Development 135, 931-940 (2008) doi:10.1242/dev.011940

Ephrin signaling establishes asymmetric cell fates in an endomesoderm lineage of the *Ciona* embryo

Weiyang Shi* and Mike Levine

Mesodermal tissues arise from diverse cell lineages and molecular strategies in the *Ciona* embryo. For example, the notochord and mesenchyme are induced by FGF/MAPK signaling, whereas the tail muscles are specified autonomously by the localized determinant, Macho-1. A unique mesoderm lineage, the trunk lateral cells, develop from a single pair of endomesoderm cells, the A6.3 blastomeres, which form part of the anterior endoderm, hematopoietic mesoderm and muscle derivatives. MAPK signaling is active in the endoderm descendants of A6.3, but is absent from the mesoderm lineage. Inhibition of MAPK signaling results in expanded expression of mesoderm marker genes and loss of endoderm markers, whereas ectopic MAPK activation produces the opposite phenotype: the transformation of mesoderm into endoderm. Evidence is presented that a specific Ephrin signaling molecule, *Ci-ephrin-Ad*, is required to establish asymmetric MAPK signaling in the endomesoderm. Reducing *Ci-ephrin-Ad* activity via morpholino injection results in ectopic MAPK signaling and conversion of the mesoderm lineage into endoderm. Conversely, misexpression of *Ci-ephrin-Ad* in the endoderm induces ectopic activation of mesodermal marker genes. These results extend recent observations regarding the role of Ephrin signaling in the establishment of asymmetric cell fates in the *Ciona* notochord and neural tube.

KEY WORDS: *Ciona*, Ephrin, MAPK, Endomesoderm

INTRODUCTION

It has been proposed that the mesoderm evolved from the ancient endoderm of diploblastic ancestors (Ball et al., 2004; Martindale et al., 2004). Indeed, portions of the mesoderm arise from a bipotential cell type, the endomesoderm, in a variety of metazoan embryos (Rodaway and Patient, 2001). For example, in *C. elegans*, the endomesoderm blastomere (EMS) is polarized by a Wnt/MAPK signal that inhibits mesoderm fate in the posterior E lineage (Maduro, 2006), whereas the anterior blastomere (MS) forms mesoderm by default. In sea urchins, Notch signaling promotes secondary mesenchyme cell formation while repressing endoderm fate (Sherwood and McClay, 1999). Heart, blood and some muscle tissues are derived from endomesoderm precursors at the marginal zone of zebrafish and frog embryos (Rodaway et al., 1999; Kimelman and Griffin, 2000; Mathieu et al., 2004).

In this study, we examine the basis for distinct endoderm and mesoderm derivatives arising from the A6.3 endomesoderm cell in the early embryo of *Ciona intestinalis*, a simple chordate. A6.3 divides at the 32-cell stage to form A7.5 and A7.6 daughter cells. A7.6 gives rise to the embryonic trunk lateral cells (TLCs), which generate blood cells and additional mesoderm derivatives during metamorphosis (Tokuoka et al., 2005). Its sibling cell, A7.5, gives rise to anterior gut endoderm. Blastomere isolation experiments suggest that the A7.6 mesoderm fate is induced by a signal emanating from ectodermal cells in the animal hemisphere (Kawaminani and Nishida, 1997). Here, evidence is presented that *Ci-ephrin-Ad* is essential for the asymmetric cell fates arising from A6.3 through local inhibition of MAPK signaling. We discuss these findings with regard to the recent demonstration that Ephrin signaling is also required for the asymmetric specification of the notochord and nerve cord (Picco et al., 2007).

Department of Molecular and Cell Biology, Division of Genetics, Genomics and Development, Center for Integrative Genomics, University of California, Berkeley, CA 94720, USA.

*Author for correspondence (e-mail: wshi@berkeley.edu)

Accepted 9 December 2007

MATERIALS AND METHODS

Embryology

Adults were collected from Half Moon Bay Harbor, California, or obtained from M-Rep (San Diego, CA) and maintained in artificial seawater (Crystal Sea Marine mix) at 18°C under constant illumination. Fertilization, dechorionation and electroporation were performed as previously described (Corbo et al., 1997a). For microinjection, morpholino oligos or mRNA was co-injected with 1 mM Texas-Red Dextran 10 kDa (Molecular Probes) and only fluorescent embryos were analyzed. For MAPK inhibition assays, embryos were grown in seawater supplemented with 2 μM U0126 (Promega). For cell division arrest experiments, seawater was supplemented with cytochalasin B to a final concentration of 2 μg/ml.

In situ hybridization and immunohistochemistry

Embryos were fixed in 4% paraformaldehyde, 0.5 M NaCl, 0.1 M MOPS pH 7.5, 2 mM $MgSO_4$, 1 mM EDTA at 4°C overnight. In situ hybridization was performed as described previously (Corbo et al., 1997b). Digoxigenin-labeled antisense RNA probes were synthesized from the following cDNA clones: *Ci-Hand-like* (citb018l16, *Ciona intestinalis* Gene Collection Release 1), *Ci-FGF8/17/18* (citb002j04), *Ci-Delta-like* (cieg005o22), *Ci-MyTF* (citb040p06), *Ci-Brachyury* (Corbo et al., 1997a), *Ci-ephrin-Ad* (ciad008n17), *Ci-TTF-1* (ciad042d09), *Ci-Snail* (cibd020p17). Alkaline phosphatase (AP) enzyme assays were performed as follows. Fixed embryos were rinsed three times in PBS, 0.1% Tween and stained in AP staining buffer (Whittaker and Meedel, 1989); staining was enhanced using 10% polyvinyl alcohol. For immunohistochemistry, rehydrated embryos were treated with 0.3% H_2O_2 for 30 minutes to quench endogenous peroxidase activity, blocked for endogenous biotin (SP-2001, Vector Labs, Burlingame, CA) and then incubated overnight in mouse anti-dpERK (1:1000, Sigma, M9692). dpERK was visualized with the VECTASTAIN Elite ABC System (Vector Labs).

Molecular biology

A constitutively active form of the Ciona FGF receptor (Ci-FGFR) was created by fusing the intracellular kinase domain of Ci-FGFR (the last 437 amino acid residues) to the first 455 amino acids from the *Drosophila torso*^{4021} mutant. The *torso*^{4021} peptide includes the extracellular and transmembrane domain, and results in the constitutive activation of the RTK receptor (Sprenger and Nusslein-Volhard, 1992). The *torso-FGFR* fusion gene was cloned into the pCS2+ vector, and capped mRNA was synthesized using the mMESSAGE mMACHINE SP6 Kit (Ambion, Austin, TX). RNA

was microinjected into 1-cell embryos at a final concentration of 0.5 μg/μl. The torso-FGFR fusion gene was also attached to ~2 kb of the *ZicL* 5′ flanking regulatory sequence, which was amplified from genomic DNA using the following primers: forward, 5′-CATATTGTAAGGTGGAGATG-3′ and reverse, 5′-TGCATAGGGACGATCAACC-3′. The resulting fusion gene is expressed in the progenitors of the notochord and nerve cord.

The entire ORF of *Ci-ephrin-Ad* was amplified from cDNA clone ciad008n17. A ~4 kb upstream regulatory sequence from the *FoxD* gene was cloned using the following primers: forward, 5′-GAAAC-GATCTTCGGCGGATC-3′ and reverse, 5′-ATATTGCACACAACACT-GCAC-3′. The resulting DNA fragment was cloned into the pCESA vector (Harafuji et al., 2002) to drive *Ci-ephrin-Ad* throughout the vegetal hemisphere. A *Ci-ephrin-Ad* morpholino (5′-GGTAGTAGGTAAATT-GAGTTGCCAT-3′) (Picco et al., 2007) was purchased from GeneTools LLC (Eugene, OR) and injected at 0.5 mM.

RESULTS

The A6.3 blastomere of 32-cell embryos is a bona fide endomesoderm cell. It divides to produce two daughter cells that form derivatives of the mesoderm (A7.6) and endoderm (A7.5) (summarized in Fig. 1C,D). Here we investigate the molecular mechanisms that distinguish A7.6 and A7.5 fate. As shown previously, the A7.6 blastomere expresses a number of transcription factors and cell signaling molecules, including *Hand-like*, *FGF8/17/18* (abbreviated to *FGF8*), *Delta-like* and *MyTF* (Imai et al., 2003; Imai et al., 2004). None of these genes is significantly expressed in the A7.5 sister cell or other regions of the endoderm (Fig. 1A), and we therefore refer to them as the A7.6 group genes (Hudson and Yasuo, 2006; Imai et al., 2006). Conversely, transcription factors such as *TTF-1* exhibit restricted expression in the endoderm, including A7.5, and are excluded from A7.6 (Fig. 1B). The A7.6 group genes and *TTF-1* were used as markers to evaluate A7.6/A7.5 specification upon experimental perturbation.

Transformation of anterior endoderm into supernumerary A7.6 cells

The presumptive endoderm provides a localized source of FGF signaling that induces adjacent mesoderm tissues such as the notochord and mesenchyme (Kim et al., 2000; Imai et al., 2002c). Inhibition of this signal causes a loss of *Brachyury* (*Ci-Bra*) expression in the presumptive primary notochord and loss of *Twist-like* genes in the mesenchyme. To determine whether FGF signaling is also important for the induction of the A7.6 fate, we employed in situ hybridization assays to examine the expression of marker genes in embryos treated with the MEK inhibitor, U0126 (Fig. 2A-E).

To our surprise, the treated embryos exhibited a striking expansion, not loss, of A7.6 group gene expression. *Hand-like*, *FGF8*, *Delta-like* and *MyTF* (Fig. 2A-D) displayed expression in both A7.6 and anterior endoderm cells, including the A7.5 blastomeres. The ectopic endoderm expression was somewhat variable, but in the most extreme cases, all anterior endoderm cells expressed A7.6 group genes. Conversely, expression of the *TTF-1* gut marker gene was completely lost upon drug inhibition (Fig. 2E). These observations suggest that MAPK signaling is essential for inhibiting A7.6 group genes within the endoderm, and thereby restricting their activities to the A7.6 lineage. FGF signaling in the anterior endoderm might inhibit the expression of A7.6 determinants (see Discussion).

Anti-dpERK antibody staining was used to visualize sites of active receptor tyrosine kinase (RTK) signaling, including FGF signaling. On the vegetal side of the embryo, staining was detected throughout the presumptive endoderm, primary notochord and posterior mesenchyme of 64-cell embryos (Fig. 2F). Staining was excluded from the A7.6 blastomere, but detected in the sister A7.5 endoderm cell. This pattern – dpERK staining in A7.5 but not A7.6 – persisted during the 110-cell stage (Fig. 2G). As expected, there was a complete loss of dpERK staining in embryos treated with the MEK inhibitor drug U0126 (Fig. 2H).

In summary, differential MAPK activation is observed in the descendants of the A6.3 endomesoderm blastomere. A7.6 group genes are expressed in the lineage that lacks MAPK activity, whereas endoderm marker genes are expressed in the sister lineage exhibiting dpERK staining. Inhibition of MAPK signaling causes expansion of A7.6 group genes throughout the anterior endoderm.

Ephrin attenuates MAPK signaling in the A7.6 lineage

The asymmetric activation of MAPK in A7.5 cannot be explained by the localization of FGF signaling, as *Ci-FGF9/16/20* is expressed in the A6.3 progenitor cell at the 32-cell stage (Hudson

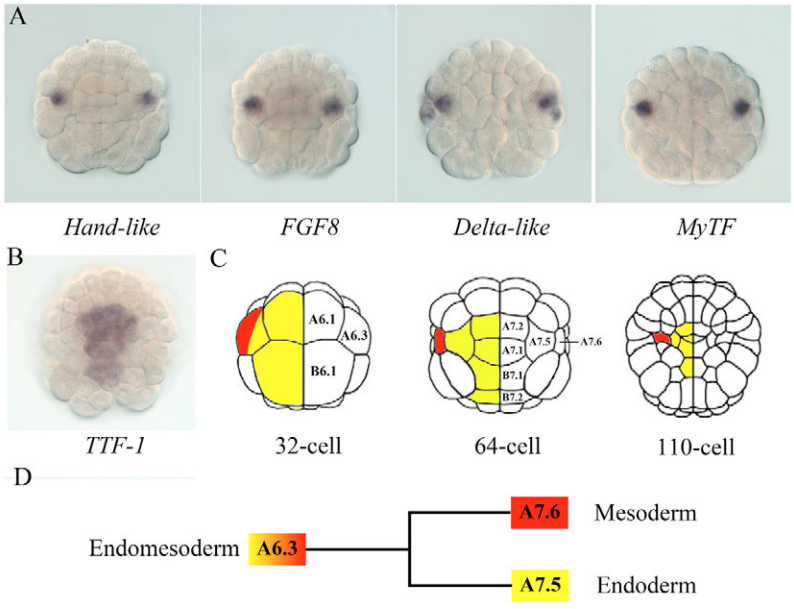

Fig. 1. A7.6 group genes mark mesoderm fate and *TTF-1* marks endoderm fate in A6.3 lineages. (**A**) A7.6-specific marker genes *Hand-like*, *FGF8*, *Delta-like* and *MyTF* are specifically expressed in A7.6 at late 110-cell stage. *Delta-like* is also expressed in animal blastomeres b7.9, b7.10 next to A7.6. (**B**) *TTF-1* is expressed in endoderm blastomeres. (**C**,**D**) Schematic of 32-, 64- and 110-cell embryos showing the mixed lineage of the A6.3 blastomere. At the 32-cell stage, A6.3 is the only endomesoderm blastomere in the *Ciona* embryo that will give rise to mesoderm (red) and endoderm (yellow) progenies.

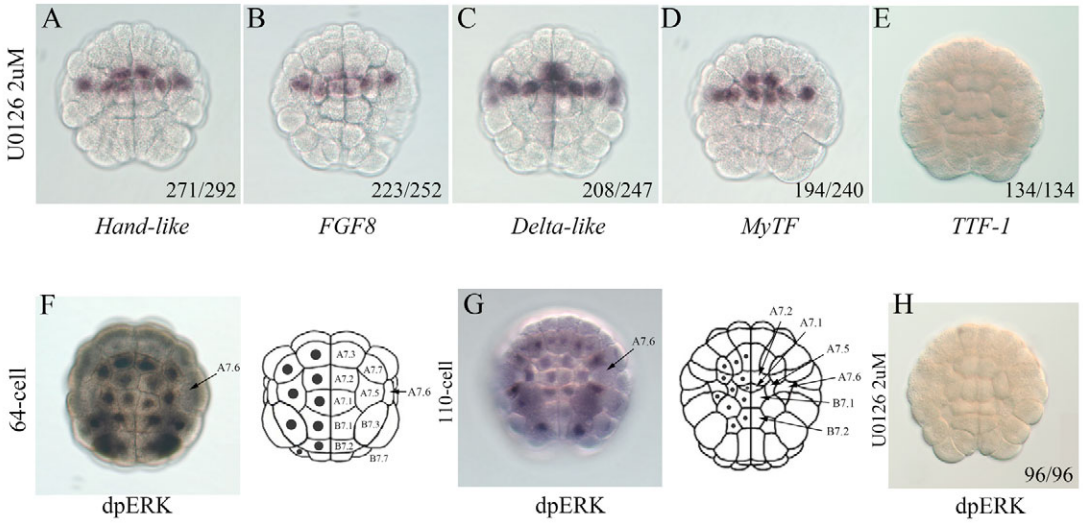

Fig. 2. Inhibition of MAPK signal causes ectopic expression of A7.6 group genes in the anterior endoderm. (**A-E**) 110-cell *Ciona* embryos treated with 2 μM U0126 at late 32-cell stage. (A-D) A7.6 group genes are ectopically expressed in the anterior endoderm blastomeres upon MEK inhibition, whereas A7.6 expression is unaffected (numbers indicate affected embryos/total embryos). Conversely, the endoderm-specific marker *TTF-1* is completely lost in the endoderm when MAPK signaling is inhibited (E). (**F**,**G**) Endogenous pattern of MAPK activation in the wild-type embryo. At the 64-cell stage (F), dpERK staining is observed in the nuclei of notochord precursors (A7.3, A7.7), all endoderm blastomeres (A7.1, A7.2, A7.5, B7.1, B7.2) and mesenchyme cells (B7.3, B7.7), but is strikingly absent from A7.6 mesoderm. This MAPK dichotomy in A7.5/A7.6 siblings persists to the 110-cell stage (G). (**H**) MAPK activation visualized by dpERK staining is completely lost in embryos treated with U0126.

et al., 2003; Nishida, 2003). Thus, some inhibitory signals from neighboring cells might be required to specifically inhibit MAPK activity in A7.6. A recent study demonstrated that Ephrin-Eph signaling is essential for the asymmetric specification of the A6.2 and A6.4 blastomeres, which form notochord and nerve cord (Picco et al., 2007). FGF9/16/20 emanating from the endoderm induces two of the daughter cells, A7.3 and A7.7, to form primary notochord cells via *Brachyury* gene activation. By contrast, Ci-ephrin-Ad inhibits FGF signaling in the sister cells, A7.4 and A7.8, that produce the future nerve cord. Disruption of *Ci-ephrin-Ad* gene activity via morpholino (MO) injection led to ectopic MAPK activation in the neurogenic lineage, ectopic activation of *Brachyury* gene expression, and a corresponding transformation of these cells into supernumerary notochord cells.

It is conceivable that a similar mechanism is used to produce asymmetric cell fates in the A6.3 lineage. As a first test of this possibility, we re-examined *Ci-ephrin-Ad* expression by in situ hybridization (Fig. 3). *Ci-ephrin-Ad* was found to be expressed in all animal hemisphere blastomeres at the 16-cell stage, including b5.3, which gives rise to the b6.5 lineage (Fig. 3A). Expression continued in a6.5, a6.6, a6.7, a6.8, b6.6 and b6.8, but diminished in b6.5 and b6.7 at the 32-cell stage. It is therefore possible that the Ci-ephrin-Ad protein is present in all animal hemisphere blastomeres at the 32-cell stage. The ANISEED three-dimensional virtual embryo reveals extensive and direct contact between A6.3 and animal hemisphere blastomeres (Tassy et al., 2006). Thus, *Ci-ephrin-Ad* appears to be expressed in the right place and time to serve as a crucial signal for A7.6 specification during the division of the A6.3 blastomere.

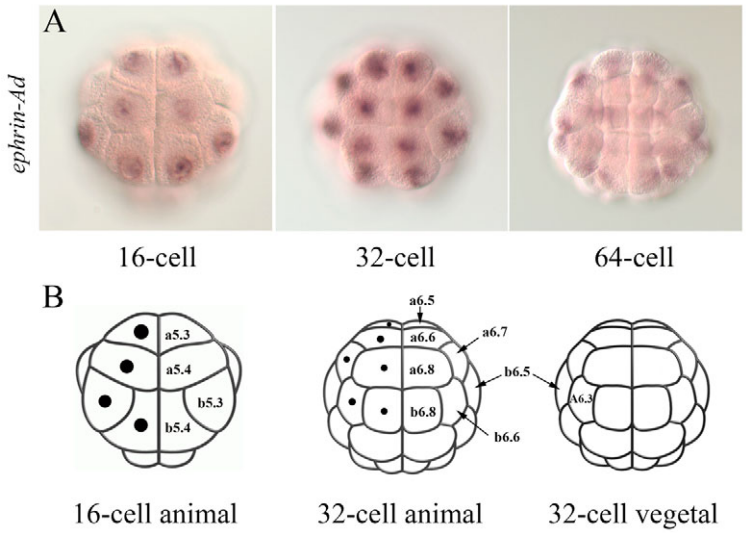

Fig. 3. *Ci-ephrin-Ad* is broadly expressed in the animal hemisphere. (**A**) *Ciona* embryos at progressively older stages hybridized for *Ci-ephrin-Ad*. (**B**) Schematic representation of *Ci-ephrin-Ad*-expressing cells in the animal hemisphere. The black dots indicate blastomeres that express the gene. The *Ci-ephrin-Ad* transcript is present in all animal blastomeres at the 16-cell stage, but diminishes in two of the progenies, b6.5 and b6.7, at the 32-cell stage. Zygotic expression further diminishes at the 64-cell stage. The relative positioning of the b6.5 and A6.3 blastomeres is shown in the vegetal view of the 32-cell embryo.

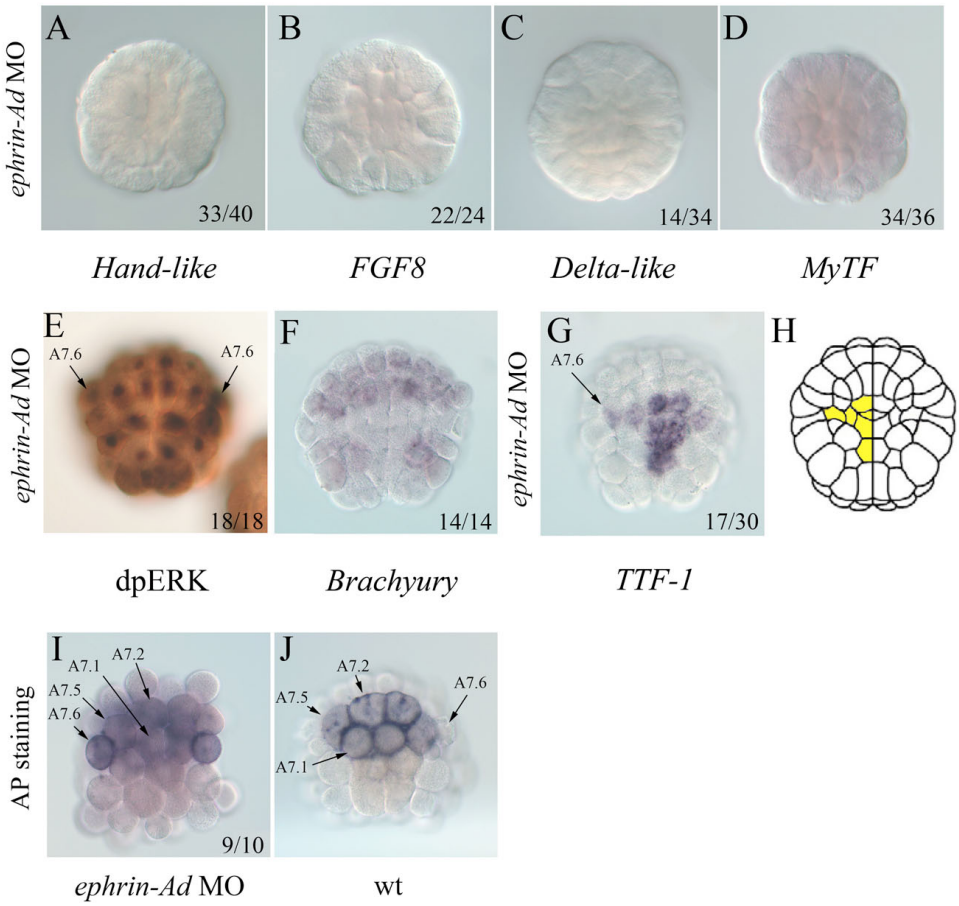

Fig. 4. *Ci-ephrin-Ad* establishes asymmetric fates in A6.3 lineages. (**A-D**) Upon *Ci-ephrin-Ad* MO injection, A7.6 group genes are severely downregulated in *Ciona* embryos. The numbers of embryos showing a complete loss of marker gene expression/total embryos are indicated. Whereas *Hand-like*, *FGF8* and *MyTF* display an almost complete loss of expression, *Delta-like* seems to be the most resilient to MO inhibition, with only ~40% loss of expression. The remaining ~60% embryos show normal or slightly reduced expression in A7.6. (**E**) When *Ci-ephrin-Ad* function is inhibited, ectopic dpERK staining is observed in the A7.6 blastomeres of all injected embryos. (**F**) *Brachyury* expression is expanded to the A-line neural tissues in MO embryos, as previously shown (Picco et al., 2007). (**G**) Ectopic expression of the endoderm marker *TTF-1* in A7.6 blastomeres of MO-injected embryos. The ectopic expression is variable and is seen on only one side of the embryo shown here. (**H**) Schematic illustrating that *TTF-1* expression (yellow) is expanded into A7.6 of MO-injected embryos. (**I**,**J**) Inhibition of *Ci-ephrin-Ad* function results in transformation of A7.6 mesoderm fate to endoderm fate. In wild-type arrested embryos (J), alkaline phosphatase (AP) activity is observed in endoderm blastomeres. For an unknown reason, the staining is consistently stronger in the anterior endoderm. (I) In *Ci-ephrin-Ad* MO-injected embryos, AP staining is also observed in A7.6 blastomeres, suggesting a fate transformation from mesoderm to endoderm.

To test whether *Ci-ephrin-Ad* is required to inhibit MAPK signaling and thereby establish mesoderm fate in A7.6, its function was disrupted via microinjection of a specific inhibitory MO. Embryos were allowed to develop to the 64- or 110-cell stage, and then hybridized with a variety of markers (Fig. 4). Upon MO injection, there was a severe loss of *Hand-like*, *FGF8*, *Delta-like*, and *MyTF* expression (Fig. 4A-D). By contrast, endoderm marker genes such as *TTF-1* exhibited ectopic expression in the A7.6 blastomere (Fig. 4G, arrow). As shown previously (Picco et al., 2007), *Brachyury* expression expands in *Ci-ephrin-Ad* MO embryos owing to loss of MAPK inhibition in the presumptive A-line CNS (Fig. 4F).

A marker for differentiated gut cells, alkaline phosphatase (AP), was used to determine whether A7.6 cells are transformed into endoderm (Whittaker and Meedel, 1989). 110-cell embryos were arrested by treatment with the cytokinesis inhibitor, cytochalasin B, and allowed to develop to the equivalent of the tailbud stage. Endogenous AP enzyme activity was visualized via standard colorimetric methods (see Materials and methods). In wild-type arrested embryos, AP activity was restricted to the endoderm, including the A7.5 blastomere, and was absent from A7.6 (Fig. 4J). However, mutant embryos injected with the *Ci-ephrin-Ad* MO exhibited AP staining in both the endoderm and A7.6 blastomere (Fig. 4I). These results raise the possibility that *Ci-ephrin-Ad* normally inhibits FGF signaling in A7.6, thereby fostering a mesoderm identity as opposed to endoderm fate.

Support for this model was obtained by examining the dpERK staining pattern in mutant embryos injected with *Ci-ephrin-Ad* MO. As shown earlier (see Fig. 2F), MAPK activity is not normally detected in the A7.6 blastomere. By contrast, mutant embryos displayed dpERK staining in A7.6, in addition to the normal sites of expression in the endoderm, primary notochord cells and mesenchyme (Fig. 4E, arrows). Thus, it would appear that the specification of asymmetric cell fates from the A6.3 lineage is similar to that seen for A6.2 and A6.4 (Picco et al., 2007). In each case, Ephrin-Ad inhibits RTK signaling in the daughter cells that are in direct contact with animal blastomeres.

FGF signaling inhibits A7.6 specification

The preceding analysis suggests that RTK signaling, as visualized by dpERK staining, is inhibited by Ephrin-Ad upon specification of the A7.6 mesoderm lineage. In the case of notochord formation, FGF signaling directly activates *Ci-Bra* expression through ETS-binding sites in the *Ci-Bra* enhancer (Matsumoto et al., 2007), and recombinant FGF protein is sufficient to induce *Ci-Bra* expression in cultured explants (Nakatani et al., 1996). Ephrin normally attenuates FGF signaling and MAPK activity in the neuronal descendants of the A6.2 and A6.4 progenitors.

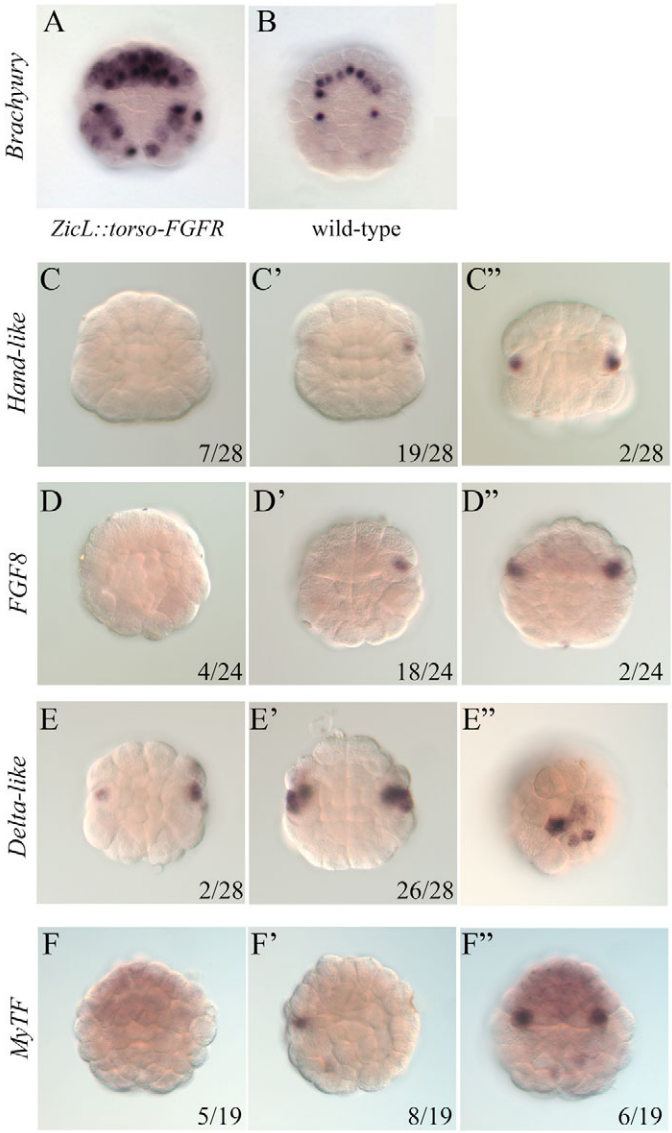

Fig. 5. Ubiquitous activation of FGF signaling inhibits A7.6 group gene expression. (**A**) The constitutively active form of *FGFR* (*torso-FGFR*) is expressed under the *ZicL* enhancer. *Brachyury* is ectopically expressed in A-line neural blastomeres, consistent with ectopic activation of the MAPK pathway. Some ectopic *Ci-Bra* expression is also observed in the muscle and mesenchyme. (**B**) *Ci-Bra* expression in wild-type *Ciona* embryo. (**C-F″**) Embryos injected at the 1-cell stage with *torso-FGFR* mRNA. Expression of A7.6 group genes is modestly reduced. To demonstrate the variable degree of reduction, embryos with no expression (C,D,F), weak expression (C′,D′,E,F′) and wild-type expression levels (C″,D″,E′,F″) are shown together with the number of embryos affected. In addition to A7.6, there are three instead of two *Delta-like*-positive blastomere cells (E″) consistently observed in injected embryos [in wild-type embryos, *Delta-like* expression in A7.8 is highly variable and only seen in ~20% of the embryos (Hudson and Yasuo, 2006), suggesting that MAPK usually activates *Delta-like* in these cells].

To determine whether Ephrin-FGF interactions are also important for establishing distinct endomesoderm fates, we examined the effect of expressing a constitutively active form of the *Ciona* FGF receptor (FGFR) in the embryo. This was created by fusing the intracellular kinase domain of FGFR with the extracellular domain of a constitutively active form of the Torso RTK in *Drosophila*, Torso^{4021} (Sprenger and Nusslein-Volhard, 1992). This strategy has been used to create constitutively active forms of a number of receptors (Galindo et al., 1995).

The *torso-FGFR* fusion gene was attached to *Ci-ZicL* regulatory sequences, which mediate expression in the progenitors of the notochord/nerve cord and muscle/mesenchyme lineages (Imai et al., 2002b). As expected, ectopic *Ci-Bra* expression was observed in the A-line nerve cord, suggesting a transformation of neural fate to notochord due to ectopic activation of MAPK signaling (Fig. 5A, compare with B). This confirms that the torso-FGFR is constitutively active and can overcome Ephrin-mediated MAPK inhibition in A-line nerve cord precursors. We then used an ~4 kb cis-regulatory sequence from the *Ci-FoxD* gene to ectopically express *torso-FGFR* in all vegetal blastomeres, beginning at the 16-cell stage. Unfortunately, the *FoxD::torso-FGFR* fusion gene did not appear to diminish expression of the A7.6 group genes, as might be predicted by sustained MAPK activity (data not shown). Perhaps the transgene is not expressed sufficiently early to interfere with Ephrin-mediated inhibition of MAPK in the A7.6 lineage.

To circumvent this potential problem, we injected full-length, capped *torso-FGFR* mRNA into 1-cell embryos, and then reared the injected embryos to the 110-cell stage. In situ hybridization assays revealed consistent reductions in *Hand-like*, *FGF8* and *MyTF* expression in A7.6 (Fig. 5C,D,F), although *Delta-like* expression was only sporadically reduced (Fig. 5E). These results are consistent with those of the *Ci-ephrin-Ad* MO experiments (Fig. 4A-D).

The incomplete inhibition of A7.6 group genes by activated FGFR is likely to be due to the repression of MAPK activation by endogenous Ephrin-Ad. To test this, we co-injected *ephrin-Ad* MO and *torso-FGFR* mRNA into 1-cell embryos and scored the expression of *Delta-like* in 110-cell embryos. *Delta-like* expression was now repressed in the A7.6 blastomere in ~80% of the embryos (Fig. 6A), whereas the *eprhin-Ad* MO or activated *FGFR* mRNA alone caused just ~40% or ~7% repression, respectively. As expected, the genes that are more sensitive to the loss of MAPK activity exhibited a complete loss in expression upon injection of the Ephrin-Ad MO and activated *FGFR* mRNA (*Hand-like*, 0/34 embryos exhibited expression in A7.6; *FGF8*, 1/31 embryos; *MyTF*, 0/32 embryos; data not shown).

The synergistic disruptions obtained by inhibiting *Ci-ephrin-Ad* and activating MAPK strongly suggest that the major role of Ephrin-Eph signaling is the suppression of FGF/MAPK in A7.6. If true, then the Ephrin-Ad MO mutant phenotype should be suppressed by the inhibition of MAPK activity. To test this prediction, we treated *ephrin-Ad* MO-injected embryos with the U0126 inhibitor at the 32-cell stage and assayed for the expression of the A7.6 group marker genes (Fig. 6B-E). The expression patterns resembled the situation seen when wild-type embryos were treated with U0126 alone (see Fig. 2A-D). A7.6 group genes were expressed in both the A7.6 blastomere and the anterior endoderm (Fig. 6B-E). These

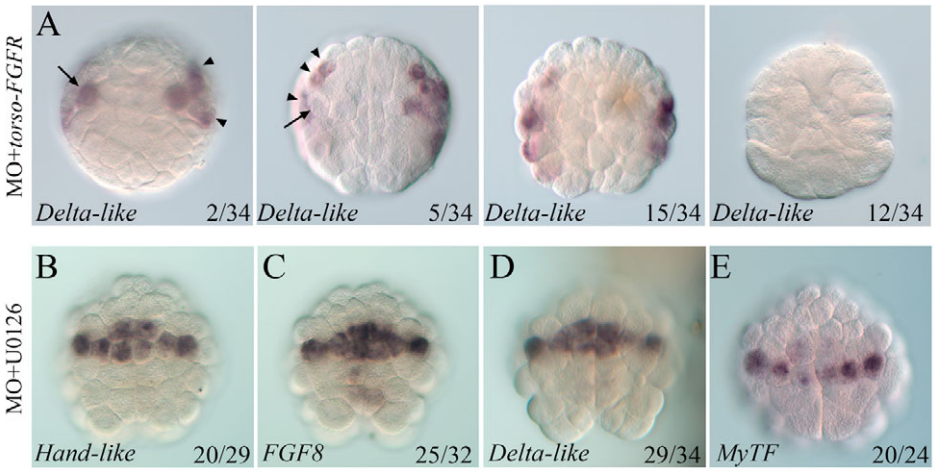

Fig. 6. Ephrin-Ad represses MAPK activity. (**A**) *Delta-like* expression is lost in both A7.6 (arrows, ~80%) or one A7.6 (~15%) of the *Ciona* embryos co-injected with *ephrin-Ad* MO and *torso-FGFR* mRNA, suggesting Ephrin-Ad and MAPK pathways function synergistically. Ectopic activation of *Delta-like* in b-line blastomeres is often observed (arrowheads). (**B-E**) Treating *ephrin-Ad* MO-injected embryos with the MEK inhibitor U0126 reverses the repression of A7.6 genes in A7.6 and ectopically activates them in the anterior endoderm. The number of affected embryos/total embryos is shown.

observations are consistent with the possibility that the sole role of Ephrin-Ad in A7.6 specification is the inhibition of MAPK activation.

Ectopic activation of Ephrin signaling expands expression of A7.6 genes

The preceding results suggest that Ephrin-expressing animal blastomeres inhibit MAPK activation in A7.6, whereas A7.5 loses contact with these blastomeres and thereby maintains MAPK activation and forms endoderm. Thus, the three-dimensional positioning of the Ephrin signal might be critical for establishing asymmetric fates among A6.3 progeny. One test of this model involved the misexpression of the Ephrin signal within the endoderm.

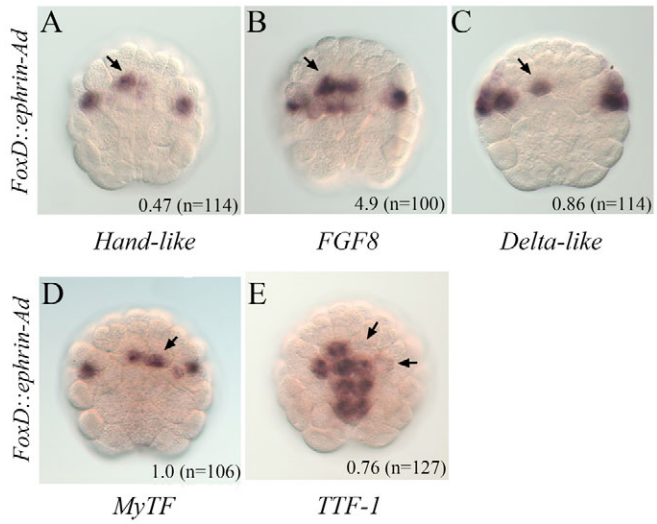

Fig. 7. Misexpression of *Ci-ephrin-Ad* in the endoderm activates mesoderm markers and represses endoderm markers. A *FoxD* enhancer was used to drive *Ci-ephrin-Ad* throughout the vegetal hemisphere at the 16-cell stage. (**A-D**) Ectopic expression of A7.6 group genes in the anterior endoderm (arrows) is observed in embryos misexpressing *Ci-ephrin-Ad*. The average number of endoderm cells misexpressing the mesoderm marker is shown. *FGF8* appears to be most responsive to misexpression of *Ci-ephrin-Ad*; the others show an average of one or fewer ectopic expressing cell. (**E**) The endoderm marker *TTF-1* is variably lost in the endoderm where *Ci-ephrin-Ad* is misexpressed, suggesting a partial transformation of endoderm to mesoderm fate.

The *Ci-FoxD* 5′ regulatory sequence was used to ectopically express the full-length *Ci-ephrin-Ad* coding region. *Ci-FoxD* is activated in the progenitors of A6.1 and A6.3 at the 16-cell stage of embryogenesis (Imai et al., 2002a). In principle, the *Ci-FoxD* enhancer should direct *Ci-ephrin-Ad* expression in the A6.3 endomesoderm cell just before its division into separate endoderm and mesoderm lineages.

Electroporated embryos expressing the *FoxD::Ci-ephrin-Ad* transgene were harvested at the 110-cell stage for in situ hybridization (Fig. 7). There was sporadic expansion of the A7.6 group genes in the anterior endoderm (Fig. 7A-D, arrows), whereas expression of *TTF-1* was lost in the anterior endoderm, including A7.5 (Fig. 7E, arrows). The mosaic expansion of A7.6 marker genes and incomplete loss of *TTF-1* probably reflect the timing of transgene expression. Expression of the *Ci-ephrin-Ad* transgene at the 16-cell stage might lead to the secretion of ectopic Ephrin-Ad protein just at the critical time when MAPK is incompletely inhibited, resulting in partial transformation of endoderm to mesoderm fate.

In summary, it appears that misexpression of *Ci-ephrin-Ad* in the vegetal blastomeres leads to inhibition of FGF signaling and MAPK activity in the endoderm. This results in a mutant phenotype similar to that obtained upon application of the U0126 MEK inhibitor (see Fig. 2).

MAPK is the critical determinant of distinct A6.3 cell fates

The b-line blastomeres that contact the A6.3 precursor cell produce localized sources of both Ephrin-Ad and Nodal. It has been suggested that Nodal signals emanating from the b6.5 lineage are the key determinant of A7.6 specification and the induction of the A7.6 group marker genes (Hudson and Yasuo, 2006). Indeed, *Nodal* MO or a drug inhibitor (SB431542) of the *Nodal* receptor completely blocks expression of the A7.6 group genes (Imai et al., 2006) (data not shown). However, it is difficult to reconcile this role for Nodal signaling with the ectopic activation of A7.6 group genes in the endoderm upon MAPK inhibition (e.g. Fig. 2A-D). To clarify this issue, we blocked *Nodal* expression in b6.5 using the *MAPK* U0126 inhibitor at different time points during development. It has been

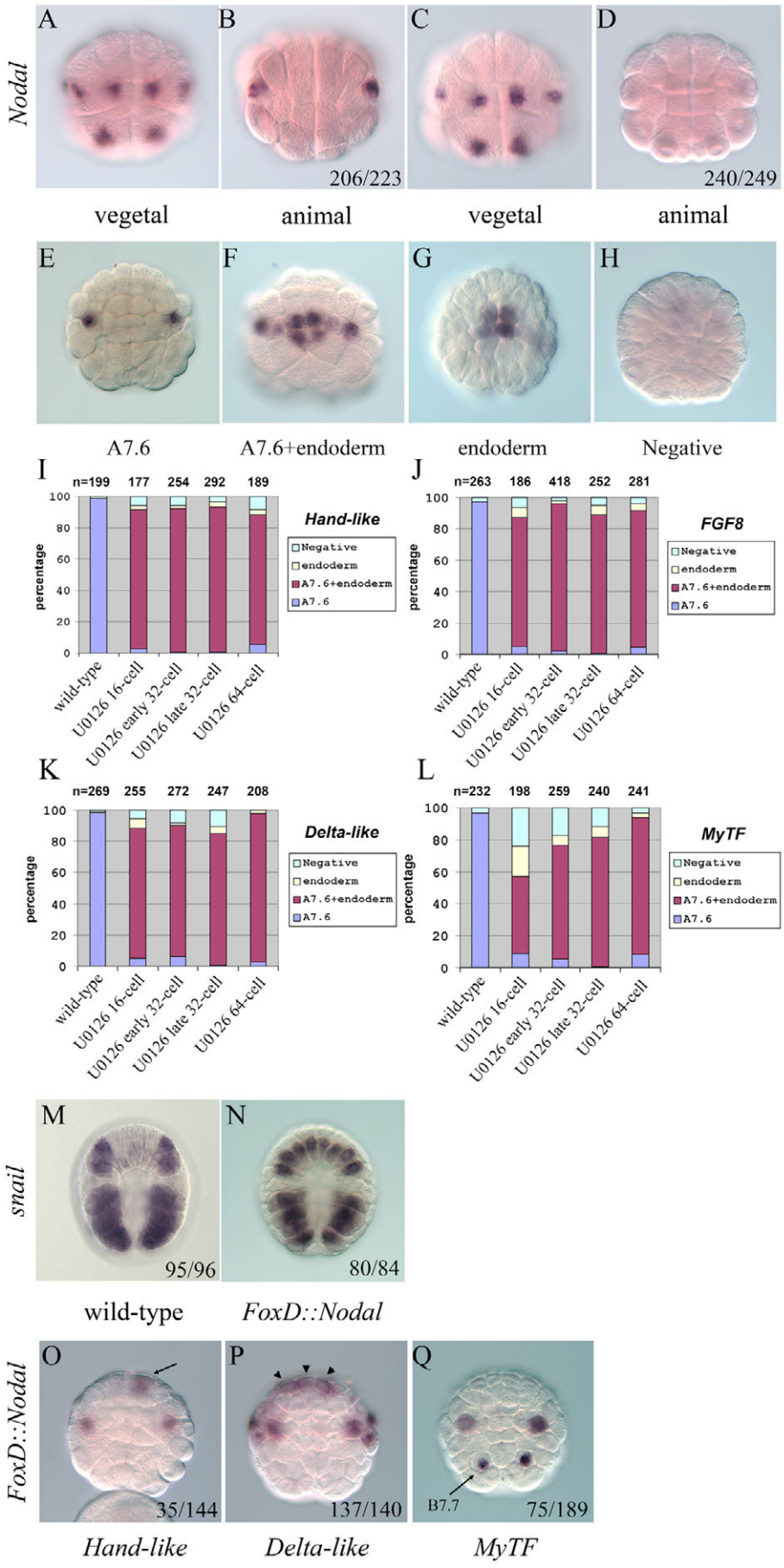

Fig. 8. A7.6 marker gene expression does not require Nodal signaling from b6.5. (**A**,**B**) In late 32-cell stage wild-type *Ciona* embryos, *Nodal* is expressed in endoderm/endomesoderm blastomeres (A) and in b6.5 (B). (**C**,**D**) In embryos treated with U0126 at early 32-cell stage, vegetal expression of *Nodal* is unaffected (C), whereas b6.5 expression is completely blocked (D). (**E-H**) Embryos grown in U0126 beginning at the 16-cell stage display variable changes in A7.6 marker gene expression. (**I-K**) *Hand-like*, *FGF8* and *Delta-like* expression in A7.6 is not affected by U0126 inhibition. (**L**) *MyTF* expression in A7.6 partially requires MAPK signaling at the 16-cell stage, but not at the 32- to 64-cell stage. (**M**,**N**) Ectopic expression of *Nodal* in the vegetal hemisphere induces ectopic *Snail* expression in the A-line neural lineage. (**O-Q**) Ectopic *Nodal* expression does not induce A7.6 gene expression in A7.5 or other endoderm blastomere, but is able to induce *Hand-like* and *Delta-like* expression in the A-line neural lineage (O, arrow; P, arrowheads) or to induce ectopic *MyTF* expression in mesenchyme lineages (Q, arrow).

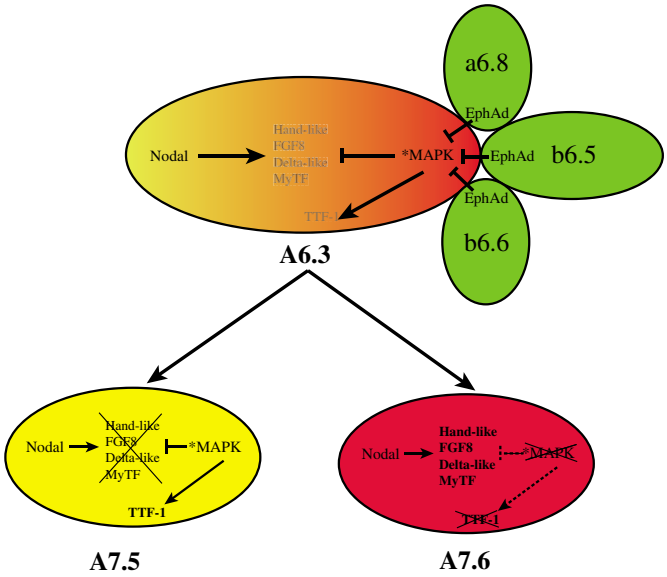

Fig. 9. A model for A6.3 endomesoderm specification in *Ciona*. In A6.3, a combination of Nodal signaling and MAPK signaling maintains an endomesoderm state. Upon cell division, MAPK is inhibited in A7.6 by Ephrin-Ad from neighboring animal blastomeres, allowing endogenous Nodal to activate A7.6 mesoderm genes. In A7.5, Nodal is unable to activate such genes owing to inhibition by MAPK. The fates of blastomeres are indicated in different colors (red, mesoderm; yellow, endoderm).

shown that *Nodal* expression in b6.5 is activated by an FGF signal from the endoderm at the 32-cell stage (Hudson and Yasuo, 2005).

Embryos treated at the early 32-cell stage with U0126 exhibited a complete loss of *Nodal* expression in b6.5, but displayed a normal pattern of *Nodal* expression in the endoderm (Fig. 8C,D, compare with A,B). These embryos often displayed normal expression of the A7.6 group marker genes (e.g. Fig. 8E). However, additional patterns were also detected, including expression in A7.6 along with ectopic expression in the endoderm (Fig. 8F), loss of A7.6 expression along with gain of expression in the endoderm (Fig. 8G), or complete loss of expression (Fig. 8H). Embryos treated with U0126 at an earlier time point, the 16-cell stage, exhibited essentially normal expression of *Hand-like*, *FGF8* and *Delta-like* in the A7.6 lineage, along with ectopic activation in the endoderm (Fig. 8I-L). Only one of the marker genes, *MyTF*, displayed a significant loss of expression in A7.6 (~40%), but the remaining embryos exhibited expression in both A7.6 and the endoderm (Fig. 8L). Thus, loss of the Nodal signal in b6.5 does not significantly alter the induction of the A7.6 group genes in the A7.6 lineage.

The preceding observations suggest that MAPK signaling in the endoderm prevents endogenous Nodal from activating the A7.6 group genes. To determine the epistatic relationship between Nodal and MAPK signaling, we ectopically expressed *Nodal* in the vegetal hemisphere using the *Ci-FoxD* enhancer. We used the *Snail* gene as a control because it is induced in the lateral neural plate by Nodal signals in b6.5 (Hudson and Yasuo, 2005). In wild-type embryos, *Snail* is expressed in the A8.15/A8.16 neural precursors (Fig. 8M). By contrast, embryos electroporated with the FoxD::Nodal transgene displayed an expanded *Snail* expression pattern that encompassed the medial rows of the A-line neural precursors, A8.7/A8.8 (Fig. 8N, compare with M). This result suggests that the *FoxD::Nodal* transgene is able to drive functional *Nodal* expression throughout the vegetal hemisphere.

We then examined the expression of A7.6 marker genes in embryos expressing the *FoxD::Nodal* transgene (Fig. 8O-Q). There was no ectopic expression in the endoderm blastomeres, suggesting that the augmented levels of Nodal signaling are unable to overcome repression by endogenous MAPK. *Hand-like* and *Delta-like* sometimes displayed ectopic expression in the A-line neural precursors (Fig. 8O,P), suggesting that high levels of Nodal are sufficient to activate their expression when MAPK signaling is absent. No ectopic *FGF8* expression was observed, whereas *MyTF* was sometimes ectopically expressed in the B7.7 lineage (Fig. 8Q, arrow).

DISCUSSION

We have presented evidence that competition between Eprhin and FGF signaling is important for the asymmetric specification of endoderm and mesoderm lineages from a common endomesoderm progenitor cell, the A6.3 blastomere. A similar mechanism was recently invoked to account for the asymmetric specification of the notochord and nerve cord from common A6.2 and A6.4 progenitors (Picco et al., 2007). In both cases, a localized Ephrin-Ad signal produced by the primitive ectoderm (the animal blastomeres) competes with FGF9 signals from the primitive gut. Those cells in extended contact with the ectoderm lack MAPK activation, whereas those cells in contact with the endoderm experience MAPK activation and follow a different fate. It is conceivable that this interplay of Ephrin and FGF signaling is used in other systems to produce asymmetric cell fates.

Ephrin-FGF competition establishes multiple *Ciona* tissues

Ephrins have been implicated in a variety of cellular processes, including axonal guidance, repulsive cell-cell interactions, and adhesion (Poliakov et al., 2004; Pasquale, 2005). Different Ephrin family members can activate (Zisch et al., 2000) or inhibit RTK signaling (Elowe et al., 2001; Miao et al., 2001) in different cellular contexts. The present study, along with the recent analysis of *Ci-Bra* regulation (Picco et al., 2007), suggest that *Ci-ephrin-Ad* functions as a localized inhibitor of FGF signaling to produce asymmetric cell fates in *Ciona*. The presumptive endoderm/endomesoderm produces a localized source of FGF9/16/20, which induces the specification of diverse mesoderm lineages, including the notochord and mesenchyme. We have presented evidence that Ephrin also controls the subdivision of the A6.3 endomesoderm.

A model for the specification of the A7.6 blastomere is summarized in Fig. 9. Previous studies have shown that *Nodal* is essential for the expression of several A7.6 marker genes, including *Hand-like* (also known as *NoTrlc*), *FGF8* and *Delta-like* (Hudson and Yasuo, 2006; Imai et al., 2006). *Nodal* is expressed in the A6.3 blastomere of 32-cell embryos, as well as in the other progenitors of the endoderm. FGF/MAPK signaling is also active in the A6.3 at this stage, as judged by anti-dpERK staining (data not shown). Ephrin-Ad produced by b6.5 (and other animal blastomeres) inhibits MAPK in A7.6, thereby permitting Nodal to activate the A7.6 group genes. Nodal signaling in A7.6 might be reinforced by Nodal expression in the b6.5 lineage. Thus, the inhibition of FGF signaling by Eprhin-Ad, along with augmented levels of Nodal signal, might be responsible for the activation of A7.6 group genes. However, we have presented evidence that Nodal in b6.5 is not essential for A7.6 group gene expression. Instead, it would appear that the combination

of endogenous Nodal in the A6.3 progenitor, along with the localized inhibition of MAPK in A7.6 by Eprhin-Ad, is the decisive determinant of A7.6 specification.

Inhibition of MAPK signaling via drug treatment or ectopic expression of Ephrin-Ad leads to misexpression of A7.6 marker genes in the anterior endoderm, where Nodal is normally inactive owing to FGF/MAPK signaling. Posterior endoderm cells also contain Nodal but fail to express A7.6 marker genes upon inhibition of MAPK. This might reflect the restricted distribution of additional activators required for A7.6 gene expression. For example, *Hand-like* is activated by the combination of Nodal signaling and the *FoxA* transcription factor (Imai et al., 2006). *FoxA* expression is restricted to the anterior endoderm, dorsal mesoderm and future CNS floorplate, but is absent from the posterior endoderm (Corbo et al., 1997b). This is consistent with the result of ectopic *Hand-like* and *Delta-like* activation in the A-line neural lineage by expression of the *FoxD::Nodal* transgene.

A7.6 serves as a signaling center

A7.6 expresses a number of localized determinants, including two crucial signaling molecules, FGF8 and Delta-like. A7.6 is located in a strategically important position within the vegetal hemisphere. It contacts components of all three germ layers: the endoderm, ectoderm and mesenchyme. The Delta-like ligand expressed in A7.6 induces the secondary notochord lineage via Notch signaling, and also induces the lateralmost neural fate (Hudson et al., 2007). Similarly, FGF8 expression is required for maintaining the primary notochord fate (Yasuo and Hudson, 2007). Because these signaling pathways require either direct cell-cell contact (Notch) or act over one or two cell diameters (FGF), it is crucial to activate the expression of Delta-like and FGF8 to A7.6, but not in its sibling A7.5 endoderm blastomere. The activities of three pathways, Ephrin, MAPK and Nodal signaling, are employed to achieve this precise asymmetric cell-fate specification event.

Specification and subdivision of the *Ciona* endomesoderm

Recent phylogenetic analysis suggests that tunicates (e.g. *Ciona*) are the closest living relatives of the vertebrates (Delsuc et al., 2006). As a result, it is possible that vertebrates employ a mechanism for the specification and subdivision of the endomesoderm that is similar to the one used in *Ciona*. The A6.3 endomesoderm cell is established by the action of a localized maternal determinant, β-Catenin (Imai et al., 2000), which activates the expression of multiple signaling molecules including *Nodal* and *FGF9*. Nodal is required to activate A7.6-specific genes such as *Hand-like*, *FGF8* and *Delta-like*. The failure of Nodal to activate A7.6 group genes in the endoderm is due to MAPK signaling. FGF signaling either directly or indirectly inhibits Nodal (e.g. Kretzschmar et al., 1999; Grimm and Gurdon, 2002). As a result, Nodal signaling is blocked in A6.3, but is activated in A7.6 owing to the localized inhibition of FGF signaling by Eprhin-Ad.

Most or all metazoan embryos possess a transient endomesoderm that generates specific mesodermal derivatives (Rodaway and Patient, 2001). In vertebrates, the presumptive endomesoderm gives rise to blood, heart and muscle (Kimelman and Griffin, 2000). Formation of the vertebrate endomesoderm depends on TGF-β signaling molecules such as Xnrs in *Xenopus* and Squint and Cyclops (Nodal-related 1 and 2, respectively – ZFIN) in zebrafish. The subsequent subdivision of the endomesoderm is not clearly understood, but might depend on FGF signaling (Rodaway et al., 1999). It remains to be seen if competitive interactions between Nodal (or some other TGF-β signaling molecule) and FGF lead to the subdivision of endomesoderm in vertebrate embryos.

We thank Lionel Christiaen for comments on the manuscript. This study was funded by NSF grant IOB 0445470 and NIH grants HD37105 and GM75049 to M.L., and from the Gordon & Betty Moore Foundation to the Center for Integrative Genomics.

References

Ball, E. E., Hayward, D. C., Saint, R. and Miller, D. J. (2004). A simple plan-cnidarians and the origins of developmental mechanisms. *Nat. Rev. Genet.* **5**, 567-577.
Corbo, J. C., Levine, M. and Zeller, R. W. (1997a). Characterization of a notochord-specific enhancer from the Brachyury promoter region of the ascidian, Ciona intestinalis. *Development* **124**, 589-602.
Corbo, J. C., Erives, A., Di Gregorio, A., Chang, A. and Levine, M. (1997b). Dorsoventral patterning of the vertebrate neural tube is conserved in a protochordate. *Development* **124**, 2335-2344.
Delsuc, F., Brinkmann, H., Chourrout, D. and Philippe, H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* **439**, 965-968.
Elowe, S., Holland, S. J., Kulkarni, S. and Pawson, T. (2001). Downregulation of the Ras-mitogen-activated protein kinase pathway by the EphB2 receptor tyrosine kinase is required for ephrin-induced neurite retraction. *Mol. Cell. Biol.* **21**, 7429-7441.
Galindo, R. L., Edwards, D. N., Gillespie, S. K. and Wasserman, S. A. (1995). Interaction of the pelle kinase with the membrane-associated protein tube is required for transduction of the dorsoventral signal in Drosophila embryos. *Development* **121**, 2209-2218.
Grimm, O. H. and Gurdon, J. B. (2002). Nuclear exclusion of Smad2 is a mechanism leading to loss of competence. *Nat. Cell Biol.* **4**, 519-522.
Harafuji, N., Keys, D. N. and Levine, M. (2002). Genome-wide identification of tissue-specific enhancers in the Ciona tadpole. *Proc. Natl. Acad. Sci. USA* **99**, 6802-6805.
Hudson, C. and Yasuo, H. (2005). Patterning across the ascidian neural plate by lateral Nodal signalling sources. *Development* **132**, 1199-1210.
Hudson, C. and Yasuo, H. (2006). A signalling relay involving Nodal and Delta ligands acts during secondary notochord induction in Ciona embryos. *Development* **133**, 2855-2864.
Hudson, C., Darras, S., Caillol, D., Yasuo, H. and Lemaire, P. (2003). A conserved role for the MEK signalling pathway in neural tissue specification and posteriorisation in the invertebrate chordate, the ascidian Ciona intestinalis. *Development* **130**, 147-159.
Hudson, C., Lotito, S. and Yasuo, H. (2007). Sequential and combinatorial inputs from Nodal, Delta2/Notch and FGF/MEK/ERK signalling pathways establish a grid-like organisation of distinct cell identities in the ascidian neural plate. *Development* **134**, 3527-3537.
Imai, K., Takada, N., Satoh, N. and Satou, Y. (2000). (beta)-catenin mediates the specification of endoderm cells in ascidian embryos. *Development* **127**, 3009-3020.
Imai, K. S., Satoh, N. and Satou, Y. (2002a). An essential role of a FoxD gene in notochord induction in Ciona embryos. *Development* **129**, 3441-3453.
Imai, K. S., Satou, Y. and Satoh, N. (2002b). Multiple functions of a Zic-like gene in the differentiation of notochord, central nervous system and muscle in Ciona savignyi embryos. *Development* **129**, 2723-2732.
Imai, K. S., Satoh, N. and Satou, Y. (2002c). Early embryonic expression of FGF4/6/9 gene and its role in the induction of mesenchyme and notochord in Ciona savignyi embryos. *Development* **129**, 1729-1738.
Imai, K. S., Satoh, N. and Satou, Y. (2003). A Twist-like bHLH gene is a downstream factor of an endogenous FGF and determines mesenchymal fate in the ascidian embryos. *Development* **130**, 4461-4472.
Imai, K. S., Hino, K., Yagi, K., Satoh, N. and Satou, Y. (2004). Gene expression profiles of transcription factors and signaling molecules in the ascidian embryo: towards a comprehensive understanding of gene networks. *Development* **131**, 4047-4058.
Imai, K. S., Levine, M., Satoh, N. and Satou, Y. (2006). Regulatory blueprint for a chordate embryo. *Science* **312**, 1183-1187.
Kawaminani, S. and Nishida, H. (1997). Induction of trunk lateral cells, the blood cell precursors, during ascidian embryogenesis. *Dev. Biol.* **181**, 14-20.
Kim, G. J., Yamada, A. and Nishida, H. (2000). An FGF signal from endoderm and localized factors in the posterior-vegetal egg cytoplasm pattern the mesodermal tissues in the ascidian embryo. *Development* **127**, 2853-2862.
Kimelman, D. and Griffin, K. J. (2000). Vertebrate mesendoderm induction and patterning. *Curr. Opin. Genet. Dev.* **10**, 350-356.
Kretzschmar, M., Doody, J., Timokhina, I. and Massague, J. (1999). A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev.* **13**, 804-816.

Maduro, M. F. (2006). Endomesoderm specification in Caenorhabditis elegans and other nematodes. *BioEssays* **28**, 1010-1022.
Martindale, M. Q., Pang, K. and Finnerty, J. R. (2004). Investigating the origins of triploblasty: 'mesodermal' gene expression in a diploblastic animal, the sea anemone Nematostella vectensis (phylum, Cnidaria; class, Anthozoa). *Development* **131**, 2463-2474.
Mathieu, J., Griffin, K., Herbomel, P., Dickmeis, T., Strahle, U., Kimelman, D., Rosa, F. M. and Peyrieras, N. (2004). Nodal and Fgf pathways interact through a positive regulatory loop and synergize to maintain mesodermal cell populations. *Development* **131**, 629-641.
Matsumoto, J., Kumano, G. and Nishida, H. (2007). Direct activation by Ets and Zic is required for initial expression of the Brachyury gene in the ascidian notochord. *Dev. Biol.* **306**, 870-882.
Miao, H., Wei, B. R., Peehl, D. M., Li, Q., Alexandrou, T., Schelling, J. R., Rhim, J. S., Sedor, J. R., Burnett, E. and Wang, B. (2001). Activation of EphA receptor tyrosine kinase inhibits the Ras/MAPK pathway. *Nat. Cell Biol.* **3**, 527-530.
Nakatani, Y., Yasuo, H., Satoh, N. and Nishida, H. (1996). Basic fibroblast growth factor induces notochord formation and the expression of As-T, a Brachyury homolog, during ascidian embryogenesis. *Development* **122**, 2023-2031.
Nishida, H. (2003). Spatio-temporal pattern of MAP kinase activation in embryos of the ascidian Halocynthia roretzi. *Dev. Growth Differ.* **45**, 27-37.
Pasquale, E. B. (2005). Eph receptor signalling casts a wide net on cell behaviour. *Nat. Rev. Mol. Cell Biol.* **6**, 462-475.
Picco, V., Hudson, C. and Yasuo, H. (2007). Ephrin-Eph signalling drives the asymmetric division of notochord/neural precursors in Ciona embryos. *Development* **134**, 1491-1497.
Poliakov, A., Cotrina, M. and Wilkinson, D. G. (2004). Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. *Dev. Cell* **7**, 465-480.
Rodaway, A. and Patient, R. (2001). Mesendoderm: an ancient germ layer? *Cell* **105**, 169-172.
Rodaway, A., Takeda, H., Koshida, S., Broadbent, J., Price, B., Smith, J. C., Patient, R. and Holder, N. (1999). Induction of the mesendoderm in the zebrafish germ ring by yolk cell-derived TGF-beta family signals and discrimination of mesoderm and endoderm by FGF. *Development* **126**, 3067-3078.
Sherwood, D. R. and McClay, D. R. (1999). LvNotch signaling mediates secondary mesenchyme specification in the sea urchin embryo. *Development* **126**, 1703-1713.
Sprenger, F. and Nusslein-Volhard, C. (1992). Torso receptor activity is regulated by a diffusible ligand produced at the extracellular terminal regions of the Drosophila egg. *Cell* **71**, 987-1001.
Tassy, O., Daian, F., Hudson, C., Bertrand, V. and Lemaire, P. (2006). A quantitative approach to the study of cell shapes and interactions during early chordate embryogenesis. *Curr. Biol.* **16**, 345-358.
Tokuoka, M., Satoh, N. and Satou, Y. (2005). A bHLH transcription factor gene, Twist-like 1, is essential for the formation of mesodermal tissues of Ciona juveniles. *Dev. Biol.* **288**, 387-396.
Whittaker, J. R. and Meedel, T. H. (1989). Two histospecific enzyme expressions in the same cleavage-arrested one-celled ascidian embryos. *J. Exp. Zool.* **250**, 168-175.
Yasuo, H. and Hudson, C. (2007). FGF8/17/18 functions together with FGF9/16/20 during formation of the notochord in Ciona embryos. *Dev. Biol.* **302**, 92-103.
Zisch, A. H., Pazzagli, C., Freeman, A. L., Schneller, M., Hadman, M., Smith, J. W., Ruoslahti, E. and Pasquale, E. B. (2000). Replacing two conserved tyrosines of the EphB2 receptor with glutamic acid prevents binding of SH2 domains without abrogating kinase activity and biological responses. *Oncogene* **19**, 177-187.