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Functional dissection of the Rho guanine nucleotide exchange factor Pebble in *Drosophila* mesoderm morphogenesis

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1 Introduction

The ability of cells to become motile and to change their position is of fundamental importance during the lifecycle of multicellular organisms. During development a group of similar cells is transformed into a variety of cell types forming the different tissues of the embryo. Many aspects of this process called morphogenesis depend on cell motility, which allows cells to take over certain positions within the developing organism. Furthermore, animals would not be able to reproduce or defend themselves against pathogens without cell migration and wounds would not heal after tissue injuries. Beside these essential functions, the deregulation of cell migration is an important feature of various diseases including tumor formation and metastasis or neurological and vasculature defects. Therefore, revealing the general principles underling these different processes is not only important to understand how developmental processes occur but is also necessary to gain insight into the molecular basis of diverse diseases in order to find starting points for the generation of drugs and therapies.

1.1 Cell motility

In multicellular organisms cell migration often originates from a group of cells that are organized in an epithelium. In order to become motile, the cells have to leave their epithelial surrounding which requires fundamental alterations of their adhesive properties. Furthermore, the cytoskeleton of the cell, including microtubule and actin network, has to be reorganized to allow greater plasticity during the migratory process. These and other prerequisites of cell motility are obtained during the central process called epithelial-mesenchymal transition (EMT; see below). Once motile, the cells have to receive signals and to communicate with their neighbours to find the correct direction and to halt at their final destination. All aspects of migration have to be tightly regulated to guarantee the integrity of epithelial cell sheets and to allow the directional movement of only a subset of cells to specific positions where they form tissues with distinct functions in the developing organism. Multiple signalling events govern the different stages of cell motility to provide best possible control mechanisms for this complex cell behaviour. Misregulation or a failure in these regulatory inputs often results in abnormal cell behaviour that could lead to generation of a tumor, for example.

As already mentioned, alteration of cell adhesion and cell shape are important features of cell motility. Two different types of cell adhesion are important for cell migration: cell-cell and

cell-substrate adhesion. Typically, cell-cell adhesion is mediated by the group of Cadherins which are Ca^{2+} dependent transmembrane adhesion molecules. This protein family can be found in both, vertebrates and invertebrates, and comprises so-called classical and non-classical Cadherins. The best studied adhesion molecule is E-cadherin, a member of the group of classical Cadherins. E-cadherin is expressed in epithelial tissue and it is a major component of the zonula adherens. The transmembrane protein forms dimers that mediate homophilic interactions with E-cadherin molecules of neighbouring cells. In the classical textbook model of cell adhesion, the Cadherins are directly connected with the actin cytoskeleton of the cells by different adaptor molecules of the Catenin family. Via these interactions, the cytoskeleton of cells in an epithelial tissue is linked to each other providing a strong mechanical connection within the epithelium (Takeichi et al., 2000; Tepass, 1999; Tepass et al., 2000). However, more recent studies indicate that the interaction between the Cadherin/Catenin-complex and the actin cytoskeleton is not based on a direct binding and might involve a more complex mechanism (Drees et al., 2005; Yamada et al., 2005).

Cell-substrate or cell-matrix interactions are very important for migratory processes as they mediate the adhesion between moving cells and their substrate. These interactions are normally weaker and persist shorter than the cell-cell adhesion interactions. Generally, this adhesion type depends on the transmembrane receptor molecule Integrin. Integrins are heterodimeric adhesion molecules consisting of an α- and a β-subunit (Gumbiner, 1996; Hynes, 2002). They are able to bind to different components of the extracellular matrix like Fibronectin for example but also to cytoskeletal proteins via the intracellular domain of the β-subunit and a variety of different anchor/linker proteins (Alberts et al., 2004). Thereby, integrins provide anchorage sites for actin filaments that drive the motion of the cells. The connection of the intracellular and the extracellular scaffold is an important function and can be regulated by the cell through modifications of the Integrin receptors that change their activity (inside-out-signalling) (Calderwood, 2004; Wegener and Campbell, 2008). Importantly, Integrins can also transmit signals in the opposite direction. Binding of extracellular ligands can induce different signal transduction pathways including the MAP kinase (Mitogen-activated protein kinase; MAPK) cascade in the moving cell that result in the expression of different genes or the inhibition of apoptosis (Boudreau and Jones, 1999).

If epithelial cells become motile, they have to undergo a process called EMT. During this event the cells lose their epithelial characteristics like their typical apicobasal cell polarity, which allows them to leave the epithelial tissue. Furthermore, the cell-cell contact sites with

the surrounding tissue are downregulated by the disassembly of their adhesive structures including adherens junction, desmosomes and gap or tight junctions (Thiery and Sleeman, 2006). The cytoskeleton of the cell needs to be reorganized to acquire a mesenchymal morphology, which can be either amoeboid or polarized in an anterior-posterior orientation (leading edge – rear end of the cells). Once motile, the cells typically interact primarily with the extracellular matrix (cell-substrate adhesion) on which they migrate in order to receive signals from the surrounding tissue. If cells move as a group of cells and not as single cells, they will be in a steady contact with each other which can be mediated by the Cadherin-Catenin system (Bryant and Mostov, 2008; Krull, 2001).

A key feature of EMT and cell movement is the reorganization of the cytoskeleton of the cell. In general, three different filament types of the cytoskeleton are distinguished. The first type is the microtubule (MT) network that is required for the organisation within the cell (the position of the different organelles) and which is also a key component that helps to keep a certain cell polarity by mediating site-directed transport of different vesicles or proteins. The second filament type is the intermediate filament system, which mainly provides mechanical stability. The third system is the actin cytoskeleton of the cell that is, together with a large set of actin interacting proteins, required for contractions, movements and shape changes of the cell. Actin filaments can be bundled in different ways depending on the cross-linking proteins that are predominantly localized to the filaments (Alberts et al., 2004). In migrating cells actin filaments generate several different protrusions of the cell surface. They can induce filopodia, which are thin finger-like protrusions of the plasma membrane that contain tight bundles of parallel actin filaments. Filopodia are normally used as antennae to probe the close environment of the cells for directional cues (Mattila and Lappalainen, 2008). A bigger, very thin extension of the membrane at the leading edge of a cell is called lamellipodium and represents the advancing site of the cell during movement. It contains a dense actin-meshwork of cross-linked filaments (Alberts et al., 2004).

Cell migration can generally be subdivided into three repetitive steps. During the first phase of migration, the cell forms filopodia- or lamellipodia-like protrusions, thereby extending into the direction of migration. These protrusions are generated by the ongoing elongation and branching of the actin filaments, which is thought to be the driving force of the movement. During the second phase, the cell forms new transient adhesion sites between the extended leading edge and the substrate, mostly via Integrins. These adhesion sites are subsequently connected to the actin cytoskeleton and become focal adhesion sites, which can exert mechanical force upon its surroundings by the myosin mediated contraction of the actin

cytoskeleton. During the third step, adhesion sites are disassembled at their rear end and the contraction of actin-myosin bundles at the back of the cells pulls this part forward (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996).

Beside this fibroblast- or keratinocyte-like migration, amoebae and neutrophils exhibit the amoeboid type of movement that involves the same three basic migration steps. Amoebas extend so-called pseudopodia, three dimensional protrusions of moderate width that attach to the substratum. Subsequently the cytoplasm flows forward into the pseudopodium and the rear end of the cell detaches and is pulled forward. This migration cycle is accompanied by dynamic changes in the viscosity of the cytoplasm that depends on differences in actin polymerisation and cross-linking of the actin filaments. The inner part of the migrating cell is filled with a more fluid cytoplasm (endoplasm) that rapidly flows into the extended pseudopodium. In contrast, the cytoplasm at the cortex is more viscous (ectoplasm) and hence it does not flow as easily. When the rear of the cell is pulled forward, the ectoplasm is transformed into endoplasm again, which facilitates its transport to the front of the cell where it is converted into ectoplasm again (Janson and Taylor, 1993; Taylor and Fechheimer, 1982).

As mentioned before, motile cells also exhibit polarity. This results in the formation of a front or leading edge side and a rear or retracting side. Such a polarization is necessary to migrate in one direction as it prevents the formation of protrusions in all directions at a time, which would result in an inefficient, random walk. Therefore, directional movement of cells involves signalling pathways that define the front/back polarity of the migrating cells. In chemotactic movements an extracellular gradient of soluble signalling molecules governs the direction of migration by activating cell surface receptors of the cell. The gradient of the external cue is translated into a polarization of the cell and a subsequent movement towards or away from the source of the signal (Ridley et al., 2003).

One of the best analyzed model systems for directed cell migration is the social amoebae *Dictyostelium discoideum*. These slime molds live as single cells until starvation triggers a signalling event that leads to the aggregation of approximately 100.000 amoebas, which subsequently form a multicellular fruiting body (Chisholm and Firtel, 2004; Garcia and Parent, 2008). Aggregation of the cells was shown to be mediated by a signal of cyclic AMP (cAMP). This chemotactic signal is emitted in periodical pulses by founder cells. The cAMP waves are detected by nearby cells via a cell surface receptor molecule (Dormann and Weijer, 2006; Weijer, 2004). Binding of cAMP to its receptor triggers activation of phosphatidylinositol 3-kinase (PI3K) at the forming leading edge and results in the localized

production of phosphatidylinositol(3,4,5)-triphosphate (PtdIns(3,4,5)P_3) and phosphatidylinositol(3,4)-bisphosphate (PtdIns(3,4)P_2). The PI3K pathway antagonizing phosphatase PTEN is active all around the cortex in non-stimulated cells, but it is absent from the leading edge in stimulated cells. This facilitates the establishment of a steep front/back polarity (Charest and Firtel, 2006; Willard and Devreotes, 2006). The high concentration of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 at the leading edge results in a recruitment of proteins to the front of the cell that can bind to these lipids via specialized protein domains called Pleckstrin homology (PH) domains. One of these downstream effectors of PI3K is the protein kinase B / Akt (PKB), which negatively regulates the assembly of myosin-II that is required for the lateral suppression of pseudopodia and the retraction of the rear of the cell. F-actin polymerization and pseudopodium propulsion at the leading edge is presumably directly regulated by other PH domain containing proteins including PhdA and different guanine nucleotide exchange factors (GEFs). The latter ones control the activation of central regulators of the actin cytoskeleton that belong to the Rho family of small GTPases (Rac GTPases; see below). By this signal transduction machinery, the extracellular gradient of the chemoattractant is translated into an internal polarity that results in actin polymerization and pseudopodium formation at the leading edge and myosinII accumulation and contraction at the rear end of the cell (Charest and Firtel, 2006; Chisholm and Firtel, 2004). As a consequence, the different *Dictyostelium* cells migrate towards the source of the cAMP signal where they subsequently aggregate and form the fruiting body (Weijer, 2004).

Another example for directed cell movement is the migration of the neural crest cells in the chicken embryo. In contrast to the previous example, neural crest cells are not guided by a gradient of a soluble chemoattractant but by other extracellular cues that are deposited in the extracellular matrix. The neural crest cells originate from the neural plate border, called neural folds. After neurulation they undergo EMT, delaminate from the neural tube and start to migrate along two major paths. Cells that take the dorsolateral pathway become melanocytes and migrate between the epidermis and the dermis while cells that take the ventral pathway will mainly form sensory and sympathetic neurons. These cells move through the sclerotome (region of the somites that forms the skeleton). Importantly, the cells only move through the anterior half of the sclerotome and do not enter the posterior half (Graham, 2003; Krull, 2001). The migration routes of the cells are mainly controlled by cues that are deposited in the extracellular space of the surrounding tissue. Some of these proteins like fibronectin, laminin or thrombospondin serve as general promoters of migration. Detection of these molecules via the integrin receptors of neural crest cells induces signalling events that promote their

migration. As thrombospondin, for example, is only expressed in the anterior part of the sclerotome, the cells prefer to migrate over the path "labelled" by this positive substrate. To avoid a migration into neighbouring tissue, receptors of the Eph family of receptor tyrosine kinases are employed. These transmembrane proteins are expressed by the migrating neural crest cells while their ligands, the ephrin proteins, are expressed in the posterior half of the sclerotome, for example. Ephrins trigger signalling events that interfere with the actin cytoskeleton of the migrating cells and therefore they impede migration. Hence the cells try to avoid regions that express this repellent and move only over the "highways" coated with the positive migration cues. These and further mechanisms ensure the correct distribution and segmental pattern of the neural crest cell derivatives (Krull, 2001; Perris and Perissinotto, 2000).

Taken together, in every example of directed cell migration the signalling events are always transmitted to the cytoskeleton of the cells and result in dramatic rearrangements of the actin filament network. Therefore, the control of the actin cytoskeleton is a key feature of cell migration and has to be tightly regulated during all phases of cell movement.

1.2 The Rho GTPase family of small G proteins

The previous chapter indicated that cell shape changes and cell motility require extensive and also dynamic reorganizations of the actin cytoskeleton. Beside migration various other cellular processes, like cell division for example, depend on complex changes in the F-actin network. Therefore, it is of utmost importance that cells are constantly capable of restructuring their cytoskeleton.

One group of proteins that are central regulators of pathways that impact on the actin cytoskeleton are Rho GTPases. These proteins belong to the Ras-superfamily of small (monomeric) G proteins. They are known to act as so-called molecular switches that mediate various effects in response to signalling events. In general, GTPases exist in two different forms, in an inactive state where they are bound to GDP and in an active, GTP bound form. The switch between both states is regulated by different proteins. Guanine nucleotide exchange factors (GEFs) are able to bind the inactive, GDP-loaded forms of the GTPases. Upon binding, they catalyze the release of the bound GDP by stabilizing the nucleotide free form of the GTPase. GTP exists in much higher intracellular concentrations than GDP. Therefore, the GTPase will eventually bind a GTP molecule leading to its activation and the dissociation of the GEF protein. The activated GTPase molecule in turn binds to a variety of

different downstream effectors (Fig. 1.1 step 1). This binding results in conformational changes and activation of the effector molecules which subsequently leads to alterations of the actin cytoskeleton (see below). Inactivation of Rho GTPases is mediated by a second group of regulatory proteins. Although Rho GTPases exhibit a very low intrinsic GTPase activity, the hydrolysis of the bound GTP would take much longer than the activity of the GTPase is required. Therefore so-called GTPase-activating proteins (GAPs) are used to turn the GTPase signalling off again in order to avoid negative effects for the cell. GAPs bind to active GTPases and enhance the low intrinsic GTPase activity of the protein leading to an efficient hydrolysis of the GTP and thereby shifting the GTPase into its inactive state again (Fig. 1.1 step 2) (Symons and Settleman, 2000; Takai et al., 2001; van Aelst and D'Souza-Schorey, 1997).

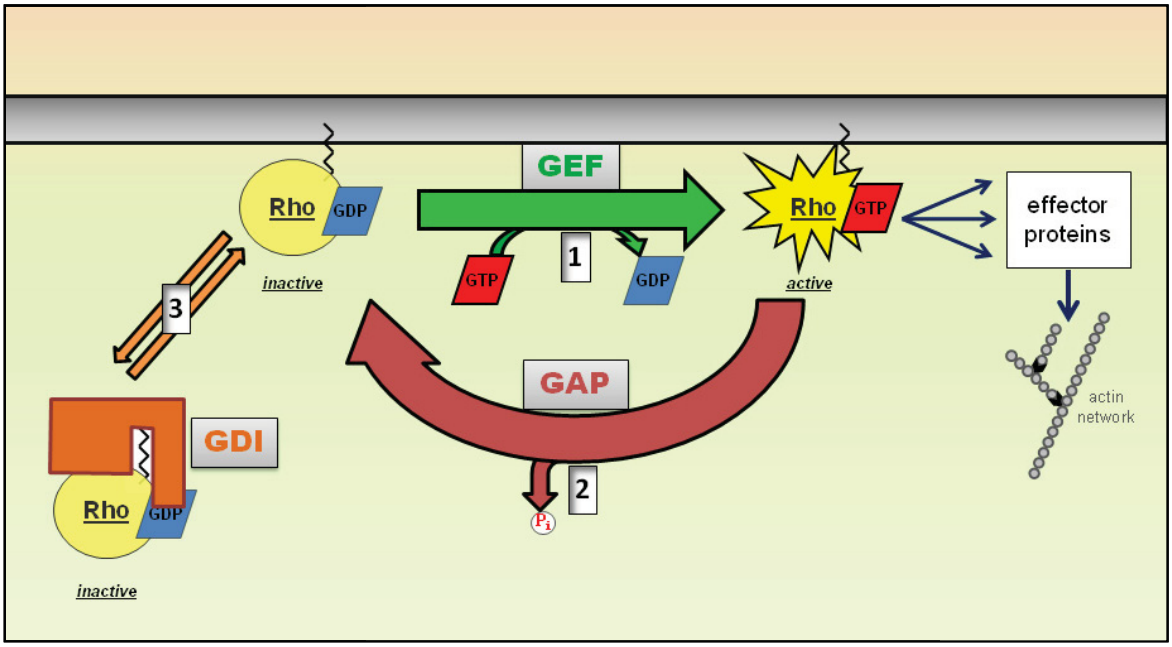

Fig. 1.1: Activation and regulation cycle of Rho GTPases.
1) Inactive Rho GTPases can be activated by GEFs, which catalyze the exchange of the bound GDP with GTP. The activated GTPase interacts with its downstream targets leading primarily to effects on the actin cytoskeleton. 2) GAP proteins enhance the intrinsic GTPase activity of the G protein resulting in its inactivation. 3) GDIs are able to bind Rho GTPases, which prevents their interaction with other regulatory proteins. By masking the membrane anchor of the GTPase they further block the membrane assoziation of the protein.

To avoid that certain GTPases can directly undergo another round of activation, a third group of regulatory proteins exists that interacts with some but not all of the different Rho GTPases. The GDP-dissociation-inhibitors (GDIs) bind and sequester inactive GTPases in the cytoplasm (Fig. 1.1 step 3). They block the exchange of the bound GDP thereby ensuring that the GTPase cannot be activated. Rho GTPases are often posttranslationally modified at their

C-terminal region by prenyl- or geranylgeranylation, in some cases also by palmitoylation. These modifications facilitate the membrane association of the G proteins and often define their localization pattern to distinct membrane compartments. As these membrane anchors are also covered by the GDIs, they further ensure that the inactive proteins are cytosolic. Furthermore, Rho GDIs sometimes target Rho GTPases to specific membrane domains or protein complexes (Ridley, 2006; Takai et al., 2001). In a few cases GDIs are also capable of binding the GTP bound form of Rho proteins, which inhibits the hydrolysis of the GTP and the inactivation of the GTPase (Hart et al., 1992).

Another way how localization and activity of some small GTPases can be regulated is via phosphorylation. In one case phosphorylation of the C-terminus was shown to prevent membrane association and to enhance the binding affinity of the GTPase for a Rho GDI (Ridley, 2006).

The three "classical" Rho GTPases that have been characterized best, are called Cdc42, Rac and RhoA (Rho1 in *Drosophila*) (Heasman and Ridley, 2008). Initial microinjection experiments in mammalian tissue culture cells using constitutive active variants of these proteins helped to reveal the distinct "standard" effects of the three main Rho GTPases. These variants are not capable of hydrolysing the bound GTP anymore; therefore they remain in the active state. Microinjection of active Cdc42 in fibroblasts was found to induce polymerisation and bundling of F-actin resulting in the extension of filopodia and microspikes (Kozma et al., 1995). Activated Rac, in contrast, triggers signalling events that result in actin polymerisation in a wider area of the cell cortex. This leads to the formation of bigger protrusion, the lamellipodia (Ridley et al., 1992). Expression of RhoA in fibroblasts induces bundling of F-actin and myosin-II filaments to contractile stress fibres on the one hand. On the other hand, RhoA was further shown to promote the rapid formation of new focal adhesion sites (Ridley and Hall, 1992).

Typically, Rho GTPases are activated by locally restricted receptor mediated signalling events, which control the exchange activity and/or localization of GEF proteins via activation of different downstream pathways (e.g. PI3K pathway) or sometimes via direct interactions between the GEF and the activated receptor. The GEF molecules in turn activate their substrate GTPases at specific intracellular sites. In general, two different effectors are employed that directly stimulate actin polymerization, the WASP/WAVE proteins (via the Arp2/3 complex) and Diaphanous-related proteins of the formin family (Ridley, 2006). However, Rho GTPases are able to interact with various other downstream effectors and therefore may respond differentially upon various signals. Furthermore, the fact that a broad

range of signalling pathways converge on the level of Rho GTPases does not only demonstrate the central role of these molecular switches but it also implies that they control processes that go beyond cell shape changes and cell motility: For example, several different Rho GTPases are involved in cell division. RhoA plays a crutial role in cytokinesis. After the chromosomal material is separated, RhoA is activated in a cortical ring in the middle of the newly forming cells. Via its downstream effector Diaphanous (Dia), RhoA controls actin nucleation and polymerisation which results in the formation of the actomyosin ring. Activation of Rho-kinase (ROCK) and Citron-kinase results in myosin activation and the contraction and ingression of the cytokinesis furrow (Piekny et al., 2005). In contrast, Rac activity needs to be repressed during furrow ingression. Genetic evidence suggests that Rac activity antagonizes Rho signalling through inhibiting Citron-kinase activity. Furthermore, it was suggested that inactivation of Rac at the site where the furrow is formed might lead to a decrease in cortical stiffness, which could in turn facilitate furrow ingression (D'Avino et al., 2005). In addition, a role for Cdc42 during mitosis was proposed for a few model systems. In this context Cdc42 activity seems to be required during metaphase. Through activating a specific isoform of Dia, Cdc42 is supposed to control biorientation and stabilization of kinetochor-MT attachments and therefore chromosome alignment (Narumiya et al., 2004; Yasuda et al., 2004). In other systems however, the complete loss of Cdc42 activity was reported not to affect mitosis rendering it uncertain whether this GTPase is indeed a general component of the mitosis machinery (Narumiya and Yasuda, 2006).

As the mitosis fuction of Cdc42 already suggests, Rho GTPases do not only control the dynamics of the actin cytoskeleton but they also influence the organization of the MT network. RhoA activity was demonstrated to have a stabilizing effect on MTs. Similarly, Rac and Cdc42 can also exhibit a stabilizing function through activation of their common target protein Pak, which in turn inactivates Stathmin, a factor promoting catastrophe events of the MT network (Watanabe et al., 2005).

Another function of Rho GTPases is to participate in membrane trafficking events. Cdc42 for example was reported to affect Golgi to Endoplasmatic Reticulum (ER) transport. By binding to the protein coat of Golgi vesicles, Cdc42 inhibits their association with the MT motor protein dynein, thereby blocking the directed transport of the vesicle. In addition, phagocytosis as well as endocytosis and secretion can also be influenced by Rho-family GTPases. Constitutively active RhoA and Rac1 mutants inhibit clathrin-mediated endocytosis, most likely by interacting with proteins that control the uncoating of vesicles (Ridley, 2006; van Aelst and D'Souza-Schorey, 1997). Other non-classical GTPases localize to endosomes

and are able to delay the trafficking of membrane receptors like EGFR to late endosomes (Gampel et al., 1999).

Rho GTPases have also been implicated in promoting transcriptional changes in the cell. Rac and Cdc42 are capable of activating the JNK pathway, MAPK cascade or the NFκB transcription factor while RhoA was found to stimulate another transcription factor, SRF (serum-response factor). Interestingly, the activation of MAPK signalling, for example, seems to be independent of alterations of the actin cytoskeleton while the effect on SRF could be linked to levels of G-actin (Hall, 2005; Perona et al., 1997; Sulciner et al., 1996). In addition, Rho GTPases are involved in different steps of cell cycle progression and furthermore they possess the ability to activate different enzymes, among them the previously mentioned PI3K (Hall, 2005). Recently, small GTPases were also reported to modulate the activity of various ion channels (Pochynyuk et al., 2007).

As mentioned in the previous chapter, chemotactic cell movement and cell polarization during migration and in epithelial tissue depends on reorganizations of the actin network and hence also on Rho GTPases. Consistent with these functions small G proteins are major regulators of EMT. Various examples demonstrate that Rac GTPases are involved in regulating Cadherin internalization and disassembly of adherens junctions by different means (Akhtar and Hotchin, 2001; Pirraglia et al., 2006; Radisky et al., 2005; Thiery and Sleeman, 2006; Yang et al., 2005). In addition, several observations imply that RhoA also plays pivotal roles for EMT in different systems. It is known that TGF-ß regulated EMT progression involves RhoA-dependent pathways affecting the regulation of the actin cytoskeleton and the stability of adherens junctions in primary mouse keratinocytes (Bhowmick et al., 2001). Furthermore, it was reported that Rho1 itself can act as a regulator of adherens junctions in *Drosophila*. Loss of Rho1 during dorsal closure leads to ectopic accumulations of *D*E-cadherin in the cytoplasm consistent with a role for Rho1 in regulating either E-cadherin transport to or recycling from adherens junctions (Fox et al., 2005). In contrast, a model for a negative RhoA regulation during EMT results from the recent work of Nakaya and colleagues (2008) that was obtained from EMT in the chick primitive streak. They propose a model, in which the disassembly of the basal membrane of an epithelium is a critical starting point of EMT. Their results indicate that the stability of the basal membrane depends on a subpopulation of basal microtubules whose stability in turn is regulated by basal RhoA. Downregulation of basal RhoA activity causes MT instability resulting in the disassembly of the basal membrane and cell ingression (Nakaya et al., 2008).

As EMT processes are not only occurring during embryonic development but are also critical steps during tumorigenesis, Rho GTPases as well as their activators, the GEF proteins, are well described proto-oncogenes who's oncogenic versions have been identified in various different tumor types (Schmidt and Hall, 2002; van Aelst and D'Souza-Schorey, 1997).

The afore mentioned examples demonstrate the wide range of cellular processes that can be regulated by monomeric GTPases. This raises the important question how a relatively small number of Rho GTPases can specifically regulate so many different processes. About 22 mammalian and 7 different *Drosophila* Rho GTPases have been described, which are often ubiquitously expressed (Raftopoulou and Hall, 2004; Ridley, 2006). It is supposed that, in this context, specificity is guaranteed by the activity of the different groups of Rho GTPase regulators, the GEFs, GAPs and GDIs. The *Drosophila* genome contains at least 23 different Rho-specific GEFs of the Dbl-family (see below) and a similar number of GAPs (Garcia-Mata and Burridge, 2007; Settleman, 2001). This indicates that the number of regulatory proteins is much higher than the actual number of GTPases suggesting that the large variety of GEFs alone is sufficient to activate distinct Rho GTPases in response to specific signalling events. As the localization and the expression patterns of the regulatory proteins are additionally not as uniform as the ones of the GTPases, these two factors provide even more possibilities to regulate the different effects triggered by small G proteins. The interplay of various GEFs and GAPs can determine specifically in which cells and at which subcellular sites Rho GTPases are activated or inactivated, ensuring spatio-temporal regulation for GTPase activity. Another level of complexity is achieved by the fact that many GEFs and even GAPs can accept more than just one substrate. Via various posttranslational protein modifications the substrate specificity of these regulatory proteins can be changed providing even more possibilities for GEF/GTPase interactions. Another important factor is the high number of effector proteins that can act downstream of distinct GTPases and define the respective cellular effects of the signalling event that led to the activation of the Rho GTPase. The idea that different specific sets of GEFs, GTPases and effector molecules can all be recruited to one scaffolding complex could be another explanation for the question how various different signalling events can be triggered specifically by a relatively small number of G proteins (Garcia-Mata and Burridge, 2007).

It is highly evident that GEF proteins play pivotal roles in regulating Rho GTPases and provide means for regulatory input that controls the spatio-temporal activity of the GTPase pathways during development and disease. Therefore, it is of great importance to understand

how GEFs themselves are controlled and regulated in order to understand the regulatory networks, which converge on the level of Rho GTPases.

1.3 Early mesoderm morphogenesis in the *Drosophila* gastrula

During the development of multicellular organisms, several morphogenetic movements occur that lead to rearrangements of the tissues of the embryo. One of the most important processes during early embryonic development is called gastrulation. After the initial cleavage divisions, blastula-stage embryos normally consist of a single epithelial cell layer. During gastrulation, this epithelial tissue is transformed into the three different germlayers ecto-, meso- and endoderm by a series of dramatic cell movements and rearrangements. It is therefore of fundamental importance for embryonic development that the different steps of gastrulation movements are highly regulated in order give the embryo its final structure.

Before gastrulation is initiated during *Drosophila* development, a blastoderm embryo is formed that consists of approximately 6000 cells, which are arranged in a single epithelium surrounding the inner yolk cell. Already at this stage, the presumptive mesoderm is specified at the ventral side of the embryo. The maternally expressed transcription factor Dorsal governs the formation of the dorso-ventral body axis during early embryogenesis. Immediately after fertilization Dorsal is distributed equally along the dorso-ventral axis. However, the induction of a spatially restricted protease cascade in the extracellular space of the egg leads to the activation of the Toll receptor only at the future ventral side of the embryo. (Amiri and Stein, 2002; Sen et al., 1998). This activation eventually triggers the release of cytoplasmic Dorsal from a complex with the inhibitory protein Cactus leading to a translocation of the transcription factor into the nucleus. The further the cells are away from the ventral signalling source, the weaker the signalling events are that trigger Dorsal activation. As a result a gradient of nuclear Dorsal is established with the highest concentration within the ventral cells (Reach et al., 1996; Roth et al., 1989; Rushlow et al., 1989; Steward, 1989; Whalen and Steward, 1993). Dorsal can induce the expression of different genes in a concentration dependent manner, as its different target genes possess binding sites of different affinities. This results in expression of distinct zygotic genes along the dorso-ventral axis and initiates the dorso-ventral specification of the embryo (Gilbert, 2003).

The mesoderm cells are specified by high concentrations of nuclear Dorsal protein. This drives the expression of the mesoderm specific transcription factors Twist (Twi) and Snail

(Sna) in an approximately 16 cells wide stripe at the ventral site of the embryo. Twi acts as an activator of mesoderm specific genes while Sna is a repressor inhibiting expression of ectodermal genes. Both genes trigger the specification of the mesoderm in concert, which is a prerequisite for the subsequent internalization of the mesodermal cells (Ip et al., 1992; Jiang et al., 1991; Leptin, 1991).

The first morphogenetic movement during gastrulation is the invagination of the presumptive mesoderm (in the stage 5 embryo (Campos-Ortega and Hartenstein, 1997)). The infolding of the epithelium is accompanied by characteristic cell shape changes. The apices of the presumptive mesoderm start to constrict while the nuclei are transported towards the basal pole of the cells. As a consequence the cells acquire a wedge-shaped structure and initiate the formation of a ventral furrow that further folds inward until the mesoderm is internalized completely (Leptin and Grunewald, 1990; Sweeton et al., 1991).

Several genes that are involved in these early cell shape changes have already been identified and it was shown that their expression depends on Twi and Sna. The secreted glycoprotein Folded Gastrulation (Fog) presumably triggers the activation of Concertina (Cta), which is an α-subunit of a heterotrimeric G protein (Costa et al., 1994; Leptin, 1994; Parks and Wieschaus, 1991). This is thought to initiate the relocation of a Rho specific GEF called RhoGEF2. RhoGEF2 is a central component of the invagination machinery as loss of its activity completely blocks furrow formation (Barrett et al., 1997; Hacker and Perrimon, 1998; Nikolaidou and Barrett, 2004). The initially MT associated GEF is released upon Cta signalling and targeted to the apical cell surface by the transmembrane protein T48. This leads to the local activation of Rho1, which initiates the formation of a contracting actomyosin ring at the apical domain of the mesodermal cells (Dawes-Hoang et al., 2005; Kolsch et al., 2007).

After invagination is complete, the mesoderm forms an epithelial tube inside the embryo. At this stage (phase 1 of mesoderm migration or “collapsing phase”; see Fig. 1.2) the cells that are basally localized within the tube start to establish contact with the underlying ectoderm. This depends on the extension of cellular protrusions that are formed by the mesoderm (Schumacher et al., 2004; Wilson et al., 2005). In consequence, the epithelial tube starts to flatten down symmetrically and the mesoderm undergoes EMT. This results in the complete disassembly of the epithelial tube and the mesoderm remains as a group of cells in the middle of the embryo. This process is coincident with a first round of mitosis in the mesoderm (Foe, 1989). During the second phase of mesoderm migration, the now mesenchymal cells start to extend filo- and lamellipodia and migrate in dorsolateral directions (Fig. 1.2). At the end of

this spreading process (phase 3) the mesoderm forms a typical monolayer on the ectoderm (Fig. 1.2) (Murray and Saint, 2007; Schumacher et al., 2004).

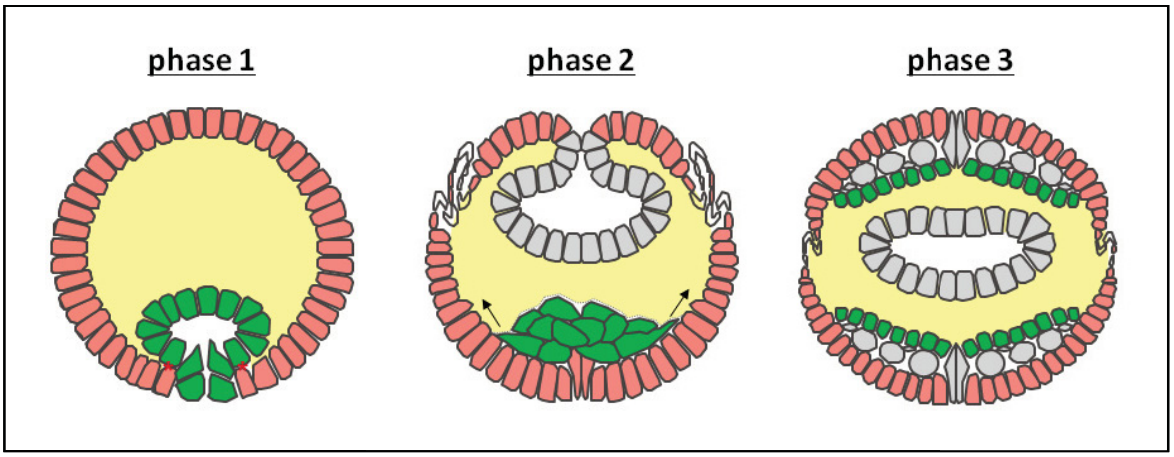

Fig. 1.2: The three phases of mesoderm migration in *Drosophila*.
Schematic representation of cross-sections through embryos during the three characteristic phases of mesodermal spreading. The ectoderm is marked in rosy, endodermal cells as well as neuronal tissue (phase 3) in grey and the yolk cell in yellow. Mesoderm cells are shown in green. Red asterisks in phase 1 highlight the positions of the initial contact between meso- and ectoderm. Phase 1: After invagination, the initial contact between the epithelial mesoderm and ectoderm is established during early stage 7 followed by EMT and a first round of mitosis. Phase 2: At early stage 8, the mesodermal cells become motile and start to spread out in dorsolateral directions. Phase 3: At the end of migration (stage 9), the mesoderm forms a monolayer on the ectoderm and the newly formed neuronal tissue. [modified after (Knust and Müller, 1998)]

During the subsequent stages of development different locally restricted signalling events specify different parts of the mesoderm along the dorsoventral axis to form distinct mesodermal derivatives (Frasch, 1995). For example, the cells that migrate to the very dorsolateral positions are determined to become heart precursor cells. Combination of Wingless (Wg, [Wnt]) and Decapentaplegic (Dpp, [Bmp]) signalling as well as activation of MAP kinase signalling through the FGF (fibroblast growth factor) receptor molecule Heartless (Htl) leads to the expression of the cell fate determinant Evenskipped (Eve) (Carmena et al., 1998; Frasch et al., 1987; Halfon et al., 2000; Knirr and Frasch, 2001). Therefore, expression of *eve* is the first marker for the presence of those pericardial cells and can be used as an indirect marker for successful spreading of the mesoderm (see below).

1.4 FGF receptor signalling during *Drosophila* mesoderm migration

The transcription factor Twi also controls the expression of genes that are required for the migratory phase of mesoderm morphogenesis. One of these genes is called *heartless* (*htl*) and it encodes an FGF receptor. Htl is expressed in all mesodermal cells and it was shown that the

spreading of the mesoderm depends on the activity of its pathway (Fig. 1.3). In embryos homozygous mutant for *htl*, the mesoderm fails to spread out properly and different mesodermal derivatives like heart precursor cells are not formed (hence: "heartless"). This is the result of the dual function of Htl during cell shape changes in the migration phase on the one hand and during the latter specification of certain mesodermal derivatives on the other hand (Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998b; Schumacher et al., 2004; Shishido et al., 1993; Shishido et al., 1997).

The earliest defect of *htl* mutant embryos can already be seen shortly after invagination, when the cells that are basally localized in the internalized epithelial tube fail to establish contact with the underlying ectoderm. After disassembly of the tube, the cells do not undergo their characteristic cell shape changes and do not spread out in dorso-lateral directions. However, in later stages, when the migratory process is already complete in wild type, the *htl* mutant cells show a moderate spreading indicating that activation of the receptor is not a general prerequisite for protrusive activity in the mesoderm. Nevertheless, the typical monolayer configuration on top of the ectoderm can never be achieved if the FGF receptor is missing (Schumacher et al., 2004).

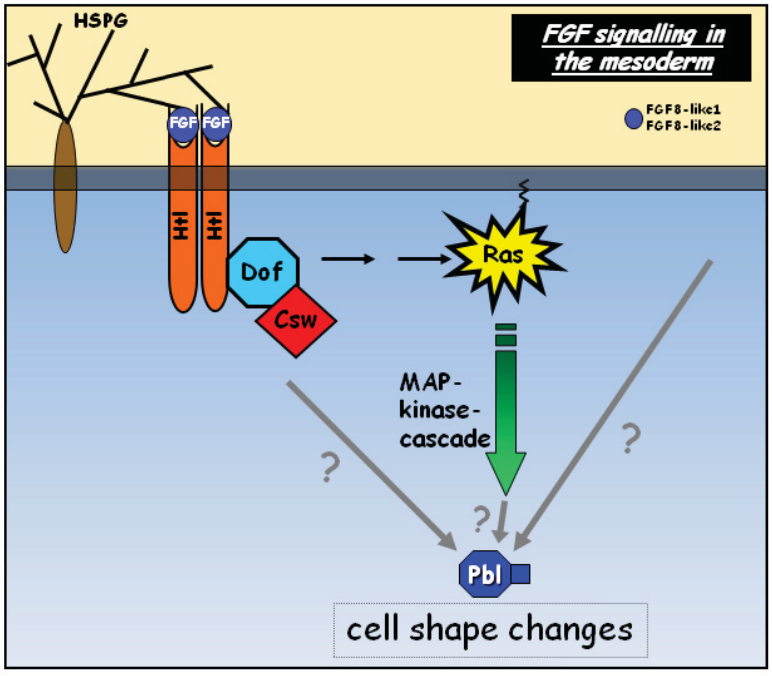

Fig. 1.3: Schematic representation of the Htl FGF pathway during mesoderm migration.
(see text for details)

The Htl FGF receptor is a so-called receptor tyrosine kinase. Upon activation by its ligands, these receptor molecules dimerize and activate each other via intermolecular tyrosine phosphorylations (Powers et al., 2000). Receptor activation leads to the recruitment of adaptor

proteins that bind to phosphorylated tyrosine residues via SH2 (Src homology 2 domain) or PTB (Phosphotyrosine binding) domains (Alberts et al., 2004). Htl possesses two ligands called FGF8-like1 (Thisbe) and FGF8-like2 (Pyramus), which show a dynamic expression pattern during mesoderm development. Initially, they are both expressed by all ectodermal cells but later FGF8-like2 expression becomes restricted to the dorsal ectoderm, which implies a possible role as an instructive signal that governs the direction of migration through the Htl pathway (Gryzik and Müller, 2004; Stathopoulos et al., 2004). However, no data exist that could indeed prove such a chemotactic role for either of the two FGFs, so far.

It was shown before that FGFs could also bind to proteoglycans. As FGFs are monomers but need to bind to two receptors that subsequently dimerize, activation can be enhanced by elevating the local concentration of ligand molecules. This is achieved by heparan sulfate proteoglycans that act as co-receptors to facilitate the FGF receptor activation. As a result, mutations in enzymes that are required for the production of heparin sulfate proteoglycans, like the two genes *sugarless* and *sulfateless*, were found to exhibit a *htl*-like mesoderm phenotype (Lin et al., 1999).

Activated Htl binds and phosphorylates the adaptor molecule Dof (Downstream of FGF receptor; also Stumps or Heartbroken) (Imam et al., 1999; Michelson et al., 1998a; Vincent et al., 1998). Active Dof in turn is capable of recruiting the phosphatase Corkscrew (Csw) to the receptor complex (Petit et al., 2004; Wilson et al., 2004). Although Csw is required for the specification function of Htl, its function during spreading or possible migration specific substrates are currently unknown (Johnson Hamlet and Perkins, 2001; Perkins et al., 1996; Wilson et al., 2005). The Dof/Csw complex is able to trigger activation of the conserved Ras/MAP kinase pathway, probably through the SH2/SH3 protein Drk (Downstream of receptor kinase; also Grb2) and the Ras GEF Sos (Son of sevenless).

MAPK activation downstream of Htl is detectable exclusively in cells that have established contact to the ectoderm during phase 1. During the migratory phase only leading edge cells can be stained for active MAPK (Gabay et al., 1997). However, activation of the MAPK pathway seems to be neither sufficient nor required for the initiation of mesoderm migration. Consistent with this idea, activated forms of Ras are not sufficient to completely rescue the defects in *htl* or *dof* mutant embryos implying the presence of another pathway downstream of Htl during mesoderm migration (Schumacher et al., 2004; Wilson et al., 2005).

1.5 The Rho GEF Pbl in cytokinesis and mesoderm migration

Another essential player during mesoderm migration is the gene *pebble* (*pbl*). In a screen for zygotically expressed genes, which are required for mesoderm migration, mutations in *pbl* were found to cause defects similar to the ones seen after loss of central components of the Htl pathway. While invagination of the mesoderm is not affected in *pbl* mutants, the first abnormalities are already visible during the first phase of mesoderm migration. Similar to *htl* mutants, the mesoderm cells fail to establish contact with the ectoderm during phase 1. After disassembly of the tube, the cells do not show a polarized morphology and remain in the centre of the embryo close to the ventral midline. The reason for this cell behavior seems to be the fact that after loss of the Pbl protein the protrusive activity of the mesodermal cells is blocked completely. In contrast to *htl* mutants, this is also the case in later stages as the cells do not spread out at all. This clearly indicates that Pbl is a key component controlling the cell shape changes and protrusive activity during mesoderm migration (Schumacher et al., 2004; Smallhorn et al., 2004).

As mentioned earlier, the formation of protrusion is dependent on extensive reorganizations of the actin cytoskeleton. These changes are not initiated in *pbl* mutants and therefore high amounts of cortical F-actin are visible after loss of the protein. Consistent with this phenotypic description, further analysis indicated that *pbl* encodes a Rho specific GEF. Therefore it represents a good candidate to transduce the signal from the Htl pathway to the cytoskeleton of the cell. Genetic interactions showed that Pbl acts either downstream of the Htl-MAPK pathway, in a second pathway downstream of the receptor or maybe in a novel parallel pathway that is also essential for the Htl mediated cell shape changes during mesodermal spreading (Fig. 1.3) (Schumacher et al., 2004).

Pbl belongs to the so-called Dbl family of GEF proteins. These exchange factors are characterized by the presence of a “tandem domain” consisting of the DH (Dbl homology) and an adjacent PH (Pleckstrin homology) domain (Fig. 1.4). The DH domain is the catalytic part of the protein that directly interacts with the substrate GTPases and facilitates the nucleotide exchange (Schmidt and Hall, 2002). The neighboring PH domain fulfills various different functions in different GEF proteins. In general, PH domains are known to be able to bind to phosphoinosites in membranes. This allows the recruitment of the respective protein to distinct regions of the cell cortex where these lipids have been enriched by previous signalling events (PI3K pathway, see chapter 1.1). Beside this membrane recruitment function, PH domains in GEFs have been implicated in contributing to the exchange activity of the DH domains (Liu et al., 1998). For some GEFs it could be shown that the PH domains

assist the DH domain in binding the substrate GTPase, thereby enhancing the GEF activity of the catalytic core (Rossman and Sondek, 2005; Rossman et al., 2002). In other cases the binding to phospholipids is not required for the subcellular localization of the GEF but it results in conformational changes in the DH-PH tandem domain that could enhance its exchange activity (Baumeister et al., 2006; Rossman et al., 2005).

The N-terminal part of the Pbl protein is characterized by the presence of two BRCT domains (BRCA1 C-terminal domain). These domains have first been identified in the breast cancer tumor suppressor protein BRCA1. BRCT domains are highly conserved through evolution and were found to act as protein-protein interaction domains. The commonly tandem-like arranged BRCT domains are phospho-protein binding domains that are frequently found in proteins involved in cell cycle checkpoint control and/or DNA damage pathways (Bork et al., 1997; Yu et al., 2003). The region N-terminal to the BRCT domains of Pbl also exhibits some characteristics of such a protein domain and it is sometimes referred to as RadECl region. This motif is only found in conjunction with BRCT domains and seems to be required for the binding of at least one interaction partner (see below) (O'Keefe et al., 2001; Somers and Saint, 2003).

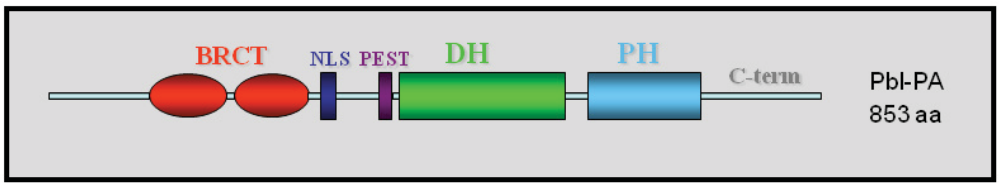

Fig. 1.4: Domain structure of the Rho GEF Pbl.
The Pbl isoform A comprises 853 amino acids and contains two BRCT domains (red), a NLS (blue) and a PEST motif (purple). As Pbl belongs to the Dbl family of exchange factors, its catalytic core consists of a DH (green) and a PH (blue) domain. The functions of the different domains are explained in the text.

The central region of Pbl harbours a nuclear localization signal (NLS) that can be recognized by special receptor proteins that mediate the nuclear import of those proteins. In addition, Pbl possesses a PEST motif (Proline-, Glutamic acid-, Serine- and Threonine-rich), which can be detected by ubiquitin ligases. Polyubiquitination marks proteins for fast degradation in the proteasome and provides an effective possibility to downregulate the activity of certain proteins. Therefore, PEST motives are frequently found in proteins with a high turn-over rate like cell cycle regulators or transcription factors, for example (Rechsteiner and Rogers, 1996). The C-terminal tail of Pbl (Carboxy-terminal to PH) does not contain any known domain structures or amino acid motives.

Originally, Pbl was identified as a protein essential for cytokinesis (Lehner, 1992; Saint and Hime, 1992). In this context, Pbl was shown to be required for the assembly of the contractile actomyosin ring in the middle of the dividing cell, which represents the driving force of cytokinesis. Subsequent analysis indicated that Pbl, as well as its mammalian homologue Ect2 (Epithelial cell transforming gene 2), is part of an evolutionary conserved pathway that activates the GTPase Rho1 [RhoA in mammals] during cytokinesis. Rho1 in turn activates other downstream factors that trigger the assembly of the contractile ring (see chapter 1.3) (Prokopenko et al., 1999; Saint and Somers, 2003). Pbl [Ect2 in mammals and LET-21 in *C. elegans*] was found to interact with the centralspindlin complex consisting of the kinesin-like molecule Pavarotti [MKLP1 in humans, ZEN-4 in *C. elegans*] and the GAP RacGAP50C [also called Tumbleweed (flybase ID FBgn0086356); MgcRacGAP in humans and CYK-4 in *C. elegans*] (Piekny et al., 2005). Pavarotti was shown to transport the GAP along astral and midbody MTs to their plus-ends in the equator region. RacGAP50C in turn recruits Pbl to the complex via interaction with its RadECl and first BRCT domain. Interaction with the centralspindlin complex results in the activation of the GEF in a cortical ring and the subsequent furrow formation at this site. As a consequence of this role, *pbl* mutant embryos generate multinucleate cells (Prokopenko et al., 1999; Somers and Saint, 2003).

Like many other mammalian GEFs, *pbl*'s homologue *ect2* was initially identified as a proto-oncogene. Deletion of the N-terminal regulatory protein domains (BRCT and NLS) was reported to generate its oncogenic form that stimulates cell proliferation in NIH 3T3 cells and leads to an adhesion independent growth of colonies. In addition, oncogenic Ect2 was also shown to activate invasive cell behavior efficiently and therefore it might exhibit different effects upon malignant transformation of cells and subsequent tumorigenesis (Miki et al., 1993; Saito et al., 2004). However, a direct function of Ect2 related to cell migration has yet to be demonstrated.

Ect2 was reported to be able to activate RhoA, Rac and Cdc42 suggesting that the GEF might act through different substrates in distinct processes (Solski et al., 2004; Tatsumoto et al., 1999). Interestingly, more recent work on HeLa cells and with *Xenopus* egg extracts implied an earlier function of Ect2 during mitosis. At least in these systems a role for Cdc42 in MT biorientation and stabilization of kinetochor-MT interactions that depends on the GEF activity of Ect2 was suggested (Narumiya et al., 2004; Narumiya and Yasuda, 2006; Oceguera-Yanez et al., 2005; Tatsumoto et al., 2003). Therefore, Ect2 seems to activate this GTPase shortly before its conserved function during cytokinesis where it activates RhoA. This result indicates that at least in these two systems, the substrate preference of Ect2 must be regulated *in vivo*

and that it has to be tightly controlled to promote proper mitosis. Nevertheless, a similar function of Ect2 has not been reported for other model systems and further reports suggest that mitosis in other cell lines is Cdc42 independent (Narumiya and Yasuda, 2006). Importantly, in *Drosophila* the only reported substrate of Pbl is Rho1. Furthermore, loss of Pbl activity was shown not to interfere with chromosome condensation, spindle assembly or spindle function (Hime and Saint, 1992; Lehner, 1992). Nevertheless, these results indicate that it is of fundamental importance to understand how GEF proteins are regulated in different cellular contexts and how they are able to trigger specific events in response to different signalling events.

1.6 Aim of work

Previous work has shown that the function of Pbl during mesodermal cell migration is independent of its conserved role during cytokinesis. Using a genetic background where cells do not enter postblastodermal mitosis, Schumacher et al. (2004) could show that the protrusive activity of the mesoderm cells is still dependent on Pbl function, indicating that even in the absence of cytokinesis defects the GEF is essential for migration of the mesoderm. Analysis of mutant alleles revealed that the GEF activity of Pbl is required for both functions as a point mutation within the DH domain that reduces its catalytic activity affects both processes. Subsequent misexpression experiments using a dominant negative version of Rho1 indicated that interference with active Rho1 efficiently blocks cytokinesis in the mesoderm but does not affect the capacity of the cells to form cellular protrusions. Therefore, it was concluded that Pbl may act through a different pathway during migration, independent of Rho1 activation (Schumacher et al., 2004; Smallhorn et al., 2004).

These findings directly pose the central questions of this thesis, how Pbl can act in two distinct processes and how its dual activity is regulated. In addition, it has to be clarified which other pathway is employed by the GEF during migration and which GTPase acts as its substrate in this context. Importantly, expression of a construct lacking the N-terminal region including both BRCT domains ($Pbl^{\Delta BRCT}$) was shown to specifically rescue migration but not cytokinesis (Smallhorn et al., 2004). This indicates that both functions of the GEF can be separated on the protein level suggesting that the specific activity of Pbl might be regulated through its different domains. Hence, a domain-function analysis of the protein will be used to examine the role of the different protein domains of Pbl in order to find possible answers to the questions raised above.

A further aim of this work is to investigate the interphase localization of the protein. So far the GEF was reported to localize exclusively to the nucleus during interphase raising the question how Pbl can influence the actin cytoskeleton in interphase mesoderm cells (Prokopenko et al., 1999; Prokopenko et al., 2000a). Therefore, the localization pattern of Pbl in interphase mesoderm cells will be analyzed in detail. The usage of tagged constructs for the mentioned domain-function analysis will further allow the utilization of the different constructs in order to identify the domains of the protein that mediate the localization of Pbl in migrating mesoderm cells.

2 Material and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were obtained from the following companies in *pro analysis* quality:
Acros (Geel, Belgium), *Baker* (Deventer, Netherland), *Biomol* (Hamburg, Germany), *Bio-Rad* (München, Germany), *Difco* (Detroit, USA), *Fluka* (Buchs, Switzerland), *Gibco/BRL LifeTechnologies* (Karlsruhe, Germany), *Macherey-Nagel* (Düren, Germany), *Merck* (Darmstadt, Germany), *Roth* (Karlsruhe,Germany), *Serva* (Heidelberg, Germany), *Sigma-Aldrich* (Steinheim, Germany), *Qiagen* (Hilden, Germany).

All solutions were prepared with dH_2O and they were autoclaved or sterile filtrated prior to use.

General solutions:
PBS (10x): 1,3M NaCl; 70mM $NaHPO_4$; 30mM NaH_2PO_4 (adjust pH to 7.4)
PBT: 1x PBS with 0.1% Tween20

Enzymes for molecular work were purchased from:
MBI Fermentas (St. Leon-Rot, Germany), *Boehringer/Roche Diagnostics* (Mannheim, Germany), *Stratagene* (La Jolla, USA) and *Promega* (Madison, USA).

For plasmid DNA preparation and for DNA extraction from agarose gels, kits from *Qiagen* (Hilden, Germany), *Macherey-Nagel* (Düren, Germany) or *Promega* (Madison, USA) were used.

2.1.2 Microscopy, image acquisition and employed software

Microscopy was performed on a Zeiss Axiophot, an Olympus BX61 as well as on Zeiss 510 Meta and Leica-SP2 confocal microscopes (*Zeiss*, Jena, Germany; *Olympus*, Watford, UK; *Leica,* Heidelberg Germany). Scanning electron microscopy was performed on a Leo Supra (*Leo Electron Microscopy Ltd.*, Cambridge, UK) and a Philips XL30 ESEM (*Phillips*, Eindhoven, Netherlands).

Images were processed using Adobe Photoshop (*Adobe Systems*, San Jose, USA), Volocity (*Improvision, a PerkinElmer Company*, Coventry, UK), the LSM software (*Zeiss*, Jena, Germany) and Canvas 8 (*Deneba Systems*, Miami, USA).
For sequence analysis DNA-Star Lasergene V6 (*DNASTAR Inc.*, Madison, USA) was used on a Macintosh system (*Apple*, Ismaning, Germany).

2.2 Molecular biology

2.2.1 Amplification of DNA molecules

2.2.1.1 Polymerase-chain-reaction

The Polymerase-chain-reaction (PCR) is a possibility to generate multiple copies of a certain DNA fragment *in vitro*. Initially, double stranded DNA containing the sequence of interest is denatured at 94°C. By using sequence specific oligonucleotides, the region of the DNA that should be subsequently amplified is flanked by these primer molecules. The binding of the primers to the template DNA occurs after every denaturing step and is called annealing. In the following step, the elongation, the primers are used as starting points for the synthesis of the complementary DNA strand. This cycle is repeated several times and leads to an exponential increase in the copy number of the amplified DNA sequence. The DNA synthesis is performed by temperature stable DNA polymerases that are not inactivated by the high temperature during the denaturing step. Beside the standard polymerase Taq another polymerase called Pfu is used especially when the PCR products are subsequently used for the cloning of expression constructs, for example. The Pfu polymerase possesses a proofreading activity and therefore it generates fewer errors when synthesizing the complementary DNA strand.
An example for a PCR reaction mix with 50µl total volume is shown below:

Xµl DNA (~ 50 - 100ng)
1µl *forward* primer (50µM)
1µl *reverse* primer (50µM)
1µl dNTP mix (10mM)
5µl 10x Pfu-polymerase-buffer
+ 0.5µl Pfu-polymerase
add dH_2O to a total volume of 50µl

The PCRs were performed in a Thermocycler PTC-200 (*MJ Research*, Watertown, USA) using the following standard program. The elongation time depends on the length of the amplified DNA molecule (the Pfu polymerase amplifies a 1kb DNA fragment in approximately one minute). The annealing temperature depends on the G/C proportion of the used primer molecules.

	duration	temperature	step
	5min	94°C	denaturation of DNA
30x	30sec	94°C	denaturation of DNA
	1min	primer-dependent	annealing (binding of the primer molecules)
	amplicon size dependent	72°C	elongation (DNA-synthesis)
	10min	72°C	final elongation step (DNA-synthesis)
	∞	4°C	end

Table 2.1: Standard-PCR-program

The amplified DNA can be purified using the NucleoSpin Extract kit from *Macherey-Nagel* or by gel extraction (see below). This extra step allows the transfer of the DNA in a medium with lower salt concentrations, which facilitates the activity of other enzymes that are used for subsequent cloning steps.

2.2.1.2 Transformation of electro- or chemocompetent bacteria

Another way to amplify certain DNA fragments is the transformation of *E. coli* cells. First, the DNA fragment is ligated into a linearized vector molecule (see 2.2.3.1) giving rise to a ring-like plasmid molecule. Then, the plasmid DNA is transformed into suitable *E. coli* cells where it is replicated together with the bacterial DNA before every cell division. Therefore, the cultivation of such transformed cells leads to an increase in bacteria numbers and hence to an amplification of the cloned DNA fragment which can subsequently be isolated from the bacteria culture again (see below).

Electrocompetent *E. coli* cells (e.g. XL1-Blue or DH5α) are transformed by electroporation using the Gene Pulser II from *Biorad* (München, Germany): For each DNA sample, a 50µl aliquot of electrocompetent cells is thawed on ice and mixed with the respective plasmid DNA (1µl of a 1:10 dilution of a ligation mix or 1µl of a 1:200 dilution of isolated DNA [Midi]). The mix is loaded into an electroporation cuvette and the transformation is performed as described in the user manual of the electroporator (using 1,8kV). Immediately after the transformation, 1 ml of prewarmed LB medium is added to the cells and the suspension is transferred into a reaction tube

In case chemocompenet *E. coli* cells should be transformed, an aliquot of the cells is thawed on ice and mixed with the appropriate amount plasmid DNA (see above). The mix is incubated for 30 min on ice before the cells are heatshocked for 30-40 seconds in a 42°C waterbath. Afterwards the cells are put back on ice for a few seconds before 1 ml of prewarmed LB medium is added.

After an one hour incubation at 37°C on a bacteria shaker, the cells are spun down (2 min at 5000rpm) and resuspended in approximately 100µl of the supernatant. The cells are plated on selective plates (100µg/ml ampicillin) and grown over night at 37°C.

LB medium: 1% bactotrypton, 0.5% bactoyeast, 1% NaCl

LB-agar: 6.3g agar for 300ml LB

2.2.2 Isolation of DNA molecules

After transformation of *E. coli* cells, single colonies are picked from the selective plates the following day. They are used to inoculate an appropriate volume of LB medium that depends on the amount of DNA that should be isolated from the culture. For Mini-preparations 2-10 ml, for Midi-preparations 25-50 ml and for Maxi-preparations 100ml and more of LB medium are used. The isolation of the DNA from the bacteria solutions is performed using the Midi kits from either *Qiagen* (Hilden, Germany) or *Macherey-Nagel* (Düren, Germany) and following the instructions in the user manual.

2.2.3 Manipulations of DNA molecules

2.2.3.1 DNA digest using restriction enzymes

Restriction enzymes are so-called endonucleases of bacterial origin. They are capable of hydrolysing the phosphodiester bonds between both strands of a DNA molecule. Enzymes

that belong to the type II of endonucleases recognize specific nucleotide motives within the DNA that are often palindromic (the sequence read from 5' to 3' and from 3' to 5' is identical). The type II restriction enzymes cleave the DNA within this palindromic sequence thereby generating either sticky ends or blunt ends that can subsequently be connected again by DNA ligases (see below). The concentration of restriction enzymes is stated in units (U) per µl. One unit is the amount of enzyme that is required to digest 1µg of DNA in one hour.
The following points should be considered when preparing a digestion mix:

- The 4-fold amount of enzyme should always be used, e.g. for the digestion of 1µg DNA, 4U of the respective restriction enzyme are added.
- The total volume of enzymes should not be more than 10% of the total reaction volume. The enzymes are kept in a glycine containing medium and it is important to keep the final glycine concentration in the reaction mix at low levels as otherwise the full activity of the restriction enzyme can be affected (when using 2,5µl enzyme the total volume of the reaction mix should be at least 25 µl).
- The 10-fold reaction buffer of the enzyme should be in a 1x concentration in the final reaction mix (the volume can be adjusted with dH_2O).

2.2.3.2 5` dephosphorylation of linear DNA

DNA fragments generated by digestion with a restriction enzyme can be inserted into a plasmid that was linearized with the same restriction enzyme. To avoid a direct re-ligation of the two ends of the plasmid molecule, the linearized vector is treated with alkaline phosphatase (calf intestine phosphatase "CIP", *Boehringer*, Mannheim, Germany). This enzyme dephosphorylates the 5' ends of DNA molecules which impedes the re-ligation of the vector molecule.
The vector should be treated with CIP directly after the restriction enzyme digest. 1µl of the enzyme is added to the digestion mix together with the appropriate volume of the 10x reaction buffer and dH_2O. After an one hour incubation at 37°C, the phosphatase is inactivated at 65°C for ten minutes. Before the DNA is used for a ligation reaction, a purification step should be performed to achieve a higher efficiency (see 2.2.5).

2.2.3.3 Ligation

The digestion with restriction enzymes generates DNA fragments with compatible ends that can be connected in a ATP dependent manner by the T4-ligase. Typically, a smaller insert is ligated into a much longer, linearized vector molecule. To achieve the best possible efficiency

of the reaction, the relative amount of vector and insert DNA should be chosen in a certain ratio:

$$\frac{100\ \text{ng (vector DNA)} \times \text{kb (insert length)}}{\text{kb (vector length)}} \times 10 = \textit{ng (amount of insert DNA that should be used for the reaction)}$$

This formula can be used to calculate the amount of insert DNA that has to be added to a reaction mix containing 100 ng of linearized vector DNA to achieve of 10-fold surplus of insert molecules (can be increased to 100-fold if ligations do not work efficiently or when cloning into the pUAST-HA vector). The total volume of the reaction mix should be kept between 10 and 30µl including the DNA, the ligase-buffer, 1µl T4-ligase (and dH_2O). The reaction is performed on 16°C over night or at 22°C for an hour or longer (T4-Ligase from *MBI Fermentas*, St. Leon-Rot, Germany). The ligase can be inactivated by an incubation step at 65°C for 10 minutes, which is thought to increase the efficiency of subsequent transformations.

2.2.4 DNA electrophoresis

DNA fragments are separated according to standard protocols on 1% TAE-agarose gels at 110V. For the visualization of the DNA ethidium bromide is added to the agarose (0.1-1 µg/ml) and the gel is imaged on an UV transilluminator. The size of the different fragments is estimated by comparison with standard fragments of known sizes (1kb ladder, *MBI Fermentas,* St. Leon-Rot, Germany).

2.2.5 Elution of DNA from agarose gels

To remove by-products from PCR reactions or to isolate DNA of a distinct size of a restriction enzyme digestion, DNA solutions are separated on a 1% agarose gel and the DNA fragments of interest are cut from the gel on an UV transilluminator using a clean razor blade. Elution of the DNA is performed using the NucleoSpin Extract Kit from *Macherey-Nagel* (Düren, Germany).

2.2.6 Sequencing

To check whether the coding sequence of the different expression constructs are inserted correctly into the respective expression vectors, the cloned constructs were sequenced. The

sequencing reactions were performed either by *SEQLAB* (Göttingen, Germany) or by the Sequencing Service of the University of Dundee (Dundee, UK).

2.2.7 Cloning of HA-tagged UAS-expression constructs

All Pbl constructs were generated using the *pbl* cDNA isoform A (Clone ID: SD01796) as template for PCRs to amplify the parts of the ORF that should be included in the expression construct. The vectors, primer and conditions used for the cloning of the different Pbl construct are listed below. Furthermore, it is stated which amino acids of the Pbl isoform A are encoded by the respective expression construct.

Name	Binding site	Sequence	Restriction site
358069	5' upstream of 1. ATG	5'-CGGAATTCGTAACTGCAGAAGATCCATG-3'	*Eco*RI
5DH GST EI	5' upstream of DH	5'-GCCAAGAATTCGATGCGC-3'	*Eco*RI
pbl5HA	3' upstream of STOP	5'-CGGAATTCCGCGTGGGTTGCACCG-3'	*Eco*RI
Pbl E	3' downstream of DH	5'-CCGGAATTCGATTCGGTCCGCCTTTTATC-3'	*Eco*RI
Pbl G GW	5' upstream of PH	5'-CACCATGTGTCCGGCGCATTTAGTG-3'	Gateway
Pbl H	3' downstream of PH	5'-CGGCCGAATTCAGTTTG-3'	*Eco*RI
Pbl I	5' upstream of C-term	5'-GGAATTCCAACCAGATGGCAGCCC-3'	*Eco*RI
Pbl M	3' downstream of BRCT2	5'-GCTCTAGAGCTCTGTGTTCGGTGT-3'	*Xba*I
Pbl N	3' downstream of PH	5'-GGAATTCCGCAGGTGTGGGCTGC-3'	*Eco*RI
Pbl N2	3' downstream of PH	5'-CGGAATTCCGGCAGGTGTGGGCTGC-3'	*Eco*RI
5' C-term-tag	5'including ATG	5'-CACCATGGAAATGGAGACCATTG-3'	Gateway
5' N-term-tag	5' excluding ATG	5'-CACCGAAATGGAGACCATTG-3'	Gateway
3'C-term-tag	3' upstream of STOP	5'-AATGCGGCCCACAACGGCC-3'	Gateway
3' N-term-tag	3' including STOP	5'-CTAAATGCGGCCCACAACGGCC-3'	Gateway

Table 2.2: Sequences of primers employed for the cloning of different Pbl constructs.
Restriction sites within the sequence are highlighted in green, START and STOP codons in red and the 5' extension for Gateway cloning is shown in blue.

construct	5'-Primer (forward)	3'-Primer (reverse)	Annealing-temperature	Elongation time	Encoded amino acids	Cloned via	Expression vector
PblA-GFP	5' C-term-tag	3' C-term-tag	51°C	3 min	1-853	Gateway	pTWG
GFP-PblA	5' N-term-tag	3' N-term-tag	51°C	3 min	1-853	Gateway	pTGW
$PblA^{I565L}$	5' C-term-tag	3' C-term-tag	51°C	3 min	1-853	Gateway	pTWH
$PblA^{R557S.L558S}$	5' C-term-tag	3' C-term-tag	51°C	3 min	1-853	Gateway	pTWH
$PblA^{D554A,H555L}$	5' C-term-tag	3' C-term-tag	51°C	3 min	1-853	Gateway	pTWH
$Pbl^{BRCT1,2}$	5' C-term-tag	Pbl M	53°C	2 min	1-304	Gateway	pTWH
$Pbl^{\Delta N\text{-}term}$ -HA (= $Pbl^{DH\text{-}PH\text{-}C\text{-}term}$)	*Cloning facility, University of Dundee*				386-853	*Eco*RI, *Xho*	pUAST-HA
$Pbl^{\Delta N\text{-}term\_V531D}$ -HA (= $Pbl^{DH\text{-}PH\text{-}C\text{-}term\_V531D}$)	*Cloning facility, University of Dundee*				386-853	*Eco*RI, *Xho*	pUAST-HA
$Pbl^{DH\text{-}PH}$	5DH GST EI	Pbl H	50°C	3 min	386-775	*Eco*RI	pUAST-HA
$Pbl^{DH\text{-}PH\_V531D}$	5DH GST EI	Pbl H	50°C	3 min	386-775	*Eco*RI	pUAST-HA
$Pbl^{DH\text{-}PH\text{-}short}$	*Cloning facility, University of Dundee*				386-722	*Eco*RI, *Xho*	pUAST-HA
$Pbl^{DH\text{-}PH\text{-}short\_V531D}$	*Cloning facility, University of Dundee*				386-722	*Eco*RI, *Xho*	pUAST-HA
Pbl^{DH}	5DH GST EI	Pbl E	55°C	2 min	386-581	*Eco*RI	pUAST-HA
$Pbl^{GFP\text{-}PH}$	PblG GW	Pbl N	53°C	2min	595-719	Gateway	pTGW
$Pbl^{C\text{-}term}$	Pbl I	pbl5HA	55°C	2 min	716-844	*Eco*RI	pUAST-HA
$Pbl^{\Delta C\text{-}term}$	358069	Pbl N2	55°C	3 min	1-720	*Eco*RI	pUAST-HA

Table 2.3: Description of cloning conditions and length of different Pbl expression constructs.
The PblA-HA construct was cloned by Sabine Schumacher (for details see (Schumacher, 2005)) and flies carrying the $Pbl^{\Delta BRCT.myc}$ construct were obtained from the R. Saint lab (Canberra, Australia) (Smallhorn et al., 2004). Highlighted constructs were cloned by the Cloning facility of the College of Life Sciences, University of Dundee. Therefore, no primer sequences etc. are mentioned for these constructs.

Constructs containing point mutations were generated using the QuikChange II Site-Directed Mutagenesis Kit (*Stratagene*). Therefore, the constructs were subcloned into either pBluescript or the pENTR vector first and only after the successful mutagenesis into the respective expression vector. The mutagenesis reactions were performed following the guidelines in the kit manual. Primers that were used for mutagenesis are listed below:

V531D mutation (Val → Asp)

V531D **GAT GAT CCG TCC GGA* TCA GCG ATT GCC CAG**

V531D_rev **CTG GGC AAT CGC TGA T*CC GGA CGG ATC ATC**

30bp 60% GC
3.3% mismatch
T_m=86.93°C

I565L mutation (Ile → Leu)

I565L **GGA GGC TCT GAA GGC CC*T CAA GCA GGT GAC ACT GC**

I565L_rev **GC AGT GTC ACC TGC TTG AG*G GCC TTC AGA GCC TCC**

35bp 60% GC
2.86% mismatch
T_m=83.95°C

R557S,L558S mutation (Arg →Ser, Leu →Ser)

R557S,L558S **GGC AAT GCG GAC CAC GGA A*GT TC*G GAG GAG GCT CTG AAG GCC**

R557S,L558S_rev **GGC CTT CAG AGC CTC CTC CG*A ACT* TCC GTG GTC CGC ATT GCC**

42bp 61.9% GC
4.76% mismatch
T_m=86.05°C

D554A,H555L mutation (Asp → Ala, His → Leu)

D554A,H555L **CGA GTA GTG GCA ATG CGG C*CC T*CG GAC GTT TGG AGG AGG C**

D554A,H555L_rev **G CCT CCT CCA AAC GTC CGA* GGG* CCG CAT TGC CAC TAC TCG**

40bp 62.5% GC
5% mismatch
T_m=85.22°C

The site-directed mutagenesis reactions were performed using the following PCR program.

Segment	Cycles	Temperature	Time
1	1	95°C	30 sec
2	15	95°C (denaturation) 55°C (annealing) 68°C (elongation)	30 sec 1 min 5:20 min

Table 2.4: PCR program for site-directed mutagenesis.

2.3 Germline transformation of *Drosophila melanogaster*

A suitable way to insert DNA constructs into flies in order to express the encoded proteins *in vivo* is the transformation of the germline. In this context, gene transfer is mediated by a modified transposable element, a so-called P-element. Transposons are mobile DNA elements that are able to 'jump' to other positions within the genome. In contrast to so-called retrotransposons, which are transcribed into RNA first and then re-transcribed into DNA again which is subsequently inserted into the genome, transposition of P-elements depends on their excision and the reintegration into the host genome. Transposable elements encode the enzymes that mediate the different steps of the transposition. P-elements use the enzyme transposase, which is capable of recognizing flanking motives of the transposon sequence, the so-called inverted repeats. The emzyme mediates the excision of the elements between these inverted repeats. The reintegration of the free P-element is also mediated by the transposase.

For germline transformations modified versions of P-elements are used. Inverted repeats and the transposase gene are separated on two different plasmids. The sequence of the DNA construct is integrated into an expression vector like the pUAST plasmid, which contains inverted repeats on each side of the multiple cloning site. For the integration into the fly genome a second plasmid is required that encodes the transposase gene (the helper plasmid, e.g. pUChs$_{\Pi}\Delta$2-3). The splitting of both elements on two different plasmids enables an integration of the construct into the genome when both plasmids are injected into the same cell. Later on, however, the construct will not be able to transpose to other regions of the genome on its own, as the transposase gene (the helper plasmid) is not integrated into the fly genome but is eventually lost during cell divisions.

2.3.1 Generation of the injection mix

After Midi-preparation using a *Qiagen* Plasmid Purification kit (produces DNA solutions with only low levels of toxic bacterial products), 4µg of the respective expression plasmid are mixed with 1µg of the pUChs$_{\Pi}\Delta$2-3 helper plasmid and the total volume is adjusted to 10µl with dH_2O. The injection mix is centrifuged 5 to 10 min at 13000 rpm prior to use in order to avoid a blocking of the injection needle by floating particles.

2.3.2 Injection into *Drosophila* embryos

For germline transformations w^{1118} embryos are used. The white eye colour of the corresponding flies allows the selection of the positive transformants by eye colour, because the construct plasmid also contains a so-called mine-white gene as a selection marker, whose expression results in a reddish eye colour in the F_1 generation.

To achieve a transformation of the germline, the plasmid mix is injected at the posterior pole of embryos prior to the cellular blastoderm stage (Campos-Ortega and Hartenstein, 1997). In this region of the embryo the future germline cells, called pole cells, are formed during the process of cellularisation. If the injected plasmids are incorporated into at least one of the pole cells, the successful integration into the genome will result in flies, whose progeny is in part derived from this germline stem cell and therefore it possesses the construct in all cells of the body (F_1 generation).

The w^{1118} flies are transferred into egg-lay bottles two to three days before the first round of injection to allow them to acclimate to this egg-lay condition. The apple juice agar egg-lay plates are at least changed twice a day when not injecting.

Injections are performed on 18°C using an inverted microscope with a 20x lens, the micromanipulator InjectMan from *Eppendorf* (Hamburg, Germany) and the air pressure-driven injecting device FemtoJet (*Eppendorf*, Hamburg, Germany). The microinjection capillaries FemtoTip II (*Eppendorf*, Hamburg, Germany) are used as injections needles. Embryos from 15-20 min egg-collections are dechorionized with diluted sodium hypochlorite (NaOCl) and washed with H_2O. The embryos are transferred on agar blocks and aligned in the same a/p orientation under a dissecting microscope. A drop of “fly glue” (see below) is pipetted on a coverslip. After drying of the glue the aligned embryos are pressed carefully against the coverslip. Then the coverslip with the embryos is put into a drying chamber (Petri dish with blue Silica gel from *Sigma-Aldrich* (Steinheim, Germany)) for 8-14 min. The exact time depends on the humidity of both the room and the Silica gel and it possibly has to be

adjusted after a few rounds of injections. The drying should result in embryos that later do neither explode nor loose too large amounts of cytoplasm upon insertion of the needle and injection of the DNA mix. After drying, the embryos are covered with Halocarbon oil 700 and the plasmid mix is injected carefully into the posterior end of the embryo under microscopic control. The injection pressure has to be adjusted that way that only small droplets of the DNA mix are injected (test in a droplet of Halocarbon oil 700). After injection the slides are placed in weighing dishes and covered with Halocarbon oil 27. The dishes are kept on 18°C in moisture boxes and hatched larvae are collected and transferred to standard fly vials several times a day 1-3 days after injection.

Hatched flies are collected and mated with uninjected w^{1118} flies. The progeny of this cross (F_1 generation) is screened for the presence of red-eyed flies (positive transformants). Through subsequent crosses with a double balancer stock, the chromosome carrying the respective insertion is identified and kept over a balancer chromosome to create a stabile transgenic line.

Part of the injections were perfomed by the following injection services: *Rainbow Transgenic Flies, Inc.* (Newbury Park, USA) and *BestGene Inc.* (Chino Hills, USA).

Preparation of "fly glue":
Scotch tape is cut into pieces and covered with heptane in a glass bottle. After incubation on a roller for at least two days, the glue can be used for the injections. However, the glue might have to be slightly diluted with fresh heptane depending on the viscosity of the solution.

2.4 Protein biochemistry

2.4.1 Protein extraction from *Drosophila* tissue

For the preparation of protein extracts embryos from a 0-12 h collection are harvested, dechorionized and homogenized in lysis buffer (25mM HEPES-KOH pH 7.6, 100mM NaCl, 5mM EDTA, 0.1% IPEGAL, 10% Glycerol) supplemented with protease inhibitors (2µg/ml of Aprotinin, Leupeptin, Pepstatin and Pefabloc). After a 30 min incubation on ice, the solution is centrifuged for 10 min at 4°C and 13000rpm. The supernatant is transferred into a fresh tube and centrifuged for additional 20-30 min at 4°C and 13000rpm. The supernatant is transferred into a new tube and the protein concentration measured using the Bradford assay ("Coomassie Plus - The Better Bradford Assay Reagent", *Pierce/Thermo Fisher Scientific*, Rockford, USA). The protein solution is kept on ice until use.

2.4.2 Expression and purification of GST-fusion proteins

GST-fusion proteins can be expressed in bacteria and purified from lysates using Glutathione Sepharose 4A beads (*Amersham / GE Healthcare*, Piscataway, USA). As GST strongly interacts with its substrate Glutathione, which is covalently coupled to the agarose beads, GST-fusion proteins are bound after incubation with the Glutathione Sepharose beads.

BL21 pLysS bacteria are transformed with the respective pGEX plasmid encoding the GST-fusion protein (see 2.2.1.2). A single colony is used to inoculate a 20ml LB medium (+ selective antibiotic) preculture, which is incubated on a 37°C shaker o/n. Prewarmed 200ml LB medium is inoculated with 4 ml of the preculture and grown until the culture reaches a OD_{600} of 0.5-1. Expression of the fusion protein is induced by the addition of IPTG to a final concentration of 0.5mM. Induction is performed for 3-4h on room temperature (RT) or o/n at 16°C. After centrifugation (10 min at 5000rpm), the pellet is resuspended in 10 ml ice-cold 1x PBS supplemented with protease inhibitors (2µg/ml of Aprotinin, Leupeptin, Pepstatin and Pefabloc) and the bacterial cell walls are ruptured by sonification (3x 40 sec; cool on ice in between). After addition of 1% (final concentration) Triton X-100 on a vortexer, the solution is incubated for 1h on a shaker at RT and centrifuged 10 min at 10000rpm. The supernatant is added to 500-800 µl Glutathione Sepharose 4A beads (previously washed 3 times with PBS) and incubated for 1h on a shaker at RT. Then the beads are washed 3 times with 10ml ice-cold PBS. The loaded beads can be kept in 1x PBS on 4°C or can be used to elute the GST-fusion protein.

2.4.3 SDS-PAGE and Western Blotting

For SDS-PAGE 5-20µg of total protein is boiled with 1xSDS sample buffer (NuPAGE LDS sample buffer, *Invitrogen*, Paisley, UK) and 1x reducing agent (NuPAGE sample reducing agent, *Invitrogen*, Paisley, UK) for 10 min and then transferred on ice. Samples are loaded onto a 4-12% Bisacrylamid gel or a 12% Bisacrylamid gel (NuPAGE Novex Mini Gels, *Invitrogen*, Paisley, UK) and the proteins are separated at 200V for 50 min in SDS running buffer (NuPAGE MOPS SDS Running Buffer, *Invitrogen*, Paisley, UK).

Separated proteins are transferred onto a Protan nitrocellulose membrane (*Schleicher & Schuell*, Dassel, Germany) in transfer buffer (0.025-0.1% SDS, 25mM Tris, 192mM Glycine supplemented with 20% methanol) at 100V for 1h at RT. After washing in TBST (150mM NaCl, 10mM Tris, pH 7.4) the membrane is blocked in 5% milk powder in TBST for 1,5-2h and incubated with primary antibody over night at 4°C. After four washing steps, the HRP-

conjugated secondary antibody is applied for 2h at RT and it is detected using the BM Chemiluminescence Blotting Substrate (POD) from *Roche Diagnostics* (Mannheim, Germany).

2.4.4 In vitro GEF-binding assay

The initial binding of a GEF protein to the inactive GTPase-GDP complex occurs with a relatively low affinity. However, after dissociation of the GDP, the GEF stabilizes the nucleotide depleted GTPases because both proteins form a very short life high-affinity complex until the GTPase binds to GTP. The high affinity of the GEF for the nucleotide depleted form of the substrate GTPase can be used for an *in vitro* GEF binding assay (Blanke and Jackle, 2006).

For this purpose, GST-GTPase fusion proteins are expressed in bacteria and purified using glutathione agarose beads (see 2.4.2). Lysates from embryos expressing an HA-tagged GEF variant [in the mesoderm] are produced (see 2.4.1). For each pull-down reaction, 45µl Glutathione agarose beads coated with the respective GTPase are equilibrated in binding buffer (25mM HEPES-KOH pH 7.6, 100mM NaCl, 0.1mM EDTA, 0.01% IPEGAL, 10% Glycerol) by 2-3 short washing steps. Then the beads are transferred into binding buffer supplemented with 5mM EDTA (25mM HEPES-KOH pH 7.6, 100mM NaCl, 5mM EDTA, 0.01% IPEGAL, 10% Glycerol) and incubated for 10 min at RT and for further 20 min at 4°C. During this incubation, Mg^{2+} ions are captured by EDTA leading to a release of the bound nucleotides from the GTPase molecules (Mg^{2+} is required for the stabilization of the GTPase-GDP/GTP complex). After two washing steps in binding buffer supplemented with 5mM EDTA, approximately 1mg total protein of embryo lysate is added to the 45µl of GTPase loaded glutathione beads. The volume is adjusted to at least 200-500µl total volume with binding buffer supplemented with 5mM EDTA to avoid the drying of beads during the subsequent incubation time: The tubes are incubated for at least 2h (better: over night!) on a roller at 4°C. After extensive washing steps (6-8x) with washing buffer (25mM HEPES-KOH pH 7.6, 500mM NaCl, 12.5mM $MgCl_2$, 0.1mM EDTA, 0.05% IPEGAL, 10% Glycerol) the samples are separated by SDS-PAGE. After Western Blotting, possibly bound GEF fusion proteins are detected using a rat-anti-HA antibody (dilution 1:500-1:800, clone '3F10' from *Roche Diagnostics*, Mannheim, Germany).

2.5 *Drosophila* genetics

Flies are kept on standard medium at either 18°C, room temperature or 25°C. Cups for egg collection are kept on 25°C for 5h (migratory stages) or for 9.5h or overnight (for staining of pericardial cell clusters). Embryos are staged according to Campos-Ortega and Hartenstein (1997).

2.5.1 Fly stocks, chromosomes and mutant alleles

The following fly stocks, alleles and chromosomes were used for this thesis:

name	genotype	source
w^{1118}	w^{1118};;	Bloomington stock centre
$Cdc42^{4}$	$Cdc42^{4}$/FM6;;	Bloomington stock centre
twiG4, CD2	twi::Gal4, twi::CD2	Bloomington stock centre
GMR::Gal4	;GMR::Gal4;	Bloomington stock centre
Rho1[l(2)k07236]	w; Rho1[l(2)k07236]/CyO;	Bloomington stock centre
twi::G4	;twi::Gal4;	Bloomington stock centre
twiG4, Dmef2G4	;twi::Gal4(2x);Dmef2::Gal4;	Bloomington stock centre
pbl^{11D}	pbl^{11D}/TM3(ftz::lacZ)	Bloomington stock centre
pbl^{3}	pbl^{3}/TM3(ftz::lacZ)	Bloomington stock centre
htl^{AB}	htl^{AB}/TM3(ftz::lacZ)	Bloomington stock centre
pbl^{NR}	w;; Df(3L)pblNR/TM3(ftz::lacZ)	Bloomington stock centre
$Rac1^{J10}$, $Rac2^{\Delta}$, FRT2A, Mtl^{Δ}	y,w;;$Rac1^{J10}$, $Rac2^{\Delta}$, FRT2A, Mtl^{Δ} /TM3(ftz::lacZ)	Bloomington stock centre
hs::Flp	y, w, hs::Flp;; cx^{D}/TM3	Bloomington stock centre
ovoD	P[ovoD1-18]3L, FRT2A/βTub85D/TM3	Bloomington stock centre
$pbl^{\Delta BRCT}$.myc	UAS::$pbl^{\Delta BRCT}$.myc/TM3(ftz::lacZ)	R. Saint, Canberra (Smallhorn et al., 2004)
$RhoL^{N25}$	UAS::RhoL.N25/CyO	Bloomington stock centre
$RhoL^{V20}$	UAS::RhoL.V20	Bloomington stock centre
$Rac1^{V12}$	y,w;;UAS::Rac1.V12	Bloomington stock centre
$Rac1^{N17}$	y,w;;UAS::Rac1.N17	Bloomington stock centre
$Rac1^{wt}$	y,w;UAS::Rac1.L;	Bloomington stock centre
$Rac2^{wt}$	EP(3)3118/TM3	Szeged *Drosophila* stock centre
If/CyO;MKRS/TM6	w;If/CyO(wg::lacZ); MKRS/TM6	Müller lab
If/CyO lz;TM6/TM3 lz	w;If/CyO(wg::lacZ); TM6/TM3(ftz::lacZ)	A. Bachmann, Düsseldorf
Rac1.Myc	y,w,UAS::Rac1.Myc;;TM3/Tm6	Bloomington stock centre
$Rho1^{V14}$	w; UAS::Rho1.V14;	Bloomington stock centre
$Rho1^{wt}$	w;; UAS::Rho1.Sph	Bloomington stock centre

Table 2.5: Employed fly stocks, mutations and balancer chromosomes.

2.5.2 The UAS/Gal4 system

The UAS/Gal4-system was developed in 1993 by A. H. Brand and N. Perrimon as a tool for temporal and spatial restricted expression of transgenes (Brand et al., 1994). The system employs the GAL4 transcriptional activator from yeast, which is expressed under the control of specific enhancer elements from endogenous *Drosophila* proteins. Transgenic lines that contain such a specific Gal4 construct are called "driver lines". The expression of the construct of interest is controlled by the Gal4 target sequence from yeast, the UAS sequence (upstream activating sequence). It is cloned 5' of the insert of interest and controls the transcription of the transgene in the so-called "effector line". If the effector line is crossed to flies of the driver line, the Gal4 protein will be expressed in the progeny of this cross in a specific pattern. This subsequently drives the expression of the target construct in the respective cells. By choosing driver lines that use enhancer elements of a protein that is normally expressed only in a subset of cells to a specific time point during development, the effector construct can be expressed in the same spatio-temporal pattern. Hence, usage of a *twist*::Gal4 driver line, for example, is a suitable tool to express a UAS-construct in the mesoderm of developing embryos.

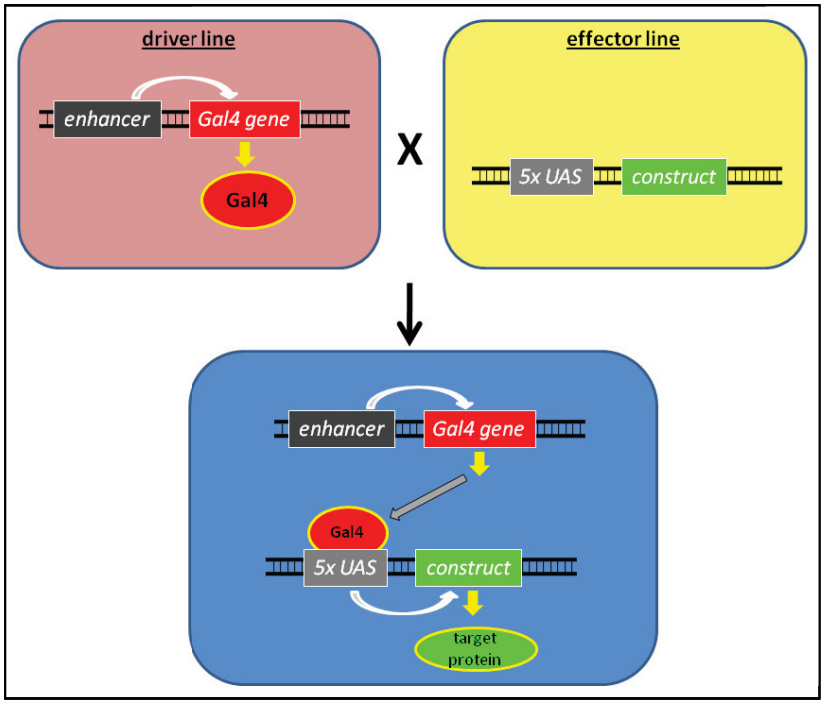

Fig. 2.1: Schematic representation of the UAS/Gal4 system.
(see text for details)

2.5.3 Crossings for the production of Rac germline clones using the FRT/Flp system

The following crosses are performed in order to generate germline clones that completely lack Rac1 and Rac2 activity and have reduced levels of Mtl (for detailed genotypes of the flystocks see 2.5.1):

♀ *y,w, hs::Flp ; ; cx^{D}/TM3* **X** +/Y; ; *ovoD, FRT2A/TM3* ♂

♀ *y,w ; ; Rac1^{J10}, Rac2$^{\Delta}$, FRT2A, Mtl$^{\Delta}$/TM6* **X** *y,w,hs::Flp/Y ; ; ovoD, FRT2A/TM3* ♂

(L3 larvae: 2xHS for 2h at 37°C)

♀ *y,w,hs::Flp/y,w ; ; Rac1^{J10}, Rac2$^{\Delta}$, FRT2A, Mtl$^{\Delta}$/ovoD, FRT2A*

X *y,w ; ; Rac1^{J10}, Rac2$^{\Delta}$, FRT2A, Mtl$^{\Delta}$/TM6* ♂

germline clone genotypes:

1. *y,w,hs::Flp/y,w ; ; Rac1^{J10}, Rac2$^{\Delta}$, FRT2A/Rac1^{J10}, Rac2$^{\Delta}$, FRT2A, Mtl$^{\Delta}$*
2. *y,w,hs::Flp/y,w ; ; Rac1^{J10}, Rac2$^{\Delta}$, FRT2A, Mtl$^{\Delta}$/Rac1^{J10}, Rac2$^{\Delta}$, FRT2A, Mtl$^{\Delta}$*
3. *y,w,hs::Flp/y,w ; ; Rac1^{J10}, Rac2$^{\Delta}$, FRT2A/TM6* [paternal rescue]
4. *y,w,hs::Flp/y,w ; ; Rac1^{J10}, Rac2$^{\Delta}$, FRT2A, Mtl$^{\Delta}$/TM6* [paternal rescue]

2.5.4 Crossings for testing genetic interactions between Pbl$^{\Delta BRCT}$ and Rho GTPases

For the genetic interactions between the Rho GTPases Rac1 and Rho1 and the Pbl construct Pbl$^{\Delta BRCT}$ two different sets of crosses have to be performed. As in both cases the crosses and therefore the genetic backgrounds are slightly different, two control crosses are set up in order to have the same genetic conditions as in the respective interaction test cross.

For the genetic interactions between Rac1 and Pbl$^{\Delta BRCT}$, the following crosses are analyzed:

twi::Gal4;pbl^{3}/TM3(ftz::lacZ) **X** *;; UAS::Pbl$^{\Delta BRCT}$,pbl^{3}/TM3(ftz::lacZ)* [control]

twi::Gal4;pbl^{3}/TM3(ftz::lacZ) **X** *UAS::Rac1.L;; UAS::Pbl$^{\Delta BRCT}$,pbl^{3}/TM3(ftz::lacZ)* [interaction]

For the interaction between Rho1 and Pbl$^{\Delta BRCT}$, a stock stably expressing the Pbl construct had to be established. Therefore, the genetic conditions of the following crosses are different from the ones above and require the additional control cross:

twi::Gal4; UAS::Pbl$^{\Delta BRCT}$,pbl^{3}/TM3(ftz::lacZ) **X** *;; pbl^{3}/TM3(ftz::lacZ)* [control]

twi::Gal4; UAS::Pbl$^{\Delta BRCT}$,pbl^{3}/TM3(ftz::lacZ) **X** *;UAS::Rho1.Sph; pbl^{3}/TM3(ftz::lacZ)* [interaction]

2.6 Histological methods

2.6.1 Used antibodies

antibody	dilution	source
primary antibodies		
rabbit α ß-Gal	1:1000	*Cappel*
mouse α ß-Gal	1:100	*DSHB*
mouse α CD2	1:500	*Serotec*
mouse α GFP	1:800	*ABCAM*
rabbit α Myc	1:35	Santa Cruz
mouse α HA	1:1000	*Roche*
mouse α Eve	1:100	*DSHB*
rat α Pbl	1:350	Müller lab (Schumacher, 2005)
rat α DN-Cadherin	1:50	*DSHB*
mouse α Rho1	1:50	*DSHB*
rabbit α Twist	1:1000	Müller lab
secondary antibodies		
donkey α rabbit Alexa488	1:200	*Molecular Probes*
goat α rabbit Cy3	1:200	*Jackson ImmunoResearch*
goat α mouse Alexa488	1:200	*Molecular Probes*
donkey α mouse Cy3	1:200	*Jackson ImmunoResearch*
goat α rat Alexa647	1:200	*Molecular Probes*
goat α rat Alexa488	1:200	*Molecular Probes*
donkey α rat Cy3	1:200	*Jackson ImmunoResearch*
goat α rabbit Biotin	1:200	*Vector Laboratories*
goat α mouse Biotin	1:200	*Vector Laboratories*
goat α rabbit-AP	1:800	*Dianova*
goat α mouse-AP	1:800	*Dianova*
fluorescent dye		
DAPI	1:1000 (stock:1mg/ml)	*Sigma-Aldrich*

Table 2.6: Antibodies and fluorescent dyes employed for the staining of embryos.

2.6.2 Immunocytochemistry

Embryos were obtained, fixed, stained and sectioned as described in here and in (Muller, 2008).

2.6.2.1 Fixation of embryos

Embryos from 5h, 9.5h or o/n egg-collections are dechorionized with diluted sodium hypochlorite (NaOCl) and washed with H_2O. Then the embryos are transferred into scintillation vials filled with 3ml fixative (4% Formaldehyde in 1x PBS or Stefanini-Fix:

1.1ml 37% Formaldehyde, 1.5ml Picric acid, 1.5ml 0.5M PIPES, 5.9ml dH_2O) and 3ml Heptane and incubated for 25 min at RT on a shaker. After discarding the lower aqueous phase (fixative) 3ml of methanol *p.a.* are added and the vial is shaken vigorously for 30 sec to pop the embryos out of their vitelline membranes. All embryos that settle down in the methanol phase (the lower one) are transferred into a reaction tube and washed three times with fresh methanol *p.a.*. Embryos in methanol can be stored at -20°C.

2.6.2.2 Antibody staining of embryos

Embryos stored in methanol are washed three times with PBT (3x 10-15min) and then blocked in 5% goat serum in PBT for 2h at RT (shaker). Then the embryos are incubated in primary antibody solution (antibodies +5% goat serum in PBT) o/n at 4°C. The following day the embryos are washed four times for 10-15 min with PBT before the secondary antibody solution (secondary antibodies [+ DAPI in case of fluorescence staining] in 5% goat serum) is applied. After a 2h incubation on a shaker at RT, three to four additional washing steps with PBT are performed.
Embryos that are stained with flurophore-conjugated antibodies can directly be mounted in Mowiol/DAPCO.

In case HRP stainings should be performed, an aliquot of the avidin-biotin- enhancer system (Vectastain ABC kit from *Vector Laboratories*, Burlingame, USA) is prepared after the first of the previously mentioned three to four washing steps. Therefore, 500µl of PBT are mixed with 5µl of solution A and afterwards 5µl of solution B are added and the solution is mixed again followed by a 30 min incubation at RT. After the last washing step, the AB solution is added to the embryos and left on a shaker for 45min (RT). After three 15 min washing steps with PBT, the staining solution is prepared: 500µl of a 1mg/ml DAB stock (3,3-Diaminobenzidine-tetrachloride, *Sigma-Aldrich,* Steinheim, Germany) are added to 500µl PBS. After addition of 2µl 10% H_2O_2, the staining solution is added to the embryos and the staining reaction is controlled under a dissecting microscope. To stop the reaction, the embryos are briefly washed twice with PBT.

In case an (additional) AP staining should be performed, the embryos are washed twice for 5 min in PBT and then two times in AP-buffer for 10 min (100mM NaCl, 50mM $MgCl_2$ 100mM Tris pH 9.5 and 0.1% Tween 20, which is added prior to use). The AP-staining solution is prepared by mixing 500µl AP-buffer with 1.7µl BCIP and 1.5µl NBT and it is added to the embryos. The staining reaction is controlled by eye under the dissecting microscope and stopped by addition of PBT.

After the HRP or AP staining embryos are washed 3x 10min with PBT and then incubated in 30%, 50%, 70%, 95% and 100% ethanol (5min each) followed by an incubation in 100% acetone for 5-10 min. Afterwards, a 1:1 solution of araldite and acetone is added to the embryos and the closed reaction tube is left o/n at 4°C. The following day the embryos are transferred onto a slide with as little araldite/acetone as possible and oriented using an eyelash. The slide is incubated o/n at 65°C. After this step, the embryos are mounted in 100% araldite, which is hardened at 65°C o/n.

50g araldite: 27.175g Durcupan component A/M and 23.705g Durcupan component B are incubated on an overhead shaker for 1h and then 1.75g Durcupan component C and 1g Durcupan component D is added and incubated on an overhead shaker for another hour.

2.6.2.3 Semi-thin sections of stained embryos

HRP- or AP- stained embryos are processed as described in 2.6.2.2. After the o/n incubation in 1:1 araldite acetone, the embryos are transferred onto a slide as well. However, this time an eyelash is used to sort the embryos and to transfer and align 1-4 embryos in a special mould filled with a bit of araldite. After orienting the embryos in the tip of these grooves, they are completely filled with araldite and the casted blocks are hardened on 65°C o/n.
Then the araldite blocks can be used to prepare 5µm thin sections of the embryos employing a microtome (OmU2, *Reichert*). The sections are mounted in araldite and hardened o/n at 65°C again.

2.6.2.4 Preparation of embryos for live cell imaging

Embryos from egg-collections of the desired age (e.g. a 30 min collection) are dechorionized with diluted sodium hypochlorite (NaOCl) and washed thoroughly with H_2O. The embryos are transferred on agar blocks and aligned in the same a/p orientation under a dissecting microscope. A drop of “fly glue” (see 2.3.2) is pipetted on a thick coverslip. After drying of the glue the aligned embryos are carefully pressed against the coverslip. Then the coverslip with the attached embryos is put into a drying chamber (Petri dish with blue Silica gel from *Sigma-Aldrich* (Steinheim, Germany)) for 5-10 min. The embryos are covered with halocarbon oil 700 and imaged on an inverse confocal microscope without adding a slide. Alternatively the embryos can also be mounted normally in halocarbon oil 27S on a slide using small spacers to avoid a squeezing of the embryos by the coverslip. After sealing the edges of the coverslip with nail polish the embryos can be imaged on a regular confocal microscope.

2.6.3 Preparation of adult fly heads for scanning electron microscopy

For the preparation of adult fly heads, flies are decapitated with a sharp razor blade and the heads are collected in reaction tubes containing PBS to prevent drying-out. For dehydration the heads are subsequently incubated in 30%, 50%, 70%, 80%, 96% and 100% ethanol *p.a.* for 5-10min each (heads should always be covered with a bit of ethanol). Then the heads are incubated o/n at 4°C or for 20min at RT in 100% acetone. Afterwards, they are incubated in a 1:1 mixture of acetone and tetramethylsilane (TMS; *Sigma-Aldrich,* Steinheim, Germany) for 30 min (TMS evaporates at RT, therefore keep on 4°C!) followed by a 30 min incubation in 1:2 acetone-TMS and a final 30 min step with pure TMS. Then most of the TMS is replaced by fresh TMS and the tube is left open o/n to allow the evaporation of the TMS and the drying of the tissue.

Heads are cut in halves with a razor blade and are mounted on stubs using adhesive tabs. Specimen are coated either with Au/Pd (65nm) using a *Cressington* 208HR sputter coater or sputtered with Au employing a Balzers Union sputter (*Balzers*, Lichtenstein; for 3 min at 25 mA, 0.1Torr) and examined using a *Philips* XL30 or a LEO Supra scanning electron microscope.

3 Results

The Rho GEF Pbl plays an essential role during two distinct biological processes. On the one hand, the protein was shown to be required for the migratory process of the mesoderm in the early *Drosophila* gastrula. On the other hand, Pbl was identified as a central component of a conserved signalling pathway during the process of cytokinesis. Although the role of Pbl in the cytokinesis pathway is relatively well understood, not much is known about the signalling pathways that are utilized by the protein to fulfill its migratory function. Previous work implicated that the known cytokinesis signalling cascade via Rho1 is rather unlikely to be activated by Pbl during migration. Therefore, a central question of this thesis was to characterize the mechanisms of Pbl function during mesoderm migration in more detail in order to get insight into the pathways that act downstream of Pbl in this context. Another important question is how the protein is regulated in order to fulfill its two distinct functions as both processes take place in the same cells in a very narrow time window [there are two mitotic divisions during the migration of the mesoderm cells (Foe, 1989)]. A possible explanation for this would be the idea that both processes depend on different isoforms of Pbl. Indeed several isoforms of the GEF have already been identified. Nevertheless, none of these isoforms has shown a mesoderm specific expression pattern in the early embryo which would be a hint for the existence of a migration specific Pbl isoform (Schumacher, 2005). In addition, expression of a transgene encoding the Pbl isoform A is sufficient to rescue both processes in a *pbl* mutant background (see below and Schumacher et al., 2004) indicating that at least this isoform is able to fulfill both functions. This renders it rather unlikely that the simple model of isoform specific functions is correct. Therefore, an alternative explanation seems to be more reasonable: both functions are mediated by different domains or different combinations of domains.

An appropriate way to assess this possibility is to perform a structure-function analysis of the Pbl protein. To this end, a set of different constructs was generated, in which specific parts of the protein were deleted or which encoded only distinct domains of Pbl (Fig. 3.1). By using suitable expression-vectors for the cloning of the constructs, all of these various constructs carried an HA- or GFP-tag, which allowed a comparison of the expression levels of the different transgenic lines and localization studies of their respective proteins.

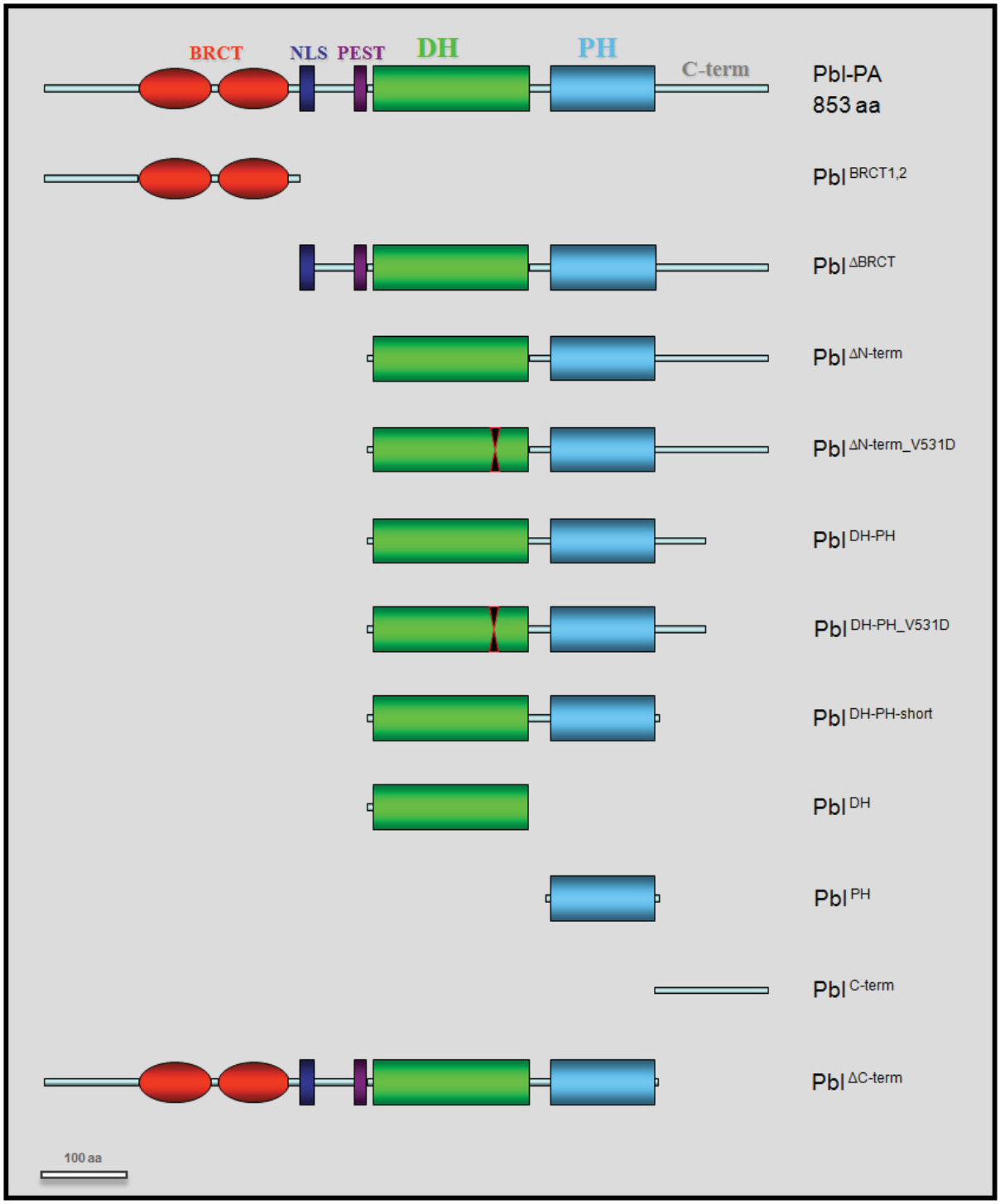

Fig. 3.1: Domain structure of different Pbl constructs used in this study.

The Pbl-PA full-length construct contains the complete open reading frame of the Pbl isoform A. Extensive truncation of the C-terminal half of the protein leads to the $Pbl^{BRCT1,2}$ construct encoding only the N-terminus including both protein-protein interaction domains. Deletion of the N-terminal BRCT domains creates the $Pbl^{\Delta BRCT}$ construct (Smallhorn et al., 2004). Additional deletion of the NLS and PEST motives gives rise to $Pbl^{\Delta N\text{-}term}$. The $Pbl^{DH\text{-}PH}$ construct encodes for the regulatory DH-PH tandem domain and a short stretch of the C-terminal tail. By contrast, $Pbl^{DH\text{-}PH\text{-}short}$ lacks the whole C-terminal part of the Pbl protein. Through missense mutation of the essential Valine at position 531 within the DH domain to aspartic acid (representing the same lesion as in the loss of function allele *pbl*[5]), catalytically inactive versions of the $Pbl^{\Delta N\text{-}term}$ and the $Pbl^{DH\text{-}PH}$ constructs were generated ($Pbl^{\Delta N\text{-}term\_V531D}$ and $Pbl^{DH\text{-}PH\_V531D}$). The Pbl^{DH} construct encodes the catalytic DH domain, while Pbl^{PH} only codes for the Pleckstrin-homology domain. The carboxy-terminal tail, which does not contain any known domain structure, is encoded by the $Pbl^{C\text{-}term}$ construct while deletion of this protein part in the full-length construct gives rise to $Pbl^{\Delta C\text{-}term}$.

To identify the domains of Pbl that are required and essential for its migration function, rescue- as well as overexpression experiments were conducted. In addition, the subcellular localization of different constructs was analyzed in detail to characterize the thus far unknown interphase localization pattern of Pbl in the mesoderm and to identify domains of the protein that are required in this context.

As shown in Fig. 3.1, most of the constructs comprise the catalytic DH domain of the GEF. The reason for this is the previous finding that point mutations, which render the DH domain inactive (*pbl*^{5} allele), block both Pbl functions (Schumacher et al., 2004; Smallhorn et al., 2004). In addition, a rescue construct that carried a deletion within the DH domain was found not to be sufficient to fulfill any of Pbl's functions anymore (Smallhorn et al., 2004). Therefore, the GEF activity is essential for both, the cytokinesis as well as the migration function of the protein and hence all constructs used for recue experiments should include this catalytic domain of Pbl.

3.1 Identification of the essential protein domains required for the migratory function of Pbl

3.1.1 Expression of a full-length Pbl transgene rescues migratory defects in *pbl* mutant embryos

The basis for a structure-function analysis of the Rho GEF Pbl was provided by the previous finding that a mesoderm specific expression of the PblA cDNA using the UAS/Gal4 system is capable of rescuing both, migration as well as cytokinesis defects in a *pbl* mutant background (Schumacher et al., 2004). By using a suitable marker that allows a quantification of the migration ability of the cells this system represents an excellent tool to identify the protein domains of Pbl that are involved in and essential for migration. To measure migration, the presence of mesodermal derivatives was scored. Embryos were stained against the transcription factor Eve, which is normally expressed only in cells that have reached the very dorso-lateral positions during the migratory process. Eve is expressed segmentally in 11 cell clusters on each side of the embryo and each cluster contains three cells (Frasch et al., 1987). Unlike Htl, Pbl is not directly involved in the specification of these pericardial cells, as even in a *pbl* loss of function background single Eve-positive cells are occasionally found (Carmena et al., 1998; Michelson et al., 1998b; Schumacher et al., 2004; Smallhorn et al.,

2004). Therefore, the lack of any Eve-positive clusters on either side of the embryo reflects earlier problems during spreading of the mesoderm and can be used as a quantitative measure for the migration potential of mesoderm cells.

Expression of an HA-tagged full-length Pbl version (PblA-HA) is capable of rescuing the defects in *pbl* mutants as it was described previously for an untagged version of the protein (Schumacher et al., 2004). Compared to the strong pbl^3 allele, which on average gives rise to only 1.7 Eve clusters per embryo, the number of pericardial cells was rescued to 18.6 clusters after mesoderm specific expression of the fusion protein (Tab. 3.1, Fig. 3.2 B,C and Fig. 3.4). Loss of Pbl function causes cytokinesis defects resulting in multinucleated cells, in which the nuclei eventually fuse and give rise to a single, relatively large nucleus (Fig. 3.2 D). After expression of the transgene however, the size of the nuclei in the three cells of each Eve-cluster was much smaller suggesting that cytokinesis was rescued under these conditions (Fig. 3.2 D'). To narrow down the region of the protein that is essential and sufficient for Pbl's migratory function different deletion constructs were subsequently expressed and their rescue capacities analyzed.

3.1.2 The BRCT domains are not essential for migration

The two N-terminal BRCT domains play essential roles during cytokinesis by mediating protein-protein interactions that lead to the localized activity of Pbl at the site were the contractile ring is formed (Somers and Saint, 2003). However, expression of a truncated Pbl version lacking these regulatory domains is still able to rescue migration (Smallhorn et al., 2004). As this $Pbl^{\Delta BRCT}$ construct was only tested with a non-mesoderm specific driver line so far and in a background that presumably was not completely "null" for *pbl* (Schumacher, 2005), the rescue experiment was repeated using the *twi::Gal4; pbl^3* driver line, which was employed for all mesoderm specific rescues in this thesis. As described previously, expression of this deletion construct indeed rescues the migration in a *pbl* mutant background, as the number of Eve-positive cell-clusters is restored to 8,3 per embryo (Tab. 3.1, Fig. 3.4). Although the rescue is not as good as the one using the full-length protein, this result suggests that for Pbl's function during migration, the BRCT domains are dispensable. Nevertheless, cytokinesis is not rescued by expression of this truncated protein (Smallhorn et al., 2004). This is reflected by the size and the shape of the Eve-positive nuclei, which still appear to be larger than normal in these embryos (Abb. 3.2 E). Therefore, in contrast to mesoderm migration, the interactions mediated by the BRCT domains are essential for Pbl's function during cytokinesis. However, this does not rule out an additional function for these domains

during migration, as the rescue with the full-length protein containing the BRCT domains is much more effective than the one using the $Pbl^{\Delta BRCT}$ construct (Fig. 3.4).

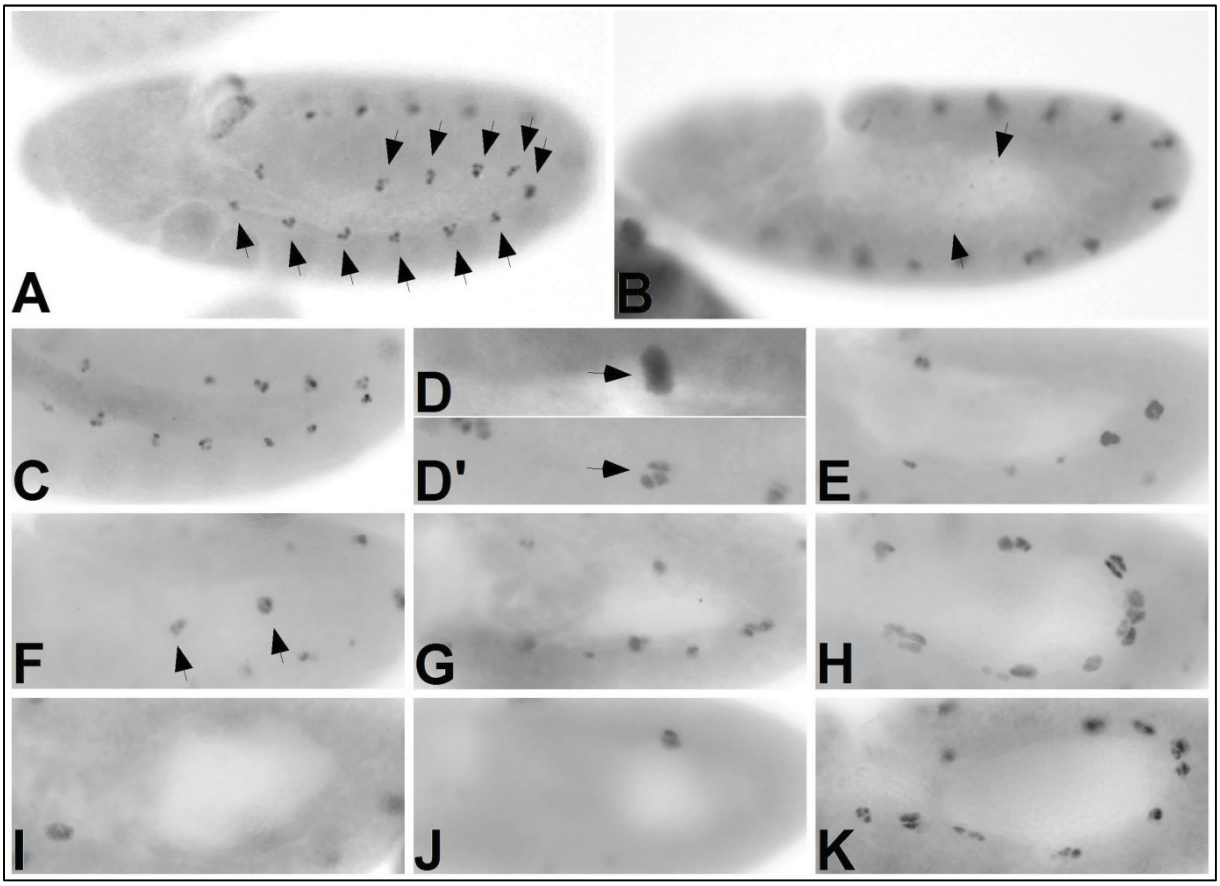

Fig. 3.2: Rescue capacity of different Pbl variants assessed by anti-Eve staining.
(A) Eve is expressed in 11 dorsal mesodermal cell clusters on each side of the embryo in wild type (arrows). (B) In *pbl*^{3} mutant embryos the number of Eve clusters is strongly reduced (dorsal positions marked by arrows). (C-K) Expression of various Pbl constructs using *twi::Gal4* reveals the DH-PH domains as the smallest entity sufficient to rescue migration in *pbl*^{3} mutant embryos. (C) Loss of Eve clusters can almost completely be rescued by expression of full-length Pbl. Cytokinesis defects in *pbl*^{3} embryos result in large, fused nuclei (D), a phenotype that is completely rescued by expression of PblA-HA (D'). (E) $Pbl^{\Delta BRCT}$ expression; (F) $Pbl^{\Delta N\text{-term}}$ expression (arrows mark Eve-positive nuclei in the mesoderm); (G) $Pbl^{DH\text{-}PH}$ expression; (H) $Pbl^{DH\text{-}PH\text{-}short}$ expression; (I) $Pbl^{DH\text{-}PH\_V531D}$ expression; (J) Pbl^{DH} expression; (K) $Pbl^{\Delta C\text{-}term}$ expression.

3.1.3 The catalytic DH-PH tandem domain is the smallest entity providing rescue activity for migration

To investigate whether further domains of the protein are dispensable for Pbl's activity during migration, another deletion construct, called $Pbl^{\Delta N\text{-}term}$, was expressed in *pbl* mutants. This construct does not only lack the BRCT domains but also the NLS and the PEST motif.

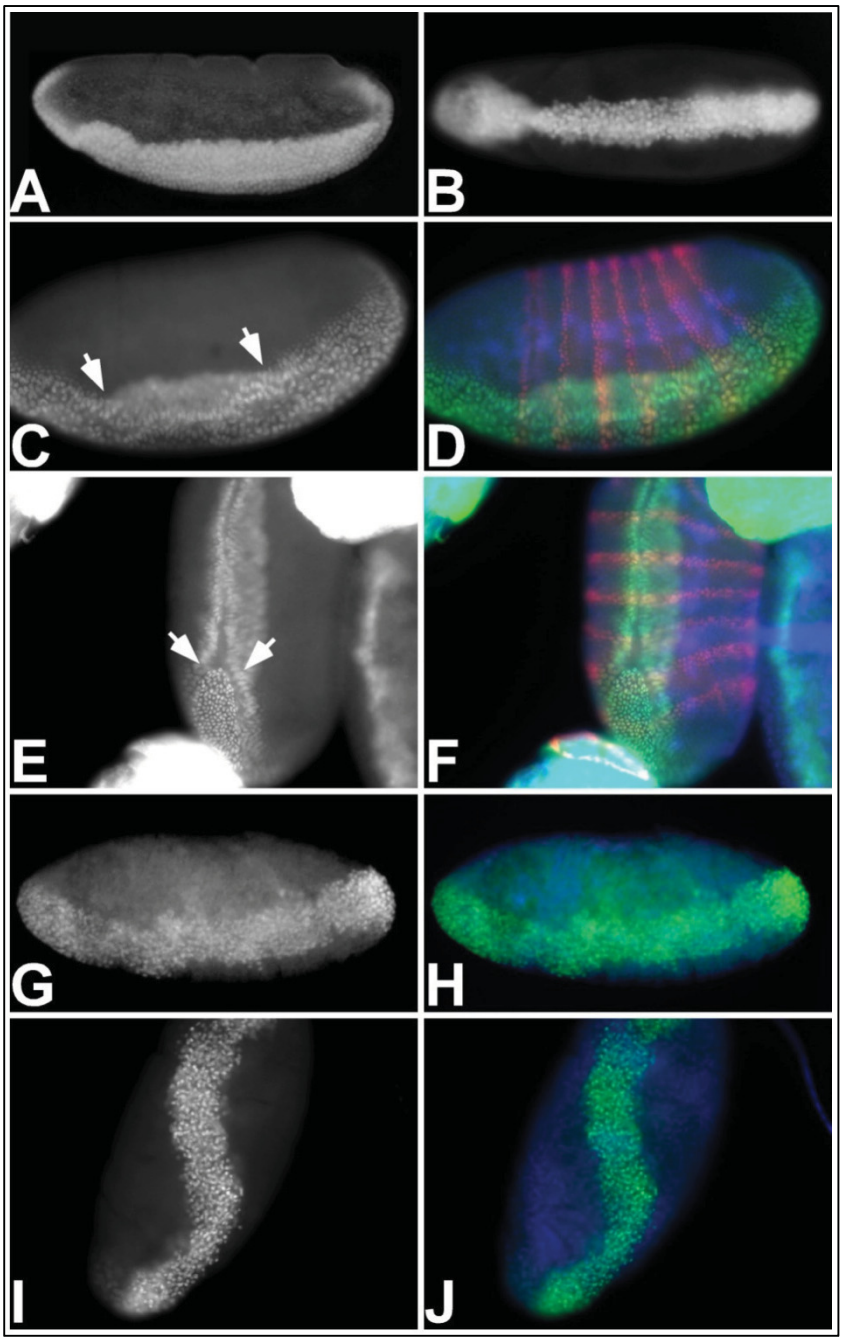

Fig. 3.3: Expression of Pbl$^{\Delta N\text{-term}}$ in a *pbl*^{3} mutant background causes dominant effects during invagination.
(A-J) Embryos were stained against Twi (A-C,E,G,I and green in merged images), DAPI (blue in merged pictures) and Eve (red in B and D). Wild type embryos at stage 6 (A) and 7 (B). Expression of Pbl$^{\Delta N\text{-term}}$ results in incomplete (arrows in C flank internalized part of the mesoderm) or misshaped invagination furrows (arrows in E). As a result, the mesoderm cells remain at the surface of the embryo (G,H) and frequently cause problems during germband elongation (I,J).

Expression of Pbl$^{\Delta N\text{-term}}$ did not lead to a rescue of mesodermal differentiation defects in *pbl*^{3} mutant embryos, as the average number of Eve-positive cell clusters was only about 3.3 (Fig. 3.2 F, Fig. 3.4, Tab. 3.1). Instead, an abnormal early mesoderm development was observed in these embryos (Fig. 3.3). During invagination of the mesoderm, the furrow was

not present over the whole length of the mesoderm (Fig. 3.3 C,D) or had an abnormal shape (Fig. 3.3 E,F). This resulted in an abnormal internalization of the mesoderm cells and even in older embryos the mesoderm remained at the surface of the embryo (Fig. 3.3 G-J). Such invagination problems never occur in *pbl* mutant embryos (Schumacher and Müller, unpublished), therefore the defects displayed here are likely to be a result of a dominant activity of the expressed construct (see below) that does not reflect an involvement of the endogenous protein in these early processes. In addition, no signs for a rescue of the cytokinesis phenotype were observed under these conditions.

A further construct lacking most of the conserved C-terminal tail of the protein and encoding only the catalytic DH-PH tandem domain showed suppression of the migration but not of the cytokinesis defects in this rescue assay (Fig. 3.2 G, Fig. 3.4). The number of Eve-positive cell clusters reached on average 8.9 (Tab. 3.1). This result indicates that a functional NLS motif is not essential for the migration function of the protein. This is an important finding, as Pbl has only been described to localize to the nucleus in interphase cells thus far (Prokopenko et al., 1999; Prokopenko et al., 2000a). If Pbl acted within the nucleus during mesoderm migration, any construct unable to enter the nucleus should fail to rescue the migration defects. Therefore, these results strongly suggest that Pbl has to be active in the cytoplasm during spreading. A detailed analysis of the localization of the Pbl protein in interphase cells can be found below (chapter 3.3).

The presence of the complete C-terminus in the $Pbl^{\Delta N\text{-}term}$ construct had a negative effect on the rescue capacity of the DH-PH domains (compare $Pbl^{\Delta N\text{-}term}$ and $Pbl^{DH\text{-}PH}$ in Fig 3.4). To rule out that the remaining part of the C-terminal tail in the $Pbl^{DH\text{-}PH}$ construct also contributes positively or negatively to the rescuing activity of the protein, the $Pbl^{DH\text{-}PH\text{-}short}$ construct, lacking the whole C-terminus (Fig. 3.1), was expressed. As for the $Pbl^{DH\text{-}PH}$ construct, expression of this Pbl variant also led to a suppression of the migration defects but to no rescue of cytokinesis (Fig. 3.2 H, Fig. 3.4). On average 7.1 Eve-positive cell clusters were found in these embryos (Tab. 3.1). This indicates that the DH-PH domains alone seem to be responsible for the rescue activity of the $Pbl^{DH\text{-}PH}$ construct. Furthermore, it can be concluded that the cause for the dominant effects seen in the $Pbl^{\Delta N\text{-}term}$ rescue experiments is most likely part of the second half of the C-terminus, which is absent in the $Pbl^{DH\text{-}PH}$ construct. The fact that the complete deletion of the C-terminal tail in $Pbl^{DH\text{-}PH\text{-}short}$ leads to a slightly weaker rescue is not necessarily a proof for a requirement of these sequences for the rescue capacity, as these small differences could also be the result from subtle differences in the expression levels of the constructs.

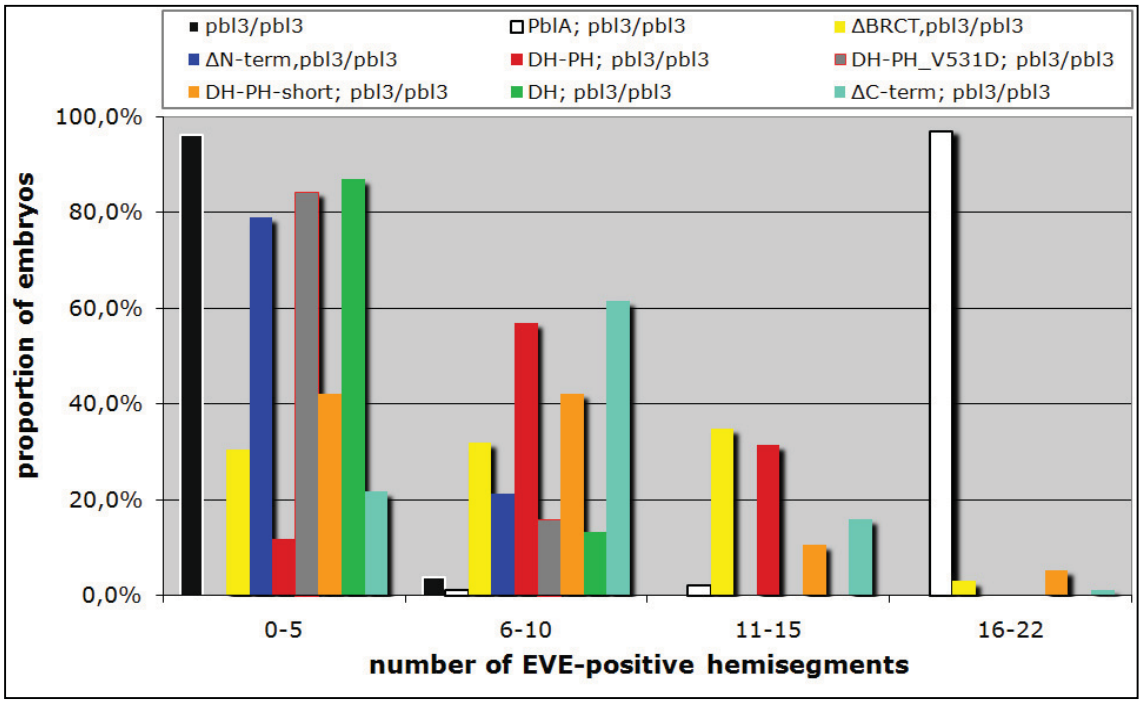

Fig. 3.4: Potential of different Pbl constructs to rescue migration in a *pbl*^{3} mutant background.
After expression of different Pbl constructs in a *pbl*^{3} mutant background the numbers of Eve-positive cell clusters were counted, organized in 4 groups (X-axis) and plotted against the relative proportion of embryos (Y-axis). The values for *pbl*^{3} homozygotes are shown in black, the full-length rescue in white, Pbl$^{\Delta BRCT}$ in yellow, Pbl$^{\Delta N\text{-term}}$ in blue, Pbl$^{DH\text{-}PH}$ in red, Pbl$^{DH\text{-}PH_V531D}$ in grey, Pbl$^{DH\text{-}PH\text{-}short}$ in orange, Pbl^{DH} in green and Pbl$^{\Delta C\text{-}term}$ in pale blue. Mean values for the different constructs are shown in table 3.1.

In order to test whether the DH domain alone, which is the part of the protein that directly interacts with substrate GTPases, is also sufficient to provide some rescue activity, the Pbl^{DH} construct was expressed in a *pbl*^{3} mutant background. As shown in Fig. 3.2 J and Fig. 3.4, this did not result in a suppression of *pbl* mesodermal differentiation defects and further cytokinesis was not rescued by the expression of the DH domain alone. These observations suggest either that the DH domain does not provide enough activity *in vivo* without the neighboring PH domain or that the PH domain is required for the localization of the GEF domain within the mesoderm cells in order to achieve a suppression of the migration defects as seen for the Pbl$^{DH\text{-}PH}$ protein. The role of the PH domain is further addressed in chapter 3.4. In addition, both the Pbl^{DH} as well as the Pbl$^{DH\text{-}PH}$ constructs did not exert any rescuing activity for cytokinesis which is consistent with the previous finding, that the presence of the BRCT domains is essential for this process (Smallhorn et al., 2004).

To address the question whether the activity of Pbl$^{DH\text{-}PH}$ depends on a functional DH domain, a point mutation was introduced. This mutation leads to the same amino acid exchange within the highly conserved CR3 region of the DH domain that was previously described to render the GEF catalytic inactive in the *pbl*^{5} loss of function allele (Liu et al., 1998; Schumacher et

al., 2004; Smallhorn et al., 2004). Introduction of this missense mutation indeed abolished the rescue capacity of the $Pbl^{DH\text{-}PH}$ construct, indicating that the activity of the catalytic core is essential and to some extent sufficient to fulfill Pbl's function during mesoderm migration (Fig 3.2 I, Fig. 3.4 and Tab. 3.1). Altogether, these experiments show that in contrast to cytokinesis, the catalytic DH-PH tandem domain is the smallest part of the protein that can exert sufficient rescue activity for mesoderm migration.

genotype	Eve-positive hemisegments	SD	n
pbl^3/pbl^3	1.7	1.7	128
$PblA\text{-}HA;\ pbl^3/pbl^3$	18.6	1.7	98
$Pbl^{\Delta BRCT};\ pbl^3/pbl^3$	8.3	4.2	69
$Pbl^{\Delta N\text{-}term};\ pbl^3/pbl^3$	3.3	2.4	123
$Pbl^{DH\text{-}PH};\ pbl^3/pbl^3$	8.9	2.9	102
$Pbl^{DH\text{-}PH\_V531D};\ pbl^3/pbl^3$	2.8	2.2	101
$Pbl^{DH\text{-}PH\text{-}short};\ pbl^3/pbl^3$	7.1	3.9	38
$Pbl^{DH};\ pbl^3/pbl^3$	2.4	2.2	106
$Pbl^{\Delta C\text{-}term};\ pbl^3/pbl^3$	7.7	2.9	88

Table 3.1: Average number of Eve-positive cell clusters after expression of various Pbl constructs in a *pbl^3* mutant background.
Mean values and their standard deviations (SD) of the number of Eve-positive hemisegments are shown for *pbl^3* mutant embryos expressing different Pbl constructs (n = number of embryos examined).

3.1.4 The C-terminal tail of Pbl is involved in but not essential for the migratory function of the full-length protein

The analysis of the $Pbl^{\Delta N\text{-}term}$ and $Pbl^{DH\text{-}PH}$ constructs showed several differences in the rescue capacity, the dominant effects as well as the localization of these two proteins, which differ only in the presence or absence of the C-terminal tail (see paragraph 3.1.3 and below in chapter 3.2 and 3.4). To examine the role of this part of the protein for the function of the full-length protein, a C-terminally truncated version, called $Pbl^{\Delta C\text{-}term}$, was generated and expressed in a *pbl^3* mutant background. While the full-length construct fully complemented the loss of endogenous Pbl function during cytokinesis, the $Pbl^{\Delta C\text{-}term}$ protein showed no obvious rescue of the defects as multinucleated cells could still be found in those embryos (see Fig. 3.2 K and Fig. 3.13 G). On the other hand, $Pbl^{\Delta C\text{-}term}$ expression led to a migration rescue in the range of the rescue levels of $Pbl^{DH\text{-}PH}$, $Pbl^{DH\text{-}PH\text{-}short}$ and $Pbl^{\Delta BRCT}$. However,

compared to the full-length protein the deletion of the C-terminal tail resulted in a significant reduction of the rescue capacity, as on average 7.7 Eve-positive cells were detected under these conditions (Tab. 3.1, and Fig. 3.4).

These observations clearly demonstrate that the carboxy-terminal tail, which does not contain any known domain structure, resembles a novel important domain for Pbl function. Although the expression of the deletion constructs $Pbl^{\Delta N\text{-term}}$ and $Pbl^{DH\text{-}PH}$ implied that at least in these truncated proteins the presence of the C-terminal tail had a negative effect on the rescue of the migration, in the context of the full-length protein the presence of the C-terminus is obviously required for both functions. Nevertheless, as $Pbl^{\Delta C\text{-term}}$ still exhibits a significant migration rescue, it can be concluded that an absolute requirement of the C-terminus can only be detected for Pbl's cytokinesis function (see below).

3.2 Misexpression of different Pbl variants gives rise to distinct dominant effects

As mentioned above, expression of the $Pbl^{\Delta N\text{-term}}$ construct in a *pbl* mutant background led to dominant effects on mesoderm development (Fig. 3.3). In order to examine in more detail whether this and other Pbl variants elicit distinct dominant effects on mesoderm morphogenesis, the constructs were misexpressed in the mesoderm of wild type embryos.

Consistent with the findings from the rescue experiments, overexpression of $Pbl^{\Delta N\text{-term}}$ had a strong impact on normal mesoderm development. In all embryos expressing the HA-tagged construct, the mesoderm cells were not internalized properly through the ventral furrow (Fig. 3.5 C, D). Instead, the mesoderm remained at the surface of the embryos, which can be appreciated best in cross-sections (Fig. 3.6 C, D). Because of these invagination problems, most embryos also displayed defects during germband elongation (Fig. 3.5 D). It has to be stressed here, that in wild type the process of invagination is not dependent on *pbl*, as this morphogenetic event is never affected in embryos lacking Pbl activity (Schumacher et al. , 2004; Schumacher and Müller, unpublished). A further defect caused by $Pbl^{\Delta N\text{-term}}$ expression is an interference with cell division. Transgene expressing cells displayed a multiple nuclei phenotype during the migratory stages of mesoderm development (Fig. 3.12 A-C), arguing for a disruption of cytokinesis in these cells.

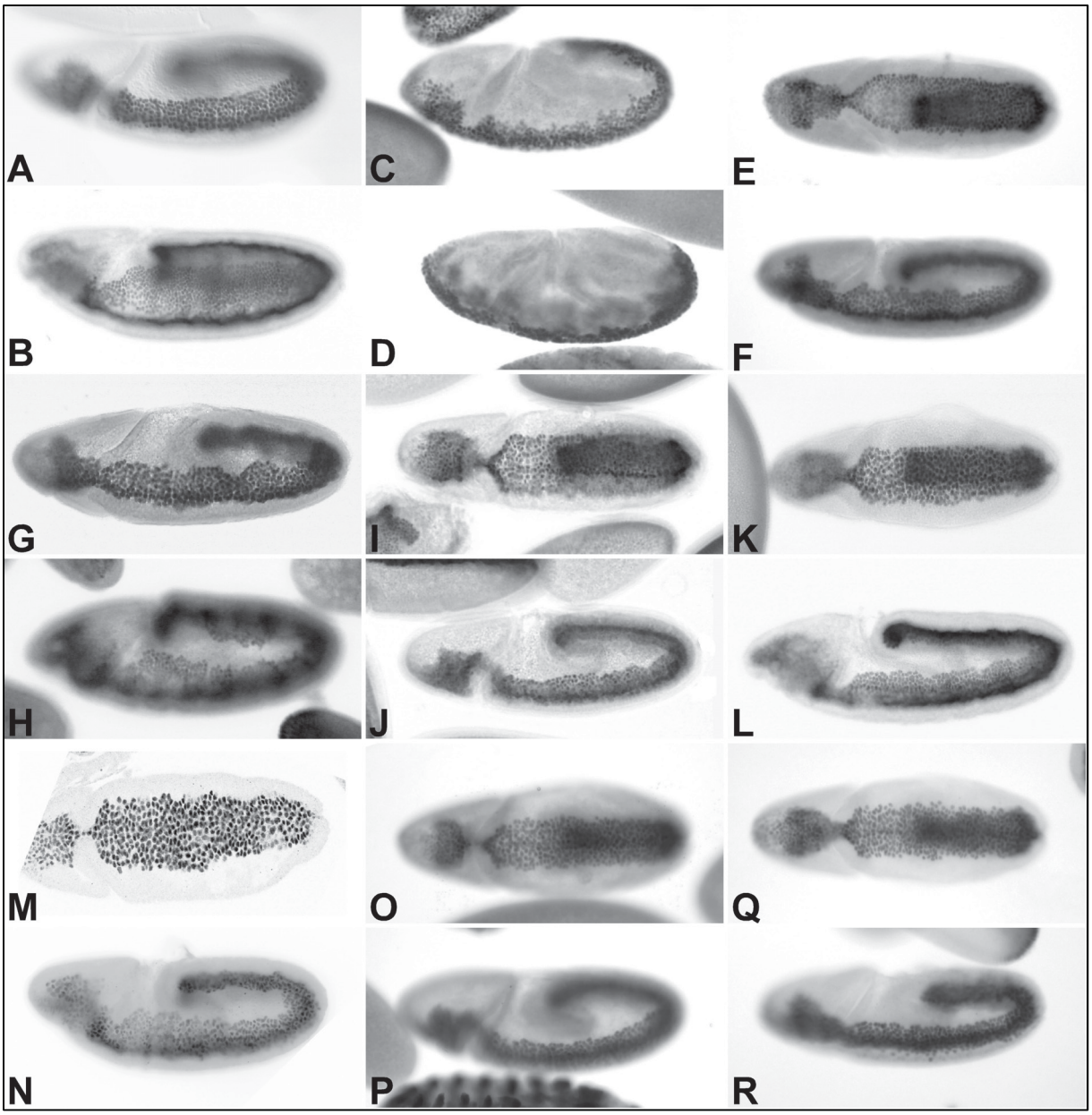

Fig. 3.5: Dominant effects of truncated Pbl variants on mesoderm morphogenesis.
(A-R) Embryos in phase 2 and late phase 2/phase 3 of mesoderm migration (see Fig. 1.2 for staging) were stained against the Twi protein and are shown in lateral (A,B,C,D,F,H,J,L,N,P,R) and ventral (E,G,I,K,M,O,Q) views. In comparison to wild type (A,B), overexpression of $Pbl^{\Delta N\text{-}term}$ using *twi::Gal4* results in embryos in which the mesoderm remains at the surface (C,D). In contrast, misexpression of $Pbl^{DH\text{-}PH}$ generates embryos with defects in mesodermal spreading (G,H). Overexpression of $Pbl^{\Delta BRCT}$ (E,F), Pbl^{DH} (I,J), $Pbl^{C\text{-}term}$ (K,L), $Pbl^{\Delta N\text{-}term\_V531D}$ (M,N), $Pbl^{DH\text{-}PH\_V531D}$ (O,P) or $Pbl^{BRCT1,2}$ (Q,R) does not cause any dominant effects.

Interestingly, expression of the relatively similar $Pbl^{\Delta BRCT}$ construct did not cause any dominant phenotypes; invagination and spreading of the mesoderm occurred like in wild type (Fig. 3.5 E, F). This construct differs from the $Pbl^{\Delta N\text{-}term}$ protein only in the additional presence of the NLS and the PEST motif. Therefore, the presence of these regulatory domains seems to be sufficient to abolish the dominant effects caused by expression of the C-terminal half of the Pbl protein.

Consistent with this conclusion, embryos expressing only the catalytic core of Pbl, which is the DH-PH tandem domain, also exhibited defects during mesoderm morphogenesis. In contrast to $Pbl^{\Delta N\text{-}term}$ however, $Pbl^{DH\text{-}PH}$ expression gave rise to embryos with problems during mesodermal spreading. Compared to wild type, the mesoderm displayed a very uneven leading edge in whole mount stainings against the Twi protein (Fig. 3.5 G, H). As a result, spreading of the mesoderm was strongly reduced at least in some regions of the embryos. In cross-sections, this irregular migration could also be observed as the cells occasionally spread out only to one side of the embryo (Fig. 3.6 E).

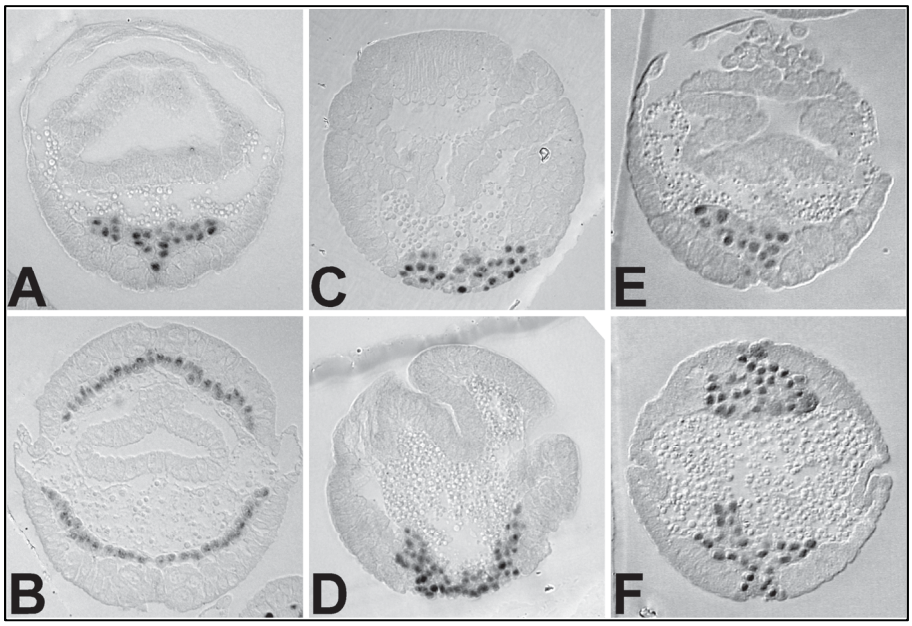

Fig. 3.6: Differential dominant effects caused by misexpression of $Pbl^{\Delta N\text{-}term}$ and $Pbl^{DH\text{-}PH}$.
(A-F) Transversal cross-sections of embryos stained against Twi in stage 8 (phase 2; A,C,E) and stage 9 embryos (phase 3; B, D, E). The sections were taken between 30% and 60% of the embryo length (anterior-posterior axis). In comparison to wild type (A,B), *twi::Gal4* overexpression of $Pbl^{\Delta N\text{-}term}$ results in invagination defects while $Pbl^{DH\text{-}PH}$ expression results in abnormal migration (E,F).

In addition, effects on the initial phase of mesoderm migration were evident after expression of $Pbl^{DH\text{-}PH}$. In wild type, the mesodermal tube starts to flatten down symmetrically to establish contact with the underlying ectoderm after invagination is complete. This process is accomplished by EMT of the mesoderm and a first round of mitosis, after which the cells eventually start to migrate in dorso-lateral directions (stage 8, Fig. 3.6 A) to form a monolayer at the end of the process (Fig. 3.6 B). After transgene expression however, mesodermal cells still formed an aggregate, pointing into the inner yolk cell of the embryo, even if the rest of the cells had already started to spread out (Fig. 3.6 F). As in *htl* mutants for example, these cells might fail to dissociate from each other and are presumably subsequently pushed to one

side of the embryo by the yolk cell. In whole mount stainings, this phenotype is probably reflected by the observed uneven distribution of mesoderm cells in later stages of development. Therefore, the later defects might result from problems already during EMT of the mesoderm, which is implicated by the presence of the unusual aggregates of mesoderm cells. A further difference to the phenotype of $Pbl^{\Delta N\text{-}term}$ overexpression is the finding that expression of $Pbl^{DH\text{-}PH}$ had no obvious impact on cytokinesis, as multinucleated cells were never observed (not shown). Importantly, raising the amount of $Pbl^{DH\text{-}PH}$ in the cells by expressing two copies of the transgene at once did not alter the mesoderm specific phenotypes suggesting that the differences in the dominant effects of $Pbl^{DH\text{-}PH}$ and $Pbl^{\Delta N\text{-}term}$ are not caused by differences in the expression levels.

When expressed in the wild type, the DH domain did not cause any obvious defects (Fig. 3.5 I, J). As this construct did also not exhibit any rescuing activity in a *pbl*^{3} mutant background, the DH domain alone does not seem to provide sufficient activity *in vivo* to cause an effect in either assay. Even if the localization of the protein to a certain subcellular compartment was required for a rescue activity (via PH or other domains, see below), a dominant effect on Rho GTPases could nevertheless be expected when misexpressed at high levels in the cytoplasm. Therefore it seems reasonable that the PH domain also contributes to the catalytic activity of the DH domain as indicated for other proteins of the Dbl family (Baumeister et al., 2006; Liu et al., 1998; Rossman et al., 2003; Rossman et al., 2005; Rossman and Sondek, 2005; Rossman et al., 2002).

The obvious difference in the effects of the $Pbl^{\Delta N\text{-}term}$ and $Pbl^{DH\text{-}PH}$ constructs raises the question why both constructs interfere with different processes. Both proteins encode the catalytic core of the GEF but $Pbl^{\Delta N\text{-}term}$ also contains the whole C-terminal tail of the full-length protein. As even the expression of multiple copies of the $Pbl^{DH\text{-}PH}$ transgene did not lead to a phenotype comparable to the one caused by $Pbl^{\Delta N\text{-}term}$, a discrepancy in the expression levels is unlikely to be the cause of these distinct effects. Therefore, the presence or absence of the C-terminus in either construct is the only major difference here. In order to test whether the C-terminal domain alone could provide a dominant activity towards early mesoderm development, the $Pbl^{C\text{-}term}$ construct was expressed. This expression did not cause any defects during invagination or the subsequent spreading of the mesoderm (Fig. 3.5 K, L) demonstrating that the dominant phenotypes are not a consequence of the presence of the C-terminal tail in the first place and therefore, the catalytic domains are essential for these effects.

To prove that the different abnormalities caused by $Pbl^{\Delta N\text{-}term}$ and $Pbl^{DH\text{-}PH}$ are indeed dependent on the GEF activity of the DH domain, mutated versions of both constructs were expressed. As anticipated, in both cases the dominant effects of the respective construct were completely abolished by introduction of the V531D mutation which renders the DH domain inactive (Fig. 3.5 M-P). This clearly demonstrates that the invagination and cytokinesis defects as well as the migration phenotypes are consequences of the exchange activity of the constructs and therefore probably reflect a misregulation of Rho GTPase activities. Consistent with this idea, it was shown for several other GEFs of the Dbl family that a deletion of regulatory domains leads to a constitutive activation of the exchange factor (Rossman et al., 2005; Whitehead et al., 1997). Interestingly, this is also the case for the oncogenic form of Pbl's mammalian homologue, Ect2 (Miki et al., 1993; Saito et al., 2004; Solski et al., 2004). Therefore, it is likely that both, $Pbl^{\Delta N\text{-}term}$ and $Pbl^{DH\text{-}PH}$, are hyperactivated variants of Pbl that lead to the dominant as well as the rescue effects by a strong activation of their GTPase substrates. If this assumption was true, one might argue that both constructs should exhibit differential activities towards distinct Rho GTPases, which causes distinct dominant effects in the end. An attractive model would be the involvement of the C-terminus in regulating the substrate preference of the catalytic core, as its presence clearly modifies the dominant phenotypes caused by the misexpression of the DH-PH tandem domain (see below and discussion).

The previous rescue assays have indicated that the BRCT domains are indispensable for Pbl's function during cytokinesis. Although deletion constructs comprising only the C-terminal half of the protein can provide rescuing activity during migration, an involvement of the two BRCT domains cannot be excluded. In comparison to the rescue achieved by expression of the full-length protein, the $Pbl^{\Delta BRCT}$ construct showed a reduced rescue capacity (Fig. 3.4 and Tab. 3.1). This discrepancy might reflect a possible role of the two BRCT domains during mesodermal spreading. Like in cytokinesis, during mesoderm spreading Pbl might be regulated in some way via protein-protein interactions that are mediated by these protein domains. To test this possibility, a transgene encoding the N-terminus as well as both BRCT domains was generated and overexpressed in the mesoderm. In the case that protein-protein interactions during migration require the BRCT domains, misexpression of these parts might generate a dominant effect. One could imagine that by flooding the cells with these domains, binding partners for the endogenous protein might be sequestered leading to an interference with proper mesoderm migration. However, expression of $Pbl^{BRCT1,2}$ did not cause any migration defects (Fig. 3.5 Q, R). Importantly, an inhibition of cytokinesis was not observed

after expression of $Pbl^{BRCT1,2}$ either, although an involvement of these domains in this context has already been shown (Somers and Saint, 2003). This could be interpreted as a hint that the expression levels of $Pbl^{BRCT1,2}$ might be too low to dominantly interfere with either process. To assess this possibility, multiple copies of the construct should be expressed in the future. Therefore, these results do not exclude a requirement for the BRCT domains during migration in the wild type, but they render it rather unlikely that these domains are involved in a very critical step during Pbl's mesoderm function (see discussion).

It should also be mentioned here, that the $Pbl^{\Delta C\text{-term}}$ construct did not cause any dominant effects when overexpressed in the mesoderm (not shown). As observed for the full-length protein, mesodermal spreading as well as cytokinesis was not affected by the misexpression of this construct. This is consistent with the idea that an amino-terminal truncation of Pbl results in the constitutive activation of $Pbl^{\Delta N\text{-term}}$ and $Pbl^{DH\text{-}PH}$ and therefore in the observed dominant effects described earlier. However, $Pbl^{\Delta C\text{-term}}$ still contains these regulatory domains and hence does not reflect an activated version of Pbl that could dominantly interfere with mesoderm morphogenesis. An alternative explanation would be that in the presence of the NLS the cytoplamic levels of the respective construct (in this case $Pbl^{\Delta C\text{-term}}$) are too low to cause dominant effects. The cytoplasmic levels and the subcellular localization of $Pbl^{\Delta C\text{-term}}$ are analyzed in chapter 3.4.3.

3.3 Localization of Pbl in interphase cells

3.3.1 Pbl localizes to the cortex of mesoderm cells

The data presented above as well as the phenotypic description of Pbl mutants favor a model in which Pbl acts to activate its substrate GTPase somewhere at the cell cortex. On the one hand, Pbl constructs lacking the NLS motif were able to rescue the migration defects in *pbl* mutants indicating that a nuclear localization is not a prerequisite for its migration function. On the other hand, Pbl was shown to control the protrusive activity of the mesodermal cells (Schumacher et al., 2004; Smallhorn et al., 2004). Therefore, one would expect Pbl to induce rearrangements of the cortical actin cytoskeleton by activating Rho GTPases at the cell periphery, if the formation of cellular protrusions was a direct effect of Pbl activity. However, earlier reports have indicated that Pbl localizes to the cell cortex only during and shortly after mitosis. During interphase, it was described to be localized within the nucleus until the cell

enters mitosis again (Prokopenko et al., 1999; Prokopenko et al., 2000a). These data raise the question how Pbl could activate Rho GTPases at the cortex in migrating mesoderm cells (see also 3.5.7), while being trapped in the nucleus during interphase.

In order to determine whether Pbl is also localized to the cell cortex area in mesoderm cells, a Pbl antiserum was generated (Schumacher, 2005). Staining of wild type embryos revealed, that although the highest amount of the protein was indeed visible within the nucleus, low amounts of Pbl at cell borders could occasionally be observed as well (Fig. 3.7 A, B). The fact that staining of the cell borders was always weak and could only be detected in part of the cells implicates that the total amount of endogenous Pbl present at the cell periphery might be very low. Therefore, another approach was taken in order to determine the subcellular localization of Pbl in the mesoderm. An HA-tagged full-length Pbl construct was generated and expressed in a *pbl* mutant background.

Expression of this construct fully complemented the loss of endogenous Pbl during cytokinesis. As previously described for the endogenous protein, PblA-HA accumulated cortically at the cleavage furrow where it could be found in a ring-like structure as the furrow further progressed (Fig. 3.7 C-K) and at the whole cell cortex shortly after mitosis (arrow in Fig. 3.7 C) (Prokopenko et al., 1999; Prokopenko et al., 2000b). In addition, lower amounts of the protein also localized to the region in the middle of the cortical Pbl ring (Fig. 3.7 F-H and I-K). This signal could be interpreted as a binding to the central spindle, where only Pbl's interaction partners during cytokinesis, the kinesin-like Pavarotti and the RacGAP50C (together referred to as centralspindlin complex), have been found in *Drosophila* so far (Somers and Saint, 2003). Surprisingly, a localization to this so-called midbody is well described for the Ect2 protein in mammalian systems and it is implicated in the spatial control of cytokinesis (Kamijo et al., 2006; Tatsumoto et al., 1999; Yuce et al., 2005). This poses the question why Pbl is excluded from the midbody region in *Drosophila* cells. On the one hand, the typical ring-like localization pattern of the GEF at the cell cortex is supposed to be the result of an interaction with the centralspindlin complex. On the other hand however, the centralspindlin complex does not only localize to astral microtubules but also to the central spindle (Adams et al., 1998; Hirose et al., 2001; Saint and Somers, 2003; Somers and Saint, 2003). Therefore, a recruitment of Pbl to the central spindle would have to be strictly inhibited in a completely unknown fashion. Although the discrepancies between the Pbl and the Ect2 localization might in part be the result of minor differences in the general cytokinesis machinery between different systems, it could also be the case that antibodies against the

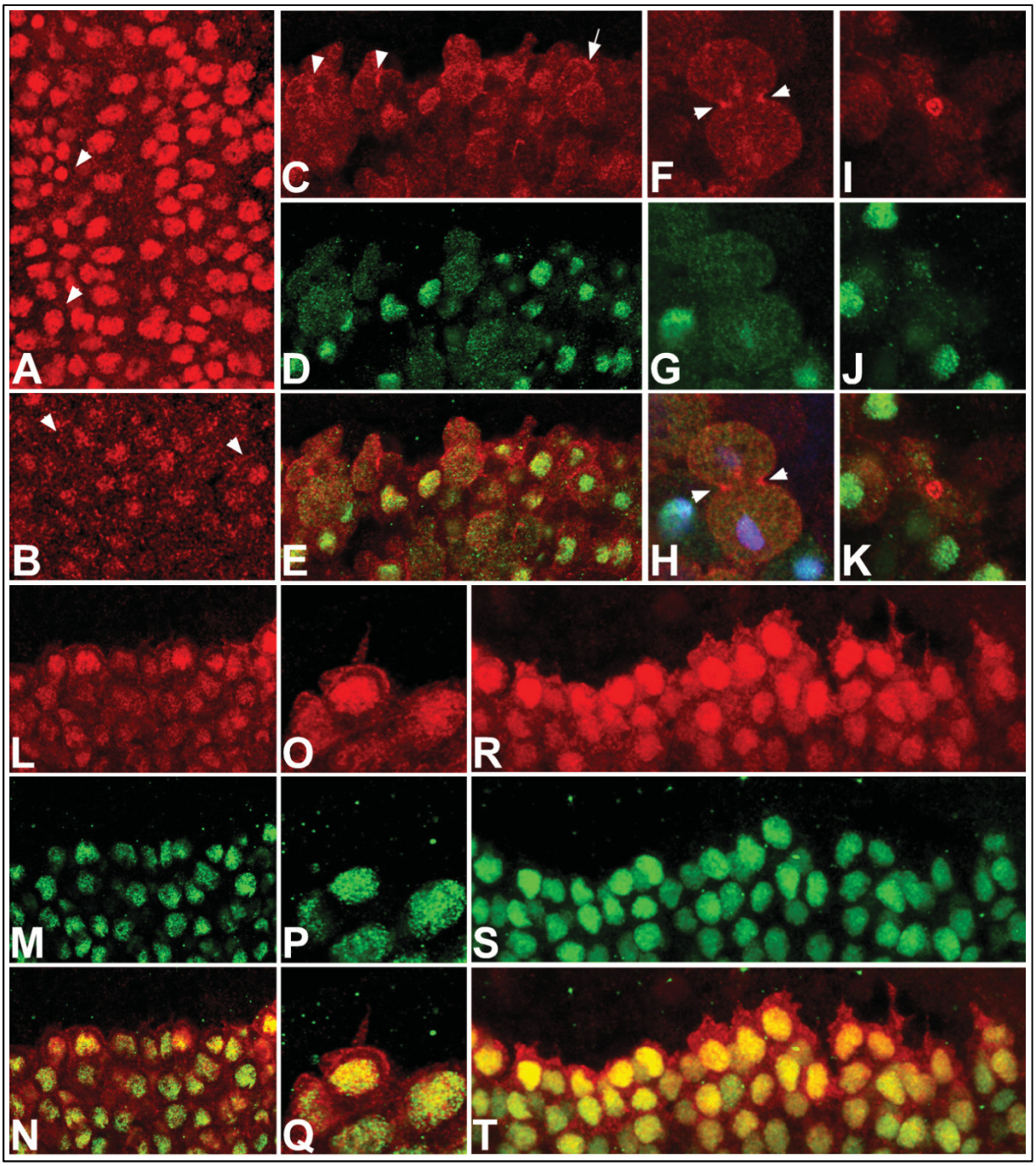

Fig. 3.7: Pbl localizes to the cortex of migrating mesoderm cells.
(A-B) Wild type embryos stained against the endogenous Pbl protein (red). A z-projection of 56 optical sections over 16 µm (A) and a single optical section (B) demonstrate prominent nuclear Pbl staining and occasional staining of the cell cortex (arrowheads). (C-T) Embryos expressing full-length PblA-HA in a *pbl*^{3} (C-E, L-Q) or wild type background (using the *twi::Gal4;Dmef2::Gal4* driver; in F-K, R-T) are stained against HA (red) Twi (green) and DAPI (blue in H). (C-D) PblA-HA localizes to the cleavage furrow in dividing cells (arrowheads) and to the cell cortex at the end of mitosis (arrow). (F-K) Higher magnifications of PblA-HA accumulations at the cleavage furrow. (L-M) In migrating *pbl*^{3} interphase mesoderm cells PblA-HA strongly accumulates in the nucleus but also at the cell cortex and cell boundaries. (O-Q) In higher magnifications the cortex association and staining of protrusions at the leading edge is clearly visible. (R-T) Z-projection of 47 optical sections in 0.16 µm intervals (7.5 µm in total) displaying the cortex accumulation of PblA-HA at the leading edge. A 3D-reconstruction of a similar data set is provided as Suppl. Movie 1.

endogenous protein are not sensitive enough to detect Pbl at the central spindle. Hence, it is important to analyze in more detail whether the HA-tagged Pbl is indeed capable of localizing to the central spindle. This should be done in conjunction with antibody stainings against microtubules and the components of the centralspindlin complex to test whether the proteins colocalize at the midbody region.

As already shown before, mesoderm specific expression of the PblA-HA construct also rescued the migration defects in *pbl* mutant embryos (Fig 3.2 C and Fig. 3.4). In interphase mesoderm cells, the majority of the protein was found inside the nucleus. Nevertheless, a subpopulation of PblA-HA was also present in the cytoplasm where it accumulated at the cell cortex. The fusion protein not only rescued the protrusive activity of pbl^3 mutant mesoderm cells but was also present within the cellular protrusions that were generated (Fig. 3.7 L-Q and wt overexpression in R-T; also see Suppl. Movie 1). These data demonstrate that although most of the protein is stored within the nucleus, a fraction of functional Pbl also localizes to the cortex and the protrusions of mesoderm cells during migration. Therefore, the GEF is at the right position to activate Rho GTPases during mesoderm morphogenesis.

3.3.2 In living hemocytes a subpopulation of GFP-tagged Pbl accumulates at the cell cortex and actin-rich structures

To follow the dynamics of Pbl's localization pattern in living embryos, a GFP-tagged full-length version was expressed in the mesoderm of wild type embryos. Unfortunately, under these conditions the expression levels were not high enough resulting in only a nuclear staining in the mesodermal cells. However, the fusion protein seemed to accumulate within the Twi expressing cells over time, which made it possible to assess the subcellular distribution of the protein in another well-characterized migrating cell type, the *Drosophila* hemocytes. These cells derive from the procephalic mesoderm and start to migrate out of the head region following various routes (Tepass et al., 1994).

In addition to a strong nuclear signal, Pbl-GFP was also evident at the cell periphery as well as in microspikes allowing the visualization of the protrusive activity of hemocytes while they move through the embryonic tissue (Fig. 3.8 and Suppl. Movie 2). Together these data indicate that a minor fraction of the total Pbl protein is able to accumulate at actin-rich structures in migrating cells, which supports the model that Pbl activates its substrate GTPase at the cell cortex during migration.

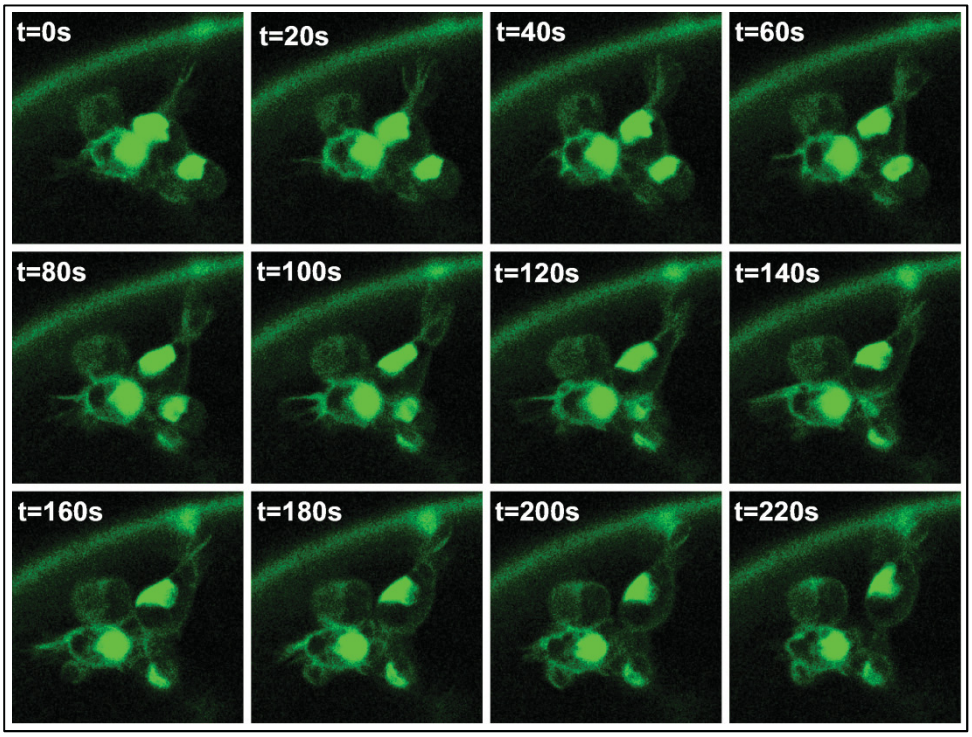

Fig. 3.8: Pbl localizes to the cell periphery and microspikes in migrating hemocytes.
Series of still images of a time-lapse sequence (20 sec intervals) of migrating hemocytes expressing GFP-Pbl during late embryogenesis. The GFP fusion protein accumulates in the nucleus but also localizes to the cortex and actin-rich microspikes in a dynamic fashion. The complete time-lapse sequence is provided as Suppl. Movie 2.

3.3.3 Pbl's overall membrane association is not dependent on the Htl FGF-signalling pathway

So far, nothing is known about the direct impact of FGF-signalling on the function of the Pbl protein. To test whether the cortical localization of Pbl depends on Htl FGF-signalling, the localization of PblA-HA was examined in *htl, pbl* double mutant embryos. Interestingly, even in the absence of a signalling input from the Htl receptor, Pbl was still able to bind to the cell periphery (Fig. 3.9), indicating that the overall cortical localization of the protein during migration is not regulated by FGF-signalling.

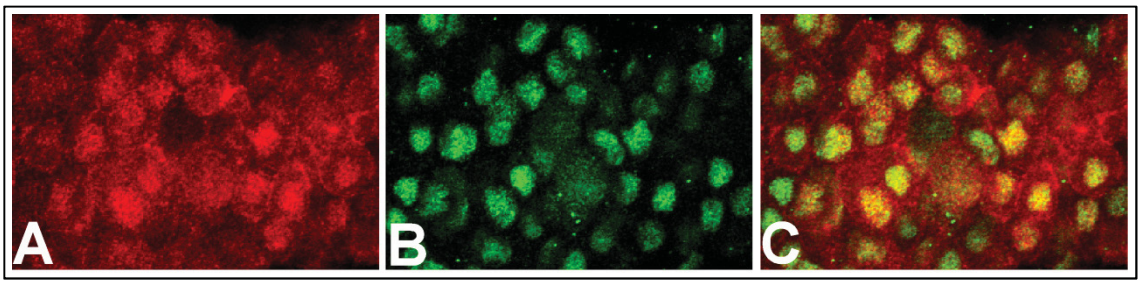

Fig. 3.9: Cortical localization of Pbl does not depend on Htl signalling.
Expression of PblA-HA in embryos double mutant for htl^{AB} and pbl^{3}. Embryos were stained against HA (red) and Twi (green). (A-C) Full-length Pbl localizes to the cell periphery even in the absence of Htl.

3.4 Identification of protein domains required for the localization of Pbl

3.4.1 The interphase localization of Pbl is not mediated by its BRCT domains

To determine which domains of Pbl are involved in cortical localization of the protein in interphase cells, different Pbl constructs were analyzed by antibody staining against their respective peptide tag. Loss of the BRCT domains in the $Pbl^{\Delta BRCT}$ construct lead to a protein that only rescued Pbl's migratory but not its cytokinesis function (see above and Smallhorn et al., 2004). In cells undergoing mitosis, $Pbl^{\Delta BRCT}$ showed a cortical localization after nuclear breakdown and release into the cytoplasm (Fig. 3.10 A). Although the staining did not reveal the exact phase of mitosis during which the construct localizes to the periphery of the cell, it can be assumed that this resembles the cortical localization that had been described for the endogenous Pbl protein during late anaphase just before the formation of the contractile ring starts (Prokopenko et al., 1999). The presented data indicate that the cortical localization of Pbl during mitosis does not depend on the presence of the two BRCT domains. In contrast, the accumulation of the protein at the cleavage furrow was never observed for the $Pbl^{\Delta BRCT}$ construct. This is not surprising, as this process is thought to depend on the interaction of Pbl with the centralspindlin complex formed by the Kinesin-like motor protein Pavarotti and the GTPase-activating-protein RacGAP50C (Somers and Saint, 2003). As the BRCT domains are deleted in this construct, the protein is not able to bind to the complex anymore and therefore it fails to accumulate at the site where the contractile ring would normally form.

Like the full-length protein, $Pbl^{\Delta BRCT}$ was highly concentrated in the nucleus of the mesoderm cells during interphase (Fig. 3. 10 B). Importantly, the construct was also found in minor amounts at the cell cortex, indicating that this truncated protein is still able to localize to all subcellular compartments where the endogenous Pbl can normally be found in interphase cells (Fig. 3. 10 B).

Expression of the complementary construct, $Pbl^{BRCT1,2}$, that consists only of the N-terminus and both BRCT domains, led to a different localization pattern. $Pbl^{BRCT1,2}$ localized to the cytoplasm of interphase cells (Fig. 3. 10 C-H). Furthermore, HA-positive dot-like structures could frequently be observed between the cells (Fig. 3. 10 C). Whether these structures resemble accumulations of the protein, for example at the midbodies of dividing cells or cells that just have completed mitosis, remains to be examined further. Altogether, the results clearly indicate that the BRCT domains are neither required nor sufficient to mediate the cortex association of Pbl in interphase mesoderm cells.

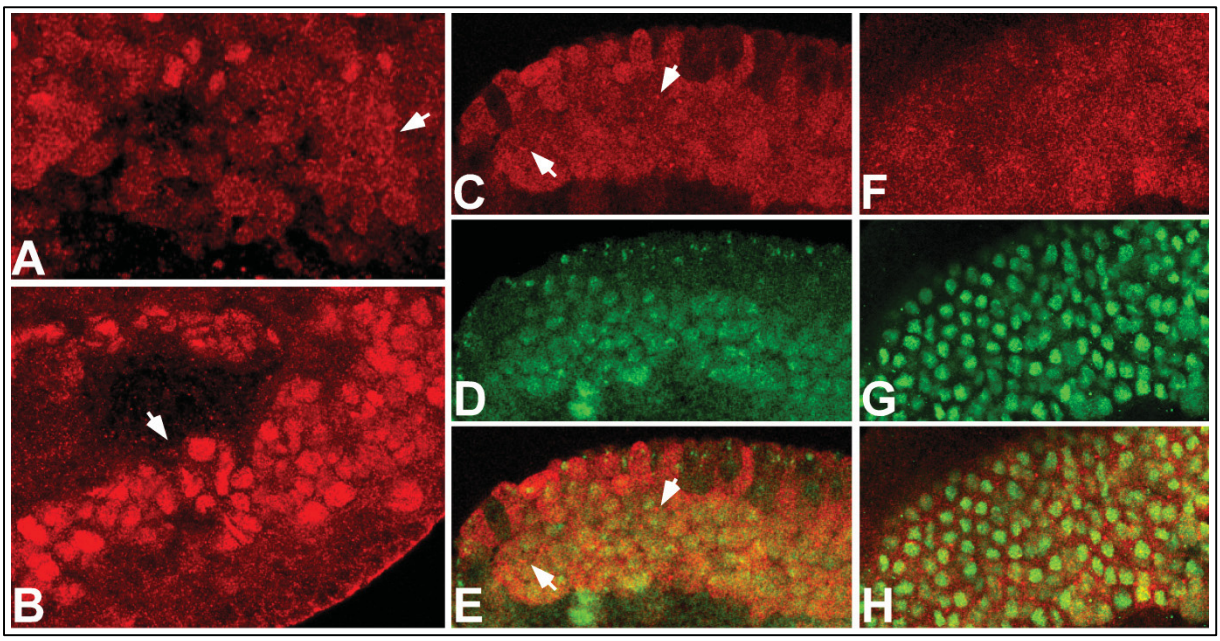

Fig. 3.10: The BRCT domains are not required for cortical association in interphase cells.
(A-B) Anti-myc staining (red) of *pbl*^{3} embryos expressing Pbl$^{\Delta BRCT}$. The construct predominantly localizes to the nucleus but is also present at low amounts at the cortex shortly after mitosis (arrow in A; note the lack of nuclear staining in the highlighted cells) and in interphase mesoderm cells (arrow in B). (C-H) Embryos overexpressing Pbl^{BRCT1,2} under the control of *twi::Gal4;Dmef2::Gal4* were stained against the HA-tag (red) and DAPI (green in D,E) or the Twi protein (green in G,H). The protein shows a diffuse cytoplasmic staining with eventual dot-like accumulations (arrows).

3.4.2 The PH domain is not the prime mediator of cortex association in the Pbl protein

Pleckstrin homology domains are known to be able to bind to phosphoinosides and in some cases they have been shown to drive membrane translocation of the respective protein by binding to membrane regions with high concentrations of their target phospholipids (Kavran et al., 1998). In the case of GEFs of the Dbl homology family, PH domains have a function in supporting the activity of the protein either through directly affecting the catalytic activity of the DH domain or through recruiting the GEF to the appropriate intracellular locations (Liu et al., 1998; Rossman et al., 2003; Rossman et al., 2002; Schmidt and Hall, 2002).

In order to test whether the PH domain of Pbl functions in targeting the protein to certain subcellular domains, the localization of different constructs was examined. In contrast to Pbl$^{\Delta BRCT}$, these constructs all lack the NLS sequence and thus did not accumulate in the nucleus. The constitutive active Pbl^{DH-PH} as well as its catalytic dead version Pbl$^{DH-PH_V31D}$ was found in punctae in the cytoplasm of mesoderm cells with a subtle enrichment at the cell cortex (Fig. 3.11 A-F). When expressed alone however, the DH domain located completely cytoplasmic suggesting that the membrane association of Pbl^{DH-PH} is driven by its PH domain (Fig. 3.11 G-I). To assess the membrane affinity of the PH domain directly, a GFP-tagged PH

construct was generated. As the expression levels were too low to retrieve data from the mesoderm of living embryos, antibody staining against the GFP-tag was performed. Although high amounts of the fusion protein were found in the cytoplasm, the protein was also detectable at the cell cortex in the mesoderm (Fig. 3.11 J-L) or when expressed in stripes by *en::Gal4* in the ectoderm of the embryos (Fig. 3.11 M). Therefore, it can be concluded that although the typical cortex localization of Pbl full-length might predominantly be mediated by another part of the protein, the PH domain is to some extend sufficient for cortical association of the expressed protein, explaining why the Pbl^{DH-PH} construct was able to rescue migration.

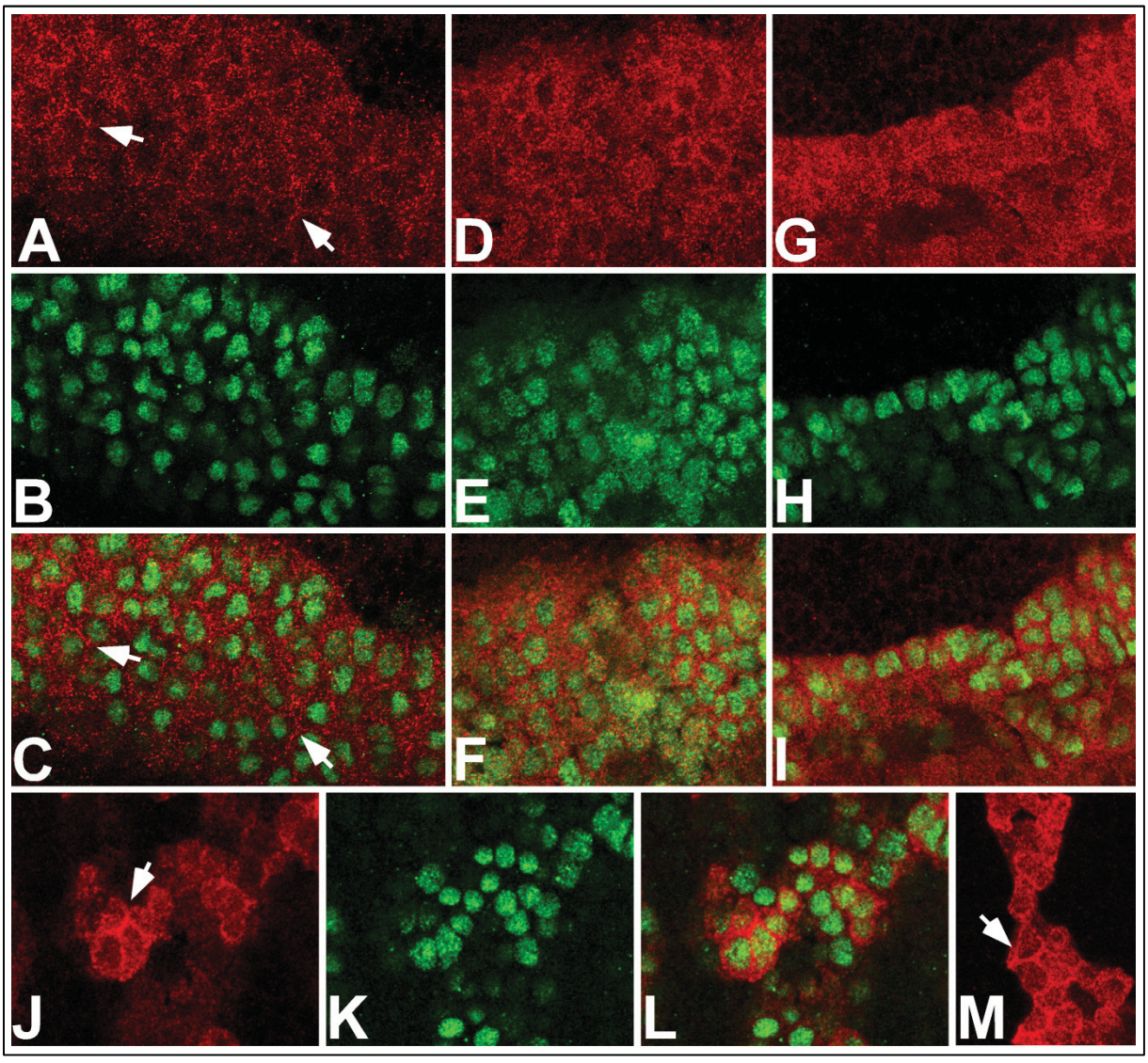

Fig. 3.11: The presence of the PH domain is sufficient for a weak cortical association.
(A-M) Embryos overexpressing different Pbl constructs were stained against Twi (green) and HA (red in A-I) or GFP (red in J-M). The Pbl^{DH-PH} (A-C) and Pbl^{DH-PH_V531D} (D-F) constructs localize to the cell cortex in a punctate fashion. Pbl^{DH} localizes to the cytoplasm (G-I). Pbl^{PH} accumulates at the cell periphery in the mesoderm (arrow in J-L) and when expressed in ectodermal cells using *en::Gal4* (arrow in M).

3.4.3 Pbl's conserved C-terminal tail is essential and sufficient for a robust membrane localization during interphase

As shown above, the Pbl^{DH-PH} construct exhibited only a weak association with the cell periphery of migrating mesoderm cells. In contrast, the Pbl$^{\Delta N-term}$ and Pbl$^{\Delta N-term_V531D}$ proteins showed a strong accumulation at the cell cortex (Fig 3.12 A-F). In both constructs, the NLS is missing and therefore the proteins are present in much higher concentrations outside the nucleus compared to Pbl$^{\Delta BRCT}$ and PblA-HA. The fact that most of the protein was cortically localized suggests that these constructs contain a part of Pbl, which is required for the robust cortex association that was already found for PblA-HA.

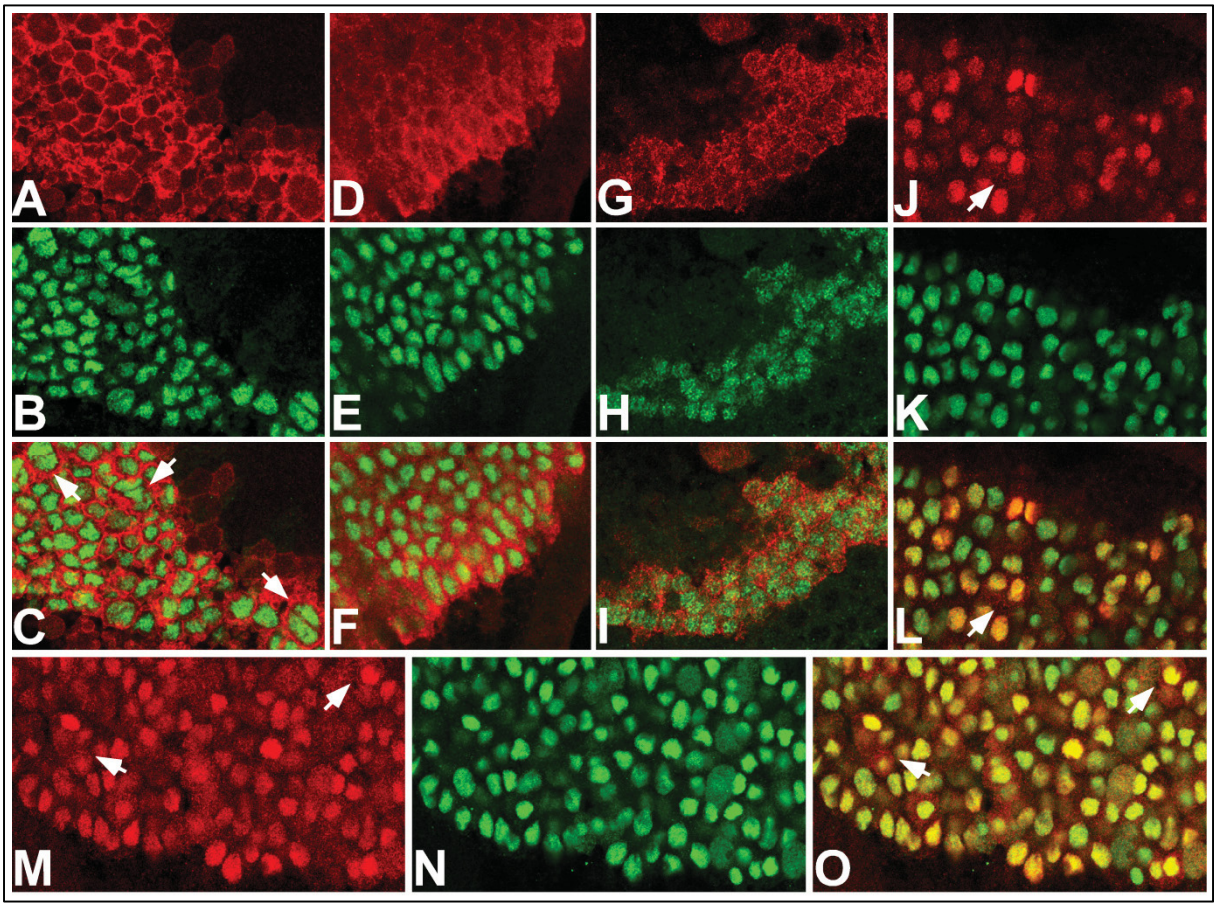

Fig. 3.12: The C-terminus is required and sufficient for cell cortex localization.
(A-O) Tagged constructs were expressed in the mesoderm by *twi::Gal4;Dmef2::Gal4* and embryos were stained against HA (red) and Twi (green). (A-C) Pbl$^{\Delta N-term}$ strongly accumulates at the cell cortex and dominantly interferes with cytokinesis (arrows indicate multinucleated cells). (D-F) Expression and localization of Pbl$^{\Delta N-term_V531D}$. (G-I) Pbl^{C-term} also shows a strong cortical localization. (J-L) Pbl$^{\Delta C-term}$ exhibits rather low cytoplasmic levels with elevated staining in the cytoplasm shortly after mitosis (arrow). Eventually, low amounts of the protein are detectable at the cell periphery (marked by arrows in M-O).

Pbl^{DH-PH} and Pbl$^{\Delta N-term}$ differ only in the presence of the entire C-terminal tail in the Pbl$^{\Delta N-term}$ construct. Thus, this so far uncharacterized part of Pbl might contain residues that are

important for the interphase localization of the protein. To test this idea, an HA-tagged form of the C-terminus was expressed. Interestingly, this Pbl$^{C\text{-}term}$ construct was also strongly enriched at the cortex of the mesoderm cells indicating that this part of the protein is indeed sufficient for a robust cortical association (Fig. 3.12 G-I).

In order to gain insight into the significance of this part for Pbl localization in the context of the full-length protein, a truncated Pbl version lacking only the C-terminal tail was expressed. Surprisingly, the Pbl$^{\Delta C\text{-}term}$ construct exhibited a rather weak cytoplasmic staining (Fig. 3.12 J-L). The protein was present in high amounts in the nucleus of mesoderm cells suggesting that the overall expression levels were comparable to PblA-HA for example. It was frequently observed that neighboring cells that had probably arisen from the same mitotic division shortly before, had higher amounts of the protein present in the cytoplasm (Fig. 3.12 J-L). Therefore, it is likely that the truncated protein is instable in the cytoplasm and only detectable in higher cytoplasmic concentration during or shortly after mitosis. Nevertheless, in a few cells that exhibited stronger cytoplasmic staining a weak cortex association could also be observed, a finding that is consistent with the previous result that the PH domain also confers a cortical localization (Fig. 3.12 M-O). However, a strong cortex association of the protein to the cell periphery could not be observed after deletion of the C-terminus. Therefore, these experiments indicate that the C-terminal tail is required and sufficient for the cortex localization of interphase Pbl. This conclusion is consistent with and explains why the Pbl$^{\Delta C\text{-}term}$ construct exhibited only a decreased rescue potential for migration (Fig. 3.4 and Tab. 3.1).

3.4.4 The C-terminus is not required for Pbl's localization to the cleavage furrow during cytokinesis

The loss of the C-terminus did not only lead to a weakening of the cortex association but also to lower protein amounts in the cytoplasm of interphase cells. Therefore, it was tested whether the cytokinesis localization pattern of the GEF is also comprised after the deletion of this protein region. Although the recruitment to the cleavage furrow is mediated by its BRCT domains, a reduced protein stability of Pbl$^{\Delta C\text{-}term}$ could cause a reduction or complete loss of Pbl from the cleavage furrow. This would explain the failure of Pbl$^{\Delta C\text{-}term}$ to rescue the cytokinesis defects in *pbl* mutants. Importantly, in mitotic cells Pbl$^{\Delta C\text{-}term}$ was still present in high concentrations in the cytoplasm and accumulated in a cortical ring during cytokinesis (Fig 3.13 A-F). Therefore, a mislocalization or degradation of the protein cannot be the cause for the failure to rescue cytokinesis. In fact, loss of the C-terminus must have an impact on the

capacity of the protein to activate Rho1 at the cleavage site. Most notably, *pbl*^{3} mutant embryos expressing Pbl$^{\Delta C\text{-term}}$ produced multiple cellular protrusions while multinucleated cells were still visible in another focus plane (Fig. 3.13 G, H). This is consistent with the

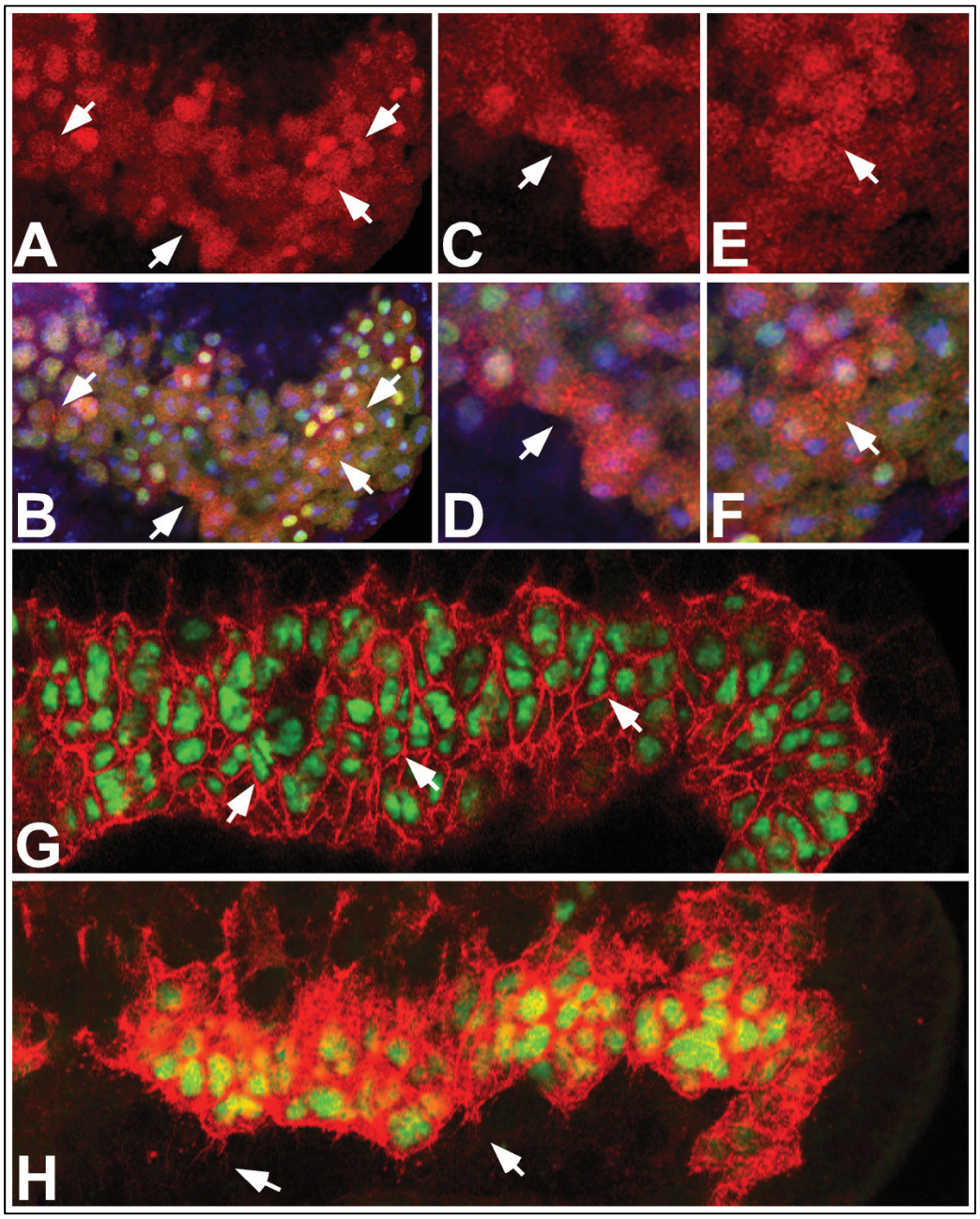

Fig. 3.13: Cytokinesis localization and differential rescue of Pbl$^{\Delta C\text{-term}}$.
(A-H) The Pbl$^{\Delta C\text{-term}}$ construct was expressed in wild type embryos that were stained against HA (red in A-F), Twi (green in merged images) and DAPI (blue in merged images). (A-C) Pbl$^{\Delta C\text{-term}}$ localizes to the midzone region in dividing cells. (C-F) Higher magnifications of Pbl$^{\Delta C\text{-term}}$ localization at the cleavage furrow in dividing cells. (G-H) Expression of Pbl$^{\Delta C\text{-term}}$ in *pbl*^{3} mutant embryos also expressing the cell surface marker *twi::CD2* (anti-CD2 in red anti-Twi in green) (Dunin-Borkowski and Brown, 1995). (G) Single optical section indicates the presence of multinucleated cells (arrows). (H) z-projection of the same embryo as in (G), focusing on the leading edge of the mesodermal cells; note the presence of protrusions at the leading edge (arrows).

above described migration rescue by $Pbl^{\Delta C\text{-}term}$ in the Eve-assay (see 3.1.4) and indicates that the ability to activate Pbl's migration substrate is not abolished after truncation of the C-terminus. However, the ability to trigger Rho1 activation seemed to be lost, suggesting an essential role for the C-terminus in Rho1 activation.

In conclusion, deletion of the C-terminal tail affects both Pbl functions differentially and is to some extent sufficient to uncouple Pbl's dual role in migration and cytokinesis. The results further suggest an involvement of the C-terminal tail in defining the substrate preference of the GEF, an hypothesis that is supported by the differential effects – invagination and cytokinesis vs. migration – of the dominant constructs upon overexpression, dependent on the presence or absence of the C-terminal tail (see 3.2).

3.5 Analysis of the GTPase pathway controlled by Pbl during mesoderm migration

3.5.1 Constitutively active Pbl interacts genetically with Rho1 and Rac GTPases in the compound eye

Thus far, the results of the rescue as well as the misexpression studies have shown, that the GEF activity is required for both, rescue and dominant effects of N-terminally truncated Pbl constructs. Furthermore, dominant effects could only be detected after loss of the N-terminal regulatory domains (BRCT, NLS and PEST). As mentioned before, deletion of regulatory domains renders the remaining catalytic parts of Dbl family GEFs constitutively active. This leads to a strong transforming activity in mammalian systems which has also been shown to be the case for Pbl's homologue, the proto-oncogene Ect2 (Miki et al., 1993; Rossman et al., 2005; Saito et al., 2004; Solski et al., 2004; Whitehead et al., 1997).

In this context it is very likely that the migration phenotypes elicited by $Pbl^{DH\text{-}PH}$ expression reflect consequences of a hyperactivation of the respective GTPase pathways that normally act downstream of Pbl during mesodermal spreading. As mentioned before, expression of a dominant negative version of Rho1 in the mesoderm only blocks cytokinesis but does not interfere with the capacity of the cells to migrate. Therefore, it was suggested that it is rather unlikely that Rho1 is required for the Pbl driven protrusive activity of mesodermal cells (Schumacher et al., 2004). This means that even if Pbl acts through another substrate than

Rho1 during migration, Pbl^{DH-PH} is obviously able to interact with that pathway as seen in both, misexpression and rescue experiments.

Based on those findings, Pbl^{DH-PH} was used to test for genetic interactions between Pbl and different Rho GTPases in order to get a hint, which other GTPase might act as Pbl's substrate during migration. A commonly used model system to study genetic interactions in the fly is the compound eye. By using a specific driver line, e.g. *GMR::Gal4*, a UAS-construct can be expressed exclusively in the eye tissue (see 2.5.2 for detailed description of the UAS/Gal4 system). In the case that expression of the construct results in defects in eye morphology, it can be tested easily, whether co-expression of another construct or heterozygosity for a particular gene modifies the severity of the dominant effect. This would indicate a genetic interaction between the constructs or between the construct and the respective gene.

Expression of Pbl^{DH-PH} in the developing eye led to a so-called rough eye phenotype. In comparison to the *GMR::Gal4* driver line alone (Fig 3.14 A), the eyes were reduced in size and the regular hexagonal arrangement of the omatidia was strongly affected (Fig 3.14 B). Importantly, expression of the catalytic dead version of the construct, Pbl^{DH-PH_V531D}, did not cause any effects on eye morphology indicating that this dominant effect again depends on the activity of the DH domain (Fig 3.14 C). Hence, the Pbl^{DH-PH} eye phenotype must also be based on a hyperactivation of substrate GTPases demonstrating that the eye system is a suitable tool to identify possible downstream pathways of Pbl *in vivo*.

Reduction of the dose of endogenous Pbl by introduction of one copy of the pbl^{3} allele in this genetic background led to a mild suppression of the Pbl^{DH-PH} induced rough eye phenotype (Fig. 3.14 D). This result further stresses the idea that the deletion construct acts as an activated form of Pbl interfering with the downstream targets of the endogenous protein. In addition, it suggests that comparable effects might be observed if the levels of substrate GTPases for Pbl were altered in this system.

Indeed, reduction of the Rho1 gene dose strongly suppressed the defects caused by Pbl^{DH-PH} expression suggesting that the dominant effects of the construct result in part from a misregulation of Rho1 pathways (Fig. 3.14 E). This interaction was expected, as Rho1 is the proposed substrate for Pbl and its mammalian homologue Ect2 during cytokinesis and therefore it can be assessed as a positive control (Prokopenko et al., 1999). On the other hand, co-expression of constitutive active or dominant negative versions of the small GTPase RhoL had no effects on the defects (Fig. 3.14 F, G). Together with the finding that heterozygosis for Cdc42 also had no impact on the rough eye phenotype (Fig. 3.14 H), this demonstrates that

the used experimental setup provides some specificity as not all Rho GTPases show an interaction.

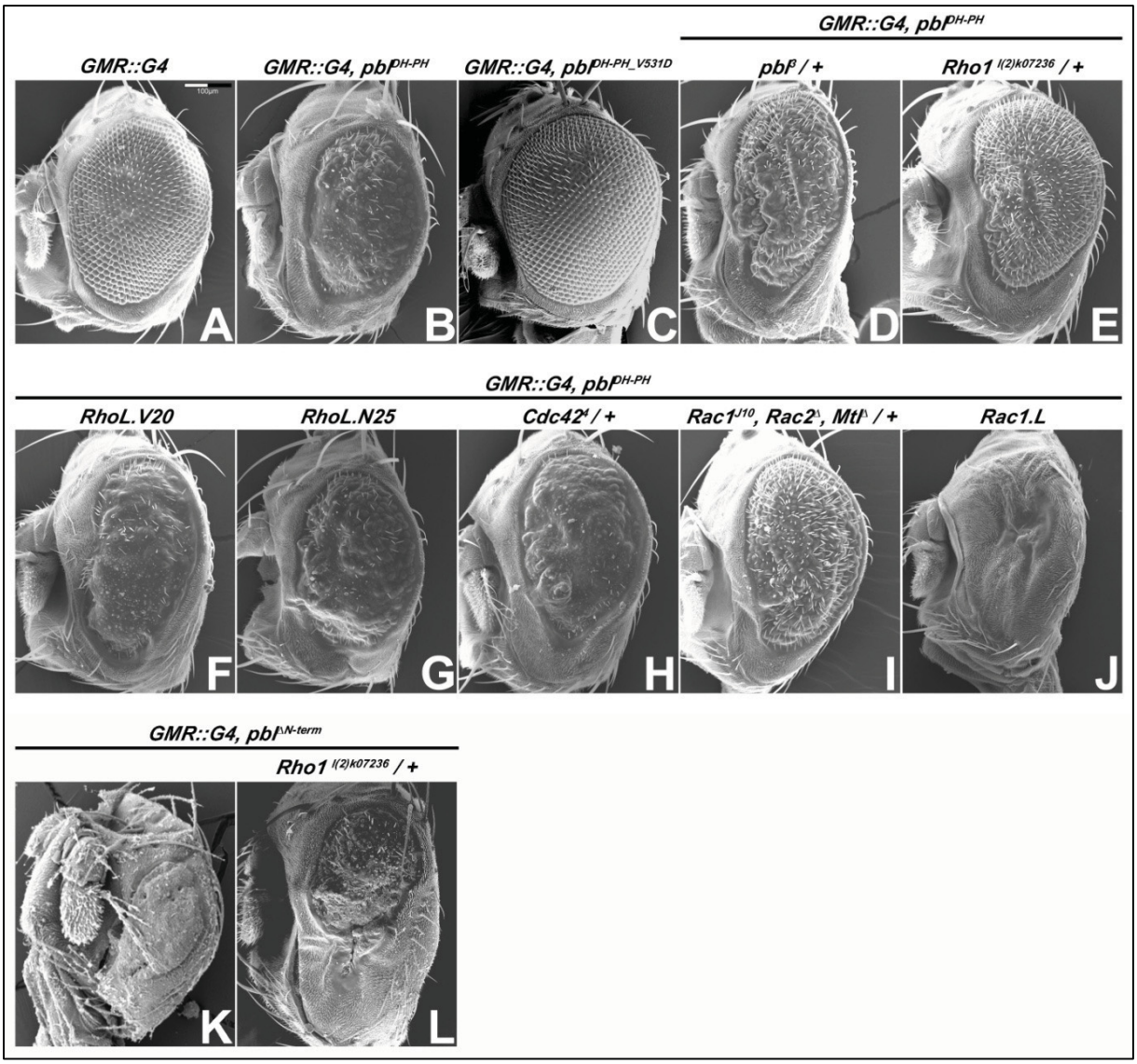

Fig. 3.14: Genetic interactions between activated Pbl and Rho1 / Rac GTPases.
In comparison to the eye specific *GMR::Gal4* driver line alone (A), expression of Pbl^{DH-PH} causes a rough eye phenotype (B). The phenotype depends on the catalytic activity of the DH domain, as the Pbl^{DH-PH_V531D} control construct does not cause any defects (C). The Pbl^{DH-PH} phenotype is partially suppressed in flies heterozygous for pbl^{3} (D). Heterozygosity for a Rho1 mutation strongly suppresses the eye defects (E) while co-expression of either dominant active $RhoL^{V20}$ (F) or dominant negative $RhoL^{N25}$ (G) has no impact on the phenotype. (H) Reduction of the dose of endogenous Cdc42 also has no effects. (I) Lowering the gene doses of all three *Drosophila* Rac GTPases leads to a suppression of the Pbl^{DH-PH} induced eye defects. Co-expression of either wild type Rac1 or Rac2 strongly enhances the phenotype as most flies die as adult pharates. The few escapers show a dramatic reduction in eye structures (J) . (K) Expression of $Pbl^{\Delta N-term}$ at 18°C leads to pharate adult lethality; flies dissected out of their pupal cases display a strong rough eye phenotype. (L) The lethality is rescued by lowering the gene dose of Rho1 and the resulting adult flies exhibit a strong rough eye phenotype.

Importantly, if the dose of all three *Drosophila* Rac GTPases was reduced, a suppression of the eye defects could be observed (Fig. 3.14 I). As for Rho1, this finding implies that the dominant effects of Pbl^{DH-PH} are caused by hyperactivation of these GTPases. By reducing the levels of potential substrates for Pbl or Pbl^{DH-PH} in the eye, the dominant effects were alleviated. Consistent with this idea, amplifying the putative substrate molecules by co-expression of either wild type Rac1 or Rac2, or directly raising the levels of active Rac1 by co-expression of an activated form ($Rac1^{V12}$) led to a strong enhancement of the phenotype (Fig. 3.14 J and not shown).
Taken together these results indicate that Pbl^{DH-PH} acts as a gain of function allele that genetically interacts not only with Rho1 but also with Rac GTPases. Therefore, Rac could be the so far unknown substrate for Pbl during migration.

To test the model that the presence of the C-terminus in $Pbl^{\Delta N-term}$ misexpression leads to selective effects on Rho1 dependent processes such as invagination and cytokinesis, this constitutive active variant of Pbl was also tested in the eye modifier system. In contrast to Pbl^{DH-PH}, eye specific expression of $Pbl^{\Delta N-term}$ led to pupal lethality. However, at lower temperatures (18°C) the flies developed to the pharate adult stage and displayed a strong rough eye phenotype when dissected out of their pupal cases (Fig. 3.14 K). In trans to a Rho1 mutation, this lethality was suppressed and flies hatched that had reduced eye structures (Fig. 3.14 L). In contrast, loss of one copy of all three Rac GTPases ($Rac1^{J10}$, $Rac2^{\Delta}$, Mtl^{Δ}) did not rescue the lethality of $Pbl^{\Delta N-term}$ expression and the rough eye phenotype of dissected pharates was not modified (not shown). These results suggest that the dominant effects of $Pbl^{\Delta N-term}$ expression in the eye are mainly based upon a hyperactivation of Rho1 and further strengthen the idea that the embryonic phenotypes of the DH-PH tandem domain in presence of the C-terminal tail are caused by misregulation of Rho1 dependent pathways.

3.5.2 Impact of dominant Rac1 constructs on mesoderm development

Although the results from the genetic analysis in the compound eye strongly suggest that the Rac GTPases might act as an alternative downstream target for Pbl, the genetic interactions were obtained in a tissue different from the mesoderm. Therefore, the overall requirement for Rac activity during mesoderm migration had to be investigated first, followed by experiments addressing a possible interaction between Rac and Pbl during this process (see 3.5.4).
As a first attempt to test whether interference with levels of active Rac in the mesoderm has an effect on the spreading behavior of the cells, dominant negative or constitutive active Rac1

constructs were misexpressed in the mesoderm of wild type embryos. Mesoderm specific expression of $Rac1^{N17}$ did not cause any defects during mesoderm morphogenesis as judged from whole mount stainings. During rescue experiments with active Rac1 however (see paragraph 3.5.4), dominant effects of $Rac1^{V12}$ on mesoderm migration were detected even in a *pbl* heterozygous background. The mesoderm spread out quite unevenly in these embryos (Fig. 3.15 A-D), which was also evident in older stages, where migration in the wild type is already complete (Fig. 3.15 E, F). In a ventral view, the defects are reminiscent of the phenotypes of *htl* mutant embryos; the mesoderm formed an irregular aggregate and exhibited a wavy morphology in Twi stainings (Fig. 3.15 A, B).

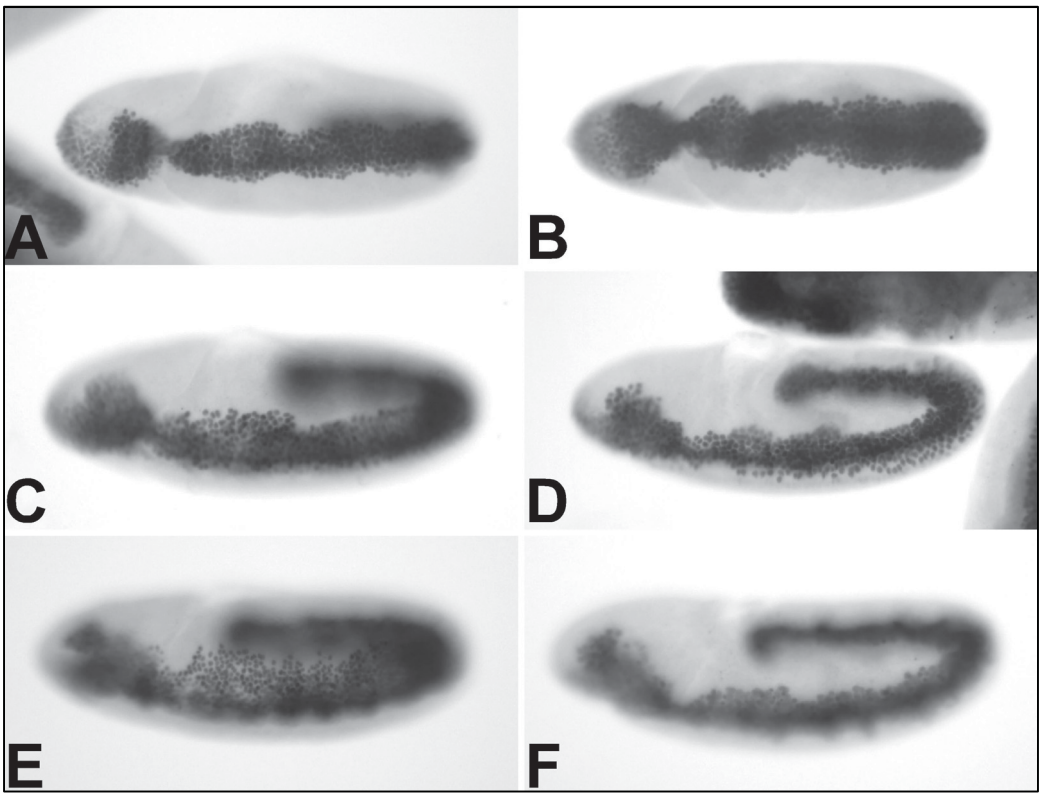

Fig. 3.15: Misexpression of $Rac1^{V12}$ causes strong defects during mesodermal spreading.
(A-F) Wild type embryos expressing $Rac1^{V12}$ in the mesoderm were stained against Twi. Ventral views (A,B) and lateral views (C,D) of embryos during the migratory phase (stage 8) and lateral views of embryos at the end of migration (late stage 9; E,F) are shown. The mesoderm exhibits a *htl*-like wavy morphology in ventral views (A,B) and spreads out very uneven along the anterior-posterior axis (C,D). This strongly abnormal spreading is also visible in older stages, where spreading should normally be complete (E,F).

Cross-sections of these embryos revealed several different abnormalities. In wild type, after invagination is complete, the internalized mesodermal tube establishes contact with the underlying ectoderm and undergoes EMT and a first round of mitosis (see chapter 1.3). Hence, in sections of stage 7 wild type embryos, the symmetrical flattening of the tube could be seen while the first cells had already entered mitosis, marked by the breakdown of the

nuclear envelope and a cytoplasmic Twi staining (Fig 3.16 A). At the same stage, the mesoderm of $Rac1^{V12}$ expressing embryos looked different. Although most cells already entered mitosis, the mesoderm still reached far into the inner yolk cell (Fig 3.16 E). The cells did not establish contact with the ectoderm; therefore, the tube did not flatten down. Furthermore, the shape of the mesoderm was still more tube-like, suggesting that EMT was at least not finished yet. Similarly to the defects described for Pbl^{DH-PH} misexpression, mesoderm cells forming aggregates were observed in stages in which lateral spreading of the mesoderm should already occur. As shown in Fig. 3.16 F and G, the severity of these defects varied from embryo to embryo. Nevertheless, in all stages examined, groups of cells forming finger-like structures that point into the yolk cell were observed (Fig 3.16 H). Given these phenotypes, a possible explanation for the defects would be an interference with EMT. As a similar effect was already seen after expression of constitutive active Pbl (Fig. 3.6), this nicely fits with the idea that Pbl^{DH-PH} leads to a misregulation of Rac in the mesoderm, causing the problems during migration. To conclude, the results of these overexpression experiments indicate that regulation of Rac activity during mesodermal spreading is essential and that misregulation can interfere with proper mesoderm migration.

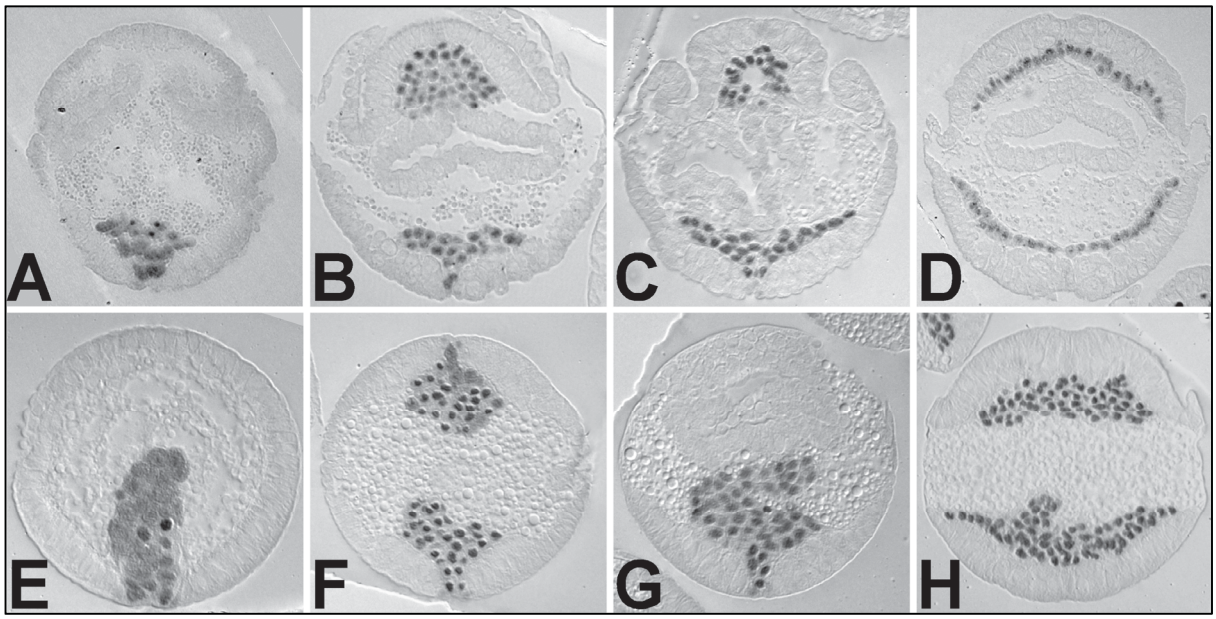

Fig. 3.16: Cross-sections of embryos misexpressing $Rac1^{V12}$ in the mesoderm.
Transversal cross-sections of wild type (A-D) and $Rac1^{V12}$ expressing embryos (E-H). (A) In wild type, the cells enter mitosis after completion of EMT and form a loose bunch of cells in the middle of the embryo. (E) $Rac1^{V12}$ expressing mesoderm cells enter mitosis while the disassembly of the mesodermal tube does not seem to be complete. In early stage 8, wild type cells start to spread out and are in close proximity to the ectoderm (B), while after $Rac1^{V12}$ expression some of the cells are still clustered together and stick into the inner yolk cells (F). (G) A more dramatic example of mesodermal cells forming an aggregate instead of spreading out on the ectoderm during late stage 8 (wt in C). At the end of the migration process $Rac1^{V12}$ expressing embryos do not reach the typical monolayer configuration of the wild type (D) but still stick out into the yolk cell (H).

3.5.3 Complete loss of Rac activity leads to strong migration defects

Expression experiments with activated Rac showed a requirement for a tight regulation of Rac activation during mesoderm migration. Nevertheless, the question whether Rac activity is indeed necessary for proper migration of the cells, is still open. To address this point, embryos were generated that lacked all Rac1 and Rac2 activity. During *Drosophila* oogenesis, the unfertilized egg becomes loaded with various mRNAs and proteins required for early embryogenesis, by so-called nurse cells. These specialized germ line cells are premeiotic cells that normally posses the genotype of the female. Hence, it is not sufficient to collect embryos homozygous mutant for Rac GTPases, as the maternal contribution would not be eliminated under these conditions. In order to obtain embryos that lack any Rac1/2 activity, germline clones must be induced that produce eggs that are maternally and zygotic homozygous mutant for the respective Rac GTPases.

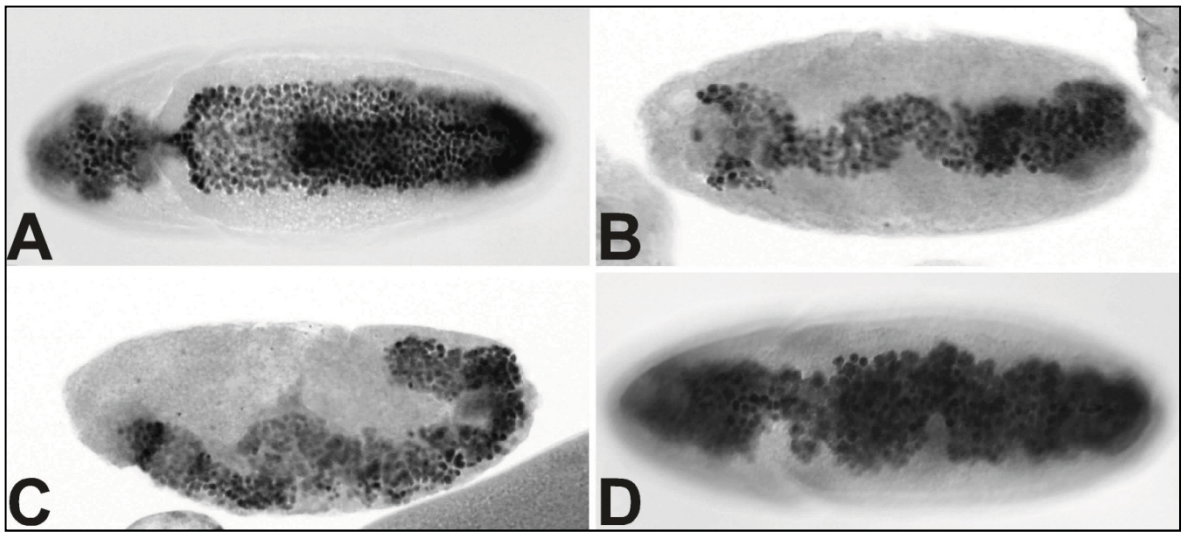

Fig. 3.17: Complete loss of Rac activity leads to strong migration defects.
Ventral (A,B,D) and lateral (C) view of stage 8 embryos stained against Twi. Unlike wild type (A), embryos lacking the complete maternal and zygotic contribution to Rac1 and Rac2 exhibit strong defects during mesoderm migration; the cells form an irregular aggregate in the middle of the embryo and fail to spread out in lateral directions (B,C). This defect is reminiscent of the phenotype seen in embryos lacking both Htl ligands, FGF8-like1 and FGF8-like2 (D).

To test whether Rac is required for mesoderm migration, germline clones were generated lacking the maternal and zygotic contribution for Rac1 and Rac2 and having a reduced maternal contribution for Mtl (see 2.5.3 for detailed genotype). The resulting embryos had strong defects already during early embryogenesis. Embryos that developed to extended germ band stages showed a complete block of mesodermal spreading (Fig 3.17 B,C). The migration

phenotype of these embryos was reminiscent of the defects seen after loss of both ligands for the Htl FGF receptor, FGF8-like1 and FGF8-like2 (Fig. 3.17 D; (Gryzik and Müller, 2004)). These observations extend earlier reports that indicated a requirement for Rac in the contact establishment of the internalized mesoderm with the underlying ectodermal (Wilson et al., 2005). Taken together, these results clearly demonstrate that Rac activity is essential for mesoderm migration, a finding that is consistent with the model that Pbl acts through the Rac pathway during this process. Furthermore, it seems to be more likely that either both Rac1 and Rac2 or only one of these GTPases are involved in migration, as the germline clones examined were not null for the third one, Mtl. Therefore, the observed phenotype seems to be the result of lacking Rac1/Rac2 activity.

3.5.4 Genetic interaction of Pbl and Rac1 in mesoderm migration

The data presented so far indicate a role for Rac in mesodermal spreading. Nevertheless, it is still unclear whether Pbl is involved in the regulation of Rac during migration, as the only data that argue for such a role were obtained from another tissue, the fly eye.

To test for genetic interactions between Rac and Pbl directly in the mesoderm of gastrulating embryos, two different approaches were taken. The first attempt included the usage of the hypomorphic pbl^{11D}allele. This allele has only a weaker migration phenotype while cytokinesis is still blocked completely. Embryos homozygous mutant for pbl^{11D} produce on average 7.7 Eve-positive cell clusters. In order to test, whether the severity of the migration phenotype can be influenced by interfering with the levels of active Rac in the mesoderm, dominant negative $Rac1^{N17}$ was expressed in the pbl^{11D} background. This had indeed an impact on migration, as the number of eve clusters was slightly lowered (Fig. 3.18, Tab. 3.2).

In a previous chapter it was already shown, that misexpression of activated Rac1 had a dominant effect on spreading of the mesoderm. To test whether this transgene would nevertheless be able to suppress the migration phenotype in *pbl* mutants partially (analogous to the pbl^{3} rescue by the constitutive actively Pbl^{DH-PH} construct), $Rac1^{V12}$ was expressed in pbl^{11D} embryos. As expected from the dominant effects described above, expression of active Rac1 also led to a significant enhancement of the migration defects that was even stronger than what was observed for $Rac1^{N17}$ (Fig. 3.18, Tab. 3.2). A similar result was obtained from the co-expression of activated Rac1 in the "Pbl^{DH-PH}, pbl^{3} rescue background" as even in this experimental set-up the number of Eve clusters dropped (Fig. 3.19). Therefore, it can be excluded that the negative effects of $Rac1^{V12}$ are a result of a specific interaction with the pbl^{11D} allele, whose mutation could not be identified yet. These findings are consistent with

and explain why previous attempts to rescue the strong *pbl*^{3} allele with either activated or wild type Rac1 had failed (Schumacher and Müller, unpublished). Obviously Rac activity is required for migration, but this activity must be tightly regulated, as artificially elevated levels of active Rac are counterproductive, which was already suggested by the dominant effects of Rac1^{V12} in a wild type background.

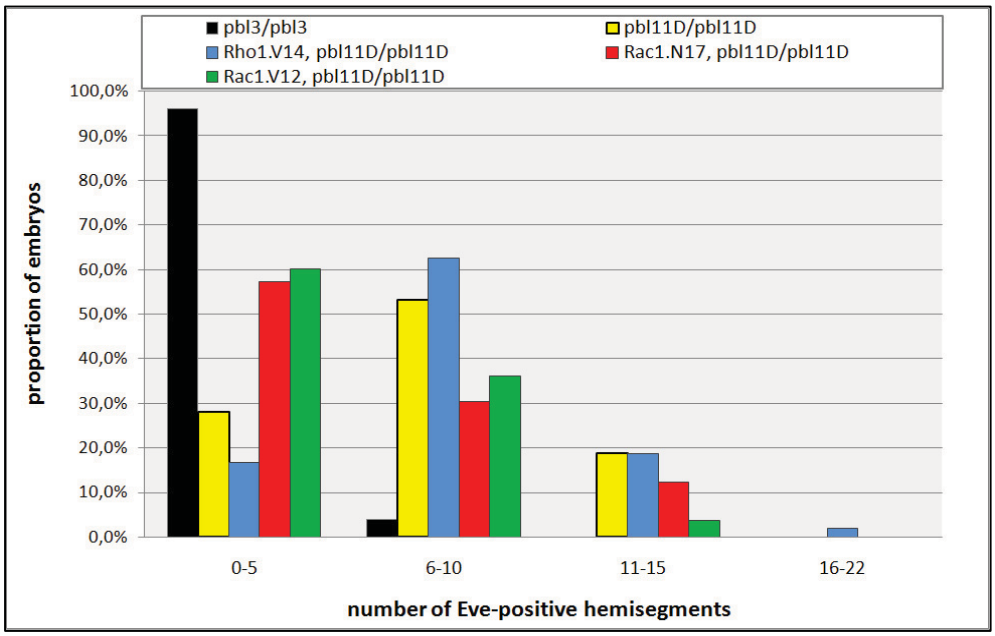

Fig. 3.18: Genetic interactions between the hypomorphic *pbl^{11D}* allele and dominant Rac1 constructs.
To test for genetic interactions between Rac1 and Pbl, embryos of different genotypes were stained against Eve and the number of pericaridial cell clusters was counted. Grouped values were plotted against the relative proportion of embryos displaying the indicated range of Eve-positive clusters. The hypomorphic *pbl^{11D}* allele (yellow) exhibits much weaker migration defects compared to *pbl^{3}* (black). Expression of Rho1^{V14} in *pbl^{11D}* homozygotes does not influence the severity of the phenotype significantly (blue), while expression of either dominant negative (Rac1^{N17} in red) or constitutive active Rac1 (Rac1^{V12} in green) enhances the migration defects.

genotype	Eve-positive hemisegments	SD	n
pbl^{11D}/pbl^{11D}	7.7	3.1	96
Rac1^{V12}, pbl^{11D}/pbl^{11D}	4.7	3.0	108
Rac1^{N17}, pbl^{11D}/pbl^{11D}	5.5	3.6	89
Rho1^{V14}; pbl^{11D}/pbl^{11D}	8.1	3.1	48

Table 3.2: Dominant Rac1 constructs enhance the migration phenotype of the hypomorphic *pbl^{11D}* allele.
Different GTPase constructs were expressed in the mesoderm of *pbl^{11D}* mutant embryos, which were subsequently stained against Eve. The mean values and their standard deviations (SD) of the number of pericardial cell clusters as well as the total amounts of examined embryos (n) are shown. The number of Eve-positive hemisegments in *pbl^{11D}* embryos differs significantly from those expressing dominant Rac1 variants (Student's test; P=3.46783E-11 for Rac1^{V12} and P=4.83543E-06 for Rac1^{N17}).

In the eye-system, not only Rac but also Rho1 showed a strong genetic interaction with Pbl^{DH-PH}. To test whether this is the case during mesoderm migration as well, activated Rho1 was expressed in the pbl^{11D} background. In contrast to $Rac1^{V12}$, $Rho1^{V14}$ did not significantly influence migration, a finding that is consistent with previous results that implicated that Rho1 is not required for the protrusive activity of mesoderm cells.

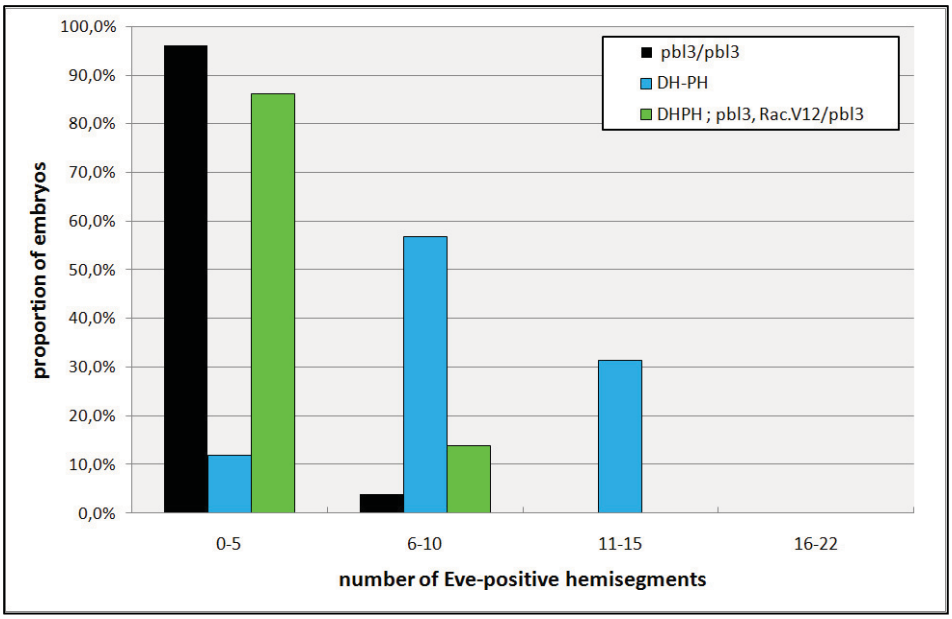

Fig. 3.19: Co-expression of constitutive active Rac1 negativley influences the migration rescue by Pbl^{DH-PH}. Embryos were stained against Eve and the number of pericardial cell clusters was counted. Expression of Pbl^{DH-PH} (blue) rescues the migration defects in pbl^{3} mutant embryos (black). This rescue capacity is lowered significantly upon co-expression of $Rac1^{V12}$ (green).

Given the previous results, a second approach was used to test for a genetic interaction of Rac and Pbl during mesodermal spreading. This time, the rescue of the pbl^{3} phenotype by $Pbl^{\Delta BRCT}$ expression was employed as a background. Misexpression of $Pbl^{\Delta BRCT}$ did not cause any dominant effects (in contrast to Pbl^{DH-PH}). As mentioned above, the presence of the NLS and the PEST motif in this construct might downregulate the cytoplasmic levels of this constitutively active Pbl variant, lowering the probability of hyperactivating substrate GTPases in a similar manner as achieved by Pbl^{DH-PH} or the direct expression of $Rac1^{V12}$. Considering these ideas, a wild type Rac1 construct was co-expressed with $Pbl^{\Delta BRCT}$ in a pbl^{3} mutant. Under these conditions, the rescue capacity was significantly enhanced compared to the rescue of the $Pbl^{\Delta BRCT}$ construct alone (Fig. 3.20 A, Tab. 3.3). In other words, the rescue is stronger the more potential substrate molecules for the Pbl construct are present in the

mesoderm. Therefore, this result strongly supports the model that Pbl acts through the activation of Rac during mesoderm migration.

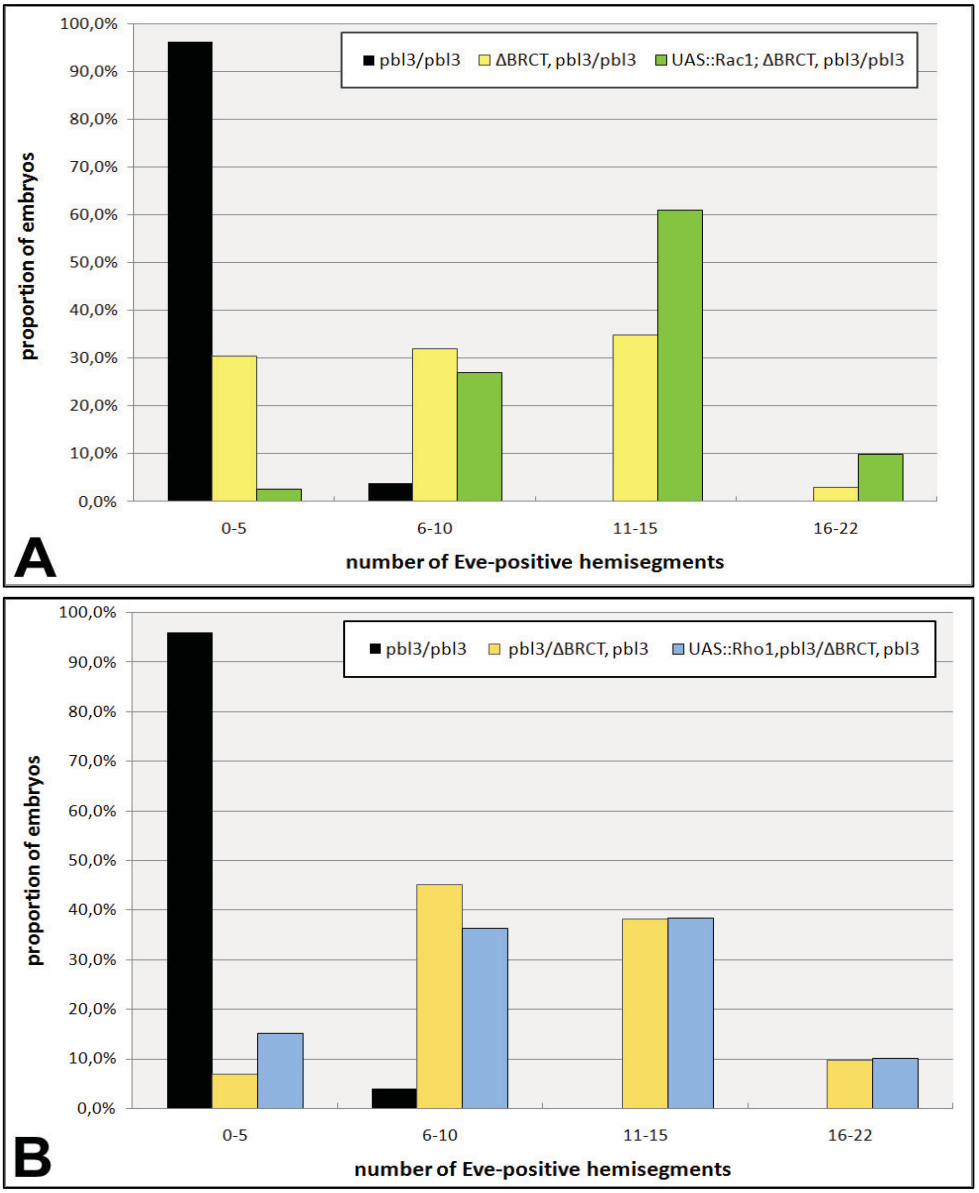

Fig. 3.20: Constitutive active Pbl interacts genetically with Rac1 but not with Rho1 during mesoderm migration.

(A,B) In order to test for genetic interactions between Pbl and Rac and Rho1, *pbl*^{3} mutant embryos expressing the Pbl$^{\Delta BRCT}$ rescue construct and wild type forms of the respective GTPase were stained (anti-Eve) and the number of pericaridial cell clusters was counted. Grouped values were plotted against the relative proportion of embryos displaying the indicated range of Eve-positive clusters. (A) The strong *pbl*^{3} phenotype (black) is partially rescued by expression of Pbl$^{\Delta BRCT}$ (pale yellow). This rescue can be significantly enhanced by co-expression of a wild type Rac1 construct (green). (B) The crosses employed for this experiment were different from the ones in (A) (see 2.5.4); the adapted control cross of Pbl$^{\Delta BRCT}$ in *pbl*^{3} embryos resulted in a slightly better rescue (pale yellow). Co-expression of wild type Rho1 does not significantly alter this migration rescue of Pbl$^{\Delta BRCT}$ (blue). Note that expression of either GTPase construct alone does not rescue migration defects in *pbl*^{3} mutant embryos (Schumacher and Müller, unpublished).

As a control for the Rac1 interaction, the experiment was repeated using a wild type form of Rho1 this time. As the crosses for this experiment were different from the ones employed before, the $Pbl^{\Delta BRCT}$ control was repeated using the identical genetic background as for the subsequent Rho1 crosses (see 2.5.4). Under these conditions, $Pbl^{\Delta BRCT}$ expression led to a slightly better rescue of the *pbl*^{3} phenotype. However, when co-expressed with wild type Rho1, the number of Eve-positive cell clusters was almost identical indicating that Rho1 does not similar to Rac1 enhance the rescue potential of activated Pbl (Fig. 3.20 B, Tab. 3.4). This result clearly demonstrates the specificity of the enhanced migration rescue by Rac, as the $Pbl^{\Delta BRCT}$ construct is probably also theoretical able to activate Rho1 analogous to what was observed for $Pbl^{DH\text{-}PH}$ and especially for $Pbl^{\Delta N\text{-}term}$ in the eye-assay. Therefore, these experiments strongly support the model that Rac but not Rho1 acts downstream of Pbl during mesodermal spreading.

genotype	Eve-positive hemisegments	SD	n
pbl^{3}/*pbl*^{3}	1.7	1.7	128
Pbl$^{\Delta BRCT}$, *pbl*^{3}/*pbl*^{3}	8.3	4.2	69
Rac1; Pbl$^{\Delta BRCT}$, *pbl*^{3}/*pbl*^{3}	11.8	2.8	82

Table 3.3: Rac1 promotes the migration rescue of *Pbl*$^{\Delta BRCT}$ in a *pbl*^{3} mutant background.
Mean values and their standard deviations (SD) of the number of Eve-positive hemisegments are shown for *pbl*^{3} homozygotes expressing the indicated constructs (n = number of embryos examined). Mesoderm specific expression of $Pbl^{\Delta BRCT}$ using *twi::Gal4* suppresses the spreading defects of *pbl*^{3} mutants. This rescue is significantly enhanced by co-expression of a wild type Rac1 construct (Student's test, P=1.55073E-08).

genotype	Eve-positive hemisegments	SD	n
pbl^{3}/*pbl*^{3}	1.7	1.7	128
pbl^{3}/ *Pbl*$^{\Delta BRCT}$, *pbl*^{3}	10.3	3.5	102
Rho1, pbl^{3} /*Pbl*$^{\Delta BRCT}$, *pbl*^{3}	10.2	4.0	99

Table 3.4: Co-expression of Rho1 does not modify the migration rescue of *Pbl*$^{\Delta BRCT}$ in a *pbl*^{3} mutant background.
Mean values and their standard deviations (SD) of the number of Eve-positive hemisegments are shown for *pbl*^{3} homozygotes expressing the indicated constructs (n = number of embryos examined). The crosses employed for this experiment differed from the one shown in Tab. 3.3 and resulted in a slightly better rescue of the *pbl*^{3} phenotype by $Pbl^{\Delta BRCT}$. Co-expression of a wild type Rho1 construct did not lead to significant alterations in the migration rescue.

3.5.5 Activated Pbl binds Rac1 and Rac2

In a guanine-nucleotide-exchange-assay (GEF-assay), which employed only the DH domain of Pbl, a robust exchange activity for Rho1 was detected. Importantly, these experiments also revealed a weaker activity of the DH domain towards Rac1 and Rac2, suggesting that the domain is able to trigger at least *in vitro* the activation of these GTPases as well (Schumacher, 2005; van Impel et al., 2009). The fact that the activity towards Rac GTPases was lower than for Rho1 might indicate that the PH domain of Pbl is required in concert with the DH domain to activate Rac GTPases with full efficiency. As mentioned in the introduction (see 1.5), PH domains often contribute to the exchange activity of GEF proteins by participating in the binding of the substrates, for example (Rossman et al., 2005; Rossman and Sondek, 2005; Rossman et al., 2002). If this was also the case for the binding of Pbl to Rac GTPases, the presence of the PH domain might facilitate and enhance the activation of Rac in an exchange assay. This idea would be consistent with the results obtained in the embryo where the DH domain had only an impact on migration if accompanied by the PH domain. In order to test this hypothesis it was intended to repeat the GEF-assay using the DH-PH tandem domain this time. Furthermore, this *in vitro* system should also allow testing for differences between the substrate preferences of $Pbl^{DH\text{-}PH}$ and $Pbl^{\Delta N\text{-}term}$. Unfortunately, this attempt failed because of the insolubility of GST- and His-tag fusions with the DH-PH tandem domains. Therefore, another strategy was chosen to further strengthen the idea that the relationship between Pbl and Rac is based on a direct physical interaction of the proteins and does not depend on an indirect activation by downstream components of an alternative Pbl dependent pathway.

The GEF mediated activation of GTPases comprises several distinct steps. The initial binding of the GEF to the inactive GTPase-GDP complex occurs with a relatively low affinity. After dissociation of the GDP however, GEF and GTPase form a very short life high-affinity complex until the GTPase binds to GTP. The high affinity of the GEF for the nucleotide depleted form of the substrate GTPase can be used for an *in vitro* GEF binding assay (Blanke and Jackle, 2006). Briefly, GST-GTPase fusion proteins were expressed in bacteria and purified using glutathione agarose beads. Lysates from embryos expressing HA-tagged constitutively active Pbl were generated. By transferring the beads into a nucleotide free buffer containing EDTA, the depletion of the bound GDP from the GTPases was achieved. These GTPases were used for a GST pull down of the GEF proteins from the lysate and bound Pbl constructs were subsequently detected by western blot analysis using an anti-HA antibody.

The embryo lysates were generated from embryos overexpressing $Pbl^{\Delta N\text{-term}}$ in the mesoderm as these lysates had the best yield of HA-tagged proteins. The obvious difference in the molecular weight between this construct and the Rho GTPases allowed a clear discrimination of the specific HA signal and an unspecific binding of the antibody to the GTPases, which were in abundance present in the respective pull down samples. The lysates were incubated with GST-Rho1 as a positive control, as Rho1 and Pbl had already been shown to interact in a yeast-two hybrid assay (Prokopenko et al., 1999). GST alone was used as a negative control. In addition, GST-Rac1 and GST-Rac2 were tested for their binding affinity towards $Pbl^{\Delta N\text{-term}}$. As indicated in Fig. 3.21, the GST pull down of $Pbl^{\Delta N\text{-term}}$ with GST-Rho1 resulted in a single signal that corresponded to a band visible only in lysates from embryos expressing $Pbl^{\Delta N\text{-term}}$ but not in wild type lysates, suggesting that the signal is not the result of an unspecific binding of the anti-HA antibody. The fact that the construct ran higher than the expected 58kDa might indicate that the Pbl variant was postranslationally modified. Consistent with this idea, the anti-Pbl antibody was also shown to detect a band above the expected molecular weight of the Pbl protein on Western Blots, suggesting that the endogenous protein might be modified as well (Schumacher, 2005). In addition, the used prestained protein ladder (BenchMark, *Invitrogen*) was found to run lower than markers from other companies when used on the same gel (e.g. the BroadRange 7-175kDa marker, *NEB*). This discrepancy might further help to explain why the $Pbl^{\Delta N\text{-term}}$ constructs was running higher than expected.

GST covered glutathione agarose beads were not able to bind the GEF efficiently. However, both Rac1 and Rac2 were also able to pull down the Pbl construct from the lysate. The fact that the signal detected on the blot for Rac1 was weaker than the ones for Rac2 or Rho1 does not necessarily reflect a major difference in the binding affinity of the Pbl construct for this GTPase. As the total amount of bound GTPases might slightly differ from one to the next experiment, this assay is not quantitative. Therefore, only qualitative but no quantitative conclusions should be drawn here.

Nevertheless, these experiments demonstrate the capacity of Pbl's catalytic core to bind Rac GTPases as well, a finding that further stresses the idea, that Pbl regulates Rac activity in a direct manner during mesoderm migration. The fact that these pull downs were possible using the $Pbl^{\Delta N\text{-term}}$ construct also demonstrates that the postulated influence of the C-terminus on the substrate preference of the protein does not generally prevent a binding of the catalytic core to the different substrate GTPases per se. In fact, the overall substrate specificity of GEFs is thought to depend on the three-dimensional structure of the DH domain, which defines the contact interphase with the different GTPases. Depending on the residues that form the

interface with the substrate, only certain GTPases 'fit' into the binding pocket formed by the DH domain and can be activated (Snyder et al., 2002). Therefore, it is more likely that modifications of GEF proteins in living cells result in shifts of the substrate preference rather than in substrate specificity. Hence, it is not surprising that the Pbl$^{\Delta N\text{-term}}$ construct is still capable of binding Rho1, Rac1 and Rac2 *in vitro* and it does not argue against an involvement of the C-terminal tail in influencing the *in vivo* substrate preference of Pbl.

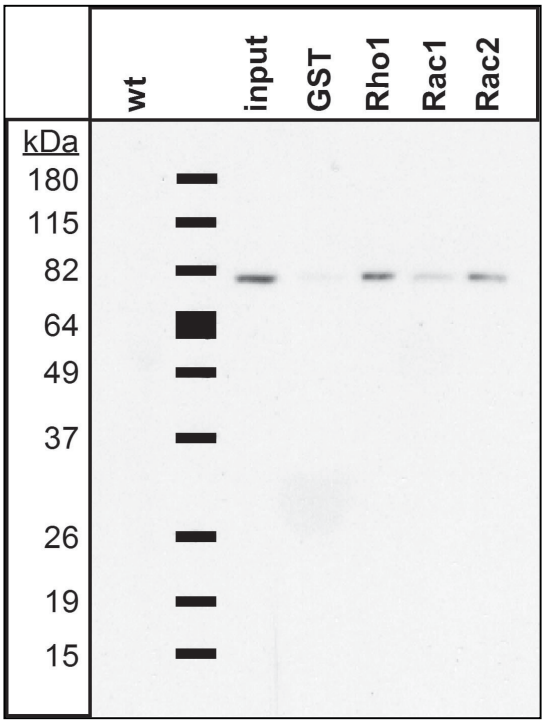

Fig. 3.21 Constitutive active Pbl binds to Rac1, Rac2 and to Rho1 *in vitro*.
Lysates from embryos overexpressing Pbl$^{\Delta N\text{-term}}$ were incubated with glutathione agarose beads coated with the indicated GST-fusion proteins (GST alone, GST-Rho1, GST-Rac1, GST-Rac2). A western blot of the respective pull down samples and the Pbl$^{\Delta N\text{-term}}$ lysate (input) was incubated with an anti-HA antibody to detect the HA-tagged Pbl construct. The specificity of the anti-HA antibody was tested using lysate from wild type embryos (wt). The Pbl$^{\Delta N\text{-term}}$ construct had an expected molecular weight of about 58 kDa.

3.5.6 Mutagenesis of distinct amino acids within the DH domain that are essential for specific substrate binding in other GEFs

A frequently used approach to study the function of a protein or the role of its different domains is to do so-called "forward genetics". In this context, alleles of the respective proteins are analyzed and compared to each other in order to find a relation between the mutations in the ORF and the resulting phenotypes. Such an approach was already taken for the *pbl* gene in order to identify mutations that would affect only the migratory or the

cytokinesis function of the protein. However, thus far no mutation could be identified that would indicate a differential requirement of certain domains in either function of the GEF (Schumacher, 2005).

A novel way to create a migration or cytokinesis specific Pbl variant is based on crystallographic studies that have revealed the structure of the complex between different Dbl family GEFs and their substrate GTPases. Using this knowledge,. Snyder et al. (2002) tried to investigate how the three-dimensional structure of a GEF domain influences the specific binding of the proteins to their substrates. In order to reveal the critical key determinants within the DH domain, they could identify single residues in the region of the strongly conserved α-helix 5 of different Dbl family proteins. By mutating these sites in some of the GEF proteins that were examined, they could indeed change the specificity of the respective DH-PH tandem domains in *in vitro* assays (Snyder et al., 2002).

The observations by Snyder et al. (2002) were used as a basis to generate Pbl variants that are only able to interact with either Rac or Rho1. In order to identify the corresponding regions within the DH domain of Pbl, a program for prediction of α-helical protein regions was employed. In the region of the fifth helical structure in Pbl's GEF domain, several amino acids were identified that could account for specificity towards Rho1 and Rac (Suppl. Fig. 4). Subsequently these amino acids were mutagenized in a Pbl full-length construct. To test whether these mutations affect the capability of the constructs to activate either Rho1 or Rac1/Rac2, the constructs were expressed in a pbl^{3} mutant background and checked for rescue of cytokinesis and migration.

The presence of Isoleucine or Lysine at one particular position within α-helix 5 was shown to change the properties of the binding pocket formed by the GEF domain. GEFs using Cdc42 as a substrate always have a Lys at this position, while specificity towards Rac1 is always linked to an Ile. Changing Lys to Ile in a GEF specific for Cdc42 for example, results in an additional *in vitro* activity towards Rac (Snyder et al., 2002).

In order to create a variant of Pbl that has a lower affinity towards Rac while the binding to Rho1 is not compromised the I565L mutation was inserted (Suppl. Fig. 4). Rescue experiments showed that the cytokinesis defects were still rescued in these embryos as in DAPI staining of stage 12 embryos the nuclei in the mesoderm are clearly smaller than the ones in the surrounding tissue (Fig. 3.22 A). As already mentioned before, problems during cytokinesis lead to multinucleated cells in which the nuclei eventually fuse to form a large, single nucleus. Therefore, DAPI staining can be used to assess the overall rescue of cytokinesis.

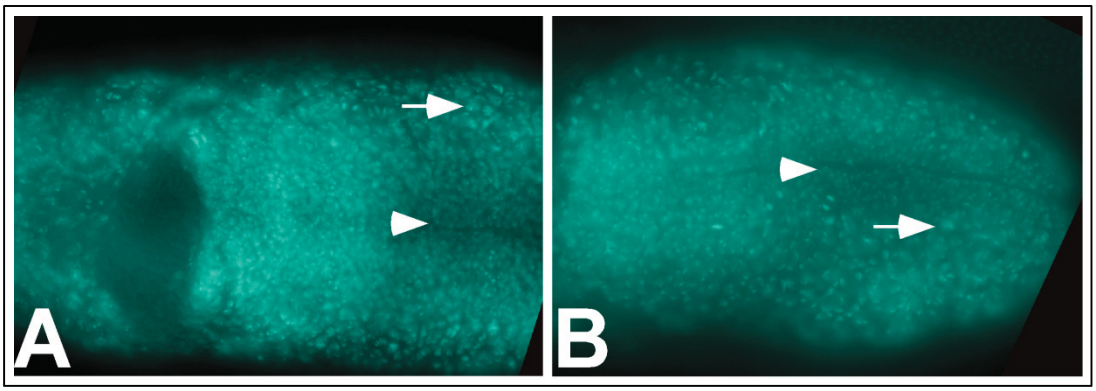

Fig. 3.22: The mutagenized PblA^{I565L} and PblA^{R557S,L558S} constructs rescue cytokinesis in *pbl*^{3} mutant embryos.
DAPI staining of stage 12 *pbl*^{3} mutant embryos expressing PblA^{I565L} (A) or PblA^{R557S,L558S} (B) in the mesoderm with *twi::Gal4*. In dorsal views, the nuclei of mesoderm cells appear much smaller and more regular in size (arrowheads in A and B) compared to the ones in the surrounding tissues (arrows in A and B), indicating that both constructs rescued cytokinesis in the mesoderm.

In contrast to cell division, the level of migration rescue was lower than what was achieved by expression of the wild type form (Fig. 3.23, Tab. 3.5). Nevertheless, it has to be pointed out that the rescue experiments for PblA^{I565L} were performed at room temperature and not at 25°C as all other rescue experiments. As the temperature has been found to exhibit a clear effect on the expression levels and thereby on the rescue properties of the constructs (see Tab. 3.5, PblA rescue on RT), part of the effects seen after replacement of Ile 565 by Lys is probably caused by this temperature effect. However, it seems to be rather unlikely that the strong differences in the migration rescue by PblA and PblA^{I565L} are exclusively based on different expression levels, as a lower temperature alone only caused a mild decrease in the number of Eve-positive cell clusters in the full-length rescue (Tab. 3.5).

To create a construct that no longer interacts with Rho1 while the ability to bind and activate Rac is not impaired, the R557S,L558S mutation was introduced to the DH domain of Pbl (Suppl. Fig 4). Expression of the resulting full-length construct in a *pbl*^{3} mutant background however also led to a rescue of the cytokinesis defects, showing that the construct still activated Rho1 efficiently *in vivo* (Fig. 3.22 B). With regard to mesodermal spreading, the rescue achieved by expression of this construct was significantly lower than for the wild type protein (Fig. 3.23, Tab. 3.5). As this experiment was performed on 25°C, introduction of the R557S,L558S mutation had a clear impact on the rescue ability of the construct with regard to mesoderm migration but it had no appreciable effect on the rescue of the cytokinesis defects. Hence, the mutation had the opposite effect as intended.

genotype	Eve-positive hemisegments	SD	n
pbl^3/pbl^3	1.7	1.7	128
PblA-HA; pbl^3/pbl^3	18.6	1.7	98
PblA-HA; pbl^{NR}/pbl^3 **(RT)**	15.9	3.8	59
$PblA^{I565L}$*;* pbl^3/pbl^3 **(RT)**	11.4	4.0	47
$PblA^{R557S,L558S}$*;* pbl^3/pbl^3	12.6	4.3	86

Table 3.5: Amino acid exchanges within the DH domain lead to a decrease in migration rescue.
Mean values and their standard deviations (SD) of the number of Eve-positive hemisegments are shown for pbl^3 homozygotes expressing the indicated constructs (n = number of embryos examined) on 25°C or room temperature (RT). Insertion of the I656L mutation into the DH domain significantly reduces the rescue capacity of the full-length construct (Student's test *PblA-HA;*pbl^3/pbl^3 to $PblA^{I565L}$*;*pbl^3/pbl^3 (RT), P=5.24249E-17; *PblA-HA;*pbl^{NR}/pbl^3 (RT) to $PblA^{I565L}$*;*pbl^3/pbl^3 (RT), P=2.32339E-08). The rescue capacity of full-length Pbl is significantly reduced by the R557S,L558S mutation (Student's test *PblA-HA;*pbl^3/pbl^3 to $PblA^{R557S,L558S}$*;*pbl^3/pbl^3, P=2.01133E-22).

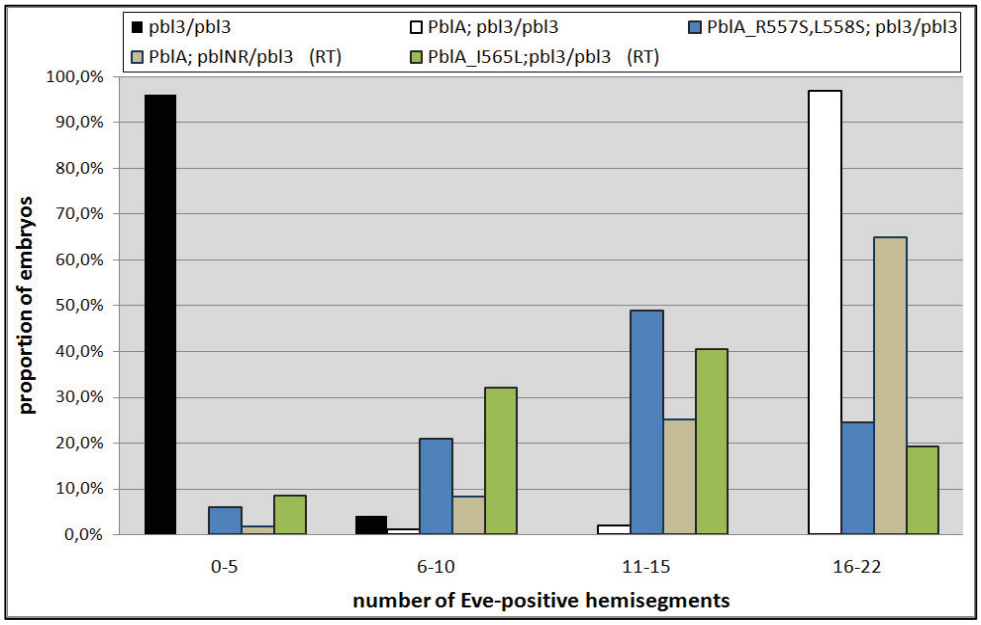

Fig. 3.23: Decrease in the migration rescue of full-length PblA after introduction of the I556L and R557S,L558S mutations.
Embryos mutant for *pbl* were stained against Eve and the number of pericardial cell clusters was counted. Grouped values were plotted against the relative proportion of embryos displaying the indicated range of Eve-positive clusters. Expression of full-length Pbl (white) rescues the migration defects in pbl^3 mutant embryos (black); the rescue is slightly weaker after expression in pbl^{NR}/pbl^3 mutant embryos at room temperature (pale brown). The rescue capacity of the full-length protein is strongly lowered in the mutated $PblA^{I565L}$ (blue) and $PblA^{R557S,L558S}$ (green) variants.

Taken together, the inserted point mutations did not generate the expected selective effects on either Rho1-dependent cytokinesis or the Rac dependent migration of the mesoderm. Nevertheless, as in both cases only a reduction in the rescue capacity for migration was

detected, the mutations seem to have a distinct effect on just one of the two Pbl functions. However, to support this conclusion a quantitative analysis of the cytokinesis rescue has to be performed to exclude the possibility that cytokinesis is not rescued to 100%. Unfortunately, further analysis of the DH domain indicated that the inserted mutations do not affect the amino acids in the DH domain that correspond to the residues identified by Snyder et al. (2002) (see discussion for details). Hence, it is possible that the inserted mutations only reduce the overall activity of the GEF domain. If migration and cytokinesis depended on different levels of active Pbl, a reduction of the exchange activity could also explain the differential rescue phenotype that was found for both constructs. Consequently, it is not clear how the different mutations led to the differential rescues of migration and cytokinesis function of Pbl.

3.5.7 Localization studies of Rho1 and Rac GTPases during mesoderm migration

One way how specificity of Rho GTPases is controlled is the spatial and temporal localization of the GEFs and GTPases at specific subcellular compartments in response to a variety of cellular cues (Rossman et al., 2005). So far, nothing is known about the localization of the different Rho GTPases in the mesoderm during the migratory process. In order to find out, whether Rho1 or Rac GTPases accumulate at special regions like the leading edge for example, different approaches were taken. To assess the localization pattern of endogenous Rac1 and Rac2 during mesodermal spreading, different antibodies against these proteins were tested. Unfortunately, none of the tested antibodies was able to detect the proteins in whole mount staining, not even when embryos overexpressing the Rac GTPases in the mesoderm were stained. Therefore, a Myc-tagged Rac1 fusion protein was expressed in the mesoderm of wild type embryos in order to check the subcellular distribution of the protein during the different phases of migration.

As shown in Fig. 3.24, the protein was visible in dots within the cytoplasm of embryos during the migratory phase (stage 8). It appeared that these punctae accumulate at the cell peripheries (Fig 3.24 A-C). However, a clear cortical localization was not detectable in all cells (Fig. 3.24 D-F). Furthermore, a specific accumulation of the protein at the leading edge of the mesoderm cells could not be observed under these overexpression conditions. In later stages however, after the spreading process was already complete, strong membrane association of the fusion protein was visible in all mesoderm cells (Fig 3.24 G-L). The fact that the protein in part localized to the cortex suggests that activation of this GTPase might occur at the plasma membrane during migration. On the other hand, as no specific subcellular membrane domains

showed an enrichment of the fusion protein it is impossible to predict whether the protein could be involved in processes at the leading edge or the rear end of the migrating cells for example. The main reason for uniform staining of the cortices might be the fact that it was not possible to detect the localization of the endogenous Rac proteins directly so that a tagged form had to be expressed. Overexpression of this form could in turn mask possible sites of accumulation of the endogenous protein as the construct might also bind to low affinity binding sites under these misexpression conditions. Alternatively, the GTPase could also be localizing to the whole cortex and spatio-temporal specificity might be mediated by site-specific activation of only a fraction of the Rac GTPases.

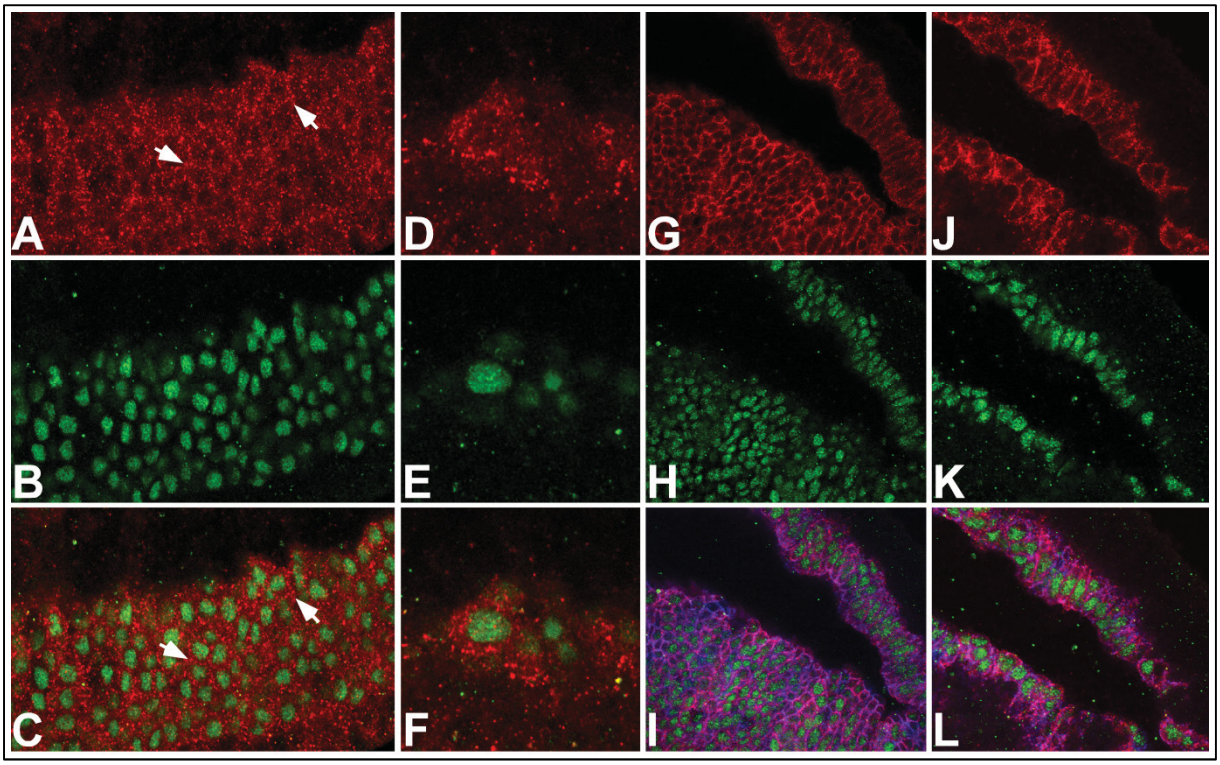

Fig 3.24: Localization of Rac1-Myc in the mesoderm of wild type embryos.
Wild type embryos expressing a Rac1-Myc construct in the mesoderm using *twi::Gal4;Dmef2::Gal4* were stained against Myc (red), Twi (green) and DN-Cadherin (DN-Cad) as a membrane marker (blue in I,L). (A-C) In stage 8 embryos, the Rac fusion protein localizes in a punctate fashion and shows enrichment at the cell cortex (arrows). (D-F) Higher magnification of mesoderm cells during stage 8 showing the dot-like accumulations of the Rac protein. At the end of migration (stage 9), the fusion protein displays a strong cortex association where it colocalizes with the membrane marker DN-Cad (G-L).

It will be interesting to check whether the dot-like Rac pattern in stage 8 embryos colocalizes with the previously mentioned punctae in which the Pbl^{DH-PH} protein localizes in mesodermal cells. This rather unusual pattern can also be interpreted as a staining of vesicles for example, which might be interesting in the context of a possible EMT function of Pbl/Rac (see discussion). Therefore, it should be assessed in the future, whether the Rac and/or Pbl^{DH-PH}

positive punctae in migrating mesoderm cells are also positive for different markers of the endo- or exocytotic vesicle system.

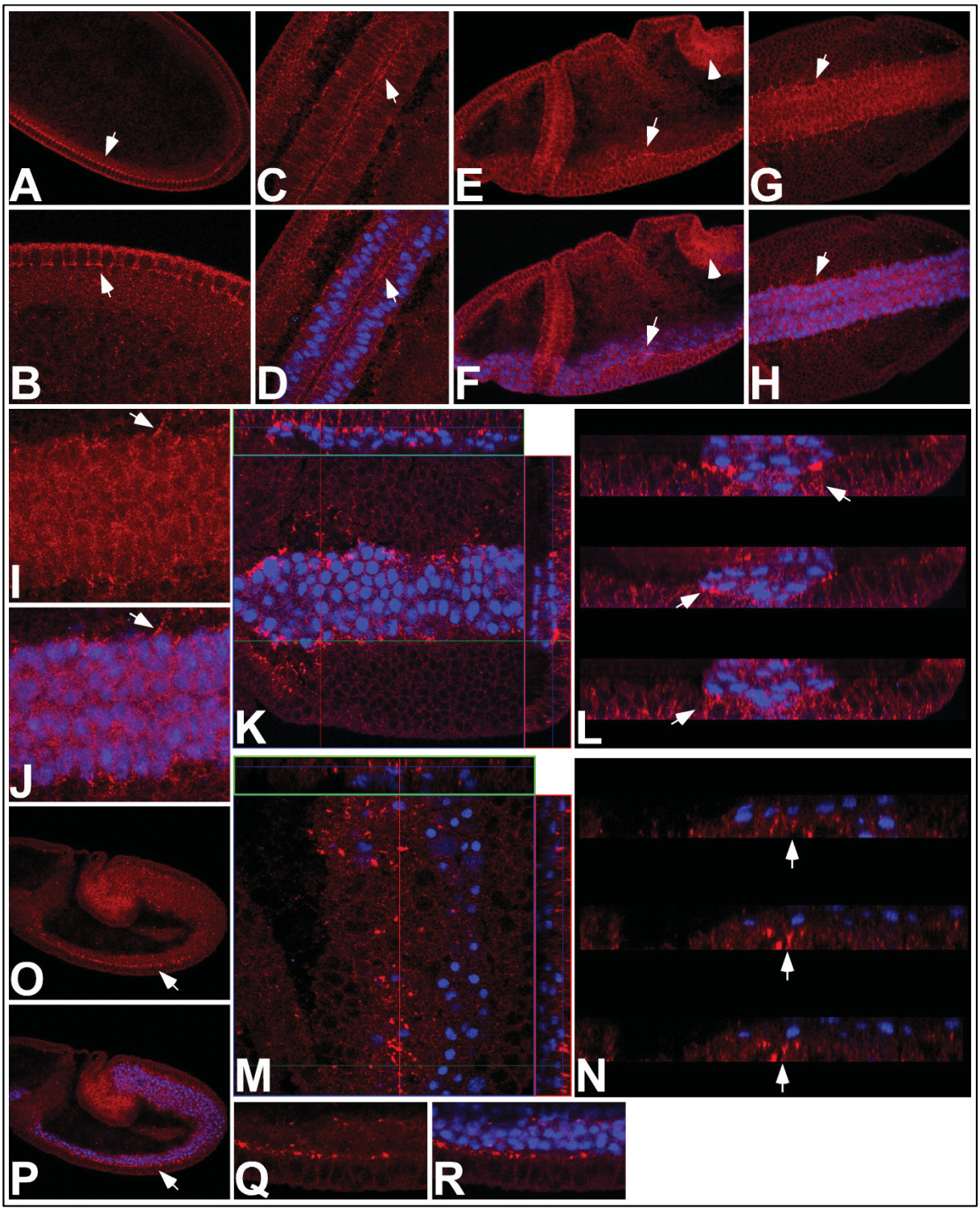

Fig. 3.25: Localization of endogenous Rho1 protein during different phases of mesoderm development. (A-R) Wild type embryos were stained against endogenous Rho1 (red) and Twi (blue). (A, B) During cellularization, Rho1 localizes to the ingressing furrow canals (arrows). (C,F) Upon invagination, the protein localizes to the cell cortex with slightly higher levels at the cell apices (arrows) as assessed in ventral (C,D) and lateral views (E,F). Rho1 also accumulates apically in the invaginating posterior midgut primordium (arrowhead in E,F). (G-J) Ventral views indicating a subtle enrichment of Rho1 at lateral sites of the mesodermal tube after

invagination is complete (arrows). (K,L) Images obtained from a z-series of 147 sections in 0.20 µm intervals (29.20 µm in total; ventral view). (K) Single section of the z-series through the mesoderm during phase 1 of migration indicates strong accumulation of Rho1 at the lateral sites of the mesoderm; virtual transversal cross-section (right box, red) of the embryo at the position marked by the coloured lines shows that these accumulations localize to the interphase of meso- and ectoderm. (L) Three virtual cross-sections of the same embryo as in (K) at different positions along the anterior-posterior axis; several protrusion-like structures and strong Rho1 accumulations are visible in all optical planes (arrows). (M,N) Images obtained from a z-series of 64 sections in 0.24 µm intervals (15.12 µm in total; ventral view). (M) During phase 2 of mesoderm migration dot-like accumulations of Rho1 can be seen at various positions in the mesoderm in a ventral view, which all localize to the interphase between ectoderm and mesoderm (corresponding transversal virtual cross-section at the top, marked by a green box; virtual lateral section at the right of main the picture, marked by a red box). (N) Series of virtual transversal cross-sections of the embryo in (M), in which the same Rho1 positive structure is marked in three sequent optical planes (arrows). (O-R) Lateral view of a stage 8 embryo showing the Rho1 accumulations between mesoderm and ectoderm during phase 2 of migration.

To get insight into the localization pattern of Rho1 during migration, wild type embryos were stained against the endogenous protein. As described earlier, the protein localized to the base of the furrow canals during the process of cellularization (Fig 3.25 A, B), indicating that the used antibody indeed recognizes endogenous Rho1, a finding that is consistent with earlier reports (Magie et al., 2002; Padash Barmchi et al., 2005).

At the onset of gastrulation, a weak accumulation of the protein was visible at the apical sites of the invaginating mesoderm cells (Fig. 3.25 C-F) as well as at the apices of the cells of the posterior midgut primordium (arrowhead in E). It is known that the RhoGEF2 protein also accumulates at these sites. This exchange factor was found to be the activator of Rho1 during the invagination processes (Barrett et al., 1997; Grosshans et al., 2005; Hacker and Perrimon, 1998; Padash Barmchi et al., 2005). After completion of invagination, Rho1 localized to the whole cell cortex of the mesoderm cells, but the staining seemed to be stronger at the lateral sites of the tube (arrows in Fig. 3.25 G-J).

In a slightly later stage when the invaginated mesodermal tube had already established the contact with the ectodermal layer and had begun to flatten down symmetrically, the accumulation of the Rho1 signal at these lateral positions within the mesoderm became more pronounced (Fig. 3.25 K). Z-stacks of the mesoderm at this stage revealed that these strong Rho1 signals were localized at the interface between the mesoderm and the underlying ectoderm (see optical cross-section at the right of K; the position corresponds to the position that is marked by the coloured lines in the main image). This accumulation was evident along the anterior posterior axis of these embryos, suggesting that this might be a specific staining. In some of the optical cross-sections it appeared that Rho1 would be located in large mesodermal protrusions into the ectodermal cell layer (see arrow in bottom image of Fig. 3.25 L). Interestingly, actin rich protrusions have recently been observed at comparable

positions during live-imaging studies with an actin-GFP fusion protein. It was shown that these protrusions are formed by mesoderm cells at the onset of EMT and that they protrude far into the ectodermal layer (Clark and Müller, unpublished data).

Later, during the spreading phase, the strong Rho1 signal at the mesoderm/ectoderm interphase could still be observed (Fig. 3.25 O-R). As during the collapse phase of the mesodermal tube, the staining was only visible in regions where the mesoderm was in contact with the ectoderm. This finding is consistent with earlier reports that have mentioned Rho1 accumulation at the site where ectodermal and mesoderm cell layers meet (Fox et al., 2005). In virtual cross-sections, the signals appeared in dot- or line-shaped accumulations that could in part be interpreted as protrusion-like structures again (Fig. 3.25 N). However, as no marker for the membrane of the mesoderm cells was used here, it is not possible to clarify whether the Rho1 staining is indeed located within mesodermal cells or whether it might reflect accumulations of the protein in the ectoderm. Furthermore, these initial observations have to be further extended in future using different markers to ensure that the described structures are indeed site-specific accumulations of the GTPase Rho1 and no artifacts (see discussion). Nevertheless, these observations render it possible that Rho1 plays a yet unknown role during mesoderm migration that should be further analyzed in the future.

3.6 Mesoderm specific expression of human Ect2

3.6.1 Human Ect2 does not rescue mesoderm migration in *pbl* mutant embryos

It is known that Pbl's cytokinesis function is conserved from fly to man. Beside this involvement in cell division, Pbl's mammalian homologue Ect2 was reported to play an essential role in some types of cancer (Miki et al., 1993). The oncogenic form of Ect2 is an N-terminally depleted version of the protein that is, analogous to some of the Pbl constructs presented here, constitutively active (Miki et al., 1993; Saito et al., 2004; Solski et al., 2004). To test whether the migratory function of Pbl is conserved during evolution as well and might therefore contribute to the transforming activity of Ect2 in cancer, a full-length human Ect2 construct was generated. When Ect2 was expressed in a pbl^3 mutant background, no obvious rescue of the migration defects could be observed. On average only 4.2 Eve-positive clusters were found after expression of the construct on room temperature (Tab. 3.6, Fig. 3.26).

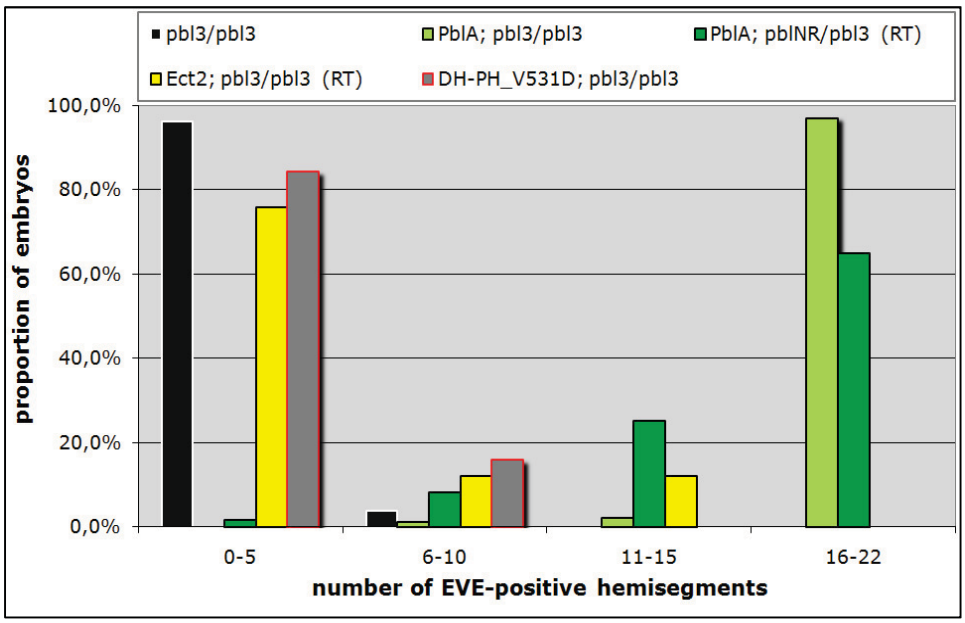

Fig. 3.26: Human Ect2 fails to rescue migration defects in *pbl* mutant embryos.
pbl mutant embryos were stained against Eve and the number of pericardial cell clusters was counted. Grouped values were plotted against the relative proportion of embryos displaying the indicated range of Eve-positive clusters. Expression of full-length Pbl rescues mesoderm migration in *pbl* mutants (black) when expressed on room temperature (dark green) or on 25°C (pale green). The human full-length Ect2 construct does not cause a significant rescue of the pbl^3 phenotype resulting in similar values as the expression of the catalytic dead $Pbl^{DH\text{-}PH\_V531D}$ control construct (grey).

genotype	Eve-positive hemisegments	SD	n
pbl^3/pbl^3	1.7	1.7	128
PblA-HA; pbl^3/pbl^3	18.6	1.7	98
PblA-HA; pbl^{NR}/pbl^3 (RT)	15.9	3.8	59
humEct2; pbl^3/pbl^3 (RT)	4.2	3.3	38

Table 3.6: Expression of human Ect2 does not rescue migration in *pbl* mutant embryos.
Mean values and their standard deviations (SD) of the number of Eve-positive hemisegments are shown for pbl^3 homozygotes expressing the indicated constructs (n = number of embryos examined) on 25°C or room temperature (RT). Expression of a full-length human Ect2 construct does not rescue mesodermal spreading in pbl^3 mutant embryos.

Although this value is higher than for pbl^3 alone, it is not a significant difference indicating that the expressed protein is not functional. As mentioned before, the temperature on which the rescue is performed can lead to variations in the expression levels of the constructs. However, as the differences in the rescue capacity of full-length Pbl on 25°C and room temperature were only subtile (see Tab. 3.5), it is very unlikely that this temperature effect is the cause for completely missing rescuing activity of the Ect2 protein. In conclusion, this result indicates that the expressed Ect2 protein is not capable of rescuing migration in *pbl*

embryos. Importantly, an appreciable rescue of the cytokinesis defects was not observed either suggesting that there might be a general problem with the activity of the human protein in *Drosophila* cells.

3.6.2 Nuclear enrichment of Ect2 in *Drosophila* embryos is rather weak

It was reported that tagged Ect2 constructs could not only be detected in the nuclei but also in the cytoplasm and at the cortex of different vertebrate cell lines during interphase (Liu et al., 2004; Liu et al., 2006). Consistent with this finding different interphase functions have been suggested for Pbl's homologues, not only in mammalian systems but also in *C. elegans*, for example (Jenkins et al., 2006; Liu et al., 2004; Liu et al., 2006; Morita et al., 2005).

In order to find out why expression of human Ect2 nevertheless failed to rescue any of the mutant phenotypes in pbl^3 embryos, the localization pattern of the protein was examined. Staining against the HA-tag showed that the fusion protein was expressed in the mesoderm as expected. However, several abnormalities were found when the subcellular localization of Ect2 was investigated. The most obvious difference to the localization pattern that was described for Ect2 in mammalian cells and Pbl in *Drosophila* is the fact that only relatively low amounts of protein were detectable in the nuclei of mesoderm cells (Fig. 3.27 A-F). Instead, the protein could be detected in high concentrations in the cytoplasm. In part of the interphase mesoderm cells, a weak cortical accumulation of Ect2 was detectable indicating that the protein is able to bind to the cortex even in *Drosophila* tissue (Fig. 3.27 A-C). In post mitotic cells the protein also showed a weak cortical localization. However, almost no accumulation of Ect2 in the nuclei was detectable in these cells (Fig. 3.27 D-I). It is therefore possible that the nuclear import of human Ect2 is less effective and hence delayed in *Drosophila* cells, which would also be consistent with the overall low nuclear staining. In addition, the characteristic localization to the centralspindlin complex during cytokinesis was never observed for Ect2 (Fig. 3.27 D-I), a finding that might explain the lack of rescue of cytokinesis defects when expressed in a pbl^3 background (not shown).

Furthermore, overexpression of Ect2 never interfered negatively with either migration or cytokinesis, a result that confirms and extends earlier findings of cytokinesis studies (Prokopenko et al., 1999). To conclude, the localization pattern of human Ect2 in *Drosophila* cells differs from what is known from human cells and from Pbl's localization and might therefore explain the failure in rescuing the functions of Pbl during migration and cytokinesis. As these results could reflect a general problem with the human NLS sequences in *Drosophila* cells, for example, they do neither confirm nor disprove a possible interphase function of Ect2 that is comparable to the role of Pbl during mesodermal spreading. An alternative way to test

this possibility in the future is to generate expression constructs of oncogenic Ect2 in order to compare possible dominant phenotypes with the data obtained in this domain-function analysis.

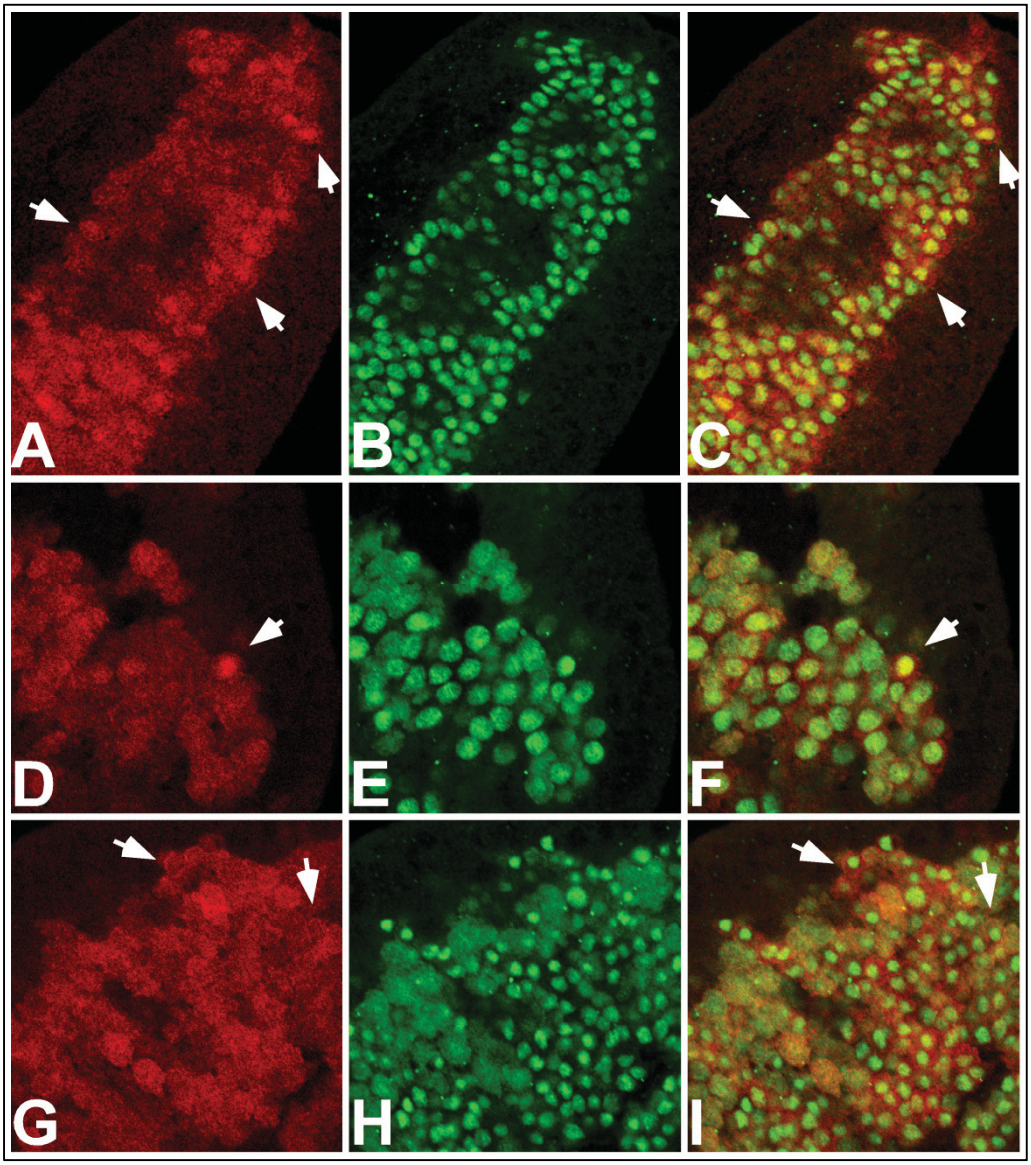

Fig. 3.27: Human Ect2 shows a weak accumulation at the cortex and in the nuclei of *Drosophila* cells.
(A-I) Expression of humEct2 in early (A-F) and late stage 8 (G-I) wild type embryos using the *twi::Gal;Dmef2::Gal4* driver line. An antibody staining against the HA-tag is shown in red and against the Twi protein in green. (A-C) The Ect2 protein shows occasionally a weak cortex association (arrows). (D-F) Only few cells display a clear nuclear accumulation of Ect2 protein (arrow; see also A-C). (G-I) In mitotic cells, no accumulations were visible at the cytokinesis furrow; note the stronger cortex association of Ect2 at the end or shortly after mitosis as assessed by Twi staining (arrows).

4 Discussion

4.1 A structure-function analysis of the Pbl protein

Cell migration is one of the key features during gastrulation of multicellular organisms and it plays an important role in illnesses like cancer. The regulation of migration is coupled to the regulation of the cytoskeleton of the cell. Therefore, it is of general importance to understand the linkage between signalling events that take place during migratory processes and the subsequent changes in cell morphology and cell behavior.

In the *Drosophila* gastrula the process of mesoderm migration has been linked to a FGF-signalling pathway involving the receptor molecule Htl (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997). The Htl receptor is capable of activating the MAP kinase cascade via Ras (Gabay et al., 1997; Vincent et al., 1998). However, MAP kinase activation does not seem to be required for early cell shape changes in the mesoderm. Furthermore, activated forms of Ras1 are not sufficient to rescue cell shape changes in *htl* mutants completely, indicating that the Ras-Raf-MAP kinase pathway is not sufficient to trigger mesoderm migration. Therefore, it is likely that a second pathway that governs the cell shape changes during the migratory process exists downstream of Htl (Schumacher et al., 2004; Wilson et al., 2005). In a genetic screen, the Rho GEF Pbl was found to play an essential role during migration. Further studies have shown that Pbl acts downstream or in parallel to the FGF pathway. As Rho GEFs control the activity of Rho GTPases, which in turn are key regulators of the actin cytoskeleton, Pbl is a good candidate to link FGF-signalling to cytoskeletal rearrangements that accompany the typical cell shape changes in migrating cells (Schumacher et al., 2004).

The function of Pbl during migration is independent of its conserved activity during cytokinesis (Schumacher et al., 2004; Smallhorn et al., 2004). In contrast to cytokinesis, nothing is known about direct upstream or downstream components of the migration pathway Pbl is involved in. Further, it is not understood how the switch between cytokinesis and migration function is regulated. As mentioned before, the involvement of different isoforms in both processes seems to be rather unlikely. Therefore, the domains present in the Pbl protein might differentially participate in the respective processes.

To test this hypothesis and to gain insight into the pathway that is employed by the GEF to trigger early cell shape changes in the mesoderm, a domain-function analysis was performed. It is known that Pbl acts in both processes as a GEF, because point mutations in the *pbl* gene

and deletions in rescue constructs that render the catalytic DH domain inactive affect both, migration and cytokinesis (Prokopenko et al., 1999; Schumacher et al., 2004; Smallhorn et al., 2004). Thus different Pbl constructs containing the catalytic domains were analyzed for their capacity to rescue migration defects in a *pbl* mutant background in order to identify the domains of the protein that are essential for its migratory function. To complete the structure–function analysis of Pbl, parts of the protein that mediate the characteristic localization pattern of full-length Pbl in interphase cells were analyzed as well.

In addition, the constructs were used for misexpression assays to examine whether and which dominant effects are caused by the respective domains. This approach is of special interest, as it is known that truncation of regulatory domains in GEFs of the DBL-homology family leads to their constitutive activation (Rossman et al., 2005; Whitehead et al., 1997). The fact that these truncated proteins normally reflect the oncogenic versions of the GEFs implies the significance of analyzing possible dominant effects in this context.

4.2 Localization pattern of Pbl in mesoderm cells

Initially, Pbl was identified as a component of the cytokinesis machinery. It was reported that the protein would localize to the cortex only during mitosis and that it accumulates in the nucleus at the end of cytokinesis (Prokopenko et al., 1999; Prokopenko et al., 2000a). This rapid transport into the nucleus was interpreted as a protection mechanism to get rid of the protein and its cytoplasmic activity after mitosis. However, the fact that Pbl is not degraded during interphase already implies that the protein might have some interphase functions as well. Initially the presence of the two BRCT domains, which are normally found in proteins linked to DNA repair functions, was interpreted as a hint of an interphase function of Pbl in the nucleus (Bork et al., 1997; O'Keefe et al., 2001).

After Pbl was shown to be required for cell shape changes and the protrusive activity of mesoderm cells during gastrulation, a cytoplasmic function for the GEF during interphase became more likely (Schumacher et al., 2004; Smallhorn et al., 2004). Consistent with this, Pbl's homologue Ect2 has recently been found to localize to the cell cortex during interphase. In *C. elegans*, Ect2 localizes in punctae at the cortex of the zygote during polarization and in different vertebrate cell lines the GEF could be detected at the cell cortex and at cell junctions of epithelial cells (Jenkins et al., 2006; Liu et al., 2004; Liu et al., 2006; Motegi and Sugimoto, 2006). To examine whether Pbl is also present in the cytoplasm during interphase, an anti-Pbl serum was generated (Schumacher, 2005). Although the antibody was able to

detect the endogenous protein, it was rather difficult to obtain a reliable cortical staining beside the strong nuclear signal. Comparable problems have also been reported from homologues of Pbl indicating that the total amount of the GEF present at the cortex of interphase cells is rather low. Therefore, tagged versions of the proteins had been used to study their subcellular localization. The same approach was taken here in order to clarify whether Pbl is indeed able to localize to the cytoplasm during interphase and to investigate whether the protein accumulates at the cortex of migrating mesoderm cells. As described in the previous chapter, the resulting fusion protein was fully functional as it rescued cytokinesis and migration after loss of endogenous Pbl activity.

During cytokinesis, the protein accumulated at all sites where Pbl has previously been reported to localize. In addition, a non-cortical staining in the central region between the newly forming daughter cells could also be observed. This might reflect a binding of the construct to the central spindle, a localization that has not been described for Pbl yet but which is well described in other species (Kamijo et al., 2006; Yuce et al., 2005). In the current model, Pbl/Ect2 is recruited to the centralspindlin complex formed by the Kinesin-like protein Pavarotti and the RacGAP50C protein, which leads to the typical localization pattern of the GEF during cell division (Saint and Somers, 2003). As this centralspindlin complex also localizes to the microtubules that form the central spindle, it remained a complete mystery why and how Pbl was excluded from this complex while being bound efficiently by the proteins in the cortex area. In addition, Ect2 had been shown to be part of the centralspindlin complex present on the central spindle, too. This localization seems to be necessary for proper cytokinesis in other systems (Somers and Saint, 2003; Tatsumoto et al., 1999; Yuce et al., 2005). The data presented here represent the first evidence that Pbl might be also able to bind to the central spindle. As the previous studies relied on Pbl antiserum or GFP-tagged constructs it is possible that low sensitivity of the Pbl antibody - as observed for the interphase localization - or low expression levels of the GFP constructs did not allow to identify the protein on the central spindle (Prokopenko et al., 1999; Prokopenko et al., 2000a; Somers and Saint, 2003). Therefore, it will be important in the future to further analyze the localization pattern of Pbl-HA during cell division using different markers like for example anti-tubulin antibodies to prove this localization pattern.

Localization studies of interphase cells were able to prove that functional full-length Pbl is present not only in the nucleus but also in the cytoplasm in a *pbl* loss of function background. Importantly the protein accumulated at the cortex and within the actin-rich protrusions formed by the mesoderm cells. Consistent with this, live imaging studies using hemocytes as a model

system could confirm the observation that the protein accumulates at actin-rich structures during cell migration. Therefore, it is likely that Pbl activates its substrates at the cell cortex in order to fulfill its migratory function. However, it was not possible to detect membrane subdomains that showed a specific enrichment of Pbl compared to the rest of the cell cortex. On the one hand, this might mean that the protein is normally present all around the cortex and is only activated locally leading to the formation of a protrusion at the site of activation. On the other hand, expression with the UAS/Gal4 system could also lead to a much higher cytoplasmic concentration of the fusion protein compared to the amount of endogenous cortical Pbl. As a result, the fusion proteins may associate with the cortex not only in regions with high affinity binding sites for Pbl but also at other parts of the plasma membrane. Therefore, it cannot be excluded that the endogenous protein localizes preferentially at sites like the leading edge of migrating cells for example. Nevertheless, the data presented here demonstrate that during interphase functional Pbl is correctly localized within the cells to activate its substrate Rac at the cell cortex. This localized activation of Rac might then induce the reorganization of the actin cytoskeleton to promote the protrusive activity of the mesoderm cells.

4.3 Role of different protein domains for the activity and localization of Pbl and Pbl constructs

The BRCT domains

Previous studies have demonstrated that the guanine nucleotide exchange activity of Pbl is required for cell migration and cytokinesis. Hence the catalytic DH domain is indispensable for both functions of the protein (Schumacher et al., 2004; Smallhorn et al., 2004). In consequence, at least one of the other domains should exhibit a differential requirement during both processes, if Pbl was regulated via the involvement of different domain combinations.

During cytokinesis, Pbl binds to the centralspindlin complex by direct association of its N-terminus and first BRCT domain with the RacGAP50C protein. The consequence of this interaction is the accumulation of Pbl at the plus ends of the microtubules in the middle of the dividing cell (Somers and Saint, 2003). Therefore, the BRCT domains of Pbl are of fundamental importance for cytokinesis. Consistent with this, previous work as well as the rescue experiments performed here indicated that in contrast to cytokinesis, the BRCT domains are dispensable for the migratory function of Pbl (Smallhorn et al., 2004). The

$Pbl^{\Delta BRCT}$ construct was sufficient to rescue migration significantly suggesting that all essential domains are included in this construct. From another point of view however, one could also argue that the full-length protein exhibits a much better rescue (see Tab. 3.1) which would imply at least a supportive role for the BRCT domains in migration. A conceivable function would be the interaction with possible upstream factors that activate or inactivate Pbl during migration, for example. Interestingly the adaptor protein Dof was identified in a yeast-2-hybrid assay as a potential binding partner of RacGAP50C (Battersby et al., 2003). Therefore, a possible scenario would be that Pbl is activated locally through recruitment to the Htl/Dof complex via RacGAP50C upon FGF-signalling. In this case, the BRCT domains would fulfill a regulatory function comparable to cytokinesis.

Another possible regulatory function for the BRCT domains could help to explain why N-terminal truncations of DBL-family GEFs lead to their constitutive activation. In this context it was previously shown, that the BRCT domains of Ect2 are able to bind to its DH domain. This interaction results in a closed conformation of the GEF, which prevents an interaction of the DH domain with its substrate GTPases (Kim et al., 2005; Saito et al., 2004). Activation of the protein and release from this autoinhibiton might be achieved upon binding of an interaction partner to the BRCT domains - as assumed for the interaction between Pbl and the centralspindlin complex during cytokinesis - or upon phosphorylation of the protein. The latter model is strengthend by results that suggest that a phosphorylation of Ect2 in the hinge region (between the second BRCT and the DH domain) would induce conformational changes, which lead to a release from autoinhibition and ultimately to the activation of the protein (Hara et al., 2006). As a comparable intramolecular inhibition was already proposed for several other GEFs, this mode of regulation might be a common feature of GEF proteins (Schmidt and Hall, 2002).

Given this autoinhibition model, a deletion of the BRCT domains should also lead to a constitutive activation of the Pbl protein. The resulting misregulation of $Pbl^{\Delta BRCT}$ could explain why the absolute rescue of this construct is below the level of the full-length protein. However, the fact that $Pbl^{\Delta BRCT}$ did not cause any dominant effects (in contrast to $Pbl^{\Delta N\text{-}term}$ or $Pbl^{DH\text{-}PH}$) can be explained by the presence of the NLS and it does not exclude a constitutive activity of the construct. It was already assumed earlier that the NLS and therefore the translocation into the nucleus after mitosis is an efficient way to get rid of Pbl activity in the cytoplasm. As a result, the cytoplasmic concentrations of $Pbl^{\Delta BRCT}$ might be close to physiological levels of Pbl and too low to negatively interfere with mesoderm development when expressed in a wild type background. This idea is supported by the finding that deletion

of the BRCT domains in Ect2 will only lead to a strong transforming activity, if the NLS is deleted simultaneously (Saito et al., 2004). In *pbl* mutants however, the cytoplasmic $Pbl^{\Delta BRCT}$ molecules may exhibit just enough activity at the cell cortex (see below) to facilitate migration. Therefore, the BRCT domains are not essential for the migratory function of the protein, although they might be involved in the regulation of Pbl activity during migration.
However, the finding that misexpression of the two BRCT domains alone does not negatively interfere with mesodermal spreading argues against an important regulatory role for these domains. If the BRCT domains would mediate protein-protein interactions that are important for the function of Pbl in the mesoderm, one might expect $Pbl^{BRCT1,2}$ to compete with the endogenous Pbl for these binding partners. In the end, this could lead to a dominant negative effect by blocking the binding of these putative interaction partners so that specific interactions with the endogenous Pbl are reduced. Alternatively, if the autoinhibition model was correct, one might also expect that the expressed BRCT domains could directly reduce the activity of endogenous Pbl through association with its DH domain. However, as $Pbl^{BRCT1,2}$ expression has also had no appreciable dominant effect on cytokinesis, a process already known to depend on the BRCT domains, this negative result is difficult to interpret and does not rule out an involvement of these protein parts in migration. In this context, it should be tested in future whether expression of multiple copies of $Pbl^{BRCT1,2}$ leads to any dominant effects, as too low expression levels of the construct might also be a possible explanation for the failure to dominantly interfere with migration or cytokinesis.
With regard to localization, $Pbl^{\Delta BRCT}$ fully resembled the interphase localization pattern of the full-length protein. Most of the protein was found in the nucleus but the construct also showed a weak association with the cell cortex in migrating mesoderm cells. Therefore, the interphase localization does not depend on the BRCT domains, a finding that is consistent with $Pbl^{\Delta BRCT}$ being sufficient to rescue mesodermal spreading. During mitosis however the typical accumulation of Pbl at the site where the cleavage furrow is formed was never observed arguing again for the essential function of the BRCT domains in mediating the interaction with centralspindlin, which is a prerequisite for the cytokinesis function of the Pbl protein. Taken together, $Pbl^{\Delta BRCT}$ obviously contains all domains that are essential for the interphase localization and function of Pbl.

The NLS and PEST motif

Consistent with a safeguarding role for the NLS, a further deletion of this sequence was reported to result in a dominant activity during cytokinesis (a $Pbl^{PEST\text{-}DH\text{-}PH\text{-}C\text{-}term}$ construct)

(Prokopenko et al., 1999). Here a similar construct was analyzed that, compared to Pbl$^{\Delta BRCT}$, also lacks the NLS and the PEST motif. Surprisingly, this Pbl$^{\Delta N\text{-term}}$ construct was unable to rescue migration any more. Instead, dominant effects could be observed even when expressed in a *pbl* mutant background. As expected, its localization pattern also differed from Pbl$^{\Delta BRCT}$. Deletion of the NLS led to a high concentration of the protein in the cytoplasm. Interestingly Pbl$^{\Delta N\text{-term}}$ accumulated strongly at the cortex of the cells, suggesting that it is still able to interact with GTPases at the cell periphery. However, as no rescue of migration but instead a dominant activity was detectable after Pbl$^{\Delta N\text{-term}}$ expression, the localization of the catalytic domains of the GEF to the cortex is not sufficient for the migration function of Pbl (see below: C-terminus and substrate preference of the DH-PH tandem domain).

These findings confirm reports that have shown that the oncogenic form of Ect2 is an analogous truncated version of the protein that exhibits a strong transforming activity. Furthermore, this dominant activity was found to be stimulated strongly by the loss of NLS (Saito et al., 2004). Consistent with the transforming potential of oncogenic Ect2, the Pbl$^{\Delta N\text{-term}}$ construct dominantly interfered with processes that in part are not even dependent on endogenous Pbl activity. In rescue as well as in misexpression experiments, Pbl$^{\Delta N\text{-term}}$ blocked the invagination of the presumptive mesoderm. Furthermore, misexpression of this construct caused defects during cytokinesis as multinucleated cells were detected in wild type embryos expressing the protein. The fact that these dominant effects vanished completely after abolishing the catalytic activity (Pbl$^{\Delta N\text{-term_V531D}}$) demonstrates that Pbl$^{\Delta N\text{-term}}$ represents a constitutive active GEF that misregulates Rho GTPases leading to the phenotypes mentioned above.

In comparison to Pbl$^{\Delta BRCT}$ these results indicate that the NLS is indeed an important and powerful regulatory domain of Pbl that helps to suppress the dominant activity of activated variants of the protein. This safekeeping and protection effect of the NLS might be further enhanced by the presence of the PEST motif in Pbl$^{\Delta BRCT}$ that could help to keep the cytoplasmic levels of this activated protein below a certain threshold and closer to physiological Pbl levels in order to avoid dominant effects. However, as a Pbl$^{PEST\text{-}DH\text{-}PH\text{-}C\text{-}term}$ construct was reported to cause defects at least during cytokinesis, the role of the NLS seems to be more important in this context.

The catalytic core and the C-terminus

Further rescue experiments indicated that the smallest entity of the protein that exhibits rescuing activity for migration is the catalytic DH-PH tandem domain. This result was not

expected after the Pbl$^{\Delta N\text{-term}}$ experiments. However, it demonstrates that the C-terminal tail of Pbl influences the rescue potential of the catalytic domains, as this is the only part of the protein that is missing in Pbl$^{DH\text{-}PH}$ compared Pbl$^{\Delta N\text{-term}}$. A further difference between both constructs was the finding that misexpression of Pbl$^{DH\text{-}PH}$ interferes negatively with the migration of the mesoderm cells. In addition, no signs for a dominant effect on cytokinesis were detectable after Pbl$^{DH\text{-}PH}$ expression indicating that the dominant activity is clearly modified by the presence of the C-terminus. The localization of both constructs was also different. While Pbl$^{\Delta N\text{-term}}$ accumulated strongly at the whole cell periphery, Pbl$^{DH\text{-}PH}$ exhibited a more punctated pattern with a weaker cortical association.

Analogous to these observations, various differences have also been reported for an Ect2 DH-PH and a DH-PH-C-term construct (Solski et al., 2004). In this study, Ect2$^{DH\text{-}PH\text{-}C\text{-}term}$ had a higher transforming activity than Ect2$^{DH\text{-}PH}$ and induced lamellipodia formation when misexpressed in a mammalian cell line. Furthermore, it was reported to accumulate at the cell periphery and along membrane ruffles. In contrast, Ect2$^{DH\text{-}PH}$ was described to localize in a dot-like pattern with only weak cortical association. Additionally Ect2$^{DH\text{-}PH}$ induced formation of actin stress fibers suggesting that it acts through a different substrate GTPase in absence of the C-terminal tail.

As mentioned before, both Pbl constructs also affected different processes depending on the presence of the C-terminus in the respective protein. On the one hand, Pbl$^{\Delta N\text{-term}}$ expression blocked cytokinesis, a process known to depend on the local activation of Rho1 in the equatorial region of the cell. This site restricted activation of Rho1 leads to the formation of the contractile actomyosin ring in the middle of the dividing cell. Furthermore, a strong impact of Pbl$^{\Delta N\text{-term}}$ on the invagination of the mesoderm was detected. Importantly, this process is also known to depend on a locally restricted activity of Rho1. In this context, RhoGEF2 activates Rho1 at the apical domains of the presumptive mesoderm cells. This activation somehow results in a subsequent constriction of the apical sites of the mesoderm cells, leading to the infolding of the mesodermal cell sheet (see introduction). As Pbl$^{\Delta N\text{-term}}$ accumulates strongly at the whole cortex of mesoderm cells, it is likely that it activates Rho1 ectopically all around the cortex leading to a completely non-polarized Rho1 activity and a blockage of both processes.

On the other hand, Pbl$^{DH\text{-}PH}$ displayed an impact on proper migration of the mesoderm that was dependent on the catalytic activity of the GEF domain (see Pbl$^{DH\text{-}PH_V531D}$). As mesoderm-specific expression of dominant negative Rho1 interferes only with mitosis but not with the spreading of the mesoderm, it was suggested, that Pbl's migratory function is

probably not dependent on Rho1 activation (Schumacher et al., 2004). Hence, it is possible that $Pbl^{DH\text{-}PH}$ misexpression affects the activity of a second substrate GTPase of Pbl during the migratory process. In this case, the presence of the C-terminal tail would define which Rho GTPase is hyperactivated by the respective constitutively active Pbl construct. Hence, a role for the C-terminus in determining or influencing the substrate preference of the catalytic DH-PH tandem domain is an attractive model, analogous to what was suggested for Ect2. It is currently unknown how this effect could be achieved on a molecular level and therefore it requires further analysis (see below).

Consistent with an important function for the C-terminus, which does not contain any known domain structure, the localization of the catalytic core within mesodermal cells was also affected by the presence of this protein region. The DH-PH domains alone showed only a weak association with the cortex and localized in punctae. Addition of the C-terminus however is sufficient to get a strong recruitment of the tandem domain to the cell periphery. When expressed alone, the C-terminus also accumulated at the cortex indicating that this protein part is indeed sufficient to mediate a robust cortex localization of the respective construct. This suggests an important role for the C-terminus in anchoring Pbl to the cortex during interphase.

To further examine the role of the C-terminus, the $Pbl^{\Delta C\text{-}term}$ construct was analyzed. The fact that the $Pbl^{\Delta C\text{-}term}$ protein was only detectable in much lower cytoplasmic levels (nuclear protein levels were comparable to PblA-HA) and that it did not accumulate strongly at the cortex could explain why the migration rescue of $Pbl^{\Delta C\text{-}term}$ was reduced compared to the full-length rescue. In contrast to the deletion constructs, the presence of the C-terminal tail seems to have an effect on the cytoplasmic stability of the full-length protein. The question why a similar effect could not be seen for the $Pbl^{DH\text{-}PH}$ and $Pbl^{\Delta N\text{-}term}$ constructs might be related to the PEST motif that was not present in these two variants. Therefore, the PEST containing full-length protein might be very instable in the cytoplasm if the C-terminal tail was missing. This could be linked to the C-terminus-mediated cortex association of the protein that may lead to increased protein stability. However, as the $Pbl^{\Delta C\text{-}term}$ was still able to rescue migration to a similar extent as $Pbl^{\Delta BRCT}$, the C-terminus dependent localization to the cortex seems to be important but not essential. This conclusion is consistent with the rescuing activity of the $Pbl^{DH\text{-}PH}$ construct (see below).

Another possible interpretation for the low cytoplasmatic $Pbl^{\Delta C\text{-}term}$ levels would be that the C-terminus contains a sequence motif that is required for the nuclear export of the protein during interphase. Deletion of this protein part in the presence of the NLS should lead to very low

levels of cytoplasmic Pbl thereby abolishing the rescue potential of this protein. Consistent with this idea, "The Eukaryotic Linear Motif resource" software (http://elm.eu.org) identifies an amino acid stretch with similarities to a NES in the C-terminus of Pbl (aa 831-841). However, as this software searches only for short linear sequences, most of the hits are presumably not statistically significant and therefore they should not be treated as factual findings. The result that a $Pbl^{NLS\text{-}PEST\text{-}DH}$ construct also localizes to the cytoplasm of mesoderm cells (data not shown) renders the NES model rather unlikely. Therefore, a role for protein stability seems to be more realistic for the function of the C-terminus in the full-length protein.

Reduced cytoplasmic levels of Pbl could affect both, the rescue of cytokinesis as well as the migration rescue. Importantly, while the migration defects were still suppressed substantially after loss of the C-terminal tail, $Pbl^{\Delta C\text{-}term}$ failed to rescue cytokinesis. This was not due to low cytoplasmatic levels of the fusion protein or a problem in localizing to the cleavage furrow. Hence, the construct was still able to activate the migratory pathway while failing to activate Rho1 at the cleavage site. Therefore, the presence of the C-terminus seems to be essential for the activation of Rho1 but dispensable for activation of the Rac pathway. This result indicates that the differential activation of GTPase pathways that was observed after deletion of the C-terminal tail in the constitutively active constructs might be equally transferable into a differential rescue of cytokinesis and migration in the full-length protein (see 4.7). In conclusion, this differential rescue by $Pbl^{\Delta C\text{-}term}$ further strengthens the model that the C-terminus might be involved in influencing the substrate preference of Pbl.

To summarize, the C-terminal tail of the Pbl protein is important for a robust cortical accumulation in interphase and it directly or indirectly influences the cytoplasmic stability of the full-length protein. Furthermore, the data presented here imply a possible role for this region in specifying the substrate preference of the catalytic domains.

The fact that $Pbl^{DH\text{-}PH}$ contains the first 55 amino acids of the C-terminal tail makes it possible to narrow the region of interest within the C-terminus down. As the deletion of these 55 amino acids in the $Pbl^{DH\text{-}PH\text{-}short}$ construct did not have a significant effect on the rescue capacity of the construct, it can be excluded that the suggested functions of the C-terminus are mediated by this first amino acid stretch. However, further analysis is required to identify the exact motif in the C-terminal tail that mediates its functions in membrane anchoring and influencing the *in vivo* substrate preference of the GEF.

The PH domain

Although the strong cortex accumulation of Pbl proteins seems to be mediated by the C-terminus, the Pbl^{DH-PH} construct was also able to rescue migration and it showed at least a weak cortex binding suggesting that an additional domain of Pbl must also have the capability to localize the protein to the periphery of the mesoderm cells. This additional region is likely to be the PH domain of the protein. First, Pbl^{DH} was found diffusely in the cytoplasm indicating that this domain alone is not sufficient to bind to the cell periphery. Second, a GFP-Pbl^{PH} fusion protein was able to accumulate at the cortex proving the ability of the domain to mediate cortex association at least to some extent. If binding to the cortex was a prerequisite for Pbl's migratory function, this PH-mediated binding to the plasma membrane is obviously sufficient to achieve a partial rescue of migration in case of Pbl^{DH-PH}. Therefore both, the C-terminus as well as the PH domain of Pbl, are capable of localizing the GEF to the cortex of the mesoderm cells. The fact that the catalytic domains of Pbl bind to the whole cortex in the presence of the C-terminus could be interpreted as a general anchoring to the membrane in a non-polarized fashion. In absence of the C-terminal tail however, the DH-PH domains only bind to specific punctae at the cortex. As the same phenomenon was reported for Ect2, it is possible that the PH domain is able to recruit the GEF to special membrane subdomains enriched in certain phosphoinosites thereby leading to subtle accumulations of the GEF at specific sites. This would be consistent with the general function of PH domains to recruit proteins to sites of the cells, where local activation of signalling pathways, involving PI3kinase for example, have generated high amounts of the respective substrate lipid (Alberts et al., 2004; Kavran et al., 1998). In this case a two phase model of cortex association for Pbl during mesoderm migration would be possible: The C-terminal tail mediates a robust general membrane binding of the GEF leading to an enhanced stabilization of cytoplasmic Pbl. Recruitment of Pbl to the leading edge for example would in turn be mediated by its PH domain leading to a polarized association. As discussed before, overexpression of the tagged constructs could mask this rather weak polarized distribution of the endogenous protein, which would explain why the Pbl^{PH} construct was detected uniformly at the cortex.

The finding that in contrast to Pbl^{DH-PH} expression of the DH domain alone did not rescue migration does not necessarily prove that cortex association of the different Pbl constructs is essential for the migration function of the GEF. Although PH domains normally serve as membrane targeting domains, their role in Dbl-family GEFs seems to go beyond a simple cortex anchoring function. For various GEF proteins it was shown that the PH domains could contribute to the catalytic activity of the DH domains in different ways. The binding of a PH

domain to phospholipids for example can stimulate the GEF activity of the neighboring DH domain *in vivo* possibly by inducing conformational changes in the DH-PH tandem domain. In this case, membrane recruitment and stimulation of the exchange activity would be coupled. Other PH domains have been shown to be directly involved in the binding of Rho GTPases thereby facilitating their activation (Baumeister et al., 2006; Liu et al., 1998; Rossman et al., 2003; Rossman et al., 2005; Rossman and Sondek, 2005; Rossman et al., 2002). These examples demonstrate that Pbl's PH domain might not only be involved in the localization of the protein but that it might also be required for an effective activation of substrate GTPases *in vivo*. Consequently, deletion constructs taking out the PH or both the PH and the C-terminal domain would not be helpful in addressing the question whether a binding to the cortex is essential for the migratory function of Pbl, as such deletions could also comprise the GEF activity directly. Hence, it is not possible to distinguish between the two possible roles of the PH domain with regard to its function for the activity of the protein. A possible way to get an idea whether cortex targeting is important would be to generate a version of $Pbl^{\Delta C\text{-term}}$ that carries another membrane anchor. If the rescue of such a construct was more efficient than the $Pbl^{\Delta C\text{-term}}$ rescue, this would demonstrate that enhancing the membrane recruitment of the construct would raise its rescuing potential. Alternatively one would have to identify the residues in the PH domain that are required for phospholipid binding but not for the binding to a GTPase for example. By mutating these residues, it should be possible to uncouple the two roles of the PH domain. However, as PH domains are in general only poorly conserved on the amino acid level, it would be rather difficult to identify critical residues in the PH domain of Pbl. In conclusion, Pbl's PH domain might not only be involved in localizing the GEF but it could also be essential for the exchange activity of the protein.

4.4 Pbl acts through the Rac signalling pathway during migration

GEF proteins of the Dbl family have been reported to contain various signal transduction modules in addition to their characteristic DH-PH tandem domain. These regulatory domains mediate interactions with other proteins that have a regulatory input on the GEF activity. In a few cases like Pbl's homologue Ect2, direct autoregulatory functions of these domains were shown, implicating that an auto-inhibitory effect might be a general characteristic of Dbl family GEFs (Hoffman and Cerione, 2002; Kim et al., 2005; Saito et al., 2004; Schmidt and Hall, 2002). In almost all members of this protein family the deletion of such regulatory

elements is a hallmark of the respective oncogenic version and it results in the constitutive activation of the protein leading to strong transforming activities (Rossman et al., 2005; Whitehead et al., 1997).

Consistent with these ideas, truncated versions of Pbl also exhibited dominant effects that are based on hyperactivation of different substrate GTPase pathways. The finding that Pbl^{DH-PH} interfered with mesoderm migration during overexpression studies and that it suppressed the migration defects in a *pbl* loss-of-function background implied that this activated Pbl version is able to interact with Pbl's substrate during migration. Therefore, Pbl^{DH-PH} was used to test for genetic interactions with the different Rho GTPases of *Drosophila*. As a result, only Rho1 and the Rac GTPases were able to modify the dominant rough eye phenotype caused by Pbl^{DH-PH} expression, indicating that both GTPases might act as substrates for Pbl's catalytic core. This result is consistent with previous reports indicating that Rho1 and Pbl can directly bind to each other in a yeast-2-hybrid assay and that the DH domain of Pbl can use both, Rho1 and Rac1/Rac2 as substrates in an *in vitro* nucleotide exchange assay (Prokopenko et al., 1999; Schumacher, 2005; van Impel et al., 2009).

Several results indicate that the Rac GTPases but not the Rho1 pathway is likely to act downstream of Pbl during mesoderm migration. On the one hand, expression of dominant negative Rho1 affects cytokinesis but not migration (Schumacher et al., 2004). On the other hand, expression of $Pbl^{\Delta N-term}$ did not show an effect on mesodermal spreading although blocking various Rho1 dependent processes, suggesting that another GTPase is required downstream of Pbl during mesoderm migration. The idea that this alternative substrate could be Rac is strengthened by the observation, that expression of constitutive active Rac1 dominantly affected mesoderm migration, while $Rho1^{V14}$ expression did not cause any defects (this work and Schumacher and Müller, unpublished). Consistent with the model that the dominant Pbl^{DH-PH} construct hyperactivates the respective downstream target during migration leading to the obtained defects, both, activated Pbl and activated Rac1 but not $Rho1^{V14}$, interfered with mesoderm migration. Hence, the fact that the constitutive activation of Pbl and Rac but not of Rho1 had similar effects can be interpreted as another hint that Rac acts downstream of Pbl during migration. Furthermore, it can be concluded that the activation state of Pbl and Rac proteins has to be tightly regulated during mesodermal spreading and thus an ectopic, non-polarized activity of the Rac GTPase pathway causes the observed abnormalities during the migratory process.

Importantly, not only too high levels but also loss of Rac activity negatively affects mesoderm migration. Germline clones that were completely null for Rac1 and Rac2 exhibited major

problems during migration that were reminiscent of the defects seen after loss of a central component of the Htl pathway, indicating that Rac activity is absolutely necessary for this process. This result is consistent with and it even extends an earlier report that showed an involvement of Rac GTPases in establishing the initial contact between mesoderm and ectoderm during EMT (Wilson et al., 2005).

Although Pbl and Rac are both required for migration it is not clear whether they act in a linear pathway and whether Rac is a direct or indirect target of Pbl. However, the fact that the DH domain of Pbl also accepts Rac1 and Rac2 as substrates *in vitro* suggests that Rac might act directly downstream of Pbl during migration. To further examine this possibility, *in vitro* GEF binding studies were performed that could demonstrate, that nucleotide depleted Rho1, Rac1 and Rac2 are able to bind $Pbl^{\Delta N\text{-term}}$ from embryo extracts, suggesting that Pbl accepts both Rho1 and Rac GTPases as substrates and that Rac is a direct downstream target of Pbl during migration. Furthermore, genetic interaction studies employing the hypomorphic allele pbl^{l1D} showed that Pbl and Rac do not only interact when expressed in the compound eye but also during mesodermal spreading. Dominant negative as well as constitutive active Rac1 variants enhanced the migration defects of these embryos. This suggests that the exact level of Rac activity in the mesoderm is of fundamental importance, because reduced as well as elevated levels of active Rac have negative effects on the process, even in a sensitized situation. Most notably, co-expression of wild type Rac1, but not of wild type Rho1, was capable of enhancing the rescue potential of the $Pbl^{\Delta BRCT}$ construct. This indicates a direct connection between the expression of activated Pbl and of wild type Rac1 as a potential substrate on the one hand and the enhanced complementation of Pbl's migratory functions in *pbl* mutant embryos on the other hand. Therefore, the results make a strong case for the Rac pathway being directly controlled by Pbl during mesoderm spreading in the *Drosophila* gastrula.

Co-expression of Rho1 did not enhance mesoderm spreading in the $Pbl^{\Delta BRCT}$ rescue assay. This finding was expected as so far no results indicated an involvement of Rho1 in mesoderm migration. In fact mesoderm specific expression of a dominant negative Rho1 construct did not impair the capacity of mesodermal cells to form leading edge protrusions while cytokinesis was blocked efficiently (Schumacher et al., 2004). Although these data argue against a direct participation of Rho1 in this context, a complete loss-of-function phenotype cannot be analyzed in the mesoderm because of the strong requirement for maternal Rho1 during oogenesis that makes the generation of germline clones impossible (Magie et al.,

1999). Therefore, it cannot be excluded that Rho1 also participates in some aspects of migration, however presumably not downstream of Pbl.

An initial indication that Rho1 might play a yet to define role during mesoderm migration was provided by the localization of the endogenous Rho1 protein. Rho1 was found to accumulate between the mesoderm and the ectoderm shortly after the mesoderm had been invaginated as a tube of epithelial cells. It was shown that the cells, which are basally localized in this tube, establish an initial contact to the ectoderm in a cellular protrusion dependent manner. This event results in the activation of the MAP kinase cascade in these cells. The contact establishment depends on the Htl pathway and the activation of MAP kinase signalling is also Htl dependent (Schumacher et al., 2004; Wilson et al., 2005). Virtual cross-sections of embryos during this first phase of migration demonstrated that Rho1 accumulates at the basal domain of the cells, which are already in contact with the ectoderm. Furthermore, the staining seems to be located in protrusions that might be formed by mesodermal cells and protrude between ectodermal cells. Therefore, it is possible that these structures resemble the early protrusions formed by the mesodermal tube to initiate the contact to the ectoderm. However, the subsequent symmetrical flattening of the tube and the EMT of the cells has been found to depend on the formation of protrusions by mesoderm cells as well. Recent live imaging studies revealed that the cells that are located at the lateral positions within the disintegrating tube form actin rich protrusions during this collapsing phase. These protrusions reach far between the ectodermal cells and can be found at various positions along the a/p axis. Nevertheless, their role for EMT and migration of the mesoderm remains elusive and at present, it is not clear whether the formation of these lateral protrusions depends on Pbl activity (Clark and Müller, unpublished). As it is hard to discriminate between both types of protrusions formed in this early migration phase, it cannot be determined yet which of these structures the Rho1 staining might reflect.

Interestingly, patches with a strong accumulation of Rho1 were also detectable during the migratory phase. Even here, the protein localized to the contact interphase of mesoderm and ectoderm. As mentioned in the results, it sometimes appeared that these patches and linear accumulations might again resemble cellular protrusions, however this was not as clear as during the early events. Interestingly, even during this phase of migration massive lateral protrusions were shown to be extended by different mesoderm cells along the d/v axis (Clark and Müller, unpublished). Therefore, these later Rho1 positive structures might also coincide with a subspecies of these lateral protrusions. Nevertheless, at least a part of these

accumulations could also reflect reminiscence of mitotic structures like the midbody as the cells undergo a second round of mitosis in this phase.

The fact that no membrane marker for the mesoderm was used makes it impossible to rule out that at least a part of the signals detected here localizes to ectodermal and not to mesodermal cells. Furthermore, it is not clear whether some Rho1 accumulations might reflect relicts from the cellularization process. During this process, ingressing membranes surround the nuclei, which were derived from a series of nuclear divisions, and the blastoderm epithelium is formed. Rho1 was reported to localize to these so-called furrow canals during cellularization (Padash Barmchi et al., 2005). It is uncertain whether these furrow canals are already closed completely at the basal end of the cells during the onset of gastrulation. Hence, it might be possible that Rho1 still localizes to these remaining basal structures leading at least in part to the strong Rho1 signals at the basal site of the mesoderm cells (which is also the basal surface of the ectoderm cells). However, the fact that similar strong accumulations were neither detectable in all mesoderm cells nor during the earlier invagination process of the mesoderm renders this possibility rather unlikely.

To conclude, it is possible that a Rho1-dependent pathway plays a yet to define role during some aspect of mesoderm migration. This option needs to be further analyzed in future, possibly employing live cell imaging techniques, which might help to detect a dynamic localization pattern for this Rho GTPase during mesoderm development.

4.5 Mutagenesis of the DH domain

If the switch between Pbl's migratory and cytokinesis functions was based upon a modification of a regulatory domain of the protein, isolating an allele that selectively affects only one of its functions could facilitate the identification of such a switch region in the protein. However, so far all attempts to isolate mutations in the open reading frame of *pbl* that only result in either cytokinesis or migration defects failed. Beside some of the deletion constructs presented here, there is only one *pbl* allele that shows a differential effect on both Pbl functions, the pbl^{l1D} allele. Embryos homozygous mutant for this hypomorphic allele were reported to exhibit moderate defects in migration while cytokinesis is blocked completely. Unfortunately, no mutation could be identified in the ORF suggesting that this allele might reflect a mutation within regulatory sequences of the gene. Therefore, no mutation that could help to separate the dual functions of the Rho GEF has been isolated so far.

One trial to generate such a function specific Pbl allele artificially was described in chapter 3.3.6. Crystallographic studies of different Dbl family GEFs have revealed that the substrate tolerance of DH domains is in part determined by distinct amino acid residues in the catalytic domain that mediate the interactions between the respective Rho GTPase and the binding pocket of the GEF. It was shown that the fifth helix in the DH domain harbours crucial residues that determine whether a certain GTPase fits into the binding pocket or not. As a result, amino acid exchanges at these sites were shown to switch the substrate specificity of the respective DH domain in *in vitro* GEF assays (Snyder et al., 2002).

To generate versions of Pbl that can interact either only with Rho1 or with Rac GTPases, different mutant full-length constructs were tested. As explained earlier the sites that were chosen for the mutagenesis had been identified using a secondary-structure-prediction program in order to find the fifth α-helix in Pbl's DH domain. This region indeed contained residues at the correct relative position to each other that argued for an interaction with Rho1 and Rac GTPases (Suppl. Fig. 4). However, as in all cases the mutagenesis resulted in decreased rescue ability for migration while the cytokinesis function did not seem to be impaired by the amino acid exchange, the structural basis for this experiment was tested again. This time two other programs for the prediction of α-helices in the DH domain were employed. Surprisingly, both programs indicated additional α-helical stretches in the sequence (see Suppl. Fig. 1). As a result, the chosen sites for mutagenesis were not within the fifth α-helix anymore suggesting that the wrong positions had been altered in the constructs. To further complicate the issue, different papers seem to use a different nomenclature depending on whether different α-helices are count as one or interpreted as distinct structures (Liu et al., 1998; Rossman et al., 2002). Therefore, a multiple alignment was generated using sequences of different Dbl family GEFs that had been used for the original crystallographic study of the GEF/substrate interactions (Suppl. Fig. 2 and 3). This alignment indicated that the initial approach taken was based on an incorrect assumption and does not affect the critical residues in α-helix 5.

The newly identified fifth α-helix also contains residues that are consistent with substrate specificity for Rho1 and Rac GTPases. On the one hand, Snyder et al. (2002) could demonstrate that positive selection of RhoA depends on a favorable electrostatic interaction of a basic residue at position 2 in the DH domain (see Suppl. Fig. 3) with Asp45 and Glu54 of RhoA. All examined GEFs that are specific for either Cdc42 or Rac1 did not contain a comparable residue at the equivalent position within α-helix 5. Interestingly, insertion of a Lys at position 2 in a GEF, which is normally specific for Cdc42, generates an additional

exchange activity towards RhoA demonstrating the importance of this polar residue at position 2. As one can see in the multiple sequence alignment in Suppl. Fig. 3, Pbl, as well as Ect2, contains this critical amino acid. A further determinant for an exchange activity for RhoA is probably a polar interaction of position 1 with Arg5 in RhoA. Pbl possesses a polar residue at this position indicating that the fifth α-helix in its DH domain harbours all described residues that are required for substrate specificity towards Rho1, a finding that is consistent with Pbl's exchange activity for this GTPase.

On the other hand, it was found that the residue at position 3 is of paramount importance for the recognition of Rac1. All GEFs specific for Rac1 have an Ile at this position, which is thought to favor a binding of this GTPase. In contrast, Cdc42 specific GEFs never have this residue at the corresponding position consistent with the idea that this Ile prevents a binding of Cdc42. Insertion of Ile at position 3 within a Cdc42-specific DH domain is sufficient to switch substrate specificity towards Rac1 emphasizing the importance of this particular amino acid (Snyder et al., 2002). As this residue is also present in Pbl and Ect2 these findings further strengthen the idea that Pbl also accepts Rac as a substrate. Therefore, the mutagenesis of these three positions within the α-helix 5 of Pbl's DH domain could indeed abolish its exchange activity for either Rho1 or Rac selectively, a finding that should be tested in the future. Nevertheless, it is not clear at present how the interactions of one GEF with several different substrates are regulated. For example human Ect2 was shown to have an *in vitro* exchange activity for RhoA, Rac and Cdc42, demonstrating that the amino acid sequence does not completely dictate the substrate specificity of the DH domain, because Ect2 harbours the Ile at position 3 but is nevertheless capable of activating Cdc42 at least *in vitro*. Therefore, it is of great importance to determine how GEFs using multiple substrates are able to switch their binding properties for distinct substrates to allow specific interactions with single GTPases *in vivo*.

Although the mutagenesis of the DH domain performed here did not affect the described critical residues in the fifth α-helix, they exhibited an effect. Interestingly, both mutations led to a reduction of the migration rescue while the overall ability to complement the loss of endogenous Pbl during cytokinesis did not seem to be disturbed. On the one hand, this selective effect on migration can be explained by the mutation of sites that are important for an interaction with Rac. Because of the mutagenesis, the DH domain might possess a lower affinity for Rac while the binding to Rho1 is not affected thereby causing a specific effect on migration. On the other hand, the mutations could lower the total activity of the GEF domain. Depending on the thresholds for active GTPases in both processes, a lower activation of Rho1

might not affect the rescue of cytokinesis, while migration might be more sensitive to a reduction of active Rac, thereby leading to a reduced rescue potential for this process. The latter idea is supported by the defects seen in the *pbl*^{E091} allele. This allele contains another amino acid exchange in the DH domain (T548S) and does not show an appreciable effect on cytokinesis while causing a mild migration defect because on average only 20.4 Eve cell clusters were found in homozygous embryos (Schumacher, 2005). As it is rather unlikely that this mutation also hit an amino acid that is essential for the specific exchange activity towards Rac, the idea that random mutations in the DH domain reduce its overall activity seems to be more reasonable. However, as the rescue of cytokinesis was not quantified for the mutagenesis constructs, it cannot be entirely excluded that there are also subtle cytokinesis defects present, which are difficult to detect.

Although it is unclear what the cause of the differential effects seen on cytokinesis and migration is, the amino acid exchanges had, as intended, only an appreciable effect on one of the two Pbl functions. Hence, it seems to be possible to separate both Pbl functions on the level of its catalytic domain by either influencing the substrate specificity or more likely by lowering the overall catalytic activity of the DH domain.

4.6 Is Pbl involved in EMT or cell migration?

Two independent groups have identified the gene *pbl* to be required for proper migration of the mesoderm. It was described that Pbl is essential for the typical cell shape changes during the different phases of migration because *pbl* mutant embryos exhibit no or only a dramatically reduced protrusive activity. Smallhorn and colleagues (2004) further described *pbl* mutant mesoderm cells to appear more closely adhered with each other, less rounded and with fewer gaps between the cells if compared to wild type tissue. Therefore, they proposed a function for Pbl already during EMT of the mesodermal tube. Schumacher et al. (2004) directly assessed the question whether EMT is blocked after loss of Pbl function by comparing wild type and mutant embryos using electron microscopy. The apical adherens junctions, a hallmark of epithelial polarity, were found to be downregulated properly even in the absence of Pbl activity. Consistent with this, immunostaining against *D*E-cadherin demonstrated that the strong apical accumulation of the protein disappears during EMT in *pbl* mutant embryos, indicating that the loss of epithelial characteristics is not affected. They concluded that Pbl is required to gain mesenchymal characteristics rather than loosing epithelial characteristics. Interestingly, even Smallhorn et al. believe that the initial down

regulation of epithelial characteristics is not impaired. However, they propose a model in which Pbl is required to further downregulate the adhesive properties of the cells in order to become motile. Both reports agree in the idea that regulation of adhesive properties of the mesoderm cells during EMT is at least one aspect of Pbl's function during mesodermal spreading (Schumacher et al., 2004; Smallhorn et al., 2004). This conclusion raises the question whether Pbl's migration function might be primarily EMT related and whether the lack of cellular protrusion might be a secondary effect therefore. In this case, Pbl's activity would not necessarily result directly in the formation of lamellipodia for example, but it would transfer the cells into a state where they are able to induce the formation of such mesenchymal structures.

Several indications exist that point towards an early function of the GEF protein during mesoderm migration. In *pbl* mutant embryos the first visible defect is already appreciable at the onset of EMT. In the wild type, cells that are basally located in the internalized epithelial tube start to form protrusions towards the ectoderm leading to an initial contact and the subsequent symmetrical flattening of the tube that is followed by its complete disassembly. In *pbl* as well as in *htl* mutants this early attachment to the ectoderm is absent (Schumacher et al., 2004; Wilson et al., 2005). However, these mutant cells are still able to subsequently downregulate their apical junctions suggesting that the initial contact between meso- and ectoderm is not required for the initiation of EMT. That this conclusion is not necessarily true results from the observation that the disassembly of the meosdermal tube is accompanied by a first round of mitosis in the wild type. Hence, the loss of epithelial characteristics could also be a general consequence of mitosis and not of an EMT process.

Importantly, cells that are homozygous mutant for the cell cycle regulator *string* (*stg*), which is the *Drosophila* homologue of cdc25, do not enter mitosis but they still show a normal dispersal of the tube and also exhibit no defects on the level of Eve-positive cell clusters (Carmena et al., 1998; Leptin and Grunewald, 1990). This clearly indicates that EMT of the mesoderm is not a consequence of mitosis in the first place suggesting the existence of a regulatory pathway. Indeed, first indications exist that imply an involvement of Htl signalling in EMT of the mesoderm. Interestingly, embryos double mutant for *htl* and *stg* display a delayed EMT, while a comparable defect could not be observed in the respective single mutants. Hence, Htl might be necessary for the induction of EMT and the loss of Htl function can be partially complemented by the downregulation of cell junctions during the first round of mitosis. Surprisingly no indications for an equal involvement of Pbl in this process could be identified yet (Otte, 2007). In conformity with an involvement of *htl* in EMT, the initial

contact establishment of the mesoderm with the ectodermal cell layer is Htl dependent and linked to the activation of the MAPK pathway. At this stage, only the cells of the mesodermal tube that initiate the contact with the ectoderm are positive for active MAP kinase in antibody stainings. From different systems it is known that MAP kinase is part of signalling pathways involved in EMT by regulating the expression of E-cadherin for example (Edme et al., 2002; Thiery and Sleeman, 2006). As activation of MAP kinase occurs downstream of Htl and it is absent in *htl* embryos therefore, it might reflect a possible link between FGF-signalling and EMT in the mesoderm. As already mentioned above, Pbl is also required for establishing the initial contact to the ectoderm. Consequently, loss of Pbl also abolishes activation of MAP kinase during this initial phase while during the following spreading phase Pbl was found to act downstream or in parallel of MAPK (Schumacher et al., 2004; Wilson et al., 2005). This suggests that Pbl, potentially by mediating the cytoskeletal rearrangements that are required for the formation of the early protrusions that are extended by the mesoderm cells towards the ectoderm, also participates in EMT. Beside the early defects in *pbl* mutant embryos, several other results could also be interpreted as a hint of an EMT function of Pbl. Expression of the constitutive active construct Pbl^{DH-PH} led to defects in mesodermal spreading. As described earlier, cells expressing the construct frequently formed aggregates extending into the yolk cell of the embryo. These tightly packed cells were visible even after part of the mesoderm had started to spread out on the ectoderm suggesting that the adhesive properties of these cells are somehow misregulated after Pbl^{DH-PH} expression.

Consistent with the model proposed here that Pbl acts through the Rac pathway during migration, misexpression of constitutive active Rac1 led to a comparable phenotype. The mesoderm cells failed to establish the initial contact with the ectoderm and did not dissociate from each other. Like in Pbl^{DH-PH} expressing embryos, aggregates of cells were visible even in very late stages of mesodermal spreading, suggesting again that the adhesive properties of the cells are changed. Reduction of Rac activity in the embryo was already reported before to negatively affect the contact establishment of ectoderm and mesoderm (Wilson et al., 2005). Therefore, reduction of Pbl and Rac activity as well as expression of activated forms of Pbl and Rac1 has comparable effects during phase 1 of mesoderm migration, suggesting that the proteins might both contribute to EMT in the wild type. In addition, either lack of activation or the hyperactivation of the pathway seems to result in similar effects demonstrating the importance of a tight regulation of the Pbl and Rac activation state.

There are several examples that demonstrate an involvement of Rac GTPases in EMT. In different systems, a role for Rac1 in the downregulation of E-cadherin was reported. In

vertebrate cells, the matrix metalloproteinase Stromelysin, which is upregulated in many cancers, was found to induce a spliced form of Rac, called Rac1b. This Rac1 isoform in turn stimulates Snail expression, a transcription factor known to repress E-cadherin (Radisky et al., 2005; Thiery and Sleeman, 2006). Furthermore, the Rac/Cdc42 downstream target PAK has been found to mediate phosphorylation of Snail leading to an enhancement of its nuclear translocation and its transcriptional functions (Yang et al., 2005).

Besides influencing indirectly the expression levels of E-cadherin, Rac also triggers the downregulation of cadherin from junctions by other means. In human keratinocytes it was shown that Rac1 mediates the recruitment of E-cadherin/catenin complexes to recycling endosomes through a clathrin independent mechanism thereby downregulating the adhesive properties of the cells (Akhtar and Hotchin, 2001). Similarly, Rac regulates salivary gland morphogenesis in *Drosophila* by modulating *D*E-cadherin/ß-catenin dependent cell-cell adhesion through dynamin-mediated endocytosis (Pirraglia et al., 2006). This indicates that mediating endocytosis of cadherins is not a feature of Rac activity evident only in a subset of vertebrate tissue culture cells but it also reflects a possibility how the small GTPase influences cell-cell adhesion in *Drosophila*. Therefore, a similar mechanism is also conceivable for EMT in the mesoderm. Interestingly, the constitutive activation of the *Drosophila* PAK homologue Mbt was recently reported to disrupt adherens junctions during eye development consistent with the involvement of vertebrate PAK1 in downregulating E-cadherin from junctions (Lozano et al., 2008; Menzel et al., 2007). These examples demonstrate that an involvement of Rac GTPases in EMT processes is a frequent observation that could also apply for mesoderm development in the fly. It is also worth mentioning here that an involvement of Pbl in endosomal trafficking of Notch pathway components was recently suggested implying a role of the GEF in vesicle transport that would fit nicely to a potential EMT function upstream of Rac (Jones, 2007).

However, the finding that Rac and Pbl are required for establishing the initial contact with the ectoderm renders it more likely that Rac activity triggers the early cell shape changes rather than being directly involved in EMT pathways that might be acting downstream of this early event (Wilson et al., 2005). Hence, an involvement of Pbl and Rac in EMT might only be restricted to the establishment of the initial contact to the ectoderm leading to the activation of MAPK via Htl signalling. MAPK activation in turn could govern the downregulation of adherens junctions etc. during the EMT process.

Another observation that argues for a requirement for Pbl in EMT comes from *D*E-cadherin stainings of *pbl* mutant embryos. During the different rescue assays performed in this thesis, it was noticed that the downregulation of *D*E-cadherin seems to be affected in *pbl* mutants. Suppl. Fig. 5 shows embryos misexpressing Pbl^{DH}. As previously mentioned, expression of this construct did not show an effect neither in overexpression nor in rescue experiments. Furthermore, embryos expressing Pbl^{DH} but being heterozygous for pbl^{3} did not show any abnormalities indicating that the expression of the fusion protein had no effects on the persistence of *D*E-cadherin in the mesoderm cells. In the wild type, *D*E-cadherin becomes downregulated in the mesoderm during disassembly of the mesodermal tube. Although the apical adherens junctions disappear, the membranes of the mesoderm cells are still in contact with each other and with the yolk cell membrane in the interior of the embryo. At this stage *D*E-cadherin as well as α- and ß-catenin become distributed among the entire surface. This is followed by the complete degradation of *D*E-cadherin in the mesoderm and the subsequent expression of *D*N-Cadherin (Oda et al., 1998). Consistent with this description, the strong *D*E-cadherin accumulation at the adherens junctions disappears after invagination even in the absence of Pbl (Schumacher et al., 2004). However, the subsequent downregulation of the adhesion protein seemed to be affected in *pbl* mutant embryos. Even after the first mitosis the cells were tightly packed in the collapsed tube in the middle of the embryo (see different sections through the same embryo in Suppl. Fig. 5 A-F). The *D*E-cadherin staining was distributed among the whole surface of mesoderm cells. However, the staining intensity suggested that the total levels of the protein were not lower, if not even higher, than in the neighboring ectoderm for example (Suppl. Fig. 5 A,B). This was also visible in parts of the mesoderm that were located further inside the embryo (Suppl. Fig. 5 C-F). Importantly, multinucleated cells were already visible in the mesoderm, indicating that the cells have already finished their first mitotic division and should have already finished EMT (arrowhead in Suppl. Fig. 5 E,F). In slightly older embryos, it was frequently observed that *D*E-cadherin accumulated at contact sides with the yolk membrane or the ectodermal cell layer (Suppl. Fig. 5 G-J). This was never seen in embryos heterozygous for the *pbl* mutation indicating that these protein accumulations are not a consequence of the transgene expression but that they depend on the loss of Pbl activity (Suppl. Fig. 5 M,N).

In a few cases, embryos displayed a folding of the mesoderm into the interior of the embryo. As such defects have not been reported for normal *pbl* mutant embryos thus far, it cannot be excluded that this defect is a result of the expression of the constructs (in this case Pbl^{DH}) or of the Gal4 protein. However, it is very striking that the Cadherin staining in this unusual

structure is also much stronger than normal, suggesting that the *pbl* mutant phenotype might indeed provide a connection to a misregulation of *D*E-cadherin levels, consistent with a function of the GEF in EMT of the mesoderm. If these observations were correct, they would indicate that, consistent with earlier reports, the initial disassembly of the adherens junctions is not disturbed in *pbl* mutants (Schumacher et al., 2004). Nevertheless, the subsequent downregulation of *D*E-cadherin, maybe through endocytosis of the protein or by repressing its expression, could be affected in *pbl* mutant embryos.

It has to be examined in the future whether the EMT function of Htl is indeed linked to the early cell shape changes and MAP kinase activation after invagination. If this is the case, the role of Pbl and its substrates in this context has to be analyzed in more detail as the mutant phenotypes clearly suggest a requirement for the GEF in this process downstream or in parallel to Htl.

4.7 Impact of Htl signalling on Pbl activity during migration

It is known that Pbl is essential for the Htl induced cell shape changes in the mesoderm. Mesoderm specific expression of an activated version of the Htl receptor (λHtl) is capable of rescuing the early cell shape changes in *htl* mutants. When expressed in a *pbl* mutant background however, λHtl expression is not sufficient to trigger the early cell shape changes anymore, indicating that Pbl's function is required downstream or in parallel of the Htl pathway in this context (Schumacher et al., 2004).

Beside these genetic indications, nothing is known about the relationship between Htl and Pbl activity during mesoderm migration. One possible model how FGF-signalling could influence Pbl or its activity during migration would be that the interphase localization of the GEF in the mesoderm depends on active Htl. It could be shown here that the full-length protein still localizes to the cell cortex in a *htl* mutant background. Loss of Htl signalling did not cause appreciable differences in nuclear exclusion of the protein or the ability to localize to the cell periphery. Therefore, it is rather unlikely that the overall interphase localization pattern of Pbl is Htl regulated. This is consistent with the fact that the full-length PblA-HA protein was also able to bind to the cortex when expressed in epithelial cells like the follicle epithelium of developing oocytes, for example (data not shown). As active Htl signalling was not reported for this cell type, cortex association of the protein in these cells cannot be dependent on an active FGF receptor. However, as already discussed before, these observations are based on transgene expressions that might result in abnormal high concentrations of tagged Pbl. This

may result in a binding to lower affinity sites, thereby masking membrane subdomains that normally show a specific enrichment in the endogenous protein. If such structures cannot be detected in the wild type, one will not be able to see whether this pattern is changed in a *htl* background either.

Another point that qualifies this localization results comes from the HA staining of the different constructs. In this context it was shown that the C-terminus is sufficient to mediate a strong binding to the cortex. The proteins were detected throughout the cell surface indicating that potential binding sites for this domain are not distributed in a polarized fashion at the cortex. The Pbl^{DH-PH} construct however localized in punctae at the cortex suggesting that the PH domain may mediate a binding to the membrane in a more site restricted fashion. Therefore, it is possible that the C-terminal tail is required for a general anchoring at the cortex while the PH domain triggers weak accumulations at specific membrane subdomains. As the effect of Htl signalling on this PH domain mediated cortex association was not examined here, an involvement of Htl signalling in localizing Pbl to certain sites at the cell cortex cannot be excluded. However, the idea that Htl-triggered modifications of the Pbl protein are necessary for a cortex association per se or a release from the nucleus does not seem to be correct.

Another possibility how FGF-signalling could influence Pbl would be that the activation of the GEF at the cortex depends on Htl. The local activation of Rac at specific sites of the membrane could also be mediated by a site-specific activation of the GEF. In this case, membrane subdomains, in which active Htl receptors cluster, might trigger phosphorylation of cortex-associated Pbl in these spots, for example, leading to a spatially restricted GTPase activation. Interestingly, a similar model has been proposed for Ect2 during cytokinesis. Several phosphorylation sites have been identified that play a role during cytokinesis. The so-called hinge region of Ect2 (between BRCT and DH domains) contains a Threonine residue (T341) that is most likely phosphorylated by Cdk1 during mitosis. This phosphorylation does not affect the localization of Ect2 but triggers a conformational change in the protein. It has been suggested previously that the BRCT domains of Ect2 bind to the C-terminal region in its inactive state. The resulting closed conformation of the protein should negatively regulate its GEF activity. Phosphorylation of T341 is thought to result in an intra-molecular release thereby leaving the midregion of the protein accessible for further phosphorylation (Hara et al., 2006; Kim et al., 2005). Cdk1 probably mediates one of these additional phosphorylations as well. The kinase was shown to phosphorylate T412 in the midregion of Ect2 leading to a

stimulation of RhoA activation by the GEF *in vivo* and providing a binding site for Plk1, a kinase also required for normal mitosis progression (Niiya et al., 2006). Interestingly, the C-terminus of Ect2 also contains a consensus phosphorylation site for Cdk1, which can be phosphorylated *in vivo*. A mutation of this residue led to a slightly reduced GEF activity towards Rac1 *in vitro* suggesting that modifications of the C-terminal domain might affect the interaction with specific substrates. However, whether a phosphorylation of this T814 is of any biological relevance *in vivo* is currently unknown (Niiya et al., 2006).

The latter finding is especially interesting when considering the function of the C-terminal tail of Pbl/Ect2. In both proteins the presence of the C-terminal tail had a clear impact on the dominant effects caused by the catalytic DH-PH tandem domain that are consistent with the activation of different substrate GTPases by the respective constructs (see Pbl^{DH-PH} and $Pbl^{\Delta N-term}$) (Solski et al., 2004). Although the overall ability to bind the different substrates is not affected by the C-terminus (pull down of Rho1, Rac1 and Rac2 with $Pbl^{\Delta N-term}$ and Solski et al. (2004)) the *in vivo* activation of distinct substrates seems to be modified by the C-terminal tail. In this context, rescue experiments with $Pbl^{\Delta C-term}$ clearly demonstrated that the C-terminus is essential for Rho1 activation during cytokinesis while being dispensable for the activation of Rac during migration. Together with the results from the misexpression studies, this finding strongly suggests a pivotal role for the C-terminal domain in regulating the substrate preference of Pbl. Therefore, this domain is a good candidate for regulatory input that affects Pbl's substrate interactions and thereby presumably the switch between its functions in migration or cytokinesis. Consequently, modifications like phosphorylation or dephosphorylation of the C-terminus represent another possibility for FGF-signalling to influence Pbl's function during migration. Consistent with this hypothesis the highly conserved C-terminal tail of Pbl contains several predicted phosphorylation sites. Hence, it is possible that either active Htl directly phosphorylates Pbl or that the activation of downstream factors like the phosphatase Corkscrew (Csw) results in the dephosphorylation of the C-terminus in the FGF receiving cells. This FGF-triggered modification could be necessary to generate a fraction of total Pbl that is capable of activating Rac at the cell cortex during migration (Fig. 4.1).

There are already several examples of receptor tyrosine kinases that activate Rho GEFs in a direct manner. In some cases, GEF proteins contain domains that allow a direct binding to the receptor leading to their activation. This does not only include protein domains like PDZ domains, but it can also be mediated by the PH domains of the GEFs (Schiller, 2006). A more common way seems to be a phosphorylation mediated recruitment or activation of Rho GEFs

by the RTKs. For example several different RTKs can phosphorylate a special Tyrosine in different Vav GEFs, resulting in their activation (Schiller, 2006). An even more striking example is the Rho GEF Ephexin1, which is required for growth cone repulsion during axon guiding. In this context, Ephexin1 mediates cytoskeletal rearrangements downstream of the EphA receptor. EphA dependent Tyrosine phosphorylation of Ephexin1 was shown to switch its substrate specificity towards RhoA thereby lowering the activation of Rac1 and Cdc42 by the GEF (Sahin et al., 2005). Importantly FGF-receptor-2 was also reported to be able to phosphorylate Ephexin1 changing its substrate preference in favor of RhoA (Zhang et al., 2007). This indicates that the postulated model of Pbl modification downstream of Htl signalling is a reasonable idea that should be tested in future.

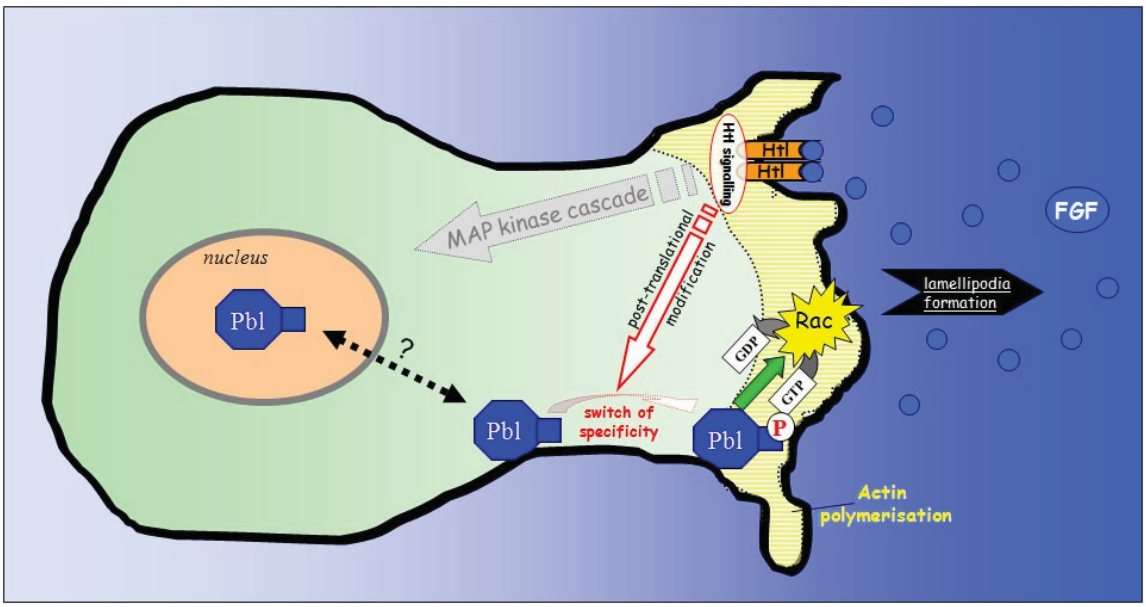

Fig. 4.1: Model for Pbl regulation during FGF-triggered mesoderm migration.
Possible model demonstrating the idea that Htl signalling influences the substrate preference of cytoplasmic Pbl by triggering post-translational modifications of its conserved C-terminus. This signalling event leads to the activation of Rac GTPases at the leading edge of migrating mesoderm cells and the formation of lamellipodia. Alternatively, modified Pbl could also act already during EMT of the mesoderm influencing vesicle transport via Rac activation, for example (not shown, see also chapter 4.6).

4.8 Pbl and its mammalian homologue Ect2

Similar to the data presented here, the oncogenic form of human Ect2 was also reported to exhibit different dominant phenotypes depending on whether its C-terminus was present or deleted in the expressed protein. Further *in vivo* analysis revealed that the C-terminus of Ect2 is important for a GEF activity towards RhoA, Rac1 and Cdc42 while the DH or DH-PH

domains alone show a strong substrate preference for RhoA only. The authors proposed that the C-terminus is involved in substrate specificity and that posttranslational modifications of this region might be necessary to control the substrate spectrum of Ect2 *in vivo* (Solski et al., 2004). These results demonstrate that Ect2 might be regulated in a similar way as proposed for Pbl (see above). This regulation would be of special interest in the context of Ect2's transforming activity. Therefore, it was tried to address the question whether Ect2 might also have a migration related function that, when misregulated, could contribute to metastasis of human cancer cells. Unfortunately, the rescue assays performed with human Ect2 protein did not show a rescue potential for mesoderm migration. However as stated earlier, the Ect2 protein expression was also unable to rescue cytokinesis, suggesting that it might not be active when expressed in *Drosophila* tissue. Previously, mouse Ect2 (mEct2) has been used in cytokinesis studies to test whether Pbl and mEct2 exhibit similar effects during mitosis. Interestingly, N-terminally truncated oncogenic mEct2 exhibited dominant effects on cytokinesis when misexpressed in embryos, indicating that this dominant mEct2 construct is also active in *Drosophila* (Prokopenko et al., 1999). Unfortunately, no rescue experiments with mEct2 were published, rendering it open whether at least the cytokinesis function can be complemented by mammalian Ect2 in a *pbl* mutant background. Therefore, the obtained data for human Ect2 do neither strengthen nor weaken the idea, that the protein could fulfill a migratory function analogous to Pbl. An approach that should be tested in future is to perform rescue assays employing dominant forms of mEct2 with or without its C-terminal tail to see whether either form exhibits any rescuing activity for migration. If a connection between Pbl activity and the role of Ect2 in cancer could be established, the *Drosophila* model would be a powerful tool to assess the mechanism by which misregulation of the GEF contributes to different aspects of cancer.

5 Summary

The multifunctional guanine nucleotide exchange factor (GEF) Pebble (Pbl) is an essential player during cytokinesis and fibroblast growth factor-triggered mesoderm migration in the *Drosophila* gastrula. During cytokinesis, Pbl activates Rho1 at the cell cortex leading to the formation of the contractile actomyosin ring. Although Pbl's role in the conserved cytokinesis pathway is well characterized, its migration-specific function is less well understood. The subcellular localization of Pbl as well as its GTPase substrate during mesoderm spreading is unknown. Furthermore, it is unclear how the switch between the dual functions of the GEF is mediated in order to guarantee a specific activation of the respective downstream pathways during cytokinesis and migration. To address these questions a domain-function analysis of the Pbl protein was conducted. This work showed that full-length Pbl localizes not only to the nucleus but also to the cell cortex and cellular protrusions in migrating cells. The PH domain and the conserved C-terminal tail are both involved in the cortical localization of Pbl.

Several lines of evidence indicated that the Rac GTPase pathway is involved in mesoderm migration and that Rac is directly activated by Pbl. First, Rac genetically interacted with activated forms of Pbl in the compound eye of the fly. Lowering the dose of Rac weakened the dominant phenotype while co-expression of extra Rac led to an enhancement. Second, co-expression of wild type Rac1 enhanced the migration rescue of a constitutively active Pbl variant in a *pbl* loss-of-function background. Third, dominant Rac constructs were able to enhance migration defects in the hypomorphic pbl^{l1D} allele. Forth, expression of constitutive active Rac1 in the mesoderm lead, analogous to the misexpression of the constitutive active $Pbl^{DH\text{-}PH}$, to an interference with proper mesoderm spreading. Fifth, loss of Rac1/Rac2 activity in the early embryo caused severe migration defects indicating the requirement for Rac GTPases in this process. Finally, biochemical data from a previous *in vitro* guanine-nucleotide-exchange-assay as well as *in vitro* GEF binding assays indicated that Rho1, Rac1 and Rac2 can all bind to the catalytic core of Pbl and that they are accepted as substrates.

Results of gain-of-function and rescue experiments both suggested an important regulatory role for Pbl's C-terminal tail for the selective activation of Rho1 vs. Rac dependent pathways. These data support a model in which post-translational modifications of Pbl, most likely at its conserved C-terminus, result in a change in its substrate preference. This enables at least a subpopulation of the GEF to trigger activation of Rac GTPases at the cell cortex thereby fulfilling its migration specific function.

5.1 Zusammenfassung

Der multifunktionelle Guaninnukleotidaustauschfaktor (GEF) Pebble (Pbl) ist eine zentrale Komponente der Zellteilungsmaschinerie. Er agiert darüberhinaus aber auch als wichtiger Faktor während der FGF-gesteuerten Zellwanderung des Mesoderms im *Drosophila* Embryo. Während der Cytokinese aktiviert Pbl die kleine GTPase Rho1 am Zellkortex, was die Bildung des kontraktilen Ringes zu Folge hat. Obwohl die konservierte Cytokinesefunktion des GEFs vergleichsweise gut untersucht ist, bleiben viele Fragen zum Verständnis seiner migratorischen Rolle offen. So ist nachwievor unklar, wo das Protein während der Migration in den Zellen lokalisiert und welche GTPase in diesem Kontext als Substrat für Pbl dient. Darüberhinaus konnte bislang ebenfalls nicht geklärt werden, wie das Umschalten zwischen beiden Funktionen reguliert wird, um jeweils eine spezifische Aktivierung des korrekten Signalweges unterhalb des GEFs zu gewährleisten.

Zur Klärung der vorgenannten Fragen wurde im Rahmen dieser Doktorarbeit eine Struktur-Funktionsanalyse des Pbl Proteins vorgenommen. Die zu diesem Zweck hergestellten Pbl-Konstrukte zeigten, dass Pbl nicht nur im Zellkern, sondern auch am Zellkortex sowie in Zellausläufern im Mesoderm lokalisiert. Diese Membranassoziierung wird hierbei durch die PH Domäne sowie den konservierten corboxyterminalen Bereich des Proteins vermittelt.

Darüberhinaus konnte im Zuge dieser Arbeit eine Reihe von wichtigen Beweisen gesammelt werden, die nahelegen, dass Pbl, im Gegensatz zur Zellteilung, während der Migration durch den Rac GTPase Signalweg agiert. So wurde Rac zum einen als Interaktionspartner von Pbl bei genetischen Interaktionsstudien im Komplexauge der Fliege identifiziert. Hierbei konnte eine Reduktion der endogenen Rac Menge die durch Expression einer aktivierten Pbl Version ausgelösten Defekte suprimieren, während die Koexpression von wildtypischen Rac1 oder Rac2 den Phänotyp noch verstärkten. Zum anderen konnten diese genetischen Interaktionen auch während der Mesodermausbreitung bestätigt werden, da die Koexpression von wildtypischen Rac1 die Rettung des *pbl* Migrationsphänotyps durch eine konstitutiv aktive Pbl Form weiter verbessern konnte. Dagegen führte die Expression dominanter Rac-Konstrukte zu einer Verstärkung der Migrationsdefekte in einem hypomorphen *pbl* Hintergrund. Ferner konnte gezeigt werden, dass die Überexpression von konstitutiv aktivem Rac1 im Mesoderm, ähnlich wie die Expression des aktivierten $Pbl^{DH\text{-}PH}$ Konstrukts, zu Defekten während der Zellwanderung führt. Die Notwendigkeit einer kontrollierten Rac Aktivierung im Mesoderm konnte ferner durch die Untersuchung von Embryonen nachgewiesen werden, welche die Rac1/Rac2 Aktivität vollständig verloren hatten; unter diesen Bedingungen waren starke Defekte während der Mesodermausbreitung sichtbar.

Schließlich bestätigten *in vitro* Bindungsstudien frühere Ergebnisse eines Guaninnukleotid-Austausch-Versuches und legen eine direkte physikalische Interaktion und somit eine direkte Aktivierung von Rac durch Pbl nahe.

Im Zuge von Überexpressionsstudien und Rettungsexperimenten konnte desweiteren eine bisher unbekannte Rolle des C-Terminus von Pbl für die selektive Aktivierung von Rho1- bzw. Rac-abhängigen Signalwegen aufgezeigt werden. Diese Ergebnisse unterstützen das Modell, dass durch etwaige posttranslationale Modifikationen, vermutlich innerhalb des konservierten C-terminalen Bereiches, die Substratpräferenz des GEFs beeinflusst werden könnte. Auf diese Weise würden solche Modifikationen des Proteins dann zumindest einer Subpopulation von Pbl Molekülen erlauben, die Rac GTPasen am Zellkortex zu aktivieren, um die migrationsspezifische Funktion des GEFs zu erfüllen.

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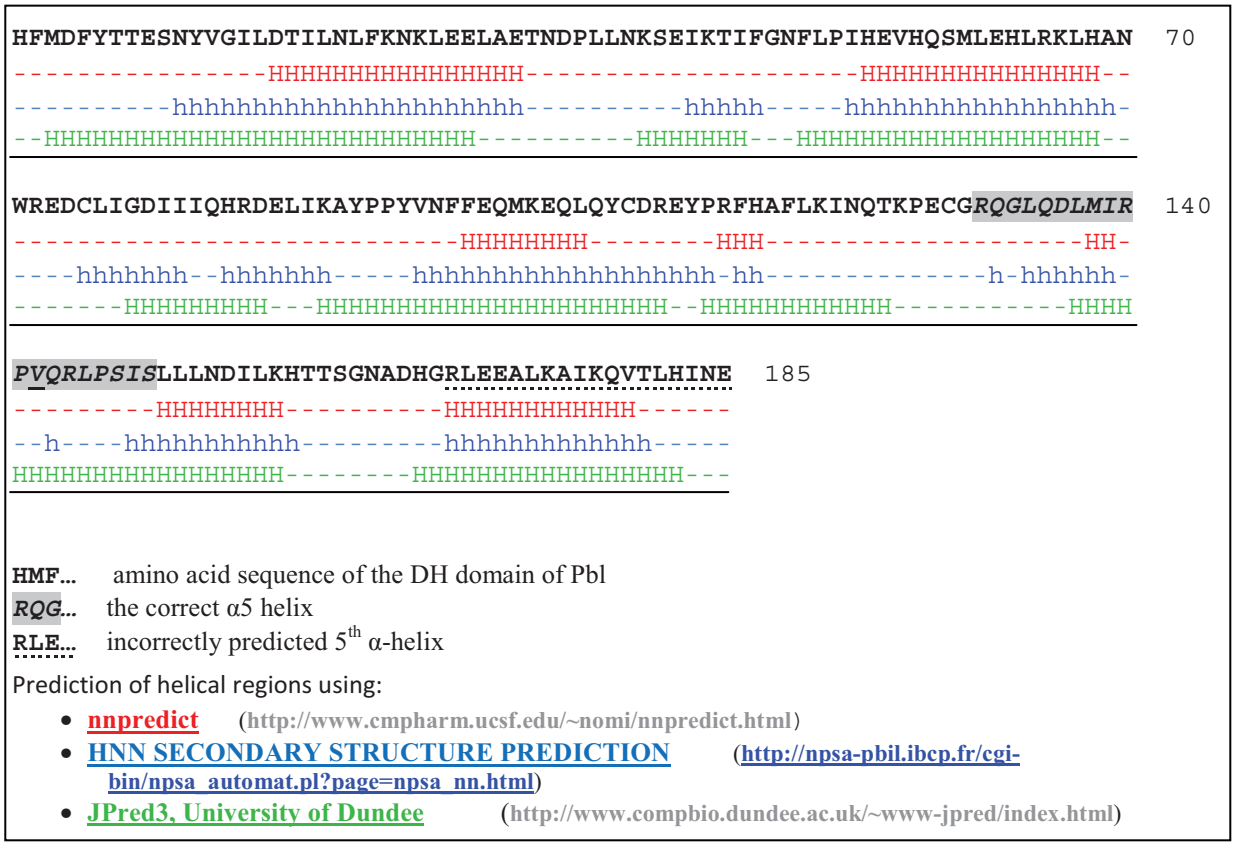
7 Supplementary Figures



Suppl. Fig. 1: Prediction of α-helical regions in the DH domain of Pbl using three different programs.
The amino acid sequence of the DH domain was examined with three different programs to predict α-helical regions. The predicted helical regions in the DH domain are shown in red for nnpredict, in blue for the HNN secondary structure prediction results and in green for JPred3. The fifth α-helix in the Pbl sequence that corresponds to the described regions of Snyder et al. (2002), is marked in grey (the previously assumed region that was taken for the design of the mutagenesis constructs is highlighted by a dotted line).

CLUSTAL W (1.83) multiple sequence alignment

http://myhits.isb-sib.ch/cgi-bin/clustalw

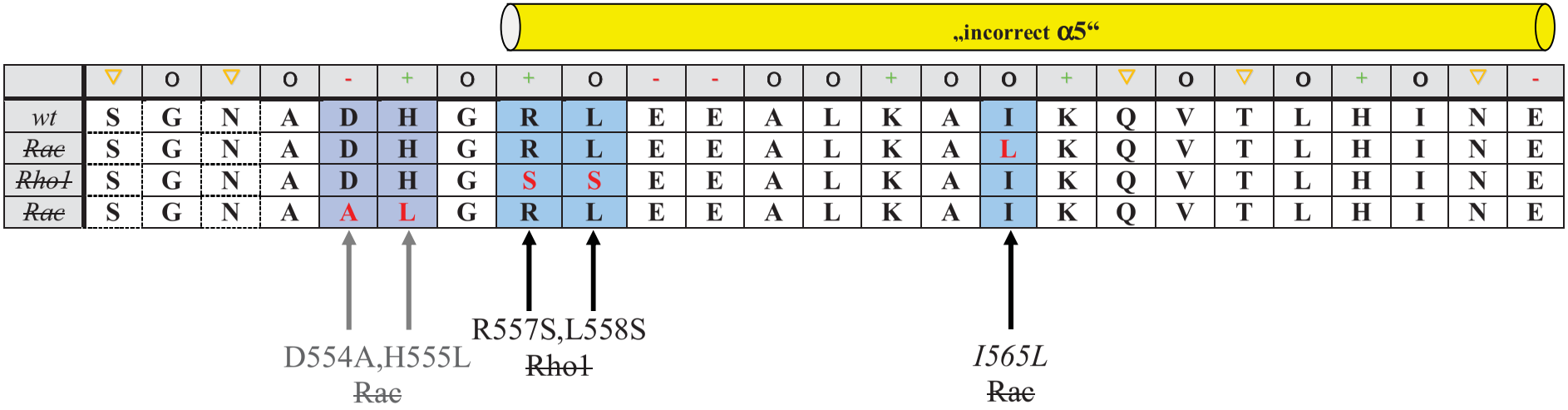
```
sw:Pbl      ASDATPAKKSMRFNHFMDFYTTESNYVGILDTILNLFKNKLEE-LAETNDPLLNKSEIKT
sw:Ect2     ----------ARWQVAKELYQTESNYVNILATIIQLFQVPLEE-EGQRGGPILAPEEIKT
sw:Dbs      ----------LRRHVMSELLDTERAYVEELLCVLEGYAAEMDNPLMAHLLSTGLHNKKDV
sw:Dbl      ----------LKNHVLNELIQTERVYVRELYTVLLGYRAEMDNPEMFDLMPPLLRNKKDI
sw:Tiam     ----------KLRKVICELLETERTYVKDLNCLMERYLKPLQK------ETFLTQDELDV
sw:ITSN     ----------KRQGYIHELIVTEENYVNDLQLVTEIFQKPLME------SELLTEKEVAM
sw:Tim      ----------KLQEVKFELIVSEASYLRSLNIAVDHFQLSTS------LRATLSNQEHQW
                           ::  :*  *:  *      :                 .:

sw:Pbl      IFGNFLPIHEVHQSMLEHLRKLHANWREDC---------------LIGDIIIQHRDELIK
sw:Ect2     IFGSIPDIFDVHTKIKDDLEDLIVNWDESK---------------SIGDIFLKYSKDLVK
sw:Dbs      LFGNMEEIYHFHNRIFLRELENYTDCPELV---------------GRCFLERMEDFQIYE
sw:Dbl      LFGNMAEIYEFHNDIFLSSLENCAHAPERV---------------GPCFLERKDDFQMYA
sw:Tiam     LFGNLTEMVEFQVEFLKTLEDGVRLVPDLEKLEKVDQFKKVLFSLGGSFLYYADRFKLYS
sw:ITSN     IFVNWKELIMCNIKLLKALRVRKKMSGEKMP-------------VKMIGDILSAQLPHMQ
sw:Tim      LFSRLQDVRDVSATFLSDLEENFENNIFS---------------FQVCDVVLNHAPDFRR
            :*     :      :

sw:Pbl      AYPPYVNFFEQMKEQLQYCDREYPRFHAFLKINQTKPECGRQGLQDLMIRPVQRLPSISL
sw:Ect2     TYPPFVNFFEMSKETIIKCEKQKPRFHAFLKINQAKPECGRQSLVELLIRPVQRLPSVAL
sw:Dbs      KYCQNKPRSESLWRQCSDCP---------FFQECQRKLDHKLSLDSYLLKPVQRITKYQL
sw:Dbl      KYCQNKPRSETIWRKYSECA---------FFQECQRKLKHRLRLDSYLLKPVQRITKYQL
sw:Tiam     AFCASHTKVPKVLVKAKTDT-----AFKAFLDAQNPKQQHSSTLESYLIKPIQRILKYPL
sw:ITSN     PYIRFCSRQLNGAALIQQKTDEAPDFKEFVKRLAMDPRCKGMPLSSFILKPMQRVTRYPL
sw:Tim      VYLPYVTNQTYQERTFQSLMNSNSNFREVLEKLESDPVCQRLSLKSFLILPFQRITRLKL
             :                              .              * . :: *.**:    *

sw:Pbl      LLNDILKHTTSGNADHGRLEEALKAIKQVTLHINEDKRRTESRMAIFDIF
sw:Ect2     LLNDLKKHTADENPDKSTLEKAIGSLKEVMTHINED--------------
sw:Dbs      LLKEMLKYSRNCEG-AEDLQEALSSILGILKAVNDS--------------
sw:Dbl      LLKELLKYSKDCEG-SALLKKALDAMLDLLKSVNDS--------------
sw:Tiam     LLRELFALTDAESEEHYHLDVAIKTMNKVASHINEM--------------
sw:ITSN     IIKNILENTPENHPDHSHLKHALEKAEELCSQVNEG--------------
sw:Tim      LLQNILKRTQPGSSEEAEATKAHHALEQLIRDCNNN--------------
            ::.::   :            *      :    *:
```

Suppl. Fig. 2: Multiple sequence alignment of various DH domains.
Multiple sequence alignment of Pbl's DH domain with the sequences of the DH domains of human Ect2, human Dbs, human Dbl, human Tiam, human ITSN and human Tim. The alignment was produced using the clustalW software (http://myhits.isb-sib.ch/cgi-bin/clustalw). Critical amino acids that should dictate the GEF specificity (Snyder et al., 2002) are marked in blue, the 5th α-helical stretch in bold (the underlined 'V' shows the position of the V531D control mutation).

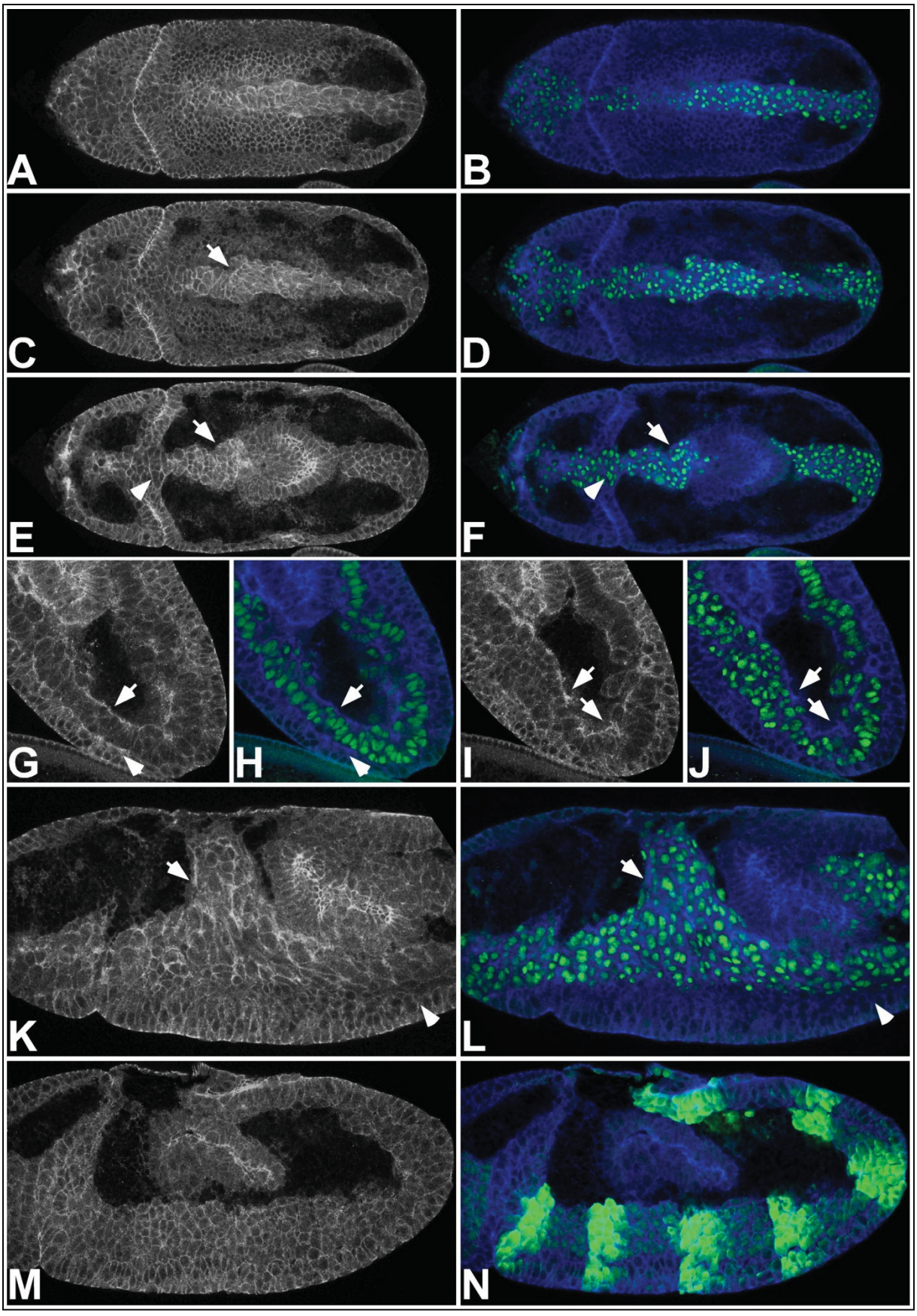
GEF		α5																						
RhoA																								
Net1		E	S	P	F	S	R	K	L	D	L	W	S	F	L	D	I	P	R	S	R	L	V	K	Y	P
Larg		S	N	P	L	C	R	R	L	Q	L	K	D	I	I	P	T	Q	M	Q	R	L	T	K	Y	P
Lfc		S	-	A	V	L	K	R	H	G	V	Q	E	C	I	L	L	V	T	Q	R	I	T	K	Y	P
Trio/ C		Q	-	R	L	G	H	R	L	Q	L	T	D	L	L	I	K	P	V	Q	R	I	M	K	Y	Q
Tim		S	D	P	V	C	Q	R	L	S	L	K	S	F	L	I	L	P	F	Q	R	I	T	R	L	K
RhoA & Cdc42																								
Dbs		Q	K	K	L	D	H	K	L	S	L	D	S	Y	L	L	K	P	V	Q	R	I	T	K	Y	Q
Dbl		Q	R	K	L	K	H	R	L	R	L	D	S	Y	L	L	K	P	V	Q	R	I	T	K	Y	Q
RhoA & Cdc42 & Rac1																								
Vav		Q	R	A	N	N	G	R	F	T	L	R	D	L	L	M	V	P	M	Q	R	V	L	K	Y	H
Vav2		K	-	V	Q	D	G	K	F	K	L	Q	D	L	L	V	V	P	M	Q	R	V	L	K	Y	H
Cdc42																								
ITSN		M	D	P	R	C	K	G	M	P	L	S	S	F	I	L	K	P	M	Q	R	V	T	R	Y	P
Fgd1		E	-	E	A	C	G	N	L	T	L	Q	H	H	M	L	E	P	V	Q	R	I	P	R	Y	E
Pem2		L	L	Q	Q	M	I	D	I	A	I	D	G	F	L	L	T	P	V	Q	K	I	C	K	Y	P
Rac1																								
Tiam 1		N	P	R	Q	Q	H	S	S	T	L	E	S	Y	L	I	K	P	I	Q	R	V	L	K	Y	P
UNC-73		-	-	G	L	E	I	N	N	E	I	A	S	L	L	I	K	P	V	Q	R	I	T	R	Y	R
Trio/ N		R	H	G	L	-	-	A	N	S	I	S	S	Y	L	I	K	P	V	Q	R	I	T	K	Y	Q
Rho1 & Rac																								
Pbl	Q	T	K	P	E	C	G	R	Q	G	L	Q	D	L	M	I	R	P	***V***	Q	R	L	P	S	I	S
Ect2	Q	A	K	P	E	C	G	R	Q	S	L	V	E	L	L	I	R	P	V	Q	R	L	P	S	V	A
position		1						2								3								

negative residue positive residue
polar residue non-polar residue

Suppl. Fig. 3: Alignment of the conserved α-helix 5 of different DH domains.
Alignment of the critical α-helix 5 regions in various GEF proteins with known substrate specificity (see grey bars). The alignment corresponds to the one published in Snyder et al. (2002) and the corresponding sequences for Pbl and Ect2 were added. Except for Dbs and Lfc (both *mus musculus*), UNC-73 (*C. elegans*) and Pbl (*Drosophila melanogaster*), all sequences are human. Negatively charged amino acid residues are marked in red, positively charged residues in green, polar residues in yellow and non-polar residues in black. The three critical positions for the substrate preference of DH domains (Snyder et al., 2002) are highlighted in blue.



Suppl. Fig. 4: Graphical representation of the mutated amino acid residues in the DH domain of Pbl^{I565L} and Pbl^{R557S,L558S}.
Amino acids in the previously misannotated fifth α-helical region of Pbl's DH domain are shown (wt). The three mutated sequences of the mutagenesis constructs are shown below and the exchanged amino acids are highlighted in red. The D554A,H555L and I565L mutations were expected to abolish interactions with Rac GTPases while the R557S,L558S mutation was expected to affect activation of Rho1. Negatively charged amino acid residues are marked by a red minus, positively charged residues by a green plus, polar residues by a yellow triangle and non-polar residues by a black circle.



Suppl. Fig. 5: Downregulation of E-cadherin during EMT in *pbl*^{3} mutant embryos.
Anti-*D*E-cadherin (*D*E-cad; white in single and, blue in merged images), anti-Twi and anti-ßGal antibody staining (both in green) are shown for homozygous (A-L) and heterozygous (M-N) *pbl*^{3} mutant embryos expressing Pbl^{DH} with *twi::Gal4* in the mesoderm. (A-F) Several single optical sections of a late stage 7/early

stage 8 embryo at three different focal planes in a ventral view. The mesoderm cells display high levels of *D*E-cad at the cell cortices (arrows). The fact that multinucleated cells are already visible demonstrates that the cells have already undergone the first round of mitosis and that EMT should be already complete therefore (arrowhead in F). (G-J) Two lateral views at different focal planes of a mid stage 8 embryo shows *D*E-cad accumulations between the mesoderm and the inner yolk cell (arrows) and the mesoderm and the ectoderm (arrowhead). (K,L) In a few cases the mesoderm folds in far into the embryo, giving rise to a strong abnormal mesoderm morphology (note the higher levels of *D*E-cad staining in the infolded region of the mesoderm (arrow) compared to the ectodermal tissue (arrowhead)). (M-N) Heterozygous embryos expressing Pbl^{DH} do not exhibit the previously described *D*E-cad accumulations suggesting that this effect is not caused by expression of the construct.

Movie files provided on extra CD

Suppl. Movie 1:
3D reconstruction of an image stack through leading edge cells overexpressing PblA-HA using the opacity renderer of Volocity. Antibody staining against HA is shown in red and against the Twi protein in green. The original image stack comprises 51 sections and covers in total 10.2µm in thickness.

Suppl. Movie 2:
Localization of PblA-GFP in migrating hemocytes after expression with *twi::Gal4*. The fusionprotein accumulates at actin rich structures. Images were taken at 20 second intervals.

8 Appendix

Abbreviations

α-...	anti-...
ß-Gal	ß-Galactosidase
Δ	delta ("deleted")
µ...	micro...(10^{-6})
A	Alanine
A	Ampere
aa	amino acid
AP	alkaline phosphatase
fig.	figure
bp	base pair
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
cDNA	coding DNA
chr.	chromosome
D	Aspartic acid
dH_2O	demineralised water
DAPI	4',6-diamidino-2-phenylindole
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
e.g.	for example ("*exempli gratia"*)
FGF	Fibroblast Growth Faktor
ftz	Fushi tarazu
GEF	Guanine nucleotide exchange factor
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
H	Histidine
HA-tag	Hemagglutinin-epitope
HRP	Horse raddish preoxidase
HS	heat shock
I	Isoleucine
k…	kilo…(10^{3})
kb	kilo-base pairs
kDa	kilo Dalton
L	Leucine
LB medium	Luria Bertani broth medium
m...	milli...(10^{-3})
M	Molarity (mol/l)
MAPK	Mitogen-activated-protein-kinase
min	minutes
mRNA	messenger-RNA

n...	nano...(10^{-9})
NBT	Nitro blue tetrazolium
OD	optical density
o/n	overnight
PAGE	Polyacryl amide gel electrophoresis
R	Arginine
rpm	revolutions per minute
RT	room temperature
S	Serine
SDS	Sodium dodecyl sulfate
sec	seconds
suppl.	supplementary
tab.	table
temp.	temperature
U	unit
UAS	Upstream Activating Sequence
UV light	ultraviolet light
V	Valine
V	Volt

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ERKLÄRUNG

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

Andreas van Impel im Februar 2009

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