The receptor-tyrosine kinase Ror2 acts as an alternative receptor or co-receptor for Wnt5a and mediates Wnt5a-induced convergent extension movements during embryogenesis in mice and Xenopus as well as the polarity and migration of several cell types during development. However, little is known about whether Ror2 function is conserved in other vertebrates or is involved in other non-canonical Wnt ligands in vivo. In this study we demonstrated that overexpression of dominant-negative ror2 (ror2-TM) mRNA in zebrafish embryos resulted in convergence and extension defects and incompletely separated eyes, which is consistent with observations from slb/wnt11 mutants or wnt11 knockdown morphants. Moreover, the co-injection of ror2-TM mRNA and a wnt11 morpholino or the coexpression of ror2 and wnt11 in zebrafish embryos synergetically induced more severe convergence and extension defects. Transplantation studies further demonstrated that the Ror2 receptor responded to the Wnt11 ligand and regulated cell migration and cell morphology during gastrulation. DnRor2 inhibited the action of Wnt11, which was revealed by a decreased percentage of Wnt11-induced convergence and extension defects. Ror2 physically interacts with Wnt11. The intracellular Tyr-647 and Ser-863 sites of Ror2 are essential for mediating the action of Wnt11. Dishevelled and RhoA act downstream of Wnt11-Ror2 to regulate convergence and extension movements. Overall, our data suggest an important role of Ror2 in stream components (8–13). Ror2, which belongs to the Ror family of receptor-tyrosine kinases, is an orphan receptor (14) that possesses an extracellular cysteine-rich domain, which closely resembles the Wnt-binding sites of the Fz proteins (14). The identification of Wnt binding domains in Ror2 suggested that noncanonical Wnt ligands may trigger alternative receptors, i.e. receptors other than Fzd (15, 16). This makes more complexes between receptors-co-receptors and noncanonical Wnt ligands on the cell membrane and even downstream components in the cytoplasm. For example, in the regulation of convergent extension movements in Xenopus gastrulation, XWnt-5A regulates XRor2 and downstream PI3 kinase and cdc42, which activate the JNK signaling cascade by mediating the expression of the transcription factors ATF2 and c-jun (17). Wnt5a-Ror2-Dishevelled (Dsh) is a core developmental pathway that controls tissue morphogenesis in early mouse embryonic development and in the progression of different types of cancers in humans (18, 19). However, whether the role of Ror2 is conserved in other vertebrates or whether Ror2 mediates the effects of other ligands in gastrulation movements is not completely understood.

In vertebrates, gastrulation is a phase of essential fundamental morphogenetic movements that facilitate the formation of the basic germ layers and the body axis during early embryonic development. During gastrulation, convergence and extension movements narrow a group of embryonic cells mediolaterally and lengthen them anteroposteriorly (1, 2). Although the precise molecular mechanisms underlying this process are not particularly well documented in vertebrates, the noncanonical Wnt pathway, also termed the planar cell polarity (PCP) pathway, plays an important role in controlling convergence and extension movements and is well conserved (3–7).

Wnt-PCP signaling is triggered by noncanonical family members of Wnt ligands, including Wnt4, Wnt5a, and Wnt11, through an interaction with the seven-transmembrane domain receptor Frizzled (Fzd) and then relayed to cytoplasmic downstream components (8–13). Ror2, which belongs to the Ror family of receptor-tyrosine kinases, is an orphan receptor (14) that possesses an extracellular cysteine-rich domain, which closely resembles the Wnt-binding sites of the Fz proteins (14). The identification of Wnt binding domains in Ror2 suggested that noncanonical Wnt ligands may trigger alternative receptors, i.e. receptors other than Fzd (15, 16). This makes more complexes between receptors-co-receptors and noncanonical Wnt ligands on the cell membrane and even downstream components in the cytoplasm. For example, in the regulation of convergent extension movements in Xenopus gastrulation, XWnt-5A regulates XRor2 and downstream PI3 kinase and cdc42, which activate the JNK signaling cascade by mediating the expression of the transcription factors ATF2 and c-jun (17). Wnt5a-Ror2-Dishevelled (Dsh) is a core developmental pathway that controls tissue morphogenesis in early mouse embryonic development and in the progression of different types of cancers in humans (18, 19). However, whether the role of Ror2 is conserved in other vertebrates or whether Ror2 mediates the effects of other ligands in gastrulation movements is not completely understood.

In this study we demonstrate that Ror2 is required for normal convergence and extension movements in zebrafish. The over-
expression of a dominant-negative Ror2 results in convergence and extension defects and zebrafish embryo eye fusion, which is a phenocopy of slb/wnt11 mutants and wnt11 knockout morphants. Ror2 interacts with Wnt11 and mediates Wnt11 signaling during gastrulation movements via Dsh and RhoA.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—RNA polymerase and RNase-free DNase were purchased from Promega (Madison, WI). Restriction endonucleases were purchased from New England Biolabs Inc. (Ipswich, MA). KOD DNA polymerase was purchased from Toyobo (Osaka, Japan). Superscript II reverse transcriptase, Moloney murine leukemia virus reverse transcriptase, oligonucleotide primers, Alexa Fluor 488, and Alexa Fluor 546 were purchased from Invitrogen. Protein A/G Plus-agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-His antibody was purchased from Cell Signaling Technology (Danvers, MA), and anti-FLAG M2 antibody was purchased from Sigma.

**Experimental Animals**—Wild type zebrafish (Danio rerio; Tuebingen strain) were maintained on a 14-h light/10-h dark cycle and fed twice daily. Embryos were obtained by natural fertilization during gastrulation movements via Dsh and RhoA. Embryos were wild-type embryos or embryos injected with wnt11 MO at the one-cell stage. When the host embryos were at the shield stage, 20–30 cells were removed from each donor embryo at the stage by using a needle into the center of lateral mesoderm of a host embryo. The host embryos were then allowed to develop until ~11 hours post-fertilization (hpf) for imaging. The images were acquired with a stereo fluorescence microscope (M165FL, Leica). The distance between Alexa Fluor 546- and Alexa Fluor 488-labeled cells in 10 embryos from each group was analyzed and calculated as previously reported (23).

For assessing ligand-receptor interactions by transplantation, donor embryos were injected with 800 pg of ror2 mRNA + 400 pg of membrane gfp mRNA + 4 ng of wnt11 MO or 800 pg of ror2-TM mRNA + 400 pg of membrane gfp mRNA + 4 ng of wnt11 MO. Host embryos were injected with 100 pg of wnt11 mRNA. At the sphere stage, 20–30 cells were transplanted into the blastodermal margin of each host embryo. At the shield stage, embryos with transplanted cells in the lateral or ventral mesoderm were selected and allowed to grow to 90% epiboly for imaging with a 63× water immersion objective on a Leica DM2500 fluorescence microscope. Cell roundness was measured by Image J software (National Institutes of Health), and...


~100 cells from each group were analyzed as previously reported (11).

Cell Culture, Western Blotting, and Co-immunoprecipitation—To prepare protein-expressing cells, HEK293T (2 × 10^6) cells were seeded in 10-cm dishes for 24 h, after which the cells were transfected with 4.5 μg of receptor Ror2-FLAG and ligand pcDNA3.1-Wnt11 using Lipofectamine 2000 (Invitrogen) separately. Five hours after transfection, the cells were trypsinized and mixed in a ratio of 1:4 (receptor cells: ligand cells) and then reseeded in a 10-cm dish for co-culture.

After 24 h of co-culture, cell lysates were prepared by lysing the cells with 800 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, and complete protease-inhibitor mixture (Roche Applied Science)) and removing the insoluble materials by centrifugation at 12,000 × g for 10 min at 4 °C. A total of 15 μl of the lysate was used as a whole-cell lysate by adding 15 μl of 2× SDS sample buffer. The remaining lysates were subjected to immunoprecipitation by incubation with 2 μg of an anti-His antibody or 2 μg of an anti-FLAG antibody at 4 °C overnight. Then 40 μl of Protein A/G Plus-agarose was added to the lysates, which were further incubated at 4 °C for 2 h. Immune complexes were precipitated by centrifugation at 1,000 × g for 2 min, washed 4 times with 1 ml of PBS buffer, and dissolved in 20 μl of 2× SDS sample buffer. The immunoprecipitates or whole-cell lysates were separated by SDS-PAGE and blotted onto PVDF membranes. The primary antibodies were as follows: anti-His and anti-FLAG M2 (24).

Statistical Analysis—Values are presented as the means ± S.E. Student’s t test was used for comparisons between two groups. One-way analysis of variance following Tukey’s multiple comparison test was used for comparisons among multiple groups. Statistical tests were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA), and significance was defined as p < 0.05 or greater.

RESULTS Zebrafish Ror2 Is Closely Related to ROR2 Proteins in Other Vertebrates and Ubiquitously Expressed during Gastrulation—To address the function of Ror2 in zebrafish development, we cloned zebrafish ror2 by searching the zebrafish genome database. Zebrafish Ror2, a member of the type-I transmembrane receptor-tyrosine kinase family, contains an extracellular immunoglobulin (Ig) domain and a Cys-rich domain. The intracellular region of zebrafish Ror2 contains a tyrosine kinase domain, a proline-rich domain (PRD), and two Ser/Thr-rich domains (Ser/Thr1 and Ser/Thr2) (Fig. 1A). The sequence alignment of zebrafish Ror2 with human, mouse, and Xenopus Ror2 proteins revealed a high level of identity, suggesting that Ror2 is a well-conserved protein in vertebrates (Fig. 1A). The phylogenetic analysis showed that the putative zebrafish Ror2 was clustered together with Ror2 of other species and separated from the Ror1 branch, another family member of Ror (Fig. 1B).
mount in situ hybridization. RT-PCR results revealed that zebrafish ror2 was maternally deposited and highly expressed at 6–9 hpf. Although ror2 expression decreased at 12–16 hpf, elevated expression levels were maintained from 20 hpf (Fig. 1C). Whole-mount in situ hybridization with a zebrafish ror2 antisense probe indicated the ubiquitous presence of ror2 transcripts at different developmental stages, and the expression occurred predominantly in the brain at 24 and 48 hpf (Fig. 1D). The ubiquitous and dynamic expression of ror2 implies that zebrafish Ror2 may play an important role in the regulation of embryonic development.

Overexpression of a Dominant-negative Ror2 in Zebrafish Embryos Impairs Convergence and Extension Movements during Gastrulation—To investigate the role of Ror2 in embryonic development, ror2 knockdown was performed using translation-blocking MOs. We evaluated the efficiency of the ror2-targeting MOs (ror2-TB1-MO and ror2-TB2-MO) by co-injecting zebrafish with the MOs and a ror2 5' UTR-GFP expression construct. Both the ror2-TB1-MO and the ror2-TB2-MO successfully blocked the expression of the GFP-tagged reporter. Subsequently, we injected ror2-TB1-MO (8 ng) or ror2-TB2-MO (8 ng) into zebrafish embryos that were at the one-cell stage. Unexpectedly, the injected embryos exhibited normal morphology at 11 hpf. The expression pattern of dlx3b (indicates the edges of the neural plate) and hgg (indicates the prechordal plate), which are markers for gastrulation convergence and extension movements, did not change (data not shown). We propose that the inability of ror2 MO knockdown to block convergence and extension movements resulted from the presence of abundant maternally deposited Ror2 protein.

To further investigate the role of Ror2 in embryonic development, a dominant-negative form of zebrafish Ror2, Ror2-TM, which contains the extracellular and transmembrane domains of the zebrafish Ror2 protein but lacks the cytoplasmic domain (Fig. 2A), was developed according to the structure from a dominant-negative mouse Ror2 used previously in cultured cells (25). Injection of ror2-TM mRNA in zebrafish embryos induced severe defects in anterior-posterior extension after gastrulation (Fig. 2B, upper row). In addition, the ror2-TM-injected embryos showed broader notochords and somites than gfp-injected embryos at 12 hpf, indicating that convergence might be impaired by Ror2 disruption (Fig. 2B, lower row). Overexpression of ror2-TM significantly increased the percentage, in a dose-dependent manner, of embryos with an enlarged angle between the anterior and posterior ends as well as a broader mediolateral distance (Fig. 2C). This defect was not due to alterations in the dorsoventral axis, which was evidenced by a normal expression pattern of the dorsoventral axis marker genes chordin (chd) and even-skipped-1 (eve1) (data not shown). At 24 hpf, the ror2-TM-injected embryos exhibited eye fusion, which was not observed in the gfp-injected control group. According to the interocular distances or cyclopia, overexpression of ror2-TM significantly increased the fusion of the eyes in a dose-dependent manner (Fig. 2D and E). Using in situ hybridization, we also evaluated the expression pattern of hgg, dlx3b, and ntl (indicates the nascent notochord) for convergence and extension movements. The ror2-TM-injected embryos were separated into three categories according to the width of the neural plate: normal (<380 µm), moderate (380–460 µm), and severe (>460 µm). According to the position of hgg anterior to dlx3b, the injected embryos were also separated into three categories: normal (hgg anterior to dlx3b), moderate (less than half of hgg expression posterior to dlx3b), and severe (more than half of hgg expression posterior to dlx3b). Compared with the gfp-injected embryos, the ror2-TM-injected embryos exhibited a wider neural plate (dlx3b) and a more posteriorly positioned prechordal plate (hgg) at the tail bud stage (Fig. 2, F–H). Collectively, these results suggest that Ror2 is required for convergence and extension movements.

Forced expression of ror2 mRNA in the zebrafish embryos also inhibited the mediolateral narrowing and anterior-posterior lengthening of the development axis, resulting in convergence and extension defects (Fig. 2I). Overexpression of ror2 mRNA significantly increased in a dose-dependent manner the angle between the anterior and posterior ends of the embryo as well as the mediolateral distance of the embryo (Fig. 2I). The expression pattern of hgg and dlx3b was also analyzed by whole-mount in situ hybridization at the tail bud stage (10 hpf). Compared with the controls, the ror2-injected embryos exhibited a more posteriorly positioned prechordal plate and a wider neural plate at the tail bud stage (Fig. 2, K–M). Taken together, these results demonstrate that overexpression of either ror2-TM or ror2 in zebrafish embryos impairs convergence and extension movements during gastrulation.

Co-knockdown or Coexpression of Wnt11 and Ror2 Synergistically Induce Severe Convergence and Extension Defects—Genetic evidence underscores the importance of Wnt in regulating convergence and extension movements during zebrafish embryo gastrulation (13). Zebrafish wnt11 is expressed in the anterior paraxial mesendoderm, and wnt11/silberblick mutants show elongated prechordal plates and shorter notochords, which correlate with a partial fusion of the eyes at later developmental stages (10, 11). Therefore, we speculate that Ror2 mediates Wnt11 activity, which regulates convergence and extension movements in zebrafish.

To evaluate whether Wnt11 and Ror2 interact, a double knockdown of wnt11 and ror2 was performed simultaneously. A low dose injection of wnt11 MO (0.25 ng) or ror2-TM mRNA (200 pg) individually generated mild convergence and extension defects (15 and 25% of the total, respectively) (Fig. 3A). The co-injection of ror2-TM mRNA and wnt11 MO resulted in enhanced convergence and extension defects (70% of the total; Fig. 3A). The altered expression patterns of hgg and dlx3b also supported the enhanced convergence and extension defects after co-injection of ror2-TM mRNA and wnt11 MO (Fig. 3, B–D). We also analyzed eye defects after co-injection of low doses of wnt11 MO and ror2-TM mRNA. A low dose of wnt11 MO or ror2-TM mRNA generated mild eye defects (8 and 20%, respectively, of the total), whereas co-injection of low doses of ror2-TM mRNA and wnt11 MO synergistically enhanced the eye defects (40% of the total) (Fig. 3E).

Furthermore, we coexpressed wnt11 mRNA and ror2 mRNA. A low dose of wnt11 mRNA (25 pg) or a low dose of ror2 mRNA (100 pg) individually generated mild convergence and extension defects (5 and 20% of the total, respectively), whereas coexpression of ror2 mRNA and wnt11 mRNA synergistically
enhanced convergence and extension defects (50% of the total) (Fig. 3f). These results were consistent with the expression patterns of hgg and dlx3b (Fig. 3, G–I). Therefore, Ror2 acts synergistically with Wnt11 in zebrafish development to regulate convergence and extension movements.

Knockdown of Ror2 or Wnt11 Changes Cell Movements and Morphology during Gastrulation—To address the interaction of Wnt11 and Ror2 in cell migration during gastrulation, a series of transplantation assays was performed. Donor embryos were injected with cMO or Ror2-TM mRNA combined with...
Ror2 Receptor Mediates Wnt11 Signaling in Zebrafish Embryos

fluorescent lineage markers. Host embryos were uninjected or were injected with wnt11 MO. Then labeled cells from ~3 hpf donor embryos were mixed and transplanted into the center of lateral mesoderm of shield-stage host embryos because cells in this region of the embryo migrate in a ventral-to-dorsal direction and extend in both anterior and posterior directions. After gastrulation of host embryos was completed, the distribution of transplanted cells was scored. As shown in Fig. 4A, the donor cells from two control MO-injected embryos showed similar convergence and extension movements in wild-type host embryos. By contrast, the ror2-TM-injected cells indicated reduced convergent movements when compared with cMO-injected cells (Fig. 4B). Whereas wnt11 was knocked down in host embryos, both of the donor cells from cMO- and ror2-TM-injected embryos showed reduced convergent movements (Fig. 4C). To quantify the migration distance of donor cells, the mean distance from the center of the embryos to the red- or green-labeled cells was measured, and the difference between these two groups of cells was calculated as previously reported (23).

In wild-type host embryos, the difference in migration distance between the ror2-TM-injected cells and the control MO-injected cells was 69 μm, which was significantly different from that in the control group (17 μm). However, in Wnt11 MO-injected hosts, there was no significant difference in the mean migration distance of ror2-TM- and control MO-injected cells (Fig. 4D). These data suggest that convergent movement could be impaired in the absence of the Ror2 receptor and the absence of the Wnt11 ligand with or without the Ror2 receptor during gastrulation. To further evaluate the interaction between Ror2 and Wnt11 at the level of individual cells during gastrulation, we determined the elongation of both ror2-overexpressing and ror2-TM-overexpressing cells in wnt11-overexpressing embryos because elongation of ectodermal and mesendodermal cells along the medio-lateral axis is regarded as a prerequisite for CE movement. Therefore, we performed another cell transplantation assay. The donor embryos were injected with ror2 or ror2-TM mRNAs combined with membrane gfp mRNA to label the cell membrane. Donor embryos were also injected with wnt11 MO to reduce antocrine effects. As shown in Fig. 5, A and B, knockdown of Wnt11 in donor embryos did not alter cell shape. The host embryos were injected with wnt11 mRNA. Cells from ~3-hpf donor embryos were transplanted into the center of the lateral mesoderm of shield-stage host embryos. At 90% epiboly, we determined the roundness of labeled cells in wnt11-overexpressing host embryos to assess the elongation and calculated as previously reported (11). As shown in Fig. 5, C–F, the ror2-overexpressing cells exhibited a significantly lower degree of roundness than ror2-TM-overexpressing cells. These data indicate that Wnt11 stimulates the elongation of Ror2-expressing cells during gastrulation.

**Inhibition of Ror2 Attenuates Wnt11 Activity in Convergence and Extension Movements as Well as Wnt11 and Ror2 Physically Interact**—We have demonstrated that co-knockdown or coexpression of wnt11 and ror2 synergistically induce severe convergence and extension defects in zebrafish. To further verify the interaction between Wnt11 and Ror2, we co-injected zebrafish embryos with wnt11 mRNA and ror2-TM mRNA to observe whether inhibition of Ror2 could attenuate Wnt11 activity in convergence and extension movements.

Injection of wnt11 mRNA (50 pg) induced distinct convergence and extension defects during gastrulation, whereas injection of ror2-TM (200 pg) induced mild convergence and extension defects (Fig. 6A). Injection of ror2-TM mRNA reduced wnt11-induced convergence and extension defects, suggesting that inhibition of Ror2 attenuates Wnt11 activity in convergence and extension movements (Fig. 6A). These results were further confirmed by the expression of hgg and dlx3b as well as eye phenotypes (Fig. 6, B–D). ROR1, a homolog of ROR2, mediates Wnt5a activity *in vitro* (26–28). To exclude the possibility that Ror2-TM also interacts with ROR1 and subsequently affects convergence and extension movements, ror2-TM and ror1 or ror2 were coexpressed, and eye defects were analyzed. The results indicated that Ror2-TM attenuates the convergence and extension defects caused by Ror2 but not Ror1 (data not shown).

To determine whether the Ror2 and Wnt11 proteins physically interact, FLAG-tagged Ror2 and His-tagged Wnt11 were generated to perform a co-immunoprecipitation assay. The results demonstrated that Ror2 was able to interact with Wnt11.
FIGURE 3. Co-knockdown or coexpression of Wnt11 and Ror2 synergistically induced severe convergence and extension defects. A, quantitation of convergence and extension defects in zebrafish embryos injected with the indicated mRNA and/or MO at 12 hpf. The embryo number of each group is shown at the top of each related column. The results are from three independent microinjection experiments. B, WISH of marker genes (hgg, dlx3b, and ntl) in gfp-injected and wnt11, ror2 knockdown or double knockdown embryos. The black double-headed arrow indicates the width of the neural plate, and the embryos were separated into three categories according to the width: normal, moderate, and severe. According to the position of hgg anterior to dlx3b, the embryos were also separated into three categories: normal, moderate, and severe. The asterisk indicates the hgg position. C, width statistics of the neural plate of each indicated group, as described in B. The embryo number of each group is shown at the top of each related column. The results are from three independent microinjection experiments. D, position statistics of hgg anterior to dlx3b of each indicated group, as described in B. The embryo number of each group is shown at the top of each related column. The results are from three independent microinjection experiments. E, quantitation of eye phenotypes in each indicated group at 24 hpf. The embryo number of each group is shown at the top of each related column. The results are from three independent microinjection experiments. F, quantitation of convergence and extension defect phenotypes in zebrafish embryos injected with the indicated mRNA at 12 hpf. The embryo number of each group is shown at the top of each related column. The results are from three independent microinjection experiments. G, WISH of marker genes (hgg, dlx3b, and ntl) in gfp, ror2, wnt11, and ror2 + wnt11 mRNA-injected embryos. The phenotype definition is described in B. H, width statistics of the neural plate of each indicated group, as described in G. The embryo number of each group is shown at the top of each associated column. The results are from three independent microinjection experiments. I, position statistics of hgg, anterior to dlx3b of each indicated group, as described in G. The embryo number of each group is shown at the top of each related column. The results are from three independent microinjection experiments.
These data together with the previous results suggest that zebrafish Ror2 interacts with Wnt11 and mediates Wnt11 activity.

Tyr-647 and Ser-863 Are Important Functional Sites of Ror2 in Convergence and Extension Movements—Upon stimulation of Wnt5a in several cell types, Ror2 is phosphorylated on Ser/Thr residues but not on Tyr residues (27, 29). In contrast, the tyrosine kinase activity of Ror2 has been shown to be required for inhibiting canonical Wnt signaling in vitro (30). Therefore, we investigated the sites of Ror2 that are critical for convergence and extension movements in zebrafish. Ser-863 in the Ser/Thr2 domain and Tyr-647 in the tyrosine kinase domain were point-mutated or double-mutated according to previously reported methods (Fig. 7A). As established earlier, co-injection of low doses of ror2 and wnt11 synergistically enhanced convergence and extension defects. This synergistic effect was seriously impaired when the Tyr-647 and/or the Ser-863 residue was mutated (Fig. 7B). These results suggest that both the Tyr-647 residue and the Ser-863 residue are crucial mediators of Wnt11 activity in convergent extension movements.

Dsh and RhoA Act Downstream of the Wnt11/Ror2 Signaling Pathway—Dsh-ΔDIX is an amino-terminal-truncated form of Dsh that contains the PDZ and DEP domains and is thought to transduce Wnt signals that regulate morphogenetic movements; however, Dsh-ΔDIX is not involved in the canonical Wnt signaling pathway (31). Previous studies demonstrated that the ubiquitous overexpression of Dsh-ΔDIX could rescue the wnt11/slb phenotype, indicating that Dsh acts pervasively downstream of Wnt11/Slb (10). Injection of rhoA mRNA effectively reversed the defects in wnt11 morphants, which provides in vivo evidence that RhoA acts downstream of Wnt11 to affect convergence and extension movements in zebrafish embryos (32). These data indicate that in zebrafish, Dsh, and RhoA may act downstream of the Wnt11-Ror2 signaling pathway. Compared with ror2-TM mRNA injection, co-injection of Xdsh-ΔDIX and ror2-TM induced a marked decrease in eye defects (Fig. 8A). Co-injection of rhoA and ror2-TM induced similar results (Fig. 8B). These results suggest that Dsh and RhoA act downstream of the Wnt11-Ror2 signaling pathway to mediate convergence and extension movements.

DISCUSSION

Vertebrate gastrulation involves massive cell movements that establish the germ layers and shape the embryonic body. During gastrulation, convergent extension movements are major cell movements and play important roles in vertebrate
**FIGURE 5. Wnt11 stimulates elongation of Ror2-expressing cells.** A–D, representative views of cells in 90% epiboly stage embryos; lateral view. Scale bar = 5 μm. A, donor embryo injected with ror2 mRNA and wnt11 MO combined with membrane gfp mRNA. B, donor embryo injected with ror2-TM mRNA and wnt11 MO combined with membrane gfp mRNA. C, Ror2-overexpressing donor cells in Wnt11-overexpressing host embryos. D, Ror2-TM-overexpressing donor cells in Wnt11-overexpressing host embryos. E and F, analysis of cell roundness of transplanted cells, as described in C and D. E, a column chart shows the roundness for each group. Values are the mean ± S.E. ***p < 0.001; unpaired t test. F, a cumulative frequency chart shows the distribution of roundness values for each group. Approximately 100 cells were measured for each group.
early embryonic development (33). In this study we report that Ror2 mediates Wnt11 activity in zebrafish gastrulation. Both the knockdown and overexpression of Ror2 in zebrafish embryos impaired convergence and extension movements during gastrulation. Furthermore, the co-knockdown or coexpression of Wnt11 and Ror2 synergistically induced severe convergence and extension defects, and Wnt11 activity in convergence and extension movements was attenuated by Ror2 inhibition. Intriguingly, we found that both the Tyr-647 and Ser-863 residues of Ror2 are important functional sites for the mediation of convergence and extension movements, which contrasts with previous reports (27, 29, 30). Ror2 acts synergistically with Wnt11 in zebrafish gastrulation to regulate convergence and extension movements, at least partially through Dsh and RhoA.

In humans, disrupted convergent extension movements result in failure of neural tube closure, which causes spina bifida, a common permanently disabling birth defect (34–36). Multiple sources of genetic evidence underscore that the Wnt-PCP pathway is a key regulator of convergence and extension movements (37, 38). In zebrafish, several mutants exhibited defective gastrulation convergence and extension movements associated with mutations in the Wnt-PCP pathway components, and the Wnt11 mutant slb exhibited compromised convergence and extension movements (10, 11, 39, 40). Wnt11 regulates cell movements in zebrafish by interacting with Fzd-7 and Flamingo (41). Whether or not other receptors or coreceptors are involved in mediating Wnt11 activity is not clear. As a non-Fzd receptor for Wnt ligands, Ror2 was able to interact with Wnt5a to regulate convergence and extension movements in *Xenopus* and mice (14, 16). In zebrafish, Wnt5a is expressed at low levels during gastrulation, and the knockdown of Wnt5a did not result in convergence and extension defects, suggesting that Wnt5a may not be the major ligand driving convergence and extension movements (23). The Wnt5b pathway provides directional signals via the Ryk receptor to regulate posterior gastrulation movements (23). RT-PCR analysis revealed that *ror2* is deposited maternally and expressed highly at 6–9 hpf, and Wnt11 was also reported as being highly expressed at the gastrulation stage (11), which suggests that Ror2 may function with Wnt11 during gastrulation.

In the present study we observed that MO-mediated *ror2* knockdown embryos were morphologically normal. This unexpected finding may have resulted from the presence of maternal Ror2 proteins (42, 43). To expand our study, the dominant-negative form of zebrafish Ror2, Ror2-TM, was used to...
inhibit the function of wild-type Ror2 (25). Overexpression of Ror2-TM inhibited the mediolateral narrowing and anterior-posterior lengthening of the development axis, resulting in convergence and extension defects. Additionally, ror2 overexpression induced convergence and extension movement defects that were similar to defects observed with Ror2 knockdown. Similar effects of overexpression and knockdown have also been reported for another noncanonical Wnt receptor, Ryk, which is also essential for convergence and extension movements (44, 45). These data are in agreement with previous reports that convergence and extension movements are sensitive to both elevated and reduced levels of their regulators (43, 46–51).

The disruption of zebrafish Ror2 function resulted in a phenocopy of the noncanonical Wnt signaling mutant silberblick/wnt11. Moreover, the coexpression of low-dose ror2-TM mRNA and wnt11 MO synergistically enhanced convergence and extension and eye defects. The co-injection of low-doses of ror2 and wnt11 mRNA induced severe convergence and extension defects, whereas these low-dose mRNAs by themselves did not affect development.

The transplantation assay showed that dorsal convergence migration was impaired in the absence of Ror2. When Wnt11 was lost, cells with or without the Ror2 receptor could not migrate normally. Moreover, Wnt11 stimulated the elongation of Ror2-overexpressing cells but not Ror2-TM-overexpressing cells. The above results indicated that Wnt11 may function as a ligand of Ror2. In addition to Wnt11, the noncanonical Wnt ligands Wnt4a and Wnt11-related are also required and act non-redundantly to control specific aspects of convergence and extension. However, their associated receptors have not been identified (13, 52).

Wnt11 overexpression-induced convergence and extension defects were attenuated by the inhibition of Ror2. These results further indicate the role of Ror2 in convergence and extension and the genetic interaction of Ror2 with Wnt11. Notably, coex-
expression of ror2-TM and wnt11 exhibited similar convergence and extension defects. Here, we cannot exclude the potential interaction of other Wnts with Ror2 to regulate convergence and extension movements in zebrafish.

A previous study demonstrated that the Cys-rich domain of Xror2 interacts with Xwnts in Xenopus (16). The conserved Cys-rich domain also exists in the zebrafish Ror2 protein. Furthermore, our results for mixed cocultured cells demonstrated that Ror2 co-immunoprecipitated with Wnt11, indicating that Wnt11 was able to interact with Ror2. Whether or not this interaction includes other proteins is still unclear. In zebrafish, Wnt11 induces the accumulation of its receptor Fzd-7 (41). In Xenopus, XFzd-7 biochemically and functionally interacts with Wnt11, and coexpression of Xror2 with Xwnt11, XFzd-7, or both synergistically inhibits convergent extension in embryos (9, 16). Cthrc1, a secreted glycoprotein, bound to Wnt proteins, Fzd proteins, and Ror2 and then enhanced the interaction of Wnt proteins and Fzd/Ror2 by forming the Cthrc1-Wnt:Fzd/Ror2 complex (24).

Wnt5a promotes the phosphorylation of Ror2 on Ser-864 in mouse embryonic fibroblasts (27). Because zebrafish Ror2Ser-863 demonstrated high conservation with mouse Ror2Ser-864, we speculate that Ser-863 is a phosphorylation site of zebrafish Ror2. Upon stimulation of Wnt5a, Ror2 is phosphorylated on Ser/Thr residues but not Tyr residues (29). In mouse embryonic fibroblasts, Wnt5a promotes the phosphorylation of Ror2 on Ser-864 by GSK3, but Ror2 Tyr kinase activity is not required for Wnt5a activation of Ror2 (27). In contrast, tyrosine kinase activity is required for Ror2 inhibition of canonical Wnt signaling in cultured HEK293T cells (30). In the present study we observed that the synergistic effect of Ror2 and Wnt11 on convergence and extension movements was seriously impaired when ror2 with Y647F or S863A alone or together was coexpressed with wnt11, suggesting that these sites are crucial mediators of convergence and extension movements in zebrafish.

Overexpression of XDs/h-ΔDIX or rhoa mRNA rescued the wnt11 phenotype, indicating that Dsh and RhoA act pervasively downstream of Slb/Wnt11 (10, 32). Our results demonstrated that XDs/h-ΔDIX and RhoA partially rescued the convergence and extension defects caused by Ror2 knockdown. It is possible that XDs/h-ΔDIX was derived from Xenopus; therefore, a lesser effect was observed. RhoA belongs to the Rho GTPase family of proteins, which have emerged as key mediators of the noncanonical Wnt signaling pathway. The three most well-studied members of this family are RhoA, Rac, and Cdc42. In Xenopus, RhoA and Rac operate in distinct signaling pathways that are integrated to control cell motility during convergent extension (53). Furthermore, cdc42 also mediates Xwnt-5a/Xror2 signaling to activate the JNK signaling pathway and thus regulates convergence and extension movements (17). The finding that XDs/h-ΔDIX and RhoA only partially rescues the Ror2 knockdown phenotype indicates that Ror2 convergence and extension movement regulation may be mediated by additional factors. Whether or not other proteins are involved in Ror2-mediated convergence and extension movement regulation needs to be investigated further.

Taken together, as illustrated in Fig. 8C, our results provide the following model: Wnt11 interacts with Ror2 via the important Ser-863 and Tyr-647 sites to at least partially activate Dsh and RhoA, which facilitates the regulation of convergent and extension movements during gastrulation in zebrafish. These results provide novel insights into convergence and extension movements in vertebrate gastrulation.

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