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STAT3 noncell-autonomously controls planar cell polarity during zebrafish convergence and extension

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Zebrafish signal transducer and activator of transcription 3 (STAT3) controls the cell movements during gastrulation. Here, we show that noncell-autonomous activity of STAT3 signaling in gastrula organizer cells controls the polarity of neighboring cells through Dishevelled-RhoA signaling in the Wnt-planar cell polarity (Wnt-PCP) pathway. In STAT3-depleted embryos, although all the known molecules in the Wnt-PCP pathway were expressed normally, the RhoA activity in lateral mesendodermal cells was down-regulated, resulting in severe cell polarization defects in convergence and extension movements identical to Strabismus-depleted embryos. Cell-autonomous activation of Wnt-PCP signaling by ΔN-dishevelled rescued the defect in cell elongation, but not the orientation of lateral mesendodermal cells in STAT3-depleted embryos. The defect in the orientation could be rescued by transplantation of shield cells having noncell-autonomous activity of STAT3 signaling. These results suggest that the cells undergoing convergence and extension movement may sense the gradient of signaling molecules, which are expressed in gastrula organizer by STAT3 and noncell-autonomously activate PCP signaling in neighboring cells during zebrafish gastrulation.

Introduction

Vertebrate gastrulation involves the fate specification and coordinated movement of three germ layers. Ample evidence links the Wnt-planar cell polarity (Wnt-PCP) pathway with regulation of the polarized cell morphology during the convergence and extension movements in gastrulation (Wallingford et al., 2002). The zebrafish Wnt-PCP–specific genes *strabismus* (*trilobite*) (Jessen et al., 2002; Park and Moon, 2002), *glypican* (*knypek*) (Topczewski et al., 2001), *wnt11* (*silberblick*) (Heisenberg et al., 2000), and *wnt5* (*pipetail*) (Rauch et al., 1997), the mutants of which exhibit shortened embryonic axes, are required for the establishment of mediolateral cell polarization that mediates the convergence and extension movements. However, it is not known what activates PCP signaling.

Several lines of evidence implicate signal transducer and activator of transcription (STAT) signaling in the establishment of cell polarity during zebrafish gastrulation and *Drosophila* eye development (Zeidler et al., 1999; Yamashita et al., 2002). We previously reported that the activity of STAT3 in the zebrafish gastrula organizer is noncell-autonomously essential for the convergence and extension movement of neighboring cells, and cell-autonomously for the anterior migration of organizer cells. Furthermore, we recently showed that Zinc transporter LIV1 is essential and sufficient for the cell-autonomous but not noncell-autonomous role of STAT3 (Yamashita et al., 2004). The noncell-autonomous requirement for STAT3 during zebrafish convergence and extension resembles the requirement for *Drosophila* STAT92E in the establishment of planar polarity during eye development, raising the possibility that PCP determination by STAT signaling may be conserved throughout evolution (Hou et al., 2002; Yamashita and Hirano, 2003). However, the molecular mechanisms of the noncell-autono-

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Key words: STAT3; Dishevelled; Rho; planar cell polarity; zebrafish

Abbreviations used in this paper: FRET, fluorescence resonance energy transfer; MO, morpholino oligonucleotide; PCP, planar cell polarity; STAT, signal transducer and activator of transcription.

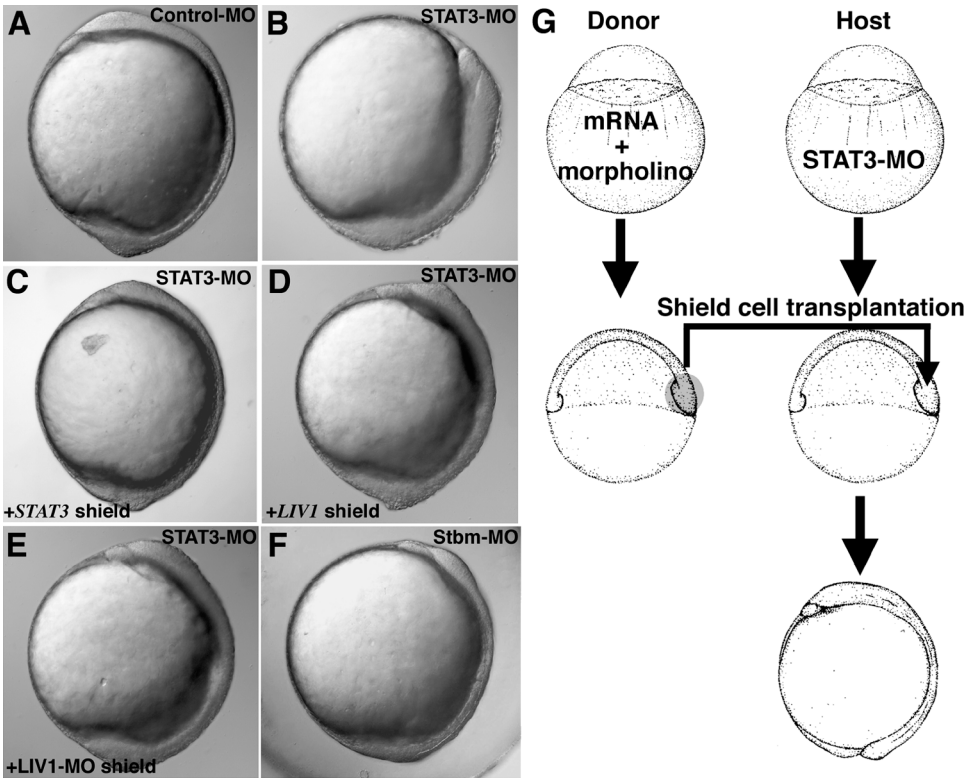

Figure 1. **Noncell-autonomous effect of STAT3 activity in organizer cells.** (A–F) Live zebrafish embryos at the one-somite stage. Animal pole is up, and dorsal is to the right. (A) STAT3D4-MO (10 ng) injected control embryos. (B) STAT3-MO (10 ng) injected embryos. (C) STAT3-MO (10 ng) injected embryos receiving shield transplantation from *STAT3* mRNA (100 pg) and STAT3-MO (10 ng) coinjected donor embryos. (D) STAT3-MO (10 ng) injected embryos receiving shield transplantation from *LIV1* mRNA (100 pg) and STAT3-MO (10 ng) coinjected donor embryos. (E) STAT3-MO (10 ng) injected embryos receiving shield transplantation from LIV1-MO (10 ng) injected donor embryos. (F) Strabismus-MO (5 ng) injected embryos. Each experiment was performed at least 10 times, and one experiment with typical results is shown. (G) Shield cells from mRNA and/or morpholino injected donors were transplanted into the shield region of a STAT3-MO injected host at the early gastrula stage.

mous action of STAT signaling are unknown for both zebrafish gastrulation and *Drosophila* eye development.

Here, we report that the lateral mesendodermal cells in the zebrafish embryos lacking STAT3 activities in gastrula organizer were defective in cell polarity and mobility during convergence and extension. Our results suggest that the downstream target of STAT3 in gastrula organizer cells may be an as-yet-unidentified secretory molecule that is able to noncell-autonomously activate Dishevelled-RhoA signaling in the PCP pathway in neighboring cells.

Results and discussion

To investigate the noncell-autonomous role of STAT3 during zebrafish convergence and extension, we analyzed the dorsal convergence movement of lateral mesendodermal cells in embryos lacking STAT3 activity using cell-tracing analysis (see Fig. 2 G). In the course of gastrulation, the lateral mesendodermal cells marked in the control embryos converged dorsally (Fig. 1 A and Fig. 2 A). In STAT3-morpholino oligonucleotide (MO)–injected embryos, convergence of the lateral mesendodermal cells toward the dorsal side was severely impaired; however, cell specification normally occurred (Fig. 1 B and Fig. 2 B; Yamashita et al., 2002). Transplantation of *STAT3* mRNA-expressing shield cells (shield cells derived from *STAT3* mRNA and STAT3-MO coinjected donor embryos; Fig. 1 G) rescues the defect in convergence and extension of STAT3-MO–injected embryos, confirming that these defects are caused by the absence of STAT3 activity in gastrula organizer cells (Fig. 1 C and Fig. 2 C). Consistent with this, transplantation of shield cells having cell-autonomous but not noncell-autonomous activity of STAT3 signaling (shield cells derived from *LIV1* mRNA and STAT3-MO coinjected donor embryos; Fig. 1 G) could not rescue the defect in convergence and extension (Fig. 1 D and Fig. 2 D), indicating that noncell-autonomous activity of STAT3 signaling in organizer cells was essential for dorsal convergence movement of lateral mesendodermal cells. A very similar convergence defect was observed in Strabismus-MO–injected embryos (Fig. 1 F and Fig. 2 F) (Sepich et al., 2000). Furthermore, transplantation of shield cells derived from LIV1-MO–injected donor embryos (Fig. 1 G), where cell-autonomous but not noncell-autonomous activity of STAT3 signaling was defective, showed normal convergence movement of lateral mesendodermal cells (Fig. 1 E and Fig. 2 E). All data showed that noncell-autonomous activity of STAT3 signaling controlled convergence and extension movements during gastrulation (Fig. 2 H).

To analyze these events more precisely, we next examined the migratory behavior of the lateral mesendodermal cells in the lateral blastoderm margin, 75° from the dorsal embryonic shield, in STAT3-MO–injected embryos or Strabismus-

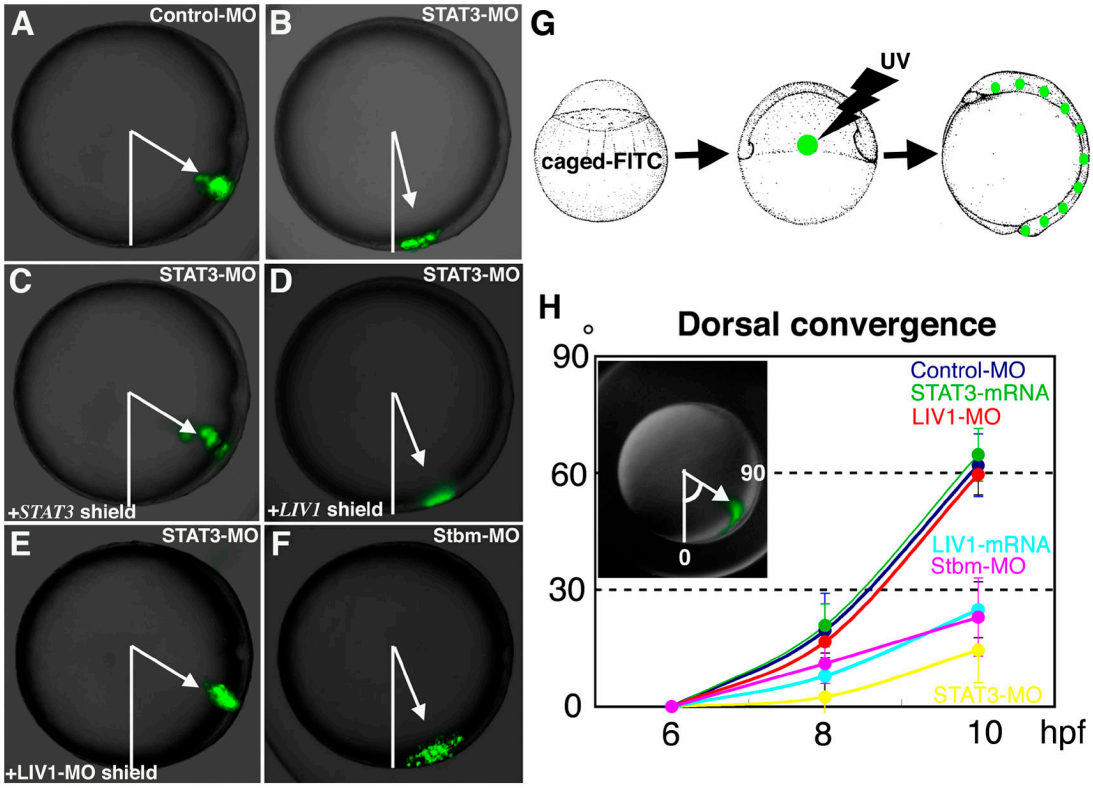

Figure 2. **Requirement of noncell-autonomous activity of STAT3 signaling for dorsal convergence movement.** (A–G) To monitor convergence movements, small groups of marginal cells located 90° from dorsal were labeled by UV-mediated activation of caged FITC at early gastrula. (A–F) Lateral mesendodermal cells that were labeled with FITC in the one-somite stage control embryos (A), STAT3-MO injected embryos (B), STAT3-MO injected embryos receiving shield transplantation from *STAT3* mRNA and STAT3-MO coinjected donor (C), STAT3-MO injected embryos receiving shield transplantation from *LIV1* mRNA and STAT3-MO coinjected donor (D), STAT3-MO injected embryos receiving shield transplantation from LIV1-MO injected donor embryos (E), and Strabismus-MO injected embryos (F). The animal pole view of the embryo is presented; dorsal is to the right. (H) Graph compares the dorsal convergence of labeled cell groups in the embryos shown in A (Control, blue), B (STAT3-MO, yellow), C (*STAT3*-mRNA, green), D (*LIV1*-mRNA, cyan), E (LIV1-MO, red), and F (Stbm-MO, magenta). Dorsal convergence was quantified by the position of the labeled cells at the indicated stage. Each experiment was performed 10 times, with the mean and SD shown in H; the results shown in A–F represent one experiment with typical results. Arrows in A–F indicate dorsal converging lateral mesendodermal cells.

MO–injected embryos. The lateral mesendodermal cells in the control-MO–injected embryos formed filopodia and lamellipodia toward the dorsal side, elongated mediolaterally, and underwent dorsal migration (Fig. 3 A). However, in STAT3-MO–injected embryos and Strabismus-MO–injected embryos, the lateral mesendodermal cells formed filopodia and lamellipodia in random directions and were defective in mediolateral elongation; as a result, dorsal migration of the lateral mesendodermal cells was severely perturbed (Fig. 3, B and F). The orientation and shape of the lateral mesendodermal cells highlighted the cell polarity defect associated with the loss of STAT3 or Strabismus function (Fig. 3, G–T). In the lateral region of the control mid-gastrula embryo, mesendodermal cells were elongated and the orientation of their long axes exhibited a strong mediolateral bias (Fig. 3, G and N). In contrast, the cells in the STAT3-MO–injected embryos were significantly less elongated (Fig. 3 H); the same result was seen in the Strabismus-MO–injected cells (Fig. 3 L). Moreover, little mediolateral bias in the orientation of the cells in the STAT3-MO–injected embryos and Strabismus-MO–injected embryos was detected (Fig. 3, O and S). Based on these observations, we propose that the disruption of polarized cell behavior is the basis for the defective convergence and extension movements in STAT3-depleted embryos as similar to Strabismus-depleted embryos.

To test the noncell-autonomy of STAT3 function in polarity determination during convergence and extension, we performed shield transplantations at the early gastrula stage and assessed the morphology of the host-derived lateral mesendodermal cells at mid-gastrulation. The lateral mesendodermal cells in the STAT3-MO–injected embryos were rounded and showed no mediolateral bias (Fig. 3, B, H, and O). However, in STAT3-MO–injected embryos receiving a shield transplant from normal donor embryos, or *STAT3* mRNA and STAT3-MO coinjected donor embryos, the lateral mesendodermal cells were elongated and mediolaterally aligned (Fig. 3, C, I, and P; unpublished data). The ability of the lateral mesendodermal cells in the STAT3-MO–injected embryos to behave normally after shield transplantation from a normal or STAT3-expressing donor indicates that STAT3 activity in the organizer cells functions noncell-autonomously in the establishment of polarity in neighboring cells during convergence and extension. To confirm this issue, we transplanted shield cells having only cell-autonomous activity of STAT3 into the shield region of STAT3-MO-injected embryos at the early gastrula stage as shown

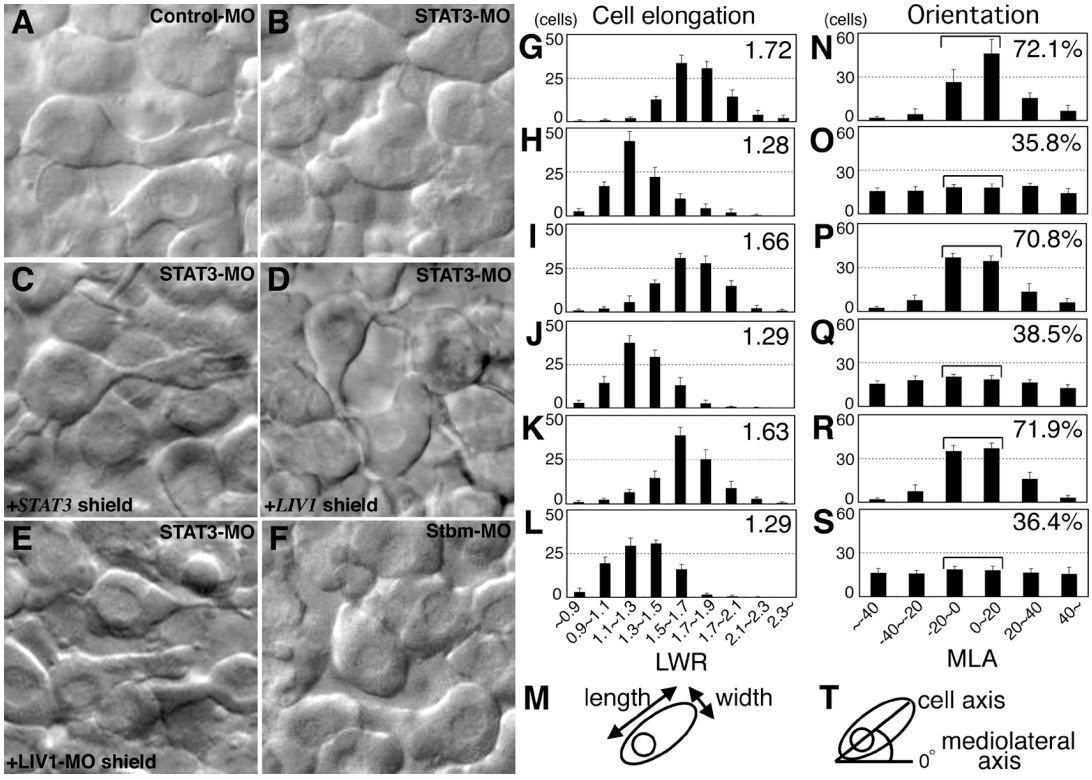

Figure 3. **Noncell-autonomous activity of STAT3 signaling is essential for the establishment of PCP during convergence and extension.** Morphology (A–F), elongation (G–M), and orientation (N–T) of lateral hypoblast cells in control embryos (A, G, and N), STAT3-MO injected embryos (B, H, and O), STAT3-MO injected embryos receiving shield transplantation from *STAT3* mRNA and STAT3-MO coinjected donor (C, I, and P), STAT3-MO injected embryos receiving shield transplantation from *LIV1* mRNA and STAT3-MO coinjected donor (D, J, and Q), STAT3-MO injected embryos receiving shield transplantation from LIV1-MO injected donor embryos (E, K, and R), and Strabismus-MO injected embryos (F, L, and S) at the 75% epiboly stage is shown; animal pole is up, and dorsal is to the right. Cell elongation is represented by measurement of the length to width ratio (G–M; LWR), and orientation is represented by measurement of the angle of the longitudinal cell axis relative to the medio-lateral embryonic axis (N–T; MLA). The numerical data in G–L and N–S indicate average LWR and percentage of medio-lateral aligned cells (MLA $< 20°$), respectively. Each experiment was performed 10 times, with the mean and SD shown in G–L and N–S; the results shown in A–F represent one experiment with typical results.

in Fig. 1 (D and G), and assessed the morphology of the host-derived lateral mesendodermal cells at mid-gastrulation. Shield cells having only cell-autonomous activity of STAT3 could not rescue the defect in cell polarity of the lateral mesendodermal cells in the STAT3-MO–injected embryos (Fig. 3, D, J, and Q). In contrast, transplantation of shield cells having only noncell-autonomous activity of STAT3 (Fig. 1, E and G) could rescue the defect in cell polarity of lateral mesendodermal cells of STAT3-MO–injected embryos (Fig. 3, E, K, and R). Together, these results indicated that the noncell-autonomous function of STAT3 in gastrula organizer is essential for the establishment of PCP in neighboring cells during convergence and extension. Next, we transplanted normal donor-derived shield cells into the shield region of Strabismus-MO–injected embryos at the early gastrula stage, and assessed the morphology of the host-derived lateral mesendodermal cells at mid-gastrulation. The defect in cell polarity of the lateral mesendodermal cells in Strabismus-MO–injected embryos was not rescued by the shield transplantation from a normal donor (unpublished data) in which STAT3 activity was intact, indicating that the noncell-autonomous function of STAT3 in polarity determination during convergence and extension requires Strabismus, which is an essential component of the Wnt-PCP pathway. Together with the similarity of the phenotype and cell behavior between STAT3-depleted embryos and Strabismus-depleted embryos, these results suggested that STAT3 activity in the gastrula organizer cells might control cell polarization during convergence and extension through the Wnt-PCP pathway in a noncell-autonomous manner.

The establishment of cell polarity is achieved through reorganization of the microtubule regulated by Rho GTPases, such as Rho, Rac and Cdc42. Rho GTPases can also modulate the actin cytoskeleton. Rho activity in migrating cells is associated with cell body contraction and rear end retraction. Rac and Cdc42 induce actin polymerization to generate lamellipodia and filopodia, respectively (Etienne-Manneville and Hall, 2002). The lateral mesendodermal cells in STAT3-MO-injected embryos formed filopodia and lamellipodia in random directions and were defective in mediolateral elongation, suggesting that activation of Rho, but not Rac and Cdc42, might be perturbed in lateral mesendodermal cells in the embryos lacking the activity of STAT3 in organizer cells. To test this, we next examined the spatiotemporal activity of RhoA in converging lateral mesendodermal cells using fluorescence resonance energy transfer (FRET)–based probes for RhoA, which consisted of truncated RhoA (aa 1–189), the RhoA-binding domain of PKN, and a pair

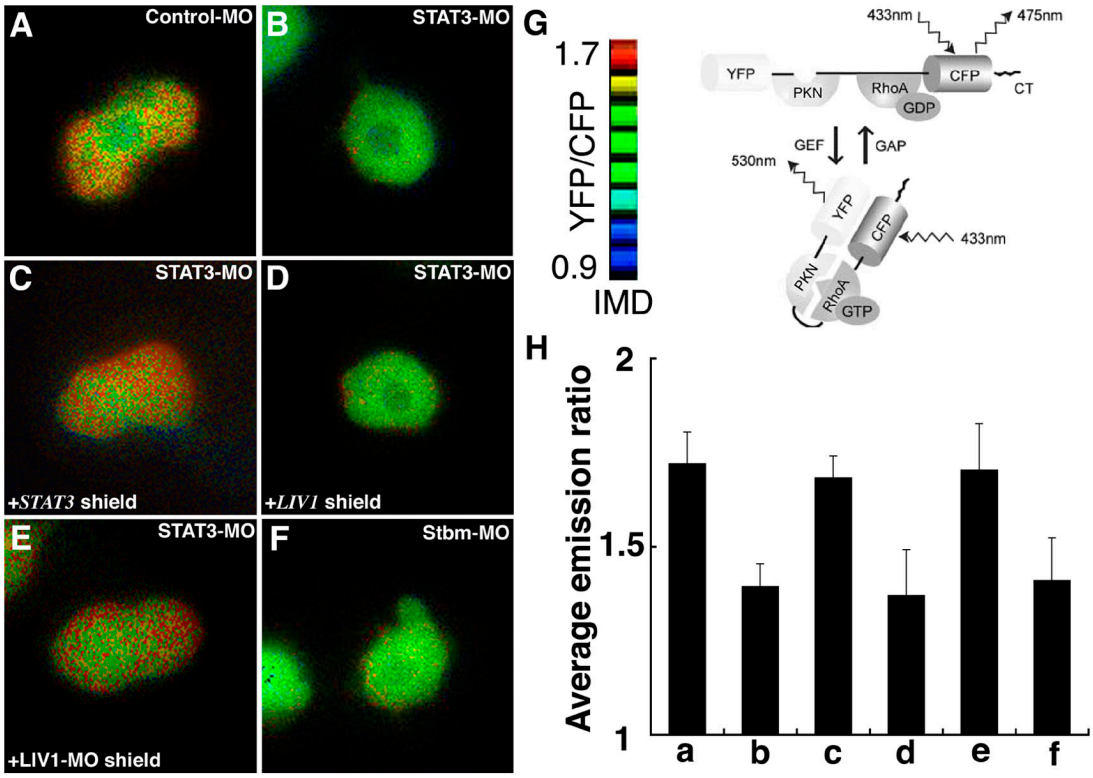

Figure 4. **Noncell-autonomous activity of STAT3 signaling is essential for the activation of RhoA to establish cell polarization during convergence and extension.** Lateral hypoblast cells expressing Raichu-RhoA of control embryos (A, a in H), STAT3-MO injected embryos (B, b in H), STAT3-MO injected embryos receiving shield transplantation from *STAT3* mRNA and STAT3-MO coinjected donor (C, c in H), STAT3-MO injected embryos receiving shield transplantation from *LIV1* mRNA and STAT3-MO coinjected donor (D, d in H), STAT3-MO injected embryos receiving shield transplantation from LIV1-MO injected donor embryos (E, e in H), and Strabismus-MO injected embryos (F, f in H); animal pole is up, and dorsal is to the right. During the mid-gastrula stage, CFP and YFP images were obtained every 2.5 min with a time-lapse microscope. The YFP/CFP ratio images were created to represent the FRET efficiency, which correlated with the RhoA activity, and are shown in intensity-modulated display (IMD) mode. Eight colors from red to blue are used to represent the YFP/CFP ratio from 0.9–1.7, with the intensity of each color indicating the mean intensity of YFP and CFP. Experiments were performed 10 times for each experiment, and similar results were obtained. (G), Schematic representations of Raichu-RhoA bound to GDP or GTP. (H) From the images in A–F, the net intensities of YFP and CFP in each cell were measured to calculate the average emission ratio.

of GFP mutants, YFP and CFP, as previously reported (Fig. 4 G) (Yoshizaki et al., 2003). We found that RhoA activation in the normally converging mesendodermal cells increased both toward the leading edge and toward the trailing edge (Fig. 4 A and H). No increase in FRET efficiency was observed at any protrusion by using a probe in which PKN was replaced by PAK as negative control (unpublished data). In STAT3-MO-injected embryos, which showed severe defect in cell polarization and dorsal convergence movement of lateral mesendodermal cells, the RhoA activity was severely perturbed (Fig. 4 B and H). The reduced RhoA activity and cell polarization defect in the converging lateral mesendodermal cells in STAT3-MO-injected embryos was rescued by shield transplantation from a normal donor, *STAT3* mRNA expressing donor, or LIV1-MO-injected donor (Fig. 4 C, E, H and unpublished data), but not from a *LIV1* mRNA and STAT3-MO coinjected donor embryos (Fig. 4 D and H), indicating that noncell-autonomous activity of STAT3 signaling in the organizer cells is essential for the activation of RhoA in neighboring cells to establish the PCP during convergence and extension. A similar defect in RhoA activation, cell polarization, and dorsal convergence movement, was observed in the Strabismus-MO-injected embryos (Fig. 4 F and H), indicating that the RhoA activity in these regions is associated with Wnt-PCP signaling. In addition, the reduced RhoA activity in lateral mesendodermal cells in Strabismus-MO-injected embryos was not rescued by shield transplantation from a normal donor (unpublished data). Together with the cell morphology analysis and the determination of RhoA activity in the converging lateral mesendodermal cells, these results suggest that STAT3 activity in the organizer cells is essential for the activation of RhoA associated with the Wnt-PCP signaling in neighboring cells, to determine the cell polarity in a noncell-autonomous manner.

To examine whether noncell-autonomous activity of STAT3 signaling activates Dishevelled-RhoA signaling in the Wnt-PCP pathway, we next examined the relationship between STAT3′s noncell-autonomous activity and Dishevelled, an intracellular modulator of the Wnt pathway (Fig. 5, A–F). An amino-terminally truncated form of Dishevelled (ΔN-Dishevelled) that contains the PDZ and DEP domains can transduce Wnt signals that regulate PCP (Heisenberg et al., 2000). As shown in Fig. 5, ΔN-Dishevelled could rescue the defect in the elongation of lateral mesendodermal cells in STAT3-depleted embryos (Fig. 5 B), but not in Strabismus-depleted embryos (Fig. 5 F), indicat-

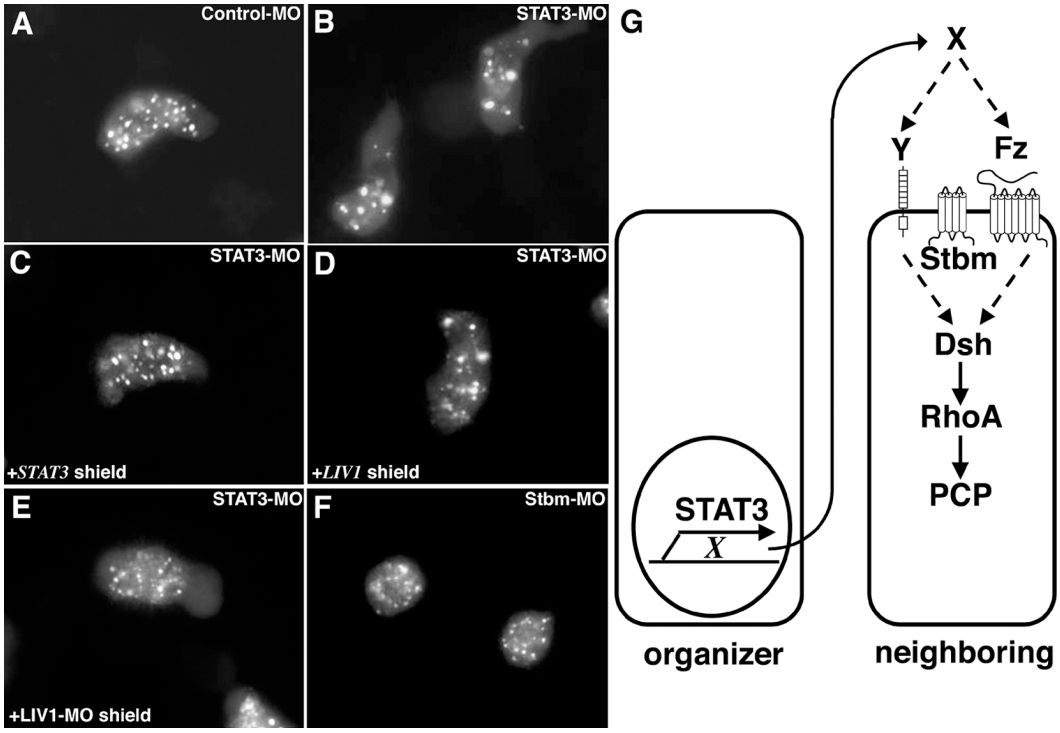

Figure 5. **Noncell-autonomous activity of STAT3 signaling is essential for the correct activation of PCP signaling through Dishevelled.** Lateral hypoblast cells expressing ΔN-Dishevelled-GFP in control embryos (A, LWR = 2.17, MLA = 70%, *n* = 100), STAT3-MO injected embryos (B, LWR = 2.31, MLA = 13%, *n* = 100), STAT3-MO injected embryos receiving shield transplantation from *STAT3* mRNA and STAT3-MO coinjected donor (C, LWR = 2.13, MLA = 72%, *n* = 100), STAT3-MO injected embryos receiving shield transplantation from *LIV1* mRNA and STAT3-MO coinjected donor (D, LWR = 2.05, MLA = 18%, *n* = 100), STAT3-MO injected embryos receiving shield transplantation from LIV1-MO injected donor embryos (E, LWR = 2.00, MLA = 73%, *n* = 100), and Strabismus-MO injected embryos (F, LWR = 1.17, MLA = 32%, *n* = 100); animal pole is up, and dorsal is to the right. Experiments were performed at least 10 times for each experiment, and similar results were obtained. (G), Model for the establishment of PCP during zebrafish convergence and extension by noncell-autonomous activity of STAT3 signaling. In gastrula organizer cells, STAT3 induces an as-yet-unidentified signaling molecule (X) that is able to noncell-autonomously activate Dishevelled-RhoA signaling in the PCP pathway in neighboring cells to establish the PCP. The converging neighboring cells may sense the extracellular gradient of signaling molecules through the receptor Frizzled (Fz), and translate this signal into the transmembrane protein Strabismus (Stbm), an intracellular modulator Dishevelled (Dsh), and its downstream effector RhoA. Alternatively, non-Frizzled receptors (Y) such as PTK7/CCK-4 may sense the gradient of signaling molecules, which are expressed in gastrula organizer by STAT3 signaling and noncell-autonomously activate PCP signaling in neighboring cells during zebrafish convergence and extension movements.

ing that Dishevelled acts permissively downstream of noncell-autonomous activity of STAT3 signaling. However, the direction of elongated cells is random, suggesting that the extracellular gradient of signaling molecules, which are expressed in gastrula organizer and activate Wnt-PCP signaling, is required for lateral mesendodermal cells to sense the direction of convergence movement. To investigate whether noncell-autonomous activity of STAT3 signaling in organizer cells can control the direction of convergence movement of lateral mesendodermal cells expressing ΔN-Dishevelled, we transplanted shield cells from *STAT3* mRNA expressing donor, *LIV1* mRNA expressing STAT3-depleted donor, or LIV1-MO-injected donor (Fig. 5 C, D and E). In the embryos receiving shield transplantation from *STAT3* mRNA expressing donor or LIV1-MO-injected donor, but not *LIV1* mRNA expressing STAT3-depleted donor, the elongated lateral mesendodermal cells expressing ΔN-Dishevelled were mediolaterally aligned, indicating that noncell-autonomous activity of STAT3 signaling in the organizer cells is required for the correct activation of the Wnt-PCP signaling and polarity establishment in neighboring cells.

Next, we tested whether STAT3 regulates the expression of the genes involved in the Wnt-PCP pathway. The expression of *dishevelled*, *strabismus*, *prickle*, *glypican*, and *frizzled* were normal in STAT3-MO–injected embryos (unpublished data), indicating that all the known cellular components of the Wnt-PCP pathway were intact in the STAT3-depleted embryos. Because STAT3 activates the Wnt-PCP signaling in a noncell-autonomous manner, we next examined the relationship between STAT3 activity and the extracellular secretory molecules acting in cell polarity determination during convergence and extension. The expression of *wnt11* and *wnt5* in STAT3-MO–injected embryos were normal during gastrulation (unpublished data), indicating that the expression of known extracellular secretory molecules for the Wnt-PCP pathway was independent of the STAT3 activity in organizer cells. Thus, despite the impaired Wnt-PCP signaling activity, all the molecules known to be involved in the Wnt-PCP pathway were expressed normally in the STAT3-depleted embryo.

Our analyses establish that STAT3 activity in organizer cells regulates cell polarization during zebrafish convergence and extension in a noncell-autonomous manner. Further-

more, our results suggest that a downstream target (Fig. 5 G, "X") of STAT3 in the gastrula organizer noncell-autonomously activates Wnt-PCP signaling in neighboring cells. Although it is not known if X functions through Frizzled and/or non-Frizzled receptors like PTK7/CCK-4 (Lu et al., 2004), our data suggest that the extracellular gradient of signaling molecules, which are expressed in gastrula organizer by STAT3 signaling, is required for lateral mesendodermal cells to sense the direction of convergence movement via the activation of Dishevelled-RhoA signaling associated with the Wnt-PCP pathway. Our data indicated that this signaling molecule is not Wnt11/Silberblick or Wnt5/Pipetail. In *Drosophila* eye morphogenesis, ommatidial polarity is determined nonautonomously by JAK/STAT signaling via the regulation of an unknown second signaling molecule (Strutt and Strutt, 1999). The ommatidia appear to sense the local gradient of the second signal and rotate accordingly. Our results raise the possibility that not only in the polarity determination of *Drosophila* ommatidia, but also in cell polarization during zebrafish convergence and extension, STAT signaling regulates the expression of a signaling molecule capable of activating the PCP pathway (Fig. 5 G).

Materials and methods

Morpholinos, mRNAs, and plasmids

Morpholinos and mRNA injections were performed essentially as described previously (Park and Moon, 2002; Yamashita et al., 2002, 2004). The cDNA sequence of NH_2 terminus–deleted *Xenopus* Dishevelled (178–736) (Heisenberg et al., 2000) was amplified by high fidelity PCR using specific oligonucleotide primers that created an EcoRI site at the 3′ end. The resulting PCR product was cloned into a unique EcoRI site located upstream of the ATG of the GFP gene in the pCS2 expression plasmid.

Cell tracing experiments and transplantation experiments

Cell tracing experiments and transplantation experiments were performed essentially as described previously (Yamashita et al., 2002).

Cell polarity analysis

Between the 75% epiboly and 90% epiboly stages, embryos were mounted in 1.0% methyl cellulose, and differential interference contrast images were collected using an upright microscope (AxioPlan-2; Carl Zeiss MicroImaging, Inc.) fitted with a CCD camera (CoolSNAP HQ; Roper Scientific), and were analyzed using MetaMorph software (Universal Imaging Corp.).

FRET analysis

The prototype of pRaichu-RhoA was described previously (Yoshizaki et al., 2003). We injected the modified version of pCS2+Raichu-RhoA (1294X) into zebrafish embryos at the one-cell stage. At the 75% epiboly stage, the lateral mesendodermal cells expressing Raichu-RhoA were imaged on an inverted microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) that was equipped with a CCD camera (CoolSNAP HQ; Roper Scientific), controlled by MetaMorph software (Universal Imaging Corp.). For the dual-emission ratio imaging of Raichu-RhoA, we used an XF88-2 CFP/YFP FRET filter set (Omega Optical, Inc.), and the ratio image of YFP/CFP was created with MetaMorph software after subtracting the background.

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