Armadillo^{Central} 1:2000; rabbit-anti-Bazooka 1:1000; guinea pig-anti *Dm*PAR-6 1:1000; rabbit-anti-nPKC_ C20; rat-anti-Ral 1:200 (this work); rabbit-anti-*D*PATJ 1:2000; mouse-anti-hRalA 1:10 (BDBiotech); rabbit-anti-Actin 1:2000 (Sigma);

The following primary antibodies were used for immunoprecipitation: mouse-anti-Armadillo7A1 1:50; affinity purified rabbit-anti-Armadillo^{Central} 1:500; mouse-anti-DIAP1 1:50; rabbit-anti-*D*PATJ 1:500.

Secondary antibodies were used as Cy2 or Cy3 conjugates with a dilution of 1:250 (Jackson ImmunoRes) and 1:250 as Alexa 647 conjugates (MolecularProbes) or 1:10000 as HRP conjugates (Jackson ImmunoRes). Secondary goat anti-mouse antibody coupled to Alcaline Phosphatase (AP) was used 1:800.

4.2.8 Genetic Methods

4.2.8.1 The Gal 4/*UAS* system

The Gal4/*UAS* system allows conditional expression of transgenes in *Drosophila* (Brand and Perimmon, 1993). The method uses the yeast transcription factor Gal4 under control of gene specific enhancers as activators and P-element insertions carrying upstream activated sequence (*UAS*) 5` of the gene of interest as effectors.
The crossing of activator and effector line allows binding of Gal4 to the *UAS* sequences upstream of the gene of interest to drive its expression.
The transgenic fly stock that allows conditional expression of Arm^{D88A} was established by the author using standard methods injecting the respective pUAST-Arm^{D88A} construct into w^{1118} embryos. Subsequently, flies carrying an insertion of this transgene on the second chromosome were crossed to DIAP1 mutant background obtaining w; *UAS*::Arm^{D88A}; th^{109}/*TM3* (*ftz::lacz*). Additionally, a third chromosomal insertion of pUAST-Arm^{D88A} and a third chromosomal insertion of *UAS::*$Arm^{wildtype}$ (BL8370) was recombined to the th^{109} chromosome obtaining w;th^{109},*UAS*::Arm^{D88A} / *TM3* (*ftz::lacz*) and w; th^{109},*UAS*::Arm^{wt} / *TM3* (*ftz::lacz*) respectively. Crossing of the transgene lines to w; th^{109}, *mat15*::Gal4 / *TM3* (*ftz::lacz*) allowed expression in the embryo. *UAS::*Arm^{D88A} should be expressed in 100% of the th^{109} homozygous embryos.

4.2.8.2 The Flp/FRT system

Xu and Rubin developed the Flp/FRT system that allows induction of mitotic recombination with a high frequency in many *Drosophila* tissues (Xu and Rubin, 1993). Therefore, the yeast enzyme Flipase is expressed by a transgene after heat shock. It binds to Flipase recombinase target (FRT) sequences and mediates mitotic recombination between two homologues chromatides carrying FRT sequences. Wildtype and mutant alleles of a gene of interest are exchanged by that. Thus, the

daughter cells obtain either two wildtype or two mutant alleles of this gene. Both daughter cells grow and give rise to a clone of cells. The wildtype chromosome is usually marked by expression of a cell autonomous marker like GFP and the mutant cells lack GFP.

For this work the following FRT chromosomes were used:

sdt^{XP96}FRT18D/FM7, baz /FM7 and *sdt^{XN05},baz^{YD97}FRT18D/FM7* were crossed to *AN365FRT18D/Y*; *hs::FLP*; AN365 is an enhancer trap insertion that allows ubiquitous expression of ß-Galactosidase.

ral^{89}FRT101/FM7 and *ral^{70}FRT101/FM7* were crossed to *ubi^{GFP}FRT101/Y*; *hs::Flp* for induction of follicle cell clones. The hypomorph allele *ral^{89}FRT101/FM7* was crossed to males carrying the dominant female sterile mutation *ovoD FRT101*; *hs::Flp* for production of germline clones.

w*;th^{109} FRT2A/TM3 ftz::lacZ* or w; *th^{6}FRT2A/TM3* were crossed to *yw hs::Flp; ubi^{GFP}FRT2A*. w; *th^{6}FRT2A* was obtained by recombining the *th^{6}* mutation affecting the RING domain of DIAP1 (Wang et al., 1999) to the FRT2A chromosome (BL-1997).

w;*rap1^{B1}FRT2A* and w;ras$^{\Delta C40}$*FRT2A* were obtained from H.A.J. Müller. *yw;rac1^{J11},rac2$^{\Delta}$FRT2A,mtl$^{\Delta}$*/TM3 and *yw*; *FRT82*B*mtl$^{\Delta}$/TM3* recombinants were obtained from Bloomington stock center (BL6678, and 6676). The mutant chromosmes were crossed to males carrying a heat shock inducible Flp transgene on the 1st or 2nd chromosome and FRT2A or FRT82B respectively.

Fly Stocks	**Origin**
w^{1118}	Lindsley and Zimm, 1992
sdt^{XP96}FRT18D/FM7	Müller and Wieschaus, 1996
baz^{Xi106} /FM7	Müller and Wieschaus, 1996
sdt^{XN05},baz^{YD97}FRT18D/FM7	Müller and Wieschaus, 1996
AN365FRT18D/Y; hs::FLP	Müller and Wieschaus, 1996
ral^{89}FRT101/FM7	This work and Gagic, 2005
ral^{70}FRT101/FM7	This work and Gagic, 2005
ubi^{GFP}FRT101/Y; hs::Flp	This work and Gagic, 2005
Fs(1) ovo^{D1} FRT101; hs::Flp	
w/w;th^{109} FRT2A/TM3 ftz::lacZ	Gagic, 2005
w/w; th^{6}FRT2A/TM3	This work

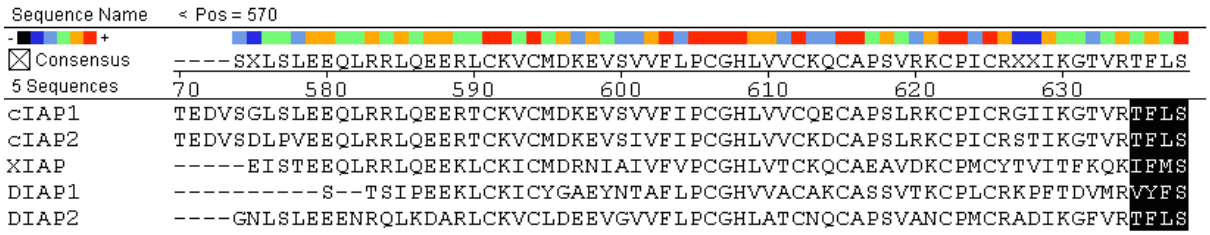
yw hs::Flp; ubi^{GFP}FRT2A	This work
w;rap1^{B1}FRT2A	Aariharan et al., 1991
w;FRT82B ras$^{\Delta C40}$	Schnorr and Berg, 1996
yw;rac1^{J11},rac2$^{\Delta}$FRT2A,mtl$^{\Delta}$/TM3	Hakeda-Suzuki et al., 2002
yw; FRT82B mtl$^{\Delta}$/TM3	Hakeda-Suzuki et al., 2002
yw hs::Flp; FRT82B ubi^{GFP}	This work
w; UAS::Arm^{D88A}/ UAS::Arm^{D88A}	This work
w; UAS::Arm^{D88A}/ UAS::Arm^{D88A}; th^{109}/TM3 (ftz::lacz).	This work
w;th^{109},UAS::Arm^{D88A} / TM3 (ftz::lacz)	This work
w; th^{109},UAS::Arm^{wt} / TM3 (ftz::lacz)	This work
w; th^{109}, mat15::Gal4 / TM3 (ftz::lacz)	This work

Table 1: Fly stocks used in this work

4.2.9 Preparation of embryos for scanning electron microscopy

To see alterations in the surface integrity of w/w;; *th^{109}/ th^{109}* embryos to embryos expressing *UAS::Arm^{D88A}* in *th^{109}* mutant background, embryos were collected for 2h, aged for 4h and then fixed in 4%PFA for 25min. Homozygous and heterozygous embryos were manually selected after antibody staining with anti-ßGal antibody and subsequent color reaction using secondary antibodies coupled to alkaline phosphatase (AP) or horse readish peroxidase (HRP) 1:800. Embryos were then incubated in 2%OsO4/4%Glutaraldehyde in PBS for 30min on ice, washed in PBS and incubated in 2%OsO4 for 1h on ice. Embryos were washed in PBS and dehydrated by a graded ethanol series of 30 %, 50%, 70 %, 96% and absolute alcohol. Embryos were washed two times in dry acetone and incubated for 30min in a 1:2 mixture and 30min in 1:1 mixture of TMS (tetramethylsilane; Sigma) and acetone and. Pure TMS was added for 30min, renewed and left to dry overnight. Probes were mounted on double stick tape, sputtered with gold and used for scanning EM using the scanning electron microscope.

5. Supplementary Figures



Supplementary Figure 1: Alignment of the IAP proteins

The protein sequence of cIAP1, cIAP2, X-linked IAP (XIAP), DIAP1 and DIAP2 was aligned using the ClustalW software integrated in the MegAlign software; (Fig.1) The C-termini of all tested IAPs are highly similar; the C-terminal most four amino acids share the consensus sequence TFLS.

6. Citation Index

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7. Abbreviations

A	Adenine
aa	Amino Acids
Ab	Antibody
Amp	Ampicillin
Arm	Armadillo
Baz	Bazooka
Bp	base pares
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C	Cytosine
°C	Celsius
Crb	Crumbs
DABCO	1,4-Diazabicyclo(2.2.2)octane
*D*αCat	*Drosophila* α-Catenin
*D*aPKC	*Drposophila* atypical protein kinase C
*D*E-Cad	*Drosophila* E-Cadherin
*Dm*PAR-6	*Drosophila melanogaster* PAR-6
Dlg	Discs large
DMSO	Dimethylsulfoxid
*D*N-Cad	*Drosophila* N-Cadherin
*D*PATJ	*Drosophila* Protein associated with tight junctions
DTT	Dithiothreitol
dNTP	Deoxyribonucleotide phosphates
dsRNA	Double stranded RNA
eg	lat exempli gratia,. For example
en	engrailed
EtOH	Ethanol
Fig.	Figure
G	Guanine
g	gramm
HRP	Horse Radish Peroxidase
kDa	kilo Dalton
LB-Medium	Luria Bertani Broth-Medium
Lgl	lethal (2) giant larvae
M	Mole
m	Milli 10^{-3}
μ	Micro 10^{-6}
Min	Minute

mRNA	Messenger RNA
n	Nano 10^{-9}
OD	Optical density
ON	Over night
PCR	Polymerase chain reaction
PDZ	PostSynapticDensity95-/discs large/zonula occludens
rpm	Rounds per minute
RT	Room temperature
Scrib	Scribbled
Sdt	Stardust
T	Thymine
Taq	Termophilus aquatiqus
TJ	tight junction
U	Unit
UAS	upstream activating sequence
UV	Ultra violet
V	Volume
ZA	Zonula Adherens

Danksagung

Diese Dissertation wurde im Institut für Genetik der Heinrich-Heine Universität Düsseldorf unter der Anleitung von Herrn PD Dr. H. Arno J. Müller angefertigt. Ihm möchte ich für den Freiraum im wissenschaftlichen Arbeiten, die stetige Unterstützung sowie die Überlassung des Themas danken.

Ebenfalls recht herzlich bedanken möchte ich mich bei Frau Prof. Dr. Elisabeth Knust für die Übernahmen des Gutachtens sowie die fortwährende Mitbetreuung und Diskussionsbereitschaft.

Ich möchte mich bei allen Mitarbeitern der AG Müller für die nette Atmosphäre, die vielen Diskussionen und den Zusammenhalt bedanken. Im Besonderen gilt mein Dank Wibke Meyer, Sirin Otte, Annika Raupbach, Thorsten Volkmann, Andreas van Impel und Anna Klingseisen die immer für einen da sind wenn man sie braucht.

Allen Mitarbeitern der AG Knust im Institut für Genetik gilt mein herzlichster Dank für die jahrelange Unterstützung und die tolle Arbeitsatmosphäre. Ins besondere möchte ich dafür Ferdi Grawe danken, der nicht nur wissenschaftlich sondern auch persönlich immer ein hervorragender Ansprechpartner gewesen ist. Dank gilt auch Andre Bachmann, der immer ein offenes aber kritisches Ohr für meine „Blottgeschichten“ hatte und der diesen Dank diesmal nicht mit einem „jo, schon gut, war ja gar nix“ abtun kann. Viel Dank geht auch an Özlem Kempkens, Susann Özüyaman und Melisande Richard.

Ebenfalls danken möchte ich Mic, Manuel und Woody für Fußball+Bierchen, Michael, Diane und Nannette für die Unterstützung in allen Lebenslagen sowie Herrn M. Hoffmann, Soya und Walter für nette Gespräche. Ich danke Christian Büchter für die technische Unterstützung.

Meinen Eltern und meiner Schwester gebührt großer Dank dass sie dies alles mit mir zusammen durchgestanden und mich zu jeder Zeit ohne Frage unterstützt haben.

Meiner Frau Mirjana danke ich für all die Liebe und Geduld die sie mir in den letzten Jahren hat zukommen lassen und sehr viel mehr.

Erklärung:

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Keßler Düsseldorf, April 2006