TGF-β signaling-mediated morphogenesis: modulation of cell adhesion via cadherin endocytosis

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The molecular mechanisms governing the cell behaviors underlying morphogenesis remain a major focus of research in both developmental biology and cancer biology. TGF-β ligands control cell fate specification via Smad-mediated signaling. However, their ability to guide cellular morphogenesis in a variety of biological contexts is poorly understood. We report on the discovery of a novel TGF-β signaling-mediated cellular morphogenesis occurring during vertebrate gastrulation. Activin/nodal members of the TGF-β superfamily induce the expression of two genes regulating cell adhesion during gastrulation: Fibronectin Leucine-rich Repeat Transmembrane 3 (FLRT3), a type I transmembrane protein containing extracellular leucine-rich repeats, and the small GTPase Rnd1. FLRT3 and Rnd1 interact physically and modulate cell adhesion during embryogenesis by controlling cell surface levels of cadherin through a dynamin-dependent endocytosis pathway. Our model suggests that cell adhesion can be dynamically regulated by sequestering cadherin through internalization, and subsequent redeploying internalized cadherin to the cell surface as needed. As numerous studies have linked aberrant expression of small GTPases, adhesion molecules such as cadherins, and TGF-β signaling to oncogenesis and metastasis, it is tempting to speculate that this FLRT3/Rnd1/cadherin pathway might also control cell behavior and morphogenesis in adult tissue homeostasis.

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During gastrulation, the three germ layers (ectoderm, mesoderm, and endoderm) establish new contacts, permitting new inductive interactions to specify the development of organ primordia. Some of the major movements that constitute gastrulation are convergence and extension, involution, and epiboly. The dynamic nature of cell–cell contacts in tissues undergoing these movements has been visualized using time-lapse video microscopy. Cells involved in convergence and extension movements are shown to continuously break and remake local adhesive contacts, via polarized membranous processes (e.g., lamellipodia), as they “slide” past one another during mediolateral intercalation [Davidson et al. 2002; Shook et al. 2004].

Distinct adhesive properties conferred by adhesion molecules constitute a key feature of cells that undergo gastrulation movements. Type I cadherins are required for proper morphogenesis in sea urchin [Miller and McClay 1997], zebrafish [Montero et al. 2005; Shimizu et al. 2005], and mouse embryos [Rietmacher et al. 1995]. Inactivation of C-cadherin, the primary mediator of adhesion in the Xenopus blastula, leads to both involution and convergent extension defects during gastrulation [Heasman et al. 1994]. By changing the functional activity of C-cadherin at the cell surface, the morphogenetic elongation of “animal cap” ectodermal explants (mimicking the convergence and extension movements of the embryonic mesoderm) is altered [Brieher and Gumbiner 1994].

The Wnt signaling network has been implicated in the regulation of cell polarization and mediolateral intercalation in both Xenopus and zebrafish convergence and extension [Heisenberg et al. 2000; Sokol 2000; Wallingford et al. 2000]. Manipulation of components of a Wnt
signaling pathway (e.g., Fz7, Silberblick/Wnt11, Disexvelled, Prickle, and Strabismus) has effects on cell polarity and disrupts convergence and extension of the mesoderm while not perturbing cell fate. Activin/nodal signaling regulates gastrulation and subsequent morphogenesis and cell movements. Mice deficient in the nodal gene arrest at early gastrulation and form no mesoderm (Zhou et al. 1993). Nodal inhibition perturbs normal gastrulation in zebrafish and *Xenopus* (Feldman et al. 1998), while stimulation of activin/nodal signaling in *Xenopus* animal cap ectoderm leads to cell movements that mimic convergence and extension (Asashima et al. 1990; Smith et al. 1990). Previous work has shown that these ectodermal cells, when challenged with activin, downregulate C-cadherin “activity” [Lee and Gumbiner 1995] and affect convergence and extension movements. At present, how activin/nodal signaling affects cadherin activity remains a mystery.

In order to better understand the mechanism underlying TGF-β-regulated morphogenesis, we conducted a DNA microarray screen to look for potential activin/nodal target genes with key roles in gastrulation. Two molecules caught our attention: Rnd1, a small GTPase previously identified in an expression screen as a molecule that displays strong anti-adhesive activity [Nobes et al. 1998; Wunnenberg-Stapleton et al. 1999], and FLRT3, a member of the Fibronectin Leucine-rich Repeat Transmembrane family of proteins [Lacy et al. 1999]. FLRT3 was also recently shown to modulate cell adhesion [Karaulanov et al. 2006] and fibroblast growth factor [FGF] signaling by physically interacting with the FGF receptor via its fibronectin [FN] type III domain [Bottcher et al. 2004]. In the process of uncovering FLRT3’s function, we made the following observations: First, FLRT3 and Rnd1 are coexpressed in the involuting equatorial cells of gastrula-stage embryos, and these molecules physically and functionally interact. Second, overexpression of FLRT3 or Rnd1 reduces cadherin-mediated cell adhesion, whereas loss of FLRT3 or Rnd1 increases cadherin-mediated cell adhesion. Third, embryos depleted of Rnd1 display significant defects during gastrulation and Rnd1 is required for FLRT3-mediated cell adhesion effect. Lastly, the changes in cell adhesion via FLRT3/Rnd1 are regulated by controlling the availability of C-cadherin protein on the cell surface, which is dependent on dynamin-mediated endocytosis. We propose that activin/nodal members of the TGF-β superfamily induce expression of FLRT3 and Rnd1 to permit cell movements within a tissue while not sacrificing tissue integrity.

Results

**FLRT3 is a direct activin/nodal target expressed in involuting mesodermal cells**

To understand how activin/nodal signaling functions in early development, we conducted a DNA microarray screen to identify genes directly regulated by signaling. Briefly, ectodermal explants (animal caps) dissected at blastula stage were treated with cycloheximide to block ongoing protein synthesis [Cho et al. 1991]. These animal caps were then stimulated with activin to induce transcription of direct targets [without activating further downstream gene expression]. Total RNAs extracted from activin-treated and untreated control caps were subjected to DNA microarray analysis using our 42,000 homemade cDNA chips (Shin et al. 2005). We found that FLRT3, encoding a potential transmembrane signaling or adhesion molecule, was reproducibly induced to high levels along with several dozen other direct activin target genes (our unpublished observations).

Whole-mount in situ analysis showed high FLRT3 expression in the forming dorsal blastopore lip of early gastrulae [Fig. 1A]. As gastrulation proceeds, FLRT3 expression gradually surrounds the entire equatorial zone, coinciding with the progression of formation of the lateral and ventral blastopore [Fig. 1B], and the expression is confined to the deep mesoderm [Fig. 1E]. Expression around the blastopore circumference persists until the blastopore is completely closed at late neurula stage [Bottcher et al. 2004; data not shown]. Overall, the expression of FLRT3 appears to coincide with the movement and rearrangement of marginal zone cells during gastrulation. As we identified FLRT3 in a screen for direct targets of activin/nodal signaling, we sought to verify that FLRT3 is indeed regulated by this pathway.

Overexpression of Cerberus-S, a truncated form of the Cerberus protein that specifically inhibits nodal signaling [Piccolo et al. 1999], inhibited FLRT3 expression in the equatorial zone [Fig. 1G]. Furthermore, we confirmed FLRT3’s status as an immediate early activin target gene by RT–PCR from animal caps [Fig. 1H]. *Xenopus* homologs of the other mammalian FLRT family members [Lacy et al. 1999], namely FLRT1 and FLRT2, were also isolated by database homology searches [Supplementary Fig. 1]. Neither of these genes are direct activin targets [Supplementary Fig. 2; Bottcher et al. 2004]. FLRT1 is maternally expressed and the expression persists during early development. FLRT2 is expressed strongly at neurula stages.

**FLRT3 overexpression causes cell death independent of FGF signaling**

FLRT3 overexpression in the marginal zone causes tissue protrusions in the tailbud-stage embryo [Bottcher et al. 2004]. When FLRT3 was ectopically expressed in the animal hemisphere, the blastocoel roof was one cell layer thick, while normally this tissue is three to four cell layers thick at this stage [Fig. 1I,J]. Histological sections of embryos microinjected with FLRT3 together with RNA encoding the lineage tracer β-galactosidase revealed that FLRT3-overexpressing cells [Fig. 1I,J, blue] had lost the ability to adhere to neighboring cells and had fallen into the blastocoel cavity. Similar results were obtained when FLRT1 or FLRT2 were overexpressed [data not shown].

As FLRT3 was shown to interact with FGF receptors [Bottcher et al. 2004], we determined whether this cell
deadhesion was due to its modulation of FGF signaling. Based on the following experiments, we conclude that FLRT3’s effect is not due to a perturbation of FGF signaling. First, expression of a dominant-negative FGF receptor at a concentration sufficient to inhibit FGF signaling failed to abrogate the cell dissociation phenotype induced by FLRT3 overexpression (Fig. 1J–L). Second, treatment of FLRT3-injected animal caps or embryos with the drug SU5402, a well-studied FGF receptor inhibitor (Mohammadi et al. 1997), failed to rescue the cell deadhesion of animal cap cells in isolated explants or in intact embryos (data not shown). Third, microinjection of basic FGF mRNA into two-cell stage Xenopus embryos did not affect the cell dissociation activity mediated by FLRT3 (data not shown).

Since the FGF receptor interaction domain of FLRT3 was previously mapped to an extracellular FN type III domain of FLRT3 (Bottcher et al. 2004), we also sought to determine whether this domain of FLRT3 was dispensable for its influence on cell adhesion. To map the required domains of FLRT3, we generated both FLRT3 proteins lacking extra- or intracellular domains and also fusion proteins constructs between FLRT3 and LIG1 (Supplementary Fig. 3A). LIG1 is a single-pass transmembrane protein possessing 10 leucine-rich repeats, which show 28% identity (50% similarity) to FLRT3 (Suzuki et al. 1996). Importantly, overexpression of wild-type LIG1 itself does not cause loss of cell adhesion and no other obvious phenotype was observed (data not shown). Using a construct comprising the N-terminal extracellular domain of LIG1 fused to the transmembrane and C-terminal cytoplasmic domains of FLRT3 (LIG1EC–FLRT3CP), we found that the cytoplasmic domain of FLRT3 is necessary for the disruption of cell adhesion (Supplementary Fig. 3C). The cytoplasmic domain of FLRT3 adjoining the transmembrane domain alone was sufficient for the disruption of cell adhesion (Supplementary Fig. 3E). Since the cytoplasmic domain of FLRT3 is responsible for the disruption of cell adhesion, but the extracellular FN type III domain is dispensable, we conclude that FLRT3 regulates cell adhesion in a manner independent from its role in FGF signaling.

FLRT3 functions upstream of Rnd1
The loss of cell adhesion by FLRT3 overexpression mimics the loss of cell adhesion we previously observed following overexpression of Rnd1, a small GTPase (Wunnenberg-Stapleton et al. 1999) that is induced by activin, albeit weakly (data not shown). Furthermore, compar-
sons of the expression patterns of these two genes by whole-mount in situ hybridization revealed that FLRT3’s expression in the forming blastopore lip is similar to that of Rnd1 (Fig. 1, cf. A,B,E and C,D,F). These similarities in overexpression phenotypes and expression patterns suggest that FLRT3 and Rnd1 might function together, and therefore we tested the hypothesis that these molecules might act synergistically. Low doses of FLRT3 (30 pg) or Rnd1 (30 pg, 100 pg) mRNAs are incapable of inducing cellular deadhesion (Fig. 2A,B; data not shown). However, coinjection of these low doses of FLRT3 and Rnd1 mRNAs together showed a strong enhancement of cellular detachment, suggesting that these molecules work together to affect cell adhesion.

Next, we examined the epistatic relationship between these two proteins. Inhibition of Rnd1 translation by microinjection of a Rnd1 morpholino antisense oligonucleotide (MO) into the equatorial zone of the four-cell stage embryo caused aberrant gastrulation movements (Fig. 2C,D). In normal embryos, at the beginning of gastrulation, the leading endomesodermal cells involute and move anteriorly under the ectoderm. However, when Rnd1 MO is injected at high concentrations, the cells fail to crawl under the ectoderm (Fig. 2D). Consequently, Rnd1 MO-injected embryos fail to gastrulate (arrest) and eventually die (data not shown). At lower concentrations of Rnd1 MO, the anterior migration of dorsal mesodermal cells is significantly retarded, resulting in bent embryos (Fig. 3; Supplementary Fig. 5B). These effects were rescued by coinjection of Rnd1 mRNA lacking a sequence complementary to the MO (Supplementary Fig. 5C). If Rnd1 is required for FLRT3-mediated cell detachment in animal caps, knockdown of Rnd1 expression should block the detachment phenotype. Since Rnd1 is expressed in the animal pole of gastrula-stage embryos, in addition to its normal expression in the mesoderm (Wunnenberg-Stapleton et al. 1999), Rnd1 MO was injected animally together with FLRT3 mRNA. Cell deadhesion induced by FLRT3 was efficient, but not completely, blocked when Rnd1 expression was inhibited (Fig. 2F,G). The partial rescue is probably due to the inability of Rnd1 MO to completely inhibit endogenous Rnd1 expression. Interestingly, a
constitutively active version of RhoA (XrhoA-V14) that antagonizes Rnd1 activity (Wunnenberg-Stapleton et al. 1999) restored adhesion upon FLRT3 overexpression (Fig. 2H), indicating that FLRT3-mediated cell adhesion activity can be cross-regulated by small GTPases. This experiment also demonstrates that the cell dissociation phenotypes resulting from overexpression of FLRT3 and Rnd1 are not associated with nonspecific “toxicity” because the overexpression phenotypes were rescued by additional overexpression of RhoA. We conclude that Rnd1 functions downstream from FLRT3 and is a mediator of FLRT3-mediated cell detachment activity.

FLRT3 and Rnd1 physically and functionally interact

We previously showed that Rnd1 acts at the cell membrane, and that this membrane association is essential for the disruption of cell adhesion in Xenopus embryos (Wunnenberg-Stapleton et al. 1999). As FLRT3 is a transmembrane protein and exerts its function upstream of Rnd1 as demonstrated above, we examined whether there might be a direct physical interaction between these two proteins. Coprecipitation analysis was performed using a GST protein fusion to the cytoplasmic domain of FLRT3, together with either in vitro translated Rnd1, RhoA, Rac, or Cdc42, as well as constitutively active forms of RhoA and Rac (Peppenlenbosch et al. 1995). Among these, only Rnd1 was successfully co-precipitated (Fig. 2I; data not shown). Converse experiments using GST-Rnd1 and GST-RhoA fusion proteins were not possible due to an inability to isolate a full-length GST-Rnd1 fusion protein from bacterial lysates. However, coprecipitation assays using embryonic extracts overexpressing both FLRT3-ΔLRR and various small GTPases showed an identical specificity (Supplementary Fig. 4).

In order to define the region of FLRT3 responsible for Rnd1 interaction, several basic amino acid sequences within the cytoplasmic domain of FLRT3 were mutated. We chose this approach since Rac GTPases interact with the basic amino acids of PAK (p21-activated kinase) (Knaus et al. 1998). Mutation of two adjacent lysine residues in the most conserved region of the cytoplasmic domain of FLRT3s (FLRT3 amino acids 580–581) (Supplementary Fig. S1) produced FLRT3-KK. This change inactivated FLRT3’s ability to cause cell detachment (data not shown) and, at the same time, significantly weakened the physical interaction between FLRT3 and Rnd1 (Fig. 2J). From these observations we conclude that FLRT3 and Rnd1 interact physically, and that this interaction is important for their function in cell detachment.

Figure 3. Rnd1 and FLRT1/3 loss-of-function phenotypes. In situ hybridization of Bra (A–F), gsc (G–I), and Xnot (M–R) in Xenopus mid-gastrula-stage embryos. A–R are dorsal external views, and A’–R’ are cross-sections bisecting through Spemann’s organizer. Note the lack of notochord extension in the mid-line of Rnd1-injected [12 ng] and FLRT1/3 MO-injected [30 ng each] embryos.
Loss of Rnd1 and FLRT3 affects convergence and extension movements in embryos

We examined FLRT3 and Rnd1 loss-of-function phenotypes in *Xenopus* gastrulae. Rnd1 MO-injected embryos displayed noticeable defects. In normal embryos, endomesodermal cells invaginate and crawl along the roof of blastocoel [Fig. 2C]. However, in embryos injected with high concentrations of the Rnd1 MO (10–12 ng per embryo), invaginating tissue often failed to move properly, as if it had lost directional cues [Fig. 2D]. At lower concentrations of Rnd1 MO [e.g., 6 ng], the involution and migration of the dorsal mesoderm occurs along the roof of blastocoel. However, the anterior migration of the dorsal mesodermal cells are significantly retarded (see below), resulting in bent embryos (75%) [Supplementary Fig. 5B]. Both of these phenotypes were fully rescued by coinjection of a Rnd1 rescue mRNA [Fig. 2E, Supplementary Fig. 5C].

In order to better understand the nature of the defects, whole-mount in situ hybridization was performed using *Brachyury* (*Bta*, circumblastoporal mesoderm and notochord), *goosecoid* (*gsc*, prechordal plate mesoderm), and *Xnot* (notochord) markers [Cho et al. 1991; Smith et al. 1991], von Dassow et al. 1993). Embryos were injected with Rnd1 MO at the four-cell stage and fixed at various stages of gastrulation. Whole-mount in situ hybridization of *Bta* and *Xnot* revealed that their expression domains were significantly altered in the absence of Rnd1. In Rnd1 MO-injected embryos, notochord fails to elongate properly and becomes confined near the closing dorsal blastopore lip of embryos [Fig. 3, cf. A and B, M and N]. Importantly, the overall expression levels of *Bta* and *Xnot* are unchanged between control uninjected and Rnd1 MO-injected embryos [Supplementary Fig. 6], suggesting that Rnd1’s effects are on convergence and extension movements, and not on cell fate specification. We also examined the expression of *gsc*, which normally resides anteriorly to the notochord [Fig. 3G,H]. In Rnd1-deficient embryos, *gsc* is still expressed anteriorly to the *Xnot* expression domain, and the expression domain of *gsc* is as broad as that of the wild type [Fig. 3, cf. G,G’ and H,H’]. The overall expression levels of *gsc* are also unchanged between uninjected and Rnd1 MO-injected embryos [Supplementary Fig. 6]. The above deficient phenotypes of Rnd1 MO-injected embryos were rescued by coinjection of rescue Rnd1 mRNA. Based on these data, we conclude that Rnd1 regulates convergence and extension movements of dorsal marginal cells, but does not affect the induction of mesoderm.

Injection of FLRT1, FLRT2, and FLRT3 MOs alone or in combination caused delays in blastopore closure when compared with control MO-injected embryos, but the embryos were still able to gastrulate (data not shown). Interestingly, a combination of FLRT1 and FLRT3 MO injection was particularly effective as the delay was more significant and resulted in bent embryos [Supplementary Fig. 5E], similar to the phenotype of low-dose Rnd1 MO injection. However, injection of a combination of FLRT1 and FLRT3 was unable to completely block gastrulation. Based on these results, we propose that in addition to FLRTs other proteins may be involved in regulating gastrulation movements. Alternatively, our MOs are ineffective in completely abolishing expression of FLRTs in early embryos.

We next examined the contribution of FLRT1 and FLRT3 to gastrulation movements since these molecules seem to have additive effects [Supplementary Fig. 5D,E]. As shown in Figure 3, inhibition of both FLRT1 and FLRT3 affected notochord elongation as revealed by changes in *Xnot* and *Bra* expression patterns [Fig. 3E,F,G,H,Q]. Interestingly, we found that FLRT1/3 MO-induced defects could be only rescued by coinjection of both FLRT1 and FLRT3 rescue mRNA [Fig. 3F,R], but not by either FLRT3 or FLRT1 rescue mRNA alone (data not shown). This suggests that FLRT1 and FLRT3 have nonredundant functions.

Potential genetic interactions between FLRT3 and Rnd1 were also examined by coinjecting FLRT3 MO together with Rnd1 MO. Our previous experiment suggested that injection of low concentrations of Rnd1 MO [5–6 ng] caused mild gastrulation defects as examined by the morphological defects and the expression patterns of *Bta*, *gsc*, and *Xnot* [Supplementary Fig. 5B,H,L,P]. We then coinjected FLRT3 MO together with Rnd1 MO, and found that the convergence extension of notochord was affected as revealed by changes in *Bta*, *gsc*, and *Xnot* expression patterns [Supplementary Fig. 5I,M,Q]. This suggests that FLRT3 and Rnd1 work in the same pathway.

Loss of Rnd1 and FLRT3 affects elongation of animal caps

Next, MO loss-of-function analyses were further characterized using animal caps [Fig. 4]. FLRT3 is very weakly expressed in animal cap ectoderm, but is induced after exposure to activin protein [Fig. 1G,H]. We hypothesized that, if FLRT3 mediates activin-induced morphogenetic movements of animal caps (mimicking its role in the marginal zone), in the absence of FLRT3 animal caps would not be able to elongate. Indeed, elongation of animal caps was strongly (but not completely) inhibited by FLRT3 MO [Fig. 4A–C] and the elongation was restored upon coinjection of a FLRT3 rescue mRNA [Fig. 4D]. Control MO did not block activin-induced animal cap elongation [Fig. 4F; data not shown], suggesting that it is the expression of FLRT3 that is critical for activin-mediated animal cap elongation. The FLRT3 MO did not alter the expression of various activin-induced mesodermal markers in animal caps [Fig. 4G]. A similar experiment was performed using the Rnd1 MO, and it also blocked activin-induced animal cap elongation [Fig. 4H,I] and was rescued by injection of a Rnd1 rescue mRNA [Fig. 4J]. We also examined the relationship between FLRT3 and Rnd1. The FLRT3 MO blocked activin-mediated elongation of animal caps, and this effect was rescued by expressing a Rnd1 rescue mRNA [Fig. 4I]. On the other hand, injection of a Rnd1 MO blocked animal cap elongation [Fig. 4I], but this phenotype was
not rescued by FLRT3 overexpression (Fig. 4K). These results are a further confirmation of our previous data (in Fig. 2), suggesting that FLRT3 functions upstream of Rnd1.

**FLRT3 and Rnd1 regulate cadherin-mediated cell adhesion**

Overexpression of either FLRT3 or Rnd1 leads to loss of cell adhesion in animal caps, but the mechanism of action in this process is unclear. Cadherins and integrins (together with FN) are two major components involved in adhesion between cells in *Xenopus* early embryos. To determine how FLRT3/Rnd1 modulates cell adhesion, we assessed the ability of FLRT3- and Rnd1-overexpressing cells to directly interact with either purified cadherin or FN. Dissociated animal cap cells were allowed to adhere to glass slides coated with either the cadherin extracellular domain or FN. Slides were then gently agitated to wash off loosely attached cells, and firmly attached cells were counted (Reintsch and Hausen 2001). While various cadherins are known to display different effects in vivo, in an in vitro attachment assay such as the one utilized here, the extracellular domains of all type I cadherins seem to function similarly [Niessen and Gumbiner 2002].

We found that cells from FLRT3- or Rnd1 mRNA-injected embryos failed to adhere to the cadherin substrate, while uninjected control or FLRT3-KK-injected animal cap cells adhered tightly (Fig. 5A,B). The adhesion of animal cap cells to the cadherin substrate is specifically mediated by cadherin interaction because preincubation of the cadherin substrate with anti-E-cadherin monoclonal antibody [DECMA-1] efficiently blocks the adhesion of animal caps to the substrate (Fig. 5C). We also found that the FLRT3/Rnd1-mediated deadhesion activity could also be rescued by cadherin overexpression (Fig. 5B), consistent with the notion that cadherin functions downstream from FLRT3/Rnd1. Next, we examined whether FLRT3 and Rnd1 are required for activin-induced changes in cadherin-mediated adhesion. Figure 5D shows that activin-treated cells injected with FLRT3 MO or Rnd1 MO bind strongly to the cadherin substrate, indicating that FLRT3 and Rnd1 mediate activin signals controlling cadherin-mediated adhesion. We note that control, FLRT3-injected, and Rnd1-injected animal cap cells all adhered to the FN substrate to the same extent (Fig. 5A), demonstrating that the reduced attachment to the cadherin substrate seen upon FLRT3 mRNA microinjection is specific. Unlike FLRT3- or Rnd1-overexpressed cells, activin-treated animal cap cells became more adherent to the FN substrate, confirming the observations of others (Howard and Smith 1993; Reintsch and Hausen 2001). As overexpression of FLRT3/Rnd1 had no impact on FN-mediated adhesion, activin-mediated enhancement of FN-mediated adhesion is likely regulated by a mechanism independent of FLRT3 and Rnd1.

We performed Rnd1 and FLRT1/3 loss-of-function experiments to examine the role of endogenous levels of these molecules in dorsal marginal zone cells. If nodal signaling increases the transcription of FLRT3 and Rnd1, which in turn reduces cadherin-mediated cell adhesion in the mesoderm of gastrulating embryos to promote cell rearrangements, inhibition of FLRT3 and Rnd1 activity should enhance cadherin interactions. Dorsal marginal explants from embryos injected with FLRT MOs or Rnd1 MO were dissociated into single cells and assayed for their adhesion properties. MO-injected (FLRT3 MO alone, FLRT1/3 MOs, and Rnd1 MO) cells showed not only increased affinity toward the cadherin substrate, but also increased affinity for each other as MO-injected cells coalesced into large aggregates (Fig. 5F,G).
shown). We also note that, while FLRT3 MO alone was able to increase animal cap cell adhesion to the cadherin substrate, FLRT1/3 MOs were more effective than FLRT3 MO alone (data not shown). Control MO did not change cellular affinity toward cadherin (Fig. 5H). MO activity was also fully reversed by coinjection of corresponding rescue mRNAs (Fig. 5I,J). From these results we conclude that FLRT1/3 and Rnd1 specifically affect cadherin-mediated cell adhesion of mesodermal cells.

Cadherin subcellular localization is influenced by FLRT3/Rnd1

How is cadherin-mediated cell adhesion regulated by FLRT3 and Rnd1? We first examined whether ectopic FLRT3 or Rnd1 expression reduced the overall expression level of cadherin proteins. Western blot analysis showed that expression levels of C-cadherin protein were unchanged between control and FLRT3-expressing samples (Fig. 6A), suggesting that the change in cadherin-mediated adhesion is not due to an expression difference. We next examined whether differences in cell surface presentation of cadherin protein could account for the observed changes in adhesion. Total amounts of C-cadherin on the cell surface were analyzed by measuring the amount of C-cadherin accessible to trypsin digestion on wild-type FLRT3-, FLRT3-KK-, or Rnd1-expressing and uninjected animal cap cells (Fig. 6B, lanes 1–4). For instance, if C-cadherin is internalized upon FLRT3 or Rnd1 expression, we expect the internalized cadherin protein to be resistant toward trypsin digestion (Fig. 6B, arrow), while cadherin exposed on the cell surface would be cleaved by trypsin, yielding a 100-kDa polypeptide (Fig. 6B, arrowhead). The amount of C-cadherin present on the cell surface was reduced significantly in FLRT3- and Rnd1-expressing cells (Fig. 6B, lanes 1,3) when compared with that of control or FLRT3-KK (Fig. 6B, lanes 2,4). Since total C-cadherin levels are unchanged (Fig. 6B, lanes 5–8), the latter results imply that a FLRT3–Rnd1 pathway elicits a change in subcellular localization of cadherin proteins. In order to examine whether the FLRT3/Rnd1 effect is cadherin specific, we examined the internalization of integrin after stripping and reprobing.
Figure 6. Expression of FLRT3 and Rnd1 promotes cadherin internalization. (A) C-cadherin expression in FLRT3-expressing cells. Western blot analysis was performed using extracts obtained from uninjected and FLRT3 mRNA-injected animal cap cells. (B) Expression of C-cadherin in animal cap explants before and after trypsin treatment. An arrow indicates native C-cadherin protein, a closed arrowhead indicates processed C-cadherin, and an open arrowhead indicates β-tubulin. The full-length integrin-β1 protein is indicated with a single asterisk (*), while the processed integrin-β1 is indicated with double asterisks (**) (C–H) Immunofluorescent staining of C-cadherin in activin-treated or untreated animal cap explants. C-cadherin (in red) is predominantly localized at the cell membrane, while the explants treated with activin show an increase of cytoplasmic cadherin (shown in C,D). (E,G) Inhibition of FLRT3 or Rnd1 expression blocks the internalization of C-cadherin even after an activin treatment. (F,H) Overexpression of FLRT3 or Rnd1 reverts the localization of C-cadherin to the cytoplasm. DAPI staining is in blue. (I) Expression of C-cadherin in deep cells of the dorsal marginal zone before and after a trypsin treatment. An arrow indicates native C-cadherin protein, and a closed arrowhead indicates processed C-cadherin. (J) Schematic diagram of a mid-gastrula-stage embryo. A boxed area in red represents the magnified regions in K–M. (K–M) Subcellular localization of C-cadherin in the organizer of a wild-type, a Rnd1 MO-injected (10 ng per embryo), and a FLRT1/3 MO-injected (30 ng each per embryo) gastrula-stage embryo (stage 11). (K) In the wild-type embryo, C-cadherin staining is predominantly on the cell surface of peripheral cells, but not in the internal cells. The boundary between the peripheral layer and the internal layer is indicated with a dotted line, and the tip of the cleft is marked with an asterisk (*). Arrowheads indicate interstitial spaces between cells.
the same Western blot. The expression of integrin is unchanged under the conditions in which C-cadherin was internalized (Fig. 6B), suggesting that the internalization of cadherin after activin treatment is specific.

We also confirmed these results by directly examining changes in cadherin subcellular localization using immunofluorescence microscopy. Animal caps were treated with activin in the presence or absence of injected FLRT3 or Rnd1 MOs, and analyzed after incubation with antibodies specific for C-cadherin. C-cadherin was predominantly localized on the cell surface of control animal cap cells [Fig. 6C], but became enriched in the cytoplasm after an activin challenge [Fig. 6D]. Importantly, C-cadherin remained on the cell surface of activin-treated cells when FLRT3 or Rnd1 expression was inhibited by MOs [Fig. 6E,G]. Furthermore, the C-cadherin localization was again found in the cytoplasm after coinjection of FLRT3 or Rnd1 rescue mRNAs with the MOs [Fig. 6F,H], confirming the results of the trypsin experiment. Thus, we conclude that FLRT3 and Rnd1 regulate cell adhesion in the gastrula via modulation of the subcellular distribution of cadherin protein.

Regulated cadherin subcellular localization in Spemann’s organizer

We next investigated the contribution of endogenous levels of FLRTs/Rnd1 to C-cadherin subcellular localization in the blastopore region of Xenopus gastrulae. Deep involuting cells were isolated from the organizer of early gastrula-stage embryos and trypsin digestion was performed to determine the subcellular localization of C-cadherin in the tissue fragments. In wild-type embryos, a noticeable amount of C-cadherin was protected from trypsin digestion, whereas in a Rnd1 MO-injected sample, most of C-cadherin was digested to the shorter 100-kDa polypeptide [Fig. 6l, lanes 1,2]. Immunofluorescence analysis [Fig. 6K,L] also revealed that C-cadherin was predominantly localized on the cell surface of prospective ectoderm and preinvoluting mesoderm (“peripheral” cells). However, in the deep “internal” [involuted] cells, the cell surface expression of C-cadherin was less apparent, and increased interstitial spaces between cells were more noticeable [Figs. 6K, 7C]. Interestingly, in Rnd1 MO- or FLRT1/3 MO-injected embryos, C-cadherin expression in the internal cells became localized on the cell surface, and cells appeared to be more tightly attached to each other [Fig. 6L,M], suggesting that the decreased abundance of cell surface C-cadherin on involuting mesodermal cells [Fig. 6K] is dependent on Rnd1 and FLRT1/3. We also performed a similar experiment using FLRT3 MO alone and obtained the similar result [data not shown], albeit weaker.

C-cadherin is internalized by endocytosis

In order to further uncover the mechanism by which FLRT3/Rnd1 regulate cadherin subcellular localization, we examined the involvement of endocytosis. Rab5 is a small GTPase localized to early endosomes. It regulates the fusion between endocytic vesicles and endosomes as well as homotypic fusion between endosomes [Olkonen and Stenmark 1997]. We examined whether C-cadherin polypeptide could associate with Rab5. Rab5 was tagged with the red fluorescent protein mCherry and coexpressed with EGFP-tagged C-cadherin in Xenopus animal cap cells. Animal cap explants were dissociated into single cells and subjected to confocal immunofluorescence microscopy [Fig. 7A]. In control uninjected animal cap cells, C-cadherin-EGFP was predominantly localized on the cell surface, while Rab5-mCherry was mostly found in the cytoplasm. In contrast, FLRT3-expressing cells showed distinct punctae where colocalization of C-cadherin and Rab5 was detected in the cytoplasm, suggesting that internalized C-cadherin is part of the endosome associated with Rab5.

In order to provide direct evidence that internalization of cadherin is dependent on endocytosis, we blocked the function of dynamin, a critical molecule involved in endocytosis. While an increase in the trypsin-resistant form of C-cadherin was detected in FLRT3-overexpressing cells [Fig. 7B, lane 2], this internalization of cadherin was blocked by dominant-negative dynamin [Fig. 7B, lane 3]. Consistent with this observation, when dominant-negative dynamin is expressed in the dorsal marginal region, C-cadherin became predominantly localized to the cell surface [cf. Fig. 7C,D]. From these results we conclude that subcellular localization of cadherin in the involuting marginal cells is regulated by dynamin-mediated endocytosis, and this process is up-regulated by FLRT3 and Rnd1.

Discussion

Activin/nodal-induced changes in cell adhesion

Cells undergoing morphogenetic changes dynamically alter their cell-adhesive properties. During gastrulation in amniote embryos, the primitive streak undergoes a process of epithelium–mesenchymal transition (EMT) allowing delaminating cells to form the mesoderm. Epithelial cells acquire the property of mesenchymal cells, become motile, and leave the epithelium. In amphibian embryos, dorsal marginal cells at gastrula stages undergo convergence and extension movements, in which cells undergo local cell rearrangements involving the continual breaking and remaking of adhesive contacts [Davidson et al. 2002; Shook et al. 2004]. In these examples cells participating in morphogenesis not only must remain attached to each other to maintain tissue integrity, but also must dynamically disassemble and reassemble adhesion complexes to “slide” past one another.

Cadherin-based cell adhesion controls cell movements and the creation of tissue architecture during development. Studies have shown that compaction of blastomeres in the early mouse embryo is associated with the activation of E-cadherin, and cells of the mammalian epiblast require down-regulation of E-cadherin to migrate through the streak [Winkel et al. 1990; Burdsal et
Xenopus animal cap explants treated with activin/nodal members of the TGF-β superfamily go through convergence and extension-driven elongation movements characteristic of gastrulation (Asashima et al. 1990; Smith et al. 1990). This morphogenetic change is accompanied by changes in two different cell-adhesive activities. Activin/nodal induces both a reduction in C-cadherin activity and an increase in FN-adhesive activity at the cell surface, implying that activin/nodal signaling alters either the expression, subcellular localization, or biochemical activities of these adhesion molecules (Zhong et al. 1999).

FLRT3/Rnd1-mediated cell adhesion signaling

We report on the discovery of an activin/nodal-mediated morphogenetic signaling pathway [activin/nodal → FLRT3 → Rnd1 → cadherin] based on the following observations: First, there is a close link between the dynamic expression patterns of both FLRT3 and Rnd1 with the propagation of nodal signaling in the gastrula. Both FLRT3 and Rnd1 are initially detected dorsally, then proceed ventrally across the embryo during gastrulation similar to the changing activity of nodal signaling itself (Lee et al. 2001; Schohl and Fagotto 2002; Hashimoto-Partyka et al. 2003). Second, both gain- and loss-of-function of FLRT3 and Rnd1 in animal caps showed similar phenotypes in cell adhesion. Third, various epistasis experiments demonstrate that Rnd1 functions downstream from FLRT3. Fourth, immunoprecipitation assays suggest that FLRT3 and Rnd1 physically interact to regulate cell adhesion, and that a FLRT3 mutation [FLRT3-KK] that loses the ability to interact with Rnd1 correlates with abrogation of its cell deadhesion activity. Lastly, both biochemical and immunofluorescence data showed that FLRT3 and Rnd1 affect endocytosis of C-cadherin, which in turn regulates the availability of C-cadherin on the cell surface to control cell adhesion.
Our last result differs from that of Brieher and Gumbiner (1994) who have shown that activin-induced changes in C-cadherin-mediated adhesion were not accompanied by noticeable changes in the amount of cadherins at the cell surface. Doses of activin used for these experiments may have contributed to the differences. In our case, embryos were injected with activin mRNA at the two-cell stage and the trypsin assay was performed 6–8 h after the injection, while Brieher and Gumbiner (1994) treated cells isolated from blastula-stage embryos with activin for 60 min, followed by 90 min of cell aggregation before trypsin digestion. Higher-dose activin treatment permits observation of global deadhesion that does not occur at lower doses. In vivo, these high doses are not required, as involuting marginal zone cells do not require complete deadhesion but only a loosening of contacts. We suggest that activin/nodal signaling induces FRLT3 and Rnd1, which then participate in reducing cadherin protein levels from the cell surface to permit cell/tissue movements that require weaker cell–cell contacts. Both our trypsin digestion and immunofluorescence data are consistent with the notion that only cells that have rounded the lip (and express FLRT3/Rnd1) have reduced cell surface cadherin.

Conservation of a FLRT3–Rnd1 pathway in vertebrates

Based on our RT–PCR and whole-mount in situ hybridization analyses, FLRT3 is the only FLRT-related gene expressed highly in the marginal mesodermal cells. However, we note that FLRT1, FLRT2, and FLRT3 all have indistinguishable cell deadhesion activities when they are overexpressed [data not shown]. Our current data also suggest that FLRT1 participates in regulating cadherin adhesion together with FLRT3, and that the activity of FLRT1 is somewhat distinct from that of FLRT3 as FLRT1- and FLRT3-depleted embryos cannot be rescued by simply providing FLRT1 or FLRT3 rescue mRNA alone. In the future, it would be important to distinguish the activity differences between FLRT1 and FLRT3.

We hypothesize that FLRT3 and Rnd1 physically interact and affect C-cadherin subcellular localization. However, the direct physical interaction is based on in vitro and in vivo overexpression data, and further work is needed to demonstrate this interaction using FLRT3- and Rnd1-specific antibodies in the future. We propose that FLRT3–Rnd1 pathway represents a conserved morphogenetic pathway regulated by activin/nodal signaling in vertebrates. Consistent with this notion, a zebrafish homolog of FLRT3 is expressed in the embryonic shield and equatorial zone during gastrulation, very similar to its expression around the blastopore lip in Xenopus [T. Schilling and K.W.Y. Cho, unpubl.]. Moreover, injection of a MO against zebrafish OEP (one-eye pinhead, a nodal coreceptor) successfully blocked FLRT3 expression. FLRT3 and Rnd1 are also expressed in other vertebrates, including man. Interestingly, human Rnd1 [also known as Rho6] causes loss of cell adhesion in mammalian cell lines when it is overexpressed [Nobes et al. 1998], and loss of Rnd1 affects growth cone retraction behavior [Oinuma et al. 2004]. In Xenopus, overexpression of Rnd1 causes cell deadhesion and Rnd1 loss-of-function disrupts gastrulation movements [Wunnenberg-Stephenson et al. 1997, the current study]. While these observations suggest that the FLRT3/Rnd1 pathway is likely to be conserved among vertebrates, further experiments are necessary to confirm the model.

It is currently unclear whether this pathway is conserved among more evolutionarily distant deuterostomes: The homologs of FLRT3 and Rnd1 cannot be easily identified in the existing databases, while activin/nodal signaling is known to be involved in regulating gastrulation in deuterostome embryos from sea urchins to mammals [Flowers et al. 2004]. Whether activin/nodal-mediated morphogenesis in these more distant deuterostomes is regulated by a FLRT3/Rnd1 pathway, or by a related pathway, is an important future question.

Mechanisms regulating and coordinating adhesive activity

The small GTPases Rac, Rho, and Cdc42 have been implicated in cadherin-mediated adhesion [Kaihara et al. 1999]. Cdc42 affects the state of cell contacts at the level of the association of β-catenin with the cadherin complex [Kuroda et al. 1998; Fukata et al. 1999]. Overexpression of constitutively active Rac results in the accumulation of E-cadherin at the regions of contact between epithelial cells [Braga et al. 1997], and activated Rac increases E-cadherin-mediated adhesion [Hordijk et al. 1997]. Here, we show that small GTPase Rnd1 alters cadherin-mediated cell adhesion by affecting the subcellular localization of C-cadherin. Controlling the amount of cadherin on the cell surface may be achieved by regulating the balance between endocytosis and intracellular transport processes. Cadherins may be cleared from the cell surface when adhesion must be reduced or broken by endocytosis. Then, the internalized cadherins could be redeployed by a recycling pathway to sites where new contacts are being formed. Consistent with this idea, we find that C-cadherin is internalized and associated with Rab5-expressing endosomes. Endocytosis of cadherin was also observed to accompany an epithelial-to-mesenchymal transformation during sea urchin gastrulation [Miller and McClay 1997]. Additionally, an intracellular pool of E-cadherin was shown to be recruited to the cell surface upon cell–cell contact in epithelial cells, suggesting an involvement of the trafficking pathway for the delivery of E-cadherin to the cell surface [McNeill et al. 1993; Adams et al. 1996].

What triggers the endocytosis of cadherin molecules? One plausible mechanism is that internalization of cadherin by FLRT3 and Rnd1 is regulated by controlling cadherin ubiquitination, similar to the ubiquitin-mediated control of cadherin endocytosis upon the stimulation of the intracellular tyrosine kinase Src [Fujita et al. 2002]. It is tempting to speculate that a ubiquitination
pathway might be playing a similar role during gastrulation, and our future experiments will explore the possibility.

Coordinating gastrulation movements in developing embryos is complex. Many signaling molecules are activated or inhibited at various stages of gastrulation, and these signaling events need to be precisely orchestrated in concert with changing signaling activities. In this regard, it is interesting to note that both FLRT3 and Rnd1 are mediator to other signaling molecules implicated in gastrulation. For instance, Rnd1 is antagonized by RhoA, a mediator of a noncanonical Wnt signaling pathway involved in planar cell polarity [Wunnenberg- Stapleton et al. 1999]. Rnd1 was also shown to interact directly with FRS2α and FRS3, which are docking proteins of FGF receptors [Harada et al. 2005]. In addition, FLRT3 was previously shown to participate in FGF signaling [Bottcher et al. 2004]. Together with these observations, we propose that the mechanisms mediated by FLRT3 and Rnd1 may represent a point of signal integration at which a component of activin/nodal morphogenetic signaling interacts intracellularly with Wnt and FGF signaling components to coordinate complex gastrulation movements.

Materials and methods

Embryological manipulations and whole-mount in situ hybridization

Embryo manipulations, microinjections, and whole-mount in situ hybridizations were as described previously [Cho et al. 1991; Blitz et al. 2000]. Animal cap elongation assays were performed in the presence of 5 ng/mL of recombinant human activin A [R&D Systems].

Antibodies

Monoclonal antibody 6B6 and rabbit polyclonal antibodies against Xenopus C-cadherin are from Dr. B. Gumbiner [University of Virginia, Charlottesville, VA]. Monoclonal antibody E7 against β-tubulin and 8C8 against integrin-β1 were from the Developmental Studies Hybridoma Bank. Monoclonal antibody DECMA-1 against E-cadherin was purchased from Sigma. Rabbit anti-FLRT3 antibodies were generated using GST-FLRT3CP fusion protein. Affinity-purified anti-FLRT3CP antibodies were obtained after depleting anti-GST antibodies using GST affinity column followed by affinity purification using GST-FLRT3CP-coupled column.

Plasmids

Xenopus FLRT3 (XL050c15 and XL106c18), Rac [XL048c18], Cdc42 [XL010d05], LIG1 [XL087m13], Rab5 [XL042g12], and βFGF [XL094k18] cDNAs were isolated from an arrayed Xenopus neurula cDNA library [http://Xenopus.nibb.ac.jp]. A full-length Xenopus FLRT1 cDNA [IMAGE #7020577, GenBank accession no. BC075290] was from American Type Culture Collection. FLRT3 fragments containing Met-Ser(1–649) and Met-Val(1–551) were subcloned to generate FLRT3(ΔWT) and FLRT3EC constructs, respectively. Two cDNA fragments containing Met-Ser(1–54) and Thr-Ser(51–649) were sequentially subcloned to generate a FLRT3-ΔLRR construct. LIG1EC-FLRT3CP was generated by replacing the Met-Ser(1–54) portion of FLRT3-ΔLRR with a fragment encoding Met-Gln(1–501) of LIG1 cDNA. BM40SP-FLRT3CP was generated by fusing BM40 signal peptide in frame with FLRT3CP. GST-FLRT3CP(KK) was generated by mutating the two lysine residues at amino acid position 580 and 581 using a PCR-based mutagenesis method. To generate a FLRT3 rescue construct [V5-FLRT3], a cDNA fragment encoding Lys–Ser(29–649) of FLRT3 was subcloned into pCS2 + V5, which contains the mouse kremlin2 signal peptide and V5 tag sequences. To generate a FLRT1 rescue construct [V5-FLRT1], a cDNA fragment encoding Thr–Thr(59–682) of FLRT1 was subcloned into pCS2 + V5. A Rnd1 rescue construct [HA-Rnd1] was generated by subcloning 3× HA tags in frame with the 5′ end of the Rnd1 ORF. To generate C-cadherin-EGFP construct, the full-length ORF of C-cadherin and EGFP were sequentially subcloned into pcDNA3.1. Xenopus Rab5a cDNA was cloned into pCS2 + mCherry plasmid [a kind gift of Dr. Roger Tsien [University of California at San Diego, La Jolla, CA], and Drs. Trevor Hoffman and Thomas Schilling [University of California at Irvine, Irvine, CA)] to generate mCherry-Rab5.

DNA microarray

DNA microarray experiments were performed as described previously [Shin et al. 2005], except that intact animal caps were treated with activin in the presence and absence of cycloheximide [Cho et al. 1991].

RT–PCR analysis and morpholino antisense oligonucleotides

RT–PCR primers are described in Supplementary Figure 5. The following MOs were generated: Xenopus FLRT3-a, 5′-AAATT CCAAGTGTCGTAATGCTAGG-3′; FLRT3-b, 5′-ATTCAGAG TTTCTGTAGACTTTGTATCTTGT GGGCTGTTTATCC-3′; FLRT1, 5′-TCGCCATCTTG TGTGTCTGCTGCTC-3′; Rnd1, 5′-TTGGTTCCT TCTGTAGACATGGTC-3′. For FLRT3 MO injection experiments, equal amounts of FLRT3-a and FLRT3-b MOs were mixed and microinjected into the animal pole region of two-cell stage embryos or to the entire marginal zone at four-cell stage embryos.

Cell adhesion assays

Cell adhesion assays were described previously [Reintsch and Hausern 2000], except that human E-cadherin Fc chimera was used instead of CEC fragment. Fibronectin and E-cadherin were purchased from Sigma. To study the adhesion of cells derived from the organizer explants to cadherin-coated surface, 10 organizer explants were isolated from gastrula-stage [stage 10] embryos. The explants were dissociated in CMFM [Cho et al. 1991] for 45 min and seeded on the cadherin-coated plates containing regular 1× MBS medium. After 2 h of incubation, cells were visualized under a microscope.

Western blot and immunoprecipitation assays

For in vivo immunoprecipitation assays, indicated mRNAs were microinjected into two-cell stage embryos. Whole embryos at stage 11 or animal cap explants from stage 9 embryos were homogenized in homogenization buffer [Fagotto and Gumbiner 1994] supplemented with 1% Triton X-100 and complete protease inhibitor cocktail [Roche]. The extracts were preincubated with protein G-Sepharose and the cleared lysates were incubated with anti-HA Sepharose beads to precipitate HA-tagged small GTPases. The precipitates were washed and subjected to SDS-PAGE, followed by a Western blot analysis using ECL Reagent Western Blotting System (Amersham).
to trypsin treatment [0.01%] for 30 min at room temperature. Subsequently, soybean trypsin inhibitor (Sigma) was added to the extracts and centrifuged. The resulting lysates were subjected to a SDS-PAGE analysis.

GST pulldown assays were performed as described previously (Ring et al. 2002). GST-FLRT3CP or FLRT3CP(KK) fusion protein was purified using glutathione beads and incubated with the indicated in vitro translated products.

**Immunofluorescence**

Animal caps or whole embryos were fixed in PBS containing 3.7% formaldehyde (PBS + FA) for 2 h, washed, and incubated in Dent's fixative. The samples were rehydrated in a graded series of methanol/PBS treatment. Embryos were bisected through the blastopore and refixed in PBS + FA for 30 min. The rehydrated animal cap explants or hemisphereod embryos were washed in PBT [PBS plus 0.1% Triton X-100, 0.2% BSA], followed by pre-incubation in PBT containing 20% goat serum, and incubation in PBT containing rabbit anti-C-cadherin pAb (1:200). The samples were washed extensively and incubated in PBT containing 20% goat serum, and incubation in PBT containing rabbit anti-C-cadherin pAb (1:200). The samples were washed extensively and incubated in PBT containing biotin-conjugated goat anti-rabbit IgG (1:100), followed by washing and incubation in PBT containing Texas-Red streptavidin (1:400, Vector Laboratories) overnight at 4°C. After further incubation in PBT containing 20% goat serum, the samples were fixed with PBS + FA, dehydrated in a graded series of ethanol wash, and embedded in Technovit 7100 glycolmethacrylate resin (Kulzer). Sectioned embryos or tissues (5 μm) were mounted on glass slides, counter-stained with DAPI, and examined under an inverted fluorescence microscope.

Further details can be obtained under “Dissection of Organizer and Animal Pole from Xenopus laevis” at http://www.jove.com/Details.htm?ID=188&VID=151.

**Confocal microscopy**

Coverwell imaging chambers (Electron Microscopy Sciences) were coated with 1× MBS medium supplemented with 4% BSA for 1 h. Injected and dissociated animal cap cells were suspended in regular 1× MBS medium, seeded onto the BSA-coated chamber, and sealed with a coverslip. Confocal images were taken with Zeiss LSM510 confocal microscopy system.

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TGF-β signaling-mediated morphogenesis: modulation of cell adhesion via cadherin endocytosis

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