The epithelial-mesenchymal transition of the *Drosophila* mesoderm requires the Rho GTP exchange factor Pebble

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Summary

***Drosophila pebble* (*pbl*) encodes a Rho-family GTP exchange factor (GEF) required for cytokinesis. The accumulation of high levels of PBL protein during interphase and the developmentally regulated expression of *pbl* in mesodermal tissues suggested that the primary cytokinetic mutant phenotype might be masking other roles. Using various muscle differentiation markers, we found that Even skipped (EVE) expression in the dorsal mesoderm is greatly reduced in *pbl* mutant embryos. EVE expression in the dorsalmost mesodermal cells is induced in response to DPP secreted by the dorsal epidermal cells. Further analysis revealed that this phenotype is likely to be a consequence of an earlier defect. *pbl* mutant mesodermal cells fail to undergo the normal epithelial-mesenchymal transition (EMT) and dorsal migration that follows ventral furrow formation. This phenotype is not a secondary consequence of failed cytokinesis, as it is rescued by a mutant form of *pbl* that does not rescue the cytokinetic defect. In wild-type embryos, newly invaginated cells at the lateral edges of the mesoderm extend numerous protrusions. In *pbl* mutant embryos, however, cells appear more tightly adhered to their neighbours and extend very few protrusions. Consistent with the dependence of the mesoderm EMT and cytokinesis on actin organisation, the GTP exchange function of the PBL RhoGEF is required for both processes. By contrast, the N-terminal BRCT domains of PBL are required only for the cytokinetic function of PBL. These studies reveal that a novel PBL-mediated intracellular signalling pathway operates in mesodermal cells during the transition from an epithelial to migratory mesenchymal morphology during gastrulation.**

Key words: Mesoderm, Cell migration, Epithelial-mesenchymal transition, EMT, *Drosophila*, Pebble, Rho, GTP exchange factor, RhoGEF

Introduction

Embryonic development comprises a series of coordinated cellular events that together produce the mature organism. The cytoskeleton plays a crucial role in many of these processes by directing cell behaviour during development. The Rho family of small GTPases, or G proteins, are key regulators of the actin cytoskeleton. In fibroblasts, for example, Rho stimulates the assembly of contractile acto-myosin filaments and associated focal adhesion complexes (Ridley and Hall, 1992), Rac induces the formation of lamellipodia and membrane ruffles (Ridley et al., 1992), whereas CDC42 induces filopodia (Kozma, 1995). Many studies have since confirmed the importance of Rho family proteins as molecular switches that control a wide range of cellular processes including shape change, adhesion and cell cycle progression (Hall, 1998).

The functional activity of the Rho family of small GTPases is regulated in vivo by proteins that control their GTP/GDP bound state. Guanine nucleotide exchange factors (GEFs) activate G proteins by catalysing the exchange of bound GDP for GTP, while GTPase-activating proteins (GAPs), inactivate G proteins by increasing their low intrinsic GTPase activity (Whitehead et al., 1997; Zalcman et al., 1999). In the active GTP-bound state, conformational changes allow the G protein to interact with downstream effectors and to generate a response. Members of a third group of regulatory proteins, the guanine nucleotide dissociation inhibitors (GDIs), bind G proteins and maintain them in an inactive soluble state by inhibiting the exchange of GDP for GTP and sequestering them from membranes (Zalcman et al., 1999).

In *Drosophila melanogaster*, seven Rho family members have been identified: RHO1 (RhoA), Rho-like (RhoL), RAC1, RAC2, CDC42, MIG2-like (MTL) and RhoBTB (Hakeda-Suzuki et al., 2002; Hariharan et al., 1995; Luo et al., 1994; Murphy and Montell, 1996). Phenotypic analysis of mutant alleles and of the effect of expressing dominant negative and constitutively active forms of these proteins have suggested roles in a wide range of developmental processes that require dynamic actin cytoskeleton reorganisation. During *Drosophila* oogenesis, for example, CDC42 and RhoL are thought to be important for the maintenance of the actin-rich ring canals that connect nurse cells and the oocyte, whereas RAC1 appears to be required throughout migration of the somatic border cells (Murphy and Montell, 1996). Reducing RHO1 levels affects the organisation of the actin cytostructure of egg chambers as well as ring canal morphology (Magie et al., 1999).

Embryogenesis also involves numerous Rho family

functions. During cellularisation, the inhibition of Rho function or activation of CDC42 disrupts the actomyosin cytoskeleton, halting cellularisation and embryogenesis (Crawford et al., 1998). The loss of RHO1 activity during dorsal closure results in abnormal cell shape changes along the dorsal midline, although closure does occur (Magie et al., 1999), whereas embryos mutant for *Rac1*, *Rac2* and *Mtl* do not complete dorsal closure, presumably owing to the lack of F-actin at the leading epidermal edge (Hakeda-Suzuki et al., 2002). Finally, numerous studies have shown that Rho family members play a crucial role in *Drosophila* neurogenesis and muscle development (Hakeda-Suzuki et al., 2002; Hassan and Vaessin, 1996; Lee and Luo, 1999; Lee and Luo, 2001; Lee et al., 2000; Luo et al., 1994; Ng et al., 2002). RHO1 is necessary for neuroblast proliferation and for limiting dendrite growth (Lee et al., 2000), whereas axon outgrowth requires low levels of Rac activity (Hakeda-Suzuki et al., 2002; Lee et al., 2000). The expression of dominant-negative and constitutively active *Rac1* or *Cdc42* in the mesoderm blocks myoblast fusion (Luo et al., 1994), and little or no myoblast fusion occurs in either a *Rac1 Rac2* double mutant or *Rac1 Rac2 Mtl* triple mutant embryos (Hakeda-Suzuki et al., 2002).

Activation of different Rho family members in specific tissues and subcellular locations is regulated by the activity of an even larger family of RhoGEFs and GAPs. Seven *Drosophila* Rho family regulators have been studied to date: RhoGEF2, RhoGEF3, GEF64C, Pebble (PBL), Trio, RnRacGAP and RacGAP50C (Barrett et al., 1997; Hacker and Perrimon, 1998; Debant et al., 1996; Guichard et al., 1997; Hicks et al., 2001; Prokopenko et al., 1999; Somers and Saint, 2003). RhoGEF2 is required for gastrulation (Barrett et al., 1997; Hacker and Perrimon, 1998), Pebble and RacGAP50C are required for cytokinesis (Prokopenko et al., 1999; Somers and Saint, 2003), whereas GEF64C and Trio are necessary for neurogenesis (Bashaw et al., 2001; Bateman et al., 2000).

Pebble (PBL), a putative Rho GTP exchange factor (RhoGEF), is required specifically for the cytokinesis phase of the cell cycle (Hime and Saint, 1992; Lehner, 1992; Prokopenko et al., 1999). In *pbl* mutant embryos, cells fail to divide at cycle 14 of mitosis resulting in embryonic lethality (Hime and Saint, 1992; Lehner, 1992). Although the cytokinetic role of PBL and its mammalian orthologue, the proto-oncogene ECT2, has been the subject of considerable analysis (O'Keefe et al., 2001; Somers and Saint, 2003; Tatsumoto et al., 1999), some aspects of the pattern of accumulation of PBL, such as the high level of protein in interphase nuclei and the tissue-specific expression patterns during development (Prokopenko et al., 2000) suggested that PBL might play roles in processes other than cytokinesis. We show here that, in addition to a failure in cytokinesis, *pbl* mutants display a defect in the epithelial to mesenchymal transition (EMT) during mesoderm development. The EMT is a general term given to the process by which cells in an epithelium lose their contacts with neighbouring cells and adopt a migratory mesenchymal morphology. An EMT is not a single event. Rather, it is a series of coordinated changes in cell-cell adhesion, cell-matrix interactions and cytoskeletal organisation. The mesodermal EMT occurs in wild-type embryos immediately after invagination, when the mesodermal cells dissociate from their epithelial neighbours and migrate dorsally, forming a monolayer over the underlying ectoderm (Leptin, 1999; Wilson and Leptin, 2000). We show here that during this process, wild-type mesodermal cells adopt a migratory morphology, extending protrusions in the direction of migration. In *pbl* mutants, mesodermal cells extend fewer protrusions and fail to migrate correctly. Furthermore, we show that this phenotype requires the GEF activity of *pbl* and is not a secondary consequence of the cytokinetic role of PBL. These observations identify a novel, PBL-dependent intracellular signalling pathway required for the transition of mesodermal cells from an epithelial to a migratory mesenchymal state during *Drosophila* embryogenesis.

Materials and methods

Drosophila stocks and crosses

The following mutations and transgenes were used in this study: pbl^2, pbl^3, pbl^5 (Jurgens et al., 1984; Prokopenko et al., 1999), *paired-GAL4* (Brand and Perrimon, 1993), *twist-GAL4* (Greig and Akam, 1993), *UAS-pbl3.2* (Prokopenko et al., 1999), *UAS-myc-pbl* (a construct encoding a myc-tagged PBL) (L. O'Keefe and R.S., unpublished), $UAS\text{-}pbl^{\Delta BRCT}$ (A. Harley and R.S., unpublished), $UAS\text{-}pbl^{\Delta DH}$ (Prokopenko et al., 1999) and *UAS-GFP-Actin* (Verkhusha et al., 1999). Expression of all *UAS pbl* constructs was confirmed by immunohistochemistry. Stocks were maintained over marked balancers where necessary.

In situ hybridisation

A 961 bp fragment corresponding to nucleotides 2179-3140 of the *pbl* cDNA (CG8114) and the full-length *twist* cDNA (gift from M. Frasch) were linearised and labelled with digoxigenin according to the manufacturer's protocol (Roche). The labelled probes were hybridised to embryos collected and fixed using standard methods and detected as described previously (Tautz and Pfeifle, 1989).

Sectioning of embryos

Stained embryos were dehydrated through an ethanol series to 100% ethanol. Embryos were then transferred to dry acetone followed by a 1:1 ratio of dry acetone and araldite (ProSciTech). Once the embryos had equilibrated, the dry acetone and araldite was replaced twice with araldite alone. The embryos were then orientated for sectioning in embedding moulds, polymerised at 60°C for 2 days and sectioned on a microtome (Sorvall). The sections were photographed using a Zeiss Axioscope and processed using Adobe Photoshop.

Antibody stains

The primary antibodies used were as follows: mouse anti-EVE (1:450) (Developmental Studies Hybridoma Bank), rabbit anti-Spectrin (1:50) (gift from D. Branton, Harvard University), mouse anti-Fasciclin 3 (1:1) (Developmental Studies Hybridoma Bank), mouse anti-Muscle Myosin heavy chain (MHC) (1:10) (Kiehart and Feghali, 1986), rabbit anti-βGal (1:500) (Jackson ImmunoResearch Laboratories) and rabbit anti-GFP (1:200) (Jackson ImmunoResearch Laboratories). Secondary antibodies used were goat anti-mouse AP (1:500), goat anti-rabbit HRP (1:500), goat anti-rabbit Cy5 (1:200) (Jackson ImmunoResearch Laboratories), goat anti-mouse Alexa 488 (1:200) and goat anti-rabbit Alexa 488 (1:200) (Molecular Probes). Embryos for fluorescence imaging were stained with Hoechst 33258 (10 μg/ml) to visualise the nuclei. Antibody stains were photographed using a Zeiss Axioscope or a DeltaVision (Applied Precision) deconvolution microscopy system and processed using Adobe Photoshop.

Embryos were stained for F-actin using Phalloidin-TRITC (Sigma). Phalloidin and anti-GFP stains were imaged using a Leica TCS SP2 Inverted Confocal System. For Fig. 8, heterozygous embryos were

chosen based on the strength of a fluorescently labelled marker on the TM3 balancer.

EVE-positive hemisegment counts and Kolmogorov-Smirnov test

The numbers of EVE-positive hemisegments for the control and the experimental embryos (n=50) at stage 11 were determined [adapted from Michelson et al. (Michelson et al., 1998)]. For the analysis, thoracic segments T1-T3 and abdominal segments A1-A8 on both sides of the embryo were scored. A hemisegment was scored as EVE positive if one or more EVE-positive mesodermal cells were present. The mean and standard error of the mean (s.e.m.) were calculated for each dataset, and graphs were drawn using Microsoft Excel. The control and experimental embryos were compared using a Kolmogorov-Smirnov test (KS-test) (Conover, 1999) to determine whether the two datasets differed significantly. In the KS-test the maximum vertical deviation between the cumulative distribution functions for the two samples is calculated to determine whether there is a significant difference.

Results

pebble expression in the presumptive mesoderm is developmentally regulated

pebble (*pbl*), a regulator of the Rho family, has previously been shown to be expressed in proliferating tissues, consistent with its role in cytokinesis (Prokopenko et al., 2000). Some observations, such as the presence of high levels of PBL protein in the nuclei of interphase cells, suggested that the primary cytokinetic phenotype of *pbl* mutants could be obscuring other roles for PBL during *Drosophila* development. As a first step in examining this possibility, the *pbl* expression pattern was re-analysed by whole-mount in situ hybridisation with a *pbl* RNA antisense probe. As reported previously, *pbl* mRNA was found to be present at high levels in pole cells at cellularisation (Fig. 1A) and zygotic expression was induced during interphase of cycle 14 (Prokopenko et al., 2000). However, induction of zygotic expression was not uniform throughout the embryo. Specifically, expression was lower in the ventral region of the blastoderm epithelium than in other parts of the embryo (Fig. 1B). *pbl* expression in the presumptive mesoderm was first observed immediately prior to invagination (Fig. 1C), the expression pattern becoming more pronounced and discrete as stage 6 progresses (Fig. 1D,E). After mesoderm invagination, *pbl* is strongly expressed in the invaginated tissue (Fig. 1F).

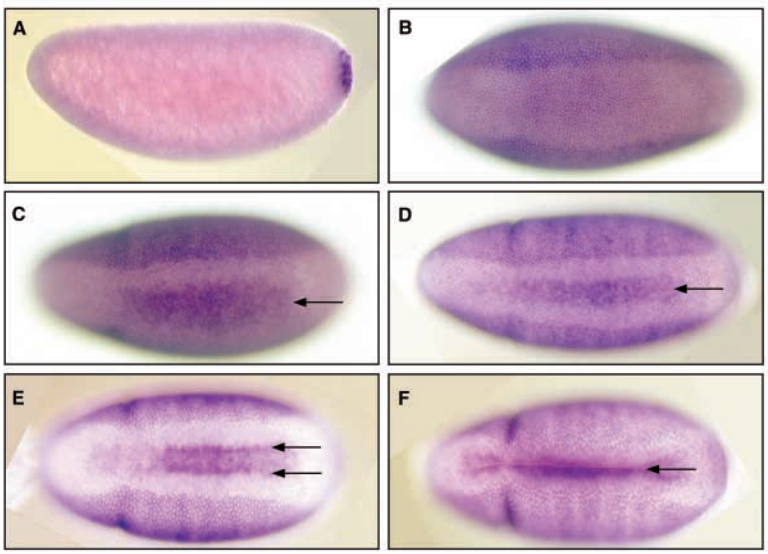

Fig. 1. *pbl* expression in the mesoderm is developmentally regulated. Whole-mount in situ hybridisation of wild-type embryos with a *pbl* RNA antisense probe. (A) During early stage 5, *pbl* transcripts are restricted to the pole cells. (B-F) Ventral view. (B) At late stage 5, *pbl* transcripts are observed on either side of the presumptive mesoderm. (C) At the onset of gastrulation, *pbl* transcripts are evident in the presumptive mesoderm (arrow). (D) This expression becomes restricted as stage 6 progresses (arrow). (E) *pbl* transcripts accumulate in a band of cells at the edge of the invaginating mesoderm (arrows). (F) As invagination proceeds, *pbl* transcripts are concentrated in the ventral furrow (arrow).

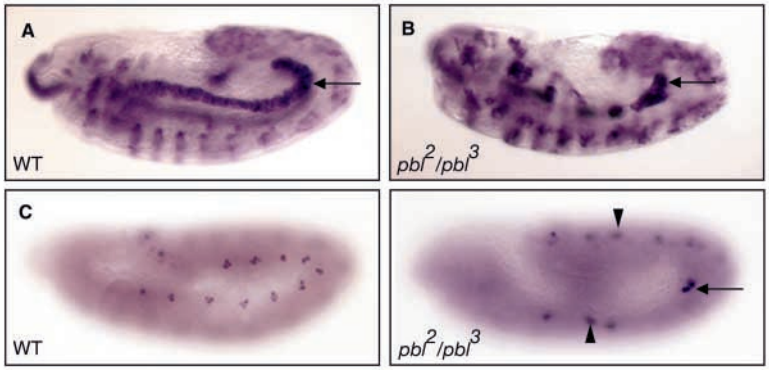

Fig. 2. *pbl* mutant embryos have reduced numbers of EVE-positive mesodermal cells. (A,B) Anti-Fasciclin 3 stains and (C,D) anti-EVE stains of wild-type (A,C) and pbl^2/pbl^3 mutant embryos (B,D). (A) At stage 12 in a wild-type embryo, the visceral mesoderm is seen as a band of cells running along the anteroposterior axis (arrow). (B) In a similarly staged pbl^2/pbl^3 mutant embryo, the visceral mesoderm is evident, although it is less organised (arrow). (C) At stage 11 in a wild-type embryo, EVE is expressed in segmentally repeated clusters of dorsal mesodermal cells. The EVE-expressing neuroblasts are out of focus in this image. (D) In a similarly staged pbl^2/pbl^3 mutant embryo, the majority of EVE-positive mesodermal cells fail to form. One EVE-positive hemisegment can be seen in this embryo (arrow). Based on their position and morphology, the remainder of the EVE-expressing cells are neuroblasts (arrowheads).

The majority of EVE-positive mesodermal cells fail to form in *pebble* mutant embryos

The mesodermal pattern of expression prompted us to explore the nature of mesoderm development in *pbl* mutant embryos. Mesoderm development is a highly dynamic process characterised by significant cell shape change and cell movement. Initially, the mesoderm is defined on the ventral surface of the blastoderm embryo by the expression of two zygotic genes, *twist* (*twi*) and *snail* (*sna*) (Leptin, 1991). During gastrulation, cells of the mesoderm primordium are internalised and undergo an epithelial-mesenchymal type of transition in which the epithelial structure breaks down, the mesodermal cells dissociate from one another and migrate

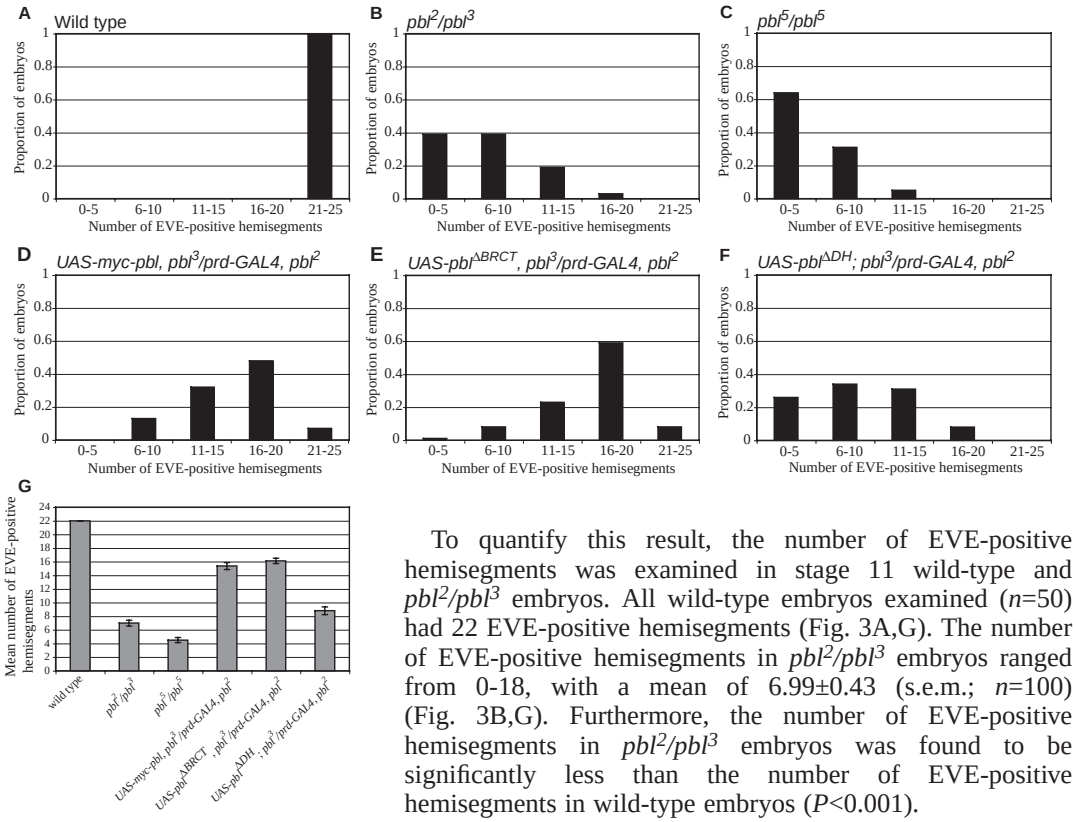

Fig. 3. The number of EVE-positive hemisegments is reduced in *pbl* mutant embryos. (A-F) Histograms of the number of EVE-positive hemisegments in wild-type (A), *pbl*^{2}/*pbl*^{3} (B), *pbl*^{5}/*pbl*^{5}(C), *UAS-myc-pbl, pbl*^{3}*/prd-GAL4, pbl*^{2} (D), *UAS-pbl*$^{\Delta BRCT}$*, pbl*^{3}*/prd-GAL4, pbl*^{2} (E) and *UAS-pbl*$^{\Delta DH}$*; pbl*^{3}*/prd-GAL4, pbl*^{2} (F) embryos. (G) The mean number of EVE-positive hemisegments in all genotypes examined. Error bars indicate the standard error of the mean (s.e.m.).

dorsally, forming a monolayer over the underlying ectoderm (Leptin, 1999). Cells in different locations then encounter intercellular signalling molecules such as Wingless (WG) and Decapentaplegic (DPP), which induce different subsets of mesodermal cells to adopt different fates (see Frasch, 1999).

To investigate whether *pbl* plays a role in mesoderm development, we used a variety of markers to examine the fate of different types of mesodermal tissues in *pbl* mutant embryos. We used anti-Fasciclin 3 and anti-Muscle Myosin heavy chain (MHC) to visualise visceral mesoderm and somatic mesoderm respectively. We also used an anti-Even skipped (EVE) antibody, which, at stage 11, stains segmentally repeated clusters of dorsal mesodermal cells, which give rise to two pericardial cells and two somatic muscles (Frasch, 1987; Carmena et al., 2002). Anti-EVE also stains a subset of cells in the CNS (Frasch et al., 1987), but these cells are located ventrally and are readily distinguishable from the pericardial cells by their location and morphology.

pbl embryos transheterozygous for the amorphic alleles *pbl*^{2} and *pbl*^{3} (*pbl*^{2}/*pbl*^{3}), were found to develop abnormal visceral mesoderm (Fig. 2A,B) and somatic musculature (data not shown). The visceral mesoderm is no longer seen as a continuous band running along the anterior to posterior axis on each side of the embryo (Fig. 2B) and the fibres of the somatic musculature are irregular in structure (data not shown). Anti-EVE staining revealed a more striking phenotype. The number of EVE-positive mesodermal cells in *pbl*^{2}/*pbl*^{3} embryos was dramatically reduced compared with wild-type embryos (Fig. 2C,D). The only cells stained with anti-EVE in many *pbl*^{2}/*pbl*^{3} embryos were neuroblasts, based on their ventral location and on their morphology (Fig. 2D).

To quantify this result, the number of EVE-positive hemisegments was examined in stage 11 wild-type and *pbl*^{2}/*pbl*^{3} embryos. All wild-type embryos examined (n=50) had 22 EVE-positive hemisegments (Fig. 3A,G). The number of EVE-positive hemisegments in *pbl*^{2}/*pbl*^{3} embryos ranged from 0-18, with a mean of 6.99±0.43 (s.e.m.; n=100) (Fig. 3B,G). Furthermore, the number of EVE-positive hemisegments in *pbl*^{2}/*pbl*^{3} embryos was found to be significantly less than the number of EVE-positive hemisegments in wild-type embryos (P<0.001).

The transition of the mesoderm from epithelium to migratory mesenchyme is aberrant in *pebble* mutant embryos

EVE expression in the mesodermal cells is confined to the dorsal mesoderm, which is dependent on induction by DPP secreted by the dorsal ectoderm (Gisselbrecht et al., 1996; Lin et al., 1999; Shishido et al., 1997). Failure to form EVE-positive mesodermal cells could result from an inability to read or transduce the DPP signal. Alternatively, it could result from a failure of mesodermal cells to migrate dorsally to a position where they would encounter the DPP signal. To investigate the latter possibility, the invaginated population of mesodermal cells in wild-type and *pbl* mutant embryos was examined by in situ hybridisation with a full-length *twist* RNA antisense probe (Fig. 4A,B).

In late stage 10 wild-type embryos, the mesodermal cells have migrated to form a uniform layer, such that the dorsalmost mesodermal cells are adjacent to the dorsalmost ectodermal cells (Leptin and Grunewald, 1990) (Fig. 4A). In late stage 10 *pbl*^{2}/*pbl*^{3} embryos, the cells do not form a uniform layer and appear aggregated. Moreover, they fail to migrate to a position adjacent to the dorsalmost ectodermal cells (Fig. 4B).

To further characterise this defect, embryos were stained for F-actin and optical cross-sections obtained. In stage 10 wild-type embryos, the mesodermal cells consistently spread into a uniform monolayer on the inner surface of the ectoderm (Fig. 4C). Mesodermal cells in the same stage *pbl*^{2}/*pbl*^{3} embryos were typically less spread out (Fig. 4D,E). There was some variability in the extent of this phenotype. In some cases, there was complete failure to dissociate from the aggregation along the midline (Fig. 4D), while in other cases a relatively uniform monolayer of binucleate cells developed (data not shown). This

variability correlates with the variability observed in the number of EVE-positive hemisegments formed in *pbl* mutant embryos. Mesodermal cells in pbl^2/pbl^3 embryos also appeared less rounded than control embryos and were tightly packed (Fig. 4E).

The use of transheterozygous pbl^2/pbl^3 embryos in our phenotypic analysis should have avoided any complications from second site mutations. However, to confirm that loss of *pbl* was the cause of the mesoderm phenotype, we used the GAL4/UAS system (Brand and Perrimon, 1993) to rescue the mutant phenotype by expression of wild-type PBL with the mesodermal-specific driver *twist-GAL4 (twi-GAL4)*. To distinguish the mesodermal cells from the adjacent ectoderm, we co-expressed GFP-Actin and wild-type PBL in a *pbl* mutant background. In stage 10 control pbl^2/pbl^3 mutant embryos expressing GFP-Actin (Fig. 4F), mesodermal cells exhibited a similar phenotype to mesodermal cells examined in pbl^2/pbl^3 embryos (Fig. 4D-E). By contrast, in stage 10 embryos also expressing PBL (Fig. 4G), mesodermal cells had dissociated and migrated to form a monolayer over the underlying ectoderm, similar to that seen in wild-type embryos (Fig. 4A,C). Expression of PBL with *twi-GAL4* rescued cytokinesis in the mesodermal cells, but not in the ectoderm (Fig. 4G′). These results demonstrate a cell-autonomous requirement for PBL in mesodermal cells as they migrate to form a monolayer. They also show that this process does not depend on PBL-dependent cytokinesis in the underlying ectodermal cells.

To further characterise the *pbl* phenotype, we used *twi-GAL4* driven GFP-Actin to examine the morphology of migrating mesodermal cells in stage 8 wild-type and pbl^2/pbl^3 embryos (Fig. 5). Embryos expressing GFP-Actin alone appeared to develop normally and gave rise to viable and fertile adults. In addition to clearly labelling cellular protrusions, cytoplasmic GFP-Actin was excluded from nuclei during interphase, allowing us to identify cells undergoing mitosis (data not shown).

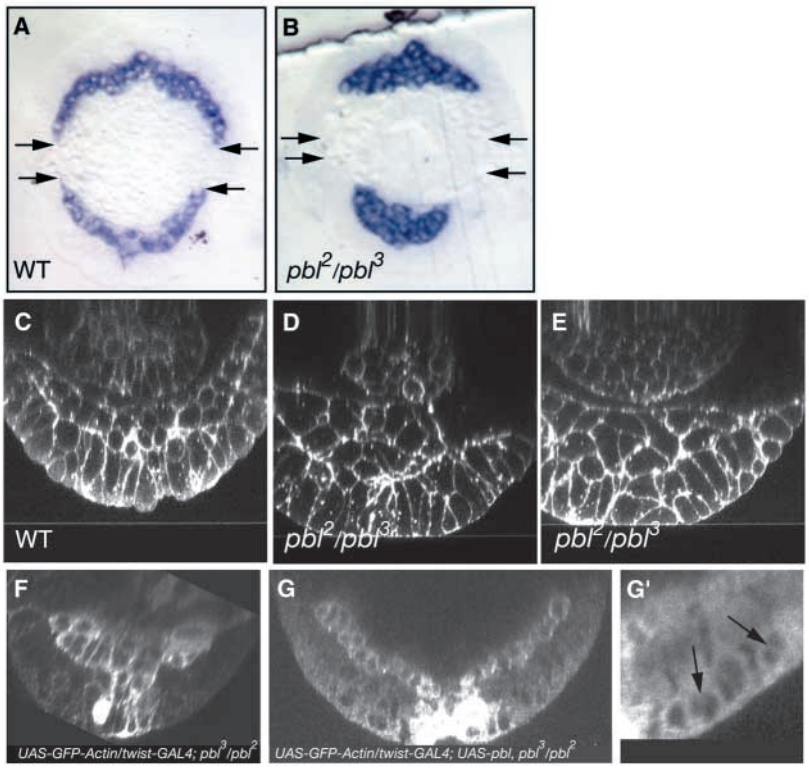

Fig. 4. *pbl* mutant embryos have a mesodermal cell migration defect. (A,B) Transverse sections of in situ hybridisations with a *twi* antisense RNA probe. (A) In late stage 10 wild-type embryos, the mesodermal cells have dissociated and migrated dorsally to form a uniform layer beneath the ectoderm (arrows indicate dorsalmost mesodermal cells, which lie adjacent to the dorsalmost epidermal cells). (B) In a similarly staged pbl^2/pbl^3 mutant embryo, the mesodermal cells appear aggregated and have failed to complete dorsolateral migration (arrows indicate the dorsalmost epidermal cells). (C) A stage 10, wild-type embryo stained for F-Actin. Mesodermal cells have formed a monolayer beneath the ectoderm. (D,E) Equivalently staged pbl^2/pbl^3 embryos, in which mesoderm spreading is defective. (F) A stage 10 pbl^2/pbl^3 embryo expressing GFP-Actin driven by *twi-GAL4* visualised with an anti-GFP antibody. Similar to D,E, the spreading of the mesoderm is defective. (G) A stage 10, pbl^2/pbl^3 embryo co-expressing wild-type PBL and GFP-Actin with *twi-GAL4* visualised with an anti-GFP antibody. The dissociation and migration of the mesodermal cells has been rescued, such that the mesodermal cells form a uniform layer beneath the ectoderm similar to wild type. (G′) The same embryo showing multinucleate cells in the ectoderm (arrows).

In wild-type embryos, mesodermal cells undergo their first mitotic divisions at early stage 8, shortly after gastrulation (Bate, 1993; Campos-Ortega and Hartenstein, 1985). The cells then disaggregate and begin to spread dorsally (Fig. 5B,C), before undergoing a second round of mitosis at stage 8/9 (Bate, 1993; Campos-Ortega and Hartenstein, 1985). To assess changes in the morphology of mesodermal cells, we examined embryos between these two rounds of mitosis (Fig. 5A). Embryos were oriented so that the leading edge of the migrating cells was parallel to the microscope stage (Fig. 5B).

In wild-type embryos, migrating mesodermal cells were polarised, with numerous protrusions evident in the direction of motion, and a more rounded profile observed on the trailing side (Fig. 5C). Wild-type cells also appeared to lose their close association with neighbouring mesodermal cells, with gaps between cells at the leading edge being common (Fig. 5C). Similarly, cells further back from the leading edge, which were not adjacent to epidermal cells (Fig. 5B), appeared rounded with gaps visible between neighbouring cells (Fig. 5E).

By contrast, mesodermal cells in a pbl^2/pbl^3 mutant background had fewer protrusions in the direction of motion, and appeared to be more closely adhered/associated with neighbouring mesodermal cells (Fig. 5D). This was particularly clear for those cells adjacent to the epidermis, but was also a feature of cells further into the mass of aggregated mesodermal cells, where cells were less rounded and more solidly packed together, leaving fewer intercellular gaps (Fig. 5F).

Thus, the failure to form EVE-positive mesodermal cells in *pbl* mutants is explained by the failure of *pbl*-deficient mesodermal cells to correctly undergo the epithelial-mesenchymal transition and subsequent dorsal migration that normally follows invagination.

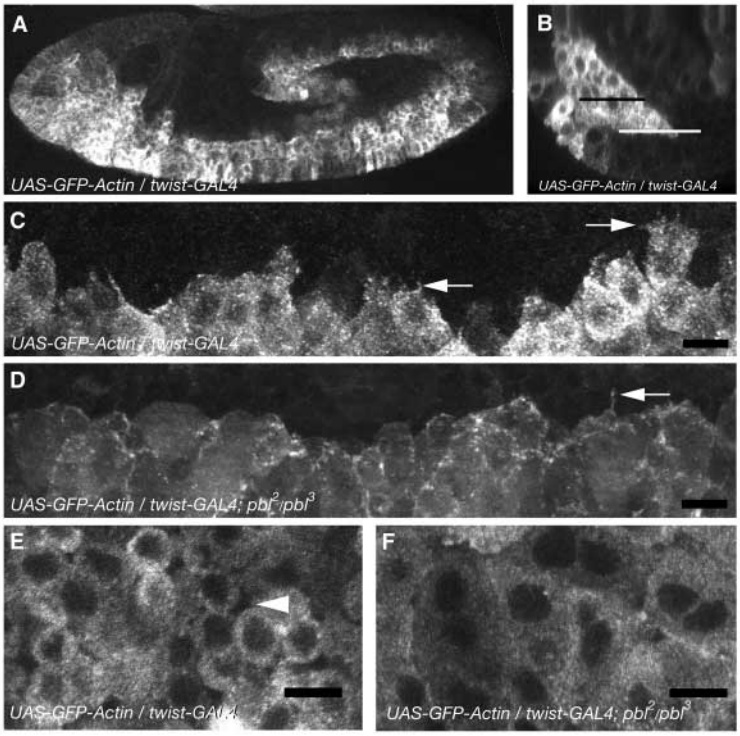

Fig. 5. The morphology of mesodermal cells is defective in *pbl* mutant embryos. Control (A-C,E) and pbl^2/pbl^3 mutant (D,F) stage 8 embryos in which GFP-Actin was expressed in mesodermal cells using the *twist-GAL4* driver and visualised with an anti-GFP antibody. (A) A *UAS-GFP-Actin/twist-GAL4* control embryo typical of the stage used in this morphological analysis, between the first two waves of mitosis in the mesoderm. (B) Cross-section of a control embryo expressing GFP-Actin in mesodermal cells. Embryos were oriented so that the leading mesodermal cells were parallel to the plane of the microscope. The white line indicates the plane of focus seen in C,D. The black line indicates a deeper plane of focus seen in E,F. (C,D) Projections of 1 μm optical sections of mid-stage 8 control (C) and pbl^2/pbl^3 (D) embryos showing the morphology of migrating mesodermal cells at the leading front. (C) Cells in a control embryo exhibit numerous protrusions (arrows) in the direction of migration and appear dissociated from each other. (D) Cells in a pbl^2/pbl^3 embryo extend far fewer protrusions (arrow) and appear more tightly adhered to their mesodermal neighbours. (E) Mesodermal cells in a control embryo appear more rounded with numerous intercellular gaps (arrowhead) present. (F) Cells in a pbl^2/pbl^3 embryo appear more tightly packed and are less rounded, with fewer intercellular gaps. Scale bars: 10 μm.

The *pebble* mesoderm phenotype is not a secondary consequence of failed cytokinesis

In *pbl* mutant embryos, cytokinesis fails during the 14th mitotic cycle, the first cycle that exhibits cytokinesis (Hime and Saint, 1992). As a result, mesodermal cells become multinucleate during the epithelial-mesenchymal transition and subsequent migration of mesodermal cells. It was possible therefore, that the mesoderm phenotypes observed were simply the consequence of an inability of the large bi- and multi-nucleate cells to undergo a normal epithelial-mesenchymal transition and/or migration. To address this possibility and to determine which domains of PBL were required for the mesodermal EMT and subsequent EVE-positive mesodermal cell formation, we used a rescue assay previously developed in our laboratory (L. O'Keefe and R.S., unpublished) in which alternating stripes of cells express *UAS-pbl* constructs under the control of the embryonic driver *paired-GAL4* (*prd-GAL4*). This assay has the advantage of having internal control stripes that show the *pbl* mutant phenotype, even when the phenotype of the alternating stripes is modified by expression of the construct. Expression of wild-type PBL with a *prd-GAL4* driver in a *pbl* mutant background results in stripes of rescue of the cytokinetic phenotype, which can be visualised in the epidermis by anti-Spectrin staining (L. O'Keefe and R.S., unpublished) (see Fig. 6E,G).

Expression of wild-type PBL using the *prd-GAL4* driver was able to rescue the number of EVE-positive hemisegments in pbl^2/pbl^3 embryos (Fig. 6A). The number of EVE-positive hemisegments in *UAS-myc-pbl, pbl*^{3}*/prd-GAL4, pbl*^{2} embryos ranged from 7-22, with a mean of 15.37±0.51 (n=60) (Fig. 3D,G). This was significantly greater (P<0.001) than the number of EVE-positive hemisegments in pbl^2/pbl^3 embryos.

At its N terminus, PBL contains two BRCT (BRCA1 C-terminal) domains. BRCT domains are found in the familial breast cancer tumour suppressor gene, BRCA1, and in many other proteins involved in DNA damage sensing and repair (Bork et al., 1997; Callebaut and Mornon, 1997). The N-terminal BRCT domain contains an extended region of homology we have named the RadECl domain (Somers and Saint, 2003).

prd-GAL4 driven expression of a construct, termed $pbl^{\Delta BRCT}$, lacking the majority of the RadECl region and the two consensus BRCT domains (Fig. 6I), failed to rescue cytokinesis in *pbl* mutant embryos (A. Harley and R.S., unpublished) (Fig. 6F,H). By contrast, the EVE-positive mesodermal cell phenotype in *pbl* mutant embryos was rescued (Fig. 6B,D). The number of EVE-positive hemisegments in *UAS-pbl*$^{\Delta BRCT}$*, pbl*^{3}*/prd-GAL4, pbl*^{2} embryos ranged from 4-22, with a mean of 16.12±0.39 (n=86) (Fig. 3E,G). Again, this was significantly greater (P<0.001) than the number of EVE-positive hemisegments in pbl^2/pbl^3 embryos.

To further characterise the effect of $PBL^{\Delta BRCT}$ on mesodermal cells we used the *twi-GAL4* driver to express both $PBL^{\Delta BRCT}$ and GFP-Actin in a pbl^2/pbl^3 mutant background. As expected, expression of $PBL^{\Delta BRCT}$ in migrating mesodermal cells did not rescue cytokinesis (Fig. 6K,L). It did, however, substantially shift the mesodermal cell morphology towards wild type, with binucleate cells often exhibiting numerous protrusions (Fig. 6J,L) and appearing more rounded and less closely adhered to each other (Fig. 6K). These data show that the failure in cytokinesis and the failure in mesoderm development in *pbl* mutants are separable.

The RhoGEF function of Pebble is required for normal mesoderm development

Mesodermal cell migration is a dynamic process that requires significant actin cytoskeleton rearrangements. The Rho family of small GTPases and their regulators are known to be required for numerous actin-based processes during *Drosophila*

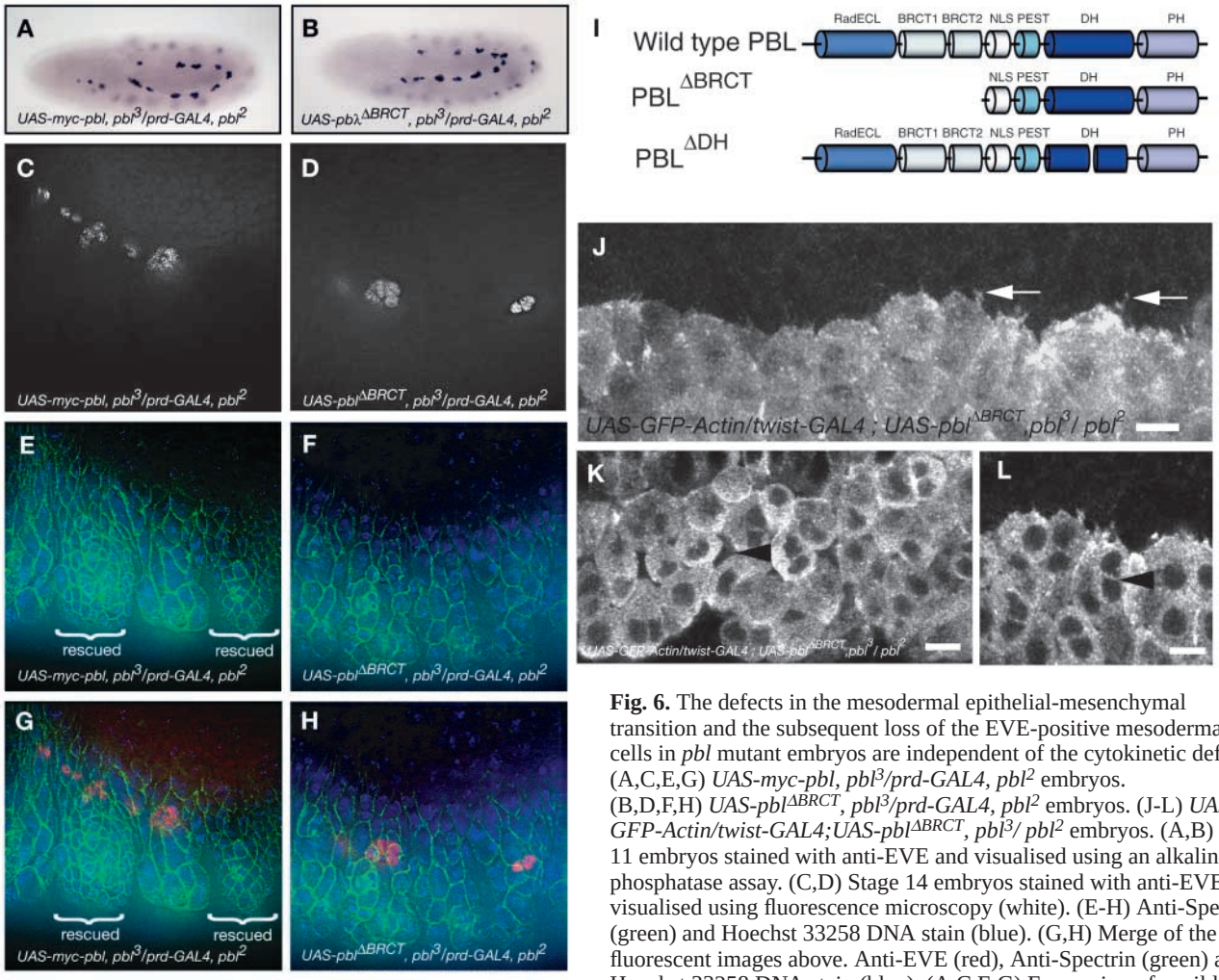

Fig. 6. The defects in the mesodermal epithelial-mesenchymal transition and the subsequent loss of the EVE-positive mesodermal cells in *pbl* mutant embryos are independent of the cytokinetic defect. (A,C,E,G) *UAS-myc-pbl, pbl*^{3}*/prd-GAL4, pbl*^{2} embryos. (B,D,F,H) *UAS-pbl*$^{\Delta BRCT}$*, pbl*^{3}*/prd-GAL4, pbl*^{2} embryos. (J-L) *UAS-GFP-Actin/twist-GAL4;UAS-pbl*$^{\Delta BRCT}$*, pbl*^{3}*/ pbl*^{2} embryos. (A,B) Stage 11 embryos stained with anti-EVE and visualised using an alkaline phosphatase assay. (C,D) Stage 14 embryos stained with anti-EVE and visualised using fluorescence microscopy (white). (E-H) Anti-Spectrin (green) and Hoechst 33258 DNA stain (blue). (G,H) Merge of the fluorescent images above. Anti-EVE (red), Anti-Spectrin (green) and Hoechst 33258 DNA stain (blue). (A,C,E,G) Expression of a wild-type copy of a *pbl* cDNA in a *pbl* mutant background rescues the EVE-positive mesodermal cell formation and the cytokinetic defect. (A) EVE-positive mesodermal cells are evident in embryos rescued with the wild-type *pbl* cDNA. (C,E,G) EVE-positive mesodermal cells are evident at a higher magnification (C,G) and rescue of cytokinesis is seen in epidermal cells in alternating stripes corresponding to the *prd-GAL4* induced wild-type *pbl* expression (E,G). (D,F,H) Pbl$^{\Delta BRCT}$ expression rescues the EVE-positive mesodermal cell defect (D,H) but fails to rescue the cytokinetic phenotype in *pbl* mutants (F,H). Note that the EVE-positive mesodermal cells in C,D are imaged at a different focal plane than the epidermis shown in E,F. (I) Schematic representations of the PBL constructs used in the EVE-positive hemisegment rescue assay. (J-L) Expression of PBL$^{\Delta BRCT}$ using the *twist-GAL4* driver alters the cellular phenotype of GFP-Actin-expressing mesodermal cells. (J) Cells extend more protrusions (arrows) in the direction of migration. (K) Cell bodies in the central mass of mesodermal cells appear more rounded (compare with Fig. 5F) and intercellular gaps (arrowhead) are present. (L) Single optical slice of cells in J showing binucleate cells (arrowhead) more clearly. Scale bars: 10 μm.

development. Therefore, we investigated whether the GEF function of PBL was required for mesodermal cell migration and subsequent EVE-positive mesodermal cell formation. GEF proteins are characterised by the presence of two domains at their C terminus, a Dbl Homology (DH) and a Pleckstrin Homology (PH) domain (Whitehead et al., 1997). Experiments with a number of RhoGEFs have revealed that point mutations and deletions within the DH domain significantly reduce the exchange activity (Hart and Roberts, 1994; Liu et al., 1998; Ron et al., 1991; Steven et al., 1998; Whitehead et al., 1995).

To address whether PBL was functioning as a RhoGEF in mesodermal cell migration, we carried out two experiments. In the first, a GEF mutated form of PBL, PBL$^{\Delta DH}$, in which amino acids 497-549 within the DH domain are removed (Fig. 6I) (Prokopenko et al., 1999), was expressed using a *prd-GAL4* driver in a *pbl* mutant background. Expression of *UAS-pbl*$^{\Delta DH}$ with *prd-GAL4* in a *pbl* mutant background failed to rescue the EVE-positive mesodermal cell formation phenotype (Fig. 7A). The number of EVE-positive hemisegments in *UAS-pbl*$^{\Delta DH}$*; pbl*^{3}*/prd-GAL4, pbl*^{2} embryos ranged from 0-18, with a mean of 8.81±0.56 (*n*=61) (Fig. 3F,G). This number is similar to the number observed in *pbl*^{2}*/pbl*^{3} mutant embryos. We conclude that the DH domain, and therefore the GEF activity, is required for EVE-positive mesodermal cell formation.

The second approach used the *pbl*^{5} allele that contains a single missense mutation in the most highly conserved region (CR3) of the DH domain (Prokopenko et al., 1999). This point mutation (valine to an aspartate at amino acid 531) has been shown in other systems to significantly reduce the nucleotide exchange activity of RhoGEFs (Liu et al., 1998; Prokopenko

et al., 1999). Consistent with this observation, *pbl*^{5} homozygous mutant embryos exhibit a strong cytokinetic phenotype. *pbl*^{5} homozygotes were found to have few EVE-positive hemisegments (Fig. 7B). The number of EVE-positive hemisegments in *pbl*^{5}/*pbl*^{5} mutant embryos ranged from 0-14, with a mean of 4.5±0.37 (*n*=100) (Fig. 3C,G). This number is even fewer than the number observed in *pbl*^{2}/*pbl*^{3} embryos.

We also examined the morphology of *pbl*^{5} mutant cells using F-actin staining and mesodermal expression of GFP-Actin. The results were comparable with *pbl*^{2}/*pbl*^{3} embryos, with mesodermal cells showing a similar range of defects in spreading (Fig. 7C), morphology (Fig. 7E) and the extent of rounding/dissociation in the body of the mesoderm (Fig. 7D). These results show that the GEF activity of PBL is required for the normal epithelial-mesenchymal transition, migratory morphology and subsequent formation of EVE-positive mesodermal cells.

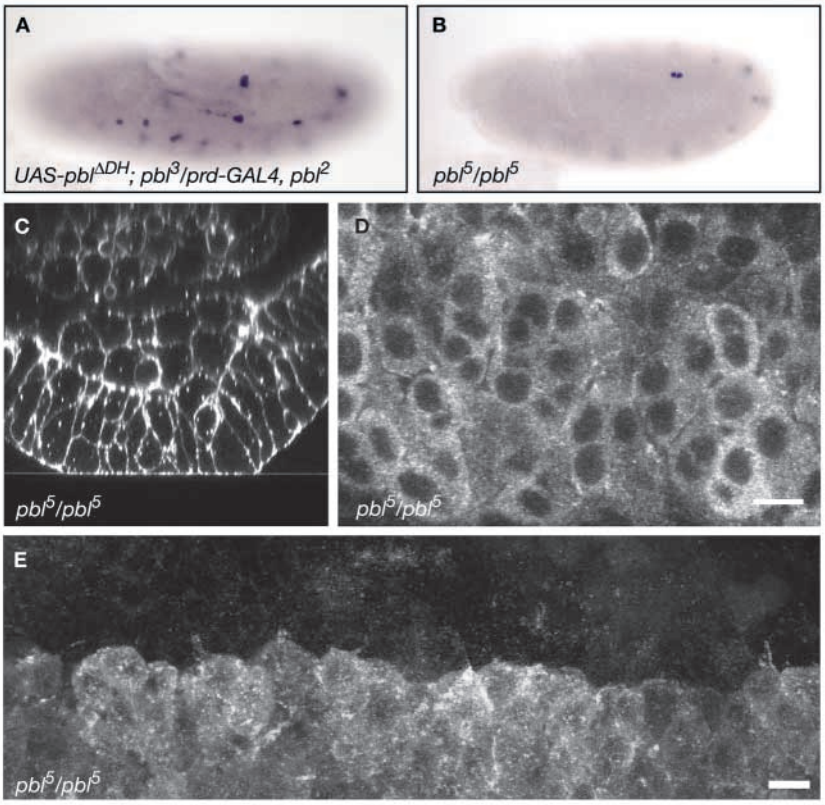

Fig. 7. The GEF function of PBL is required for the transition of the mesoderm from an epithelium to migratory cells during *Drosophila* gastrulation. (A,B) The majority of EVE-positive hemisegments fail to form in *UAS-pbl*$^{\Delta DH}$*; pbl*^{3}*/prd-GAL4, pbl*^{2} (A) and *pbl*^{5}/*pbl*^{5} embryos (B). (C) Cross-section of a stage 10 *pbl*^{5}/*pbl*^{5} embryo stained for F-actin showing a typical failure of the mesoderm to disaggregate and spread dorsally. (D,E) *UAS-GFP-Actin/twist-GAL4; pbl*^{5}/*pbl*^{5} embryos show similar morphology to *UAS-GFP-Actin/twist-GAL4; pbl*^{2}/*pbl*^{3} embryos (compare with Fig. 5D,F). Scale bars: 10 μm.

The HTL/MAPK pathway is activated in *pebble* mutant embryos

Heartless (HTL), a receptor tyrosine kinsase (RTK) of the fibroblast growth factor receptor (FGFR) subfamily is required for the mesoderm EMT, where it is known to activate the conserved Ras/MAP kinase pathway (reviewed by Michelson et al., 1998). In *htl* mutant embryos, mesodermal cells fail to dissociate from each other following invagination and fail to migrate dorsally (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997). Mesoderm migration also fails in embryos mutant for three other genes: *Downstream-of-FGFR* (*Dof*) (Vincent et al., 1998), *Sugarless* and *Sulphateless* (Lin et al., 1999). In each case, the failure in mesoderm migration is accompanied by a failure in the activation of the Ras1/MAPK pathway (Lin et al., 1999; Vincent et al., 1998).

To investigate whether the *pbl* mutant phenotype was also due to a failure in the activation of the HTL/MAPK pathway, *pbl* mutant embryos were stained with an antibody directed towards the dual phosphorylated form of MAP kinase (dp-ERK) (Fig. 8) (Gabay et al., 1997a; Gabay et al., 1997b). In wild-type embryos following gastrulation, dp-ERK is expressed in the dorsalmost mesodermal cell rows on each lateral surface of the embryo, a staining pattern that is HTL dependent (Fig. 8A) (Gabay et al., 1997a; Gabay et al., 1997b). In *pbl* mutant embryos, dp-ERK staining is seen in the dorsalmost mesodermal cell rows similar to wild type (Fig. 8B). This result shows that PBL function is not required for HTL-dependent activation of the MAP kinase signalling pathway, and that the mesoderm migration defect in *pbl* mutants is not due to a failure in the activation of the MAPK pathway.

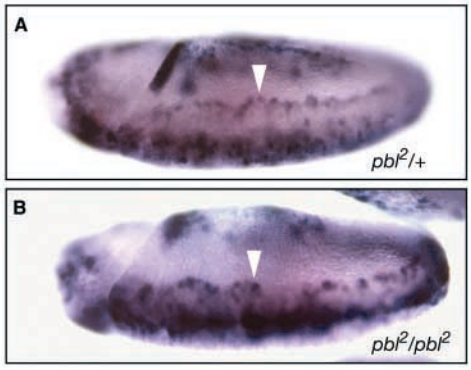

Fig. 8. PBL is not required for activation of the HTL/MAPK pathway. Ventrolateral view of stage 8 embryos heterozygous (A) and homozygous (B) for *pbl*^{2}. Activation of the MAPK pathway, visualised using an antibody specific to dpERK, the dual-phosphorylated form of the MAPK (Gabay et al., 1997a; Gabay et al., 1997b), is seen in the cells at the leading front (arrowheads) of the mesoderm during dorsal migration.

Discussion

The regulation and reorganisation of the actin cytoskeleton by the Rho family of small GTPases is central to the control of cell behaviour during embryonic development. We have shown that Pebble (PBL), a putative exchange factor for Rho, is

necessary for the transition of the *Drosophila* mesoderm from an epithelial to a mesenchymal layer of cells following ventral furrow formation. In wild-type mesodermal cells, this transition involves a series of events that includes dissociation of cells of the invaginated ventral furrow, settlement onto the ventral ectoderm and spreading of the cells dorsally to the edge of the ectoderm (reviewed by Leptin, 1999). In *pbl* mutant embryos, the initial loss of epithelial structure occurs, but the cells remain more tightly adhered to their neighbours and extend very few protrusions, failing to disperse and to migrate dorsally.

The Pebble RhoGEF is an essential component of an intracellular signalling pathway required for acto-myosin reorganisation during cytokinesis (Hime and Saint, 1992; Lehner, 1992; Prokopenko et al., 1999). Although cell division occurs during the epithelial-mesenchymal transition of mesodermal cells, there appears to be no causal connection between cell division and mesodermal cell behaviour. Cells in *string* (*stg*) mutant embryos, for example, arrest in G2 phase of cycle 14 (Edgar and O'Farrell, 1989) immediately prior to the stage at which the cytokinetic defect becomes evident in *pbl* mutant embryos. However, mesoderm spreading and subsequent EVE-positive mesodermal cell formation occurs in *string* (*stg*) mutant embryos (Carmena et al., 1998; Leptin and Grunewald, 1990), while it is aberrant in *pbl* mutant embryos. It is true that the nature of the cell cycle arrest in these two mutants is very different. *stg* mutant cells are mononucleate and arrested in G2 phase, while *pbl* mutant cells become binucleate, then multinucleate, undergoing apparently normal mitotic divisions without cytokinesis (Hime and Saint, 1992; Lehner, 1992). However, a causative role for cytokinesis failure in the mesoderm phenotype was ruled out by the observation that expression of a site-directed mutant form of *pbl* that lacks the N-terminal BRCT domains rescues the mesoderm phenotype but not the cytokinetic phenotype of *pbl*. We conclude, therefore, that the *pbl* mesodermal function appears to be distinct from its cytokinesis function.

It is well documented that mesoderm development, in particular the invagination, dissociation and migration of mesodermal cells, requires significant cytoskeletal mediated cell shape changes (Leptin and Grunewald, 1990). The first regulatory components implicated in these processes were RHO1 and the RHO1 activator, RhoGEF2, which are required for ventral furrow formation (Barrett et al., 1997; Hacker and Perrimon, 1998). However, very little is known about the control of the cytoskeleton in the dispersion and dorsal migration of mesodermal cells that follows ventral furrow formation.

Two lines of evidence suggest that PBL is required to reorganise the actin cytoskeleton in order for the cells to dissociate and migrate. First, we observed that the protrusions normally found at the leading edge of the dispersing ventral furrow cells were greatly reduced in *pbl* mutant embryos. Second, we found that the PBL RhoGEF activity is required for this process. We are yet to determine which Rho family small GTPase is activated by PBL in mesodermal tissues. Genetic analysis indicates that PBL acts in vivo as a GEF for RHO1 during cytokinesis (O'Keefe et al., 2001; Prokopenko et al., 1999). Rho activity is generally thought to stimulate acto-myosin contractile activity or to promote stress fibre formation in association with cell-cell or cell-matrix connections (Hall, 1998; Omelchenko et al., 2002; Ridley and Hall, 1992). Our observations that protrusions are greatly reduced in *pbl* mutant embryos, is more reminiscent of a loss of Rac and/or CDC42 activity than Rho activity. It is possible that PBL could be modified to target Rac and/or CDC42 in migrating mesodermal cells.

In addition to a reduction in the number of protrusive structures, *pbl* mutant mesodermal cells are less rounded and appear more tightly adhered to each other. The epithelial to mesenchymal transition during mesoderm development is known to involve a reduction in levels of DE-Cadherin (Oda et al., 1998). It is possible therefore, that PBL may play a role in reducing adhesion between mesodermal cells. In vertebrates, Rho family GTPases are known to positively regulate cadherin-based adhesion (Braga et al., 1997; Fukata et al., 1999). Their role in *Drosophila* is less clear. During tracheal morphogenesis, RAC1 appears to be required to negatively regulate cadherin adhesion to allow cell rearrangements to occur (Chihara et al., 2003). RHO1, however, appears to play a positive role. Zygotic loss of RHO1 causes mislocalisation of DE-Cadherin (Magie et al., 2002) and expression of dominant negative RHO1 can reduce DE-Cadherin levels and cell-cell adhesion (Bloor and Kiehart, 2002). However, overexpression of RHO1 in the wing imaginal disks can induce a type of EMT in which cells drop out of the epithelium, express lower levels of DE-Cadherin and can become displaced from their neighbours (Speck et al., 2003). One possibility therefore, is that PBL is acting through RHO1, or perhaps RAC1, to reduce adhesion between mesodermal cells allowing them to spread out over the epidermis. The delamination of neural crest cells from the neural tube, another example of an epithelial-mesenchymal transition, has been found to require RhoB (Liu and Jessell, 1998). It is possible therefore, that these biologically and evolutionarily different processes may be driven by related mechanisms.

PBL joins a small set of factors that have been shown to be required for mesodermal cell behaviour immediately following ventral furrow formation. These include Heartless (HTL), a *Drosophila* FGF Receptor homologue (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997), the Sugarless (SGL) and Sulfateless (SFL) enzymes required for the synthesis of extracellular glycosaminoglycans that facilitate FGF signalling (Lin et al., 1999), and intracellular factors acting downstream of HTL, including Downstream of FGFR (DOF) and Ras1 (Michelson et al., 1998; Vincent et al., 1998). PBL is unique in not being required for activation of the HTL/MAPK pathway. Whether PBL acts downstream of this pathway or in a parallel pathway also required for the mesoderm EMT is yet to be determined.

In conclusion, our studies identify a novel, PBL-mediated mechanism required for the epithelial to mesenchymal transition of mesodermal cells of the ventral furrow. The epithelial-to-mesenchymal transition is an important feature of animal development and it is also central to the spread of cancers. It will therefore be important to determine whether orthologues of PBL, such as the mammalian proto-oncogene ECT2, are playing roles in related processes.

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