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Regulation of the Rac GTPase pathway by the multi-functional Rho GEF Pebble is essential for mesoderm migration in the *Drosophila* gastrula

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The *Drosophila* guanine nucleotide exchange factor Pebble (Pbl) is essential for cytokinesis and cell migration during gastrulation. In dividing cells, Pbl promotes Rho1 activation at the cell cortex, leading to formation of the contractile actin-myosin ring. The role of Pbl in fibroblast growth factor-triggered mesoderm spreading during gastrulation is less well understood and its targets and subcellular localization are unknown. To address these issues we performed a domain-function study in the embryo. We show that Pbl is localized to the nucleus and the cell cortex in migrating mesoderm cells and found that, in addition to the PH domain, the conserved C-terminal tail of the protein is crucial for cortical localization. Moreover, we show that the Rac pathway plays an essential role during mesoderm migration. Genetic and biochemical interactions indicate that during mesoderm migration, Pbl functions by activating a Rac-dependent pathway. Furthermore, gain-of-function and rescue experiments suggest an important regulatory role of the C-terminal tail of Pbl for the selective activation of Rho1- versus Rac-dependent pathways.

KEY WORDS: *Drosophila*, Gastrulation, Mesoderm migration, Rho GEF

INTRODUCTION

Gastrulation represents the first major morphogenetic event in the development of most multicellular animals. One key aspect of gastrulation is the specification of the presumptive mesoderm cells and their morphogenetic rearrangements within the embryo by dramatic cell movements. In *Drosophila*, these mesoderm movements can be divided into two major stages: internalization and migration (Costa et al., 1993). Mesoderm cells are derived from a population of ventral cells of the blastoderm epithelium. Internalization involves actin-myosin-mediated apical constriction that promotes formation of a ventral furrow and internalization of the mesoderm as an epithelial tube-like structure (Leptin and Grunewald, 1990; Sweeton et al., 1991). Once internalized, the cells undergo epithelial to mesenchymal transition and mitotic divisions. During the following migration stage, the multilayered cell aggregate spreads out to form a monolayer. At this time, mesoderm cells begin to differentially express transcription factors that identify distinct fates along the dorsal/ventral axis of the embryo (Jagla et al., 2001; Furlong, 2004; Stathopoulos and Levine, 2004).

The genetic control of gastrulation has been attributed to the function of a limited number of genes. Internalization is controlled by targets of the zygotically active transcription factors Twist (Twi) and Snail (Sna) (Leptin and Roth, 1994; Leptin, 1999; Seher et al., 2007). Cell signalling through the secreted glycoprotein Folded Gastrulation and the transmembrane protein T48 are both implicated in local activation of Rho1 at the apical cell cortex of invaginating mesoderm cells (Costa et al., 1994; Leptin and Roth, 1994; Barrett et al., 1997; Kolsch et al., 2007). Migration of the mesoderm depends on signalling via the FGF receptor Heartless (Htl) and its two FGF8-like ligands, Thisbe (Ths; FGF8-like1) and Pyramus (Pyr; FGF8-like2) (Shishido et al., 1993; Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997; Gryzik and Müller, 2004; Stathopoulos et al., 2004). In most developmental contexts, Htl acts through the adaptor protein Stumps (Sms) via the conserved Ras/Raf/MAP kinase pathway (Michelson et al., 1998a; Vincent et al., 1998; Imam et al., 1999). However, targets of MAPK with a role in mesoderm migration remain elusive, and genetic evidence suggests that activation of MAPK by Htl is neither required nor sufficient for the early morphogenetic events occurring during early mesoderm spreading (Schumacher et al., 2004; Wilson et al., 2005).

A major unresolved issue is how signalling from the FGF receptor is transduced to trigger changes in cell behaviour, which eventually results in the collective cell movements to form a monolayer. Guanine nucleotide exchange factors (GEF) activate Rho GTPases and provide entry points for the regulation of Rho activity in different signalling contexts (Rossman et al., 2005). RhoGEF2 and Rho1 promote the recruitment and assembly of cytoplasmic myosin that drives apical constriction during ventral furrow formation (Barrett et al., 1997; Hacker and Perrimon, 1998; Nikolaidou and Barrett, 2004; Dawes-Hoang et al., 2005). Another GEF called Pebble (Pbl) is indispensable for Htl-triggered cell shape changes and thus represents an excellent candidate that links FGF signalling to the modulation of cell shape (Schumacher et al., 2004; Smallhorn et al., 2004).

Pbl is the single fly orthologue of the human proto-oncogene *ect2* and plays an evolutionarily conserved role in cytokinesis. Pbl localizes to the cell cortex and activates Rho1, which acts through its effector Diaphanous to promote formation of the contractile actin-myosin ring (Piekny et al., 2005). The two functions of Pbl, cytokinesis and cell migration, can be separated genetically: Pbl function is still required for cell migration in a genetic background in which no mitosis occurs, indicating that Pbl plays independent roles in cytokinesis and cell migration (Schumacher et al., 2004).

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Whereas protein interactions of Pbl during cytokinesis appear to be highly conserved, to date nothing is known about the mechanisms of Pbl function in mesoderm migration. Pbl belongs to a large family of GEFs that contain a Dbl-homology (DH) domain, which harbours catalytic activity (Whitehead et al., 1997). The function of Pbl in cell migration involves activation of Rho GTPases, as a point mutation in the highly conserved CR3 region within the DH domain compromises its catalytic activity and exhibits equally severe defects as *pbl* null alleles (Whitehead et al., 1997; Liu et al., 1998; Schumacher et al., 2004; Smallhorn et al., 2004). The only currently known Pbl substrate, Rho1, is unlikely to be involved in mesoderm migration, because Rho1 dominant-negative constructs fail to block mesoderm spreading while efficiently inhibiting cytokinesis (Schumacher et al., 2004).

In the present paper, we define domains of Pbl involved in regulating mesoderm migration. We provide evidence that the catalytic tandem DH-PH domain is essential for mesoderm migration and interacts with Rho1, Rac1 and Rac2. Mis-expression of the tandem DH-PH domain interferes with normal mesoderm migration. Biochemical assays suggest that the interaction between Pbl and Rac is direct. We further show that Pbl localizes to the cell cortex of migrating cells and that the conserved C-terminal tail and the PH domain are important for this cortical localization. These data suggest that Pbl acts through the Rac pathway during mesoderm migration in *Drosophila*.

MATERIALS AND METHODS

Drosophila genetics

Flies were kept under standard conditions. The following stocks were obtained from the Bloomington stock centre: w^{1118}, *twi::Gal4(2x); Dmef2::Gal4*, *GMR::Gal4*, pbl^{11D}/*TM3[ftz::lacZ]*, pbl^{3}/*TM3[ftz::lacZ]*, $rho1^{l(2)k07236}$/*CyO*, $Cdc42^{4}$/*FM6*, *yw;*$Rac1^{J10}$*,*$Rac2^{\Delta}$*,FRT2A,*Mtl^{Δ}/*TM3[ftz::lacZ]*, *Df(2R)ED2238/CyO[ftz::lacZ]*, *yw,hs::Flp;*cx^{D}/*TM3*, *w;P[ovo*$^{D1\text{-}18}$*]3L,FRT2A/*βTub^{85D}/*TM3*, *UAS::*$pbl^{\Delta BRCT\_myc}$/*TM3[ftz::lacZ]*, *UAS::*$RhoL^{N25}$/*CyO*, *UAS::*$RhoL^{V20}$, *UAS::*$Rac1^{V12}$, *UAS::*$Rac1^{N17}$, *UAS::Rac1.L*, *UAS::Rho1.Sph* and *EP(3)3118/TM3*.

All rescue assays were performed using virgins from a *twi::Gal4; pbl*^{3}*/TM3[ftz::lacZ]* stock. Genetic interactions of Pbl with Rac1 and Rho1 were examined using a *UAS::*$pbl^{\Delta BRCT}$*,*pbl^{3} recombinant chromosome crossed in trans to pbl^{3} with *UAS::Rac1.L* on the second chromosome or in trans to a recombinant *UAS::Rho1.Sph,*pbl^{3} chromosome, respectively. These experiments required distinct crosses to control for the genetic background: for the Rac1 experiment, *twi::Gal4;*pbl^{3} crossed to *UAS::*$pbl^{\Delta BRCT}$*,*pbl^{3} was used as control; for the Rho1 control experiment, *twi::Gal4;UAS::*$pbl^{\Delta BRCT}$*,*pbl^{3} was crossed to pbl^{3}.

Molecular biology

The *pbl* cDNA constructs were generated through PCR amplification using the *pbl-RA* cDNA as a template. Fragments were inserted in frame into the *pUAST-HA* vector to create C-terminal fusions of the HA epitope. The Pbl-GFP and GFP-Pbl^{PH} constructs were generated using the Gateway system (Invitrogen) and cloned into the pTGW or pTWG expression vectors (DGRC, Bloomington). The Pbl constructs encode the following amino acids of the Pbl-A protein: Pbl-A 1-853, $Pbl^{\Delta N\text{-}term}$ 386-853, $Pbl^{DH\text{-}PH}$ 386-775, Pbl^{DH} 386-581, Pbl^{PH} 595-719, $Pbl^{C\text{-}term}$ 716-844 and $Pbl^{\Delta C\text{-}term}$ 1-720. The $Pbl^{DH\text{-}PH\_V531D}$ and $Pbl^{\Delta N\text{-}term\_V531D}$ constructs were generated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) to generate a single amino acid exchange (Pbl-A Val^{531} to Asp) of the respective construct.

Biochemistry

GST fusion proteins were expressed from pGEX plasmids in BL21DE *E. coli* cells. After lysis in 50 mM Tris-HCl (pH 8), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, the fusion proteins were purified by affinity chromatography (wash buffer, 50 mM Tris-HCl pH 8, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT; elution buffer, 50 mM Tris-HCl pH 8, 50 mM NaCl, 20 mM glutathione, 1 mM DTT). The GEF assay was performed as described previously (Grosshans et al., 2005). Briefly, 0.2 μM GST-GTPases were loaded with [8-^{3}H]GDP (Amersham). The ^{3}H-GDP loaded GTPases were incubated as duplicates with 0.1 μM of the corresponding GEF in the presence of GTP at 25°C for 20 minutes. After nitrocellulose filtration, the radioactivity bound on the filter was determined by liquid scintillation counting.

Immunocytochemistry and microscopy

Embryos were obtained, fixed, stained and sectioned as described previously (Müller, 2008). Microscopy was performed on a Zeiss Axiophot, an Olympus BX61 as well as Zeiss 510 Meta and Leica-SP2 confocal microscopes. Images were processed using Adobe Photoshop and Volocity (Improvision). Heads of adult flies were prepared for scanning electron microscopy as described (Meyer et al., 2006). The following antibodies were used: mouse-anti-Eve, mouse-anti-βGal (both at 1:100, DSHB), rabbit-anti-βGal (1:5000, Cappel), mouse-anti-HA (1:1000, Roche), mouse-anti-GFP (1:800, ABCAM), rabbit-anti-Myc (1:35, Santa Cruz), mouse-anti-CD2 (Serotec), rabbit-anti-Twi (1:1000) and rat-anti-Pbl (1:350). Pbl antiserum was generated against a GST-Pbl-A fusion protein. A 1.6 kb fragment of *pbl-RA* cDNA (encoding amino acids 1-532 of Pbl-A) was cloned into pGEX-4T-2. The corresponding GST fusion protein was used to immunize rats (Eurogentec, Belgium).

RESULTS

Domain-function analysis of Pbl in cell migration

Pbl is a modular multi-domain protein (Fig. 1). The amino (N)-terminal part contains two BRCT domains, which act as protein-protein interaction domains and are required to localize Pbl to the cleavage furrow during cytokinesis (Somers and Saint, 2003). The central region of Pbl contains a PEST sequence and a nuclear

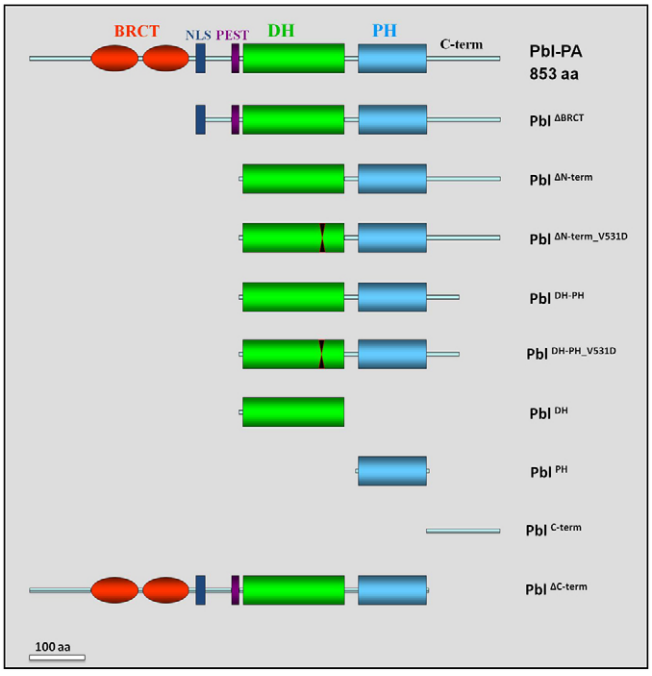

Fig. 1. Domain organization of Pbl constructs. All constructs are derived from a cDNA encoding the Pbl-A isoform. The extent of the constructs is indicated. The domains from N to C terminus are BRCT (BRCA1 carboxy-terminal domain), NLS (nuclear localization sequence), PEST (rich in proline, glutamic acid, serine and threonine), DH (Dbl homology), PH (Pleckstrin homology domain) and C-term (carboxy-terminal tail). $Pbl^{\Delta N\text{-}term\_V531D}$ and $Pbl^{DH\text{-}PH\_V531D}$ represent catalytically inactive variants. Scale bar: 100 amino acids.

localization signal, whereas the C-terminal half harbors the catalytically essential DH domain associated with a PH domain, also called tandem DH-PH domain.

The full-length *pbl* cDNA, when expressed in the mesoderm using the UAS-Gal4 system, rescues both the migration and cytokinesis defect of *pbl*-null mutants and thus provides an excellent assay for identifying domains of the protein required for Pbl function (Schumacher et al., 2004) (Fig. 2A-C). As a quantitative measure of mesoderm migration, we scored the segmental expression of *even-skipped* (*eve*) in a cluster of dorsal mesoderm cells (Frasch et al., 1987). Expression of *eve* in the dorsal mesoderm represents a reliable marker for proper dorsal mesoderm migration in *pbl* mutants because, unlike Htl, Pbl is not directly involved in the activation of *eve* expression in those cells (Carmena et al., 1998; Michelson et al., 1998b; Schumacher et al., 2004; Smallhorn et al., 2004).

A point mutation (V531D) in the DH domain that is known to compromise its catalytic activity abolished the activity of Pbl in cytokinesis and cell migration (Liu et al., 1998; Schumacher et al., 2004; Smallhorn et al., 2004). To identify other functionally important protein domains of Pbl, we tested the rescue potential of a range of tagged deletion constructs (Fig. 1). Expression of Pbl$^{\Delta BRCT}$, which has both N-terminal BRCT domains deleted, with *twi::Gal4* was unable to rescue cytokinesis, but still rescued migration at ~55% compared with wild type (Fig. 2D,J; Table 1) (Smallhorn et al., 2004). Similarly, a construct lacking the conserved C-terminal tail, Pbl$^{\Delta C\text{-term}}$, did not rescue cytokinesis, but was still able to partially rescue the migration defect to a similar extent as Pbl$^{\Delta BRCT}$ (Fig. 2I,J; Table 1; see below). These data indicate that neither domain alone plays an essential role, because in the absence of either domain there is still a partial rescue. However, as the rescue is not complete, both the BRCT domains and the C-terminal tail must be important for Pbl function in mesoderm migration.

Deletions of N-terminal regulatory domains extending beyond the NLS and PEST sequences create variants of Pbl that are characterized as oncogenic forms of Ect2 as they promote transformation in mammalian cells (Rossman et al., 2005; Saito et al., 2004) (Fig. 1). Expression of Pbl$^{\Delta N\text{-term}}$ in the mesoderm of *pbl*^{3} homozygotes did not rescue the mesoderm differentiation defects (Fig. 2E,J; Table 1). Moreover, even in heterozygous embryos expressing Pbl$^{\Delta N\text{-term}}$ the mesoderm cells failed to internalize (see below). By contrast, Pbl$^{DH\text{-}PH}$ lacking the conserved C-terminal tail was able to suppress the *pbl* mesoderm defect (Fig. 2F,J; Table 1). The V531D point mutation completely abolished the rescuing activity of Pbl$^{DH\text{-}PH}$; both constructs were expressed at very similar levels (Fig. 2G,J; Fig. 5G,H,K,L). Importantly, the DH domain alone did not exhibit any rescue activity (Fig. 2H,J). Thus, the activity of the tandem DH-PH domain of Pbl requires both a functional DH domain and the presence of the PH domain. Moreover, the rescue capability of the tandem DH-PH domain was dependent on the absence of the C-terminal tail, suggesting that this domain might impinge on the activity of the DH-PH domain.

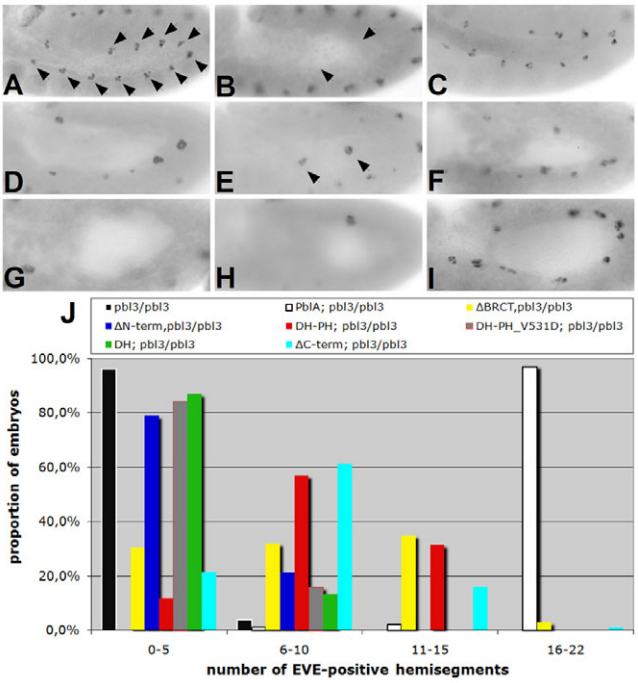

Fig. 2. Rescue potential of mesoderm-spreading defects in *pbl* mutants by Pbl constructs. (**A**) Eve is expressed in 11 segmental dorsal mesodermal cell clusters in the wild type (arrowheads). (**B**) In *pbl*^{3} mutants, the number of Eve clusters is strongly reduced (dorsal positions marked by arrowheads). Transgenic *UAS::Pbl*^{n} constructs were expressed in *pbl*^{3} mutants using *twi::Gal4*. (**C**) Expression of full-length Pbl almost completely rescues *pbl*^{3} mutant embryos. (**D**) Pbl$^{\Delta BRCT}$ expression. (**E**) Pbl$^{\Delta N\text{-term}}$ expression; arrows indicate Eve-expressing mesoderm cells. (**F**) Pbl$^{DH\text{-}PH}$ expression. (**G**) Pbl$^{DH\text{-}PH_V531D}$ expression. (**H**) Pbl^{DH} expression. (**I**) Pbl$^{\Delta C\text{-term}}$ expression. (**J**) Quantification of suppression by the various constructs: (*pbl*^{3} homozygotes, black; Pbl-A, white; Pbl$^{\Delta BRCT}$, yellow; Pbl$^{\Delta N\text{-term}}$, dark blue; Pbl$^{DH\text{-}PH}$, red; Pbl$^{DH\text{-}PH_V531D}$, grey; Pbl^{DH}, green; Pbl$^{\Delta C\text{-term}}$, pale blue). The graph depicts the relative proportion of embryos that exhibit *eve-positive* hemi-segments in the various genotypes indicated (values are shown in Table 1).

Table 1. Suppression of *pbl*^{3} mutant mesoderm phenotype

Genotype	Eve-positive hemisegments	s.d.	*n*
pbl^{3}/*pbl*^{3}	1.7	1.7	128
Pbl$^{A\text{-}HA}$; *pbl*^{3}/*pbl*^{3}	18.6	1.7	98
Pbl$^{\Delta BRCT}$; *pbl*^{3}/*pbl*^{3}	8.3	4.2	69
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$^{\Delta N\text{-term}}$; *pbl*^{3}/*pbl*^{3}	3.3	2.4	123
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

Mean values and standard deviations (s.d.) of the number of Eve-positive hemisegments are shown for *pbl*^{3} homozygous embryos expressing Pbl constructs as indicated (*n*=number of embryos examined). Fig. 2J shows a graph of the relative proportions.

Differential dominant phenotypes of oncogenic forms of Pbl

In addition to the different rescue potentials of Pbl$^{\Delta N\text{-term}}$ and Pbl$^{DH\text{-}PH}$, we noticed that these constructs also exhibited distinct dominant phenotypes. Expression of Pbl$^{\Delta N\text{-term}}$ in a wild-type background blocked invagination and the cells failed to undergo cytokinesis (Fig. 3C,D,O,P; Fig. 5F). As null mutants of *pbl* do not exhibit any defects in mesoderm invagination (S.S. and H.A.J.M., unpublished), Pbl$^{\Delta N\text{-term}}$ exhibits an abnormal activity interfering with that process. By contrast, expression of Pbl$^{DH\text{-}PH}$ exhibited defects in mesoderm spreading, whereas cytokinesis was unimpaired (Fig. 3I,J,Q,R). The expression levels of the constructs were in a similar range and even when the level of Pbl$^{DH\text{-}PH}$ was increased using multiple copies of transgenes, the occurrence of phenotypic classes did not change (Fig. 5) (A.v.I. and H.A.J.M.,

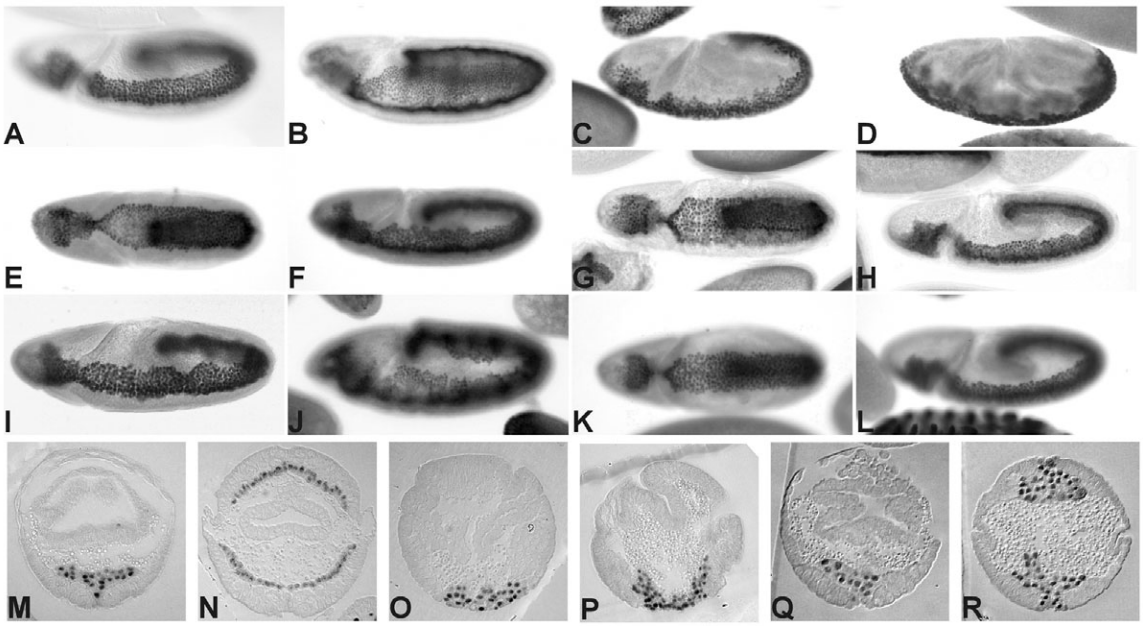

Fig. 3. Dominant effects of truncated Pbl constructs on mesoderm morphogenesis. (**A-R**) Embryos were stained with anti-Twi antibody and are depicted either as whole mounts (A-L) or transverse cross-sections [M-R; sections were taken between 30% and 60% embryo length (anterior-posterior axis) at early stage 8 (M,O,Q) and stage 9 (N,P,R)]. Lateral and ventral views are shown as whole mounts at early stage 8 (A,C,E,G,I,K) or late stage 8 (B,D,F,H,J,L). Pbl constructs were expressed in the mesoderm using *twi::Gal4; Dmef::Gal4*. In comparison with the wild type (A,B,M,N), overexpression of $Pbl^{\Delta N\text{-term}}$ results in embryos in which the mesoderm cells remained at the surface (C,D,O,P). Overexpression of $Pbl^{\Delta BRCT}$ (E,F) or Pbl^{DH} (G,H) does not interfere with early mesoderm development. Overexpression of $Pbl^{DH\text{-}PH}$ mainly results in defects during mesoderm spreading (I,J,Q,R). (K,L) The catalytic loss-of-function $Pbl^{DH\text{-}PH\_V531D}$ mutant as a control.

unpublished). Introducing the V531D mutation into either $Pbl^{DH\text{-}PH}$ or $Pbl^{\Delta N\text{-term}}$ abolished the dominant activity (Fig. 3K,L and data not shown). Expression of $Pbl^{\Delta BRCT}$, $Pbl^{\Delta C\text{-term}}$ or of the DH domain alone in the mesoderm of wild-type embryos had no adverse effects on development (Fig. 3E-H). Similarly, expression of the C terminus alone did not have any effect on development (data not shown). In summary, the distinct dominant mis-expression phenotypes of $Pbl^{\Delta Nterm}$ and $Pbl^{DH\text{-}PH}$ support the idea that the C-terminal tail plays an important role in modulating the activity of the tandem DH-PH domain.

The C-terminal tail and the PH domain are important for cortical localization of Pbl

Activation of Rho GTPases is thought to occur by recruiting GEFs to specific subcellular locations. We therefore reasoned that one possible means by which the C-terminal tail might promote Pbl activity would be by controlling its localization. Thus far Pbl has been reported to accumulate at the cleavage furrow during cytokinesis and in the nucleus during interphase (Prokopenko et al., 2000). When endogenous Pbl function was complemented by expression of HA-tagged Pbl-A, we found prominent localization of HA-Pbl to the cytokinesis furrow and the nucleus (Fig. 4G-O). Importantly, HA-Pbl was also associated with the cell cortex and cell protrusions of migrating mesoderm cells (Fig. 4A-F; see Movie 1 in the supplementary material). By contrast, our Pbl antiserum revealed prominent staining of the nuclei, but only very weak staining of cell borders in wild-type embryos, suggesting that the fraction of total Pbl protein at the cell cortex might be low (Fig. 4P,Q). To examine the dynamics of Pbl distribution in vivo, we generated eGFP-tagged Pbl. In mesoderm cells, eGFP-Pbl was present in the nuclei but expression was too low to detect cortical Pbl. However, in migrating haemocytes, levels of eGFP-Pbl were much higher and the protein was localized to the cell periphery and actin-rich microspikes as well as the nucleus (Fig. 4R; see Movie 2 in the supplementary material). Taken together, these data demonstrate that in addition to its prominent nuclear localization, a subpopulation of Pbl localizes to the cell cortex and actin-rich structures.

We next sought to determine the domains that are required for cortical localization of Pbl in mesoderm cells. $Pbl^{\Delta BRCT}$ was localized similarly to wild-type Pbl, whereas the two BRCT domains alone localized to the cytoplasm (Fig. 5A,B) (A.v.I. and H.A.J.M., unpublished). Thus, the BRCT domains appear not to be involved in the association of Pbl with the cell cortex in interphase cells. $Pbl^{\Delta Cterm}$ was present at high levels in the nucleus, but low amounts in the cytoplasm and cell cortex (Fig. 5C,D). The importance of the C-terminal tail for the cortical localization was even more evident in constructs lacking N-terminal PEST and NLS sequences, in which cytoplasmic levels are accumulating. $Pbl^{\Delta Nterm}$ exhibited a strong accumulation at the cell cortex (Fig. 5E,F). Even when the C-terminal tail alone was expressed it was enriched at the cell cortex of mesoderm cells, suggesting that this domain is to some extent sufficient for cortical localization (Fig. 5O,P).

Despite the importance of the C-terminal domain, constructs lacking this domain still exhibit some cortical localization. $Pbl^{DH\text{-}PH}$, which lacks the C-terminal tail, was also localized at the cell periphery in a conspicuous punctate fashion – similar to that described for the tandem DH-PH domain of Ect2 in mammalian cells (Fig. 5G,H) (Solski et al., 2004). This result suggested that the PH domain might contribute to membrane association of Pbl. Indeed, the DH domain alone was localized in the cytoplasm, indicating that the PH domain is required for the punctate cortical localization of $Pbl^{DH\text{-}PH}$ (Fig. 5M,N). Moreover, a Pbl PH-GFP fusion protein was enriched at the cell cortex,

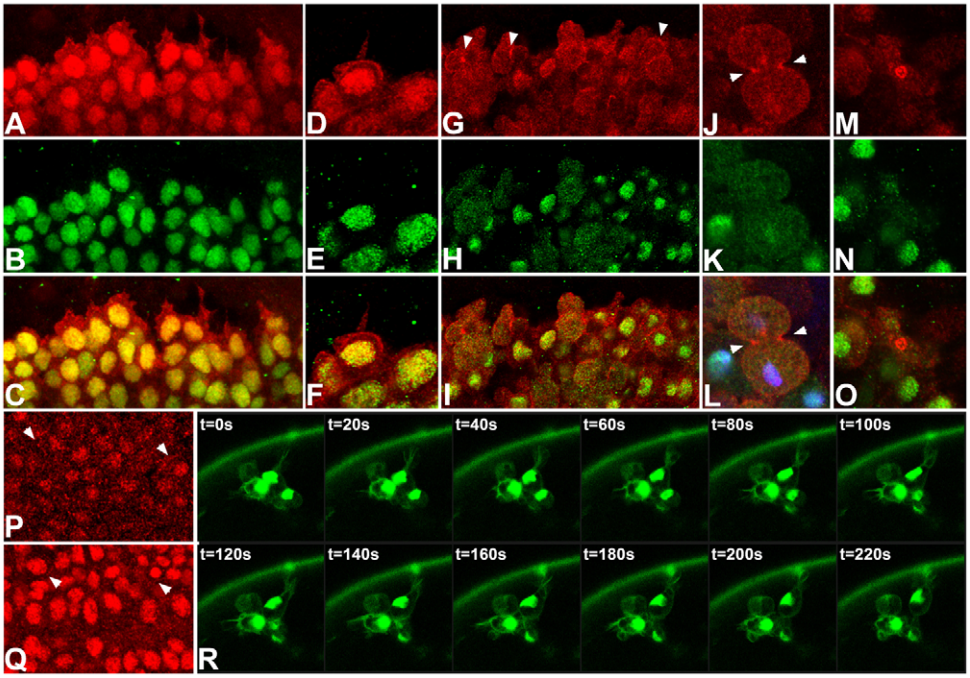

Fig. 4. Cell cortex localization of Pbl in interphase cells. Embryos were fixed and stained with anti-HA (red in A,D,G,J,M) and anti-Twi (green in B,E,H,K,N), anti-Pbl antibodies (red in P,Q) or DAPI (blue in L). Merged images are shown in C,F,I,L,O. (**A-C**) Pbl-HA was overexpressed in the mesoderm using the *twi::Gal4, Dmef::Gal4* driver. The images represent a z-projection of 47 optical sections in 0.16 μm intervals (7.5 μm total). A 3D reconstruction of a similar data set is provided as Movie 1; note strong localization of Pbl-HA to the nucleus and staining in cell protrusions of the leading edge (A,C). Pbl-HA expressed in mesoderm cells of *pbl*^{3} homozygous embryos by *twi::Gal4*. (**D-F**) Cell protrusions at the leading edge are stained. Accumulation of HA staining is seen in dividing cells. (**G-I**) Arrowheads indicate cells in different stages of cytokinesis; note accumulation of staining at cell cortex of dividing mesoderm cells. (**J-O**) High magnifications of Pbl-HA staining to highlight localization to the cleavage furrow of dividing mesoderm cells; arrowheads indicate Pbl-HA accumulation at the cleavage furrow and the central spindle. (**P**,**Q**) Staining of endogenous Pbl protein with anti-Pbl antibodies. Single optical section is depicted in P, note only subtle cortical association (arrowheads) of staining with the antibodies. Projection of *z*-series (56 sections over 16 μm) in Q demonstrates prominent nuclear localization of Pbl and occasional staining of the cell cortex (arrowheads). (**R**) Still images (at 20-second intervals) of a time-lapse sequence of Pbl-GFP in haemocytes during late embryogenesis; note that Pbl-GFP localizes to the cell cortex and actin-rich microspikes in a dynamic fashion (see Movie 2 in the supplementary material).

suggesting that the PH domain was to some extent sufficient to mediate cortical localization (Fig. 5Q,R). In summary, these localization studies indicate that both the C-terminal tail and the PH domain are involved in the localization of Pbl to the cortical cytoplasm.

Genetic interactions of gain-of-function Pbl constructs with Rho1 and Rac1,-2

As Pbl$^{\Delta Cterm}$ can still partially rescue mesoderm defects in *pbl* mutants, cortical localization through the C-terminal tail appears to be important but not essential for the activity of Pbl in cell migration. By contrast, Pbl$^{\Delta Cterm}$ was unable to rescue cytokinesis in *pbl* mutants (Fig. 6G-J). The failure of Pbl$^{\Delta Cterm}$ in rescuing cytokinesis was not due to a requirement for subcellular localization. Pbl$^{\Delta Cterm}$ was localized to the cleavage furrow of dividing cells as in the wild type (Fig. 6A-F). These data indicate that the C-terminal tail is required for the activation of Rho1 during cytokinesis and suggest that the C-terminal domain might play a more direct role in regulating the activity of the DH domain. Thus, the Pbl$^{\Delta Cterm}$ construct uncouples the dual functions of Pbl, in cytokinesis and cell migration, and supports the previous model that Pbl activates a different Rho pathway during mesoderm migration (Schumacher et al., 2004).

We sought to determine the Rho GTPase specificity of Pbl in vivo by testing genetic interactions in the developing eye using *GMR::Gal4*. The dominant activities of Pbl^{DH-PH} and Pbl$^{\Delta Nterm}$ were both dependent on a functional DH domain. Thus, the overexpression phenotypes are most probably consequences of over-activating the respective Rho GTPase pathway downstream of Pbl. As Pbl^{DH-PH} is able to partially rescue the mesoderm defect in *pbl* mutants, it represents an excellent tool with which to identify the substrate of Pbl in cell migration through testing genetic interactions with Rho GTPases. Expression of Pbl^{DH-PH} results in a rough eye phenotype that is characterized by a reduction of the size of the eye and highly abnormal ommatidial structures (Fig. 7A,B). Expression of Pbl$^{DH-PH_V531D}$ did not produce any phenotype, indicating that the Pbl^{DH-PH} rough eye phenotype is a result of overactivation of downstream Rho GTPase pathways (Fig. 7C). Moreover, expression of Pbl^{DH-PH} in a *pbl*^{3} heterozygous background mildly suppressed the rough eye phenotype (Fig. 7D). Therefore, Pbl^{DH-PH} probably acts in the normal Pbl pathway, but is hyperactive. Hence, it should be possible to suppress the eye phenotypes similarly by reducing the expression level of the target GTPases of Pbl.

Pbl^{DH-PH} interacted with Rho1, as a reduction of the *Rho1* gene dose resulted in suppression of the rough eye phenotype (Fig. 7E). This result was expected, as it has been shown before that Pbl can directly bind Rho1 (Prokopenko et al., 1999). Co-expression of dominant versions of *RhoL* or heterozygosity of a loss-of-function mutation in *cdc42* did not modify the rough eye phenotype (Fig. 7F-H). However, in flies heterozygous for a triple mutation in

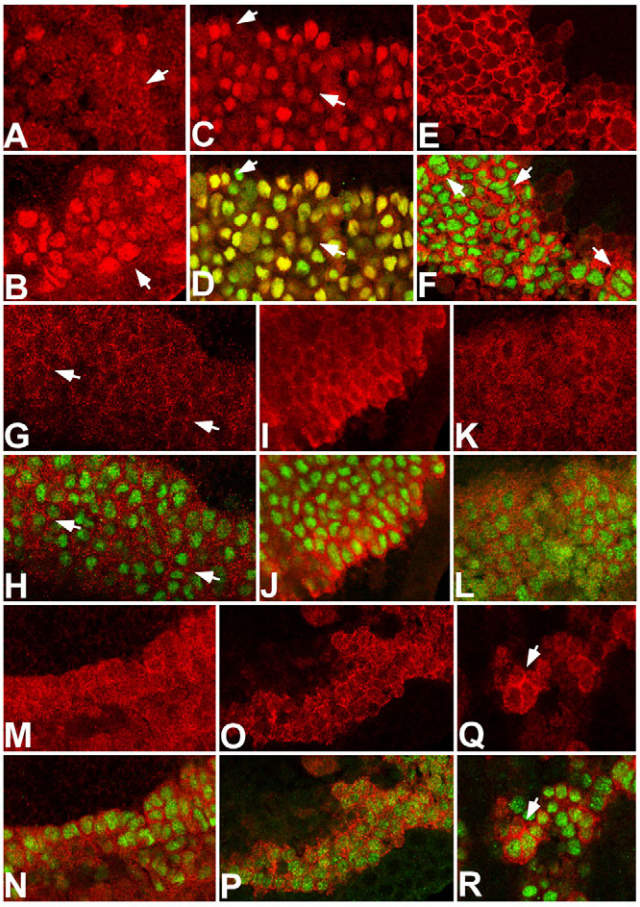

Fig. 5. Localization of Pbl constructs. Tagged Pbl constructs were expressed in mesoderm cells by *twi::Gal4,Dmef::Gal4*. The following antibody staining was used to detect tagged proteins: anti-Myc (red in A,B), anti-HA (red in C-P) and anti-GFP (red in Q,R). Anti-Twi staining (green) marks the mesoderm cells and merged images are shown in D,F,H,J,L,N,P,R. (**A**,**B**) $Pbl^{\Delta BRCT}$ accumulated in the nucleus and low amounts were also detected at the cell cortex (arrows). (**C**,**D**) $Pbl^{\Delta C\text{-term}}$ localizes to the nucleus and the cytoplasm with very low cortical protein localization (marked by arrows). (**E**,**F**) HA-tagged $Pbl^{\Delta N\text{-term}}$ accumulates prominently at the cell cortex; note that this construct also interferes with cytokinesis (arrows indicate multi-nucleated cells). (**G**,**H**) The HA-tagged $Pbl^{DH\text{-}PH}$ is present at the cortex in a punctate fashion (arrows). (**I**,**J**) Expression and localization of $Pbl^{\Delta N\text{-term\_V531D}}$. (**K**,**L**) Expression and localization of $Pbl^{DH\text{-}PH\_V531D}$. (**M**,**N**) Pbl^{DH} localizes to the cytoplasm. (**O**,**P**) $Pbl^{C\text{-term}}$ localizes to the cell cortex. (**Q**,**R**) Localization of $Pbl^{PH\text{-}GFP}$ in mesoderm cells; note accumulation at the cell cortex (arrows).

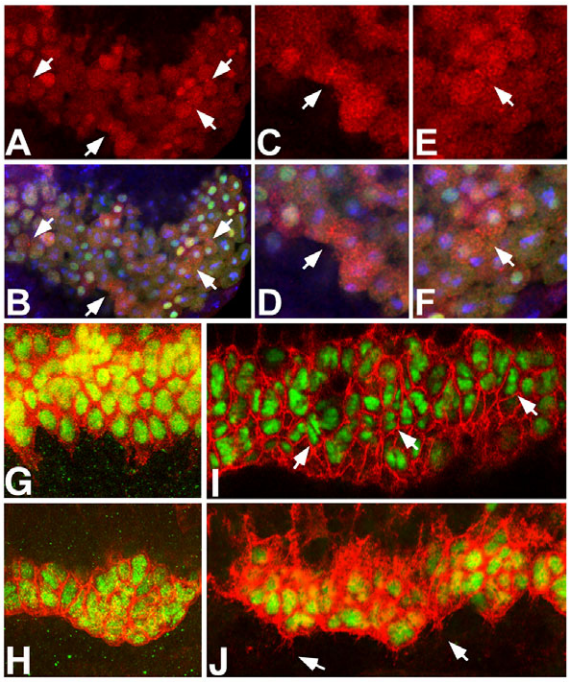

Fig. 6. Differential rescue and localization of $Pbl^{\Delta C\text{-term}}$. $Pbl^{\Delta C\text{-term}}$ was expressed in wild-type (A-F) embryos and stained with anti-HA antibodies (red, A-F) Twi antibodies (green) and DAPI (blue); merged images are shown in B,D,F. (**A-F**) Accumulation of $Pbl^{\Delta C\text{-term}}$ at the cell cortex in dividing cells (marked by arrows in A,B). (C-F) High magnification of $Pbl^{\Delta C\text{-term}}$ localization at the cleavage furrow of dividing cells (arrows). (**G**,**H**) *twi::CD2* (red) and Twi (green) in wild-type (G) and pbl^{3} homozygous (H) embryos; as shown previously cellular protrusions are absent in pbl^{3} mutants (Schumacher et al., 2004). (**I**,**J**) Expression of $Pbl^{\Delta C\text{-term}}$ in pbl^{3} homozygous embryos also expressing *twi::CD2*. (I) Single optical section indicates multinucleated cells (arrows). (J) *z*-projection (12 sections at 0.4 μm; 4.9 μm total) of the same embryo as in I showing the leading edge of migrating mesoderm cells; note cellular protrusions at the leading edge (arrows in J).

Drosophila Rac GTPases ($Rac1^{J10}$, $Rac2^{\Delta}$ and Mtl^{Δ}), the $Pbl^{DH\text{-}PH}$ rough eye phenotype was strongly suppressed (Fig. 7I). Moreover, co-expression of either Rac1 or Rac2 with $Pbl^{DH\text{-}PH}$ strongly enhanced the rough eye phenotype (Fig. 7J; data not shown). These results suggest that overexpression of $Pbl^{DH\text{-}PH}$ in the eye promotes activation of Rac GTPases. We conclude that $Pbl^{DH\text{-}PH}$ behaves as a gain-of-function allele and exhibits genetic interactions consistent with activation of Rho1 and Rac pathways.

Expression of $Pbl^{\Delta Nterm}$ in the embryo affected two Rho1-dependent processes, cytokinesis and invagination, suggesting that this construct might specifically overactivate the Rho1 pathway in the cell. Unfortunately, expression of $Pbl^{\Delta Nterm}$ in the eye results in lethality at pupal stages. However, at a lower temperature (18°C), lethality occurred at the pharate adult stage [0% eclosion (n=43); Fig. 7K]. The lethality is suppressed by removal of one functional copy of *Rho1*, as those flies eclosed and displayed a strong rough eye phenotype [20% eclosion (n=54); Fig. 7L]. No suppression of the $Pbl^{\Delta Nterm}$ lethality was observed in flies heterozygous for $Rac1^{J10}$, $Rac2^{\Delta}$, Mtl^{Δ} [0% eclosion (n=42)]. These results indicate that $Pbl^{\Delta Nterm}$ specifically activates the Rho1 pathway and support the idea that the embryonic phenotype produced by $Pbl^{\Delta Nterm}$ is caused by overactivation of the Rho1 pathway.

The DH domain promotes nucleotide exchange activity for Rho1, Rac1 and Rac2 in vitro

The genetic interactions demonstrated that the tandem DH-PH domain of Pbl activates Rho1 and Rac GTPases. To determine whether Pbl is capable of directly interacting with Rac GTPases, we performed functional guanyl-nucleotide exchange assays using GST fusion proteins of Rho1, Rac1, Rac2, Mtl, RhoL and Cdc42, the DH domain of Pbl, and the first DH domain of Trio as a control. The GTPases were loaded with ^{3}H-GDP and incubated with the respective DH domain or GST as a control in the presence of GTP. The release of ^{3}H-GDP reflects a measure of the exchange activity

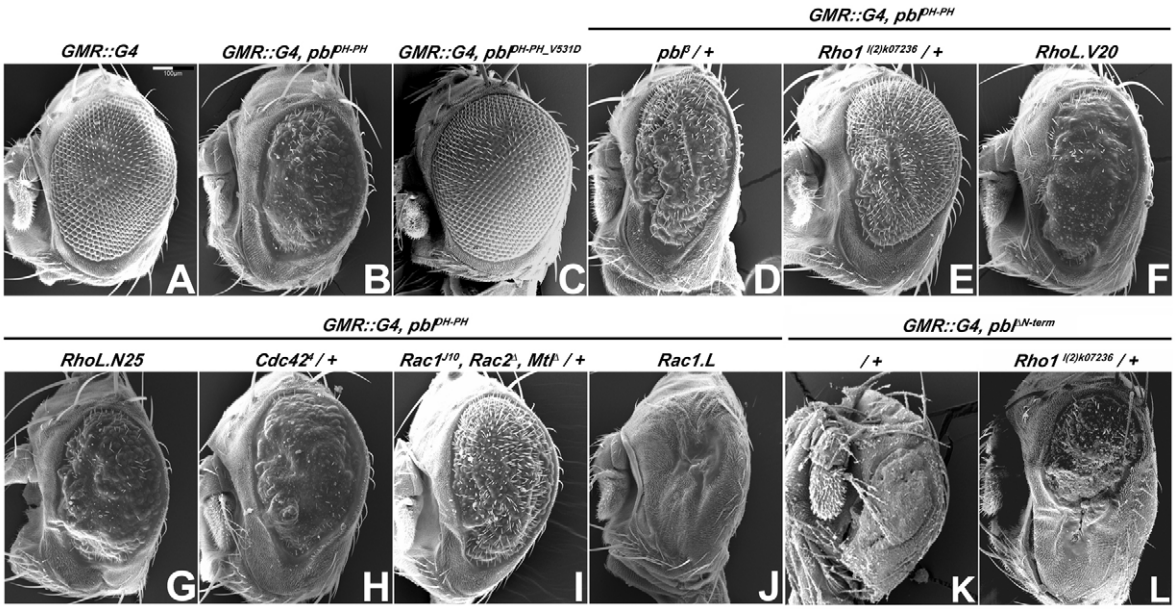

Fig. 7. The tandem DH-PH domain of Pbl interacts with Rho1 and Rac GTPases. (**A**,**B**) Expression of Pbl$^{DH\text{-}PH}$ using the eye-specific *GMR::Gal4* driver (A) leads to a rough eye phenotype (B). (**C**) This phenotype depends on the catalytic activity of the DH domain, as Pbl$^{DH\text{-}PH_V531D}$ does not promote a rough eye phenotype. (**D**) The phenotype is partially suppressed in *pbl^{3}* heterozygotes. (**E**) The Pbl$^{DH\text{-}PH}$ rough eye phenotype is suppressed in flies heterozygous for a loss-of-function mutation in *rho1*. (**F-H**) Co-expression of dominant versions of RhoL (dominant active RhoL^{V20} and dominant negative RhoL^{N25}) (F,G) or lowering the dose of *cdc42* (H) has no impact on the phenotype. (**I**) Reducing the gene doses of all three *Drosophila* Rac GTPases, *Rac1*, *Rac2* and *Mtl*, suppresses the eye defects caused by Pbl$^{DH\text{-}PH}$ expression. (**J**) Co-expression of wild-type Rac1 leads to an enhancement of the phenotype; most flies die as pharate adults and a few escapers hatched and failed to develop any eye structures. (**K**) Expression of Pbl$^{\Delta N\text{-}term}$ at 18°C leads to pharate adult lethality; animals dissected out of their pupal cases exhibit a strong rough eye phenotype. (**L**) The lethality caused by expression of Pbl$^{\Delta N\text{-}term}$ is rescued in a *Rho1* heterozygous background and the adult flies exhibited a strong rough eye phenotype.

of a specific DH domain towards a given GTPase. The first DH domain of Trio, an exchange factor for Rac GTPases, exhibited a strong preference for Rac1, Rac2 and Mtl, whereas Trio did not promote nucleotide exchange for Rho1 or Cdc42 and showed a weak activity for RhoL (Fig. 8). GST-Pbl^{DH} promotes GDP exchange from Rho1, consistent with our genetic data and previously reported binding studies (Prokopenko et al., 1999). Strikingly, we also detected an activity of GST-Pbl^{DH} for Rac1 and Rac2 (Fig. 8). The fact that the activity for Rac1 and Rac2 was weaker than for Rho1 might reflect a requirement of the PH domain in promoting full activity or specificity of the DH domain of Pbl. The insolubility of the bacterial GST-Pbl$^{DH\text{-}PH}$ fusion protein prohibited us from directly testing this possibility. Together, these data indicate that the DH domain of Pbl is able to use Rac1 or Rac2 as a substrate and in conjunction with the genetic interactions suggest that Pbl promotes exchange activity towards multiple substrates, including Rac GTPases.

Regulation of Rac GTPases is essential for mesoderm spreading

The genetic and biochemical data are consistent with the model that Pbl functions through activation of the Rac pathway to promote mesoderm spreading. As the compound eye represents a heterologous system, we first wanted to investigate whether the genetic interactions between Pbl and Rho1 and Rac also occurred in the embryonic mesoderm. We therefore tested whether Rho1 or Rac1 variants are able to enhance the moderate phenotype produced by the weak loss-of-function allele *pbl^{11D}*. Expression of a dominant-negative construct (*Rac1^{N17}*) enhanced the mesoderm phenotype of *pbl^{11D}* (Table 2). Overexpression of constitutively active *Rac1^{V12}*, but not *Rho1^{V14}* enhanced the mesoderm phenotype of *pbl^{11D}* mutant embryos, consistent with an adverse effect upon over-activation of the Rac pathway (Table 2).

In a second set of experiments, we asked whether Rac1 was able to enhance rescue activity of Pbl$^{\Delta BRCT}$. Overexpression of Pbl$^{\Delta BRCT}$ provides enough activity to suppress the *pbl^{3}* mesoderm phenotype substantially without producing a dominant phenotype, suggesting that this construct is present in the cells at near physiological levels (Fig. 2J; Fig. 3E,F; Table 1). Co-expression of wild-type Rac1 together with Pbl$^{\Delta BRCT}$ leads to a significant enhancement of the rescue of *pbl* mutants by Pbl$^{\Delta BRCT}$ (Table 3). When wild-type Rho1 is co-expressed with Pbl$^{\Delta BRCT}$, there was no change in the strength of the rescue of the *pbl* phenotype by Pbl$^{\Delta BRCT}$ (Table 4). This experiment indicates that Rac1 interacts with Pbl$^{\Delta BRCT}$ and can

Table 2. Genetic interaction of Rac1 with *pbl* in the embryo

Genotype	Eve-positive hemisegments	s.d.	*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

Mean values and standard deviations (s.d.) of the number of Eve-positive hemisegments are shown for *pbl^{11D}* homozygous embryos and *pbl^{11D}* homozygotes expressing Rac^{V12}, Rac^{N17} or Rho1^{V14} in the mesoderm using *twi::Gal4* (*n*=number of embryos examined). The number of Eve-positive hemisegments between *pbl^{11D}* mutant and *pbl^{11D}* mutant embryo expressing either of the Rac1 mutant forms was significantly different (Student's *t*-test; *P*=3.46783E-11 for Rac1^{V12} and *P*=4.83543E-06 for Rac1^{N17}).

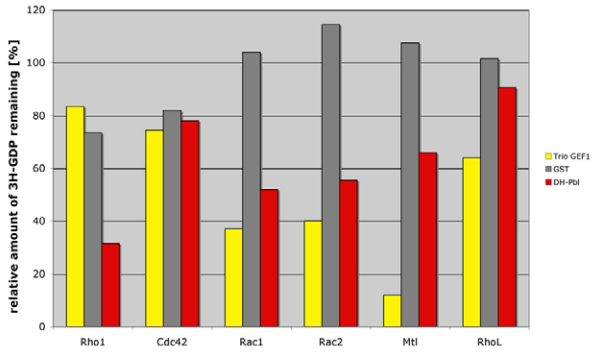

Fig. 8. Exchange activity of Pbl^{DH} in vitro. Results of GEF assays are plotted as relative amounts of ^{3}H-GDP bound to indicated GST-Rho GTPase fusion proteins after a 20-minute incubation at 25°C with GST-DH fusion proteins of Pbl (red) or Trio (yellow) and unlabelled GTP. GST (grey) was used as a control. Note activity of the Pbl DH domain towards GDP exchange for Rho1, Rac1 and Rac2.

promote its ability to rescue the *pbl*^{3} migration defect. Together, the genetic interactions strongly support a role of Pbl to activate the Rac pathway in mesoderm spreading.

We next asked whether mesoderm spreading depends on Rac GTPases and analyzed maternal-zygotic mutants lacking *Rac1* and *Rac2* with reduced maternal *Mtl* expression (Hakeda-Suzuki et al., 2002; Ng et al., 2002). In *Rac1 Rac2 Mtl* mutant embryos, the mesoderm never migrated dorsally, as assessed by Twi staining (Fig. 9A,B). The phenotype is similar to the mesoderm spreading defects seen in embryos lacking both FGF ligands *FGF8-like1* and *FGF8-like2* (Fig. 9C) (Gryzik and Müller, 2004). These results extend previous findings that embryos with reduced maternal expression of Rac GTPases fail to initiate mesodermal-ectodermal contact after invagination (Wilson et al., 2005). Moreover, when expressed in the mesoderm of wild-type embryos, *Rac1*^{V12} affects mesoderm spreading (Fig. 9D-H). These data indicate that tight spatiotemporal regulation of the Rac pathway plays an important role in mesoderm migration.

DISCUSSION

The Rho GEF Pbl provides one of the few molecular links between the proximal FGF receptor signalling events and the regulation of cell shape changes. We have previously characterized the loss-of-function phenotype of *pbl* mutants, showing that Pbl acts in a pathway downstream or in parallel to Htl-dependent MAP kinase activation (Schumacher et al., 2004). Here, we used genetics and biochemistry to determine the regulation of Pbl and its downstream Rho GTPase pathways in migrating cells. Our data demonstrate that Pbl partially localizes to the cell cortex of mesoderm cells and functionally interacts with Rac GTPases in this process.

We show that the tandem DH-PH domain of Pbl is essential for cell migration and employs not only Rho1, but also the Rac pathway. Several lines of evidence strongly suggest that Pbl acts through Rac GTPases during mesoderm migration. The dominant rough eye phenotype induced by Pbl^{DH-PH} is sensitive to gene doses of Rac GTPases. Expression of constitutively active or dominant-negative Rac1 but not Rho1 enhances the mesoderm phenotype in the hypomorphic *pbl*^{11D} allele. Moreover, co-expression of Rac1, but not of Rho1, promotes the suppression of mesoderm migration defects by Pbl$^{\Delta BRCT}$ in *pbl*-null mutants. In addition, we provide biochemical data that strongly suggest the Rac pathway as a direct target of Pbl.

Pbl has previously been reported to localize to the nucleus in interphase cells. Nuclear localization was interpreted as a means of storing the protein until rapid release at mitosis (O'Keefe et al., 2001). In cultured cells and *C. elegans* zygotes, homologues of Pbl localize at the cell cortex, e.g. cell junctions or the anterior cortex in the nematode zygote (Liu et al., 2004; Jenkins et al., 2006). We detected functional Pbl-HA in the nucleus and the cytoplasm, including membrane protrusions. These data are consistent with the model that Pbl activates Rac GTPases at the cell cortex during cell migration.

Our study identified two domains, the conserved C-terminal tail and the PH domain, as candidates to mediate the association of Pbl with the cell cortex in interphase cells. The use of N-terminally deleted constructs facilitated these studies, because the respective proteins were excluded from the nucleus as they lack the NLS. Either domain alone is sufficient to localize to the cell cortex, and deletion studies suggest that both domains are crucial for cortical localization. We propose that the PH domain and the C-terminal tail might cooperate in localizing Pbl to the cell cortex. DH domain associated PH domains are essential for GEF function and are known to promote binding to specific membrane subdomains enriched in phosphoinositides (Lemmon, 2008). An attractive model therefore is that the PH domain provides specificity by targeting Pbl to membrane domains enriched for particular phospholipids, whereas the C-terminal tail functions in anchoring Pbl to the cell cortex. In addition, binding to phospholipids might promote the specific exchange activity of the tandem DH-PH domain, as described for other Dbl family GEFs (Snyder et al., 2001; Rossman et al., 2003).

It is difficult to address the issue of whether cortical localization is important for the function of Pbl in mesoderm migration. The reduced rescuing capability of Pbl$^{\Delta C\text{-term}}$ is consistent with a correlation of cortical localization through the C-terminal domain

Table 3. Rac1 promotes rescue of *pbl* loss of function mutant in a Pbl$^{\Delta BRCT}$ overexpression background

Genotype	Eve-positive hemisegments	s.d.	*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

Mean values and standard deviations (s.d.) of the number of Eve-positive hemisegments are shown for *pbl*^{3} homozygous embryos and *pbl*^{3} homozygotes expressing wild-type Rac1 protein in the mesoderm using *twi::Gal4*; (*n*=number of embryos examined). The number of Eve-positive hemisegments between *pbl*^{3} mutant expressing Pbl$^{\Delta BRCT}$ and *pbl*^{3} mutant embryo expressing Pbl$^{\Delta BRCT}$ and Rac1 was significantly different (Student's *t*-test; *P*=1.55073E-08).

Table 4. Rho1 does not promote rescue of *pbl* loss-of-function mutant in a Pbl$^{\Delta BRCT}$ overexpression background

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

Mean values and standard deviations (s.d.) of the number of Eve-positive hemisegments are shown for *pbl*^{3} homozygous embryos and *pbl*^{3} homozygotes expressing wild-type Rho1 protein in the mesoderm using *twi::Gal4* (*n*=number of embryos examined). The crosses employed for this experiment were different from the experiment described in Table 3 and resulted in slightly better rescue of the *pbl*^{3} phenotype by Pbl$^{\Delta BRCT}$ (see Materials and methods). The number of Eve-positive hemisegments between *pbl*^{3} homozygotes expressing Pbl$^{\Delta BRCT}$ and *pbl*^{3} homozygotes expressing Pbl$^{\Delta BRCT}$ and Rho1 was largely unimpaired.

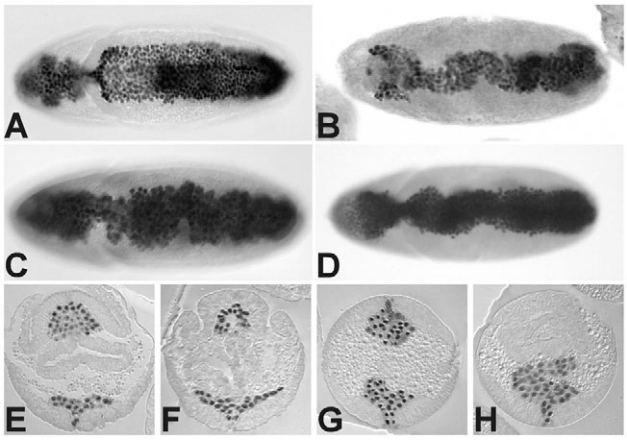

Fig. 9. Regulation of Rac GTPases is essential for mesoderm migration. (**A-D**) Ventral views of embryos (during stage 8, germ band extension) stained against Twi. Unlike wild type (A), embryos lacking the maternal and zygotic contribution of Rac1 and Rac2 show strong defects during mesoderm migration; the cells fail to spread out laterally and form a tight aggregate (B). These defects are reminiscent of the phenotype found after loss of both the Htl ligands *FGF8-like1* and *FGF8-like2* (C). A similar, although slightly weaker, phenotype can be observed after expression of constitutive active Rac1, $Rac1^{V12}$, in the mesoderm (D). (**E-H**) Cross-sections of early (G) and late stage 8 (H) embryos expressing $Rac1^{V12}$ show strongly abnormal mesoderm spreading compared with wild-type embryos (E,F, stage 8 early and late, respectively).

and the function of Pbl in cell migration. A more stringent experiment would involve the generation of a construct that lacks the PH and C-terminal domains for membrane association. However, as PH domains are essential for DH domain function in vivo, deletion of the PH domain will abolish activity in any case, as we have shown for the constitutively active DH-PH construct. Such an analysis would require a way to uncouple the activities of the PH domain that promote the exchange activity and membrane-phospholipid binding. It will therefore remain important to determine whether the function of the PH domain involves its interaction with lipid substrates or directly promotes the activity of the DH domain in migrating cells.

The inhibition of invagination and cytokinesis by $Pbl^{\Delta Nterm}$ is probably caused by disruption of the local activation of Rho1 at the cell cortex. During invagination and cytokinesis, the Rho1 pathway is activated locally: in the apical domain of the mesoderm cells to trigger apical constriction or at the cell equator of the dividing cell to promote assembly of the contractile ring. As $Pbl^{\Delta Nterm}$ strongly accumulates at the cortex in a non-polarized fashion, it might activate Rho1 ectopically throughout the cell cortex and thereby overriding any polarizing cues for local activation.

The dramatic differences in the overexpression phenotypes of Pbl^{DH-PH} or $Pbl^{\Delta Nterm}$ suggest an important function of the C-terminal tail in controlling the biochemical activities of the tandem DH-PH domain. Strikingly, $Pbl^{\Delta Nterm}$ genetically interacts with Rho1, but not with Rac GTPases, supporting the idea that the C-terminus promotes the exchange activity towards Rho1. We propose that in the mesoderm cells this activity of the C-terminal domain is antagonized to activate the Rac rather than to the Rho1 pathway. In the presence of the NLS and PEST motifs, the cytoplasmic levels of Pbl are low and allow for this regulation to occur, whereas the oncogenic forms lacking these motifs are present in the cytoplasm at high levels and might escape regulation. Thus, constructs that lack the C-terminal tail promote interaction with Rac and rescue Rac-dependent mesoderm migration. This model is also supported by the observation that the C-terminal domain is essential for Rho1 activation, but not for Pbl localization in dividing cells. The same construct, $Pbl^{\Delta C-term}$, is still able to rescue Rac-dependent migration defects. Thus, deletion of the C-terminal tail uncouples activation of Rho1- from Rac-dependent processes and suggests that in the absence of the negative interaction with the C-terminal tail, the tandem DH-PH domain promotes activation of Rac.

Although many receptor tyrosine kinases signal through Rho GTPases, only few FGF receptors have been reported to regulate Rho GEFs (Schiller, 2006). One attractive model is that FGF signalling mediates post-translational modification of the C-terminal tail to trigger the switch in the differential interaction with Rho1 and Rac GTPases. The sequence of the C-terminal tail contains several conserved putative phosphorylation sites that might represent targets for FGF signalling. Interestingly, the exchange factor specificity of oncogenic *ect2* for GTPase substrates depends on the C-terminal tail of the protein (Solski et al., 2004). Identification of proteins that interact with the C-terminal domain might shed light on its role in controlling selectivity for distinct GTPase pathways. Such studies will be important to advance our understanding of the mechanism of the transforming potential of Pbl, as well as its mechanism of action in cell polarity and cell migration.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/5/813/DC1

References

Barrett, K., Leptin, M. and Settleman, J. (1997). The Rho GTPase and a putative RhoGEF mediate a signaling pathway for the cell shape changes in Drosophila gastrulation. *Cell* **91**, 905-915.

Beiman, M., Shilo, B. Z. and Volk, T. (1996). Heartless, a Drosophila FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. *Genes Dev.* **10**, 2993-3002.

Carmena, A., Gisselbrecht, S., Harrison, J., Jimenez, F. and Michelson, A. M. (1998). Combinatorial signaling codes for the progressive determination of cell fates in the Drosophila embryonic mesoderm. *Genes Dev.* **12**, 3910-3922.

Costa, M., Sweeton, D. and Wieschaus, E. (1993). *Gastrulation in Drosophila: Cellular Mechanisms of Morphogenetic Movements*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Costa, M., Wilson, E. T. and Wieschaus, E. (1994). A putative cell signal encoded by the folded gastrulation gene coordinates cell shape changes during Drosophila gastrulation. *Cell* **76**, 1075-1089.

Dawes-Hoang, R. E., Parmar, K. M., Christiansen, A. E., Phelps, C. B., Brand, A. H. and Wieschaus, E. F. (2005). folded gastrulation, cell shape change and the control of myosin localization. *Development* **132**, 4165-4178.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the even-skipped protein of Drosophila. *EMBO J.* **6**, 749-759.

Furlong, E. E. (2004). Integrating transcriptional and signalling networks during muscle development. *Curr. Opin. Genet. Dev.* **14**, 343-350.

Gisselbrecht, S., Skeath, J. B., Doe, C. Q. and Michelson, A. M. (1996). heartless encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the Drosophila embryo. *Genes Dev.* **10**, 3003-3017.

Grosshans, J., Wenzl, C., Herz, H. M., Bartoszewski, S., Schnorrer, F., Vogt, N., Schwarz, H. and Muller, H. A. (2005). RhoGEF2 and the formin Dia control

the formation of the furrow canal by directed actin assembly during Drosophila cellularisation. *Development* **132**, 1009-1020.
Gryzik, T. and Müller, H. A. (2004). FGF8-like1 and FGF8-like2 encode putative ligands of the FGF receptor Htl and are required for mesoderm migration in the Drosophila gastrula. *Curr. Biol.* **14**, 659-667.
Hacker, U. and Perrimon, N. (1998). DRhoGEF2 encodes a member of the Dbl family of oncogenes and controls cell shape changes during gastrulation in Drosophila. *Genes Dev.* **12**, 274-284.
Hakeda-Suzuki, S., Ng, J., Tzu, J., Dietzl, G., Sun, Y., Harms, M., Nardine, T., Luo, L. and Dickson, B. J. (2002). Rac function and regulation during Drosophila development. *Nature* **416**, 438-442.
Imam, F., Sutherland, D., Huang, W. and Krasnow, M. A. (1999). stumps, a Drosophila gene required for fibroblast growth factor (FGF)- directed migrations of tracheal and mesodermal cells. *Genetics* **152**, 307-318.
Jagla, K., Bellard, M. and Frasch, M. (2001). A cluster of Drosophila homeobox genes involved in mesoderm differentiation programs. *BioEssays* **23**, 125-133.
Jenkins, N., Saam, J. R. and Mango, S. E. (2006). CYK-4/GAP provides a localized cue to initiate anteroposterior polarity upon fertilization. *Science* **313**, 1298-1301.
Kolsch, V., Seher, T., Fernandez-Ballester, G. J., Serrano, L. and Leptin, M. (2007). Control of Drosophila gastrulation by apical localization of adherens junctions and RhoGEF2. *Science* **315**, 384-386.
Lemmon, M. A. (2008). Membrane recognition by phospholipid-binding domains. *Nat. Rev. Mol. Cell Biol.* **9**, 99-111.
Leptin, M. (1999). Gastrulation in Drosophila: the logic and the cellular mechanisms. *EMBO J.* **18**, 3187-3192.
Leptin, M. and Grunewald, B. (1990). Cell shape changes during gastrulation in Drosophila. *Development* **110**, 73-84.
Leptin, M. and Roth, S. (1994). Autonomy and non-autonomy in Drosophila mesoderm determination and morphogenesis. *Development* **120**, 853-859.
Liu, X., Wang, H., Eberstadt, M., Schnuchel, A., Olejniczak, E. T., Meadows, R. P., Schkeryantz, J. M., Janowick, D. A., Harlan, J. E., Harris, E. A. et al. (1998). NMR structure and mutagenesis of the N-terminal Dbl homology domain of the nucleotide exchange factor Trio. *Cell* **95**, 269-277.
Liu, X. F., Ishida, H., Raziuddin, R. and Miki, T. (2004). Nucleotide exchange factor ECT2 interacts with the polarity protein complex Par6/Par3/protein kinase Czeta (PKCzeta) and regulates PKCzeta activity. *Mol. Cell. Biol.* **24**, 6665-6675.
Meyer, W. J., Schreiber, S., Guo, Y., Volkmann, T., Welte, M. A. and Müller, H. A. (2006). Overlapping functions of argonaute proteins in patterning and morphogenesis of Drosophila embryos. *PLoS Genet.* 2, e134.
Michelson, A. M., Gisselbrecht, S., Buff, E. and Skeath, J. B. (1998a). Heartbroken is a specific downstream mediator of FGF receptor signalling in Drosophila. *Development* **125**, 4379-4389.
Michelson, A. M., Gisselbrecht, S., Zhou, Y., Baek, K. H. and Buff, E. M. (1998b). Dual functions of the heartless fibroblast growth factor receptor in development of the Drosophila embryonic mesoderm. *Dev. Genet.* **22**, 212-229.
Müller, H. A. (2008). Immunolabeling of embryos. *Methods Mol. Biol.* **420**, 207-218.
Ng, J., Nardine, T., Harms, M., Tzu, J., Goldstein, A., Sun, Y., Dietzl, G., Dickson, B. J. and Luo, L. (2002). Rac GTPases control axon growth, guidance and branching. *Nature* **416**, 442-447.
Nikolaidou, K. K. and Barrett, K. (2004). A Rho GTPase signaling pathway is used reiteratively in epithelial folding and potentially selects the outcome of Rho activation. *Curr. Biol.* **14**, 1822-1826.
O'Keefe, L., Somers, W. G., Harley, A. and Saint, R. (2001). The pebble GTP exchange factor and the control of cytokinesis. *Cell Struct. Funct.* **26**, 619-626.
Piekny, A., Werner, M. and Glotzer, M. (2005). Cytokinesis: welcome to the Rho zone. *Trends Cell Biol.* **15**, 651-658.
Prokopenko, S. N., Brumby, A., O'Keefe, L., Prior, L., He, Y., Saint, R. and Bellen, H. J. (1999). A putative exchange factor for Rho1 GTPase is required for initiation of cytokinesis in Drosophila. *Genes Dev.* **13**, 2301-2314.
Prokopenko, S. N., Saint, R. and Bellen, H. J. (2000). Tissue distribution of PEBBLE RNA and pebble protein during Drosophila embryonic development. *Mech. Dev.* **90**, 269-273.
Rossman, K. L., Cheng, L., Mahon, G. M., Rojas, R. J., Snyder, J. T., Whitehead, I. P. and Sondek, J. (2003). Multifunctional roles for the PH domain of Dbs in regulating Rho GTPase activation. *J. Biol. Chem.* **278**, 18393-18400.
Rossman, K. L., Der, C. J. and Sondek, J. (2005). GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol.* **6**, 167-180.
Saito, S., Liu, X. F., Kamijo, K., Raziuddin, R., Tatsumoto, T., Okamoto, I., Chen, X., Lee, C. C., Lorenzi, M. V., Ohara, N. et al. (2004). Deregulation and mislocalization of the cytokinesis regulator ECT2 activate the Rho signaling pathways leading to malignant transformation. *J. Biol. Chem.* **279**, 7169-7179.
Schiller, M. R. (2006). Coupling receptor tyrosine kinases to Rho GTPases-GEFs what's the link. *Cell Signal.* **18**, 1834-1843.
Schumacher, S., Gryzik, T., Tannebaum, S. and Müller, H. A. (2004). The RhoGEF Pebble is required for cell shape changes during cell migration triggered by the Drosophila FGF receptor Heartless. *Development* **131**, 2631-2640.
Seher, T. C., Narasimha, M., Vogelsang, E. and Leptin, M. (2007). Analysis and reconstitution of the genetic cascade controlling early mesoderm morphogenesis in the Drosophila embryo. *Mech. Dev.* **124**, 167-179.
Shishido, E., Higashijima, S., Emori, Y. and Saigo, K. (1993). Two FGF-receptor homologues of Drosophila: one is expressed in mesodermal primordium in early embryos. *Development* **117**, 751-761.
Shishido, E., Ono, N., Kojima, T. and Saigo, K. (1997). Requirements of DFR1/Heartless, a mesoderm-specific Drosophila FGF-receptor, for the formation of heart, visceral and somatic muscles, and ensheathing of longitudinal axon tracts in CNS. *Development* **124**, 2119-2128.
Smallhorn, M., Murray, M. J. and Saint, R. (2004). The epithelial-mesenchymal transition of the Drosophila mesoderm requires the Rho GTP exchange factor Pebble. *Development* **131**, 2641-2651.
Snyder, J. T., Rossman, K. L., Baumeister, M. A., Pruitt, W. M., Siderovski, D. P., Der, C. J., Lemmon, M. A. and Sondek, J. (2001). Quantitative analysis of the effect of phosphoinositide interactions on the function of Dbl family proteins. *J. Biol. Chem.* **276**, 45868-45875.
Solski, P. A., Wilder, R. S., Rossman, K. L., Sondek, J., Cox, A. D., Campbell, S. L. and Der, C. J. (2004). Requirement for C-terminal sequences in regulation of Ect2 guanine nucleotide exchange specificity and transformation. *J. Biol. Chem.* **279**, 25226-25233.
Somers, W. G. and Saint, R. (2003). A RhoGEF and Rho family GTPase-activating protein complex links the contractile ring to cortical microtubules at the onset of cytokinesis. *Dev. Cell* **4**, 29-39.
Stathopoulos, A. and Levine, M. (2004). Whole-genome analysis of Drosophila gastrulation. *Curr. Opin. Genet. Dev.* **14**, 477-484.
Stathopoulos, A., Tam, B., Ronshaugen, M., Frasch, M. and Levine, M. (2004). pyramus and thisbe: FGF genes that pattern the mesoderm of Drosophila embryos. *Genes Dev.* **18**, 687-699.
Sweeton, D., Parks, S., Costa, M. and Wieschaus, E. (1991). Gastrulation in Drosophila: the formation of the ventral furrow and posterior midgut invaginations. *Development* **112**, 775-789.
Vincent, S., Wilson, R., Coelho, C., Affolter, M. and Leptin, M. (1998). The Drosophila protein Dof is specifically required for FGF signaling. *Mol. Cell* **2**, 515-525.
Whitehead, I. P., Campbell, S., Rossman, K. L. and Der, C. J. (1997). Dbl family proteins. *Biochim. Biophys. Acta* **1332**, F1-F23.
Wilson, R., Vogelsang, E. and Leptin, M. (2005). FGF signalling and the mechanism of mesoderm spreading in Drosophila embryos. *Development* **132**, 491-501.