# Signaling pathways controlling primordial germ cell migration in zebrafish

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## Summary

**During their migration, zebrafish primordial germ cells (PGCs) rely on directional cues provided by the chemokine SDF-1a, whose receptor is CXCR4b. The molecular mechanisms whereby CXCR4b activation is interpreted intracellularly into directional migration are not known. Here we investigate the role of two important biochemical pathways – G-protein-dependent and phosphoinositide 3-kinase (PI3K)-dependent signaling – in directing PGC migration in zebrafish. We show that G proteins of the Gi family are essential for directional migration but not for PGC motility. Inhibition of PI3K signaling in PGCs slows down their migration and leads to abnormal cell morphology as well as to reduced stability of filopodia. Invariably, during directed PGC migration, the distribution of the products of PI3K activity – phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)$P_3$] and/or phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)$P_2$] – is not polarized, and reducing the level of these 3-phosphoinositides does not affect the ability of PGCs to migrate directionally. We therefore conclude that Gi-dependent signaling is essential for directional migration, whereas the PI3K pathway is important for the actual motility of PGCs.**

Supplementary material available online at
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Key words: *Danio rerio*, PGC, Chemotaxis, Cell migration, PH domain, G protein, PI3K, SDF-1, CXCR4, Chemokine

## Introduction

Chemotaxis is defined as the ability of cells to sense and migrate along an external chemical gradient. This process requires the integration of different cell responses that include motility, polarization and directional sensing (Devreotes and Janetopoulos, 2003; Iijima et al., 2002). Directed cell migration in response to external cues plays an essential role in development and homeostasis, as well as in diseases caused by aberrant migration such as chronic inflammation and cancer. Therefore, the characterization of the molecular machinery controlling directional migration and motility in vivo is both an important theme in development and is highly pertinent to clinical research.

An in vivo system that has emerged as an excellent model for studying directional cell migration is that of primordial germ cells (PGCs). In many organisms, PGCs actively migrate from the position where they are specified towards the region of the gonad, where there they will differentiate into gametes (Starz-Gaiano and Lehmann, 2001; Wylie, 2000). In zebrafish too, PGCs actively migrate while responding to directional cues emanating from the somatic tissues on their way to the gonad (Doitsidou et al., 2002; Raz, 2003; Weidinger et al., 1999; Weidinger et al., 2002). Previous work has demonstrated that directional migration of PGCs in zebrafish requires the function of the chemokine SDF-1a (Doitsidou et al., 2002) and its seven-transmembrane receptor CXCR4b (Doitsidou et al., 2002; Knaut et al., 2003). Using modified antisense oligonucleotides (morpholinos) to inhibit the translation of RNAs encoding either the receptor or the ligand abrogates directional PGC migration and thus leads to ectopic localization of these cells (Doitsidou et al., 2002). Activated chemokine receptors transduce signals from the cell surface to various intracellular downstream effectors through the heterotrimeric G proteins they are coupled to. Each of these heterotrimeric G proteins is composed of a guanine-nucleotide-binding $\alpha$ subunit of the G$\alpha$i subfamily and a dimer consisting of $\beta$ and $\gamma$ subunits. A role for Gi proteins in chemokine-directed chemotaxis has been demonstrated in vitro in different cell types (Luther and Cyster, 2001; Thelen, 2001).

Downstream to G proteins and central to directional cell migration in many migrating cells are proteins of the phosphoinositide 3-kinase (PI3K) family. A significant insight into the importance of PI3K in controlling directional cell migration and cell polarity has come from studies carried out in *Dictyostelium discoideum* and neutrophils. In such studies, activation of PI3Ks results in the production of 3′-phosphorylated phosphoinositides – phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)$P_2$] and phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)$P_3$] – at the leading edge of migrating cells in response to receptor activation and G-protein signaling (Chung et al., 2001; Devreotes and Janetopoulos, 2003; Iijima et al., 2002; Merlot and Firtel, 2003; Parent, 2004). The resulting asymmetric localization of PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$ serves to recruit proteins that contain the pleckstrin homology (PH) domain to the leading edge, which is thought to activate downstream responses such as actin

polymerization and, ultimately, the establishment of cellular polarity (Chen et al., 2003; Lemmon et al., 2002; Parent, 2004)

In this study, we have investigated the molecular pathways controlling PGC migration in zebrafish embryos, an experimental system that is particularly amenable to studies of cell migration within the intact organism. We show that PGC directional migration depends on the function of Gi proteins, indicating that signaling by the CXCR4b receptor functions through the activation of Gi subunits. Whereas motility per se does not depend on Gi activation, optimal PGC migration requires functional PI3Ks. Reduction in PI3K signaling leads to slower PGC migration and correlates with unusual cell morphology and reduced filopodia stability. Lastly, we show that, in contrast to findings in *Dictyostelium* and neutrophils, PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ are not enriched in the leading edge of migrating PGCs. We therefore suggest that the PI3K pathway is important for overall PGC motility in zebrafish, but not for directional migration of these cells.

## Materials and Methods

### Fish strains and experimental manipulations

Wild-type zebrafish (*Danio rerio*) of AB genetic background and a loss-of-function mutant in the *cxcr4b* locus (*odysseus*) (Knaut et al., 2003) were used and maintained as previously described (Westerfield, 1995). The following amounts of mRNA were injected into 1-cell-stage zebrafish embryos: 150 pg of *GFP-nos1*-3′UTR and *vasa-GFP-nos1*-3′UTR; 240 pg of *PH$^{Crac}$-EGFP-nos1*-3′UTR and *PH$^{TAPP1}$-GFP-nos1*-3′UTR; 300 pg of *PH$^{GRP1}$-EGFP-nos1*-3′UTR; 510 pg of *dnPI3K-nos1*-3′UTR; and 0.9 pg of *PTX-nos1*-3′UTR. *PH$^{Akt}$-GFP-nos1*-3′UTR was injected at 120 pg for analysis on non-confocal light microscopy and 240 pg for confocal microscopy analysis. Knockdown of CXCR4b was achieved by injecting a morpholino antisense oligonucleotide directed against *cxcr4b* at the 1-cell stage (Doitsidou et al., 2002). For experiments using the general PI3K inhibitor LY294002 (Sigma), embryos were incubated in Danieau's solution containing 60 μM of the drug from 60% ebiboly until 24 hours post fertilization (hpf).

### Construction of RNA expression vectors and RNA synthesis

*GFP-nos1*-3′UTR contains the mGFP open reading frame (ORF) (Siemering et al., 1996) fused to the 3′UTR of *nanos-1* (*nos1*) as previously described (Köprunner et al., 2001). Injection of RNA generated from this construct results in specific GFP expression in the PGCs.

*dnPI3K-nos1*-3′UTR encodes a PI3K dominant-negative version of the bovine p85α regulatory subunit cloned upstream of the *nos1* 3′UTR to allow for specific expression in the PGCs. The encoded protein lacks the binding site for its catalytic p110 subunit and is also known as ΔP85 (Hara et al., 1994).

*PH$^{Crac}$-EGFP-nos1*-3′UTR encodes the PH-containing domain of Crac (Parent et al., 1998) fused in frame to EGFP cloned upstream of the *nos1* 3′UTR.

*PH$^{Akt}$-GFP-nos1*-3′UTR encodes the PH domain of Akt (Varnai and Balla, 1998) fused to mGFP and cloned upstream of the *nos1* 3′UTR.

*PH$^{GRP1}$-EGFP-nos1*-3′UTR encodes EGFP fused in frame to the PH domain of GRP1 (Klarlund et al., 1997) and cloned upstream of the *nos1* 3′UTR.

*PH$^{TAPP1}$-GFP-nos1*-3′UTR encodes EGFP fused in frame to the C-terminal PH domain of human TAPP1 (Marshall et al., 2002) and cloned upstream of *nos1* 3′UTR.

*PTX-nos1*-3′UTR encodes the bacterial pertussis toxin subunit1 (Chaffin et al., 1990) cloned upstream of the *nos1* 3′UTR.

*vasa-GFP-nos1*-3′UTR encodes an in frame fusion protein of Vasa and mGFP followed by the 3′UTR of *nos1* (Doitsidou et al., 2002) that localizes GFP to the perinuclear granules in PGCs (Wolke et al., 2002).

*EGFP*-F-*nos1*-3′UTR encodes a farnesylated EGFP that is localized to the plasma membrane (Weidinger et al., 2002).

Capped sense RNAs were synthesized in vitro using the mMessage Machine Kit (Ambion).

### Quantification of the PGC phenotype

The PGC migration phenotype in treated embryos was evaluated at either the 6-7-somite stage or at 24 hpf, a stage in which all PGCs should have reached their final target. Embryos were injected with either control RNA (a truncated version of the human CD14 linked to the *nos*1 UTR), *PTX-nos1*-3′UTR or *dnPI3K-nos1*-3′UTR and co-injected with *GFP-nos1*-3′UTR, thereby labeling PGCs that received the injected RNAs with GFP. The PGC phenotype was assayed by counting the number of ectopic GFP-labeled PGCs either in live embryos or in fixed embryos by using a *GFP* antisense RNA probe.

### Whole-mount in situ hybridization

Two-color in situ hybridization was performed as previously described (Jowett and Lettice, 1994) with modification (Hauptmann and Gerster, 1994; Weidinger et al., 2002). PGCs were labeled using an antisense probe against *nos1* (Köprunner et al., 2001) and the *sdf-1a* expression pattern was visualized using a *sdf-1a* antisense probe (Doitsidou et al., 2002).

### Time-lapse analysis

For low magnification (10× objective) time-lapse analysis, PGCs were labeled by microinjecting 1-cell-stage wild-type and experimental embryos with 150 pg *GFP-nos1*-3′UTR (Köprunner et al., 2001). Movies and PGC migration speed were generated using MetaMorph software (Universal Imaging) controlling a Zeiss Axioplan 2 microscope. Images were captured at intervals of 1 minute. The speed of migrating PGCs was not corrected for the surrounding morphogenetic movements. For high-magnification (63× objective) time-lapse analysis, a TCS LS Leica confocal was used. In this case, images were captured either every 5 or 10 seconds as indicated in the figure legends.

### Cell morphology analysis

To analyze the cell shape of PGCs in experimental and wild-type embryos, embryos were injected with 150 pg farnesylated *EGFP-nos1*-3′UTR, at the 1-cell stage. At 9 hpf, 1-micron-deep Z sections were made through PGCs using a 63× objective and a zoom of 1.9 on a TCS LS Leica confocal microscope. From each Z section, the outline of the PGC was traced and then overlaid to depict the cell shape in two dimensions using Leica confocal software.

### Filopodia and protrusion analysis

Embryos were injected with 150 pg farnesylated *EGFP-nos1*-3′UTR at the 1-cell stage. At 9-11 hpf, high-magnification time-lapse microscopy was performed using a Leica confocal microscope. 10-minute movies were produced with images captured every 10 seconds. The stability of a filopodia was defined as the number of sequential frames a given filopodium was present. The average length of a filopodia represents the average of values obtained for individual filopodia in each of the consecutive frames they were visible. The *P*-value was calculated using the Student's *t*-test.

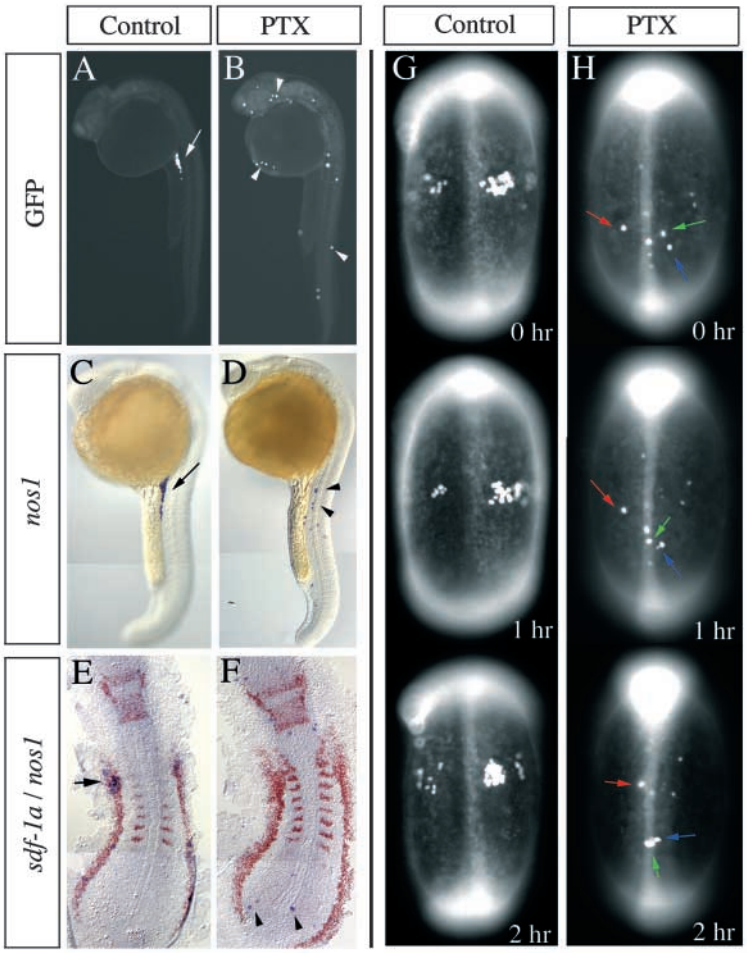


**Fig. 1.** Expression of pertussis toxin (PTX) affects directional PGC migration but not cell identity or motility. (A,B) Expression of PTX results in PGCs localized to ectopic positions by 24 hpf (arrowheads in B), as compared with control embryos where PGCs are found in their proper position (arrow in A). Yet, the ectopic PGCs properly stabilize the *GFP-nos1*-3′UTR RNA and express GFP. (C,D) Ectopic PTX-expressing PGCs show normal expression of *nos1* mRNA (arrowheads in D) similar to control PGCs (arrow in C). (E,F) 6-somite-stage embryos that had been injected with *PTX-nos1*-3′UTR show normal somatic development as determined by the expression of *sdf-1a* mRNA (red stain). At this stage, the majority of PGCs (marked by *nos1* mRNA, blue stain) in PTX-injected embryos are located outside *sdf-1a*-expressing regions (arrowheads in F) in contrast to control embryos (E), where the majority of PGCs are located within regions of *sdf-1a* expression. (G,H) PTX-expressing PGCs are motile. Images taken from time-lapse movies of control embryos (G; see also Movie S1 in supplementary material) and PTX-injected embryos (H; see also Movie S2 in supplementary material) at three different time points encompassing 2 hours of development starting at the end of gastrulation. In H, three individual PGCs are marked using red, green and blue arrows to illustrate the motility of these cells with respect to somatic tissue. Embryos are shown in a dorsal view with anterior up.

## Results

### Directional PGC migration depends on the activity of Gi proteins

It has been previously demonstrated that directional migration of PGCs requires the function of the chemokine receptor CXCR4b and its ligand SDF-1a. To characterize the biochemical pathways that control the directionality of PGC migration in zebrafish, we initially wished to determine whether the CXCR4b receptor signals through Gi proteins. For this purpose, we expressed pertussis toxin (PTX), a potent inhibitor of Gi proteins (Luther and Cyster, 2001; Thelen, 2001), preferentially in the PGCs using the 3′ untranslated region (UTR) of the *nanos-1* (*nos1*) gene (Köprunner et al., 2001) and examined the effect of this treatment on PGC migration.

Unlike control embryos, in which by 24 hours post fertilization (hpf) PGCs have reached their target of the presumptive gonadal region (Fig. 1A), PGCs expressing PTX were randomly distributed throughout the embryo with virtually no PGC found at the proper position (Fig. 1B). Whereas the observed phenotype resembles that of CXCR4b receptor knockdown, the effect of PTX expression on PGC migration could have been the result of (1) a deleterious effect on the PGC differentiation state or (2) a perturbation of the production and/or distribution of SDF-1a owing to low-level PTX expression in somatic cells. To address the first possibility, we examined a distinct feature of PGCs; namely, their ability to protect specific mRNAs from degradation and to promote their translation by virtue of the 3′UTR elements of either *vasa* or *nos1* (Köprunner et al., 2001; Wolke et al., 2002). Co-injection of *GFP-nos1*-3′UTR and *PTX-nos1*-3′UTR mRNA reveals that PGCs found at ectopic

**Table 1. Average stability of filopodia and pseudopodia in PTX- and dnPI3K-injected embryos**

| | Filopodia | | | Pseudopodia | |
|---|---|---|---|---|---|
| | Average number per minute | Average persistence (seconds) | Average length (μm) | Average number per minute | Average persistence (seconds) |
| Control | 2.0±2.4 | 64±3.5 | 3.1±0.3 | 1.2±1.6 | 42±1.9 |
| PTX | 2.2±4.3 | 39±3.3† | 2.3±0.2 | 1.2±2.3 | 39±1.4 |
| dnPI3K | 2.3±5.6 | 29±3.3‡ | 2.0±0.2§ | 1.0±1.0 | 43±3.2 |

Cellular processes were analyzed in 10-minute time-lapse confocal movies of 11 control PGCs (e.g. Movie S6 in supplementary material), 8 PTX-expressing PGCs and 10 PGCs expressing dnPI3K (e.g. Movie S7 in supplementary material). Whereas the dynamic characteristics of pseudopodia were not significantly altered by the treatments, more-pronounced changes were observed for the filopodia stability and length (significant *P* values are provided). Owing to the methodology used, where only one confocal plane was captured at each time point, the actual number of filopodia and pseudopodia that cells produce differ from the number provided in the table. Therefore, the presented values do not reflect the absolute number of these cell protrusions.

†$P<10^{-3}$; ‡$P<10^{-5}$; §$P<2\times10^{-2}$.

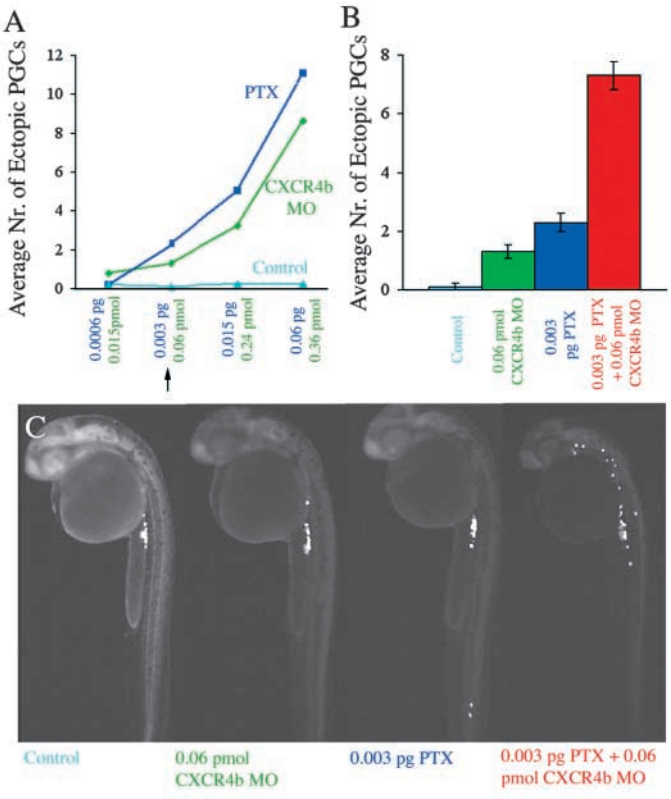


**Fig. 2.** Synergistic interaction between pertussis toxin and CXCR4b. (A) The severity of PGC migration phenotype induced by PTX RNA or CXCR4b morpholino (MO) is concentration dependent. Different amounts either of control RNA and control morpholino (turquoise), CXCR4b morpholino (green) or PTX RNA (blue) were injected. The arrow indicates the amount used for the synergism experiment. (B) Injections of control RNA and control morpholino (turquoise, *n*=67 embryos), CXCR4b morpholino (green, *n*=64 embryos) or *PTX-nos1*-3′UTR RNA (blue, *n*=84 embryos) result in 0.1, 1.3 and 2.3 average number of ectopic PGCs per embryo at 24 hpf, respectively. Injection of both CXCR4b morpholino and *PTX-nos1*-3′UTR RNA (red, *n*=110 embryos) results on average in 7.3 ectopic PGCs per embryo. (C) Representative images of 24 hpf embryos from the experiment presented in (B).

regions express GFP, as do control embryos that were not injected with the toxin (Fig. 1A,B). In addition, the RNA of two PGC-specific markers, *nos1* (Köprunner et al., 2001) and *h1m* (Müller et al., 2002), are normally expressed in PGCs treated with PTX (Fig. 1C-F and data not shown), indicating that PGCs are properly differentiated. Lastly, PTX-treated PGCs appear to be structurally intact as they contain perinuclear granules, a PGC-specific structure, as visualized by following the distribution of the Vasa-GFP fusion protein (arrow in Fig. 4E) (Wolke et al., 2002). To test whether low levels of PTX expression in somatic tissues leads to general somatic patterning defects that would affect PGC migration, we examined the expression pattern of *sdf-1a* during somitogenesis stages in PTX-injected embryos (Doitsidou et al., 2002). As a representative stage, we examined embryos at the 6-7-somite stage and found that embryos injected with *PTX-nos1*-3′UTR show normal expression of *sdf-1a* RNA, indicating that the somatic tissue is properly differentiated (Fig. 1F). However, contrary to control-injected embryos, PGCs of PTX-injected embryos are found outside the *sdf-1a* expression domain, suggesting that they are no longer able to respond to the SDF-1a signal. To determine the cellular basis for the PTX-induced phenotype, we followed the cells by time-lapse microscopy. This analysis revealed that PTX-treated PGCs are motile and are able to migrate with respect to neighboring somatic cells but do not exhibit directional migration towards the normal intermediate and final targets (Fig. 1G,H; Movies S1 and S2 in supplementary material). Whereas analysis of cellular processes did not reveal an effect of PTX on either the number of pseudopods or filopodia, we did observe a significant decrease in the average stability of filopodia in the treated cells (Table 1).

To substantiate further the notion that the CXCR4b signaling pathway acts through Gi proteins, we tested whether Gi proteins and CXCR4b interact synergistically to facilitate PGC directional migration in vivo. Indeed, low-level expression of PTX combined with a mild CXCR4b morpholino treatment resulted in a PGC phenotype that is more severe than the additive effect of each manipulation alone (Fig. 2).

These results indicate that the PGC phenotype we observe in PTX-treated embryos is identical to that of loss of CXCR4b both with respect to the final phenotype and to the preservation of motility. These findings are consistent with the idea that CXCR4b and Gi proteins act in the same pathway and that the activation of this pathway is pivotal for directional migration but not for general cell motility.

## Directed PGC migration and polarization are not correlated with polarized distribution of PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ on the cell membrane

The PI3K pathway has been shown to be important for directed cell migration in the single-cell organism *Dictyostelium* and in neutrophils (reviewed by Devreotes and Janetopoulos, 2003; Iijima et al., 2002). As this pathway plays a key role during chemokine-dependent chemotaxis (Curnock et al., 2002; Curnock and Ward, 2003; Sotsios and Ward, 2000), it prompted the question of whether activation of PI3K specifies directionality of PGC migration in zebrafish.

We initially determined the localization of PH-domain-containing proteins during directed PGC migration as studies from *Dictyostelium* and neutrophils have shown that these proteins are localized to the leading edge in response to an increase in PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ levels in chemotaxing cells (Chen et al., 2003; Iijima et al., 2002; Lemmon et al., 2002; Merlot and Firtel, 2003). We examined the localization of GFP fused to the PH domain derived from four different proteins: CRAC, Akt, GRP1 and TAPP1 (referred to as $PH^{CRAC}$-GFP, $PH^{Akt}$-GFP, $PH^{GRP1}$-GFP and $PH^{TAPP1}$-GFP, respectively). Whereas the PH domain of CRAC and Akt can bind both PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$ (Franke et al., 1997; Iijima et al., 2002), the PH domain of GRP1 binds specifically to PtdIns(3,4,5)$P_3$ whereas the PH domain of TAPP1 binds to PtdIns(3,4)$P_2$ (Gray et al., 1999; Klarlund et al., 1997; Marshall et al., 2002). $PH^{CRAC}$-GFP, $PH^{Akt}$-GFP, $PH^{GRP1}$-GFP and $PH^{TAPP1}$-GFP were each expressed in PGCs and their distribution analyzed using time-lapse confocal microscopy. We found that $PH^{CRAC}$-GFP and $PH^{Akt}$-GFP proteins were stably localized to the membrane of the PGCs (Fig. 3A-C for $PH^{CRAC}$-GFP and Fig. 3D,G for

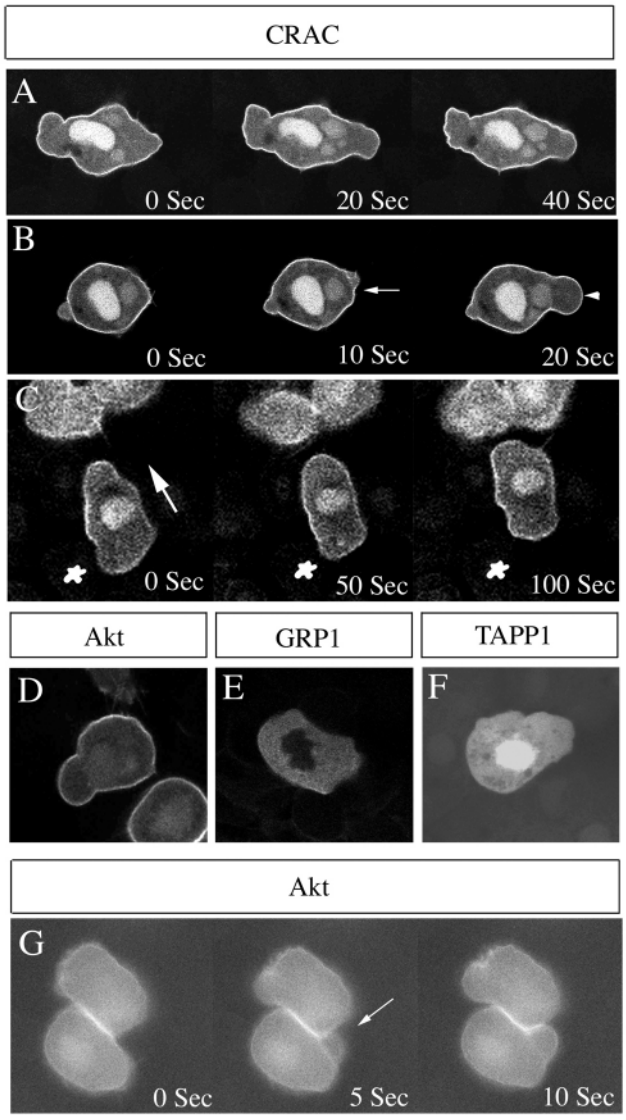


**Fig. 3.** Localization of the PH-GFP fusion proteins in wild-type PGCs. (A,B,C) Images are taken from 63× time-lapse movie showing PGCs labeled with $PH^{CRAC}$-GFP at the indicated time points. A PGC in (A) shows stable localization of $PH^{CRAC}$-GFP to the membrane (Movie S3 in supplementary material). (B) No differential membrane localization can be observed either prior to the formation of a protrusion (indicated by an arrow) or at the site of the protrusion itself (indicated by an arrowhead; Movie S3 in supplementary material). (C) No polarized membrane localization of $PH^{CRAC}$-GFP can be seen in a PGC undergoing directed migration relative to somatic cells (Movie S4 in supplementary material). The arrow marks the direction of migration of the PGC whereas the white symbol marks a neighboring somatic cell serving as a reference point. (D) A PGC showing a uniform membrane localization of $PH^{Akt}$-GFP, whereas the PGC in (E) shows weak and spotty membrane expression of $PH^{GRP1}$-GFP. The PGC in (F) shows uniform distribution of $PH^{TAPP1}$-GFP. (G) Non-confocal images of PGCs labeled with $PH^{Akt}$-GFP. The arrow marks a forming protrusion showing no increased fluorescence. Brighter fluorescence is mostly apparent at regions of cell-cell contact (Movie S5 in supplementary material).

$PH^{Akt}$-GFP) and that this localization was dependent upon PI3K activity (Fig. 4B). The lack of dynamic alteration in the distribution of PH-GFP does not support the idea that they serve as a second messenger that mediates directional migration cues to the PGCs. Furthermore, no differential localization of $PH^{CRAC}$-GFP or $PH^{Akt}$-GFP on the membrane was observed either before the formation of protrusions or at the site of the protrusions themselves (Fig. 3B and G; Movie S3 in supplementary material). Similarly, polarized $PH^{CRAC}$-GFP and $PH^{Akt}$-GFP distribution were not detected in migrating cells where the fusion proteins were distributed all around the membrane with occasional fluctuations in intensity in random positions (Fig. 3C; Movie S4 in supplementary material). Expression of $PH^{GRP1}$-GFP, a fusion protein recognizing specifically PtdIns(3,4,5)$P_3$, in PGCs resulted in weak and spotty membrane localization. Yet, in this case too, no specific polarization of the GFP signal was observed (Fig. 3E). Similarly, the $PH^{TAPP1}$-GFP fusion protein did not display any preferential localization to sites of cellular protrusions and showed no polar distribution within the cell or on the membrane (Fig. 3F). These findings differ from those of Knaut et al., who describes $PH^{Akt}$-GFP restriction to the site of lamellipodium protrusion in migrating zebrafish PGCs (Knaut et al., 2003). Non-confocal microscopy, which was used in the other study, is a method prone to artifacts stemming from differences in membrane thickness and morphology, especially along surfaces of cell-cell interaction. Nevertheless, upon repeating the same experiment using light microscopy where a lower amount of RNA could be introduced into PGCs, thus avoiding potential saturation of binding sites on the membrane, we did not detect $PH^{Akt}$-GFP accumulation at sites of cellular

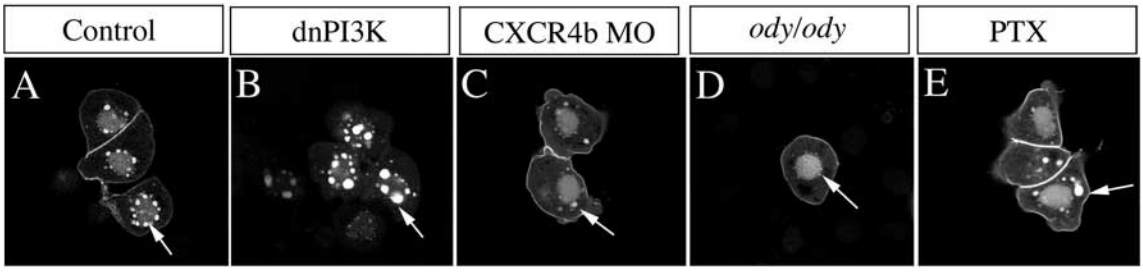


**Fig. 4.** The localization of $PH^{CRAC}$-GFP to the membrane of PGCs is independent of SDF-1a/CXCR4b signaling. Localization of $PH^{CRAC}$-GFP in (A) control PGCs, (B) cells expressing dnPI3K, (C) cells expressing CXCR4b morpholino (MO) or (E) PTX, as well as in PGCs in (D) an *ody/ody* mutant embryo. All cells also express the Vasa-GFP fusion protein, which labels the perinuclear granules. Arrows in A-E indicate the perinuclear granules.

protrusions. However, we did detect a high signal at positions of cell-cell contacts (Fig. 3G; Movie S5 in supplementary material).

Finally, the notion that PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ distribution on the membrane is irrelevant for directed PGC migration is further supported by the observation that their distribution is not altered in cells defective for directional migration. PGCs expressing PTX or cells lacking the CXCR4b receptor due to morpholino knockdown or the *ody* mutation in the *cxcr4b* locus show $PH^{CRAC}$-GFP distribution similar to that of wild-type cells (Fig. 4C-E).

## PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ are required for proper PGC migration

Although based on the results presented above the spatial distribution of PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ by itself cannot be responsible for transmitting directional cues for PGC migration, it remains possible that these molecules are important for the migration process. To address this possibility, we analyzed the migration of PGCs expressing a dominant-negative form of the regulatory subunit of class 1a PI3K (dnPI3K) (Hara et al., 1994), thereby inhibiting the function of class 1a of PI3K, which has been shown to be ubiquitously expressed in the zebrafish gastrula (Montero et al., 2003). Little or no membrane-localized $PH^{CRAC}$-GFP was detected in cells expressing dnPI3K, reflecting a strong depletion of membranal PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ (Fig. 4B). Thus, our treatment directed against class1a PI3K effectively reduces the level of PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ on the cell membrane, allowing us to determine the role of these phosphoinositides in PGC migration.

Importantly, many PGCs treated with dnPI3K were found in ectopic positions at 24 hpf (Fig. 5B). Nevertheless, in contrast to knockdown of the SDF-1a/CXCR4b pathway or injections of PTX, where virtually no PGCs arrived at their target, injection of dnPI3K led to a milder phenotype. Specifically, 63% of dnPI3K-injected embryos exhibited a PGC migration phenotype (Fig. 5G) in which on average 39% of PGCs were found in ectopic positions by 24 hpf. When embryos were monitored during earlier developmental stages, a subset of PGCs could be found outside the *sdf-1a* expression domain (Fig. 5D). Importantly, the ectopic PGCs are properly differentiated and viable as judged by the expression of *nos1* mRNA (Fig. 5D,F), by their capacity to localize Vasa-GFP to the perinuclear granules and by their normal number (Fig. 4B and data not shown). Furthermore, the somatic tissues relevant for PGC migration develop normally as determined by the mRNA expression of *sdf-1a* and the general morphology of the embryo (Fig. 5D). In agreement with the results presented above for dnPI3K, using the general PI3K inhibitor LY294002 we observe a similar PGC migration phenotype albeit with lower penetrance. We attribute the milder effect of the drug to the low dose that can be applied without severely affecting embryonic development (data not shown).

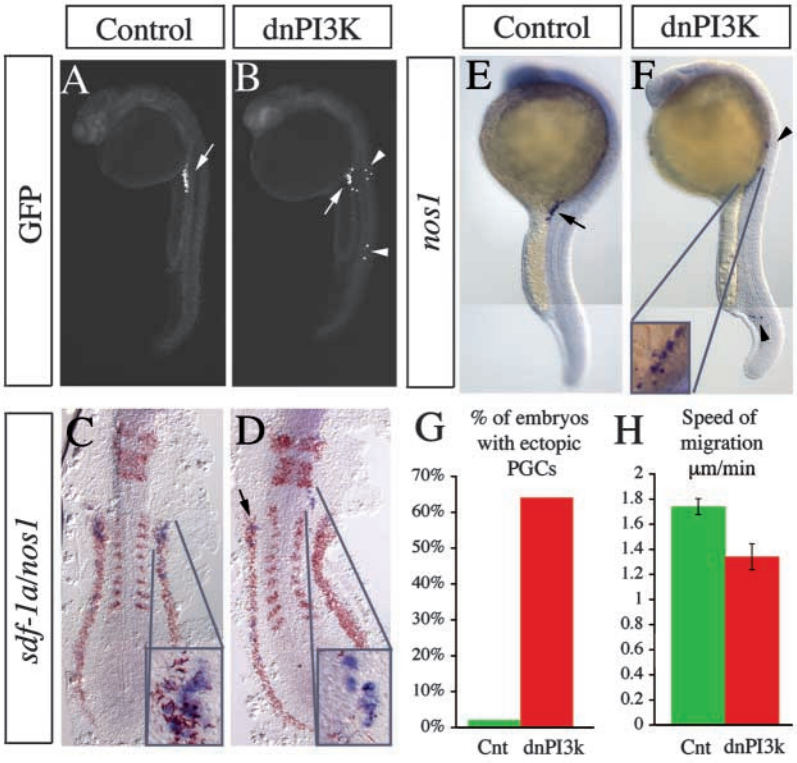


**Fig. 5.** Expression of dnPI3K in PGCs results in their localization to ectopic positions. (A,B) Expression of *dnPI3K-nos1*-3′UTR results in PGCs in ectopic positions at 24 hpf (arrowheads in B) as compared with control embryos where PGCs are located in their proper positions (arrow in A). Embryos were co-injected with *GFP-nos1*-3′UTR to label the PGCs. (C,D) *dnPI3K-nos1*-3′UTR-injected embryos show normal morphology (D) as demonstrated by in situ hybridizations of embryos at the 6-7-somite stage stained with *sdf-1a* (red) and *nos1* (blue) and compared with a control embryo (C). High-magnification inset in C shows PGCs in regions of *sdf-1a* expression in control embryos whereas inset in D shows a subset of PGCs in a region of non-*sdf-1a* expression in dnPI3K-injected embryos. Arrows in B,D and high-magnification inset in F indicate the presence of PGCs in their proper position in dnPI3K-injected embryos. (E,F) PGCs located in ectopic positions in dnPI3K-injected embryos show normal expression of *nos1* mRNA (arrowheads in F) similar to PGCs in control embryos (arrow in E). (G) Graph illustrating the percentages of embryos with ectopic PGCs. 2% of control embryos (green bar) had ⩾2 PGCs located in ectopic locations at 24 hpf as compared with 63% of *dnPI3K-nos1*-3′UTR injected embryos (red bar) showing ⩾2 ectopic PGCs by 24 hpf. (H) Graph illustrating the average speed of PGC migration in control embryos (green bar) and *dnPI3K-nos1*-3′UTR-injected embryos (red bar). Control PGCs migrate at an average speed of 1.7 μm/min ($n$=23) compared with PGCs that express dnPI3K, which migrate at a rate of 1.3 μm/min ($n$=21; $P$<0.05).

## PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ are important for PGC morphology, filopodia stability and proper migration speed

To determine the basis for the ectopic localization of PGCs depleted of PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$, we monitored the morphology and the activity of cellular protrusions in the course of PGC migration. Analysis of the three-dimensional shape of cells whose membrane was labeled

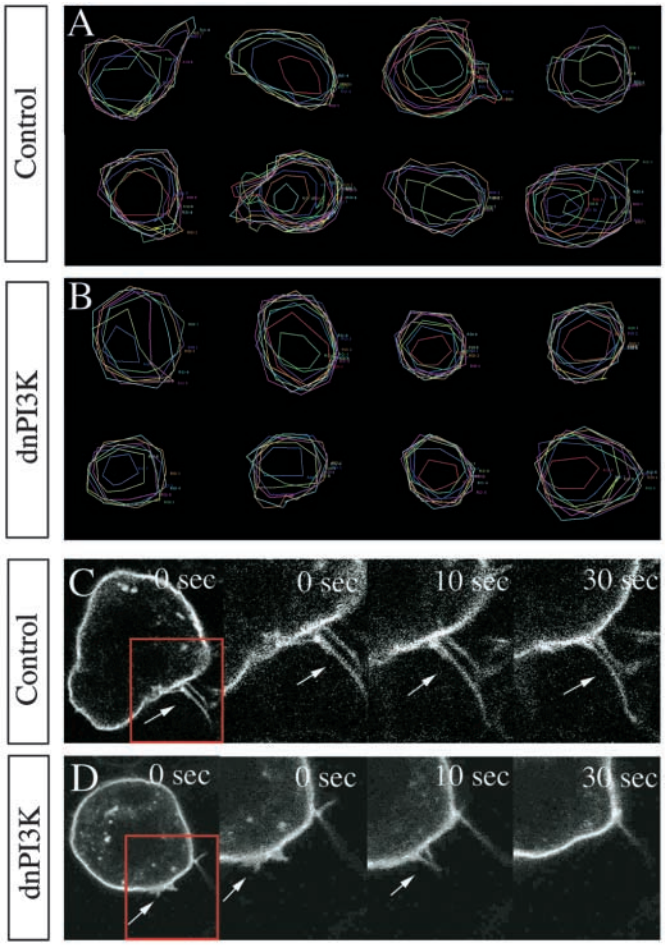


**Fig. 6.** PI3K activity affects PGC morphology and filopodia stability. (A) The outline of 8 control PGCs and (B) 8 PGCs expressing dnPI3K. The outlines of PGCs were generated as described in the Materials and Methods. Expression of dnPI3K in PGCs reduces filopodia stability and length (D; Movie S7 in supplementary material), as compared with control embryos (C; Movie S6 in supplementary material). Arrows in C and D indicate filopodia. The area demarcated in red is enlarged in the right panels.

with GFP revealed a striking difference in morphology between control and dnPI3K-expressing PGCs. Whereas control cells display a polarized elongated shape (Fig. 6A), the majority of dnPI3K-expressing PGCs exhibited a less polarized and more round cell shape (Fig. 6B). To characterize the morphological abnormalities in more detail, we analyzed the properties of the cellular protrusions in treated cells using high-magnification confocal microscopy. We saw no effect on either the formation or stability of pseudopodia in PGCs depleted for PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ (Table 1); however, we observed a clear effect on filopodia protrusions. Expression of dnPI3K leads to a significant reduction in the length and the stability of each filopodia (Fig. 6C,D; Table1; Movies S6 and S7 in supplementary material). Specifically, whereas the average persistence of a wild-type filopodium is 66 seconds and its average length is 3 μM, PGCs depleted for PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ form shorter-lived filopodia with an average persistence of only 29 seconds and an average length of 2 μM (Table 1). By contrast, expression of dnPI3K had no effect on the number of individual filopodia formed by a PGC. To test the possible functional relevance of these morphological abnormalities, we measured the migration speed of treated PGCs between 8 and 12 hpf and found it to be significantly slower compared with wild-type cells (Fig. 5H).

In conclusion, we found no evidence for a crucial role for the PI3K pathway in PGC directional migration, differentiation or maintenance of the PGC fate. However, we did identify a role for this pathway in attaining normal cell morphology and filopodia stability, which are likely to be essential for achieving optimal migration speed. Together, we propose that the highly dynamic expression of *sdf-1a* during early development coupled with the random migration starting point of the PGCs and slower cell migration are translated into a partial migration phenotype as some of the dnPI3K-expressing PGCs lose their track and end up in ectopic positions.

## Discussion

The ability of cells to migrate towards their targets depends on biochemical pathways that are important for cell motility, directionality of migration and for the interaction between the migrating cells and their environment. In this work, we demonstrate that directed PGC migration in zebrafish requires the function of Gi proteins, whereas the PI3K pathway plays a permissive role that is essential to attain proper cell morphology, filopodia stability and migration speed.

### Directional PGC migration requires G proteins of the Gi family

Directional migration of zebrafish PGCs depends on signaling by the SDF-1a/CXCR4b ligand-receptor pair (Doitsidou et al., 2002). In this study, we show that zebrafish PGC migration also requires functional Gi proteins, as inhibition of these gives rise to a phenotype indistinguishable from that of CXCR4b- or SDF-1a-knockdown, suggesting that signaling through Gi proteins acts downstream of CXCR4b receptor activation. Interestingly, loss of either chemokine signaling or the function of Gi proteins abrogates directional migration but not cell motility, demonstrating that the mechanisms underlying these processes can be uncoupled.

The inherent motility of zebrafish PGCs in the absence of a stimulus is not unique. For example, *Dictyostelium* cells migrate in random directions in the absence of an attractant (Devreotes and Zigmond, 1988; Gerisch, 1987). Similarly, when unstimulated neutrophils are treated with μ-calpain inhibitors, these cells become motile (Lokuta et al., 2003). Thus, in these examples, motile behavior constitutes an intrinsic feature of the cells. Although relatively little is known about the molecular mechanism underlying PGC motility in zebrafish, it has been shown that this process depends on the function of the PGC-specific RNA-binding protein Dead End as depletion of this protein leads to migration arrest of PGCs (Weidinger et al., 2003).

### The PI3K pathway and PGC migration

Using PH-GFP fusion proteins to monitor the localization of PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ within the PGCs, we

observed that directed migration of this cell type is not associated with polarized distribution of PtdIns(3,4,5)$P_3$ and/or PtdIns(3,4)$P_2$ on the cell membrane, thus suggesting that the PI3K pathway is not employed for PGC directional migration. Consistent with our findings, emerging evidence suggests that the PI3K pathway is not universally required for directional migration and rather could play different roles depending on the cellular context (Ward, 2004). Nevertheless, the PI3K pathway does play a role in PGC migration as PGC morphology, migration speed and filopodia dynamics are affected in cells in which this pathway is inhibited. It is possible that the uniform and constitutive activation of PI3K constitutes a component of the motility program of the cell and is therefore important for attaining optimal migration speed. Consistent with such a role for the PI3K pathway are results obtained in studies of migrating rat primary astrocytes (Machide et al., 2000). In this system, activation of the PI3K pathway by the chemokinetic factor hepatocyte growth factor promotes reorganization of the actin cytoskeleton and leads to migration of astrocytes, albeit in a non-directional manner (Machide et al., 2000). The PI3K pathway could also be important for regulating the adhesiveness between PGCs and the matrix on which they migrate. A role for the PI3K pathway in controlling cell-substrate adhesion has been demonstrated by in vitro studies using hepatic stellate cells (HSCs) (Reif et al., 2003). Treatment of HSCs with a pharmacological inhibitor of PI3K, LY294002, results in a decrease of both cell migration and cell-substrate adhesion. This finding provides a direct link between PI3K activity and cell-substrate adhesion, which could be important for attaining proper migration speed and cell morphology.

The relevance of the PI3K pathway for human disease has been highlighted by a recent study where a correlation between high PI3K (referred to as PIK3CA) activity and the onset of tumor invasiveness in different types of cancers was observed (Samuels et al., 2004). Although the precise mechanisms responsible for PIK3CA function in this context are not known, it is possible that an effect on cell motility could account for the increased metastasis. This suggestion would be consistent with our results and with studies demonstrating that fibroblasts deficient for the PI3K phosphatase PTEN display both an increased cell motility and migration speed (Liliental et al., 2000). Further studies aimed at identifying the intracellular signals controlling directional migration and cell motility would thus be important not only for understanding PGC migration but also for gaining insight into other cell migration processes in development and disease.

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