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Sphingosine-1-phosphate receptors regulate individual cell behaviours underlying the directed migration of prechordal plate progenitor cells during zebrafish gastrulation

Masatake Kai[1], Carl-Philipp Heisenberg[2] and Masazumi Tada[1,*]

During vertebrate gastrulation, cells forming the prechordal plate undergo directed migration as a cohesive cluster. Recent studies revealed that E-cadherin-mediated coherence between these cells plays an important role in effective anterior migration, and that platelet-derived growth factor (Pdgf) appears to act as a guidance cue in this process. However, the mechanisms underlying this process at the individual cell level remain poorly understood. We have identified *miles apart* (*mil*) as a suppressor of defective anterior migration of the prospective prechordal plate in *silberblick* (*slb*)/*wnt11* mutant embryos, in which E-cadherin-mediated coherence of cell movement is reduced. *mil* encodes Edg5, a sphingosine-1-phosphate (S1P) receptor belonging to a family of five G-protein-coupled receptors (S1PRs). S1P is a lipid signalling molecule that has been implicated in regulating cytoskeletal rearrangements, cell motility and cell adhesion in a variety of cell types. We examined the roles of Mil in anterior migration of prechordal plate progenitor cells and found that, in *slb* embryos injected with *mil*-MO, cells migrate with increased motility but decreased directionality, without restoring the coherence of cell migration. This indicates that prechordal plate progenitor cells can migrate effectively as individuals, as well as in a coherent cluster of cells. Moreover, we demonstrate that Mil regulates cell motility and polarisation through Pdgf and its intracellular effecter PI3K, but modulates cell coherence independently of the Pdgf/PI3K pathway, thus co-ordinating cell motility and coherence. These results suggest that the net migration of prechordal plate progenitors is determined by different parameters, including motility, persistence and coherence.

KEY WORDS: Gastrulation, Cell movement, Wnt, S1P, Pdgf, Zebrafish

INTRODUCTION

Progenitor cells destined to form ectodermal, mesodermal and endodermal fates undergo several different cell movements that shape the embryonic body axis during vertebrate gastrulation. Different populations of mesodermal cells have unique cell behaviours underlying directional and co-ordinated cell movements. For example, prechordal plate progenitor cells undergo directed anterior migration as a small group of coherent cells, whereas in the presumptive notochord and presomitic mesoderm cells undergo co-ordinated rearrangement, called convergence and extension (C&E), as a large sheet of cells (reviewed by Heisenberg and Tada, 2002; Solnica-Krezel, 2005).

In *Xenopus*, once the mesoderm has involuted, the most anterior dorsal mesodermal cells migrate directionally toward the animal pole across the blastocoel roof of the ectoderm using fibronectin as a substrate (Nagel and Winklebauer, 1999). In zebrafish, the first internalised axial hypoblast cells that become fated to the prechordal plate move as a cohesive sheet of cells toward the animal pole of the embryo using the epiblast as a substrate (Montero et al., 2005; Ulrich et al., 2003). The cohesive property of prechordal plate progenitor cells is thought to provide a mechanism for effective directed migration (Ulrich et al., 2005), and is reminiscent of the collective migration seen in border cells in *Drosophila* and in lateral line progenitor cells in zebrafish (reviewed by Lecaudey and Gilmour, 2006; Rorth, 2007). However, the mechanism underlying this process remains poorly understood, in particular is collective migration really important or can cells migrate as individuals?

[1]Department of Anatomy and Developmental Biology, University College London, Gower Street, London, WC1E 6BT, UK. [2]Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauserstrasse 108, 01307 Dresden, Germany.

*Author for correspondence (e-mail: m.tada@ucl.ac.uk)

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The identification of directional cues for the anterior migration of prechordal plate progenitor cells has fascinated developmental biologists for several decades. The best characterised is platelet-derived growth factor (Pdgf) in *Xenopus*, as Pdgf signalling is required for the orientation of cells toward the animal pole and for their directionality (Nagel et al., 2004). By contrast, Pdgf and its key intracellular transducer PI3K are necessary for cell polarisation and motility, but not for directionality, in zebrafish (Montero et al., 2003). Despite the migration defects in embryos with compromised Pdgf pathway activities, prechordal plate progenitor cells are largely still capable of undergoing directed migration in *Xenopus* and zebrafish, suggesting the existence of another cue(s) in this process.

What are the genetic pathways involved in the regulation of coherence of cells during anterior migration of the presumptive prechordal plate? E-cadherin and Rab5-mediated endocytosis regulate the cell coherence of prechordal plate progenitors, and these are mediated by Wnt11 (Ulrich et al., 2005), a member of the non-canonical Wnt/planar cell polarity (PCP) pathway (reviewed by Tada et al., 2002; Seifert and Mlodzik, 2007). Indeed, *silberblick* (*slb*)/*wnt11* mutant embryos exhibit reduced anterior migration of prechordal plate progenitor cells (Ulrich et al., 2005). The expression of *E-cadherin* (*cdh1*) in the presumptive prechordal plate is regulated by *snail* genes that are required for anterior migration of the prechordal plate in zebrafish (Blanco et al., 2007). Furthermore, Liv1 (Slc39a6 – Zebrafish Information Network), a zinc transporter, controls nuclear localisation of Snail, and is required for anterior migration of the prospective prechordal plate during zebrafish gastrulation (Yamashita et al., 2004). Moreover, the secreted Wnt antagonist Dkk1 modulates anterior migration of mesodermal cells by interacting with Knypek/Glypican4, a cofactor for Wnt11, in zebrafish (Caneparo et al., 2007).

The bioactive lipid, sphingosine-1-phosphate (S1P), is a signalling molecule that acts through binding to a family of seven-pass transmembrane, G-protein-coupled receptors (S1PRs) that have been implicated in the regulation of cytoskeletal rearrangements, cell motility and cell adhesion in a variety of cell types (reviewed by Spiegel and Milstien, 2003). In addition to the role for S1P as a ligand, it acts as a second messenger within the cell, and its intracellular levels are regulated by the balance between its production by Sphingosine kinases and its degradation by S1P lyases (reviewed by Alvarez et al., 2007). However, little is known about the functions of S1P and its receptors in regulating directed cell migration in the embryo.

In this study, we sought to search for a novel genetic pathway that controls the collective migration of prechordal plate progenitor cells at the onset of zebrafish gastrulation. In our morpholino (MO)-based screen, we have identified *miles apart* (*mil*) as a genetic suppressor of defective migration of prechordal plate cells in *slb/wnt11* mutant embryos. *mil* was initially isolated as a heart mutant with a cardia bifida phenotype, and it encodes a S1P receptor, Edg5, also called S1pr2 (Kupperman et al., 2000). We analysed the cell behaviour of the presumptive prechordal plate based on DIC time-lapse movies of the living zebrafish gastrula, and found that, in *slb* embryos injected with *mil*-MO, the cells migrated with increased motility but decreased directionality, without restoring the coherence of cell migration in *slb* embryos. Furthermore, we showed that Mil controls cell motility through the Pdgf/PI3K pathway but modulates individual cell behaviours underlying cell coherence separately from this pathway. These results highlight the unexplored role of the motility and coherence of individual cells, regulated by the Mil/S1P signal, in the directed migration of prechordal plate progenitors.

MATERIALS AND METHODS

Embryo maintenance

The maintenance of fish and the collection of embryos were performed essentially according to Westerfield (Westerfield, 2000). The allele we used was homozygous slb^{tx226}, which had been selected for *slb* carriers that consistently showed the defective movement of the prechordal plate, and *slb* mutant embryos were obtained from in-crossing of the homozygotes. The embryos were kept at 28.5°C for time-lapse analysis or at 31°C for in situ hybridisation.

Microinjections and constructs

The sequences of morpholinos used in this study were: *mil*-MO, 5′-CCGCAAACAGAGCCGAACTAGTCAT-3′ (ATG-MO) (Matsui et al., 2007); and *edg1*-MO, 5′-TTAGGTCATCCATGGTTTGCACTGG-3′ (ATG-MO). *mil*-MO and *edg1*-MO were, respectively, injected at concentrations of 4.3 ng/nl and 6.2 ng/nl. *mil*-MO injection phenocopied the cardia bifida condition in the *mil* mutant (87.5%, *n*=56). To validate the specificity of the *edg1*-MO, RNA encoding GFP tagged with amino acid sequences that include the sequence corresponding to the *edg1*-MO, or irrelevant sequences (ILK) as a negative control, was co-injected with *edg1*-MO, and *GFP* expression was examined at dome stages. *GFP* expression from the construct containing the sequence corresponding to the *edg1*-MO was suppressed (*GFP*-positive: 0%, *n*=19), whereas *GFP* expression was retained in the negative control (*GFP*-positive: 100%, *n*=36). Together with the ability of the *edg1*-MO to cancel the *edg5*-morphant phenotype when co-injected (see Fig. 6B,E,H to compare with Fig. 1B,F,I), these data validate the specificity of the *edg1*-MO.

For overexpression studies, the following constructs were used: pCS2-dn-PI3K, pCS2-p110CAAX and pCS2-PH-GFP [all described previously by Montero et al. (Montero et al., 2003)]. As for the *mil* construct, a PCR-amplified full-length fragment from a gastrula library was cloned into pCS2+, and RNA for injection was made after linearisation with *Not*I. All of the RNAs were synthesised in vitro essentially as described (Smith, 1993).

In situ hybridisation

Antisense RNA probes were synthesised with a digoxigenin RNA-labelling kit (Roche), using plasmids containing cDNA for *ntl* (Schulte-Merker et al., 1994), *hgg1* (Thisse et al., 1994), *dlx3* (Akimenko et al., 1994), *hlx1* (Fjose et al., 1994) and *sprouty4* [(Furthauer et al., 2004) originally published as *sprouty2*]. Whole-mount in situ hybridisation was carried out as described previously (Barth and Wilson, 1995).

Time-lapse imaging of embryos

For time-lapse imaging, embryos were manually dechorionated at the shield stage and mounted in 0.8% low-melting-point agarose (Sigma) in embryo medium. For DIC observation, images were taken at 1 frame per minute for 1 hour, with approximately 18 *z*-levels spaced 3 μm apart, using a 40× water immersion lens on an Axioplan 2 (Zeiss) compound microscope and a Hamamatsu Orca ER digital camera. Two-photon microscopy was performed as described by Montero et al. (Montero et al., 2003).

Cell movement analysis

The positions of five randomly chosen cells were plotted every minute, using OpenLab5.0 software (Improvision). The ‘speed’ of a cell was measured as μm/minute using gross length of migration. The ‘persistence’ of a cell was calculated by the quotient of net migration per gross migration over every 14 minutes. Assuming that cells change their relative positions more dynamically in less coherent conditions, the ‘coherence^{-1}’ of migration of a group of cells was defined as the rate of change in cell-cell distance per length of cell migration. Here, the change of distance between two cells was determined, then normalised for each cell by dividing by its net migration (see Fig. S1 in the supplementary material). This calculation was applied to all 10 combinations among five cells, every 9 minutes. For each experimental condition, three to five embryos from independent experiments were analysed. To test the significance between two mean values, Welch’s *t*-test based on an unequal variance was applied and described as ‘mean±s.e.m.’.

Analysis of cell shape

Approximately 25 cells at the leading edge from three to five embryos were randomly chosen from time-lapse images, and, of those, cells with a distinctive cellular protrusion were used for analysis with ImageJ software. The ‘length’ and ‘angle’ of the largest protrusion were determined by assuming a line from the centre of the nucleus to the tip of the protrusion: the length being its length in μm, and the angle being measured from the direction of general migration (i.e. the upper-vertical axis). Here, the mean and standard deviation of angles are calculated in cosine, as measured anticlockwise from the upper-vertical axis. White dots indicate the central position of the nucleus, and green lines show the length and angle of a protrusion. Red ‘fans’ represent the average length and angle (in degrees) of the protrusions, including their standard deviations (respectively depicted by its height and open angle). To test the significance in the length between two mean values, Welch’s *t*-test based on an unequal variance was applied and described as ‘mean±s.e.m.’.

Cell culture

Cell culture experiments were carried out according to Montero et al. (Montero et al., 2003) with slight modification. In brief, 50 embryos injected with 100 pg *cyclops* RNA together with the RNA(s) indicated in each experiment were manually dechorionated at the dome stage, and dissociated in L-15 medium containing 1×trypsin-EDTA (Biowhittaker). After stopping the enzymatic reaction by the addition of chick serum, cells were harvested by centrifugation at 150 ***g*** for 2 minutes and re-suspended in 2.5 ml of fresh L-15 medium, containing 1 mg/ml insulin, 0.3 mg/ml L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were then cultured for 1 hour at 25°C on a plastic Petri dish coated with 50 μg/ml fibronectin (Sigma), prior to treatment with 50 ng/ml Pdgf-AA (Sigma).

Fluorescence intensity analysis

The grey value along a line of 3-pixel weight was measured using ImageJ software. The *x*-axis of the graph represents the distance, while the *y*-axis indicates the relative signal intensity (as normalised by total intensity), along the line.

RESULTS

Mil/Edg5 as a suppressor of the Slb/Wnt11 gastrulation phenotype and a negative regulator of prospective prechordal plate movement

In *silberblick* (*slb*)*/wnt11* embryos, prechordal plate progenitor cells move anteriorly, but slower than those in wild-type (WT) embryos because of the reduced cohesion of cells; cell cohesion is thought to be required for prechordal plate progenitor cells to undergo collective migration (Montero et al., 2005; Ulrich et al., 2005). In order to identify genetic cascades that regulate anterior migration of the prospective prechordal plate, we have undertaken a candidate approach in which morpholino antisense oligonucleotides (MOs) are injected into *slb* homozygous embryos as a sensitised background to detect either enhancement or suppression of the *slb* phenotype. During the course of this approach, we identified *miles apart* (*mil*) as a suppressor of the *slb* phenotype. *mil* was originally isolated as a mutant exhibiting a bifurcated heart, and encodes a sphingosine-1-phosphate (S1P) receptor, Edg5 (Kupperman et al., 2000). When the position of the anterior prechordal plate was visualised with respect to the anterior edge of the neural plate at the end of gastrulation, the presumptive prechordal plate of the *slb* embryo was located more posteriorly than that of the WT embryo (Fig. 1A,C,E,G) (Heisenberg et al., 2000). Strikingly, the deficit in anterior movement of the *slb* prechordal plate was largely rescued by injection of *mil*-MO (Fig. 1C,D,G,H; 65.4%, *n*=52). When WT embryos were injected with *mil*-MO, the shape of the prospective prechordal plate was slightly flatter and wider, and its positioning was slightly more anterior than in WT embryos (Fig. 1A,B,E,F). In addition, the anteroposterior length of the posterior prechordal plate was increased in embryos with compromised Mil functions in WT and *slb* embryos (Fig. 1I-L; compare with Fig. 1A-D and Fig. S2 in the supplementary material). In contrast to the prechordal plate, *slb* embryos exhibit a shorter and thicker notochord than do WT embryos, implying defective C&E movements in the presumptive notochord (Heisenberg et al., 2000), but this defect was not rescued by injection of *mil*-MO (Fig. 1O,P).

This observation raises the possibility that Mil activity might regulate anterior migration of the presumptive prechordal plate but not of the prospective notochord, where cells undergo C&E. If this were the case, anterior movement of the lateral mesoderm would be unaffected in *mil* morphants. To test this, we examined the expression of *sprouty4* to see to what extent lateral mesodermal cells have migrated at 60% epiboly (Caneparo et al., 2007). Despite the fact that axial mesodermal cells migrated faster in the *mil* morphant, anterior movement of lateral mesodermal cells was unchanged when compared to the WT embryo (90.3%, *n*=31; Fig. 1M,N). These results suggest that Mil activity is required only for prechordal plate progenitor cells to undergo directed anterior migration, and not for other populations of mesodermal cells.

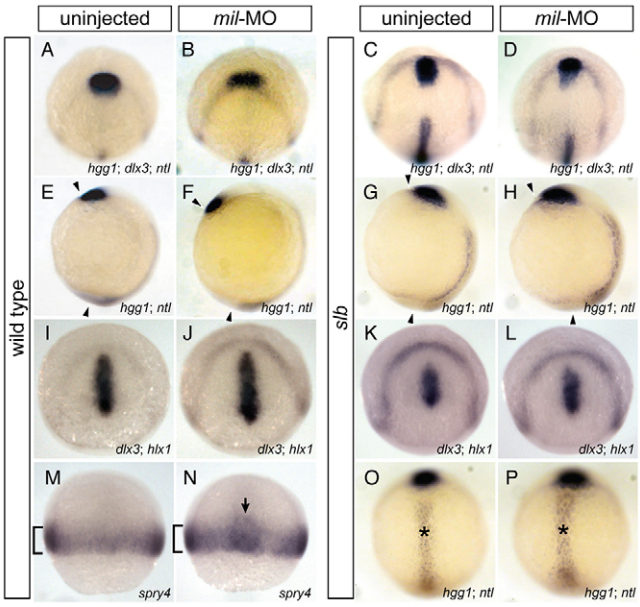

Fig. 1. Abrogation of Mil function facilitates anterior migration of the presumptive prechordal plate. In situ hybridisation (**A-H**,**O**,**P**) of *dlx3* (anterior border of the neural plate), *hgg1* (anterior prechordal plate) and *ntl* (notochord) at tailbud stage, (**I-L**) of *dlx3* and *hlx1* (posterior prechordal plate) at tailbud stage, and (**M**,**N**) of *spry4* (presumptive mesoderm) at 60% epiboly. (A-D) Dorso-animal views, (E-H) lateral views, (I-L) dorso-animal views and (M-P) dorsal views of wild-type (WT) embryos (A,E,I,M), WT embryos injected with 4.3 ng *mil*-MO (B,F,J,N), *slb* embryos (C,G,K,O) and *slb* embryos injected with 4.3 ng *mil*-MO (D,H,L,P). In *mil*-MO-injected embryos, anterior migration of the presumptive prechordal plate was promoted (B,F), as compared to WT (A,E), with regards to relative positions of the prechordal plate in relation to the anterior border of the neural plate (A-D), and to angles between the prechordal plate and the tailbud, indicated by arrowheads (E-H). Injection of *mil*-MO rescued the reduced migration of the anterior prechordal plate in the *slb* embryo (C,D,G,H), but did not alter the wider notochord (O,P, indicated by asterisks). By contrast, the altered position of the posterior prechordal plate correlated with that of the anterior prechordal plate (K,L). (M,N) The enhancement of migration was not observed in the lateral mesoderm (brackets), but was specific to the prospective prechordal plate (arrow).

Prechordal plate cells can migrate individually if their motility is increased and their coherence is decreased

In order to explore the cellular mechanisms by which the abrogation of Mil function restores the reduced migration of prechordal plate progenitor cells in *slb* embryos, we tracked the cells based on DIC time-lapse movies at the onset of gastrulation, and analysed the cell behaviours that underlie collective migration. We extracted three parameters: first, speed, by the total length of their paths over the time; second, persistence, by the gained distance over the total length of their paths; and third, coherence, by changes in relative distances between pairs of cells over time for all the combinations of five cells (a total of 10 combinations per time point).

Cell tracking analysis revealed that the reduced migration of *slb* hypoblast cells is closely associated with a lower persistency in directed migration and a reduced coherence of cell migration (Fig. 2A-F,J; see Movies 1 and 2 in the supplementary material) (Ulrich et al., 2005; Witzel et al., 2006). Surprisingly, when Mil function is compromised, *slb* hypoblast cells migrated faster than did WT cells, but more randomly and with more space between the cells, thus maintaining a lower cell cohesion than WT cells (Fig. 2G-I,J; see Movie 3 in the supplementary material). Occasionally, we observed that an isolated single cell popped out but then travelled back into the group of cells (Fig. 2H; observed in four movies out of five). These results indicate that *slb* cells with compromised Mil function have acquired more motility, but that their directionality and coherence remain as low as *slb* cells. Consistent with this, leading edge cells of the *slb* embryo with compromised Mil function formed

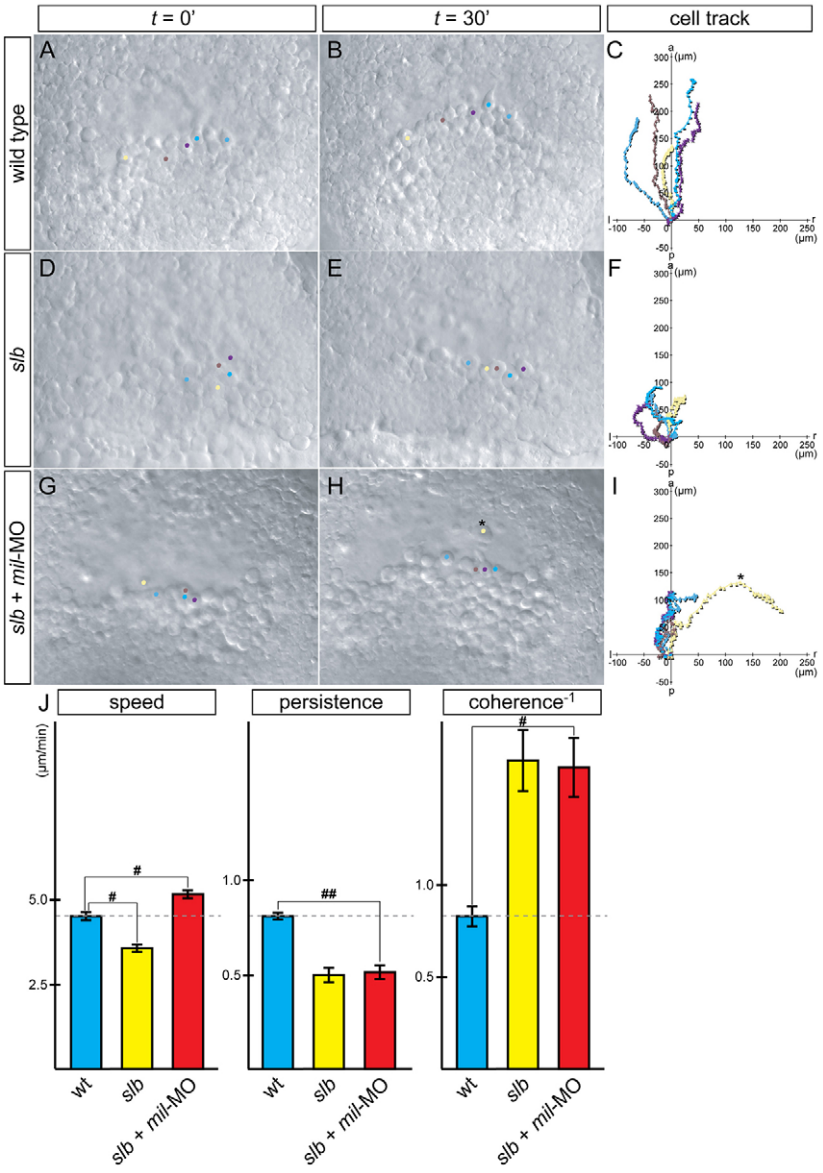

Fig. 2. *slb* prechordal plate progenitors migrate individually with increased motility and reduced cohesion when Mil function is compromised. (**A-I**) Time-lapse analysis and tracking of cell movement in migrating prechordal plate progenitors of WT embryos (A-C), *slb* embryos (D-F) and *slb* embryos injected with 4.3 ng *mil*-MO (G-I). Five cells were randomly chosen from each embryo, highlighted in different colours and tracked by merging each starting point to 0 (C,F,I). Twenty-five cells in total were accumulated from five independent experiments, to analyze for speed, persistence and coherence (J). In *slb* embryos, cell motility was significantly reduced (D-F). In *slb* embryos injected with *mil*-MO, the cells showed increased motility, and the cluster of cells appeared to be less coherent (G-I); the cells occasionally popped out from the cluster and travelled individually (H,I, asterisk). Such cell behaviour was never observed in WT embryos. (**J**) Summary of analysis of parameters: speed, persistence and coherence. Statistically significant differences are indicated ($^{\#}P<0.05$, $^{\#\#}P<0.005$).

longer lamellipodia than did both *slb* cells and WT cells, but were unable to stabilise their processes toward the direction of movement when compared with WT cells (Fig. 3A-C).

We further sought to identify differences in behaviours of *slb* cells with compromised Mil function when compared with WT cells, and investigated involution of the axial hypoblast in a sagittal view. Whereas both WT and *slb* hypoblast cells internalised as three layers of cells, as shown previously (Montero et al., 2005; Ulrich et al., 2005) (Fig. 4A and Movie 8 in the supplementary material), *slb* cells with compromised Mil function underwent internalisation as one or two layers of cells (Fig. 4B and Movie 9 in the supplementary material). Similarly, abrogation of Mil function in the WT embryo leads to the scattered cell behaviour phenotype during internalisation, as seen in the *slb* embryo with compromised Mil function (see Movie 10 in the supplementary material). Consistent with the observation from DIC movies (Fig. 2G,H), there was more space in between the hypoblast cells in *slb* embryos injected with *mil*-MO than in WT embryos, confirming that prechordal plate progenitor cells migrate individually in *slb* embryos when Mil function is compromised.

Despite the fact that *slb* cells with compromised Mil activity retain a lower coherence of cell migration to the direction of motion, they can gain the net distance as efficiently as WT cells due to their acquisition of greater motility (Fig. 5). In this extreme circumstance, cells appear be capable of migrating individually rather than in a cluster of coherent cells.

Edg1 and Mil/Edg5 reciprocally regulate directed migration of the presumptive prechordal plate

It has been shown that the two structurally related S1P receptors, Edg1 (also called S1pr1) and Edg5, are positive and negative regulators, respectively, of cell migration in lymphocytes and endothethial cells (e.g. Yamaguchi et al., 2003). To test this possibility in directed migration of the prospective prechordal plate, we first injected WT embryos with *edg1*-MO. Opposite to the *mil*-morphant phenotype, directed migration of the anterior and posterior prechordal plate was perturbed in *edg1* morphants (Fig. 1B,F,J and Fig. 6A,D,G). Consistent with their opposing activities in cultured cells, the reduced migration of *edg1*-morphant prechordal plate cells was rescued by co-injection of *mil*-MO (Fig. 6B,E,H). Next, we tested whether *edg1* is

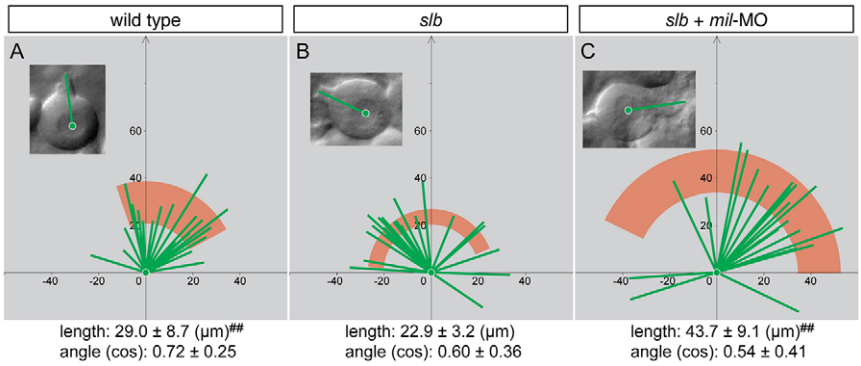

Fig. 3. *mil* is required for the stabilisation of cellular processes towards the direction of migration. The orientation and length of lamellipodia-like cellular processes of leading edge cells in a WT embryo (**A**), *slb* embryo (**B**) and *slb* embryo injected with 4.3 ng *mil*-MO (**C**). The morphology of a cell from each group is highlighted in the insets. The length of the long axis of cells and their orientation with respect to the direction of movement (*y*-axis) were calculated and plotted, and the angle from the *y*-axis was calculated as cosine. Statistically significant difference is indicated ($^{\#\#}P<0.005$).

an enhancer of the *slb* phenotype as opposed to *mil* being identified as a suppressor. When *edg1* function was compromised, *slb* embryos exhibited a more posteriorly displaced prechordal plate but the notochord was unaffected (Fig. 1C,G,K, Fig. 6C,F,I, data not shown). These results suggest that Edg1 and Mil act in a mutually antagonistic manner to regulate directed migration of the presumptive prechordal plate, and that Edg receptors control directed migration of prechordal plate progenitors but not of other mesodermal cells.

Mil can modulate cell Pdgf-induced cell polarisation

Pdgf signalling through the intracellular mediator PI3K is one genetic pathway that has been shown to regulate the directed migration of mesendoermal cells in zebrafish and *Xenopus* (Montero et al., 2003; Nagel et al., 2004; Symes and Mercola, 1996). Therefore, we investigated a possible interaction of the Mil/S1P signal with the Pdgf/PI3K pathway in directed migration of prechordal plate progenitor cells. We hypothesised that Mil controls directed migration by negatively regulating the Pdgf/PI3K pathway, as inhibition of the Pdgf/PI3K pathway leads to a reduction in cell migration (Montero et al., 2003), and this phenotype is the opposite of the *mil* morphant. To test this hypothesis, we first investigated process formation in response to Pdgf in dissociated mesendodermal cells from zebrafish blastulae. Although mesendodermal cells formed lamellipodia-like long processes when treated with Pdgf, Pdgf treatment of cells overexpressing *mil* RNA resulted in a reduction in the frequency and length of processes (Fig. 7A,B). Next, to test whether Edg5 inhibits the intracellular effecter PI3K, we monitored the membrane localisation of PH-GFP induced by Pdgf, and of membrane-RFP as a reference. Pdgf-induced membrane localisation of PH-GFP was inhibited by the presence of *mil* RNA (Fig. 7C-F). These results indicate that the Mil/S1P signal might control cell polarisation through the Pdgf/PI3K pathway.

Mil modulates coherence of cell migration independently from Wnt11 and PI3K

The possible interaction of Mil with the Pdgf signal, as well as with Wnt11, inspired us to identify which parameters (coherence, directionality and motility) are regulated by Mil through or in parallel with these pathways in the directed migration of prechordal plate progenitors. Because Mil can modulate cell adhesion underlying coherence of cell migration in a fibronectin-dependent manner (Matsui et al., 2007), we hypothesised that the reason why *slb* cells (when Mil function is compromised) can move faster than WT cells even though they retain a lower coherence of cell migration might be because Mil can modulate cell cohesion independently of Slb/Wnt11 activity. To test this, we injected WT embryos with *mil*-MO and analysed the cell behaviour of prechordal plate progenitors. Cells with compromised Mil function migrated persistently but less coherently than did WT cells (see Fig. S1 and Movie 4 in the supplementary material; see also Fig. 8C). If Mil modulates cell cohesion through PI3K, prechordal plate progenitor cells with increased PI3K activity would lead to phenotypes similar to that of *mil*-morphant cells. To test this possibility, we expressed RNA encoding a constitutively active form of PI3K (p100CAAX) in the WT embryo to examine the cell behaviour of migrating prechordal plate progenitors. In contrast to abrogation of Mil function, the expression of *p100CAAX* RNA did not cause cells to change their cohesive properties in WT embryos (Montero et al., 2003) (see Fig. S4 and Movie 5 in the supplementary material; see also Fig. 8C). Conversely, a reduction in the motility of WT cells expressing dominant-negative (dn)-PI3K was not due to a lower coherence of cell migration (see Fig. S4 and Movie 6 in the supplementary material; see also Fig. 8C). These results suggest that Mil regulates the coherence of cell migration independently of Wnt11 activity and PI3K.

Mil regulates cell motility through the Pdgf/PI3K pathway

We further tested whether Mil-mediated cell motility is dependent upon the Pdgf/PI3K pathway. As the phenotype caused by dn-PI3K shows reduced migration and is opposite apparently to that of the *mil*

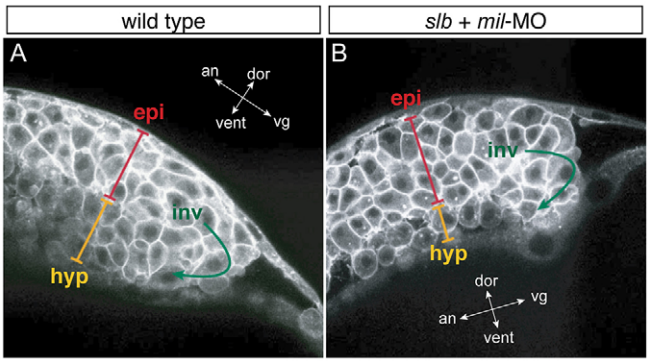

Fig. 4. *slb* cells with compromised Mil function undergo involution differently to WT cells. Multi-photon confocal analysis of involution cell behaviour through the shield region. Lateral views of WT embryo (**A**) and *slb* embryo injected with 4.3 ng *mil*-MO (**B**) at 60% epiboly. In the hypoblast layer of the *slb* embryo injected with *mil*-MO, cells are internalised as one- or two-cell thick layers, occasionally with space in between the cells, whereas in the WT embryo hypoblast cells are internalised as approximately three-cell thick layers throughout and are tightly packed. epi, epiblast layer; inv, involution; hyp, hypoblast layer. The orientation of embryos is shown as indicated along the animal (an)-vegetal (vg) axis and the dorsal (dor)-ventral (vent) axis.

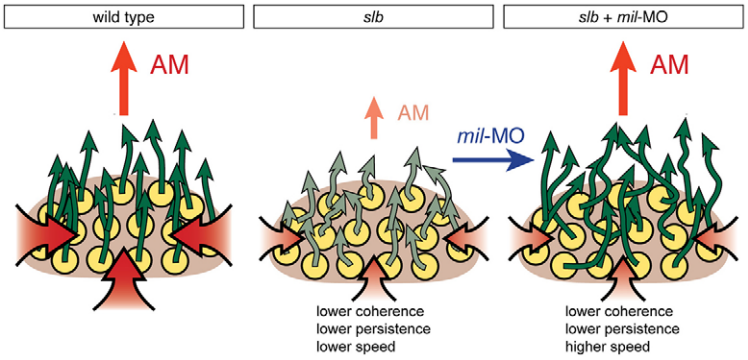

Fig. 5. Model of how *slb* cells with compromised Mil function can restore the net migration of prechordal plate progenitor cells. Normally, prechordal progenitors move persistently as a coherent cluster of cells. In *slb* embryos, the cells undergo reduced net migration due to a decrease in their cohesion and persistence. When injected with *mil*-MO, the *slb* cells are capable of undergoing directed migration as efficiently as WT cells, such that even under a circumstance in which coherence of migration is reduced, if cell motility is increased, it largely overrides the reduced directed migration. The length and size of arrows reflect the speed and efficiency of the migration of cells with respect to coherence and persistence.

morphant, we performed epistasis analysis of the interaction between the two signals by examining details of the cell behaviours based on DIC time-lapse movies. Prechordal progenitor cells expressing dn-PI3K migrated much slower than did WT cells, while their directionality was largely unaffected (Montero et al., 2003) (Fig. 8A,C; see also Movie 6 in the supplementary material). The reduced motility of cells expressing dn-PI3K was not reversed by knocking down Mil function, which suggests that Mil regulates cell motility through PI3K (see Fig. S4 and Movie 7 in the supplementary material; see also Fig. 8C). However, these cells migrated much less persistently than did cells with reduced PI3K activity only. The lower persistency of cell movement, when both PI3K and Mil functions are compromised, correlated with the randomised orientation of lamellipodia of leading edge cells with respect to their direction of motion (Fig. 8C). These results prompt us to propose that Mil controls the directed migration of prechordal plate progenitor cells in both a Pdgf/PI3K-dependent and -independent manner.

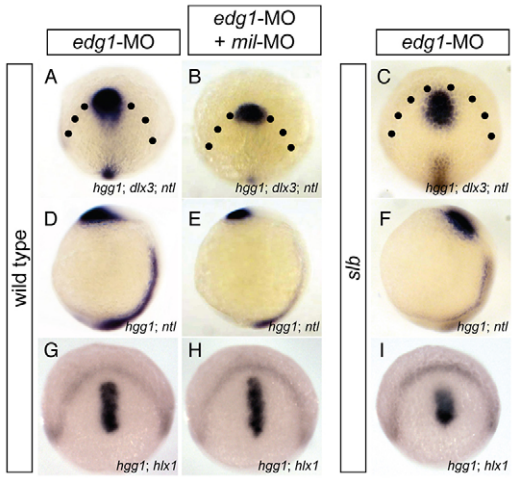

Fig. 6. Edg1 acts antagonistically against Mil in the directed migration of prechordal plate progenitor cells. (**A-F**) In situ hybridisation of *dlx*, *hgg1* and *ntl* of WT embryos injected with 6.2 ng *edg1*-MO alone (A,D) or co-injected with 4.3 ng *mil*-MO (B,E) at tailbud stage, and of *slb* embryos injected with 6.2 ng *edg1*-MO (C,F). (**G-I**) In situ hybridisation of *dlx3* and *hlx1* of WT embryos injected with *edg1*-MO alone (G) or co-injected with *mil*-MO (H) at tailbud stage, and of *slb* embryos injected with *edg1*-MO (I). (A-C,G-I) Dorso-animal views; (D-F) lateral views. *edg1*-MO injection reduced anterior migration of the anterior (A,D) and posterior (G) prechordal plate, compared with WT (see Fig. 1A,E,I), and enhanced the *slb* prechordal plate phenotype (C,F,I) (see Fig. 1C,G,K). Co-injection of *edg1*-MO and *mil*-MO cancelled out the effect of one another and often gave rise to an embryo reminiscent to WT (B,E,H).

DISCUSSION

Do prechordal plate progenitor cells undergo collective migration?

In this study, we demonstrate that prechordal plate progenitor cells are able to migrate efficiently as individual cells in the gastrula when Mil function is compromised, rather than as a cluster of coherent cells, and that this cell behaviour is more prominent in the *slb/wnt11* embryo. This brings into question the previous view that prechordal plate progenitors undergo collective migration as a group of cohesive cells that presumably creates a force to drive directed migration (Montero et al., 2005; Ulrich et al., 2005).

Cases for which collective migration in the embryo have been well documented are lateral line primodia in zebrafish and border cells in *Drosophila*. In contrast to the mesenchymal nature of prechordal plate progenitor cells in zebrafish, lateral line primodia cells undergo collective migration as a sheet of epithelia, such that only a few front-line cells lead their migration while the rest of the cells follow the leader cells (Haas and Gilmour, 2006). By contrast, border cells in *Drosophila* undergo collective migration as mesenchymal cells by changing their position within the cluster in the absence of leader cells (Prasad and Montell, 2007). There is no evidence that leader cells exist within prechordal plate progenitor cells, but we observed that cells located at the leading edge retain their position at least for the duration of the time-lapse movies (e.g. Movies 1 and 8 in the supplementary material). This suggests that prechordal plate progenitor cells are capable of migrating as individual cells, and that this might be similar to the traditional case of individual cell migration during chemotaxis in *Dictyostelium* (reviewed by Dormann and Weijer, 2006; Franca-Koh et al., 2006).

Prechordal plate progenitor cells can migrate as isolated single cells in extreme situations. Interestingly, single cells do not travel alone far away from the group of prechordal plate progenitors and in all cases they come back to the group. This raises the possibility that the isolated cell might lose its identity as a presumptive prechordal plate progenitor; for example, *goosecoid* expression could have been lost. In MZoep embryos, a single WT cell can internalise but is incapable of migrating anteriorly (Carmany-Rampey and Schier, 2001), presumably its anterior mesendodermal cell identity has been lost. Alternatively, the isolated cell might lose motility through the loss of a community effect that normally maintains cells as a group. However, it is difficult to distinguish between these possibilities, as it is technically challenging to visualise cell identity in the living embryo in such a short period. Also, it will be interesting to test whether or not cells with higher motility and lower directionality can migrate individually when they are isolated from surrounding cells with lower motility and lower coherence.

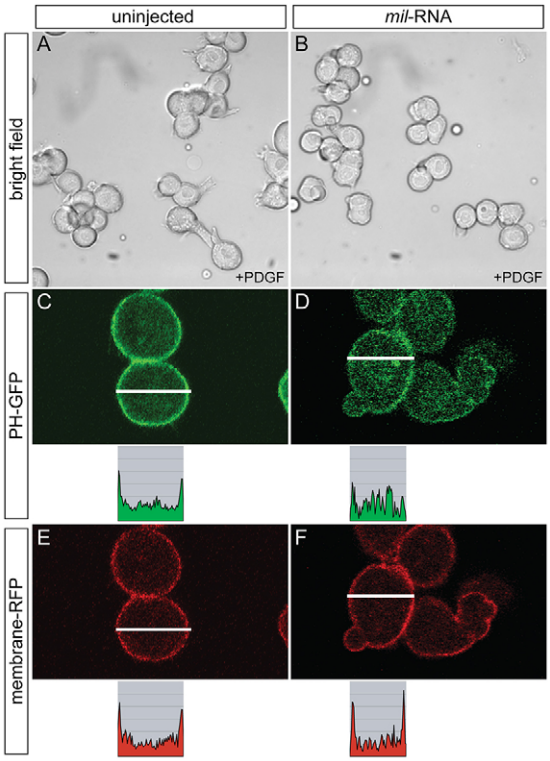

Fig. 7. Mil negatively regulates Pdgf-induced process formation. (**A**,**B**) WT embryos were injected with 100 pg *cyclops* RNA alone (A) or together with 150 pg *mil* RNA (B) at the one-cell stage, and were dissociated at sphere stage. The dissociated cells were then maintained on fibronectin-coated dishes and treated with 50 ng/ml of Pdgf 30 minutes prior to observation. The frequency of Pdgf-induced lamellipodia-like cellular processes decreased when *mil* RNA was overexpressed (22.2%), compared with those of WT cells (64.7%, B). Similarly, the length of processes in cells overexpressing *mil* RNA was reduced (27.3±4.8 μm, *n*=18) compared with those of WT cells (40.3±9.9 μm, *n*=17). (**C-F**) WT embryos were injected with 100 pg *cyclops* RNA along with RNAs coding for PH-GFP (125 pg) and membrane-RFP (125 pg) in the absence (C,E) or presence (D,F) of 150 pg *mil* RNA, and were dissociated for culture as described above. In controls (C,E), the cells responded to Pdgf stimulation and recruited PH-GFP to the plasma membrane. In cells overexpressing *mil* RNA, PH-GFP was dispersed in the cytoplasm (D), compared with the referential signal on the membrane (F). Analysis of the relative intensity of fluorescent signals along the lines is shown below each image (C-F).

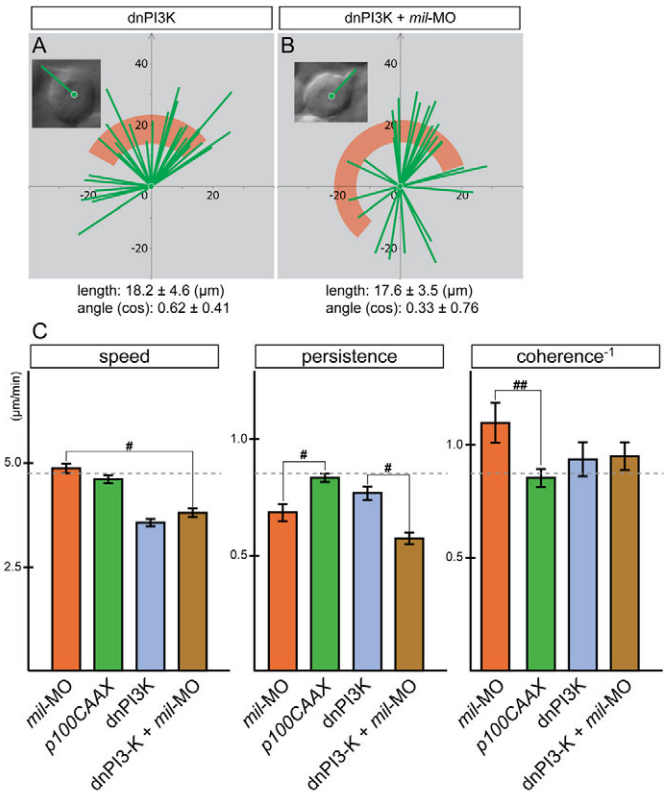

Fig. 8. *mil* requires PI3K to modulate cell motility and process formation but not directionality. (**A**,**B**) The orientation and length of cellular processes of WT embryos injected with 250 pg dn-PI3K alone (A) or with 4.3 ng *mil*-MO (B). The morphology of the cells is highlighted in the insets. The length of the long axis of cells and their orientation with respect to the direction of movement (*y*-axis) were calculated and plotted, the angle from the *y*-axis was calculated as cosine. (**C**) Summary of analysis of parameters: speed, persistence and coherence. Statistically significant differences are indicated ($^{\#}P$<0.05 and $^{\#\#}P$<0.005).

Does Mil/S1P mediate the non-canonical Wnt/PCP pathway?

We have identified *mil/edg5* as a suppressor of the *slb/wnt11* phenotype in this study. This raises the intriguing possibility that Mil might act in the non-canonical Wnt/PCP pathway. However, several lines of evidence do not support this possibility. First, although abrogation of Mil function with MOs restores the anterior migration of prechordal plate progenitors in *slb* embryos, it fails to rescue defects in the *slb* presumptive notochord. Second, while injection of *dkk1*-MO facilitates anterior migration of the prechordal plate in WT embryos, as in the *mil* morphants, it cannot rescue the defective anterior migration of the *slb* prechordal plate (Caneparo et al., 2007), suggesting that Dkk1-regulated migration is dependent upon Slb/Wnt11 function in this context. Indeed, it has been shown that Dkk1 acts in the non-canonical Wnt pathway by physically and functionally interacting with Knypek/Glypican4, a known co-factor of Wnt11 (Caneparo et al., 2007; Topczewski et al., 2001). Third, the cardia bifida phenotype caused by *mil*-MO injection is still observed in the *slb* homozygous background (data not shown). These results support the notion that the Mil signal acts independently of the non-canonical Wnt pathway.

What molecule(s) mediates downstream of the Mil/S1P signal? In mammalian cultured cells, it has been shown that $G\alpha_{12/13}$ is activated by EDG5 (Gonda et al., 1999). Importantly, abrogation of $G\alpha_{12/13}$ function leads to impaired mediolateral elongation of mesendodermal cells undergoing convergence of the lateral mesoderm and extension of the notochord during zebrafish gastrulation (Lin et al., 2005). However, it implies that $G\alpha_{12/13}$ is unlikely to mediate EDG5/S1P signalling directly, as embryos with altered EDG5/EDG1 activities exhibit little defect in mesodermal cells undergoing convergence and extension. It remains to be investigated which heteromeric G protein mediates signalling downstream of Mil/S1P in the directional migration of prechordal plate progenitors. In addition, how different G protein-coupled receptors (GPCRs) regulate the movements of distinct cell populations during gasrulation needs to be addressed, as another GPCR Agtrl1b predominantly mediates the movements of the lateral mesoderm of the zebrafish gastrula (Scott et al., 2007; Zeng et al., 2007).

Does S1P act as a directional cue or together with directional cue(s)?

The best candidate for a directional cue is Pdgf, but it appears that Pdgf regulates anterior migration of the lateral mesoderm as well as of the anterior axial mesoderm in *Xenopus* and zebrafish (Nagel et al., 2004) (data not shown), whereas the Mil/S1P signal is more specific to prechordal plate progenitor cells in zebrafish. Do Edg receptors regulate directional migration cell-autonomously within the prospective prechordal plate? Considering the fact that the expression of both *mil* and *edg1* is ubiquitous during gastrulation (Kupperman et al., 2000) (data not shown), the localisation of the bioactive lipid S1P might be restricted to either the presumptive prechordal plate or the overlying neurectoderm. Although it will be challenging to visualise the short life of the bioactive lipid in the embryo, the temporal and spatial localisation of its producing and degrading enzymes, such as Sphingosine kinase and S1P lyase, respectively, might allow us to speculate where a potential gradient of the bioactive lipid is, as in the case for the gradient of retinoic acid in the zebrafish hindbrain (White et al., 2007). Alternatively, S1P receptors might be required cell-non-autonomously in the overlying neurectoderm, as it appears that there is correlation of tissue movements between the presumptive prechordal plate and the hypothalamus (see Fig. S3 in the supplementary material). To clarify the apparent interdependency of the two tissue movements, we will need to manipulate the movement of one tissue to see possible alteration in the other tissue. This will require the use of a transgenic approach rather than a transplantation one, as transplanted cells normally disperse in the host and thereby lose coherence of cell movement, which might mediate the interaction of two distinct populations.

Recent searches for transcriptional targets of Pdgf in cultured cells revealed that Pdgf upregulates *sgpl1* (*sphingosine-1-phosphate lyase 1*), which encodes an enzyme that degrades active S1P (Chen et al., 2004). Importantly, embryonic fibroblasts from *sgpl1*$^{-/-}$ mice in cultures showed reduced migration in response to Pdgf (Schmahl et al., 2007), indicating that the ability of cells to inactivate active S1P is required for the proper response to Pdgf signalling to mediate cell motility. Moreover, Pdgf stimulates the production of S1P by activating and translocating Sphingosine kinase, an S1P-producing enzyme, to the membrane in lymphocytes (Hobson et al., 2001). This further suggests a possible auto-regulatory loop between Pdgf and S1P signals. Therefore, it will be fascinating to investigate this issue in directed migration in the gastrulating embryo.

Recent studies in medaka revealed that maternal-zygotic *fgfr1* mutants exhibit defective migration of the axial mesoderm but not of the lateral mesoderm (Shimada et al., 2008), and this phenotype is reminiscent of the *edg1*-morphant phenotype, raising the possibility that the Mil/S1P signal might also co-operate with the Fgf signal. It will be intriguing to test this possibility in zebrafish, as Fgf is capable of activating PI3K as well as Pdgf. Furthermore, the question of how prechordal plate cells acquire greater motility and/or can sense directional cues more efficiently than the other mesodermal cells remains unsolved.

How does the Mil/S1P signal regulate cell motility, directionality and coherence?

We demonstrated that overexpression of *mil* RNA inhibits Pdgf-induced cell polarisation in dissociated mesendodermal cells by interfering with Pdgf-mediated activation of PI3K, and that the facilitated migration of the *mil* morphant is blocked totally by dn-PI3K. These results indicate that Edg5 can act upstream of PI3K to negatively regulate the Pdgf pathway. However, cell-tracking analysis of *mil* morphants and of embryos with increased or reduced

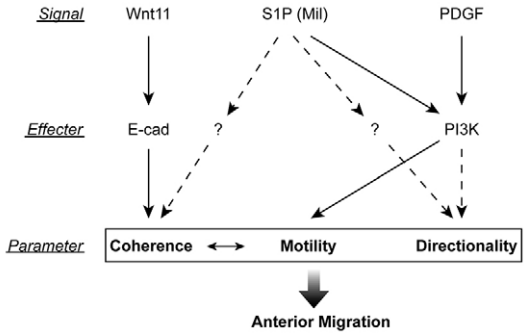

Fig. 9. Model summarising how the Mil/S1P signal regulates the anterior migration of prechordal plate progenitors. The Mil/S1P signal primarily regulates the anterior migration of prechordal plate progenitors by modulating cell motility and polarisation through PI3K, a key effecter of Pdgf (Montero et al., 2003), and it mediates directionality together with the Pdgf pathway through an unknown mechanism. Furthermore, the Mil/S1P signal is seemingly able to modulate the coherence of cell migration independently of Wnt11 function, which is required for E-cadherin-dependent cell cohesion (Montero et al., 2005). However, it is still unknown whether Mil modulates cell cohesion/adhesion through E-cadherin. There is interdependency between parameters, at least between coherence and motility, as shown in Fig. 5. Arrows indicate regulation based on published and present results; dashed arrows indicate possible co-operation between the signalling cascades.

PI3K activity revealed that coherence of cell migration mediated by Mil is likely to be independent of PI3K activity (Fig. 9). By contrast, the directionality of migrating cells is totally lost in the *mil* morphants when PI3K is blocked, implying that Pdgf and Edg signalling might co-operate to mediate the directionality of prechordal plate progenitor cells (Fig. 9). This is different from the mode of regulation of directionality in *Xenopus*, as blocking the Pdgf signal is sufficient to lose the directionality of anterior axial mesodermal cells (Nagel et al., 2004). Taken together, these results suggest that Mil modulates cell motility through the Pdgf/PI3K pathway, but that it modulates coherence independently of the pathway.

Does Mil modulate cell cohesion passively as a consequence of increased cell motility or instructively by unknown mechanisms? We favour the latter possibility for several reasons. First, abrogation of Mil function leads to the reduced coherence of cell migration in WT embryos, whereas WT embryos with increased or decreased PI3K activity do not show any changes in coherence of cell migration. This implies that cell motility and coherence are independently regulated. Second, when E-cadherin-mediated cell cohesion was decreased in *slb* embryos, *slb* embryos with compromised Mil function acquired more motility than WT embryos. Moreover, even E-cadherin-morphant cells, when Mil function was compromised, were able to migrate as efficiently as WT cells (data not shown), suggesting the presence of a Mil mediator of cell coherence that functions independently of E-cadherin-dependent cell adhesion. Third, abrogation of Mil function in the WT embryo leads to the scattered cell behaviour phenotype during internalisation, as seen in the *slb* embryo with compromised Mil function (see Movie 10 in the supplementary material). This supports the notion that Mil modulates cell cohesive properties independently of Slb activity (Fig. 9). Fourth, the *mil* cardia bifida phenotype might be explained by the modulation of adhesive properties through the interaction with fibronectin (Matsui et al.,

2007). Whether or not Mil modulates the cell cohesive property underlying the directed migration of collective cells in the embryo will require further investigation.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/18/3043/DC1

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