Ptenb Mediates Gastrulation Cell Movements via Cdc42/AKT1 in Zebrafish

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Abstract

Phosphatidylinositol 3-kinase (PI3 kinase) mediates gastrulation cell migration in zebrafish via its regulation of PIP2/PIP3 balance. Although PTO3 kinase counter enzyme PTEN has also been reported to be essential for gastrulation, its role in zebrafish gastrulation has been controversial due to the lack of gastrulation defects in pten-null mutants. To clarify this issue, we knocked down a pten isoformal, ptenb by using anti-sense morpholino oligos (MOs) in zebrafish embryos and found that ptenb MOs inhibit convergent extension by affecting cell motility and protrusion during gastrulation. The ptenb MO-induced convergence defect could be rescued by a PI3-kinase inhibitor, LY294002 and by overexpressing dominant negative Cdc42. Overexpression of human constitutively active akt1 showed similar convergent extension defects in zebrafish embryos. We also observed a clear enhancement of actin polymerization in ptenb morphants under cofocal microscopy and in actin polymerization assay. These results suggest that Ptenb by antagonizing PI3 kinase and its downstream Akt1 and Cdc42 to regulate actin polymerization that is critical for proper cell motility and migration control during gastrulation in zebrafish.

Introduction

Gastrulation is a morphogenetic process involving cell migration and rearrangements to establish three germ layers: ectoderm, mesoderm, and endoderm [1]. In zebrafish, three distinct morphogenetic cell movements occur during gastrulation, including epiboly, involution, convergence and extension [2]. Gastrulation starts after the blastula stage when embryo proper appears as a mass of cells situated on top of yolk cells. The yolk sphere is then forming a dome cap that pushes the mass of blastomeres to become thinner and start to spread over the yolk sphere in a process called epiboly. After 50% of the yolk sphere is enclosed by the blastoderm, the front runner cells at the putative dorsal side begin to involute retrogradely toward the future anterior part to form mesoderm and endoderm progenitor cells. At about midgastrulation, convergence and extension movements occur to narrow medio-lateral and elongate anterior-posterior of body axis, respectively, that is essential to set up the dorsal-ventral and anterior-posterior axes [3,4,5]. These gastrulation cell movements are well demonstrated to be mediated by cell adhesion and cytoskeleton rearrangement [6,7,8].

Cell adhesion and cytoskeleton rearrangement can be associated to the metabolism of membrane lipids. One of the key enzymes for metabolizing membrane lipid is phosphoinositide 3-kinase (PI3 kinase). PI3 kinase can phosphorylate the D3 position hydroxyl group of the inositol ring of phosphatidylinositol-4,5-diphosphate (PIP2). Phosphorylation of PIP2 results in phophatidylinositol-3,4,5-triphosphate (PIP3) whose signaling is involved in cell proliferation, migration, survival, and apoptosis via Akt/PKB signaling [9]. Blocking PI3 kinase causes convergence and extension defects with reduced directional protrusions in leading cells of mesoderm in zebrafish [10]. This implies the possible involvement of PIP2/PIP3 balance in gastrulation cell movements. A counter enzyme of PI3 kinase is PTEN (Phosphatase and TENsin homolog deleted on chromosome 10, also named MMAC1 and TEP1), a famous tumor suppressor gene [11,12]. Its mutations have been reported in numerous human cancers, like brain cancer, breast cancer, prostate cancer [13], melanoma [14], and some autosomal dominant cancer syndromes, like Cowden’s disease [15], Bannayan-Zonana [16], and Lhermitte-Duclos disease [17]. PTEN has a phosphatase domain [18], which negatively regulates PI3 kinase/Akt pathway by dephosphorylating PIP3 [19,20]. It regulates cell polarity during cell migration [21] by antagonizing PI3 kinase for a balance control of PIP2/PIP3 to mediate chemotaxis [22,23]. It is thus intriguing to us to see how PTEN functions in concert with PI3 kinase during embryogenesis.

In mouse, fruit fly and chicken, PTEN is known to regulate cell migration, cell cycle length, and cell survival during early embryogenesis [24,25,26]. Pten+/− knockout mice die at around embryonic day 7.5 [24,27] and altering pten expression before midblatula transition causes gastrulation delay in Xenopus embryos [28]. It appears that Pten is an indispensable gene during embryogenesis. However, the effects of Pten on the dynamic

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gastrulating cell movements have not been examined because of experimental constraints. Zebrafish is a well-established model to study the dynamic processes of gastrulation cell movements [29]. There are two zebrafish pten isoforms, ptena and ptenb. Each isoform has two alternatively splicing forms. Using the morpholino (MO) knockdown approach, Croushore et al. [30] showed that ptena or ptenb MO-injected embryos exhibit distinct morphological defects in 24–48 hours post fertilization (hpf), but the effects of those MOs on gastrulation were not described. Comparing genomic synteny, it reveals that zebrafish ptenb and the human PTEN have the conserved locus. It suggests that ptenb is probably the ortholog of human PTEN [31]. Thus, we set out to examine the effects of Ptenb on gastrulation cell movements and found that knockdown of ptenb by MO cell non-autonomously disturbs epiboly and convergent-extension cell movements during gastrulation in zebrafish.

Results
Ptenb expresses maternally and exists throughout early embryogenesis
Previous studies have reported that ptenb is ubiquitously expressed in zebrafish early embryos at a few selected stages [30,32], but its expression patterns at most cleavage and gastrulation stages are still lacking. RT-PCR analysis showed that ptenb mRNA was indeed expressed in every early embryonic stage examined, reduced at 30% epiboly then gradually recovered afterward (Fig. 1A). Whole-mount in situ hybridization (WISH) analysis revealed ubiquitous expression patterns of ptenb in embryos up to 18-somite stage (Fig. 1B). The maternal and ubiquitous expression patterns of ptenb during early embryonic stages suggested that it may play a pivotal role during early embryogenesis.

Figure 1. Expression patterns of zebrafish ptenb. (A) Expression of ptenb at designated developmental stage was examined by RT-PCR analysis of an 1183-bp ptenb fragment, and a 530-bp β-actin fragment was used as an internal control. hpf: h post fertilization. (B) Representative photographs of embryos fixed at designated stages and underwent whole-mount in situ hybridization against ptenb. Scale bar: 200 µm.

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Specificity and potency examination of translation-blocking ptenb MO

Two published antisense translational blocking MOs [30] were adopted to study the role of Ptenb during gastrulation. These two MOs bind to ptenb mRNA 5’ untranslated region (5’ UTR) at non-overlapping sites as indicated in Fig. 2A. To further confirm the specificity and translational blocking efficiency of the first ptenb MO (tMO1), we co-injected plasmids of a PCS2+ construct containing the tMO1 binding site (PCS2+_ptenb 5’ UTR) to check the tMO1 translation blocking ability. Embryos (n = 148) injected with the PCS2+_ptenb 5’ UTR plasmids alone expressed green fluorescent protein (GFP) in a mosaic pattern with a high ratio of 87.2±0.23% (N = 3) at 40% epiboly stage (Fig. 2C). By contrast, co-injection of 5 ng tMO1 (n = 160) completely blocked the expression of GFP (Fig. 2E). These results indicated that ptenb tMO1 specifically and potently blocks zebrafish ptenb translation.

Knockdown of ptenb impairs gastrulation

To understand the role of Ptenb during early embryogenesis, we injected embryos with tMO1 and examined its effect on embryonic development. A standard control MO (StdMO), which has a minimum effect on embryonic development, was used as a MO control. The MO-injected embryos will be called morphants. The epibolic progression in control. The MO-injected embryos will be called morphants. The epibolic progression in control. The MO-injected embryos will be called morphants. The epibolic progression in control. The MO-injected embryos will be called morphants. The epibolic progression in control. The MO-injected embryos will be called morphants. The epibolic progression in control. The MO-injected embryos will be called morphants. The epibolic progression in control. The MO-injected embryos will be called morphants. The epibolic progression in control. The MO-injected embryos will be called morphants. The epibolic progression in control. The MO-injected embryos will be called morphants. The epibolic progression in control. The MO-injected embryos will be called morphants.

Figure 2. ptenb MO target sites and potency examination. (A) Partial mRNA map of ptenb. The black and white boxes represent part of the coding sequence (CDS) with ATG translation initiation site and 5’ untranslated region (UTR), respectively. The ptenb tMO1 and tMO2 binding sites are located at the 5’ UTR as shown. The potency of tMO1 to reduce ptenb expression was checked by co-injecting 150 pg PCS2+_ptenb 5’ UTR plasmids, which containing the tMO1 binding site, with or without 5 ng tMO1. Representative photographs of control (B,C) and morphant embryos (D,E) under bright (B,D) and dark fields (C,E) are shown. These experiments were repeated 3 times. doi:10.1371/journal.pone.0018702.g002

Ptenb knockdown effects can be alleviated by inhibiting PI3 kinase

To examine the effect of tMO1 on dorsal axis extension, we measured the angle between the anterior end of prechordal plate (as revealed by WISH against ctsl1b) and the posterior end of myod. The tMO1 morphants showed notable increase in angle (i.e. the inhibition of dorsal axis extension) compared to that of StdMO morphants (Fig. 6A). The tMO1 MO-induced extension defect could also be partially rescued by ptenb mRNA co-injection as shown in Fig. 6B. Since the ctsl1b probe-labeled prechordal plates were observed in both control embryos and morphants (Fig. 6A), it appeared that the mesendodermal involution was not affected by the loss of Ptenb.

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rescued the impaired somite-convergent defect in tMO1 morphants (Fig. 7G). However, it appeared to be less potent to rescue the extension defect induced by tMO1 at the dosage tested (Fig. 7F, G). We attempted to use higher dosages up to 30 μM of LY294002, but the decorinated embryos were too fragile to be examined under those conditions. These results indicated that *ptenb* may regulate zebrafish convergent movement by antagonizing PI3 kinase.

### Figure 3. *ptenb* MOs delays epibolic progression.

Knockdown of *ptenb* by MOs caused delay in epibolic movement in a dose-dependent manner. (A) Embryos were injected with standard MO (StdMO), tMO1, 5-bp mismatched tMO1 (mis-tMO1) and tMO2 at designated amount, incubated to 10 hpf and the percentages of embryos at each epiboly stage are shown. (B) Embryos were injected with 15 ng of StdMO or tMO1 in the absence or presence of *ptenb* mRNAs (50 pg), incubated and their degrees of epiboly progression are shown. Each treatment was repeated at least 6 times.

| MO          | Percentage of Embryos (%) |
|-------------|----------------------------|
| tMO2 15 ng  | 95%↑ epiboly               |
| mis-MO 15 ng| 85-95% epiboly             |
| tMO1 15 ng  | 85%↓ epiboly               |
| tMO1 10 ng  |                            |
| tMO1 15 ng  |                            |
| StdMO 15 ng |                            |

Ptenb regulates cell movements via modulating cell protrusive activity

To examine how *ptenb* regulates convergence and extension cellular movements, we analyzed cell migration in different regions of gastrulating zebrafish embryos. As shown in movie S1 and S2, the leading edge hypoblast cells of a StdMO morphant formed different types of protrusion but mainly the long and thick
lamellapodia at the lateral side where cells converge actively toward the dorsal side (arrow, Fig. 8A). In 4 trials, there was no significant difference in the number of lamellapodia formed per cell between StdMO (0.140 ± 0.006/min, n = 28) and tMO1 (0.168 ± 0.011/min, n = 29) morphants. By observing 29 lamellapodia at 75% epiboly stage in 4 StdMO morphants, we found these lamellapodia persisted for 5.8 ± 0.6 min (Fig. 8C). More than half of these lamellapodia (60.7 ± 1.0%, Fig. 8D) pointed toward anterodorsal direction. In contrast, although cells in tMO1 morphants also formed lamellapodia (n = 32) (Fig. 8B), the average persistence time was shorter (4.2 ± 1.0 min, N = 4) compared with that in StdMO morphants (Fig. 8C) and only 46.2 ± 5.0% (N = 4, Fig. 8D) of lamellapodia extended toward the anterodorsal direction.

The behaviors of prechordal plate leading edge cells at 90% epiboly were also analyzed for extension abnormality. In StdMO morphants, prechordal plate cells formed multiple layers and migrated as a sheet with the leading edge cells forming protrusions at front and adhered cells in the back. The leading edge cells (n = 17) were quite active, they formed protrusions, including filapodia, lamellapodia, and blebbing, and migrated fast over the yolk sphere at a velocity of 2.3 ± 0.2 μm/min (N = 5, movie S3). The leading-edge cells of tMO1 morphants (n = 18) also had the same protrusions as those in StdMO morphants but their velocity of leading-edge cell migration was significantly slower at 1.5 ± 0.2 μm/min (N = 5, p<0.01, movie S4).

Pt nbr functions non cell-autonomously for gastrulation cell migration

To assess cell-autonomy of Pt nbr function in mediating migration of lateral hypoblast cells, we performed cell transplantation experiments by using rhodamine dextran as a cell tracer. Blastomeres from StdMO or tMO1 morphants were transplanted to host embryos treated without or with tMO1, and examined under confocal microscopy or epifluorescent microscopy. Under confocal microscopy, cells from a StdMO morphant showed highly protrusive activities with well formed protrusions when transplanted into an untreated host embryo ("STD > UT", arrow, Fig. 9A; movie S5) whereas protrusions were shorter and less well formed in those cells transplanted to a tMO1 morphant host ("STD > MO", arrows, Fig. 9B; movie S5). Moreover, cells from a tMO1 morphant displayed a more polygonal shape ("MO > UT", Fig. 9C; movie S5) when transplanted into an untreated host compared to those cells with a rounder shape and a reduced number of protrusions in a tMO1 morphant host ("MO > MO" Fig. 9D; movie S5). To examine the migration velocity and direction of transplanted cells in different hosts, we took time-lapse movies under epifluorescent microscopy for 6 embryos in each treatment and monitored about 8–10 cells in each embryo. The STD > UT cells traveled faster and longer than those STD > MO cells (Fig. 9E). The curvilinear velocity (Vcl, curvilinear distance/time) of STD > UT cells was 0.0257 ± 0.0031 μm/sec (n = 61), but was only 0.0202 ± 0.0024 μm/sec (n = 50), for the STD > MO cells.

Figure 4. pt nbr MOs specifically impairs gastrulation in a dose-dependent manner. Knockdown of pt nbr by MOs caused gastrulation defects in graded severity. Representative photographs of pt nbr morphants with different severity are shown in (A) normal (B) intermediate and (C) severe. (D) Embryos were injected with StdMO, tMO1, mis-tMO1 and tMO2 at designated amount, incubated to 10 somite stage and the percentages of embryos with different severity are shown. (E) Embryos were injected with 15 ng of StdMO or tMO1 in the absence or presence of pt nbr mRNAs (50 pg), incubated and their degrees of gastrulation defect are shown. Each treatment was repeated at least 4 times.

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Overall intensity of F-actin was enhanced without disturbing actin distribution patterns (Fig. 10B). To further confirm the increase of F-actin in tMO1 morphants, we measured the F-actin contents of these embryos by using an actin polymerization assay [36]. In 3 experiments, tMO1 morphants exhibited ~1.3 fold of fluorescence compared to that of control embryos (Fig. 10C). These results demonstrated that knockdown of ptenb results in an increase in actin polymerization.

Dominant-negative Cdc42 rescues ptenb MO-induced convergence and extension defects

Small GTPases Rac1 and Cdc42 were known downstream of PTEN in in vitro studies [35]. To examine whether Cdc42 and/or Rac1 are involved in the Ptenb-mediated regulation in gastrulation cell movements, dominant negative, T17NCdc42 or T17N Rac1 were used to see their effects on the ptenb MO-induced convergent extension defects. Co-injection of T17NCdc42 mRNAs (25 pg) with StdMO (10 ng) did not change the somite width, but increased the extension angle (N = 4, Fig. S1A). In contrast, T17NCdc42 mRNAs could partially restore the somite width and extension angle of tMO1 morphants (Table 1, N = 4). Similar experiments were performed using T17NRac1 (Table 1, N = 5).

Overexpression of human constitutively active AKT1 causes convergence and extension defects

Knockdown of ptenb would lead to increase of downstream Akt/PKB activity, to investigate whether the elevation of AKT1/PKB activity may result in similar ptenb MO-induced phenotypes. We overexpressed human constitutively active akt1 (caakt1) in zebrafish embryos to observe its effect on convergent extension. Embryos injected with 1× buffer and caakt1 mRNAs at 50, 100, or 200 pg were collected and subjected to WISH against myod and css1b. As shown in Table 2, lower doses (50 and 100 pg) of human caakt1 mRNAs caused slight convergence extension defects, but profound inhibition on convergent extension was observed in embryos treated with 200 pg caakt1 mRNAs. These results implied that the ptenb MO-induced convergent extension defects might be due to the elevation of AKT1 activity.

Pttenb MO causes convergence and extension defects in ptenb−/− embryos

The lack of notable early embryonic defect in ptenb−/− mutant fish is in contrast to our observations described above that might be due to the presence of maternal pten transcripts [32]. To examine this possibility, we had obtained heterozygous ptenb+/− embryos, raised to adults to give rise to F1 offspring. Those F1 fish were screened by PCR for the presence of mutated ptenb 2/2 embryos. As shown in Table 2, these embryos were significantly increased in the presence of ptenb tMO1. These data implied that the presence of maternal pten transcripts was sufficient to complete normal gastrulation in ptenb−/− embryos.

Figure 5. ptenb MOs cause convergence defect during gastrulation. Embryos were injected with designated amount of MO, incubated to 9 to 11 somites and subjected to WISH against myod. The somite width of treated embryos was measured as indicated in (A). The average somite width and number of embryos used (n) in each treatment are shown in (B). These experiments were repeated at least 4 times. Values between groups with a significant difference (p<0.05) are denoted by different superscript letters.

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Discussion

The necessity of PTEN in embryogenesis has been demonstrated in several animal species including mice, chickens and fruit flies [24,25,26]. However, the role of Pten in zebrafish gastrulation has been controversial. In this study, we demonstrated that \textit{ptenb} may regulate zebrafish gastrulation cell movements by controlling actin polymerization and directed cell migration via antagonizing PI3 kinase and downstream Cdc42 and Akt activity.

\textbf{Ptenb regulates convergence and extension during gastrulation}

PI3 kinase has been shown to be required for cell polarization of gastrulating mesendodermal cells by overexpressing dominant-negative form of PI3 kinase or using the PI3 kinase inhibitor LY294002 [10]. The authors unequivocally demonstrated the necessity of maintaining PIP2/PIP3 balance in mesendodermal cell polarity of directed migration during gastrulation in zebrafish. It is logical to hypothesize that the negative regulator of PI3 kinase, PTEN, should also be necessary for regulating gastrulation cell movements in zebrafish, but no gastrulation defects was observed in zebrafish \textit{pten} mutants [37]. Based on the \textit{pten} mutant study, Pten appears to play no role during gastrulation in zebrafish. However, the presence of maternal \textit{pten} messages in early mutant embryos cannot be excluded. Secondly, the lack of functional Pten in the zebrafish \textit{pten} mutants have not be demonstrated due to the lack of antibodies which can recognize zebrafish Pten. Therefore, we took an alternative approach by using \textit{ptenb} MOs which have been previously shown to have specifically lowered \textit{pten} translation and enhanced AKT activity [30] to analyze their effects on gastrulation cell movements in zebrafish. Our results clearly indicated that block of \textit{ptenb} activity affects convergent extension movements during gastrulation in zebrafish.

\textbf{Zebrafish Ptenb regulates gastrulation in coordination with PI3 kinase}

Pten is a known counter enzyme of PI3 kinase by dephosphorylating PIP3 to PIP2 [9,18], and also acts via downstream effectors AKT/PKB to regulate early developmental processes [22,25,28]. To examine the coordination of PI3 kinase and Ptenb in regulating zebrafish gastrulation, we showed that LY294002 could rescue convergence defect in \textit{ptenb} morphants. It clearly demonstrated that PI3 kinase and PTEN linkage is required to modulate cell movements during zebrafish gastrulation.

\textbf{Zebrafish Ptenb regulates protrusive activities of lateral mesendodermal cells but not prechordal plate cells during gastrulation}

In the LY294002 rescue experiment, the result suggested that \textit{ptenb} coordinates with PI3 kinase to regulate gastrulation cell movements presumably via their regulation on PIP2/PIP3 balance. Activated PI3 kinase can increase PIP3 concentration, which would localize at the frontal edge of protrusions and induce the directional migration, and Pten acts at the rear edge of cells to dephosphorylate PIP3 to PIP2 then restrict the spatial expression of PIP3 in the cell [22,23]. Our time-lapse recordings clearly demonstrated that active protrusive activity is required for cell movements during zebrafish gastrulation. The \textit{ptenb} MO-induced...
gastrulation defect was not due to the change in protrusion numbers. Instead, the directionality and persistency of protrusion were more relevant. Lamellapodia were formed at the leading edge of lateral hypoblast cells and these protrusions were mainly pointing anterodorsally to the direction of cell movement. However, tMO1 morphants' lateral hypoblasts exhibited interference in persistence and directionality of protrusions. In the lateral hypoblasts of tMO1 morphants, the increase of lamellapodia turnover was evident by the reduction of lamellapodia persistence. At the same time, the directionality was also affected by decreasing the number of protrusions pointed anteriodorsally. The directional defect was also observed at the transplantation experiment. StdMO cells transplanted to untreated embryos revealed a better linearity than the tMO1 morphant cells transplanted to tMO1 hosts. The abnormality of protrusion persistence and migration directionality was not observed at prechordal plate cells, this result might be due to the fact that prechordal plate cells migrate as multicellular sheets and cell-cell are highly contact. The rear area of the leading edge cells were in close contact with other cells that might help to maintain cell polarity in the absence of Ptenb [38].

Zebrafish Ptenb mediates gastrulation cellular movements via controlling actin polymerization and downstream Akt1 and Cdc42

In vitro cell studies have shown that, the increase of PIP3 concentration in cells activates Rac1 and Cdc42 which would lead to actin polymerization and lamellapodia and filapodia formation [35,39]. Rac1 has further reported to be important in Drosophila mesoderm migration during gastrula [40] and zebrafish convergence by controlling lamellapodia formation [41]. In our results, dominant negative Cdc42 and to a less extent of dominant negative Rac1 also showed to rescue convergent extension defects in ptenb morphants. AKT/PKB is another known downstream factor of PI3 kinase. The binding of AKT/PKB to PIP3 can recruit it to membrane for the regulation of actin arrangement and protrusion formation [42]. Knockdown of ptenb should lead to an increase of PIP3 and subsequent elevation of AKT activity in zebrafish embryos. The elevated AKT activity might disturb the actin cytoskeleton, protrusion formation and cell movements. Over-expression of cadkl1 showed convergence and extension defects. These results

| Treatment | StdMO (n) | tMO1 (n) | tMO1 + LY294002 (n) |
|-----------|-----------|----------|---------------------|
| somite width/embryo diameter | 0.209 ± 0.006° (120) | 0.232 ± 0.006° (119) | 0.213 ± 0.005° (94) |
| extension angle (°) | 82.7 ± 0.71° (75) | 96.2 ± 0.78° (92) | 93.8 ± 0.94° (110) |

Figure 7. Rescue of ptenb MO-induced convergence defect by inhibiting PI3 kinase. Embryos were injected with 10 ng of StdMO (A,D) or tMO1 (B,E), incubated without (A,B,D,E) or with 5 μM of LY294002 (C,F) to 9 to 11 somites and subjected to WISH against myod and ctsl1b. The somite width (solid line), embryo diameter (dash line) (A–C) and extension angle (E–F) of treated embryos were measured as indicated. The average somite width/embryo diameter ratio, extension angle and sample number in each treatment are shown in (G). These experiments were repeated 5 times. Values between groups with a significant difference (p<0.05) are denoted by different superscript letters. doi:10.1371/journal.pone.0018702.g007
demonstrated that Ptenb regulates zebrafish convergence and extension during gastrulation via PI3 kinase-Akt pathway that is consistent with the rescue of ptenb tMO1 morphants by the PI3 kinase inhibitor.

Gastrulation cell movements are tightly regulated by actin cytoskeletons. Here, we showed that actin polymerization was enhanced about two folds in the ptenb tMO1 morphants than the control embryos. These results suggest that zebrafish Ptenb downregulates small GTPases Rac1 and Cdc42, which further rearrange actin polymerization, then controls the convergence and extension during gastrulation.

**ptenb MO induces convergence and extension defects in ptenb−/− embryos**

The lack of gastrulation defect in the pten mutants argues a role of PTEN in zebrafish and the authors also questioned that the effects of pten MOs on zebrafish early embryos reported by Croushore et al. [30] may not be due to the specific loss of Pten [32]. However, we demonstrated the specificity of ptenb MO used with the following evidences: (1) Co-injection of ptenb tMO1 and ptenb 5’ UTR-GFP plasmid showed no fluorescence signal compared to ptenb 5’ UTR-GFP injection alone. (2) Ptenb tMO1 caused convergence and extension in a dose-dependent manner. (3) The defects of ptenb morphants could be rescued by exogenous ptenb mRNAs, LY294002 and dominant-negative Cdc42. (4) Since the generation of functional truncated Ptenb protein could not be excluded in the pten mutants [32], the ptenb−/− embryos was treated with ptenb tMO1 and these mutant embryos showed similar morphological defects with wild type embryos. In addition to our results, Croushore et al., [30] had shown that ptenb tMO1 inhibited Ptenb protein in vitro translation assay and elevated phospho-Akt (pAkt). Those results supported that the ptenb-induced gastrulation defects observed were specifically due to the loss of ptenb.

In summary, we provide the first in vivo evidence that Ptenb coordinates with PI3 kinase to modulate downstream AKT1 and Cdc42 for rearranging actin polymerization and protrusion formation that can lead to proper cell migration to regulate convergence and extension cell movements during gastrulation in zebrafish as schematically depicted in Fig. 11.

**Materials and Methods**

**Ethics Statement**

All animal handling procedures were approved by the use of laboratory animal committee at National Taiwan University, Taipei, Taiwan (IACUC Approval ID: 97 Animal Use document No. 55).

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**Figure 8.** Cell protrusions of lateral hypoblast cells are affected by ptenb MO. Embryos were injected with 10 ng of StdMO (A) or ptenb tMO1 (B), imaged under DIC microscopy and recorded between 75% to 85% epiboly stages. Representative snapshots are shown and arrows are pointing to typical cell protrusions. By examining a 1-h recording, the average persistent time of each protrusion (C) and the percentage of protrusions pointed anterodorsally (D) were calculated and shown for each treatment. *: p<0.05, scale bars: 25 μm.

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**Figure 9.** ptenb functions non cell-autonomously to regulate the cell protrusions and cell migration during gastrulation. (A–D) Rhodamine labeled blastomeres were transplanted from embryos injected with 10 ng of StdMO (STD) or tMO1 (MO) with rhodamine dextran to untreated hosts (UT) or tMO1 morphant hosts (MO). Host embryos were then imaged (animal pole on the top and vegetal pole at the bottom) under confocal microscopy and representative snapshots are shown in (A) STD>UT: StdMO treated cells in an untreated host. (B) STD>MO: StdMO treated cells in a tMO1 morphant. (C) MO>UT: tMO1 treated cells in an untreated host. (D) MO>MO: tMO1 treated cells in a tMO1 morphant. Arrows indicate the representative cellular protrusions in each embryo. (E) Cell transplantations were performed as described in (A–D), imaged under epifluorescent microscopy and time-lapse movies were taken for each treatment. The transplanted cells were traced and their curvilinear velocity (Vcl) and strait line velocity (Vsl) were calculated and shown. Values between groups with a significant difference (p<0.05) are denoted by different letters. Scale bar: 25 μm.

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Zebrafish maintenance and embryo collection

Wild-type TU/AB zebrafish were inbred and maintained in a 14-hr light/10-hr dark cycle and 28.5°C incubator. Embryos were collected by natural spawning and raised in 0.36 Danieau’s buffer (diluting by 1/6 Danieau’s buffer: 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, and 5.0 mM HEPES (pH 7.6), with double distilled water) until observation or fixation. The definition of embryo stage was according to Kimmel et al. [43], and the stages are indicated as hours post-fertilization (hpf).

RNA isolation and RT-PCR analysis

Zebrafish RNAs of selected embryonic stages were isolated by TriSolution Reagent Plus (GeneMark, Atlanta, Georgia, USA). RT-PCR was applied for cDNA synthesis according to the manufacturer’s instructions. cDNAs were generated from total RNAs and served as templates for an 1183-bp fragment of Ptenb amplification with the following primers: 5'-GGCTGCGATCATAAGGAAAT-3' (forward) and 5'-CTGTTCTTGCATGACTGTTCA-3' (reverse). A 530-bp β-actin amplified with primers: 5'-TTGGTATGGGACAGAAAGACAGCTAC-3' (forward) and 5'-AAGGCCACATAGCAGAGCTTC-3' (reverse) was used as a RT-PCR control.

Morpholino oligonucleotide microinjections

All antisense morpholino oligonucleotides (MOs) were custom made by Gene Tools, LLC (Philomath, OR). Standard control MO (Std MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3') has no homology sequence to any known zebrafish sequence.

Table 1. The ptenb MO-induced convergent extension defects can be rescued by dominant-negative Cdc42.

|                | Somite width (µm) | Extension angle (°) |
|----------------|-------------------|---------------------|
|                | (n)               | (n)                 |
| StdMO 10 ng    | 204.9±1.7a        | 71.6±1.8a           |
|                | (155)             | (157)               |
| ptenb tMO1 10 ng | 228.9±4.5b       | 113.9±8.7b          |
|                | (105)             | (105)               |
| ptenb tMO1 10 ng+T17NCdc42 25 pg | 212.6±2.6a     | 98.9±3.6a           |
|                | (85)              | (87)                |
| StdMO 10 ng    | 192.0±4.6a        | 71.2±4.0a           |
|                | (124)             | (124)               |
| StdMO 10 ng+T17NCdc42 25 pg | 192.6±4.0a    | 79.3±4.6a           |
|                | (92)              | (92)                |
| StdMO 10 ng    | 172.9±4.5a        | 75.5±8.3a           |
|                | (75)              | (75)                |
| ptenb tMO1 10 ng+T17NRac1 50 pg | 195.6±4.4a   | 115.0±1.9b          |
|                | (91)              | (92)                |
| ptenb tMO1 10 ng+T17NRac1 50 pg | 188.7±5.0b   | 110.5±4.5b          |
|                | (112)             | (114)               |

Embryos were injected with StdMO and ptenb tMO1, in the presence or absence of T17NCdc42 or T17NRac1 mRNAs, subjected to WISH against myod and cts1b and somite width and extension angle of embryos were measured (N≥4).
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which was used. Two published ptenb translational blocking MOs, tMO1 and tMO2, were used with the following sequences: Ptenb tMO1 (5'-GGCTGATCAGAAGGCAGTTTTTTCCCACAGC-3'), which targeting from −74 to −51 of the 5' UTR region; Ptenb tMO2 (5'-GGCTGATCAGAAGGCAGTTTTTTCCCACAGC-3'), which targeting from −25 to −1 [30]. A 5-base mismatch tMO1 was designed (5'-CAGAAGGACATCTTCC-3'), the mismatched bases were indicated by subscripts. All MOs were dissolved in sterile mini Q water to 1 mM stocks and stored at room temperature. MOs were diluted to proper working concentration by 1x Danieau’s buffer with 0.5% phenol red and keep in room temperature. Thin-wall 3.5 inch glass capillaries (1.14×0.50 mm, O.D.xI.D., World Precision Instrument, Sarasota, FL) were pulled using a horizontal puller (P-97, Sutter Instrument, Navato, CA). Embryos at one-cell stage were stationary at an injection tough on a 100-mm 1% agar plate. MOs were diluted as described at desired concentrations and loaded into a pulled capillary. The tip of loaded capillary was forced through the chorion and into the yolk cells to reach the junction between yolk cells and blastodisc where the MO was ejected by using an oil Nanoliter injector (Nanoliter 2000, World Precision Instruments) at 2.3 nl. After injection, embryos were take out from the injection troughs and cultured in 0.3× Danieau’s buffer at 28.5°C until being examined.

Cloning and preparing mRNA

After RNA isolation, ptenb cDNA was obtained by RT-PCR using primers: 5'-CCGCTCGAGATGCGTGCAGTATAAAAGAATTTTG-3' (forward) and 5'-GAATTTGCAAACTTTAGAATGCTTCC-3' (reverse), then cloned into pGEM-T easy vector. The clone was further subcloned into an expression vector, pET28a (Novagen, CA). The clone was further subcloned into pGEM-T easy vector. The clone was further subcloned into an expression vector, pET28a (Novagen, CA). The clone was further subcloned into pGEM-T easy vector. The clone was further subcloned into an expression vector, pET28a (Novagen, CA). The primer with mutation sites were used to obtain the dominant negative zebrafish wild type Cdc42 and Rac into pGEM-T easy, the primers with mutation sites of Rac were: 5'-GATGTTGCAGTTTGTGCTCTAACTGCTCTA-3' (forward); 5'-GATGTTGCAGTTTGTGCTCTAACTGCTCTA-3' (reverse). The primers with mutation sites of Cdc42 (T17NCdc42) and Rac1 (T17NRac1) constructs by performing PCR again. The primers with mutation sites of Cdc42 are: 5'-GATGTTGCAGTTTGTGCTCTAACTGCTCTA-3' (forward); 5'-GATGTTGCAGTTTGTGCTCTAACTGCTCTA-3' (reverse). The primers with mutation sites of Rac are: 5'-GGCTGATCAGAAGGCAGTTTTTTCCCACAGC-3' (forward); 5'-GGCTGATCAGAAGGCAGTTTTTTCCCACAGC-3' (reverse). The Tn7Cdc42 and Tn7Rac1 were linearized by Sall and Apal I then transcribed into mRNA by mMESSAGE mACHINE® SP6 and T7 Kits, respectively. The human constitutively active human Akt1 (caakt1) was constructed by Gateway cloning to make pCSDEST-myAkt1 from a myrAkt1 delta4–129 plasmid (Addgene, Cambridge, MA). The caakt1 plasmids were digested by SacII and mRNAs were synthesized by SP6 Kit.

Whole-mount in situ hybridization (WISH)

Embryos were fixed in desired stages in 4% paraformaldehyde in phosphate-buffered saline overnight, dechorionated manually by fine forceps and stored in 100% methanol at −20°C until use. Antisense digoxigenin (DIG)-labeled RNA riboprobes were synthesized following the manufacture instructions (Roche Applied Science, Penzberg, Germany). The ptenb construct [45] was linearized by XbaI and transcribed by T7 RNA polymerase (Roche Applied Science, Penzberg, Germany). The ctsl1b construct [46] was linearized by NotI and transcribed by T7 RNA polymerase. In situ hybridization and detection were performed according to Thise et al. [47] with a phosphatase-coupled anti-DIG antibody. The processed embryos were then transferred to 100% glycerol and photographed by using Nikon CoolPIX 995 digital camera.

Measurement and counting of embryos

For the convergence and extension assay, ptenb MOs or STD MO treated embryos were incubated at 28.5°C until 8 to 11-somite stages for morphological observation under stereo. After the morphology classification, the embryos were fixed and subjected to WISH against ptenb to measure the width of ptenb signal of the widest somite among the last three somites. Furthermore, the extension defects were characterized by measuring the angle between prechordal plate staining using ctsl1b and the vegetal end of ptenb signal. All experiments were repeated at least three times and the measurements were made using tools in Adobe Photoshop CS4.
DIC time-lapse cell migration recording

To monitor the migration of lateral hypoblast leading edge cells (marginal deep cells), the embryos at 75% epiboly stage were dechorionated with 0.01 mg/mL protease (Sigma, St. Louis, MO) and mounted in 0.8% low-melting agarose (Amresco, Solon, OH). The migrating cells were recorded using a 40× water immersion objective under a Leica DM5000 B DIC microscope (Leica Microsystems, Wetzlar, Germany). One hour long movies were recorded at 30-sec intervals by a CoolSNAP fx CCD camera (Roper Scientific, Tucson, AZ). The movies were acquired and analyzed by Simple PCI Imagine System software (Compix, Sewickley, PA). The protrusions which merged from cell body with an angle $\alpha \approx 135^\circ$ and a width $d < 2 \mu m$ were defined as a lamellapodium extension. To track transplanted cells, 1 hour long time-lapse recordings with 10-sec intervals were taken and analyzed by using the Simple PCI software for migration path, velocity and linearity (the shortest distance between the start and end points of the movement divided by the total distance moved) analyses.

PI3-kinase inhibitor (LY294002) treatment

Embryos treated with Ptenb tMO1 were dechorionated at 30% epiboly stage and treated with 5 $\mu$m LY294002 (Calbiochem, Darmstadt, Germany) from 30% epiboly to 90% epiboly in E3 media and transferred to 0.3× Danieau’s buffer until 7 somite stage and collected for further in situ hybridization examination.

Actin polymerization assay

The filamentous actin (F-actin) of embryo was determined by fluorometric assay which based on the binding affinity of rhodamine phalloidin and F actin [48,49]. Embryos injected with different MOs were fixed at shield stage in 3% formaldehyde in Actin Stabilizing Buffer (ASB) [50] overnight. The embryos were then incubated in 150 mM glycine-ASB for 3 hours, washed by ASB, and then labeled by 165 nM rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR). Control embryos were incubated by 20 mg/mL unlabeled phalloidin for 50 minutes prior to rhodamine phalloidin staining. All samples in a group of 60 embryos were then washed in ASB and extracted in dark with methanol and homogenized by 24G needles, and incubated with rotating for 36 hours at 4°C. The fluorescence signals were detected from the extractant and measured by using a fluorospectrophotometer (Hitachi F-2000, Tokyo, Japan) with an excitation wavelength of 565 nm and an emission wavelength of 580 nm [36].

Cell transplantation

For transplantation preparation, donor embryos were injected with 0.5% rhodamine dextran and Std MO 10 ng or Ptenb IMO1 10 ng respectively. Donor embryos were transplanted into wild type and Ptenb morphant host separately as described previously [51]. Transplanted cells were further recorded by using Leica DM5000 B microscope and Leica TCS SP5 Confocal Microscope Imaging System.

ptenb$^{--}$/zebrafish screening

Heterozygous ptenb$^{++}$/zebrafish embryos were kindly provided by Jeroen den Hertog (Hubrecht Institute, Utrecht, The Netherlands). Those embryos were raised to adults and mated to produce F$_1$ fish. Nested PCR was performed to select homozygous ptenb$^{--}$/zebrafish according to Faucherre et al. [32].

Statistical analysis

All experimental values are presented as mean ± standard error and were analyzed by unpaired-sample Student’s t-test in Microsoft Excel. N indicates the number of experiments repeated; n indicates the total sample number in one experimental condition. Different superscript lettering between values stands for a significant difference at p<0.05.

Supporting Information

Figure S1  Embryos were injected with 10 ng of StdMO with or without 25 pg of T7 NCdc42 mRNAs and treated as described in Table 1. The somite width and extension angle of each embryo were measured and shown (A). Embryos were injected with 10 ng of StdMO without (B) or with 50 pg of T7 NRac1 mRNAs (C), incubated to 10-somite stage and photographed. The percentages of normal and abnormal embryos in each treatment are shown (D). (PDF)
Movie S1  Time-lapse imaging of cellular migration and protrusions in lateral hypoblast cells of a StdMO-injected morphant. Shown here is a 1-h at 30-s intervals DIC time-lapse image sequence of a 75% epiboly stage embryo-injected with 10 ng StdMO. The animal pole is on the top and the dorsal side is to the left. (AVI)

Movie S2  Time-lapse imaging of cellular migration and protrusions in lateral hypoblast cells of a ptenb MO1-injected morphant. Shown here is a 1-h at 30-s intervals DIC time-lapse image sequence of a 90% epiboly stage embryo-injected with 10 ng ptenb tMO1. The animal pole is on the top and the dorsal side is to the left. (AVI)

Movie S3  Time-lapse imaging of cellular migration and protrusions in involuting prechordal plate cells of a StdMO-injected morphant. Shown here is a 5-min movie at 5-s intervals DIC time-lapse image sequence of a 90% epiboly stage embryo-injected with 10 ng StdMO. The prechordal plate cells were migrating anteriorly to the top. (AVI)

Movie S4  Time-lapse imaging of cellular migration and protrusions in involuting prechordal plate cells of a ptenb tMO1-injected morphant. Shown here is a 3-min movie at 5-s intervals DIC time-lapse image sequence of a 90% epiboly stage embryo-injected with 10 ng ptenb tMO1. The prechordal plate cells were migrating anteriorly to the top. (AVI)

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Author Contributions

Conceived and designed the experiments: C-MY YCL SJL. Performed the experiments: C-MY YCL. Analyzed the data: C-MY YCL SJL. Contributed reagents/materials/analysis tools: CDH. Wrote the paper: C-MY YCL SJL.

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