Essential roles of $\text{G}_\alpha^{12/13}$ signaling in distinct cell behaviors driving zebrafish convergence and extension gastrulation movements

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$\text{G}_\alpha^{12/13}$ have been implicated in numerous cellular processes, however, their roles in vertebrate gastrulation are largely unknown. Here, we show that during zebrafish gastrulation, suppression of both $\text{G}_\alpha^{12}$ and $\text{G}_\alpha^{13}$ signaling by overexpressing dominant negative proteins and application of antisense morpholino-modified oligonucleotide translation interference disrupted convergence and extension without changing embryonic patterning. Analyses of mesodermal cell behaviors revealed that $\text{G}_\alpha^{12/13}$ are required for cell elongation and efficient dorsalward migration during convergence independent of noncanonical Wnt signaling. Furthermore, $\text{G}_\alpha^{12/13}$ function cell-autonomously to mediate mediolateral cell elongation underlying intercalation during notochord extension, likely acting in parallel to noncanonical Wnt signaling. These findings provide the first evidence that $\text{G}_\alpha^{12}$ and $\text{G}_\alpha^{13}$ have overlapping and essential roles in distinct cell behaviors that drive vertebrate gastrulation.

Introduction

Gastrulation is a pivotal phase of vertebrate development during which the body plan is established via a complex series of the morphogenetic movements. Vertebrate gastrulation consists of three main morphogenetic processes: epiboly, internalization of presumptive mesendoderm, and convergence and extension (C&E). In zebrafish gastrulae, C&E movements narrow the germ layers mediolaterally and elongate them anteroposteriorly to define embryonic axes. Mesodermal mediolateral cell intercalation, as well as directed dorsal and anterior cell migration contribute to this morphogenetic processes (Warga and Kimmel, 1990; Trinkaus et al., 1992; Jessen et al., 2002; Glickman et al., 2003). How this diversity of gastrulation cell behaviors is generated remains poorly understood.

Noncanonical Wnt signaling, an equivalent of the planar cell polarity signaling in Drosophila melanogaster (Wnt-PCP), is a major regulator of the mediolateral cell polarization required for cell intercalation in frog and fish, and fast dorsal migration in fish gastrulae (Keller, 2002; Myers et al., 2002b; Wallingford et al., 2002). Mutants of several genes involved in this pathway, such as trilobite (strabismus; Jessen et al., 2002), knypek (glypican4/6; Topczewski et al., 2001), silberblick (wnt11; Heisenberg et al., 2000) display shortened axis and defective mesodermal cell polarization. Recent evidence indicates that Heterotrimeric G proteins may participate in the Wnt/Ca²⁺ branch of the noncanonical pathway, which involves intracellular Ca²⁺ release and activation of PKC (Sheldahl et al., 1999; Malbon et al., 2001). The Wnt signaling pathway is activated by the binding of Wnt ligands to the Frizzled receptors, which have seven transmembrane domains, a structural characteristic of G protein–coupled receptors (GPCRs). There is evidence that like GPCRs, Frizzled receptors may activate G proteins to mediate their signal transduction. In cultured cells, coupling of the Frizzled receptor to $\text{G}_\alpha$, $\text{G}_\text{q}$, and $\text{G}_\text{o}$ has been reported (Liu et al., 1999; Liu et al., 2001; Ahumada et al., 2002). In addition, it has been shown that G proteins are involved in Wnt signaling pathways that mediate gastrulation. Expression of pertussis toxin (which ADP-ribosylates $\text{G}_\alpha$ and $\text{G}_\text{q}$, and uncouples them from their cognate receptors) disrupts tissue separation during Xenopus laevis gastrulation, an effect also seen with Xfz7 depletion. Moreover, PKC can rescue the defect in tissue separation in both Xfz7-depleted and PTX-injected...
embryos, suggesting that PTX-sensitive G proteins and PKC are involved in *Xenopus* gastrulation movements (Winklbauer et al., 2001). In addition, PKCα and PKCβ are activated by Frizzled receptors, possibly through G proteins and Dishevelled to regulate C&E movements in *Xenopus* (Kuhl et al., 2001; Kinoshita et al., 2003). Furthermore, Goi is required for both the canonical Wnt and PCP signaling in *Drosophila* (Katanava et al., 2005). Recently, it has been reported that Gβγ subunits may also play important roles in C&E movements. In *Xenopus* gastrulae, inhibition of Gβγ signaling by overexpression of Go, and Go, (which sequester free Gβγ) rescued C&E defects that resulted from activation of Wnt11/Xfz7 (Penzo-Mendez et al., 2003). In addition, inhibition of Gβγ signaling in the *Xenopus* dorsal marginal zone resulted in gastrulation arrest. However, exactly which Gα-proteins are involved in Wnt-PCP-mediated gastrulation remains unknown.

G proteins consist of four classes; Go, Go, Go, and Go (Simon et al., 1991). Go, subunits are the most divergent G protein family and have been implicated in numerous cellular processes such as Rho-mediated cytoskeletal rearrangements, thereby affecting cell shape and migration (Buhl et al., 1995; Gohla et al., 1999; Sugimoto et al., 2003). Studies in *Drosophila* indicate that Gα signaling plays a role in gastrulation, as inactivation of the *Drosophila* Goα homologue, concertina, impairs cell shape changes underlying mesoderm internalization during gastrulation (Parks and Wieschaus, 1991). In mice, disruption of Goα gene led to embryonic death at midgestation, due to the failure of endothelial cells to form an organized vascular system (Offermanns et al., 1997). In addition, Go, have been shown to induce primitive endoderm formation in mouse F9 cells (Lee et al., 2004). However, the role of Go in vertebrate gastrulation has not been analyzed.

Here, we used zebrafish as a model to investigate the role of Go in early vertebrate embryogenesis. Using dominant negative receptor blocking peptides and antisense morpholino oligonucleotides (MOs), we demonstrate that Gα and Gα have overlapping and essential roles in C&E. Cell movement analyses show that Gα, signaling regulates slow dorsal migration of lateral mesoderm cells independent of noncanonical Wnt signaling. In the notochord, Gα, are required for mediolateral cell intercalation, acting cell-autonomously, and likely in parallel to noncanonical Wnt signaling. Our studies for the first time suggest a central role for Gα in signaling in generating the diversity of gastrulation cell behaviors in vertebrate embryos.

## Results

**Cloning and characterization of zebrafish gna12 and gna13 genes**

One gene encoding Gα12 (referred to as gna12) and two paralogues (gna13a and gna13b) encoding Gα13 were found in zebrafish. Gα13a and Gα13b share 81% identical and 91% similar amino acid residues with each other, and have 90–93% sequence similarity to human Gα13. The zebrafish Gα12 protein shares 81% identical and 91% similar amino acid residues with human Gα12 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200501104/DC1).

In cultured cells, mammalian Gα, induce stress fiber formation via a RhoA/Rho kinase (Rok)-dependent pathway (Buhl et al., 1995; Gohla et al., 1998). To evaluate if zebrafish Gα, have similar activities, wild-type (WT) and constitutively active Gα, mutant proteins were transiently expressed in human embryonic kidney (HEK) cells. Cells overexpressing either WT human or zebrafish Gα, or Gα, (Fig. 1, C, E, and F) displayed stress fibres even in the absence of agonist stimulation. Formation of stress fibres was blocked by pretreatment with 10 μM of Rok inhibitor, Y-27623 (Fig. 1 H; Uehata et al., 1997). These results indicate that like their human counterparts, zebrafish Gα, can promote actin rearrangements in cultured cells, through a RhoA/Rok-dependent pathway.

**Expression of zebrafish gna12 and gna13 genes**

Whole-mount in situ hybridization revealed that gna12 and both gna13 transcripts are maternally deposited (Fig. 2, A–C). Accordingly, high levels of Gα12 and Gα13 proteins were detected at the 8 cell stage by immunohistochemistry using antibodies that recognize the last 11 aa of Gα12 or Gα13 (not depicted). During blastula and gastrula stages, transcripts of all
three genes are present ubiquitously throughout embryo (Fig. 2, D–I). By 1–2 dpf, the expression becomes confined to anterior body regions (Fig. 2, J–O).

**Interference with Gα12/13 function disrupts gastrulation movements**

To investigate the function of the zebrafish Gα12 and Gα13 proteins in embryonic development, we used two strategies to inhibit Gα12/13 signaling. First, we overexpressed peptides encoding the last 11 or 50 COOH-terminal aa of both proteins, referred to as CT-peptides. The COOH-terminal region of G protein α subunits has been shown to be a major binding site of G proteins to their cognate receptors (Hamm, 1998). Overexpression of CT-peptides in cells or mice has been shown to block competitively the receptor sites that normally bind to G proteins, leading to specific blockade of the respective Gα signaling (Akhter et al., 1998; Gilchrist et al., 1999, 2001; Feldman et al., 2002; Arai et al., 2003). Strikingly, compared with their control siblings, embryos injected with synthetic RNAs encoding the CT-peptides (the last 11 aa) of Gα12 or Gα13 (Gα12-CT, Gα13-CT) exhibited shortened body axes with broader notochords and somites (Fig. 3, C–F), as well as delayed epiboly (unpublished data). At 3 dpf, these embryos remained shorter, and frequently displayed synophthalmia or cyclopia (Fig. 3, P–S), phenocopying defects often associated with defective C&E movements in silberblick (wnt11) or trilobite (strabismus) noncanonical Wnt signaling mutants (Heisenberg et al., 2000; Jessen et al., 2002). Examination of expression patterns of tissue specific markers confirmed that embryos expressing Gα12-CT or Gα13-CT displayed broader neural plate and notochord. Furthermore, prechordal mesoderm was positioned more posteriorly with respect to the anterior edge of the neural plate during early segmentation stage (Fig. 3, I–L), suggestive of impaired anterior migration of this cell population (Heisenberg et al., 2000; Topczewski et al., 2001; Jessen et al., 2002). Both severity and penetrance of the observed phenotypes increased with the dose of Gα12-CT or Gα13-CT RNAs. Whereas 15% of embryos injected with 0.5 ng Gα12-CT or Gα13-CT RNA showed morphology consistent with impaired C&E movements, 60% and 70% of embryos injected with 1 and 1.5 ng Gα12-CT or Gα13-CT RNA showed similar C&E defects, respectively (Fig. 3 M). These data suggest that Gα13 and Gα12 both function in C&E movements. In contrast, C&E defects were not observed in embryos injected
with up to 2 ng RNA encoding Ga12 CT peptide (a different class of Ga subunit; Fig. 3, A, B, G, and H), suggesting the specific involvement of Ga12/13 in gastrulation. Similarly, overexpression of the COOH-terminal 50 aa of Ga12 or Ga13, conjugated with HA-tag, produced similar gastrulation defects (not depicted). Anti-HA immunohistochemistry showed these longer peptides were robustly expressed in the cytosol, with slight enrichment on the membrane (Fig. 3, N and O).

In a complementary approach, we used antisense MOs (Nasevicius and Ekker, 2000) to block Ga12/13 translation in zebrafish. The endogenous Ga12 and Ga13 in zebrafish blastulae were detected predominantly on the cell membranes and in a punctate pattern in the cytosol (Fig. 4, A and C). When embryos were injected with MO (4 ng) targeting gna12 transcript, the expression of Ga12 was strongly reduced (Fig. 4 B). Likewise coinjection of MOs targeting gna13a and gna13b transcripts (4 ng each) decreased the level of both Ga13 proteins at late blastula stage (Fig. 4 D). Together, these results demonstrated that the MOs we designed effectively blocked gna12 or gna13 translation. Injections of up to 20 ng/embryo of MO against any single gna12/13 transcript (either gna13a or gna13b alone or in combination, or gna12 alone) had no obvious effect on embryonic development probably due to functional redundancy between Ga12 and Ga13 in zebrafish (Fig. 4 E and not depicted). However, when the embryos were injected with a mixture of MOs against gna13a, gna13b, and gna12 (3MOs, 4 ng each), 76% of embryos showed impaired C&E movements as judged by alteration of embryonic morphology and gene expression patterns (Fig. 4, F and H; n = 214). The phenotypes resulting from MO interference were very similar to those caused by overexpression of CT-peptides. To test whether the above gastrulation defects are due to specific interference of MOs with Ga12/13 function, we coinjected a subthreshold dose of human GNA13 RNA along with three MOs. Because the MO targeted nucleotide sequence of the human gene diverges from the sequences of zebrafish homologues, it cannot be blocked by the MOs used here. Injection of 10–20 pg human GNA13 RNA had no obvious effect on zebrafish morphogenetic movements of epiboly and C&E (unpublished data). However, when this amount of human GNA13 RNA was coinjected with the combination of the three MOs, the percentage of embryos with gastrulation defects decreased from 76% to 20% (Fig. 4 H). Whereas 75% of embryos (n = 120) injected with the three MOs exhibited a very short body axis, and some degree of brain degeneration by 1 dpf, coinjection of human Ga12 largely suppressed the axis extension defects with 75% embryos showing an almost normal body length (Fig. 4, I–K; n = 138). As shown by morphometric analysis, embryos coinjected with three MOs exhibited a reduced body length of 2015 ± 50 μm (n = 22), compared with embryos injected with a single MO (2962 ± 10 μm, n = 10). By contrast, embryos coinjected with human GNA13 RNA and three MOs showed significantly restored body length (2432 ± 47 μm, n = 22, P = 7.6 × 10⁻⁸). However, only modest suppression of brain degeneration was observed. These results indicate that the morphogenetic defects are a specific consequence of the interference with Ga12/13 function, whereas the neural degeneration phenotype might be a nonspecific defect, often associated with MO injection (Nasevicius and Ekker, 2000). In addition, zebrafish and human Ga13 share a conserved activity in gastrulation. Interestingly, the effects of CT peptides and MOs were synergistic. Although very few embryos showed C&E defects when injected with moderate doses of either Ga12-CT RNA (9%, 500 pg, n = 126) or gna13a-MO (0%, 5 ng, n = 120), coinjection of both resulted in 65% embryos displaying C&E defects (65%, n = 73). Comparable results were found when embryos were coinjected with Ga12-CT RNA and gna13b-MO or Ga13-CT RNA and gna12-MO (unpublished data).

Similar C&E defects were also observed in embryos overexpressing WT Ga12a, Ga12b, or Ga12 proteins, and occurred in a dose-dependent manner (Fig. 4 G and not depicted). Co-injection of Ga12/13 specific MOs suppressed gastrulation defects resulting from overexpression of Ga12/13 (not depicted). This indicates that the phenotypes caused by Ga12/13 are due to specific interference with their functions, and provides further support for the effectiveness of these MOs. Together these results show that both reduction and excess Ga12/13 function impair the C&E gastrulation process.

Interference with Ga12/13 function does not alter cell fate specification during gastrulation

Gastrulation defects might be a consequence of altered embryonic patterning and consequent changes in cell movements, or might be due to defects in cell movements alone (Myers et al., 2002b). Therefore, we tested whether dorsoventral patterning is affected in Ga12/13-CT or MO injected embryos by analyzing expression of dorsoventral patterning genes, bmp4 and chordin (Hammerschmidt and Mullins, 2002). Our results revealed that bmp4 expression was not altered in early and late gastrulae injected with three MOs (Fig. 4 M, n = 34, and not depicted). Likewise, expression of chordin gene encoding a Bmp antagonist was confined to its normal dorsal expression domain during early gastrulation in embryos injected with the combination of 3MO (Fig. 4 O, n = 32), or with RNAs encoding Ga12/13-CT peptides (not depicted). Moreover, embryos injected with CT peptide RNA or 3MO displayed normal expression of several cell type specific markers at late gastrulation, consistent with normal cell fate specification (Fig. 3, I–L; Fig. 4 F). Finally, cell tracing experiments revealed that the labeled lateral mesodermal cells acquired somitic fates in Ga12/13-depleted embryos (not depicted), consistent with their positions in the early gastrula (Sepich et al., 2000). Based on these results, we conclude that morphogenetic defects observed in Ga12/13-depleted embryos are likely not associated with significant patterning or cell fate changes during gastrulation.

Ga12/13 are required for efficient directed cell migration during early dorsal convergence movements

Shortened anteroposterior and enlarged mediolateral dimensions of the embryonic axes in Ga12/13-depleted gastrulae could be a consequence of defective C&E movements (Sepich et al., 2000). Recent studies reveal that convergence movements in
zebrafish mesoderm are accomplished by a stereotyped sequence of cell behaviors, including slow and fast directed cell migration (Jessen et al., 2002). To investigate if any of the convergence cell behaviors were altered in \(G_{\alpha}^{12/13}\)-depleted embryos, we performed Nomarski time-lapse analyses in WT (6 embryos, 144 cells) and 3MO-injected (6 embryos, 134 cells) embryos at midgastrulation. Analysis of total cell speed, accounting for movement in all directions, revealed that in \(G_{\alpha}^{12/13}\)-depleted gastrulae, cells moved at a reduced speed (70% of WT total speed; \(P = 9.8 \times 10^{-27}\); Fig. 5 B). Interestingly, the net dorsal speed of \(G_{\alpha}^{12/13}\)-depleted cells was especially strongly compromised, accounting only for 28% of the WT net dorsal speed (\(P = 2.1 \times 10^{-12}\), Fig. 5 B). Further analysis of cell migration paths revealed that, similar to the WT cells, \(G_{\alpha}^{12/13}\)-depleted cells migrated predominantly dorsally (Fig. 6 A). However, compared with WT, these cells more frequently changed their movement direction (Fig. 6, A and B), at the expense of movement in the dorsal direction (Fig. 6 C). To determine how efficiently WT and 3MO cells corrected their path direction when they were off-course, we examined cells moving toward dorsal, animal, ventral or vegetal direction (Fig. 6, D–G). We found that in WT embryos, cells moving dorsally largely maintained this direction in the next step. Moreover, WT cells that had been moving in the animal or vegetal direction turned toward dorsal in the next movement step, very few cells from these populations moved away from dorsal. By contrast, equivalent cell populations in embryos injected with 3MO were less persistent in dorsal movement (Fig. 6 D). Moreover, when these cells moved in animal or vegetal direction, they less frequently corrected their movement toward dorsal compared with.
Movement direction change in degrees. (Inset) A–C represent positions of WT and 3MO-injected embryos at 80% epiboly. (A) Representative cell shape was drawn for these selected cells at every 2.5-min interval. Nomarski time-lapse analyses were performed on lateral mesodermal cells. Cell movement directions during the midgastrulation (with LWR of 1.4 ± 0.26, 182 cells, 6 embryos; P = 0.7) and at tailbud (with LWR of 1.67 ± 0.28, 102 cells, 6 embryos, P = 0.2; Fig. 5 D). Interference with G_{12/13} function for moderate elongation of lateral mesodermal cells is manifest already at midgastrulation, and thus before the noncanonical Wnt signaling is thought to become essential during convergence (Jessen et al., 2002). Collectively, these results indicate that G_{12/13} function is required for normal cell elongation and effective directed migration during C&E movements.

**Interference with G_{12/13} function disrupts mediolateral cell intercalation**

Mediolaterally oriented intercalation of cells at the dorsal midline drives robust axial extension in zebrafish (Crawford et al., 2003; Glickman et al., 2003) and *Xenopus* gastrulae (Shih and Keller, 1992). During gastrulation, notochord precursor cells elongate, align mediolaterally and intercalate between one another to lengthen the notochord anteroposteriorly and narrow its mediolateral dimension, decreasing from the initial width of 4–5 to 1–2 cells (Glickman et al., 2003). To investigate whether G_{12/13} signaling is required for mediolateral intercalation of midline cells, we analyzed shape (LWR) and orientation of notochord cells at the 4 and 6 somite stage. We found that at the 4 somite stage, the WT notochord was one to two cells wide, and cells were aligned mediolaterally (at an average angle of 6 ± 5° relative to a line perpendicular to the embryonic axis as represented by the notochord) and were well elongated with LWR of 3.24 ± 1.19 (166 cells, 6 embryos; Fig. 7, A–C). At the 6 somite stage, notochord cells were further elongated with LWR of 4.84 ± 1.92 and aligned mediolaterally with an angle of 4 ± 3° (241 cells, 8 embryos; Fig. 7, A and B). In contrast, in G_{12/13}-depleted embryos at the 4 somite stage, the notochord was two or three cells wide revealing an intercalation defect, and these cells were rounder, exhibiting an average LWR of 2.25 ± 0.79 (254 cells, 9 embryos, P = 3.3 × 10^{-18}; Fig. 7, A and D). However, these cells still aligned mediolaterally but at a slightly greater angle of 11 ± 12° (P = 4.2 × 10^{-5}; Fig. 7, B and D). At the 6 somite stage, notochord cells in G_{12/13}-depleted embryos continued to show impaired elongation and orientation defects with LWR of 2.95 ± 1.2 and angle of 8 ± 8° relative to the me-
diolateral axis (202 cells, 6 embryos, \( P = 5.1 \times 10^{-31} \); Fig. 7, A and B). These results indicate \( \alpha_{12/13} \) signaling is also required for elongation and intercalation of axial mesodermal cells, and consequently for C&E of embryonic axis.

To test the cell-autonomy of \( \alpha_{12/13} \) function in notochord C&E, we transplanted cells at blastula stage from donors injected with rhodamine-dextran alone or with 3MOs into WT hosts injected with \( \text{mgfp} \) RNA and 3MOs or \( \text{mgfp} \) RNA alone, and determined shape and orientation of cell bodies in notochord at 4 somite stage. We found that transplanted \( \alpha_{12/13} \)-depleted cells exhibited rounder shapes (with LWR of 2.50 \( \pm \) 1.21, 39 cells, 7 embryos, \( P = 4.49 \times 10^{-10} \)) and more random orientation (with angle of 13.91 \( \pm \) 11.66, \( P = 7.0 \times 10^{-7} \)) even in WT environment (with LWR of 4.01 \( \pm \) 1.47 and angle of 6.84 \( \pm \) 6.66, 167 cells; Fig. 7, G-J). Conversely, WT cells displayed normal elongated shape and orientation (with LWR of 4.15 \( \pm \) 1.26 and angle of 7.43 \( \pm \) 5.89, 43 cells, 5 embryos; Fig. 7, K, M, and N). These results reveal a cell-autonomous requirement for \( \alpha_{12/13} \) in mediolateral cell elongation during C&E of notochord.

The relationship between \( \alpha_{12/13} \) and the noncanonical Wnt signaling during zebrafish gastrulation

The noncanonical Wnt signaling pathway mediates mediolateral cell polarization underlying normal C&E movements (Keller, 2002; Myers et al., 2002b; Wallingford et al., 2002). The morphological changes in embryos with altered \( \alpha_{12/13} \) signaling are strikingly similar to those reported for mutants of \( \text{slb (wnt11)} \) and \( \text{knypek (glypican4/6)} \) that resulted from the disruption of the noncanonical Wnt signaling (Heisenberg et al., 2000; Topczewski et al., 2001). Moreover, \( \alpha_{12/13} \)-depleted mesodermal cells also exhibited impaired cell elongation during late gastrulation (Fig. 5), similar to embryos overexpressing dominant negative Rok2 and \( \text{trilobite} \) and \( \text{knypek} \) mutants (Topczewski et al., 2001; Jessen et al., 2002; Marlow et al., 2002). However, our studies showed that \( \alpha_{12/13} \) are required for two types of directed cell migration for which noncanonical signaling does not appear to be required: early slow convergence and epiboly (Fig. 5; unpublished data), suggesting that...
Gα12/13 mediate these movements independent of Wnt signaling. To elucidate the functional relationship between Gα12/13 signaling and noncanonical Wnt signaling during gastrulation, we analyzed the effect of modulation of Gα12/13 signaling on noncanonical Wnt signaling mutant phenotypes.

The small GTPase Rho, is the main effector of Gα12; and Gα13 (Buhl et al., 1995) and is also implicated in noncanonical Wnt signaling (Habas et al., 2001). Accordingly, Rho downstream mediator Rok2 can partially suppress the slab (wnt11) gastrulation defects (Marlow et al., 2002). To address whether zebrafish Gα12/13 can modulate Wnt11 signaling during gastrulation, we injected RNAs encoding Gα13 to enhance, or Gα13-CT to inhibit the function of Gα13, in homozygous slab (wnt11)β216e216 embryos (Heisenberg et al., 2000). However, neither various levels of excess nor deficit of Gα13 signaling could suppress the gastrulation defects in slab (wnt11)−/− embryos. Rather, both perturbations of Gα13 signaling exacerbated the slab (wnt11)−/− phenotype (unpublished data). We also performed molecular epistasis experiments by injecting embryos obtained from kny+/H9251 heterozygotes, carrying a null mutation in the glypican 4/6 gene (Topczewski et al., 2001), with a small dose of synthetic RNAs encoding Gα12, or MOs against gna13 or three MOs against both gna13 and gna12. Neither overexpression of Gα13 nor down-regulation of Gα13 signaling suppressed kny+/− C&E defects. Instead, depletion of Gα12/13 signaling by injection with three MOs enhanced the kny+/− defects of neuroectoderm convergence and of anterior prechordal mesendoderm migration (Fig. 7, G–J). Finally, the ex-

Discussion

In this study, we have provided evidence that Gα12 and Gα13 play overlapping and essential roles during zebrafish gastrulation. Thus, overexpression of Gα12/13 CT-peptides to uncouple Gα12/13 from their cognate receptors, or reducing the level of Gα12/13 by MO translation interference resulted in C&E defects. Overexpression of either Gα12 or Gα13 CT-peptides caused C&E defects with similar efficiency suggests that signal transduction through both Gα12 and Gα13 is essential for these gastrulation movements. However, based on several considerations, we believe that Gα12 and Gα13 are functionally redundant in zebrafish gastrulation. First, our MO translation interference experiments showed that gastrulation defects were only observed in embryos injected with the combination of the three MOs to inhibit protein synthesis of Gα12, Gα13a, and Gα13b, but not in embryos injected with the same amount of any single MO. Second, it is well established that Gα12 and Gα13 can regulate similar physiological processes via similar signaling pathways (Dhanasekaran and Dermott, 1996; Sah et al., 2000). Indeed, we showed previously that either Gα12 or Gα13 CT-peptide could inhibit thrombin receptor-stimulated stress fiber formation in HMEC cells (Gilchrist et al., 2001). Similar results were reported for LPA-mediated stress fiber formation in fibroblasts (Sugimoto et al., 2003). It is possible that Gα12 and Gα13 interact with the same receptors in zebrafish gastrulae to regulate C&E. If their binding sites on the receptors are overlapping, it is conceivable that either Gα12 or Gα13 CT-peptide is sufficient to block completely signal transduction from both Gα12 and Gα13. Finally, the functional redundancy of Gα12 and Gα13 in vertebrates is also supported by the findings that compound Gα13 and Gα12 mutant mice die earlier (e8.5) than Gα13 null mice (e9.5), whereas Gα12 knockout mice are viable (Offermanns, 2001). Together, these findings strongly argue for overlapping functions of Gα12 and Gα13 in vertebrates.

In vivo time-lapse analyses revealed that Gα12/13 signaling is required for several distinct gastrulation cell behaviors, including dorsalward migration and intercalation during C&E movements. However, Gα12/13 signaling does not appear to act as a general motility factor. Indeed, mesendoderm internalization occurred without any obvious defects in Gα12/13-depleted gastrulae (unpublished data). Therefore, we conclude that Gα12/13
signaling affects only a subset of morphogenetic events in zebrafish gastrula. We cannot, however exclude the possibility that there is a residual activity in embryos injected with Moes and or CT-peptides, permitting other morphogenetic processes that require lower levels of Gα12/13 signaling.

We demonstrated that in Gα12-depleted embryos, mediolateral cell elongation of slowly migrating cells at midgastrulation is impaired, whereas this cell behavior is normal in the trid mutant (Jessen et al., 2002). Moreover, our preliminary analyses of lateral mesodermal cells in embryos overexpressing α13 revealed normal cell elongation (unpublished data). This suggests that the role of Gα12/13 signaling in the regulation of cell elongation is distinct from that of noncanonical Wnt signaling, where either increased or decreased pathway activity impairs elongation of mesodermal cells (Wallingford et al., 2000). Consistent with this hypothesis, our mosaic analyses indicate that mediolateral cell elongation requires only cell-autonomous Gα12/13 activities, whereas noncanonical Wnt signaling regulates cell elongation both cell-autonomously and nonautonomously (Jessen et al., 2002; Marlow et al., 2002). In addition, ectopic Gα12/13 activity cannot suppress the knt+/- and slb+/- phenotypes (Fig. 5 and not depicted) and Fz+/- morphant phenotypes (not depicted). Collectively, these results strongly argue that Gα12/13 do not act as components of a linear noncanonical Wnt signaling pathway to mediate cell polarization. Identification of the ligands and receptors that regulate gastrulation behaviors acting upstream of Gα12/13 will be our next main focus.

Recent studies indicate that a number of distinct cell behaviors contribute to vertebrate gastrulation (Elul and Keller, 2000; Jessen et al., 2002; Myers et al., 2002b; Montero et al., 2003). How is this diversity of cell behaviors generated? In some gastrula regions, cells are engaged in more than one cell behavior, suggesting that cells are competent to respond to several cues. Our work implicates Gα12/13 as key mediators of many different gastrulation cell behaviors: slow and fast dorsoconvergent movements (Fig. 5 and not depicted; Jessen et al., 2002; Myers et al., 2002b; Montero et al., 2003). Given that Gα12/13 can be activated by a variety of GPCR/ligands, it is tempting to hypothesize that these proteins may underlie interaction with signals in different regions of fish gastrulae to generate the distinct gastrulation cell behaviors. In the lateral region, cells become influenced by a dorsally provided attracting system that initiates convergence movements. Evidence suggests that β-catenin activates the STAT3 pathway in the dorsal gastrula organizer to produce a long range dorsal attractant, which could interact with Gα12/13 signaling to mediate slow dorsal convergence movements (Yamashita et al., 2002). In the dorsolateral region, Gα12/13 may also interact with the noncanonical Wnt signaling to generate high mediolateral elongation underlying fast dorsal migration. Finally in the dorsal region, Gα12/13 and noncanonical Wnt signaling could interact with yet to be identified regulators to mediate intercalation behavior. In conclusion, we establish a central role for Gα12 and Gα13 proteins in mediating several distinct cell behaviors that drive vertebrate gastrulation. Identification of extracellular cues that are integrated by Gα12/13 to mediate individual gastrulation cell behaviors is an important future goal for this research into the molecular mechanisms of morphogenesis.

Materials and methods

Zebrafish maintenance

WT zebrafish of AB*, India, TL and hybrid backgrounds, slbβ216/β216, knt+/- (Heisenberg et al., 2000; Topczewska et al., 2001), zebrafish strains were maintained as described previously (Solnica-Krezel et al., 1994). Embryos were obtained from natural mating and staged according to morphology as described previously (Kimmel et al., 1995).

Cloning zebrafish gna12/13 and generation of Gα12/13 COOH-terminal peptide constructs

Zebrafish gna12 and gna13 cDNAs were cloned by RT-PCR and then subcloned into the pCS2 expression vector. The conserved glutamine at residue 226 of Gα13 was changed to leucine to generate a constitutively active form of Gα protein (Kotah et al., 1998) using the QuiKChange mutagenesis kit (Stratagene). To generate constructs encoding the last 11 COOH-terminal aa of Gα13 (GRMHLQRYELL), Gα12 (LQENLKDIMLQ), and Gα13 (LHDNLQKQYELL), two synthetic short complimentary oligonucleotides encoding the peptide sequences were obtained for each gene. The forward and reverse oligonucleotides were annealed, and subcloned into pCS2 vector. These constructs were designated as Gα12-CT, Gα12-CT, and Gα13-CT (one peptide was used to block function of both Gα13 and Gα13 because they have identical COOH-terminal sequences). Longer forms of CT peptides encoding the last 50 aa of COOH terminal of Gα13 and Gα12, which included a HA-tag at the NH2 terminus, were constructed by PCR. All constructs were verified by DNA sequencing.

Microscopy

Live embryos for still photography were mounted in 1.5–2% methylcellulose at 28°C, whereas embryos processed for whole-mount in situ hybridization were mounted in 75% glycerol/PBT. Embryos were photographed using an AxiohoZ2 microscope (Carl Zeiss Microlmaging, Inc.) and an Axioacam digital camera (Carl Zeiss Microlmaging, Inc.). For confocal imaging, embryos were mounted in 75% glycerol/PBT and a laser scanning inverted microscope (model LSM 510; Carl Zeiss Microlmaging, Inc.) with a 40x lens and 2x digital zoom was used. All images acquired were compiled and edited using Adobe Photoshop and Illustrator software.

In situ hybridization

Sense and antisense RNA probes for gna12, gna13a, and gna13b were synthesized using the NH2-terminal EST clones as templates. Antisense RNA probes hag1, dix3, krox20, shh, deltaC, ntl, bmp4, and chordin were synthesized as described previously (Jessen et al., 2002). Whole-mount in situ hybridization was performed as described previously (Thommes and Thiess, 1998), except that BM purple (Roche) was used for the chromogenic reaction. Sense probes produced no signal.

Cell culture stress fiber formation assay

HEK cells were transiently transfected with GFP or with G protein constructs as indicated. To block ROCK activity, ROCK inhibitor, Y-27623 was added at 10 μM to media after transfection (Uehata et al., 1997). Stress fiber formation assay was performed as described previously (Glichrist et al., 2001). Anti-Gα12 or Gα13 antibodies ([1:100] generated against the last 11 AAs of human Gα12 or Gα13 (Hallak et al., 1994) were used to identify the G protein–expressing cells. Cells were mounted in Vectashield mounting medium (Vector Laboratories) and confocal images were acquired as described in Microscopy.

Whole-mount immunostaining

Zebrafish embryos were fixed in 4%PFA/PBS/4% sucrose at shield and whole mount immunohistochemistry was performed as described previously (Topczewska et al., 2001). Primary anti-Gα12 or Gα13 antibodies and Cy2-conjugated pAb ([1:100] were used. No signal was detected when the primary antibodies were preincubated with the peptides encoding the COOH-terminal 11 residues of the Gα12 or Gα13, respectively, or when the only the secondary antibody was used. Confocal images were acquired.

RNA and antisense MO injections

Capped sense RNAs encoding the Gα12, Gα13, Gα13 CT-peptides, mGFP (Walligford et al., 2000) or a full-length zebrafish Gα13a and human
Gn12/13 were synthesized using mMessage Machine system [Ambion]. RNAs were injected into embryos at 1–2 cell stage.

Antisense MOs (GeneTools) targeted against the zebrafish gna12, gna13a, and gna13b transcripts were designed according to the manufacturer’s suggestions and injected into embryos at 1 cell stage. Two distinct MOs were designed to target either the sequences overlapping the ATG initiation codon (MO1) or the S’ untranslated sequences (MO2) of gna13a transcript. For gna13b and gna12, one MO against sequence overlapping the translation start site was designed for each transcript.

Time-lapse and cell shape analysis

Nomarski time-lapse images of lateral gastrula mesodermal cells at mid-gastrulation (80% epiboly) were collected as described previously (Myers et al., 2002a). Dechorionated zebrafish embryos were mounted in 0.8–1% low melting point agarose in 30% Danieau’s buffer (100% Danieau’s buffer: 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM CaCl2, 5 mM Hepes, pH 7.6), to view the lateral mesoderm (90° from the dorsal midline). The microscope room was maintained at 28°C during recording. Single focal plane time-lapse recordings were collected at 30-s intervals using DIC optics and a 20x objective (0.5 NA Plan Neofluor) on an Axioptop2 microscope (Carl Zeiss MicroImaging, Inc.) and an Axiocam digital camera (Carl Zeiss MicroImaging, Inc.). Images were analyzed using ObjectImage software (Norbert Vischer, http://simon.bio.uva.nl/object-image.html). Data was exported to Excel [Microsoft] where cell migration speed, path, direction, turning angle, and LWRs were determined. Activity and distribution of paxillin, focal adhesion kinase, and cadherin indicate cooperative roles during zebrafish morphogenesis. Mol. Biol. Cell. 14:3065–3081.

Transplantation experiments

For cell autonomy analyses, cells from embryos injected with dextran-Rho-damamine or together with 3MO were transplanted into host embryos in Danieau’s buffer (100% Danieau’s buffer: 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM CaCl2, 5 mM Hepes, pH 7.6) and stained with anti-Rhodamine, then LWR and the angle of the long axis relative to a line perpendicular to the embryonic axis as represented by the notochord were analyzed using ObjectImage software.

Statistical analysis

Data are presented as the mean ± 1 SD. Statistical analyses were performed using unpaired t-tests unequal variance. In all analyses, the asterisk indicates $P < 0.001$.

Accession nos.

GenBank/EMBL/DDBJ accession nos. for the zebrafish gna12, gna13a, and gna13b are AY386359, AY386360, and AY386361, respectively.

Online supplemental material

Fig. S1 shows the sequence alignment of human and zebrafish Gna12/13. Two synthetic short complementary oligonucleotides encoding the peptide sequences were obtained for gna12, gna13, and gna5. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200501104/DC1.

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