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Phosphoinositide 3-Kinase Is Required for Process Outgrowth and Cell Polarization of Gastrulating Mesendodermal Cells

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Summary

Background: During vertebrate gastrulation, cell polarization and migration are core components in the cellular rearrangements that lead to the formation of the three germ layers, ectoderm, mesoderm, and endoderm. Previous studies have implicated the Wnt/planar cell polarity (PCP) signaling pathway in controlling cell morphology and movement during gastrulation. However, cell polarization and directed cell migration are reduced but not completely abolished in the absence of Wnt/PCP signals; this observation indicates that other signaling pathways must be involved.

Results: We show that Phosphoinositide 3-Kinases (PI3Ks) are required at the onset of zebrafish gastrulation in mesendodermal cells for process formation and cell polarization. Platelet Derived Growth Factor (PDGF) functions upstream of PI3K, while Protein Kinase B (PKB), a downstream effector of PI3K activity, localizes to the leading edge of migrating mesendodermal cells. In the absence of PI3K activity, PKB localization and cell polarization are strongly reduced in mesendodermal cells and are followed by slower but still highly coordinated and directed movements of these cells.

Conclusions: We have identified a novel role of a signaling pathway comprised of PDGF, PI3K, and PKB in the control of morphogenetic cell movements during gastrulation. Furthermore, our findings provide insight into the relationship between cell polarization and directed cell migration at the onset of zebrafish gastrulation.

Introduction

Gastrulation in vertebrates starts with the internalization of mesodermal and endodermal cells that eventually leads to the formation of the three germ layers, ectoderm, mesoderm, and endoderm. Embryological studies have described two major types of cell movements underlying the internalization of mesendodermal progenitor cells [1]. In *Xenopus*, mesendodermal progenitor cells internalize as a cohesive sheet of cells, which usually is referred to as "involution." In contrast, mesendodermal progenitor cells in chicken, mice, and zebrafish undergo an epithelial-to-mesenchymal transition and internalize by "ingression" of single mesenchymal cells. In zebrafish, mesendodermal cell ingression is followed by anterior migration of these cells toward the animal pole [2, 3]. Cells giving rise to axial mesendodermal structures such as the prechordal plate and notochord move as a tightly packed group of cells, while paraxial mesendodermal progenitor cells migrate as loosely associated mesenchymal cells [2, 3].

The molecular mechanisms, which underlie mesendodermal cell internalization and anterior migration, have only begun to be unraveled. In zebrafish, components of the nodal signaling pathway such as the nodal-related signals Squint and Cyclops, the EGF-CFC protein One-eyed-pinhead, and the nodal antagonist Lefty are essential for mesendodermal cell specification and internalization (for a review, see [4]). Genetic mosaic studies have demonstrated that nodal signaling can cell autonomously function in this process, indicating that mesendodermal cell ingression is regulated independently in single cells [5]. Furthermore, anterior migration of ingressing mesendodermal progenitor cells is regulated by Slb/Wnt11-mediated noncanonical Wnt signaling. Slb/Wnt11 is needed in mesendodermal progenitor cells for the preferential orientation of cellular processes in the individual movement directions of these cells. This finding suggests that Slb/Wnt11-mediated orientation of cellular processes controls the directed migration of mesendodermal progenitor cells at the onset of gastrulation [6].

Phosphoinositide 3-Kinases (PI3Ks) are key mediators of intracellular signaling required for such diverse processes as cell proliferation and growth, pinocytosis, vesicle trafficking between intracellular organelles, and chemotaxis (for reviews, see [7, 8]). There are three different classes of PI3K enzymes; of these, class I PI3Ks have been associated with cell polarization and directed cell migration. Insight into the mechanisms by which PI3Ks regulate cell polarization derives mainly from studies in mammalian neutrophils and *Dictyostelium* cells (for reviews, see [9, 10]). There, class I PI3Ks are activated upon stimulation of G protein-coupled chemoattractant receptors. Once activated, PI3Ks catalyze the production of 3′-phosphorylated phosphoinositides at the leading edge of those cells, followed by the activation of several downstream effectors such as small GTPases (which can also function as upstream activators of PI3Ks; [9–11]) and the serine/threonine Protein Kinase B (PKB, also known as Akt) [9–13]. PKB binds directly, via its Pleckstrin Homology domain (PH domain), to Phosphoinositide 3,4,5-triphosphate (PIP3) at the leading edge and activates downstream kinases such as PAKa (P21-Activated Kinase), which eventually leads to the phosphorylation of Myosin II and subsequent polarization of the cytoskeleton [10, 14, 15].

It has been suggested that PI3Ks are involved in patterning and morphogenesis during vertebrate gastrulation [16, 17]. In *Xenopus*, class I PI3Ks can function both

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within the Fibroblast Growth Factor (FGF) and Platelet Derived Growth Factor (PDGF) pathways and are needed for the induction and morphogenesis of mesodermal tissues during gastrulation and the spreading of mesodermal cells in culture [16–19]. PDGF can function as an upstream activator of class I PI3Ks in various cell types, including cultured endothelial cells and fibroblasts. Upon activation, PI3Ks are thought to signal through both small GTPases and PKB and to trigger cytoskeletal changes such as lamellipodia and filopodia formation, a decrease in actin stress fibers and a reduction in focal adhesion complexes in these cells [9, 10, 20–22]. However, it remains to be established which role PDGF and/or PI3Ks play in vivo for cell polarization and directed cell migration during vertebrate gastrulation.

In this study, we have analyzed the cellular function of PI3Ks for the morphology and movement of mesendodermal (prechordal plate) cells at the onset of zebrafish gastrulation. We show that PI3K function is required for cell polarization and the subsequent formation of polarized cellular processes in these cells. We further demonstrate that PDGF functions upstream of PI3Ks in these processes and that PI3K activity leads to a localized accumulation of PKB at the leading edge of migrating prechordal plate cells. In the absence of PI3K activity, prechordal plate cells show strongly reduced cellular polarity, followed by slower but still highly directed movement of these cells toward the animal pole. Our study reveals a novel role for PI3K signaling in cell polarization, process formation, and cell migration during vertebrate gastrulation.

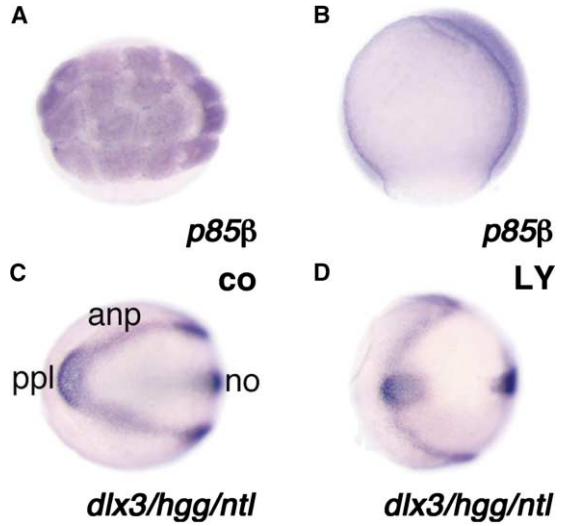

Figure 1. PI3K Is Ubiquitously Expressed during Zebrafish Gastrulation and Is Required for Normal Convergence and Extension Movements

(A and B) Expression of *p85*β mRNA at the (A) 16-cell stage and at (B) 90% epiboly (9 hpf). (A) Animal and (B) lateral views with dorsal oriented toward the right.

(C and D) Convergence and extension of the embryonic axis at bud stage in (C) control (co) embryos and (D) embryos treated with 30 μM LY294002 between sphere stage (4 hpf) and 70% epibloy (7 hpf). The notochord (no) was stained for the expression of *notail* (*ntl*), the prechordal plate (ppl) was stained for the expression of *hatching gland gene 1* (*hgg1*), and the anterior edge of the neural plate (anp) was stained for *dlx3*. Animal views have the anterior oriented toward the left.

Results

Phosphoinositide 3-Kinase (PI3K) Activity Is Required for the Formation of Polarized Cellular Processes in Prechordal Plate Progenitors

PI3Ks are essential intracellular signaling mediators determining cell polarization and directed cell migration (for reviews, see [9, 10]). To determine if PI3Ks are also needed for morphogenesis during zebrafish gastrulation, we analyzed the function of PI3K in regulating the movement and morphology of single cells within the zebrafish gastrula.

We first determined if PI3Ks are expressed during zebrafish gastrulation. The regulatory p85 subunits of PI3K are essential components controlling class IA PI3K activity and subcellular localization [7, 8]. *p85*β mRNA is expressed ubiquitously throughout gastrulation (Figures 1A and 1B and data not shown), indicating that class IA PI3Ks have a function during zebrafish gastrulation. In addition, the p85α and p110α subunits are also ubiquitously expressed (data not shown), suggesting that both regulatory and catalytic PI3Ks subunits are present during gastrulation.

To further investigate if PI3Ks are needed for cell movement and morphology during gastrulation, we exposed embryos to 30 μM of the PI3K inhibitor LY294002 (LY; [23]) from the onset of gastrulation (50% epiboly) to mid-gastrulation stages (80% epiboly) and analyzed the phenotype at the end of gastrulation. This concentration of LY has previously been shown to specifically and effectively block PI3K activity in *Xenopus* embryos [16]. Treated embryos showed a severely shortened and broadened body axis at bud stage that is indicative of reduced convergence and extension movement (Figures 1C and 1D). This phenotype suggests that PI3Ks are required for normal cell movements during zebrafish gastrulation.

In wild-type embryos, ingression of mesendodermal progenitor cells around the circumference of the germ ring, followed by anterior migration of these cells toward the animal pole, constitute the main morphogenetic cell movements at the onset of gastrulation [2, 3]. We have previously shown that mesendodermal prechordal plate progenitors exhibit numerous long pseudopodia preferentially oriented in the direction of their migration [6]. To test if PI3Ks are required for the formation of polarized cellular processes in prechordal plate progenitors at the onset of gastrulation, we used two different approaches to interfere with PI3K function. We treated embryos with the PI3K inhibitor LY, and we injected mRNA coding for a dominant-negative, kinase-dead version of the catalytic p110β subunit of PI3K (*dnPI3K*), a p110 subunit that has previously been shown to be specifically required for cell polarization and migration in macrophages [24].

By exposing embryos to 30 μM LY at 50% epiboly, a concentration that strongly reduces the production of Phosphoinositide 3,4,5-triphosphate (PIP3) in those embryos (Figure S1 in the Supplemental Data available with this article online), we found that ingressing prechordal

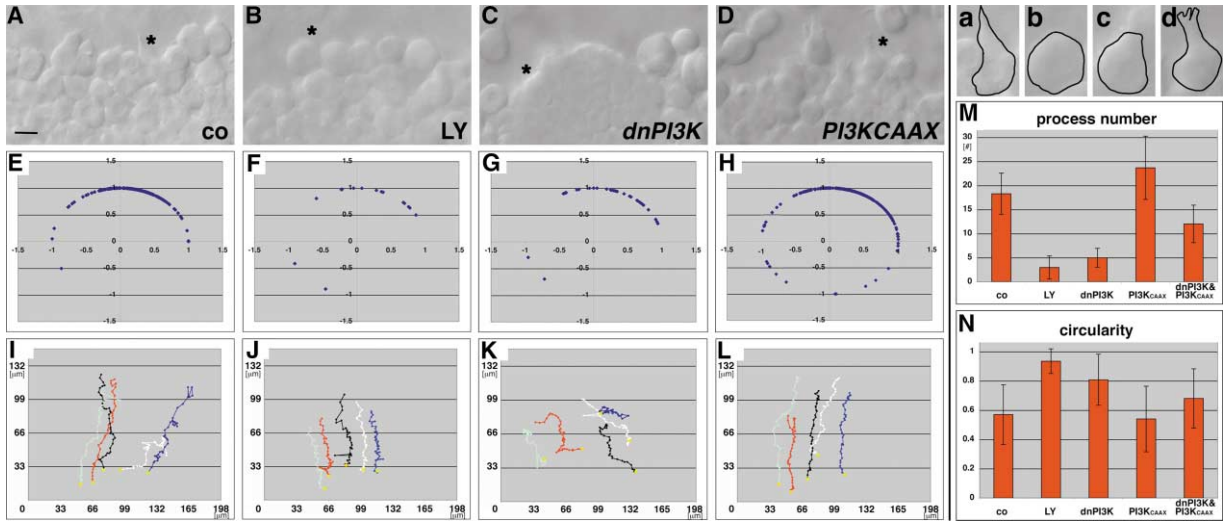

Figure 2. PI3K Activity Is Required for the Formation of Polarized Processes in Prechordal Plate Progenitors at the Onset of Gastrulation

(A–L) (A–D) Morphology and orientation of (E–H) cellular processes and (I–L) single cell tracks of prechordal plate progenitors at shield stage (6 hpf) in (A, E, and I) control (co) embryos, (B, F, and J) embryos treated with 30 μM PI3K inhibitor LY294002 (LY), (C, G, and K) embryos injected with 250 pg/embryo of *dnPI3K* mRNA, and (D, H, and L) embryos injected with 125 pg/embryo of *p110CAAX* mRNA. (A)–(D) are dorsal views at the prechordal plate with the animal oriented toward the top (see Movies 1–4 in the Supplemental Data). In (E)–(H), the orientation of cellular processes is shown relative to the *y* axis (+y = in direction of animal pole) and *x* axis (left-right axis). In (I)–(L), the migration tracks of five single cells at the leading edge of the prechordal plate are shown over a period of 1 hr and 40 min. The positions were determined every 2.5 min. The starting point of each track is marked in yellow. All panels ([A]–[L]) contain the data from the analysis of one exemplary movie per treatment. In total, five movies per treatment were analyzed. (a–d) High-magnification views of exemplary cells (marked by asterisk in [A]–[D]) from (a) control embros, (b) LY-treated embryos, (c) *dnPI3K*-injected embryos, and (d) *p110CAAX*-injected embryos. The cell outlines were artificially traced.

(M) Average number of cellular processes from 25 randomly chosen cells at the leading edge of the prechordal plate; cells were analyzed at 11 consecutive time points (time interval = 10 min) in 5 independent movies in control (co) and LY-treated embryos (LY) and in embryos injected with *dnPI3K, p110CAAX*, and a mixture of *dnPI3K* and *p110CAAX*. Note that both the differences between the co values and the *dnPI3K* and LY values as well as the difference between the *dnPI3K* and *dnPI3K/p110CAAX* values are significant ($p < 0.05$).

(N) Cell circularity ("roundness"; rnd = $4\pi A/p^2$, with A = area and p = perimeter) from 10 randomly chosen cells at the leading edge of the prechordal plate measured at 11 consecutive time points (time interval = 10 min) in 5 independent movies in control (co) and LY-treated embryos (LY) and in embryos injected with *dnPI3K, p110CAAX*, and a mixture of *dnPI3K* and *p110CAAX*. Note that both the differences between the co values and the *dnPI3K* and LY values as well as the difference between the *dnPI3K* and *dnPI3K/p110CAAX* values are significant ($p < 0.05$).

The scale bar in (A) represents 20 μm.

plate progenitors were less elongated compared to similar cells from untreated control embryos and lacked most of their cellular processes (Figures 2B, 2F, 2M, and 2N). In the few cases when cells formed cellular processes, these processes were mainly of lamellipodial character, whereas, in similar cells of control embryos, the majority of processes showed pseudopodial and filopodial character (Table 1). In contrast, the preferential orientation of the few remaining cellular processes was unchanged between LY-treated and control cells (Figure 2F). Surprisingly, although cells from LY-treated embryos showed hardly any morphologically recognizable polarization (Figure 2b), the direction of cell migration toward the animal pole was largely unchanged (Figure 2J). However, inhibitor-treated prechordal plate progenitors moved slower than their untreated controls (1.14 ± 0.17 μm/min in LY-treated embryos versus 1.46 ± 0.31 μm/min in control embryos; $p < 0.05$), indicating that PI3Ks are required for the velocity of prechordal plate progenitor cell migration. Similar defects in respect to cell morphology and movement were also seen in paraxial mesendodermal cells after treating embryos with LY (data not shown).

To test if the phenotype obtained by treating embryos with LY is specific to an inhibition of PI3K activity, we injected 250 pg *dnPI3K* mRNA into 1-cell-stage embryos and analyzed the movement and morphology of prechordal plate progenitors at the onset of gastrulation. Cells overexpressing *dnPI3K* were less elongated and showed both a significantly lower number of cellular processes (Figures 2C, 2G, 2M, 2N, and 2c) and defects in the velocity and direction of cell migration as compared to control cells (1.22 ± 0.28 μm/min in *dnPI3K*-injected embryos versus 1.46 ± 0.31 μm/min in control embryos; $p < 0.05$; Figure 2K). In contrast, the orientation and character of the few remaining cellular processes were largely unchanged as compared to control cells (Figure 2G; Table 1). This phenotype is similar, although slightly weaker, than the one observed after treating embryos with LY, which confirms the specificity of this treatment. The defect in the direction of cell migration observed in *dnPI3K*-injected, but not LY-treated, embryos (compare Figure 2K with 2J) is likely to be due to *dnPI3K* interfering with an earlier patterning function of PI3K [16]. Notably, embryos injected with *dnPI3K*, in contrast to LY-treated embryos, often exhibited differ-

Table 1. Changes in the Relative Distribution, in Percent, of Different Types of Cellular Processes in Prechordal Plate Progenitor Cells after Interfering with PDGF/PI3K Signaling

	Filopods[a]	Pseudopods[b]	Lamellipods[c]	Arborization[d]
Control	17	32	46	5
dnPI3K	13	32	49	6
LY	8	18	74	0
p110CAAX	21	39	26	14
AG	10	22	63	5

In general, cellular protrusions were counted as such if the angels between the protrusions and the cell body were $<135°$. AG, PDGF receptor inhibitor AG1295; LY, PI3K inhibitor LY294002.
[a] Filopods were defined as cellular protrusions that have a higher length than average width and an average diameter that is less than 2 μm.
[b] Pseudopods were defined as cellular protrusions that have a higher length than average width and an average diameter that is greater than 2 μm.
[c] Lamellipods were defined as cellular extensions that have a higher average width than length and a total length that is greater than 2 μm.
[d] Arborizations were defined as pseudopods that showed an arborization at their ends.

ent ranges of dorsalization at the onset of gastrulation (Figures S2 and S3 in the Supplemental Data), which previously has been shown to interfere with normal cell movements during gastrulation [25].

PI3Ks might function in prechordal plate progenitors by either allowing these cells to form polarized cellular processes (permissive function) or by actively inducing the formation of polarized cellular processes (instructive function). To distinguish between these two possibilities, we injected 125 pg *p110CAAX* mRNA into 1-cell-stage embryos and analyzed cell movement and morphology at the onset of gastrulation. *p110CAAX* codes for a membrane-anchored form of the p110α catalytic subunit shown to increase the activity of PI3K [26]. Prechordal plate progenitors overexpressing *p110CAAX* exhibited a significantly higher number of cellular processes (Figures 2D and 2M), which also often showed a more extensive arborization at their ends (Figure 2d and Table 1). The preferential orientation of these processes toward the animal pole was significantly reduced but not completely abolished as compared to control embryos (78% of all processes oriented toward the animal pole in *p110CAAX*-overexpressing cells versus 97% in control cells; $p < 0.05$; Figure 2H). This finding indicates that activation of PI3K can instructively determine the outgrowth, shape, and orientation of cellular processes. Despite the overproduction of cellular processes, neither the direction nor the velocity of prechordal plate progenitor cell migration was affected in *p110CAAX*-injected cells (1.32 ± 0.18 μm/min in *p110CAAX*-injected embryos versus 1.46 ± 0.31 μm/min in control embryos; $p > 0.05$; Figure 2L).

As a further test for the specificity of the prechordal plate progenitor cell phenotype obtained by the overexpression of *dnPI3K*, we also tried to rescue the *dnPI3K* phenotype by coexpressing the membrane-anchored p110 catalytic subunit of PI3K (*p110CAAX*). Coexpression of *dnPI3K* with *p110CAAX* led to a rescue of both the number of cellular processes formed and the elongation of prechordal plate progenitors as compared to *dnPI3K* expression alone (Figures 2M and 2N); this finding suggests that PI3Ks are specific regulators of cellular process formation in these cells.

Altogether, these results indicate that inhibition of PI3K activity leads to prechordal plate progenitors being less polarized and showing a lower number of cellular processes. However, neither activation nor inhibition of PI3K activity appears to affect the ability of those cells to move in a directed and coordinated way, while inhibition of PI3K activity reduces the velocity of migration of these cells.

PI3Ks Are Required for the Localization of Protein Kinase B (PKB) to the Leading Edge of Prechordal Plate Progenitors

In mammalian neutrophils and *Dictyostelium* cells, PKB binds via its PH domain to Phosphoinositide 3,4,5-triphosphate (PIP3), a product of PI3K activity, and induces downstream effectors. These events occur at the leading edge of migrating cells and thereby control cell polarization and directed cell migration [9, 10]. To test if PKB is also localized in prechordal plate progenitors, we injected mRNA coding for a fusion construct of the PH domain of PKB with Green Fluorescent Protein (GFP), which has previously been shown to constitute a reliable readout for subcellular PKB localization [11, 13, 27]. In control embryos, PH-GFP is strongly localized to the plasma membrane of prechordal plate progenitors at sites of cell-cell contact. Moreover, in cells at the leading edge of the prechordal plate, and predominantly in cells that are only loosely associated with their surrounding cells, PH-GFP often was asymmetrically distributed from the leading to the rearing edge during process formation and cell elongation (Figures 3A–3F). To exclude that this localization of PH-GFP is a sole secondary consequence of unequal membrane accumulations, we also overexpressed a membrane-anchored form of GFP (Gap43-GFP) in these cells. In contrast to PH-GFP, Gap43-GFP showed no preferential localization to the leading edge of prechordal plate progenitors, indicating that this localization is specific to PH-GFP (data not shown).

When embryos were treated with 30 μM LY at the onset of gastrulation or injected with *dnPI3K* mRNA at the 1-cell stage, the membrane localization and asymmetric distribution of PH-GFP were greatly diminished (Figures 4A–4C), indicating that PI3Ks are required for the localization of PKB to the membrane of prechordal plate progenitors. We also tested if overexpression of *p110CAAX* can affect the membrane localization of PH-GFP. Overexpression of 125 pg *p110CAAX* mRNA led to an increased accumulation of PH-GFP at the membrane

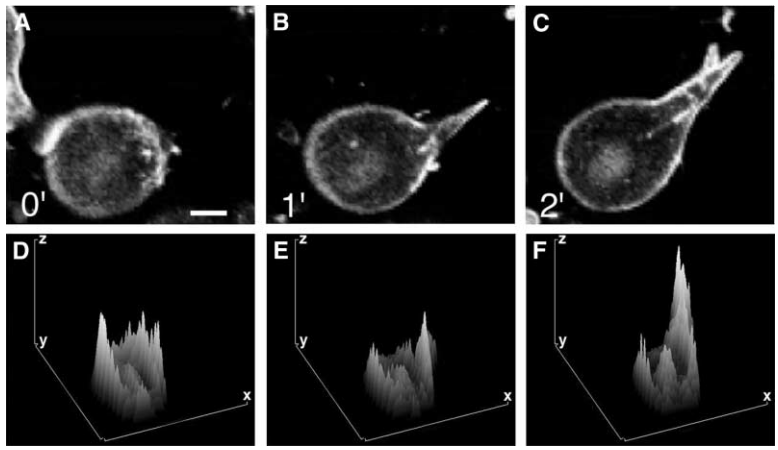

Figure 3. PKB Localizes at the Leading Edge of Prechordal Plate Progenitors at the Onset of Gastrulation

(A–C) Dynamic PH-GFP localization in prechordal plate progenitor cells during process formation at three consecutive time points (A = 0; B = +1 min; C = +2 min) at shield stage (6 hpf) (see Movie 5 in the Supplemental Data).

(D–F) Surface plots quantifying the dynamic changes in the distribution of PH-GFP labeling during process formation in the prechordal plate progenitor cell shown in (A)–(C). *x* and *y* axes = pixel localization in the corresponding pictures from (A)–(C); *z* axis = counts of pixel intensity.

The scale bar in (A) represents 10 μm.

(Figures 4A and 4D), and this increased accumulation supports the notion that PI3Ks direct the membrane localization of PKB.

Experiments, primarily in cell culture, have shown that Actin localizes at the leading edge of migrating cells and that PI3K/PKB is involved in this localization [15, 24]. To determine if Actin is localized to the leading edge of prechordal plate progenitor cells and if this polarized distribution depends on PI3K/PKB activity, we determined the distribution of F-Actin-GFP in prechordal plate progenitor cells in control, LY-treated, and *dnPI3K*- and *p110CAAX*-injected embryos. While in control and *p110CAAX*-injected embryos Actin-GFP is localized to the leading edge of prechordal plate progenitor cells, no such localization was detected in similar cells from LY-treated and *dnPI3K*-injected embryos (Figures 4E–4H). This suggests that PI3K/PKB activity is required for the polarized distribution of Actin in these cells.

Platelet Derived Growth Factor (PDGF) Functions Upstream of PI3Ks to Induce the Formation of Polarized Cellular Processes in Prechordal Plate Progenitors

PDGF has been shown to be required upstream of PI3Ks for the spreading of *Xenopus* mesodermal cells in culture [17]. Furthermore, *PDGF-A* mRNA is ubiquitously

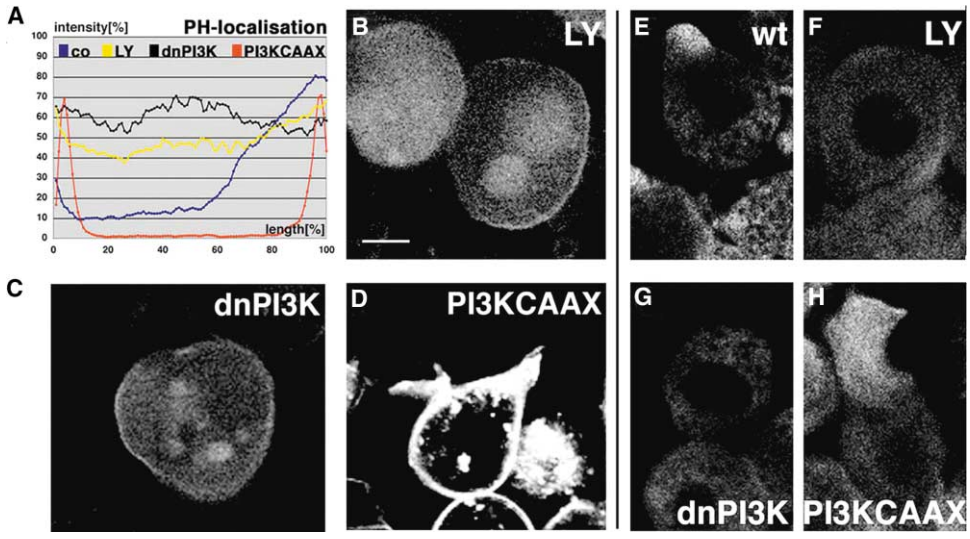

Figure 4. PKB and Actin Are Localized at the Leading Edge of Prechordal Plate Progenitors in a PI3K-Dependent Way

(A) A plot diagram showing the average distribution of PH-GFP in prechordal plate progenitor cells along the primary axis of cell elongation in control embryos (blue), embryos treated with 30 μM of the PI3K inhibitor LY294002 (yellow), injected with 250 pg/embryos of *dnPI3K* mRNA (black), and injected with 125 pg/embryo of *p110CAAX* mRNA (red) at shield stage (6 hpf). The *x* axis shows the relative positions along the axis of measurement normalized for all cells to a total length of 100% (with 100% at the leading edge). For round cells, the axis of measurement was oriented in the overall direction of cell migration. The *y* axis shows the relative intensities, with the brightest spot per cell normalized to 100%. Twenty-five cells from five different embryos were quantified per treatment.

(B–D) PH-GFP localization in prechordal plate progenitors of embryos (B) treated with 30 μM LY, (C) injected with 250 pg/embryos of *dnPI3K* mRNA, and (D) injected with 125 pg/embryo of *p110CAAX* mRNA at shield stage (6 hpf) (see Movies 6–8 in the Supplemental Data).

(E–H) Actin-GFP localization in prechordal plate progenitor cells from (E) control embryos and (F) embryos treated with 30 μM LY, (G) injected with 250 pg/embryos of *dnPI3K* mRNA, and (H) injected with 125 pg/embryo of *p110CAAX* mRNA at shield stage (6 hpf).

The scale bar in (B) represents 10 μm.

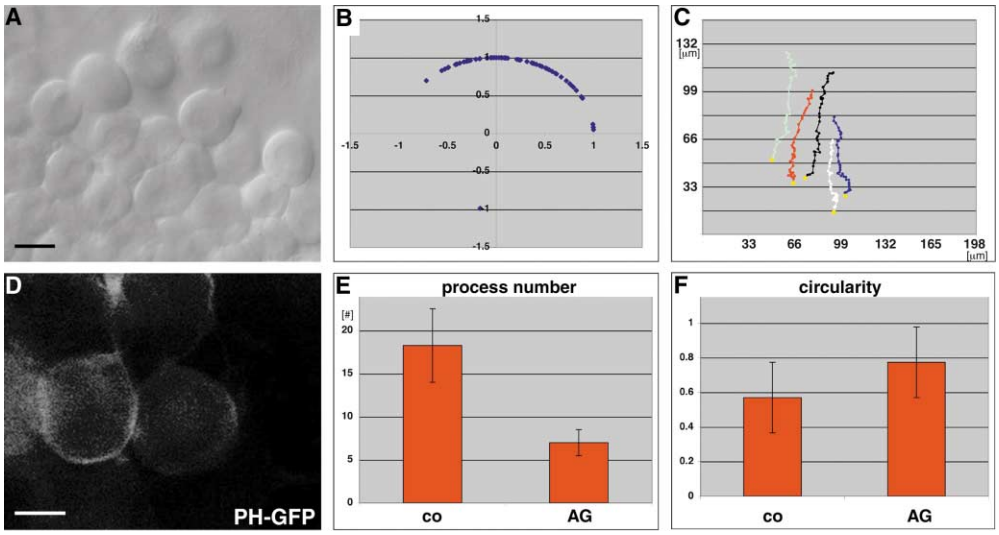

Figure 5. PDGF Is Required for the Formation of Polarized Cellular Processes and the Membrane Localization of PKB in Prechordal Plate Progenitors at the Onset of Gastrulation

(A–C) (A) Morphology and orientation of (B) cellular processes and (C) single cell tracks of prechordal plate progenitors in embryos treated with 10 μM of the PDGF receptor inhibitor AG1295 (AG) at shield stage (6 hpf). (A) is a dorsal view at the prechordal plate; the animal view is oriented toward the top (see also Movie 9 in the Supplemental Data). In (B), the orientation of cellular processes is shown relative to the *y* axis (+y = in the direction of animal pole) and *x* axis (left-right axis). In (C), the migration tracks of five single cells at the leading edge of the prechordal plate are shown over a period of 1 hr and 40 min. The positions were determined every 2.5 min. The starting point of each track is marked in yellow. All panels [(A)–(C)] contain the data from the analysis of one exemplary movie. In total, five movies have been analyzed.

(D) PH-GFP localization in prechordal plate progenitors of embryos treated with 10 μM of the PDGF inhibitor AG at shield stage (6 hpf).

(E) Average number of cellular processes from 25 randomly chosen cells at the leading edge of the prechordal plate; cells were analyzed at 11 consecutive time points (time interval = 10 min) in 5 independent movies in control (co) and AG-treated embryos. Note that the difference between the co and AG values is significant ($p < 0.05$).

(F) Cell circularity ("roundness"; rnd = $4\pi A/p^2$, with A = area and p = perimeter; for details, see also the Experimental Procedures) of 10 randomly chosen cells at the leading edge of the prechordal plate measured at 11 consecutive time points (time interval = 10 min) in 5 independent movies in control (co) and AG-treated embryos. Note that the difference between the co and AG values is significant ($p < 0.05$).

The scale bar in (A) represents 20 μm; the scale bar in (D) represents 10 μm.

expressed throughout zebrafish gastrulation [28]. We therefore tested if PDGF might function upstream of PI3Ks to induce the formation of cellular processes in zebrafish prechordal plate progenitors at early gastrula stages.

To be able to selectively interfere with a possible function of PDGF in prechordal plate progenitor cell morphogenesis at the onset of gastrulation, we made use of the highly specific PDGF receptor inhibitor AG1295 (AG) [22, 29]. Treating embryos at the onset of gastrulation with 10 μM of the PDGF inhibitor AG, a dose that specifically and effectively blocks PDGF activity in various cell types ([22, 29] and this study, see below), did not affect the induction of prechordal plate cell fates (data not shown), but instead resulted in prechordal plate progenitors being less polarized and forming a significantly lower number of cellular processes (Figures 5A, 5E, and 5F; see also Figure 2). The remaining cellular processes in AG-treated cells showed a shift from pseudopodial and filopodial character to predominantly lamellipodial character (Table 1), while their orientation was largely unchanged (Figure 5B). This phenotype is similar but also weaker than the one observed after treating embryos with LY, which might also explain why, in contrast to LY-treated cells, the velocity of cell migration was not significantly reduced in AG-treated prechordal plate progenitors (1.32 ± 0.23 μm/min in AG-treated embryos versus 1.46 ± 0.31 μm/min in control embryos; $p > 0.05$; Figure 5C).

The similarity between the phenotypes observed in mesendodermal progenitors after inhibiting PDGF and PI3K activities suggests that PDGF and PI3K function in the same signaling pathway. As a more direct proof that PDGF functions upstream of PI3K, we determined if the activity of PI3Ks to catalyze the production of PIP3 and to localize PKB to the plasma membrane of prechordal plate progenitors is blocked in the presence of the PDGF receptor inhibitor AG. In embryos treated with 10 μM AG at the onset of gastrulation, the total level of PIP3 was significantly downregulated (Figure S1), and PH-GFP showed a reduced membrane localization as compared to untreated controls (Figure 5D; see also Figure 3). This indicates that PDGF functions upstream of PI3Ks in respect to the production of PIP3 and the membrane localization of PKB in prechordal plate progenitors at the onset of gastrulation.

PDGF-Mediated PI3K Activity Controls the Outgrowth of Cellular Processes in Zebrafish Mesendodermal Cells in Culture

To more directly determine the function of PDGF/PI3K signaling for the formation of cellular processes and to prove the specificity of the results obtained with the PDGF receptor inhibitor AG, we developed primary cultures of zebrafish mesendodermal cells (for details, see the Experimental Procedures). After 1 hr in vitro, cells usually started to cluster together and extended short

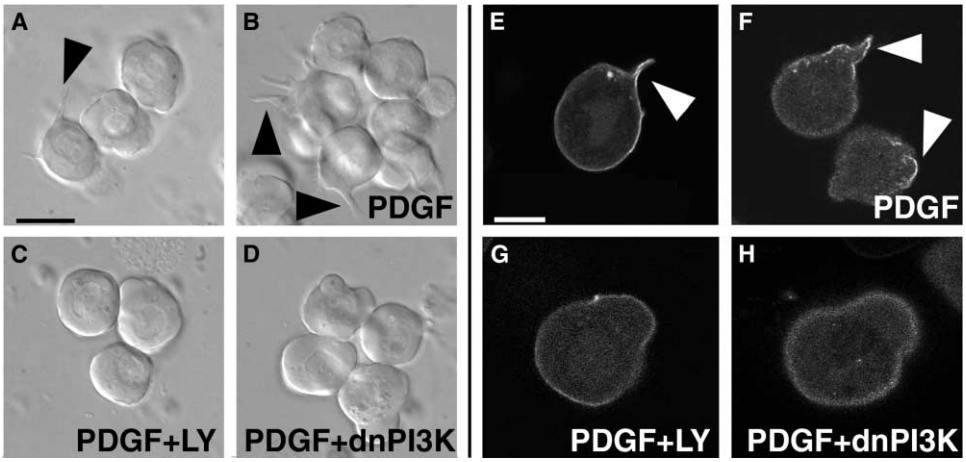

Figure 6. PDGF Signals via PI3K to Induce the Formation of Cellular Processes and to Promote the Membrane Localization of PKB in Zebrafish Mesendodermal Cells in Culture

(A–D) Morphology of mesendodermal cells kept for 2 hr in (A) control (co) medium, (B) in the presence of 50 ng/ml PDGF-AA, (C) in the presence of 50 ng/ml PDGF-AA and 30 μM of the PI3K inhibitor LY294002 (LY), and (D) after injection of 250 pg of *dnPI3K* mRNA and in the presence of 50 ng/ml PDGF-AA. The arrowheads point at cellular processes.

(E–H) PH-GFP localization in mesendodermal cells kept for 2 hr in (E) control medium, (F) in the presence of 50 ng/ml PDGF-AA, (G) in the presence of 50 ng/ml PDGF-AA and 30 μM LY, and (H) after injection of 250 pg *dnPI3K* mRNA and in the presence of 50 ng/ml PDGF-AA. The arrowheads point at sites of PH-GFP localization.

The scale bar in (A) represents 20 μm; the scale bar in (E) represents 10 μm.

cellular processes on the outside of such clusters (Figure 6A). Adding 50 ng/ml PDGF to the culture medium strongly enhanced the number and stability of cellular processes originating from such clusters (average number of processes per cell: 0.58 ± 0.14 in the presence of PDGF versus 0.39 ± 0.19 in control medium; $p < 0.05$; average time needed for the extension and subsequent retraction of one cellular process: 2.45 ± 1.87 min in the presence of PDGF versus 2.10 ± 1.41 min in control medium; $p < 0.05$; Figure 6B). This finding indicates that PDGF can promote the formation and stabilization of cellular processes in mesendodermal cells in culture.

To investigate the epistatic relationship between PDGF and PI3Ks, we determined if the effect of PDGF on cellular process formation can be blocked in the presence of PI3K inhibitor or *dnPI3K*. PDGF-induced process formation was effectively inhibited by the addition of 30 μM of the PI3K inhibitor LY to the culture medium or, alternatively, by overexpressing *dnPI3K* in these cells (no processes found) (Figures 6C and 6D); this finding indicates that PI3Ks act downstream of PDGF. Similarly, the localization of PH-GFP to the membrane at sites of cellular process formation was effectively promoted in the presence of PDGF and was strongly reduced by either adding PI3K inhibitor to the culture medium or overexpressing *dnPI3K* in these cells (Figures 6E–6H).

PDGF might either promote cellular process formation in cultured mesendodermal cells without interfering with process orientation and cell polarization or it might actively polarize these cells. To distinguish between these possibilities, we created a local gradient of PDGF-A by placing beads coated with PDGF-A into cultures of mesendodermal cells and observing the behavior of cells in response to such a gradient. Mesendodermal cells that were located close to a PDGF-A (0.25 μg/μl)-coated bead moved toward the bead and formed multiple long processes in the direction of this bead (see Movie 10 in the Supplemental Data available with this article online). In contrast, mesendodermal cells close to a control bead did not show any preferential orientation of their processes and movement directions (Movie 10). This indicates that a local gradient of PDGF-A is able to polarize the movement and protrusive activity of mesendodermal cells in culture.

Discussion

In this study, we show that Phosphoinositide 3-Kinases (PI3Ks) are required for cellular process formation and cell polarization in prechordal plate progenitors during their migration from the germ ring toward the animal pole. Platelet Derived Growth Factor (PDGF) functions upstream of PI3Ks, while the serine/threonine Protein Kinase B (PKB) is a downstream target (for a schematic illustration, see Figure 7). Prechordal plate progenitors in which PI3K activity is blocked lack most of their cellular processes and do not accumulate Actin and PKB at their leading edge, indicative of a loss of cell polarization. This loss of cell polarization is accompanied by slower but still highly directed cell movements, indicating that PI3K-mediated cellular process formation and cell polarization is required for the velocity but not the direction of mesendodermal cell migration at the onset of gastrulation.

The Role of PI3K for Cell Polarization of Prechordal Plate Progenitors

PI3Ks play important roles in the control of cell polarization and directed cell migration in different cell types in vitro (for reviews, see [9, 10]). However, the relevance of those findings for the function of PI3Ks in a developing

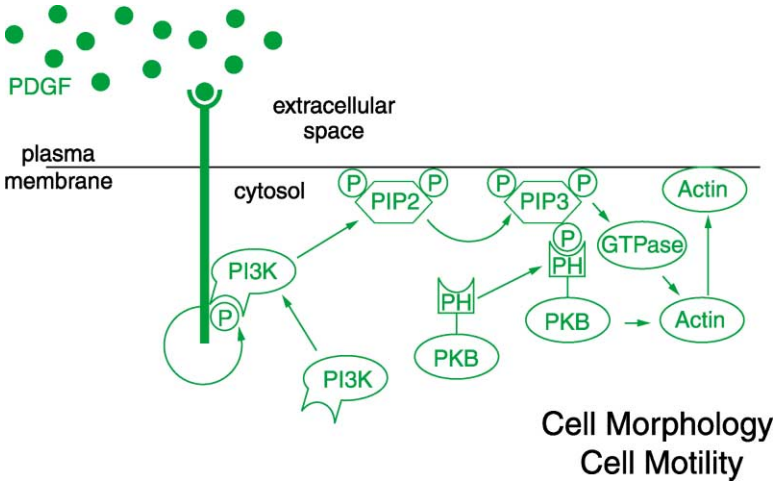

Figure 7. A Schematic Diagram Illustrating a Molecular Pathway Comprised of PDGF, PI3K, and PKB Required for Polarization and Migration of Prechordal Plate Progenitor Cells at the Onset of Zebrafish Gastrulation

PDGF binds and activates its receptor(s); PI3K binds to the activated (and phosphorylated) receptor and in turn catalyzes the phosphorylation of PIP2 to PIP3 in the plasma membrane; PKB binds via its PH domain to PIP3, triggering the localized accumulation of Actin and subsequent cell polarization/cellular process formation and motility. PDGF, Platelet Derived Growth Factor; PI3K, Phosphoinositide 3-Kinase; PIP2, Phosphoinositide 4,5-diphosphate; PIP3, Phosphoinositide 3,4,5-triphosphate; PKB, Protein Kinase B; P, Phosphate; PH, Pleckstrin Homology domain; GTPase, small GTPases (Rho, Rac, and Cdc42).

organism has not yet been tested. The observation from this study that PI3K activity is required for prechordal plate progenitor cells to polarize and that the failure to polarize in the absence of PI3K activity is accompanied by slower movements of these cells provides the first clear evidence for an involvement of PI3Ks in cell polarization and directed cell migration in the gastrula embryo. Moreover, our observation that PKB is localized to the leading edge of prechordal plate progenitors and that this localization is dependent on PI3K activity indicates that PI3Ks control cell polarization in vivo by using similar downstream effectors as previously shown in cultured cells such as mammalian lymphocytes and *Dictyostelium* cells [9–11, 13]. However, in contrast to those in vitro studies, PI3K activity is not required for the direction of cell migration in prechordal plate progenitors, indicating that the specific functions of PI3Ks in cell polarization and migration can substantially vary depending on the particular cellular environment. The implications of those findings are discussed below.

Interestingly, a strong reduction of PI3K activity in prechordal plate progenitors not only leads to a considerable decrease in the total number of cellular processes formed, but also to a shift from pseudopodial and filopodial character to predominantly lamellipodial character in the few remaining cellular processes. Similarly, an overactivation of PI3K signaling leads to an increase in the number of cellular processes (filopods and pseudopods) and a concomitant more extensive branching of those processes. This suggests that PI3Ks promote the formation and arborization of cellular processes with filopodial and pseudopodial character in prechordal plate progenitor cells at the onset of gastrulation. A similar function of PI3K has also recently been observed in sensory neurons in vitro where PI3K signaling promotes axonal elongation and branching [30].

In *Xenopus* gastrulation, PDGF signaling has been shown to be required for survival and/or migration of involuting anterior mesodermal cells in vivo and to induce, via PI3K, the spreading of *Xenopus* mesodermal cells in culture [17, 18]. The findings of this study and our own preliminary observations with *morpholino* antisense oligonucleotides against PDGF-A support these observations, demonstrating that PDGF is required for mesendodermal cell induction/survival and, upstream of PI3K, for mesendodermal cell movement and morphology at the onset of gastrulation.

It has further been speculated that PDGF/PI3K exerts its function in mesodermal cell spreading by regulating the adhesion of those cells to their substrate, although no direct evidence for such a function has yet been provided [17, 18]. We now show that PDGF/PI3K signaling, in addition to its role in mesodermal cell spreading, is needed for the polarization and branching of cellular processes of prechordal plate progenitors in vivo and that this function of PDGF/PI3K signaling is accompanied by the asymmetric localization of PKB (PH-GFP) to the leading edge of those cells. These findings provide novel insight into both the role of PDGF/PI3K for mesendodermal cell morphogenesis (polarization, process branching) and the downstream targets (PKB, Actin) of PDGF/PI3K signaling in this process. Interestingly, PI3Ks in epithelial cells are needed for the localization of PKB to the plasma membrane at sites of cell-matrix and cell-cell interactions [27]; this finding suggests that PI3Ks regulate cell adhesion in these cells via the localization of PKB at sites of preferential attachment. Furthermore, PDGF has been shown to promote cell adhesion and spreading of migrating fibroblasts by regulating the intracellular transport of integrins from the rear to the front of those cells [31], a process that appears to also be dependent on PI3K activity [32]. It is therefore conceivable that PDGF/PI3K signaling controls process formation in prechordal plate progenitors, either directly or indirectly (see also below), by the localized activation of PI3Ks at sites of preferential cell-cell and cell-matrix interactions. A better understanding of the potential role of cell adhesion in this process will require the identification and characterization of the molecular targets of PDGF/PI3K signaling in prechordal plate progenitors at the onset of gastrulation.

It remains to be established whether PDGF itself is polarizing prechordal plate progenitor cells or if it is allowing these cells to polarize in response to an unrelated polarizing signal. The finding from this study that the introduction of beads coated with PDGF-A in cultures of mesendodermal cells causes cells next to these beads to extend processes toward the source of PDGF

indicates that PDGF has the potential to polarize those cells. However, the observation that PDGF-A is expressed ubiquitously during zebrafish gastrulation [28] argues against a spatially restricted production of PDGF. It is still possible that PDGF protein, in contrast to its mRNA, shows a graded distribution at the onset of gastrulation due to temporal and spatial differences in protein translation, processing, and secretion. A more direct visualization of PDGF protein distribution through the generation of specific antibodies and/or the introduction of PDGF-GFP fusion proteins will be needed to answer this question.

The Role of PI3Ks for Directed Migration of Prechordal Plate Progenitors

A surprising outcome of this study is that blocking the function of PI3K in prechordal plate progenitors leads to a strong reduction in cell polarization and process outgrowth, while the ability of those cells to migrate in a directed way is not affected. This suggests that PI3K-mediated cell polarization is not an essential prerequisite for directed migration of prechordal plate progenitors at the onset of zebrafish gastrulation and challenges the common view that cell polarization is needed for directed cell migration [12, 33].

It is conceivable that other mechanisms than cell polarization contribute to the directed migration of prechordal plate progenitors. For instance, prechordal plate progenitors might be simply pushed away from the germ ring by newly ingressing cells at the germ ring margin rather than actively migrating in the direction of the animal pole. Taking into account that ingression of new prechordal plate progenitors is restricted to a narrow region at the germ ring margin and that mesendodermal cells simultaneously internalize around the circumference of the germ ring [3], prechordal plate progenitors would permissively be pushed away from the margin toward the animal pole of the gastrula. This assumption is supported both by the outcome of this study and by recent studies, which show that transplantations of single wild-type cells into the germ ring margin of *MZoep* mutants that lack any mesendodermal cell ingression leads to the cell-autonomous ingression of these transplanted cells [5]. However, single transplanted cells do not show any anterior movement toward the animal pole, suggesting that this movement requires the simultaneous ingression of other mesendodermal cells.

If the direction of cell migration is determined independently of cell polarization and process formation, what could be the role of cell polarization and motility (extension and retraction of cellular processes) for the migration of prechordal plate progenitors at the onset of gastrulation? The observation that in the absence of cell polarization and process formation prechordal plate progenitors display slower cell movements indicates that cell motility serves as a "fine-tuning" mechanism for these cells to facilitate movement and explore their cellular environment. Similar observations have been made by recent studies in zebrafish and *Xenopus* showing that in both gain- and loss-of-function situations for different components of the Wnt/PCP pathway, a reduction in cell elongation and/or polarization leads to less stable and slower cell movements during gastrulation but does not interfere with the net movement direction of these cells (for reviews, see [34, 35]).

It is likely that the PDGF/PI3K pathway and the Wnt/PCP pathway interact to control prechordal plate progenitor morphology and movement at the onset of gastrulation. Both pathways use small GTPases such as RhoA and Cdc42 as downstream effectors, and both of them are thought to directly modulate cytoskeletal elements such as Myosin and Actin ([9–12, 34, 35]; see also Figure 7). However, it is also possible that these pathways function independently of each other by regulating different aspects of cell polarization and process formation. For instance, the PDGF/PI3K pathway might determine global cell polarization, while the Wnt/PCP pathway is involved in the fine regulation and stabilization of this polarity. In support of this view is the observation that in the absence of PDGF/PI3K signaling, prechordal plate progenitors lack clear cell polarity (this study), while, in mutants of the Wnt/PCP pathway such as *slb/wnt11*, the direction of cell polarization, but not polarity itself, is lost, [6].

In addition to the Wnt/PCP pathway, STAT3, a member of the Janus Kinase (JAK)/signal transducers and activators of transcription (STATs) cascade, has also been shown to be required for the anterior migration of prechordal plate progenitors during zebrafish gastrulation [36]. In analogy to the situation in *Dictyostelium*, where both Dd-STAT and PI3K synergistically regulate different aspects of cell migration [37], STAT3 and PI3Ks might function cooperatively to regulate prechordal plate progenitor cell polarization and directed cell migration at the onset of zebrafish gastrulation. Future studies will have to address how these different pathways interact to regulate specific aspects of cell migration during vertebrate gastrulation.

Experimental Procedures

Embryo Maintenance

All embryos were obtained from zebrafish TL and AB wild-type lines, grown at 31°C, and manipulated in E3 zebrafish embryo media.

mRNA Injections

For mRNA synthesis and overexpression, the following constructs were used: pCS2-*p110CAAX* was obtained by cloning the BamHI/XhoI insert from pLHA110CAAX into pCS2+. pCS2-p110β(D919A) was obtained by cloning the XbaI insert from pCMV5-p110β(D919A) (obtained from Marino Zerial) into pCS2+. pCS2-PH(Akt)GFP was obtained by cloning the EcoRI/XhoI insert from a pCS2-PH-GFP-nos construct (obtained from Erez Raz) into pCS2+. For *cyclops* and *actin-GFP* mRNA synthesis, a pCS2-ndr2 [38] and a pCS2-actin-GFP construct (obtained from Lukas Roth), respectively, were used. mRNAs were synthesized by using the Ambion mMessage mMachine Kit. For overexpression, 125 pg *PH(Akt)GFP*, 125 pg *p110CAAX*, 250 pg *p110β (D919A)*, 100 pg *Cyclops*, and 100 pg *actin-GFP* mRNAs were injected into 1-cell-stage embryos.

Inhibitor Treatments

To block PI3K and PDGF activity, we used LY294002 (Calbiochem), a specific inhibitor of PI3K activity [23], and AG1295 (Calbiochem), a specific inhibitor of PDGFR activity [22, 29]. For time-lapse imaging, embryos were usually treated from 50% epiboly to shield stage in 30 μM LY294002 or 10 μM AG1295 and then mounted in 0.8% low-melting-point agarose in E3 as described below.

In Situ Hybridization

Whole-mount in situ hybridization was performed as previously described [39]. The zebrafish *p85*β cDNA was isolated by low-stringency cDNA library screening (GenBank Accession Number AY232630). For *p85*β in situs, an antisense RNA probe was synthesized from a EcoRI-linearized, full-length *p85*β plasmid by using T7 RNA polymerase.

Embryo Mounting, DIC Time-Lapse Imaging, and Image Analysis

Embryos at shield stage were manually dechorionated and mounted in 0.8% low-melting-point agarose in E3 containing 30 μM LY 294002 or 20 μM AG1295 depending on the experimental setup. For DIC time-lapse imaging, we recorded 20 Z-stacks (2.5 μm steps) at 200 consecutive time points (30-s intervals) by using a Zeiss Axioplan 2 microscope with a 40× water immersion lens and Openlab 3.1 (Improvision) imaging software. The images were analyzed by using Image-J software. For cell movement tracks, we defined the cell nucleus as the center of the cell. For determining cell circularity, we traced the outline of a cell in two dimensions, including all recognizable cellular protrusions, and calculated the degree of "roundness" by using $rnd = 4\pi A/p^2$, with A = area and p = perimeter. For the analysis of the significance between two mean values, a student's t test with a two-tailed distribution was chosen, either paired or based on an unequal variance between the two mean values.

Confocal Imaging

For confocal time-lapse imaging, we used a Nikon TE 300 Microscope at a BioRad Radiance 2000 Multiphoton Confocal Microscope with a 60× water immersion lens. For data acquisition, we used the LaserSharp 2000 program version 1.4 on a Windows NT-based PC. Images were taken by scanning a 104.2 μm × 104.2 μm area with 50 lines per second.

Cell Culture

Embryos at the 1-cell stage were injected with 100 pg *cyclops* mRNA [35] to confer mesendodermal character to most of the cells (as determined by the expression of *notail*) and were enzymatically dechorionated at dome stage by using 10 mg/ml pronase in sterilized fish water. After several washing steps in sterile fish water, embryos were incubated in DMEM containing 0.5 mg/ml trypsin and 0.2 mg/ml EDTA for 5 min at 37°C. After stopping the trypsin reaction with serum, the embryos were dissociated into single cells by pipetting them several times through a glass Pasteur pipette. Cells were harvested by centrifuging them at 500 rpm for 5 min and were then transferred to fresh DMEM medium containing 1 mg/ml insulin, 0.3 mg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, cultured on plastic petri dishes coated with 10 μg/ml fibronectin at 28°C, and imaged after 2 hr of incubation. Depending on the experiment, 50 ng PDGF-AA (Sigma) and/or 30 μM LY were added to the culture medium.

Supplemental Data

Supplemental Data including both time-lapse movies information about levels of PIP3 production and dorsoventral patterning of the gastrula after blocking PI3K function are available at http://www.current-biology.com/cgi/content/full/13/15/1279/DC1/.

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Accession Numbers

The GenBank accession number for the zebrafish *p85*β cDNA reported in this paper is AY232630.