The RhoGEF Pebble is required for cell shape changes during cell migration triggered by the *Drosophila* FGF receptor Heartless

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Summary

The FGF receptor Heartless (HTL) is required for mesodermal cell migration in the *Drosophila* gastrula. We show that mesoderm cells undergo different phases of specific cell shape changes during mesoderm migration. During the migratory phase, the cells adhere to the basal surface of the ectoderm and exhibit extensive protrusive activity. HTL is required for the protrusive activity of the mesoderm cells. Moreover, the early phenotype of *htl* mutants suggests that HTL is required for the adhesion of mesoderm cells to the ectoderm.

In a genetic screen we identified *pebble* (*pbl*) as a novel gene required for mesoderm migration. *pbl* encodes a guanyl nucleotide exchange factor (GEF) for RHO1 and is known as an essential regulator of cytokinesis. We show that the function of PBL in cell migration is independent of the function of PBL in cytokinesis. Although RHO1 acts as a substrate for PBL in cytokinesis, compromising RHO1 function in the mesoderm does not block cell migration. These data suggest that the function of PBL in cell migration might be mediated through a pathway distinct from RHO1. This idea is supported by allele-specific differences in the expressivity of the cytokinesis and cell migration phenotypes of different *pbl* mutants. We show that PBL is autonomously required in the mesoderm for cell migration. Like HTL, PBL is required for early cell shape changes during mesoderm migration. Expression of a constitutively active form of HTL is unable to rescue the early cellular defects in *pbl* mutants, suggesting that PBL is required for the ability of HTL to trigger these cell shape changes. These results provide evidence for a novel function of the Rho-GEF PBL in HTL-dependent mesodermal cell migration.

Supplemental data available online

Key words: *Drosophila*, Cell migration, Gastrulation, FGF-receptor, RhoGEF

Introduction

The spatiotemporal pattern of cell migration in the embryo is crucial for cells to receive and send out signals essential for correct specification and differentiation of tissues. The ability of cells to become motile and to migrate into certain target territories of the organism must be appropriately controlled in order to prevent misplacement of cells (Forbes and Lehmann, 1999; Lauffenburger and Horwitz, 1996; Montell, 1999; Winklbauer and Keller, 1996). Cell migration in the developing organism is likely to be regulated at multiple levels and involves the integration of signal recognition, signal transduction, cell substratum interactions and the cytoskeleton. Fibroblast-growth-factor receptors (FGFR) have been implicated in early steps of directional cell migration in development (Szebenyi and Fallon, 1999). One function of FGFRs in vivo is to recognize chemotactic gradients of FGF that direct cell migration in the embryo (Sato and Kornberg, 2002; Sutherland et al., 1996; Yang et al., 2002).

How are signals from FGFR activation transduced to the cytoskeletal and adhesive systems of the cell to elicit the migratory behavior of the cell? One of the best-characterized FGFR signaling pathways involves the Ras GTPase, which among other targets promotes activation of MAP kinase (Campbell et al., 1998). MAP kinase phosphorylates a variety of protein targets including transcription factors, other protein kinases, phospholipases or cytoskeletal proteins. For example, MAP kinase promotes phosphorylation of myosin light chain (MLC) kinase, a regulator of the contractile actin-myosin system (Klemke et al., 1997). Although many downstream components of FGFR signaling pathways have been identified, the targets that regulate cell migration are not well understood.

Migratory cells form a variety of characteristic cellular protrusions, most prominently filopodia and lamellipodia (Lauffenburger and Horwitz, 1996). The formation of these protrusions in response to extracellular stimuli is controlled by small GTPases of the Rho family: Rho, Rac and CDC42 (Hall, 1998). Mammalian cell culture systems have provided ample evidence that Rho GTPases represent the key molecules in transducing extracellular signals to the actin cytoskeleton (Schmidt and Hall, 1998). Guanine nucleotide exchange factors (GEFs) act upstream of Rho GTPases and promote their local activation in the cell. Although FGFRs might be required and sufficient for the formation of filopodial protrusions, as shown for tracheal cell migration in *Drosophila* (Ribeiro et al., 2002), the molecules that connect FGFR signaling pathways to

the regulators of the cytoskeleton during morphogenesis are yet to be defined.

Mesoderm migration in the early *Drosophila* embryo provides a good model system to study FGFR-dependent cell migration (Wilson and Leptin, 2000). Migration of the mesoderm is an important precondition for the regional specification of different mesodermal derivatives. The most dorsal mesodermal fates are controlled by combinatorial action of multiple signaling pathways and intrinsic factors. These inputs govern the formation of a subset of dorsal mesodermal derivatives, marked by the expression of *even skipped* (*eve*) (Carmena et al., 1998; Halfon et al., 2000; Knirr and Frasch, 2001). In mutants in which mesoderm migration is affected, e.g. in embryos mutant for the FGFR homolog Heartless (HTL), mesoderm cells are unable to receive appropriate signals. As a consequence, the dorsal *eve*-expressing mesoderm cells are not specified in *htl* mutant embryos (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997).

Although the ligand of HTL is yet unknown, the synthesis of proteoglycans is required for HTL activation (Lin et al., 1999). The signal transduction pathway downstream of HTL involves the product of the *downstream of FGF* (*dof*; *stumps* – FlyBase) gene (Imam et al., 1999; Michelson et al., 1998a; Vincent et al., 1998). Genetic experiments suggest that DOF functions as adaptor protein and operates downstream of the receptor and upstream of signals triggered by RAS1. Furthermore, activation of MAP kinase was observed in a subgroup of migrating mesoderm cells and was shown to depend on HTL and DOF function (Gabay et al., 1997; Vincent et al., 1998).

We show that HTL is necessary for protrusive activity of migrating mesoderm cells. The early attachment of mesoderm cells to the ectoderm is affected in *htl* mutants, suggesting that HTL is required for the adhesion of mesoderm cells to the ectoderm. Our data provide evidence that HTL exerts a permissive function in mesoderm migration. In a genetic screen, we identified the Rho GEF Pebble (PBL) as an essential molecular component of mesoderm migration. Like HTL, PBL is required for specific cell shape changes and protrusive activity of the mesoderm cells during migration. The function of PBL in cell migration is independent of the well-known function of PBL in cytokinesis (Hime and Saint, 1992; Lehner, 1992; Prokopenko et al., 1999). Overexpression of HTL or a constitutively active form of HTL using the *twist* (*twi*) promoter, are sufficient to trigger late cell shape changes in the mesoderm, but are unable to initiate early cell shape changes in *pbl* mutants. These results indicate a novel function of PBL in HTL triggered cell migration in the *Drosophila* gastrula.

Material and methods

Drosophila stocks

The following stocks were used and obtained from the Bloomington stock Center unless otherwise indicated: *Tp(3;2)C309/TM2*, *C(3)se*, *Df(3L)pbl^{NR}/TM3(ftz::lacZ)*, *Df(3L)pbl^{XI}/TM3(ftz::lacZ)*, *twi::Gal4(2x)*, *twi::Gal4(2x); Dmef2::Gal4* (Ranganayakulu et al., 1998), *UAS::pbl$^{3.2}$* (Prokopenko et al., 1999), *C(1)DX;twi::Gal4;Y* (N. Brown, Cambridge, UK), *twi::CD2 cn*, *htl^{AB42}/TM3(ftz::lacZ)*, *htl^{YY26}/TM3(ftz::lacZ)*, *pbl^{3}/TM3(ftz::lacZ)*, *pbl^{11D}/TM3(ftz::lacZ)* (C. Lehner, Bayreuth, Germany), *pbl^{5}/TM3(ftz::lacZ)* (Rob Saint, Canberra, Australia, *stg^{7M}/TM3(ftz::lacZ)*, *UAS::Rho1^{N19}*, *UAS::Rho1^{V14}* (M. Mlodzik, New York, USA), *UAS::RAS1^{V12}*, *UAS::λhtl*, *UAS::htl^{M}*. More information on translocation stocks and compound chromosomes used in the screen are provided elsewhere (Müller et al., 1999).

Microscopy, antibodies and immunocytochemistry

Embryos were obtained, staged, fixed and immunolabeled as described (Müller and Wieschaus, 1996). For f-actin staining, embryos were fixed with 37% formaldehyde/heptane for 5 minutes followed by devitellinization using 80% ethanol. For cross-sections, fluorescently labeled embryos were embedded in Technovit 7100 (Hereaus, Germany) following the manufacturer's instructions. Sections (7 μm) were obtained with a Reichert Jung Microtome and mounted on slides. As anti-bleaching procedure, sections were incubated for 20 minutes in 50 mM DABCO in PBS and mounted in DABCO/MOWIOL. Embryos were genotyped using balancer chromosomes carrying *lacZ* transgenes. Fluorescence microscopy was performed with a Leica-TCS NT confocal microscope. For whole-mount staining embryos were double labeled with appropriate secondary antibodies conjugated to alkaline phosphatase or biotin. Biotinylated secondary antibodies were detected using the ABC-kit from Vectastain (Vector, USA). Whole-mount stained embryos were dehydrated in ethanol and acetone and embedded in araldite. Embryos were then either mounted and oriented in araldite on microscope slides or embedded for sectioning. Sections were cut at 5 μm and mounted in araldite. Light microscopy was performed on a Zeiss Axiophot. Images were processed using Adobe Photoshop on an Apple Computer.

Preparation of embryos for transmission electron microscopy was performed as described by Müller and Wieschaus (Müller and Wieschaus, 1996) with few modifications. For genotyping, the embryos were prefixed at the interphase of 25% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 and heptane for 25 minutes. The embryos were then hand-peeled and rinsed in X-Gal staining buffer (0.15 M NaCl; 1 mM $MgCl_2$; 0.01 M sodium phosphate buffer, pH 7.2; 3.3 mM $K_3Fe(CN)_6$; 3.3 mM $K_4Fe(CN)_6$). For X-Gal staining, embryos were incubated overnight with 10% 5-Br-4-Cl-3-Indolyl-β-D-galactoside in X-Gal buffer at 16°C. The embryos were then sorted and processed as described. Micrographs were taken at a Zeiss EM109 transmission electron microscope.

The following antibodies were used: mouse anti-dpERK (Sigma); rabbit anti-Twist (Siegfried Roth, Cologne); rabbit anti-βGal (Cappel); mouse anti-βGal (Promega); mouse anti-EVE, mouse anti-Engrailed (EN), mouse anti-Neurotactin (NRT; DSHB, Iowa); mouse anti-CD2 (Serotec, Germany), Phalloidin-Alexa568 (Molecular Probes, USA), rat anti-DE-Cadherin and rat anti-Dα-Catenin (Hiroki Oda, Japan).

Results

Cell shape changes during mesoderm migration

In *Drosophila* the mesoderm originates from a ventral population of cells in the monolayered blastoderm epithelium (Costa et al., 1993). At the onset of gastrulation these cells are first internalized through an invagination of the epithelium. After internalization, the cells undergo mitosis, lose their epithelial characteristics, and start to spread as an aggregate between the central yolk sac and the basal cell surfaces of the ectoderm (Oda et al., 1998).

To follow cell shape changes of mesoderm cells, we used a transgene driving expression of the transmembrane protein CD2 from rat under the control of the *twist* (*twi*) promotor (*twi::CD2*) (Dunin-Borkowski and Brown, 1995). *twi::CD2* is already expressed during invagination and represents a cell-surface marker specific for the mesoderm. Mesoderm

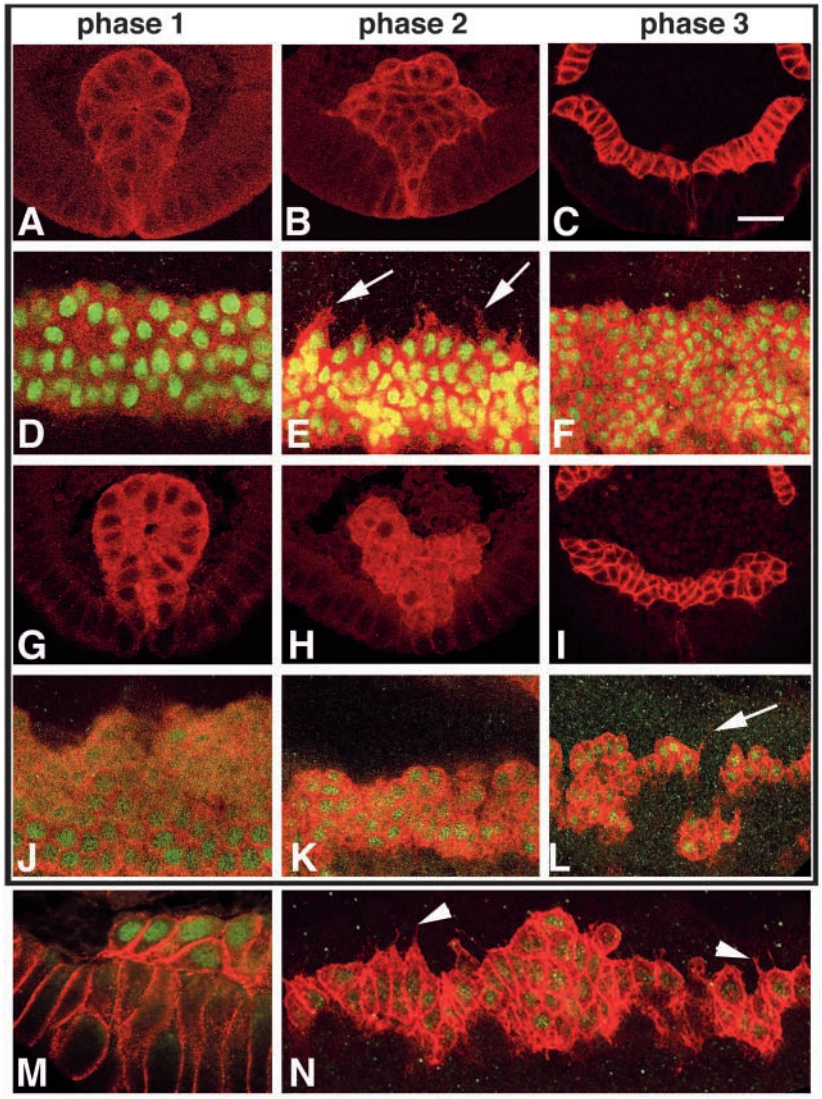

Fig. 1. HTL is required for cell shape changes during mesoderm migration. Embryos expressing *twi::CD2* were stained with anti-CD2 antibodies (red) and anti TWI antibodies (green) and either visualized as whole mounts after optical sectioning by confocal microscopy (D-F,J,K,L,N) or cross-sectioned after plastic embedding (A-C,G-I). (M) Wild-type embryo stained against NRT (red) and TWI (green). Scale bar: 20 μm for A-L. (A-F) Wild-type embryos. (A,D) Phase 1. (B,E) Phase 2; long cellular protrusions are marked with arrows in E. (C,F) Phase 3. (G-L) Embryos homozygously mutant for htl^{AB42}; (G,J) phase 1; note that some mesoderm cells are out of the focal plane, because they are not attached to the ectoderm (J). (H,K) Phase 2; note that cells remain rounded and no cell shape changes occur. (I,L) Phase 3; some cells extend protrusions (arrow in L). (M) Cross-section through the migrating mesodermal aggregate of a wild-type embryo during phase 2. The cells adopted a polarized morphology, with the front attached to the ectoderm and the rear attached to the neighboring cells. (N) htl^{AB42} mutant embryo after mesoderm migration (stage 10). Note the formation of cellular protrusions of cells attached to the ectoderm (arrowheads).

migration can be divided into three phases with characteristic cell shape changes (Fig. 1). After invagination, the mesoderm initially forms an epithelial tube (Fig. 1A,D). At phase 1 of migration, the surface of the mesoderm cells appears relatively smooth (Fig. 1D). After disassembly of the epithelial tube and mitosis, phase 2 begins, in which the mesodermal aggregate migrates out in dorsolateral direction (Fig. 1B,E). Cells at the leading edge of the aggregate are stretched along the dorsoventral axis and extend multiple cellular protrusions. The longest cellular protrusions often measure half to two-thirds the size of a cell diameter (to a length of 10-15 μm in fixed samples). Cross-sections revealed that not only the leading edge cells exhibit this polarized morphology, but that the cells immediately following the leading edge cells frequently also extend in dorsolateral direction (Fig. 1M). In this work, we will be using the term ‘protrusive activity’ to describe the formation and/or the dynamics of the filoform and lamelliform protrusions that we observed in our fixed preparations. The protrusive activity is specific for the migratory phase, because when the cells have reached their final positions (phase 3) and form a coherent monolayer, large extensions are absent and only few filoform protrusions were observed (Fig. 1C,F).

Examination of embryos homozygously mutant for *htl* revealed that HTL is required for cell shape changes during phase 1 and 2 of mesoderm migration. In phase 1, the mesodermal epithelial tube extends further into the interior of the embryo when compared with wild type (Fig. 1A,G; see below). During phase 2, the leading edge cells did not extent dorsolaterally (Fig. 1H,K). This phenotype is not simply explained by the possibility that *htl* mutant mesoderm cells were not able to contact the ectoderm, because cells directly apposed to the ectoderm also failed to extend (Fig. 1H). In phase 3, mesoderm cells of *htl* mutant embryos did not establish a monolayer configuration (Fig. 1I). Interestingly, during and after phase 3, *htl* mutant mesoderm cells exhibit directional protrusions suggesting that some migration might occur at these stages (Fig. 1L,N). This result indicates that HTL is not generally required for protrusive activity of the mesoderm cells. As reported earlier, this late migration in *htl* mutants is never able to rescue the defects in mesoderm differentiation, most probably because of a second requirement of HTL for mesoderm differentiation (Michelson et al., 1998b).

Our observations suggest that HTL is required for the early interaction of the mesoderm with the ectoderm. By analyzing

sections, we observed that wild-type cells at the base of the mesodermal tube are attached to the basal surfaces of the ectoderm (Fig. 2A). By contrast, *htl* mutant mesoderm cells at the respective stage and position failed to establish contact to the ectoderm (Fig. 2C). This phenotype correlates well with a misalignment of the mesodermal tube in *htl* mutants (Fig. 2B,D; Fig. 1J). We conclude that HTL is required for the effective attachment of mesoderm cells to the ectoderm, which might promote the protrusive activity of mesoderm cells during migration.

Identification of *pebble* as a novel player in mesoderm migration

htl and *dof* represent the only zygotically expressed genes that have thus far been described to be essential for mesoderm migration. To obtain a better insight into the genetic control of mesoderm migration, we performed a genetic screen to identify zygotically expressed genes involved in mesoderm migration (T.G. and H.A.J.M., unpublished).

Three loci mapped to the third chromosome and were characterized using chromosomal deletions and available point mutations. Two loci corresponded to the genes *htl* and *dof*, respectively. Embryos lacking the chromosomal interval 61A to 68 (based on breakpoints of the chromosomal translocation *T(2;3)C309*) displayed defects in mesoderm migration (Fig. 3A-C). Genetic mapping revealed that small overlapping chromosomal deletions, which exhibited the phenotype, all removed the gene *pbl* (data not shown). Analysis of a strong loss-of-function point mutation in *pbl*, pbl^3, indicated that *pbl* is required for mesoderm morphogenesis. Embryos homo- or hemizygously mutant for pbl^3 show a dramatic reduction in the number of EVE-positive mesoderm cells at the extended germband stage (Fig. 3D,E). These results demonstrate a thus far unrecognized function for *pbl* in mesoderm differentiation.

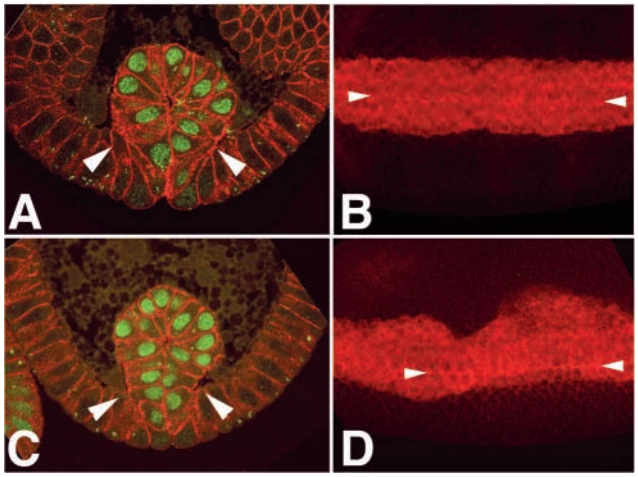

Fig. 2. HTL is required for the attachment of the mesoderm to the ectoderm. Embryos (stage 7; phase 1 of migration) were fixed and stained with anti-NRT (red) and anti-TWI (green) antibodies (A,C) or with anti-CD2 antibodies (B,D). (A) In the wild type, the mesodermal tube is attached to those ectodermal cells that lie adjacent to the basal cells of the tube (arrowheads). (B) The invaginated mesoderm cells are aligned along the midline of the embryo, indicated with arrowheads. (C) In embryos homozygously mutant for htl^{AB42}, the basal cells of the mesodermal tube fail to adhere to the ectoderm. (D) Misalignment of mesoderm cells in *htl* mutants with respect to the ventral midline (arrowheads in B,D). (B,D) Projections of a stack of confocal images covering a total of 25 μm from the ventral surface of the embryo to the interior.

We next examined whether the defects in mesoderm differentiation in *pbl* mutants are based upon a requirement of *pbl* for cell migration. While internalization of the mesoderm occurred normally, the characteristic cell shape changes during mesoderm migration were blocked in *pbl* mutants (Fig. 3F,G). As in *htl* mutants, the cells at the base of the mesodermal tube were unable to attach to the ectoderm. In phase 2, *pbl* mutant cells were in close apposition to the ectoderm, but failed to extend dorsolaterally and no protrusions were observed. Distinct from *htl* mutants, however, protrusive activity was also blocked during phase 3 in *pbl* mutants, suggesting that PBL represents an essential component of the mechanisms driving protrusive activity of the cells (Fig. 3H). To further characterize the cellular protrusions in our fixed preparations, we examined f-actin distribution in the mesoderm cells of wild-type and *pbl* mutants. We found that the protrusions in wild-type embryos contain f-Actin. In *pbl* mutant mesoderm cells an f-actin rich cell cortex was equally distributed at the circumference of the cells with no protrusions present (Fig. 3I,J). These results indicate that PBL is required for cell shape changes during migration of the mesoderm cells, which might involve the reorganization of the cortical f-actin cytoskeleton.

It remained possible that the defects in *pbl* mutants in cell migration might be a secondary consequence of a failure in the epithelial/mesenchymal transition of the mesoderm after invagination. During epithelial/mesenchymal transition cells loose their epithelial polarity and gain mesenchymal character. We compared the presence of adherens junctions in the mesoderm cells in wild type, *htl* and *pbl* mutant embryos by electron microscopy. Apical adherens junctions, a hallmark of apicobasal polarity in epithelial cells, are lost from the mesoderm cells during phase 1 in the wild type as well as in embryos homozygous for *htl* or *pbl* (see Fig. S1 at http://dev.biologists.org/supplemental). Similarly, immunolabeling with antibodies against the ectodermal epithelial marker *D*E-cadherin or the adherens junction marker Dα-Catenin showed that downregulation of epithelial characteristics occurs normally in *pbl* and *htl* mutants (see Fig. S2 at http://dev.biologists.org/supplemental). In summary these results indicate that PBL is not required for the loss of epithelial character of mesoderm cells, but that PBL is essential for the gain of mesenchymal characteristics of mesoderm cells after invagination.

The requirement of PBL for cell migration is independent of its function in cytokinesis

pbl encodes a RHO1-GEF most similar to the vertebrate *ect2* proto-oncogene (Miki et al., 1993; Prokopenko et al., 1999; Tatsumoto et al., 1999). Both *pbl* and *ect2* are required for the assembly of the contractile actin ring during cytokinesis. Interfering with the function of PBL or ECT2 results in a failure of cytokinesis and the generation of multinucleate cells (Fig. 3G,H). Because mutations in *pbl* affect cell shape changes before mitoses in the mesoderm occur, we suspected that the requirement of *pbl* for mesoderm migration might be independent from its cytokinesis function. To determine, whether the defects in mesoderm migration in *pbl* mutants are direct rather than a secondary consequence of the failure in

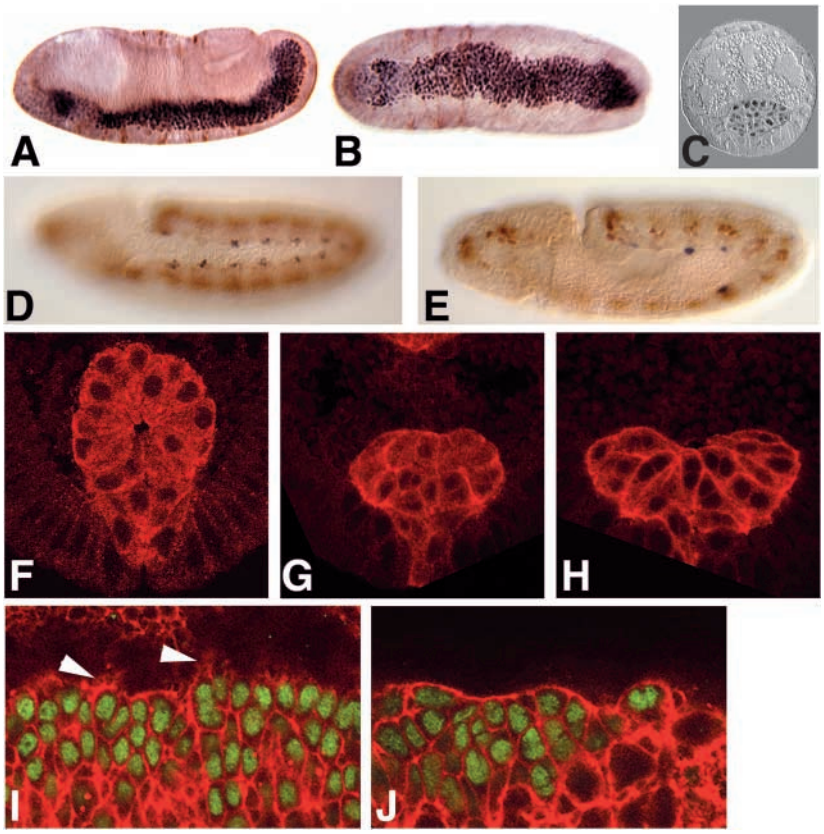

Fig. 3. Identification of *pbl* as novel zygotic factor required for mesodermal cell migration. (A-C) Embryos at extended germband stages carrying a synthetic chromosomal deletion produced by *C(3)se* females crossed to *T(3;2)C309/TM3* males (uncovering genomic segment 61 to 68) were stained with anti-EN (brown) and anti-TWI (black) antibodies. The reduction of EN stripes is due to deletion of the *hairy* locus in this genomic interval and was used as independent marker for the genotype. (A,B) Lateral (A) and ventral (B) views of whole-mount staining. (C) Cross-section demonstrates that the mesoderm cells fail to migrate in these embryos. Wild-type embryos (D) and pbl^{3} homozygous embryos (E) at stage 11 were stained for EVE (blue) and TWI (brown) protein. In *pbl* mutants the number of EVE-positive hemisegments is strongly reduced. (F-H) Cell shape changes were analyzed by examining *twi::CD2* expression in pbl^{3} homozygotes; cell shape changes are blocked in *pbl* mutant embryos. (I,J) F-actin staining (red) of wild-type (I) and pbl^{3} homozygous embryos. TWI staining is seen in green. F-actin-rich protrusions are indicated with arrowheads in I.

cytokinesis, we analyzed the *pbl* phenotype in division-defective embryos.

Postblastoderm mitotic divisions are controlled by zygotic expression of the cell cycle regulator String (STG) (Edgar and O'Farrell, 1990). As mesoderm migration and specification of EVE-positive mesoderm cells occur normally in *stg* mutant embryos, this mutation provides a genetic condition to assay cytokinesis-independent functions of *pbl* (Fig. 4B,E) (Carmena et al., 1998; Leptin and Grunewald, 1990). The cytokinesis defect of *pbl* was completely blocked by *stg* (Fig. 4F). In *pbl stg* double mutant embryos, migration of the mesoderm and specification of dorsal mesodermal derivatives was impaired similar to *pbl* single mutants (Fig. 4C,F). Moreover, cell shape changes in phase 2 occur normally in *stg* mutant embryos, but protrusive activity of the mesoderm cells was blocked in *pbl stg* double mutants (Fig. 4E,F). These results indicate that the activity of *pbl* is required for mesoderm migration even in the absence of mitosis and thus in the absence of cytokinesis defects. We therefore conclude that PBL has independent functions in cytokinesis and cell migration, respectively.

Distinct requirements for PBL and RHO1 in cytokinesis and cell migration

As the functions of PBL can be separated genetically, we wanted to investigate whether the two functions might involve different molecular mechanisms.

Genetic data indicate different requirements of the PBL protein for its two functions. We detected a significant difference in the strength of the migration phenotype of two distinct mutant alleles of *pbl*. Embryos homozygous for the strong loss of function allele pbl^{3} exhibit on average only one hemisegment that contains EVE-positive mesoderm cells, compared with 22 EVE-positive hemisegments in the wild type (Fig. 5A,B; Table 1). By contrast, embryos mutant for pbl^{11D} exhibit an average of 12 hemisegments that contain EVE-positive cells and few pbl^{11D} homozygous embryos (2/45) even contained the wild-type number of EVE-positive cells (Fig. 5C; Table 1). Importantly, pbl^{3} and pbl^{11D} homozygotes exhibit equally penetrant defects in cytokinesis (Fig. 5D,E) (see also Cui and Doe, 1995; Weigmann and Lehner, 1995). These allele-specific differences demonstrate distinct requirements of the PBL protein in cell migration and cytokinesis, and suggest that the migratory function of PBL might be acting through a mechanism distinct from the cytokinesis function.

During cytokinesis, PBL performs a conserved function as a GEF for RHO1 in the formation of the contractile ring (Prokopenko et al., 1999; Prokopenko et al., 2000b; Tatsumoto et al., 1999). Expression of a constitutively active form of RHO1 ($Rho1^{V14}$) in the mesoderm cells of *pbl* mutants was unable to rescue the migration defects in *pbl* mutant embryos (data not shown). This negative result is difficult to interpret, because expression of $Rho1^{V14}$ did not rescue the cytokinesis defects in *pbl* mutants either. We therefore tested the role of RHO1 in mesoderm migration directly by expressing a dominant-negative version of RHO1 ($Rho1^{N19}$) in the mesoderm (Strutt et al., 1997). Although expression of *UAS::*$Rho1^{N19}$ with *twi::Gal4* led to cytokinesis defects of mesoderm cells similar to *pbl* mutants, specification of EVE-positive mesoderm cells remained unimpaired (Fig. 5F,H). Importantly, mesoderm cells expressing $Rho1^{N19}$ still showed the characteristic protrusive activity during phase 2 of migration (Fig. 5G). The fact that expression of $Rho1^{N19}$ led

to a fully penetrant cytokinesis defect suggests that the function of endogenous RHO1 is efficiently blocked by the expression of the dominant-negative form (Fig. 5H). Although it might be possible that small amounts of RHO1 activity might be sufficient to support cell migration, further raising the level of *Rho1^{N19}* in the mesoderm, by using *twi::Gal4* in combination with a *Dmef2::Gal4* driver did not affect the presence of EVE-positive mesoderm cells (Fig. 5I,J).

These data support our conclusion that the defects in cell migration in *pbl* mutants are not a secondary consequence of a failure in cytokinesis. The finding that cell migration is not affected in *Rho1^{N19}*-expressing cells suggests that RHO1 function is dispensable for mesoderm migration. In summary, we propose that the two functions of PBL are independent and might involve distinct molecular mechanisms in cytokinesis and cell migration, respectively.

Requirement of PBL for cell migration is specific for mesoderm cells

Mesoderm migration depends on the mesoderm-specific expression and activity of both the HTL receptor and its putative cytoplasmic adaptor DOF (Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998a; Shishido et al., 1993; Vincent et al., 1998). By contrast, *pbl* is expressed in all cells of the embryo and is required in all cells for cytokinesis in postblastoderm embryos (Hime and Saint, 1992; Lehner, 1992; Prokopenko et al., 2000a).

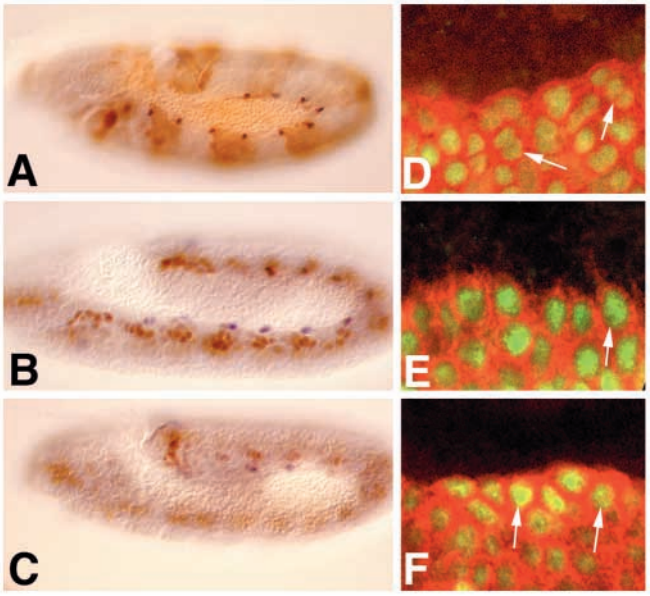

Fig. 4. The function of PBL in cell migration is independent of its function in cytokinesis. (A-C) Embryos at stage 10 stained for EVE (blue) and TWI and βGal (brown). In the wild-type (A) and *stg^{7M}* homozygous embryos (B), eleven *eve*-positive hemisegments are stained on either side of the embryo. In *pbl^{3}*, *stg^{7M}* double mutant embryos (C), the number of *eve*-positive hemisegments is strongly reduced, reminiscent of *pbl* single mutant embryos. (D-F) Cell shape changes in *twi::CD2; pbl^{3}/pbl^{3}* (D), *twi::CD2; stg^{7M}/stg^{7M}* (E), and *twi::CD2; pbl^{3},stg^{7M}/pbl^{3},stg^{7M}* double mutant (F) embryos as visualized with anti CD2 (red) and anti TWI (green) staining. Protrusive activity in phase 2 is absent in *pbl* and *pbl,stg* double mutant embryos, while it can still be observed in *stg* single mutants. Note bi-nucleated cells are present in *pbl* mutants (arrows in D), but are absent in *stg* and *pbl,stg* double mutants (arrows in E,F).

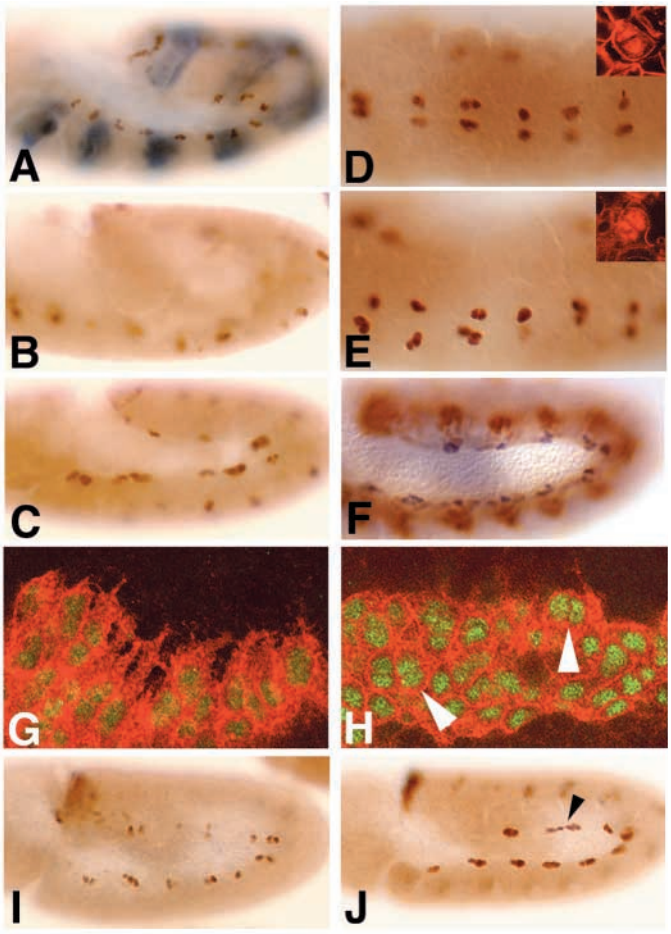

Fig. 5. Different requirements for PBL and RHO1 in cell migration and cytokinesis. (A-C) *eve* expression in the wild type (A) and in embryos homozygously mutant for *pbl^{3}* (B) or *pbl^{11D}* (C). Although a strong reduction of EVE-positive cells is seen in the *pbl^{3}* mutants (B), *pbl^{11D}* mutants (C) exhibit a significantly higher number of EVE-positive hemisegments (see also Table 1). (D,E) Cytokinesis defects in *pbl^{3}* (D) and *pbl^{11D}* (E) homozygous embryos. Ventral view of embryos at extended germband stage (stage 11) stained with antibodies against EVE. Segmental expression of *eve* in the central nervous system can be seen as described before (Cui and Doe, 1995; Weigmann and Lehner, 1995). The EVE-positive cells represent products of an incomplete cytokinesis of neuroblast 7-1. Inserts show confocal sections of anti-EVE and anti-NRT stained embryos to label cell outlines (both antibodies in red); note the presence of two nuclei in the neuroblasts. (F) Expression of *Rho1^{N19}* in the mesoderm affects cytokinesis, but not migration. *twi::Gal4; UAS::Rho1^{N19}* embryo stained with anti-EVE (blue) and anti-TWI (brown). Embryos expressing *UAS::Rho1^{N19}* exhibit normal mesoderm differentiation indicated by the normal pattern of EVE-positive cells. Formation of cellular protrusions in *twi::Gal4; UAS::Rho1^{N19}* embryos is normal (G; *twi::CD2* (red), anti-TWI (green)). (H) Cytokinesis in such embryos is blocked as binucleated cells (arrowheads) can be seen in a different focal plane of the embryo shown in (G). (I,J) EVE expression in *twi::Gal4; Dmef2::Gal4/UAS::Rho1^{N19}* embryos at stage 10 (I) and 11 (J). Note some irregularity in the arrangement of EVE-expressing cells (arrowhead in J).

Table 1. Quantitation of Eve-postive mesoderm cells

Genotype	*eve*-positive hemisegments
Oregon R	22 (s.d.=0; *n*=20)
htl^{AB42}/htl^{AB42}	0 (s.d.=0; *n*=20)
pbl^{3}/pbl^{3}	1.4 (s.d.=1.2; *n*=136)
pbl^{11D}/pbl^{11D}	11.7 (s.d.=3.5; *n*=45)
twi::Gal4; UAS::htl^{M} htl^{AB42}/htl^{AB42}	22 (s.d.=0; *n*=20)
twi::Gal4; UAS::λhtl htl^{AB42}/htl^{AB42}	12.2 (s.d.=2.9; *n*=27)
twi::Gal4; UAS::htl^{M} $pbl^{3}/Df(3L)pbl^{NR}$	0.4 (s.d.=0.7; *n*=45)
twi::Gal4; UAS::λhtl $pbl^{3}/Df(3L)pbl^{NR}$	6.5 (s.d.=2.5; *n*=17)

The number of Eve-positive hemisegments was determined from stained embryos of the indicated genotypes at stages 10 to 12. Mean values are shown and the number (*n*) of embryos and the standard deviation (s.d.) are indicated.

We sought to assess the cell-type specific requirements of PBL during mesoderm migration by rescuing the *pbl* mutant phenotype in a tissue-specific manner. A *UAS::pbl* transgene containing the full-length *pbl* cDNA was expressed in the mesoderm cells in a *pbl* mutant background using *twi::Gal4*. The cytokinesis defect and the mesoderm migration defect of *pbl* mutants was completely rescued by mesoderm-specific expression of *pbl* (Fig. 6A-C). Moreover, expression of EVE in dorsal mesoderm cells was restored (Fig. 6D,E). The cells in the ectoderm of such embryos still exhibited cytokinesis defects, indicating that the activity of the transgene was specific for mesoderm cells and that the function of PBL in the ectoderm is not essential for mesoderm migration (Fig. 6B). We therefore conclude that *pbl* acts in a mesoderm autonomous fashion to allow proper migration of the cells.

PBL activity is required for HTL function in triggering cell shape changes

Because the function of *pbl* in mesoderm migration is specific for mesoderm cells, we next investigated how PBL function relates to signals derived from HTL. Molecular and genetic epistasis has shown that HTL activates the conserved Ras/Raf/MAP kinase pathway during mesoderm migration and differentiation (Gabay et al., 1997; Michelson et al., 1998a; Michelson et al., 1998b; Vincent et al., 1998). To assess the function of PBL in HTL-dependent migration, we asked whether *pbl* is required for the activation of downstream components of the HTL pathway by measuring MAP kinase activation in *pbl* mutants. Strikingly, MAP kinase is still activated in *pbl* mutants in a pattern similar to that of wild-type embryos (Fig. 7H,I). This result indicates that PBL function is dispensable for HTL-dependent activation of MAP kinase. We therefore conclude that PBL is unlikely to act upstream of the HTL receptor and its signaling cascade.

The mesoderm defects of *htl* homozygous embryos can be completely rescued by expression of *UAS::htl* using *twi::Gal4* (Fig. 7A,B) (Michelson et al., 1998b). When, instead, the constitutively active form of HTL, λHTL, is expressed under the same conditions, the cellular defects in early mesoderm migration were rescued, but mesoderm differentiation defects were only partially rescued (Fig. 7C,D; Table 1) (Michelson et al., 1998b). These results indicate that expression of λHTL with the Gal4/UAS system is sufficient to trigger the early cell shape changes in *htl* homozygously mutant embryos.

To test whether *pbl* is required for HTL triggered cell shape changes in mesoderm migration, we overexpressed wild-type or constitutively active forms of HTL in a *pbl* mutant background. Both, expression of HTL or λHTL, were unable to trigger early cell shape changes in *pbl* homozygous embryos (Fig. 7E and data not shown). Although HTL or λHTL expression failed to rescue phase 1, some mesoderm spreading was observed in phase 3 and later (Fig. 7G). Because the rescue in phase 3 was rather moderate and variable, we examined mesoderm differentiation by analyzing the number of EVE-positive hemisegments. Mesoderm differentiation defects in *pbl* homozygotes are rescued significantly by expression of λHTL but not by expression of HTL (Fig. 7F; Table 1). This result suggests that mesoderm migration might be delayed in these embryos, in a way that might render signaling events leading to EVE expression ineffective. In addition, the partial rescue of *pbl* mutants by λHTL might reflect a second function of the receptor in differentiation of dorsal mesodermal derivatives (Michelson et al., 1998b). These results are

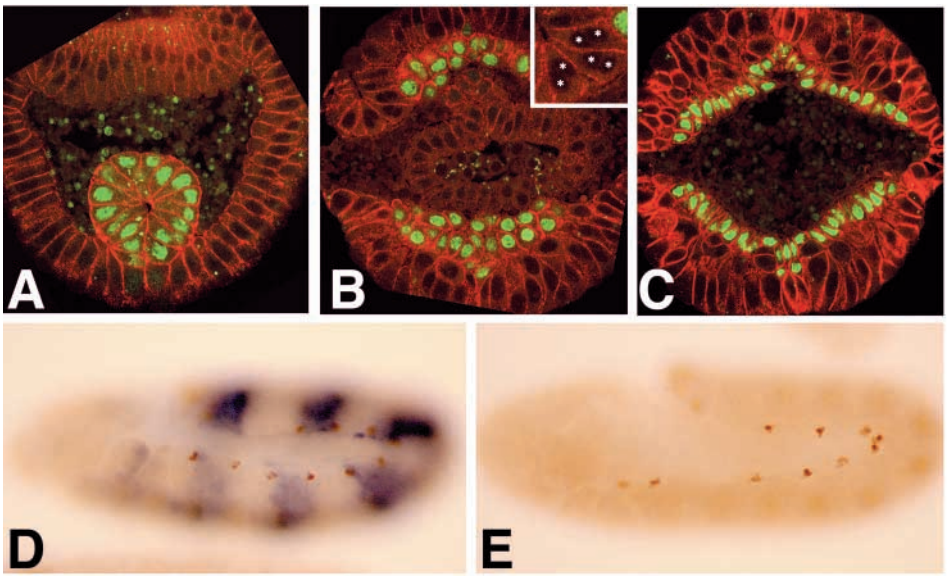

Fig. 6. The function of PBL in mesoderm migration is mesoderm autonomous. Embryos hemizygous for pbl^{3}, expressing *twi::Gal4*, *UAS::*$pbl^{3.2}$ were stained with anti-NRT (red) and anti-TWI (green) antibodies and sectioned. Embryos are shown in phase 1 (A), phase 2 (B) and phase 3 (C) of migration. A complete rescue of cell shape change defects in *pbl* mutants is seen. Note that cytokinesis is occurring normally in the mesoderm while still blocked in the ectoderm (insert in B; the asterisks mark nuclei). (D,E) Differentiation of EVE-positive mesoderm cells is rescued by expression of *UAS::*$pbl^{3.2}$ with *twi::Gal4* in pbl^{3} hemizygously mutant embryos. (D) Stage 11 embryo containing at least one wild-type copy of the *pbl* gene; the blue staining indicates presence of the *TM3 (ftz::lacZ)* chromosome. (E) Stage 11 embryo of the genotype *twi::Gal4/UAS::*$pbl^{3.2}$*;* $pbl^{3}/Df(3L)pbl^{NR}$.

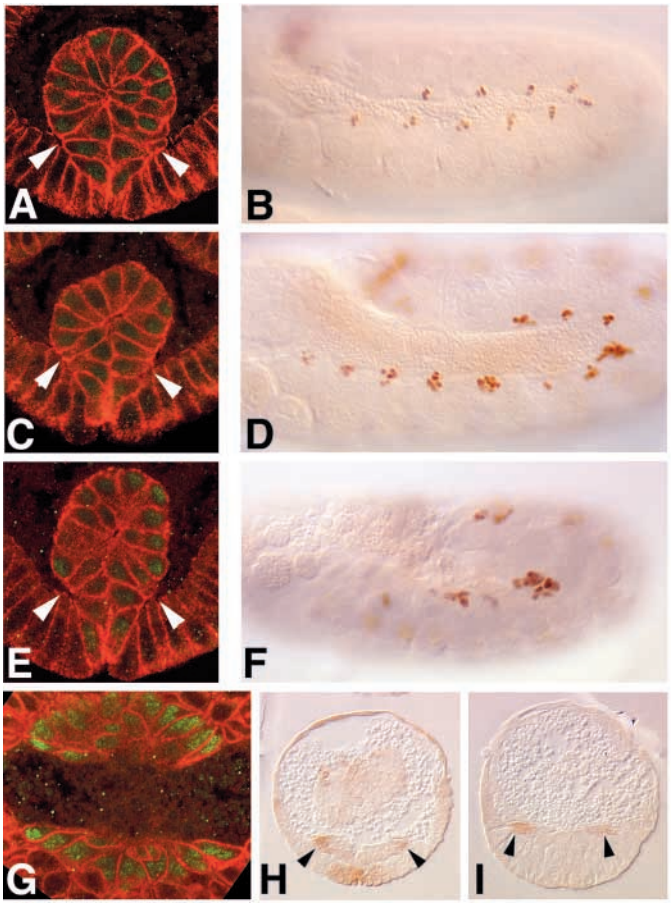

Fig. 7. Requirement of PBL for HTL-triggered cell shape changes. (A,C,E) Sections of embryos at stage 7 stained with anti-NRT (red) and anti TWI (green) antibodies. The contacts of the mesoderm with the ectoderm cells are marked with arrowheads in A,C. (B,D,F) Lateral surface views of embryos at stage 11 stained with anti-EVE antibodies. (A,B) htl^{AB42} homozygous embryos overexpressing full-length HTL protein (*twi::Gal4;* htl^{AB42}, *UAS::*htl^{M}/htl^{AB42}). (C,D) htl^{AB42} homozygous embryos overexpressing constitutively activated HTL (*twi::Gal4;* htl^{AB42}, *UAS::λhtl/*htl^{AB42}). (E-G) pbl^{3} hemizygous embryos overexpressing the activated form of HTL (*twi::Gal4;* pbl^{3}, *UAS::λhtl/Df(3L)*pbl^{NR}); embryo in G is at stage 9. Arrowhead in E indicates the failure of mesoderm cells to contact the ectoderm. (H,I) Cross-sections of wild-type (H) and pbl^{3} homozygous mutant (I) embryo at stage 8 stained with anti-dpERK antibodies. Arrowheads indicate mesoderm cells at the leading edge that stain for dpERK.

consistent with a role of PBL for early cell shape changes triggered by HTL. The fact that λHTL rescues some late defects in *pbl* mutants suggests the presence of additional mechanisms required for mesoderm migration, which might be independent of *pbl*.

Discussion

Cellular functions of HTL in mesoderm migration

HTL exerts different functions in mesoderm morphogenesis (Michelson et al., 1998b). We analyzed the requirements of HTL for cell shape changes during mesoderm migration. Our analysis of cell shape changes revealed that the cells extend in the direction of their migration and form long cellular protrusions. These observations provide evidence that the leading cell rows of the mesodermal aggregate migrate directionally in dorsolateral direction.

Although the ligand of the HTL receptor is unknown, it seems unlikely that signals mediated by HTL provide the only directional cues for mesoderm migration. Localization of activated forms of MAP kinase in the leading edge cells during mesoderm migration suggests that local activation of the receptor might be important for proper migration (Gabay et al., 1997). In addition, activation of HTL in a ligand-independent fashion throughout the mesoderm is unable to completely rescue the *htl* mutant phenotype (Michelson et al., 1998b) (this paper). Thus local activation of the receptor occurs in vivo and is essential for proper mesoderm migration. We show that HTL is required for protrusive activity only during phase 1 and 2 of mesoderm migration. However, HTL activity is not essential for the protrusive activity of the cells per se, because cells do extend dorsolaterally during phase 3 in *htl* mutant embryos. These data demonstrate that HTL activation is unlikely to provide the only directional cue in mesoderm migration. The results presented in this paper suggest that HTL signaling provides temporal information for protrusion formation during phases 1 and 2, and might be therefore acting as a permissive factor during mesoderm migration.

PBL is required for protrusive activity of mesoderm cells also in phase 3 and later. It is therefore possible that PBL function might be required in a more general way for the cell to extend protrusions. The specificity of PBL for protrusive activity is also supported by the fact that loss of epithelial characteristics is unaffected in *pbl* mutant embryos. Although the specific mechanism of PBL function in cell migration is currently unknown, it is important to note that not all morphogenetic movements are compromised in *pbl* mutants. For example, cephalic furrow formation, invagination of the ventral furrow and germband extension movements, which all depend on a functional cytoskeleton are normal in *pbl* mutant embryos (data not shown). We therefore propose that PBL might constitute an important component for cytoskeletal changes, which are triggered by FGFR signaling events.

The relation of PBL function to HTL signal transduction

Of the multiple responses generated downstream of FGFR activation, only little is known of the molecular pathways by which FGFRs trigger cell shape changes in vivo. The Rho GEF PBL represents a good candidate for mediating cell shape changes triggered by HTL signaling. Importantly, the early phenotypes of *htl* and *pbl* mutants are almost identical, indicating that both gene products are required in a narrow time window for early cell shape changes after invagination of the mesoderm. Furthermore, in both mutants this phenotype is completely penetrant, indicating that the gene products do not act in a redundant fashion.

The function of PBL for mesoderm migration is specific for mesoderm cells. Because *htl* is expressed only in the mesoderm at this stage of development, PBL might be involved in the presentation of the receptor or its unknown ligand and thus acting upstream, or PBL might be involved in downstream events triggered by the HTL signaling cascade. If PBL was

acting upstream of HTL, signaling events downstream of HTL should be blocked in such mutants. By contrast, here we show that PBL is dispensable for activation of MAP kinase in the early mesoderm cells. These results suggest that PBL does not act upstream of HTL and favor a model in which PBL acts downstream of the HTL signaling cascade.

The present results render it unlikely that PBL is directly involved in a signaling pathway downstream of HTL FGFR. In contrast to *htl* mutants, no cell shape changes and no protrusive activity was observed in *pbl* mutant mesoderm cells in phase 3. In addition, the *pbl* null mutant phenotype still allows a few cells to undergo *eve* expression, probably owing to the larger cells and abnormal cytoarchitecture in the division defective embryos. This is in contrast to *htl* loss of function mutants where EVE-positive mesoderm cells are never observed. If *pbl* was essential for signaling downstream of the HTL receptor, the phenotype of *htl* and *pbl* mutants should be more similar with respect to mesoderm differentiation; for example, the phenotypes of *htl* and *dof* mutant embryos are identical (Vincent et al., 1998; Michelson et al., 1998a). We therefore propose that PBL might represent a regulator of the cytoskeleton or adhesive mechanisms of the cell, which provide targets of the HTL signaling cascade to trigger cell shape changes.

Although the activation of MAP kinase in the mesoderm depends on HTL, it is not known whether this is a direct response or whether it is indirect, i.e. MAP kinase may not be directly activated by HTL itself, but through interactions of the mesoderm with the ectoderm. In this case, activation of MAP kinase would be a response rather than a cause of the cell shape changes. The phenotype of *pbl* mutants, however, argues against the latter possibility, because it shows that in the absence of cell shape changes, MAP kinase can still be activated. This result also suggests that activation of MAP kinase alone cannot account for the cell shape changes that occur. This idea is supported by the fact that activated forms of RAS1 are unable to completely rescue the defects in mesoderm migration of *htl* or *dof* mutant embryos, including the defects in cell shape changes in phase 1 and 2 (data not shown) (Vincent et al., 1998; Michelson et al., 1998a; Michelson et al., 1998b; Iman et al., 1999). These results suggest the presence of a signaling pathway acting in parallel to the Ras/Raf MAP kinase pathway to be involved in mesoderm migration.

A novel function for PBL in cell migration

The *pbl* gene was originally identified and characterized as an essential factor for cytokinesis (Hime and Saint, 1992; Lehner, 1992; Prokopenko et al., 1999). We describe a function of PBL in interphase cells that can be genetically separated from its requirement for cytokinesis.

Two lines of evidence indicate that the function of PBL in cell migration is mediated through a pathway different from the cytokinesis pathway. First, expression of a dominant-negative form of RHO1, which blocks cytokinesis in the mesoderm has no effect on mesoderm migration, cell shape changes associated with it or expression of differentiation markers specific for dorsal mesoderm derivatives. Second, a mutation in *pbl*, pbl^{11D} exhibits significantly weaker defects in mesoderm differentiation compared to the strong loss of function mutation pbl^{3}. These allele-specific differences indicate distinct requirements of the PBL protein for its two functions, because both alleles exhibit identical cytokinesis defects and only differ in mesoderm differentiation defects significantly. We therefore propose that the function of PBL for cell migration might not involve RHO1 and might therefore be using another mechanism.

How does PBL act in cell migration and which GTPase represents its substrate? Both PBL and its mammalian orthologs belong to the Dbl family of Rho-GEFs, which promote activation of Rho GTPases through a conserved Dbl-homology (DH) domain (Prokopenko et al., 2000b). The DH domain is required for both functions of PBL, because a missense mutation in *pbl*, called pbl^{5}, in which an amino acid exchange renders the DH domain inactive, exhibits equally strong defects in cell migration and cytokinesis (S.S. and H.A.J.M., unpublished) (M. Smallhorne, M. Murray and R. Saint, personal communication). Data from yeast two-hybrid assays, as well as genetic interactions indicate that PBL binds to and interacts with RHO1 (Prokopenko et al., 1999). During cytokinesis, PBL is proposed to locally activate RHO1, which then interacts with its effector Diaphanous, a *Drosophila* homologue of the Formin family of actin regulators (O'Keefe et al., 2001; Prokopenko et al., 2000b; Somers and Saint, 2003). Although PBL appears to interact with RHO1, but not with CDC42 or RAC1, mammalian homologs of PBL promote GTP/GDP exchange of the GTPases RHO1, RAC1 and CDC42 (Tatsumoto et al., 1999). Because these discrepancies might reflect differences in the sensitivity of the assays applied, it remains to be determined which substrate PBL uses for its function in cell migration.

Although we have detected a role of PBL in FGFR triggered cell migration, it is currently unclear how general the requirement of PBL is for the protrusive activity of migrating cells. Interestingly, mutations in *pbl* have been discovered in a screen for genes required for the development of the peripheral nervous system (Salzberg et al., 1994). These mutants affected the correct migration of the axons in the PNS without obvious defects in cytokinesis. It will therefore be interesting to assess the function of PBL in a variety of migrating cells to further characterize its potential role as a mediator of cell shape changes triggered by extracellular signals.

Note added in proof

In this paper, we state that the ligand of HTL FGFR is unknown. While this paper was in press, two papers were published describing the identification of two novel genes encoding FGF-like growth factors in *Drosophila*, consistent with being ligands of HTL (Stathopoulos et al., 2004; Gryzik and Müller, 2004).

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