

LvNotch signaling plays a dual role in regulating the position of the ectoderm-endoderm boundary in the sea urchin embryo

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SUMMARY

The molecular mechanisms guiding the positioning of the ectoderm-endoderm boundary along the animal-vegetal axis of the sea urchin embryo remain largely unknown. We report here a role for the sea urchin homolog of the Notch receptor, LvNotch, in mediating the position of this boundary. Overexpression of an activated form of LvNotch throughout the embryo shifts the ectoderm-endoderm boundary more animally along the animal-vegetal axis, whereas expression of a dominant negative form shifts the border vegetally. Mosaic experiments that target activated and dominant negative forms of LvNotch into individual blastomeres of the early embryo, combined with lineage analyses, further reveal that LvNotch signaling mediates the position of this boundary by distinct mechanisms within the animal versus vegetal portions of the embryo. In the animal region of the embryo, LvNotch signaling acts cell autonomously to promote endoderm formation more

animally, while in the vegetal portion, LvNotch signaling also promotes the ectoderm-endoderm boundary more animally, but through a cell non-autonomous mechanism. We further demonstrate that vegetal LvNotch signaling controls the localization of nuclear β -catenin at the ectoderm-endoderm boundary. Based on these results, we propose that LvNotch signaling promotes the position of the ectoderm-endoderm boundary more animally via two mechanisms: (1) a cell-autonomous function within the animal region of the embryo, and (2) a cell non-autonomous role in the vegetal region that regulates a signal(s) mediating ectoderm-endoderm position, possibly through the control of nuclear β -catenin at the boundary.

Key words: Sea urchin, Notch signaling, LvNotch, Boundaries, Ectoderm, Endoderm

INTRODUCTION

In combination with classical manipulation studies, recent molecular analyses have begun to elucidate the cellular and molecular mechanisms that pattern the sea urchin animal-vegetal (A-V) axis during early development (reviewed by Davidson et al., 1998; Logan and McClay, 1998; Wessel and Wikramanayake, 1999; Angerer and Angerer, 2000; Etensohn and Sweet, 2000). This patterning process establishes three fundamental tissues along the A-V axis: mesoderm at the vegetal pole, endoderm overlying the mesoderm and ectoderm in the animal region of the embryo. A key event in this process is the placement of the boundary that divides the endoderm and ectoderm. This border separates the endoderm tissue that invaginates into the blastocoel during gastrulation from ectoderm tissue that remains outside to cover the embryo and larva.

Embryological studies have suggested that cell-cell interactions play an important role in specifying the boundary between the ectoderm and endoderm. Lineage studies have revealed that the ectoderm-endoderm border does not correlate with early cleavage divisions, suggesting instead that cell-cell interactions establish this boundary (Logan and McClay, 1997;

Ransick and Davidson, 1998). Supporting this notion, blastomere isolation experiments have shown that interactions between animal blastomeres suppress endoderm forming potential in presumptive ectoderm cells (Henry et al., 1989). In addition, blastomere removal and transplantation studies have indicated that the micromeres, the vegetal-most cells in the 16-cell stage embryo, initiate a vegetal-to-animal wave of inductive signaling required for both normal overlying secondary mesenchyme cell (SMC) specification in the early blastula, and endoderm specification in the late blastula to early gastrula stage (Horstadius, 1973; Khaner and Wilt, 1991; Ransick and Davidson, 1993; Ransick and Davidson, 1995; Ransick and Davidson, 1998; Sweet et al., 1999; McClay et al., 2000).

Recent work has begun to reveal the molecular mechanisms that regulate the position of the ectoderm-endoderm boundary. A sea urchin BMP2/4 homolog is expressed in presumptive ectoderm in the blastula embryo, and appears to influence ectoderm-endoderm boundary position by suppressing endoderm formation within presumptive ectoderm cells (Angerer et al., 2000). In addition, β -catenin, a component of the Wnt signaling pathway (reviewed in Wodarz and Nusse, 1998), may also have a role in mediating the position of the ectoderm-endoderm boundary. Nuclear β -catenin signaling is

required for several early aspects of endo-mesodermal specification in cleavage stage embryos (Wikramanayake et al., 1998; Logan et al., 1999; Huang et al., 2000; McClay et al., 2000; Vonica et al., 2000). At the late mesenchyme blastula stage, nuclear β -catenin is present specifically within the nuclei of presumptive endoderm cells bordering the presumptive ectoderm (D. R. Sherwood, PhD thesis, Duke University, Durham, NC, 1997; Logan et al., 1999), suggesting a possible later function in regulating the position of the ectoderm-endoderm boundary.

The sea urchin homolog of the Notch receptor, LvNotch, may also have a role in mediating the position of the ectoderm-endoderm boundary. LvNotch signaling is activated within presumptive SMCs by underlying micromeres during early development, (Sherwood and McClay, 1999; Sweet et al., 1999; McClay et al., 2000), suggesting that LvNotch may be a component of the micromere-initiated vegetal-to-animal cascade of inductive signaling that influences the formation of endoderm. In addition, LvNotch protein is expressed dynamically within both presumptive ectoderm and endoderm cells in the blastula embryo (Sherwood and McClay, 1997), indicating that LvNotch could also function in these tissues to regulate the position of the ectoderm-endoderm boundary. While the Notch pathway has not yet been implicated in establishing germ-layer boundaries in other organisms, Notch signaling has been shown to mediate the formation of other types of boundaries, such as in *Drosophila* limb development and vertebrate somite formation (Irvine, 1999; Rawls et al., 2000). It is thus important to understand the possible role of LvNotch signaling in positioning the ectoderm-endoderm boundary in the sea urchin, both to gain a broader understanding of how Notch signaling is used for establishing boundaries, as well as elucidating the molecular mechanisms that pattern the sea urchin A-V axis.

In this study, we have investigated the role of LvNotch in mediating the position of the ectoderm-endoderm boundary using activated and dominant-negative forms of the receptor combined with lineage and mosaic analyses. We first demonstrate that activation of LvNotch signaling throughout the embryo shifts the ectoderm-endoderm boundary more animally along the A-V axis, whereas a loss or reduction of LvNotch signaling moves the boundary vegetally. Mosaic analyses of LvNotch function further show that LvNotch signaling has at least two distinct roles in specifying the position of the ectoderm-endoderm boundary. LvNotch signaling appears to function cell autonomously in the animal region of the embryo to promote endoderm formation more animally, while LvNotch signaling in cells vegetal to the ectoderm-endoderm border regulates a cell non-autonomous signal(s) that also establishes the endoderm higher along the A-V axis. Finally, we show that vegetal LvNotch signaling cell non-autonomously regulates nuclear β -catenin localization at the boundary, suggesting that vegetal LvNotch signaling may control the position of the boundary through the regulation of the Wnt pathway.

MATERIALS AND METHODS

Animals

Adult *Lytechinus variegatus* were obtained from Jennifer Jackson

(Beaufort, NC), and from Susan Decker (Hollywood, FL). Gametes were harvested and fertilized as described (Hardin et al., 1992). Embryos were cultured at 21-23°C in artificial sea water (ASW).

mRNA preparation and injection into zygotes

All LvNotch DNA constructs have been described (Sherwood and McClay, 1999), and were used as templates to generate in vitro transcribed 5' capped mRNAs using the T3 mMessage mMachine kit (Ambion). mRNAs were passed through Microspin G-50 columns (Pharmacia) to remove free nucleotides, precipitated and resuspended in double distilled H₂O. mRNA concentrations were determined, then mixed with glycerol (40% v/v) and injected into fertilized eggs (2.0-6.0 pg/zygote) as described (Mao et al., 1996; Sherwood and McClay, 1999).

mRNA/fluorescein dextran injection into eight-cell-stage embryos

Preparation of eight-cell stage embryos for injection into single blastomeres was identical to that described above, except that eggs were fertilized in 5 mM p-aminobenzoic acid (PABA), which was washed out immediately after fertilization. Treatment with PABA softened the fertilization membrane, allowing the embryos to be freed from the membranes, which adhered to the injection dish. To lineage and identify injected blastomeres, fluorescein dextran (M_r 40 \times 10³, Molecular Probes) was added to the mRNA/glycerol mixture at a concentration of 1.5 mg/ml. All mRNAs were injected at approximately 0.4-0.8 pg/blastomere at the eight-cell stage. Western analysis, whole-mount immunofluorescence, and the phenotypes of injected zygotes all indicated that fluorescein dextran did not affect the translation of injected mRNAs nor the phenotypes produced (see Fig. 7; data not shown). Similar to *Lytechinus pictus* embryos (see Henry et al., 1989), we noted that in *Lytechinus variegatus*, the third cleavage plane was usually slightly subequatorial. Thus, for consistency, we only injected blastomeres in eight-cell-stage embryos from batches of eggs producing embryos with slightly subequatorial third cleavage divisions. After injection, embryos were transferred to 50 mm petri dish lids coated with 1% agar, and analyzed under fluorescence at the 16-cell stage for embryos with healthy mesomere/mesomere or macromere/micromere pairs containing the mRNA/fluorescein dextran mix. These 16-cell-stage embryos were either labeled with DiI (below), or transferred to individual wells (coated with 1% agar) in a 96-well plate for culturing.

DiI labeling and lineaging of mesomeres and macromeres

Individual mesomeres and macromeres in 16-cell-stage embryos were labeled iontophoretically with DiI (5 mg/ml in ethanol; Molecular Probes; see Logan and McClay, 1997) on 1% agar coated dishes. The percentage of individual mesomeres and macromeres that normally contribute to the gut and ectoderm, respectively, was inferred from the lineage results of Logan and McClay (Logan and McClay, 1997). DiI- and fluorescein dextran-labeled plutei were imaged using a cooled CCD camera (Princeton Instruments) on a Leica DMRB microscope and Metamorph software.

Animal cap isolation

To target *LvN^{act}* mRNA into animal halves, eggs were fertilized in 5 mM PABA and injected with *LvN^{act}* mRNA as described above. Following injection, PABA was washed out of the injection plate and embryos were cultured until the eight-cell stage. The embryos were then transferred to a 1% agar-coated dish containing hyaline extraction media (Fink and McClay, 1985). After a 2 minute incubation, embryos were transferred to an agar-coated dish containing Ca²⁺-free seawater, where animal and vegetal halves of embryos were separated using a fine glass needle. Animal halves were identified by the distinctive pattern of mesomere cleavage divisions, and cultured in 96-well plates containing ASW. Embryoids resulting from these animal caps were fixed with methanol after 48 hours of

development as described (Sherwood and McClay, 1999), and stained with the Endo1 monoclonal antibody (Wessel and McClay, 1985).

Immunolocalization, cell counts and image analysis

Late mesenchyme blastula embryos injected with *LvN^{act} Δ ANK5* and *LvN^{act}* were fixed with 2% paraformaldehyde in ASW, followed by rapid permeabilization with 100% methanol as described (Sherwood and McClay, 1999). To identify the *LvN^{act}* or *LvN^{act} Δ ANK5* proteins, embryos were stained with the intracellular directed *LvNotch* polyclonal antibody, rabbit anti-ANK at 1:1000 dilution (Sherwood and McClay, 1997), which revealed the overexpressed intracellular domain of *LvNotch*, but not endogenous *LvNotch*. The number of cells expressing *LvN^{act}* or *LvN^{act} Δ ANK5* was determined by optically sectioning stained embryos with a Zeiss 510 laser-scanning confocal microscope and counting the total number of nuclei containing these proteins. Nuclear β -catenin was visualized with a guinea pig anti- β -catenin polyclonal antibody as previously described (Logan et al., 1999). *LvN^{act}*/ β -catenin double stained images were obtained by sequential confocal sectioning of double-labeled embryos, and these images were overlaid using Adobe Photoshop.

RESULTS

LvNotch signaling influences the position of the ectoderm-endoderm boundary

To determine whether *LvNotch* is involved in positioning the ectoderm-endoderm boundary, *LvNotch* signaling was perturbed within embryos by injecting fertilized eggs with mRNA encoding a constitutively activated (*LvN^{act}*) or dominant negative (*LvN^{neg}*) form of the receptor (see Sherwood and McClay, 1999). The activated form of *LvNotch* consists solely of the intracellular domain of the receptor, whereas the dominant negative form contains the extracellular and transmembrane domains of *LvNotch*, but lacks the intracellular domain. We have previously shown that after injection into fertilized eggs, the protein products of both constructs are expressed through the late mesenchyme blastula stage (Sherwood and McClay, 1999), the time at which molecular markers and manipulation studies suggest the sea urchin ectoderm-endoderm boundary is established (Davidson et al., 1998; Logan and McClay, 1998).

To assess whether *LvNotch* signaling affects the position of the ectoderm-endoderm boundary, we combined lineage analysis of individually labeled mesomeres at the 16-cell stage with injection of *LvN^{act}* and *LvN^{neg}* mRNA (Fig. 1A). The fate of mesomeres is a sensitive indicator of the position of the ectoderm-endoderm border. Lineage studies have indicated that the 16-cell stage mesomeres are usually positioned slightly animally to the ectoderm-endoderm boundary and give rise solely to ectoderm (Logan and McClay 1997). However, the mesomeres are close enough to the boundary such that approximately 16% of these cells randomly contribute progeny to both the ectoderm and the endoderm (the gut tissue in pluteus larvae). A shift in the ectoderm-endoderm boundary animally would thus be expected to increase and a vegetal shift of the border to decrease the number of mesomeres that contribute progeny to the gut.

In close agreement with previous lineage studies (Logan and McClay 1997), labeled mesomeres contributed progeny to larval gut tissue in approximately 20% of embryos injected with glycerol or with *LvN^{act} Δ ANK5*, a control construct similar to *LvN^{act}* but missing 14 amino acids required for signaling

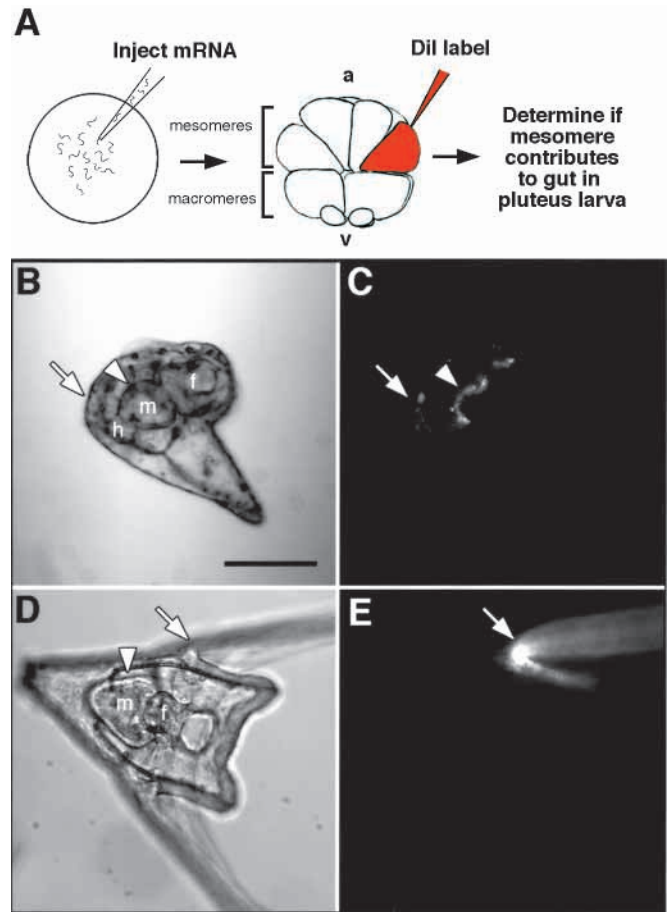


Fig. 1. Activation and inhibition of *LvNotch* signaling throughout the embryo shifts the ectoderm-endoderm boundary. (A) Zygotes were injected with *LvNotch* mRNA constructs and allowed to develop. At the 16-cell stage, individual mesomeres were labeled with DiI, and the distribution of the descendants of these cells determined in 36–42 hour pluteus larvae. (B,C) Nearly all labeled mesomeres from embryos injected with *LvN^{act}* contributed progeny to the gut (Table 1). A lateral view of a larva from a *LvN^{act}* injected embryo (B) shows that the DiI labeled mesomere (C) contributed descendants to the aboral ectoderm (arrow), and all three endoderm-derived gut compartments (h,m,f in B; arrowhead). (D,E) Almost no labeled mesomeres from embryos injected with *LvN^{neg}* contributed descendants to the gut (Table 1). A larva from a *LvN^{neg}* injected embryo (D) shows that the DiI labeled mesomere (E) contributed progeny to the ectoderm along the left arm (arrow), but not to the gut (arrowhead). The characteristic smaller size of larvae from *LvN^{act}* injected embryos (B) and larger appearance of larvae from *LvN^{neg}* injected embryos (D) was probably the result of a respective decrease and increase in the amount of ectoderm present after the shift in the ectoderm-endoderm boundary, as the amount of ectoderm in the embryo is thought to regulate the size of sea urchin larvae (Ettensohn and Malinda, 1993). a, animal pole; f, foregut; h, hindgut; m, midgut; v, vegetal pole. Scale bar: 100 μ m.

(Sherwood and McClay, 1999; Table 1). The injection procedure and protein overexpression thus had no effect on the lineage of mesomeres. Injection of *LvN^{act}*, however, dramatically influenced mesomere fate: individually labeled mesomeres contributed progeny to the gut in greater than 90% of injected embryos (Table 1; Fig. 1B,C). Furthermore,

Table 1. Influence of LvNotch signaling on mesomere fate

Percentage of lineaged mesomeres (16-cell stage) that contributed progeny to the larval gut*				
Glycerol injected (n)	<i>LvN^{act}ΔANK5</i> injected (n)	<i>LvN^{act}</i> injected (n)	<i>LvN^{full}</i> injected (n)	<i>LvN^{neg}</i> injected (n)
20% (9/44)	22% (5/23)	93% (14/15)‡	48% (14/29)‡	2% (1/48)‡

*Contribution of lineaged mesomeres to the gut was determined in 36-42 hour pluteus larvae. For each treatment, the data was pooled from four to ten independent experiments performed on approximately equal numbers of embryos.

‡Percentage is significantly different compared with respective controls ($P < 0.02$; Fisher's exact test): *LvN^{act}ΔANK5*-, *LvN^{act}*- and *LvN^{full}*-injected animals were compared with glycerol-injected embryos, while *LvN^{neg}*-injected animals were compared with both glycerol- and *LvN^{full}*-injected embryos.
n, number of embryos examined.

progeny of mesomeres that contributed to the gut in *LvN^{act}* injected embryos were typically found in all three gut compartments ($n=12/14$ cases where the mesomere contributed descendants to the gut). In contrast, in *LvN^{act}ΔANK5*- and glycerol- (control) injected embryos, mesomere descendants found in the gut were never distributed throughout the gut. Rather, the descendants were usually confined to the hindgut ($n=9/14$ combined cases where the mesomere contributed progeny to the gut), which arises from endoderm cells nearest the ectoderm-endoderm boundary (Ruffins and Etensohn, 1996).

To determine whether endogenous LvNotch signaling participates in the positioning of the ectoderm-endoderm boundary, mesomere lineages in *LvN^{neg}*-injected embryos were also examined. Expression of *LvN^{neg}* throughout the embryo had an opposite effect on mesomere fate compared with *LvN^{act}*: only one labeled mesomere contributed progeny to the gut (2% of cases; Table 1, Fig. 1D,E), and these progeny were restricted to the hindgut. Injection of mRNA encoding full-length LvNotch receptor, *LvN^{full}*, did not reduce the percentage of mesomeres contributing to gut tissue, demonstrating that the *LvN^{neg}* protein has a specific dominant negative function, rather than acting as a nonspecific sink for ligands of other signaling pathways that may also bind to the extracellular domain of Notch proteins (see Rebay et al., 1991; Wesley, 1999). Indeed, overexpression of *LvN^{full}* increased the percentage of mesomeres that contributed progeny to the gut compared with *LvN^{act}ΔANK5*- and glycerol- (control) injected embryos (Table 1), indicating that full-length LvNotch overexpression activates the pathway involved in ectoderm-endoderm boundary positioning. Taken together, these results show that LvNotch influences the position of the ectoderm-endoderm boundary by promoting endoderm formation more animally in the embryo: activation of LvNotch signaling positions the ectoderm-endoderm boundary more animally along the A-V axis, whereas loss of LvNotch signaling shifts the border vegetally.

LvNotch has distinct functions in the vegetal and animal portions of the embryo in mediating ectoderm-endoderm boundary position

Several possible mechanisms exist by which LvNotch may regulate the position of the ectoderm-endoderm boundary. Previous work has demonstrated that the vegetal-most cells, the micromeres, initiate a sequential vegetal-to-animal wave of inductive signaling at the 16-cell stage that specifies overlying

SMCs and endoderm during early development (reviewed in Davidson et al., 1998). Recent studies indicating that micromere signaling induces overlying SMC fate by activating LvNotch in adjacent presumptive SMCs (Sweet et al., 1999; McClay et al., 2000), suggests the possibility that LvNotch signaling within the presumptive SMCs is a component of the inductive signaling wave. Alternatively, LvNotch signaling could function cell autonomously within the presumptive endoderm cells to establish the ectoderm-endoderm boundary more animally. LvNotch protein is expressed at high levels along the apical domain of presumptive endoderm cells in the late blastula embryo (Sherwood and McClay, 1997), coincident with the time that the ectoderm-endoderm boundary is thought to be established (Davidson et al., 1998; Logan and McClay, 1998). Finally, it is possible that LvNotch signaling could act cell non-autonomously within the ectoderm to position the boundary. LvNotch is expressed at low levels throughout the presumptive ectoderm (Sherwood and McClay, 1997), and molecular and cellular studies have suggested that the ectoderm also has an important role in positioning the boundary (Henry et al., 1989; Angerer et al., 2000). Based on these possible distinct signaling functions for LvNotch, a mosaic analysis was performed to elucidate the mechanism(s) by which LvNotch signaling regulates the position of the ectoderm-endoderm boundary.

Vegetal LvNotch signaling mediates the position of the ectoderm-endoderm boundary cell non-autonomously

To address whether vegetal LvNotch signaling influences the position of the ectoderm-endoderm boundary by controlling another inductive signal, the lineage of uninjected mesomeres overlying *LvN^{act}*- and *LvN^{neg}*-injected macromere/micromere pairs was determined (Fig. 2A). mRNAs were co-injected with the lineage marker fluorescein dextran into single blastomeres at the eight-cell stage, the last stage at which we found consistent mRNA injection into single blastomeres technically possible. Injected embryos were then followed to the 16-cell stage when the asymmetric fourth cleavage division allowed embryos with injected macromere/micromere pairs to be identified. Individual mesomeres overlying injected macromeres were then labeled with DiI to follow the fate of their descendants (Fig. 2A). If vegetal LvNotch signaling regulates another inductive signal that positions the ectoderm-endoderm boundary, mesomeres overlying macromeres with increased levels of LvNotch signaling (*LvN^{act}* injected) would be expected to contribute to gut tissue at a higher frequency than controls. Conversely, mesomeres overlying macromeres with reduced LvNotch signaling (*LvN^{neg}* injected) would be predicted to contribute to the gut less frequently.

Labeled mesomeres overlying *LvN^{act}*-injected macromeres contributed to the gut in greater than 95% of embryos versus 24% in *LvN^{act}ΔANK5*-injected controls (Table 2; Fig. 2B,C). Furthermore, descendants of mesomeres that overlay *LvN^{act}*-injected macromeres contributed to more gut tissue than in controls. Labeled descendants were sometimes found in all gut compartments ($n=4/26$ cases where mesomere contributed descendants to the gut), whereas in *LvN^{act}ΔANK5* macromere-injected embryos, mesomere descendants were typically restricted to the hindgut ($n=8/10$ cases where the mesomere contributed progeny to the gut) and never found in the foregut.

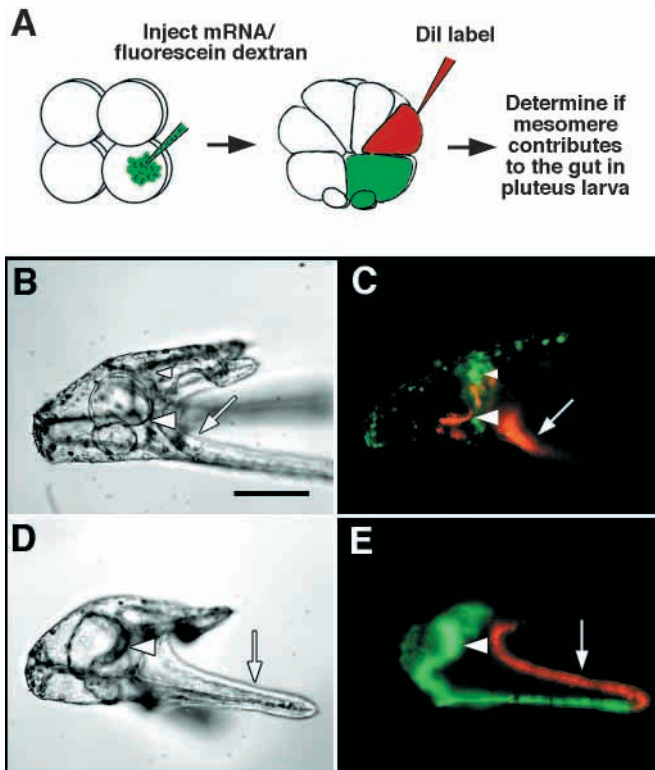


Fig. 2. Alteration of vegetal LvNotch signaling shifts the ectoderm-endoderm boundary cell non-autonomously in overlying animal cells. (A) LvNotch mRNAs were co-injected with the lineage tracer fluorescein dextran into individual blastomeres at the eight-cell stage. Embryos containing injected macromere/micromere pairs were isolated at the 16-cell stage, and single mesomeres overlying injected macromeres were labeled with DiI. The fates of DiI labeled mesomeres were then determined in pluteus larvae. (B,C) Activation of LvNotch signaling vegetally within macromere/micromere pairs increased the percentage of overlying uninjected mesomeres that contributed progeny to gut tissue (Table 2). A larva that had a DiI-labeled mesomere (C, red) labeled over a *LvN^{act}* injected macromere/micromere pair (C, green) is shown. The overlying mesomere contributed cells both to the gut (large arrowhead), and oral ectoderm (arrow). The injected micromere gave rise to primary mesenchyme cells (PMCs), which are not visible because of decreased staining after fusion with uninjected PMCs (see Hodor and Ettensohn, 1998). The injected macromere gave rise predominantly to SMCs (scattered green cells within the larvae) in response to activated LvNotch, as well as a small portion of foregut tissue (small arrowhead). (D,E) In contrast, inhibition of endogenous vegetal LvNotch decreased the percentage of overlying mesomeres that contributed progeny to the gut (Table 2). Shown is an example of a larva (D) that had a DiI labeled mesomere (E, red) labeled over a *LvN^{mes}* injected macromere/micromere pair (E, green). The overlying mesomere contributed progeny only to ectoderm, along the right arm (arrow). The macromere has given rise to tissue in all three gut compartments (arrowhead) and ectoderm, but not SMCs. Scale bar: 100 μ m.

Inhibition of LvNotch signaling in macromere descendants via injection of *LvN^{mes}* resulted in overlying mesomeres contributing cells to the gut in only 2% of cases, compared with 24% in *LvN^{act} Δ ANK5*- and 35% in *LvN^{full}*-injected controls (Table 2; Fig. 2D,E). These results indicate that endogenous vegetal LvNotch signaling acts cell non-autonomously to

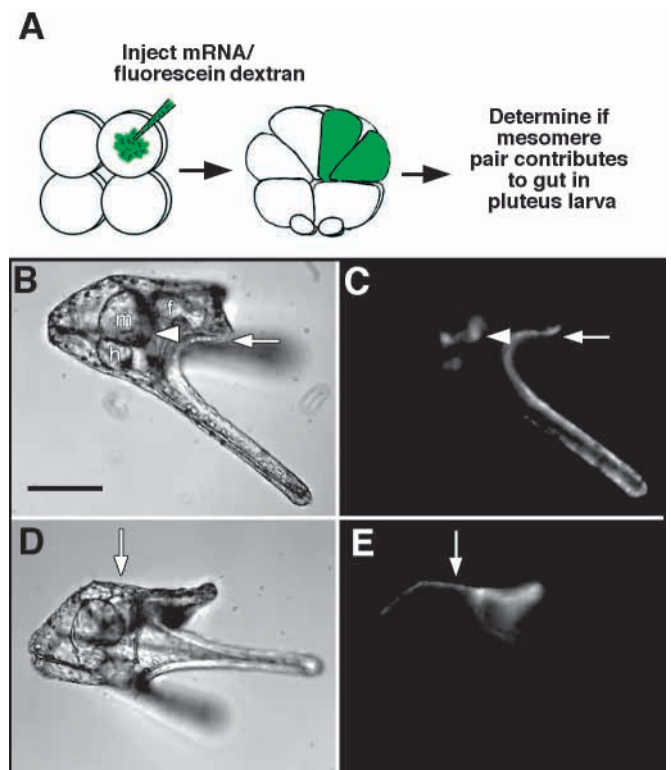


Fig. 3. Perturbation of LvNotch signaling within the animal region of the embryo alters ectoderm-endoderm boundary position. (A) LvNotch mRNAs were co-injected with the lineage tracer fluorescein dextran into blastomeres at the eight-cell stage. Embryos in which injected blastomeres gave rise to pairs of mesomeres at the 16-cell stage were isolated, and the fate of the injected mesomere pairs determined in pluteus larvae. (B,C) Activation of LvNotch signaling within mesomeres increased the percentage of these cells that contributed progeny to the gut compared with controls (Table 2). An example of a pluteus larva (B) in which the *LvN^{act}* injected mesomere pair (C) contributed descendants to ectoderm along the ciliated band (arrow) and right arm, as well as gut tissue up to the midgut/foregut boundary (arrowhead). (D,E) Conversely, inhibition of endogenous LvNotch signaling within mesomeres reduced the number of mesomeres that contributed descendants to the gut compared with controls (Table 2). A representative pluteus larva is shown (D); the mesomere pair injected with *LvN^{mes}* (E) contributed progeny only to aboral ectoderm (arrow) and not the gut. Scale bar: 100 μ m.

influence ectoderm-endoderm boundary position, regulating another inductive signal that promotes endoderm formation in overlying cells.

LvNotch signaling also functions in mesomeres to regulate ectoderm-endoderm boundary position

To determine whether LvNotch also functions within the animal region of the embryo to promote endoderm formation more animally, LvNotch mRNA constructs and fluorescein dextran were co-injected at the eight-cell stage, and embryos with injected mesomere pairs at the 16-cell stage were followed (Fig. 3A). Based on the known lineage of mesomeres (Logan and McClay, 1997), these constructs would be expressed primarily within the presumptive ectoderm; however, the vegetal-most extent of these clones would be

Table 2. Mosaic analysis of LvNotch function in ectoderm-endoderm boundary positioning

Lineage followed	Percentage of lineaged mesomeres or mesomere pairs (16-cell stage) that contributed progeny to the larval gut*			
	<i>LvN^{act}ΔANK5</i> injected (n)	<i>LvN^{act}</i> injected (n)	<i>LvN^{full}</i> injected (n)	<i>LvN^{neg}</i> injected (n)
Mesomere overlying injected macromere	24% (10/41)‡	96% (26/27)§	35% (7/20)	2% (1/43)§
Pairs of mesomeres injected with mRNA	36% (15/41)	64% (37/58)§	52% (13/25)	12% (5/43)§
Mesomere adjacent to injected mesomere pair	N.D.	17% (6/35)¶	N.D.	N.D.

*Contribution of lineaged mesomeres to the gut was determined in 36-42 hour pluteus larvae. For each treatment, the data was pooled from 5 to sixteen independent experiments performed on approximately equal numbers of embryos.
‡,¶The percentage of individual mesomeres contributing to the gut obtained in ‡ was used as a control for comparison with the percentage found in experiment ¶, and was not found to be significantly different ($P=0.57$; Fisher's exact test).
§Percentage is significantly different compared with respective controls ($P<0.01$; Fisher's exact test): *LvN^{act}*- and *LvN^{full}*-injected animals were compared with injected *LvN^{act}ΔANK5* embryos, while *LvN^{neg}*-injected animals were compared with both *LvN^{act}ΔANK5*- and *LvN^{full}*-injected embryos.
n, number of embryos examined; N.D., not determined.

situated near or at the ectoderm-endoderm boundary. We reasoned that if LvNotch signaling functions either cell non-autonomously within the ectoderm or acts cell autonomously within endoderm cells to promote endoderm formation more anally, that perturbation of LvNotch signaling within mesomere pairs would alter the frequency at which they contributed cells to the endoderm.

LvN^{act}-injected mesomere pairs contributed cells to the gut nearly twice as frequently as observed in *LvN^{act}ΔANK5*-injected controls (64% versus 36%; Table 2; Fig. 3B,C). Descendants of *LvN^{act}*-injected mesomere pairs found in the gut also contributed more extensively to gut tissue, contributing to all gut compartments in seven of 37 cases. In four cases, mesomere descendants even formed ectopic gut tissue composed exclusively of fluorescently labeled cells (Fig. 4A-C), suggesting that LvNotch signaling may function cell autonomously within the descendants of mesomeres to promote endoderm formation. In contrast, descendants of *LvN^{act}ΔANK5*-injected mesomere pairs were usually confined to the hindgut ($n=12/15$ cases), and never found in the foregut or in ectopic protrusions of gut tissue. Demonstrating a required role for LvNotch in promoting endoderm formation in mesomere descendants, mesomere pairs injected with *LvN^{neg}* contributed descendants to the gut 12% of the time, a significantly lower frequency compared with *LvN^{act}ΔANK5*- and *LvN^{full}*-injected controls (Table 2; Fig. 3D,E). Taken together, these lineage results indicate that LvNotch signaling also functions within the animal region of the embryo to promote endoderm formation, possibly through a cell-autonomous mechanism.

LvNotch promotes endoderm formation cell autonomously in mesomere descendants

Although the above experiments were suggestive of a cell autonomous mechanism for LvNotch signaling within the

mesomere descendants, they did not rigorously exclude the possibility of a cell non-autonomous role for LvNotch in these cells. We, thus, tested the possibility that LvNotch signaling influences the position of the ectoderm-endoderm boundary through the control of a cell non-autonomous lateral signal by DiI labeling uninjected mesomeres that neighbor *LvN^{act}*-injected mesomere pairs (Fig. 5A). If LvNotch signaling within the animal region of the embryo promotes the formation of endoderm by regulating a lateral signal, uninjected mesomeres neighboring *LvN^{act}*-injected mesomeres would be expected to contribute descendants to the gut at a higher frequency than controls. The descendants of DiI-labeled mesomeres that neighbor *LvN^{act}*-injected mesomeres, however, contributed to the gut at a similar frequency as control embryos (Table 2; Fig. 5B,C), strongly suggesting that LvNotch signaling within the animal region does not regulate a lateral signal.

We next examined whether LvNotch promotes endoderm formation within the animal region of the embryo by controlling a cell non-autonomous signal that acts along the A-V axis. Uninjected macromeres underlying *LvN^{act}*-injected mesomere pairs were labeled with DiI (Fig. 5D), and the ectoderm contribution of these cells was determined (Fig. 5E). Previous lineage studies have indicated that individually labeled macromeres contribute cells to the ectoderm in most cases (90% or greater, see Logan and McClay, 1997). If LvNotch signaling controls a signal in the animal region that acts along the A-V axis to promote endoderm formation, macromeres underlying *LvN^{act}*-injected mesomeres should be converted to an endoderm fate, and thus contribute descendants to the ectoderm at a reduced frequency or number. The percentage of cases in which macromere descendants contributed cells to the ectoderm, however, was not significantly different between macromeres underlying control injected *LvN^{act}ΔANK5* and experimentally injected *LvN^{act}* mesomere pairs (95% versus 91%; $n=18/19$ and

Fig. 4. Ectopic gut tissue from constitutive activation of LvNotch signaling in mesomeres consists solely of *LvN^{act}* injected cells. (A-C) Lateral and enlarged anal (inset) views of a pluteus larva in which the mesomere pair injected with *LvN^{act}* gave rise to aboral ectoderm and additional gut tissue (arrow) attached to the normal hindgut (arrowhead). Note that the ectopic gut tissue in (A) is composed exclusively of fluorescent *LvN^{act}* injected cells (B, images overlaid in C). Scale bars: 100 μ m (25 μ m in the insets).

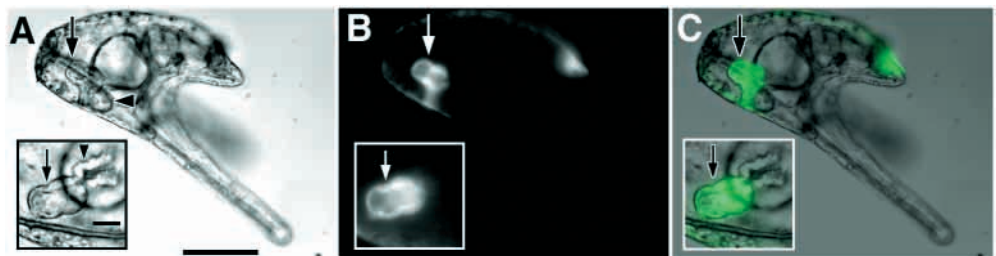
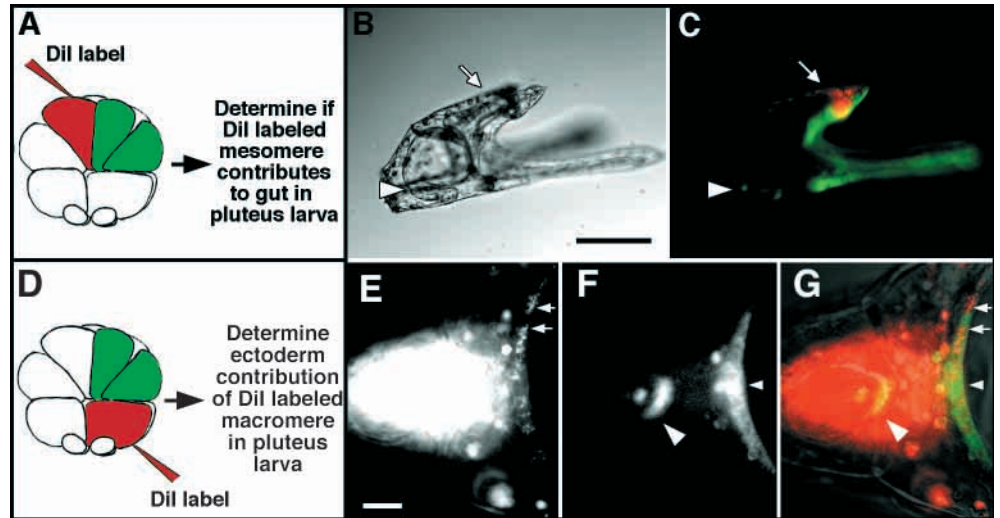


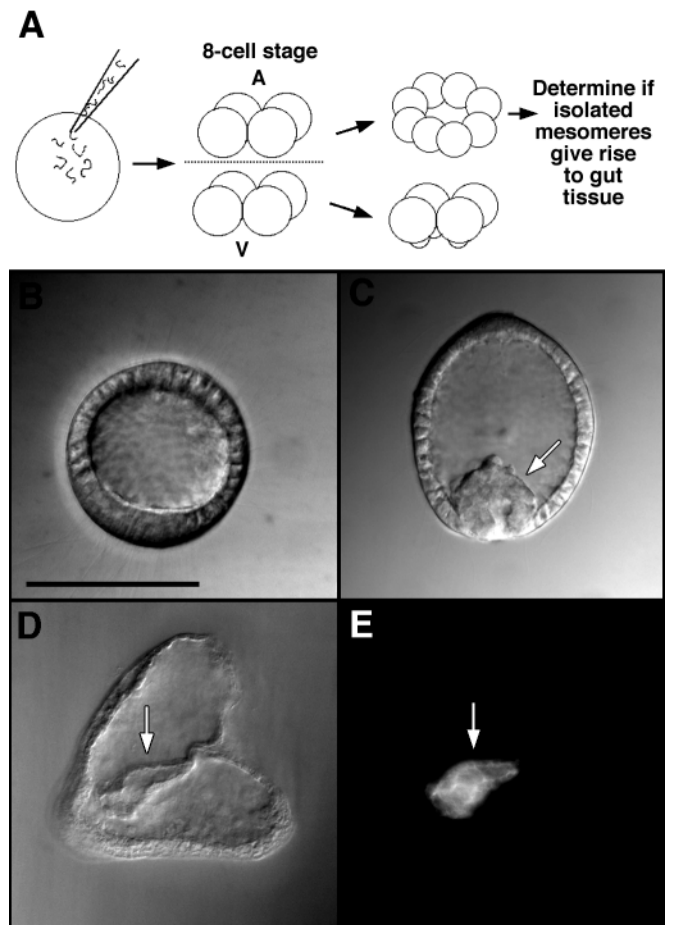
Fig. 5. The position of the ectoderm-endoderm boundary is not altered in cells neighboring *LvN^{act}*-injected mesomere pairs. (A) Uninjected mesomeres neighboring *LvN^{act}*/fluorescein dextran-injected mesomere pairs were lineage labeled with DiI. In contrast to *LvN^{act}*-injected mesomeres, neighboring DiI-labeled cells did not contribute progeny to the gut at an increased frequency (Table 2). (B,C) Lateral view of a pluteus larva (B) shows that the *LvN^{act}*-injected mesomere pair (C, green) contributed progeny to the gut tissue (arrowhead) and oral ectoderm, while the DiI labeled mesomere (C, red) gave rise only to aboral ectoderm (arrow). (D) Uninjected macromeres underlying pairs of *LvN^{act}* or *LvN^{act} Δ ANK5*/fluorescein dextran-injected mesomeres were labeled with DiI. No difference in ectoderm contribution was found for macromeres underlying *LvN^{act}*- versus *LvN^{act} Δ ANK5*-injected mesomeres in pluteus larvae. (E-G) Anal view of a larva showing the lineage of a DiI labeled macromere underlying a pair of mesomeres injected with *LvN^{act}*/fluorescein-dextran. (E) The DiI-labeled macromere has given rise primarily to gut (overexposed and out of focus fluorescence in center and left); however, the macromere has also contributed to a number of anal ectoderm cells bordering the ciliated band (arrows; individual cells clearly distinguished by perinuclear membrane staining). (F) The pair of *LvN^{act}*- and fluorescein dextran-injected mesomeres has given rise to ectoderm (small arrowhead; mostly along oral surface and not in view – similar to mesomere in C), as well as gut tissue (large arrowhead). (G) Overlay of DiI-labeled macromere descendants (red), *LvN^{act}*- and fluorescein dextran-injected mesomere descendants (green), and DIC image (gray). Scale bar: 100 μ m in B,C; 25 μ m in E-G.



21/23, respectively; $P=1.0$, Fisher's exact test). Moreover, the mean number of ectoderm cells contributed by macromere descendants underlying *LvN^{act} Δ ANK5*- and *LvN^{act}*-injected mesomeres was not significantly different (27.5 ± 3.3 versus 26.6 ± 5.4 ; $n=19$ and 23 , respectively; $P=0.9$, two-sample t -test), offering compelling evidence that LvNotch signaling within the animal region of the embryo does not promote endoderm formation by regulating a cell non-autonomous signal along the A-V axis.

As a direct test of whether LvNotch signaling can function cell autonomously within mesomeres to promote endoderm formation, we isolated animal caps from untreated and *LvN^{act}*-injected embryos (Fig. 6A). In untreated embryos, isolated animal caps form ciliated epithelial embryoids that are devoid of endoderm (Fig. 6B; Horstadius, 1973). Animal halves from embryos injected with *LvN^{act}*, however, formed endoderm tissue in 51% of the cases examined ($n=18/35$): invagination of archenteron tissue was observed by 24 hours of development, and this tissue expressed the hindgut/midgut marker, *Endo1*, by 48 hours of development (Fig. 6C-E). Together with the lineage experiments, these animal cap isolation results are indicative of a cell-autonomous role for LvNotch signaling within the animal region of the embryo in promoting endoderm formation.

Fig. 6. Isolated animal caps containing constitutively activated LvNotch form gut tissue. (A) Zygotes were injected with *LvN^{act}* and cultured to the eight-cell stage, when animal and vegetal halves were separated. (B) A 24 hour untreated animal cap has developed into a ciliated ectodermal vesicle, containing no endoderm. (C) In contrast, an archenteron has begun to invaginate in a 24 hour animal cap containing *LvN^{act}*, which (by 48 hours) has given rise to gut tissue (D, arrow) that expresses the hindgut/midgut marker *Endo1* (E, arrow). Scale bar: 100 μ m.



Perturbation of LvNotch signaling does not alter cell proliferation within descendants of injected macromeres or mesomere pairs

Notch signaling has been shown in several developmental contexts to influence cell proliferation (Go et al., 1998; Johnston and Edgar, 1998; Carlesso et al., 1999; Walker et al., 1999). We therefore examined whether the vegetal or animal effects of LvNotch signaling on ectoderm-endoderm boundary positioning might include alterations in proliferation, either through an expansion of cells over or retraction of cells from the boundary. Macromere/micromere and mesomere pairs were injected with *LvN^{act}* or *LvN^{act}ΔANK5* mRNA as described above (see Figs 2A, 3A). As *LvN^{act}* and *LvN^{act}ΔANK5* localize tightly to the nucleus in all cells that express these proteins (Sherwood and McClay, 1999), we looked at whether activation of LvNotch signaling alters proliferation by examining the number and position of cells containing nuclear-localized *LvN^{act}* and *LvN^{act}ΔANK5* proteins at the late mesenchyme blastula stage (Fig. 7, Table 3). This stage was chosen since the ectoderm-endoderm boundary is thought to be established at this time (Davidson et al., 1998; Logan and McClay, 1998). Furthermore, the expression of *LvN^{act}* in injected blastomeres is lost shortly after the late blastula stage, indicating that the direct effects of altering LvNotch signaling must occur prior to or near this time. The mean number of descendants of injected macromeres and mesomere pairs expressing *LvN^{act}*, as well as the distribution of these cells along the A-V axis, however, was not significantly different from embryos containing the *LvN^{act}ΔANK5* protein (Table 3). Thus, LvNotch signaling does not appear to regulate the position of the ectoderm-endoderm boundary either vegetally or animally by influencing cell proliferation.

Interaction of LvNotch signaling and nuclear β-catenin at the ectoderm-endoderm boundary

In the sea urchin, nuclear localized β-catenin, a component of the Wnt signaling pathway (Wodarz and Nusse, 1998), is present in presumptive endoderm cells bordering the presumptive ectoderm in the late mesenchyme blastula stage (D. R. Sherwood, PhD thesis, Duke University, Durham, NC, 1997; Logan et al., 1999). Formation of the dorsal-ventral

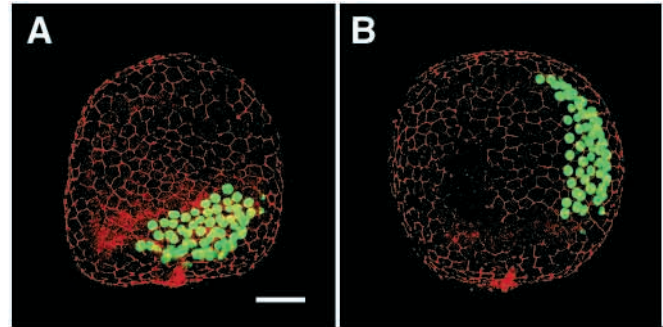


Fig. 7. Distribution and number of *LvN^{act}*- and *LvN^{act}ΔANK5*- injected macromere and mesomere pair descendants in the late mesenchyme blastula embryo. Embryos are shown viewed along the A-V axis and co-immunostained for the *LvN^{act}* protein product (green), which localizes to the nucleus, and β-catenin (red), which localizes to all epithelial adherens junctions, thus outlining the shape of the embryo. The number and distribution of injected macromere or mesomere pair descendants containing *LvN^{act}* or *LvN^{act}ΔANK5* was determined by examining the number and distribution of nuclei containing the *LvN^{act}* or *LvN^{act}ΔANK5* proteins. (A) An example of *LvN^{act}* distribution in an embryo in which a macromere/micromere pair was injected with *LvN^{act}*. (B) A typical example of *LvN^{act}* distribution in an embryo in which a mesomere pair was injected with *LvN^{act}*. Embryos injected with *LvN^{act}* showed similar nuclear distributions and numbers of cells expressing the *LvN^{act}* protein, as compared with those injected with *LvN^{act}ΔANK5* (Table 3). Scale bar: 25 μm.

boundary in the *Drosophila* wing requires the coordination of both Wnt and Notch signaling (Diaz-Benjumea and Cohen, 1995; Rulifson and Blair, 1995; Neumann and Cohen, 1996; de Celis and Bray, 1997; Micchelli et al., 1997). Therefore, the relationship of LvNotch signaling to the localization of nuclear β-catenin at the ectoderm-endoderm boundary was analyzed to determine if the Wnt and Notch signaling pathways cooperate to mediate the position of this boundary.

We first asked whether LvNotch signaling regulates the localization of nuclear β-catenin at the ectoderm-endoderm boundary by determining β-catenin distribution in embryos injected with *LvN^{act}* and *LvN^{neg}*. Paralleling the effects on

Table 3. Alteration of LvNotch signaling does not affect cell proliferation in descendants of injected macromeres or mesomeres

Number and distribution of descendants in injected macromere and mesomere pairs*				
mRNA injected	Number of macromere descendants‡ (n)	Animal-most extent of macromere descendants§ (n)	Number of mesomere descendants (n)	Vegetal-most extent of mesomere descendants¶ (n)
<i>LvN^{act}ΔANK5</i>	71.8±2.9 (16)	41.9±2.2% (19)	111.7±3.8 (16)	66.8±2.0% (18)
<i>LvN^{act}</i>	75.0±3.2 (21)	43.6±1.7% (21)	111.4±3.7 (17)	68.4±1.7% (18)

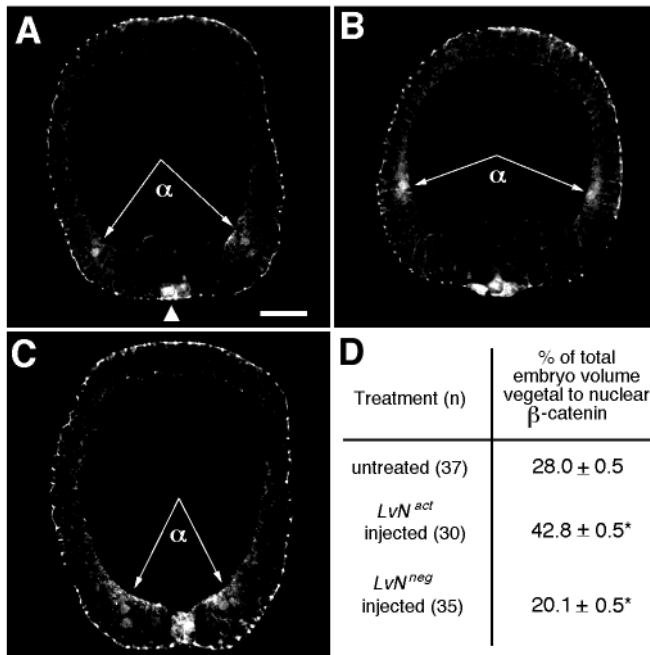
*Embryos with injected macromere or mesomere pairs were cultured to the late mesenchyme blastula stage (13–14 hours post-fertilization), fixed and stained with intracellular directed LvNotch antibody to determine the number and distribution of cells containing *LvN^{act}* or *LvN^{act}ΔANK5* protein products (see also Materials and Methods, and Fig. 7). Data were pooled from three to four independent experiments for each treatment.

‡Although our injection scheme placed mRNA into micromeres as well as macromeres (see Fig. 2A), the micromere descendants (primary mesenchyme cells) lost *LvN^{act}ΔANK5* and *LvN^{act}* protein expression shortly after ingress in the mid-blastula embryo, and thus were not counted at this later stage.

§The animal-most extent of macromere descendants was calculated by determining the distance of the animal-most cell that expressed nuclear *LvN^{act}* or *LvN^{act}ΔANK5* from the vegetal pole and dividing this by the total length of the embryo along the A-V axis. This value was then expressed as a percentage of the total length along the embryo.

¶The vegetal-most extent of mesomere descendants was calculated by determining the distance of the vegetal-most cell that expressed nuclear *LvN^{act}* or *LvN^{act}ΔANK5* from the animal pole and dividing this by the total length of the embryo along the A-V axis. This value was then presented as a percentage of the total length along the embryo. The mean number and distribution (± s.e.m.) of macromere or mesomere descendants injected with *LvN^{act}* or *LvN^{act}ΔANK5* within every column was not significantly different ($P>0.47$; two-sample *t*-test).

n, number of embryos examined.

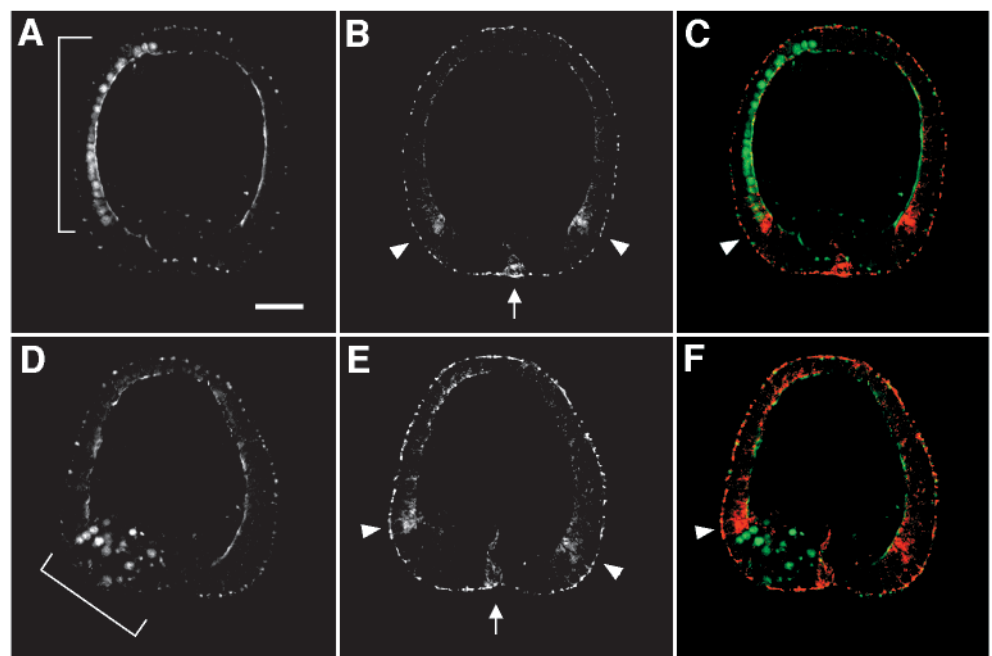


ectoderm-endoderm position, injection of *LvN^{act}* shifted nuclear localized β -catenin more animally along the A-V axis, leading to an approximate 50% increase in the volume of the embryo vegetal to nuclear β -catenin, compared with untreated controls (Fig. 8A,B,D). Furthermore, injection of *LvN^{neg}* shifted nuclear β -catenin localization lower such that the volume of the embryo vegetal to nuclear β -catenin decreased by approximately 30% (Fig. 8C,D). These results demonstrate that LvNotch signaling regulates the nuclear localization of β -catenin, and suggest that LvNotch may at least in part mediate

the position of the ectoderm-endoderm boundary through the control of β -catenin localization at the border.

As experiments in the previous sections indicated that LvNotch promotes endoderm formation both cell autonomously within the animal region of the embryo and cell non-autonomously vegetally, we next asked whether both signaling functions of LvNotch regulated nuclear β -catenin localization at the boundary. *LvN^{act}* mRNA was placed into macromere/micromere and mesomere/mesomere pairs (as in Figs 2A, 3A), and the relationship of the nuclear-localized *LvN^{act}* protein was compared with nuclear β -catenin distribution in late mesenchyme blastula embryos. Injection of *LvN^{act}* into pairs of micromeres did not shift the localization of β -catenin at the ectoderm-endoderm boundary ($n=35/35$

Fig. 9. Vegetal LvNotch signaling shifts the localization of nuclear β -catenin at the ectoderm-endoderm boundary cell non-autonomously. (A-C) *LvN^{act}* was injected into pairs of mesomeres placing activated LvNotch at or slightly above the ectoderm-endoderm boundary. Co-immunostaining of late mesenchyme blastula embryos with an intracellular directed LvNotch antibody and with a β -catenin antibody revealed the *LvN^{act}* protein in nuclei of injected cells (A, bracket), and nuclear β -catenin at the ectoderm-endoderm boundary (B, arrowheads) as well as in the small micromeres (B, arrow). An overlay (C) of *LvN^{act}* (green) and β -catenin (red) staining shows that *LvN^{act}* did not shift the localization of nuclear β -catenin animally, even when expressed in cells directly neighboring nuclear β -catenin (arrowhead; compare left and right sides of embryo). (D-E) In contrast, an example of an embryo with *LvN^{act}* injected into a macromere/micromere pair (bracket, D) shows that the localization of nuclear β -catenin (E; arrowheads) was shifted animally on the side of the embryo containing *LvN^{act}*. An overlay (F) of *LvN^{act}* (green) and β -catenin (red) staining reveals the shift of nuclear β -catenin (arrowhead) into cells that do not contain activated LvNotch. Scale bar: 25 μ m.



embryos), even when LvN^{act} directly bordered or overlapped nuclear β -catenin (Fig. 9A-C). Injection of LvN^{act} into vegetal macromere/micromere pairs, however, led to a clear shift in the localization of nuclear β -catenin more animally into neighboring cells lacking LvN^{act} ($n=30/33$ embryos; Fig. 9D-F). Vegetal $LvNotch$ signaling can thus regulate nuclear β -catenin localization cell non-autonomously, and may control the position of the ectoderm-endoderm boundary by regulating a signal(s) that positions nuclear β -catenin at the border.

DISCUSSION

The results presented in this work demonstrate that $LvNotch$ signaling influences the position of the ectoderm-endoderm boundary in the sea urchin embryo. Overexpression of a constitutively activated form of $LvNotch$ throughout the embryo shifted the ectoderm-endoderm boundary animally, whereas expression of a dominant-negative form of $LvNotch$ shifted the boundary vegetally. Together with our previous studies that show a role for $LvNotch$ signaling in specifying SMC fate (Sherwood and McClay, 1999), these results demonstrate the importance of $LvNotch$ in the overall patterning of the sea urchin A-V axis. Given that cell proliferation is not altered by perturbation of $LvNotch$ through the mesenchyme blastula stage, whereas the SMCs (Sherwood and McClay, 1999), as well as the ectoderm-endoderm boundary (Fig. 8), are affected at this time, our results further demonstrate that $LvNotch$ signaling alters the fates of cells along the A-V axis, rather than expanding or retracting territories through changes in cell proliferation (summarized in Fig. 10A).

Notably, when the position of the ectoderm-endoderm boundary was shifted by altered $LvNotch$ signaling throughout the embryo, the ectoderm and endoderm tissues appeared to accommodate to the change in the amount of territory. For example, the shift in the ectoderm-endoderm territory animally by constitutively activated $LvNotch$ did not expand the hindgut tissue (which forms from endoderm cells adjacent to the boundary) relative to other endoderm derivatives (see Fig. 1). Rather, our lineage results indicated that the patterning of the entire endoderm territory was affected. Similarly, the ectoderm territory near the ectoderm-endoderm boundary (the anal ectoderm), also was not specifically altered by shifts in the boundary (D. R. S., unpublished).

Vegetal $LvNotch$ signaling regulates ectoderm-endoderm boundary position cell non-autonomously

Our mosaic studies showed that $LvNotch$ signaling acts in cells vegetal to the ectoderm-endoderm boundary to cell non-autonomously promote the position of endoderm more animally in overlying cells. Although our analysis could not precisely indicate which vegetal cells use $LvNotch$ to send the

cell non-autonomous signal, a likely possibility is the presumptive SMCs. Embryological experiments have shown that the vegetally localized micromeres initiate a sequential vegetal-to-animal cascade of inductive signaling necessary for the specification of SMCs and endoderm (Ransick and Davidson, 1995; Sweet et al., 1999; McClay et al., 2000). Significantly, micromeres appear to activate $LvNotch$ signaling in the overlying SMC precursors to specify the SMC fate in the early blastula stage (Sweet et al., 1999; McClay et al., 2000). Thus, activation of $LvNotch$ within the SMC precursors may couple SMC specification and the expression of a cell non-autonomous signal(s) that helps to define the animal boundary of the endoderm. Consistent with this idea, constitutive activation of $LvNotch$ in vegetal cells converted most of these cells into SMCs

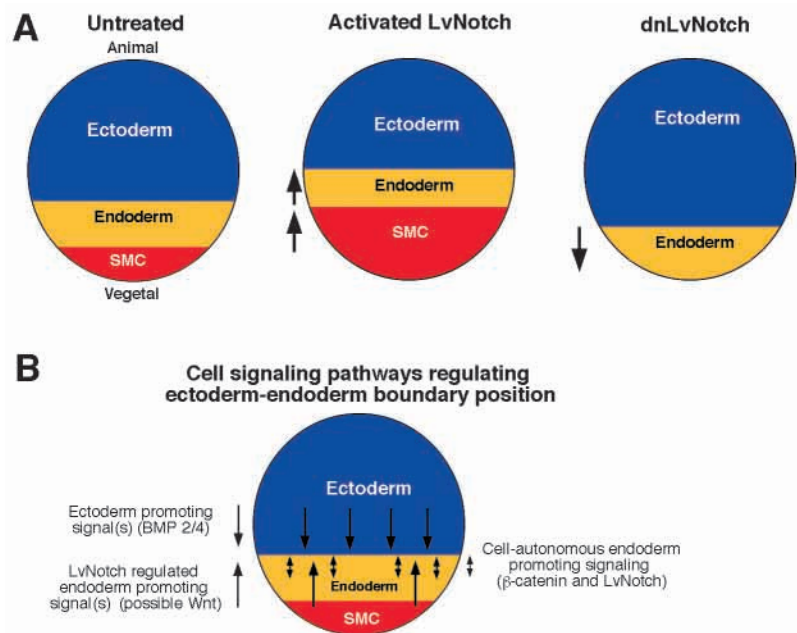


Fig. 10. Schematic summarizing the effects of perturbing $LvNotch$ signaling along the A-V axis and the relationship of $LvNotch$ to other signaling pathways that regulate ectoderm-endoderm boundary position in the sea urchin embryo. (A) A surface fate map of an untreated mesenchyme blastula stage embryo showing (from vegetal to animal pole) the SMC, endoderm and ectoderm territories. Activation of $LvNotch$ signaling throughout the embryo expands the SMC territory at the expense of neighboring presumptive endoderm cells (Sherwood and McClay, 1999), and shifts the endoderm territory animally at the expense of presumptive ectoderm (this study). Inhibition of $LvNotch$ signaling eliminates the specification of SMCs in the mesenchyme blastula embryo (Sherwood and McClay, 1999) and shifts the endoderm territory vegetally, thus expanding the amount of ectoderm in the embryo (this study). Activation or inhibition of $LvNotch$ has no effect on primary mesenchyme cell specification, which are not shown as they have ingressed inside the blastocoel at this time (Sherwood and McClay, 1999). (B) Summary of signaling pathways that affect the position of the ectoderm-endoderm boundary at the late mesenchyme blastula stage when the ectoderm-endoderm boundary is thought to be established (Logan and McClay, 1998). BMP 2/4 signaling in the animal region of the embryo has been shown in the sea urchin *Strongylocentrotus purpuratus* to promote ectoderm formation and inhibit endoderm specification (Angerer et al., 2000). In the sea urchin *Lytechinus variegatus* $LvNotch$ signaling and nuclear β -catenin signaling promote endoderm formation at the boundary (this study and M. Ferkowicz and D. M., unpublished). In the vegetal region of the embryo $LvNotch$ regulates the expression of a signal (possibly a Wnt homolog) that promotes endoderm formation in overlying cells (this study). dn $LvNotch$, dominant negative $LvNotch$; SMC, secondary mesenchyme cell.

and increased the non-autonomous signal; whereas inhibition of LvNotch reduced or eliminated SMC specification, and appeared to diminish or abolish the non-autonomous signal within vegetal cells (Fig. 2B-E; Table 2).

These mosaic studies also revealed one of the molecular targets of vegetal LvNotch signaling: the localization of nuclear β -catenin at the ectoderm-endoderm boundary in the late blastula embryo. Vegetal overexpression of activated LvNotch specifically shifted both the ectoderm-endoderm boundary and nuclear β -catenin localization more animally into overlying uninjected cells. Previous studies have demonstrated that vegetal nuclear β -catenin signaling is dynamic and required for several early aspects of endomesodermal specification (Wikramanayake et al., 1998; Logan et al., 1999; McClay et al., 2000). It is therefore possible that a later function of nuclear β -catenin within endoderm cells bordering the ectoderm is to mediate the recruitment of the animal-most endoderm cells. Supporting this notion, blocking entry of nuclear β -catenin within mesomeres, which normally give rise to ectoderm and sometimes endoderm cells at the boundary, prevents mesomere descendants from ever contributing cells to the endoderm (M. Ferkowicz and D. M., unpublished). Vegetal LvNotch signaling may therefore regulate the ectoderm-endoderm boundary through its effects on β -catenin localization at the border. Given that translocation of β -catenin to the nucleus is a downstream consequence of Wnt signaling (Wodarz and Nusse, 1998), one candidate for the cell non-autonomous signal regulated by vegetal LvNotch is a Wnt ligand. The isolation of several Wnt homologs expressed during early sea urchin development is consistent with this possibility (Ferkowicz et al., 1998), and it will be important in the future to determine which, if any, are regulated by vegetal LvNotch signaling.

LvNotch signaling has a distinct, cell autonomous function within the animal region of the embryo in promoting endoderm formation more animally

Our mosaic studies further revealed that LvNotch signaling within animal cells also promotes the formation of endoderm more animally. Unlike vegetal LvNotch signaling, however, these effects appeared to be confined to the cells that contain altered LvNotch signaling. No cell non-autonomous effects on the position of the ectoderm-endoderm boundary were detected in untreated cells neighboring mesomere descendants in which LvNotch signaling was perturbed. Furthermore, ectopic endoderm tissue induced by expressing constitutively activated LvNotch in animal cells consisted solely of cells containing activated LvNotch. Animal caps, which in untreated embryos form ectodermal vesicles devoid of endoderm (Horstadius, 1973), were also induced (in approximately 50% of cases) to form endoderm derivatives by expression of constitutively activated LvNotch. Taken together, these observations offer compelling evidence that LvNotch signaling functions via a distinct, cell-autonomous mechanism in the animal region of the embryo to promote endoderm formation.

The cell autonomous nature of the effects of perturbing LvNotch signaling on endoderm formation suggests that LvNotch signaling probably functions within the presumptive endoderm in mesomere descendants. Consistent with this possibility, endogenous LvNotch is specifically expressed at high levels at the adherens junctions and along the apical

domain of presumptive endoderm cells at the mesenchyme blastula stage (Sherwood and McClay, 1997), placing it in a position to interact with classical transmembrane ligands for Notch (reviewed in Kimble and Simpson, 1997) or a soluble processed ligand (Qi et al., 1999). It is important to note, however, that overexpression of dominant negative LvNotch did not eliminate endoderm formation, but rather shifted it more vegetally (see Fig. 1; Fig. 2D,E; Sherwood and McClay, 1999). Thus, cell autonomous LvNotch signaling may only be required within the more animal regions of the presumptive endoderm (i.e. close to the ectoderm-endoderm boundary). Furthermore, activation of LvNotch throughout the embryo did not extend endoderm tissue to the animal pole, suggesting that additional factors may confer or actively restrict the ability of LvNotch signaling to cell autonomously promote endoderm formation in the animal region. This could also explain why some isolated animal cap embryoids and injected mesomere pairs failed to form endoderm in response to constitutive activation of LvNotch signaling. For example, other endoderm specification factors required in cells for LvNotch to promote endoderm formation might not always have been present within the mesomere descendants. In support of this possibility, mesomere isolation experiments have indicated that there is considerable variability between different embryos and different batches of embryos in whether maternal vegetal determinants extend into mesomeres (Henry et al., 1989).

Another indication that the animal and vegetal signaling functions of LvNotch act distinctly in influencing the position of the ectoderm-endoderm boundary was the observation that unlike vegetal LvNotch signaling, activation of LvNotch within the animal region did not influence nuclear β -catenin localization at the boundary. These findings are consistent with both signaling functions of LvNotch working independently to promote endoderm formation more animally. Alternatively, it is possible that β -catenin localization at the boundary could mediate an interaction between both signaling functions of LvNotch. For example, nuclear-localized β -catenin could regulate the presentation of a ligand that activates LvNotch signaling directly at the border. The observation that activation of LvNotch within mesomeres fails to stimulate nuclear entry of β -catenin also implies that LvNotch signaling near the ectoderm-endoderm boundary promotes endoderm formation through a β -catenin-independent signaling mechanism. This is not unprecedented, as micromeres transplanted to the animal pole also induce endoderm without stimulating nuclear β -catenin entry (Logan and McClay, 1999).

This study provides the first undertaking of a mosaic analysis that examines the molecular mechanisms guiding ectoderm-endoderm boundary positioning in the sea urchin embryo, and contributes to our understanding of the signaling pathways that are likely to coordinately regulate the positioning of this boundary (summarized in Fig. 10B). It will be important in the future to develop more refined techniques to perturb Notch signaling, in order to better define when and where LvNotch functions, and to further address how LvNotch signaling is coordinated with other signaling pathways (e.g. BMP and Wnt) to position the ectoderm-endoderm boundary. Nevertheless, these experiments offer important new approaches to extend our understanding of ectoderm-endoderm boundary positioning in the sea urchin embryo, and in the case of LvNotch signaling, clearly demonstrate distinct functions

for LvNotch in the animal and vegetal regions of the embryo in positioning this boundary.

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REFERENCES

- Angerer, L. M., Oleksyn, D. W., Logan, C. A., McClay, D. R., Dale, L. and Angerer, R. C. (2000). A BMP pathway regulates cell fate allocation along the sea urchin animal-vegetal axis. *Development* **127**, 1105-1114.
- Angerer, L. M. and Angerer, R. C. (2000). Animal-vegetal axis patterning mechanisms in the early sea urchin embryo. *Dev. Biol.* **218**, 1-12.
- Carlesso, N., Aster, J. C., Sklar, J. and Scadden, D. T. (1999). Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. *Blood* **93**, 838-848.
- Davidson, E. H., Cameron, R. A. and Ransick, A. (1998). Specification of cell fate in the sea urchin embryo: summary and some proposed mechanisms. *Development* **125**, 3269-3290.
- de Celis, J. F. and Bray, S. (1997). Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the *Drosophila* wing. *Development* **124**, 3241-3251.
- Diaz-Benjumea, F. J. and Cohen, S. M. (1995). Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal-ventral compartment boundary of the *Drosophila* wing. *Development* **121**, 4215-4225.
- Ettensohn, C. and Malinda, K. M. (1993). Size regulation and morphogenesis: a cellular analysis of skeletogenesis in the sea urchin embryo. *Development* **119**, 155-167.
- Ettensohn, C. A. and Sweet, H. C. (2000). Patterning the early sea urchin embryo. *Curr. Top. Dev. Biol.* **50**, 1-44.
- Ferkowicz, M. J., Stander, M. C. and Raff, R. A. (1998). Phylogenetic relationships and developmental expression of three sea urchin *Wnt* genes. *Mol. Biol. Evol.* **15**, 809-819.
- Fink, R. D. and McClay, D. R. (1985). Three cell recognition changes accompany the ingression of sea urchin primary mesenchyme cells. *Dev. Biol.* **107**, 66-74.
- Go, M. J., Eastman, D. S. and Artavanis-Tsakonas, S. (1998). Cell proliferation control by Notch signaling in *Drosophila* development. *Development* **125**, 2031-2040.
- Hardin, J., Coffman, J. A., Black, S. D. and McClay, D. R. (1992). Commitment along the dorsoventral axis of the sea urchin embryo is altered in response to NiCl₂. *Development* **116**, 671-85.
- Henry, J. J., Amemiya, S., Wray, G. A. and Raff, R. A. (1989). Early inductive interactions are involved in restricting cell fates of mesomeres in sea urchin embryos. *Dev. Biol.* **136**, 140-153.
- Hodor, P. G. and Ettensohn, C. A. (1998). The dynamics and regulation of mesenchymal cell fusion in the sea urchin embryo. *Dev. Biol.* **199**, 111-124.
- Horstadius, S. (1973). *Experimental Embryology of Echinoderms*. Oxford: Clarendon Press.
- Huang, L., Li, X., Dayal, H., Wikramanayake, A. H. and Klein, W. H. (2000). Involvement of Tcf/Lef in establishing cell types along the animal-vegetal axis of sea urchins. *Dev. Genes Evol.* **210**, 73-81.
- Irvine, K. D. (1999). Fringe, Notch, and making developmental boundaries. *Curr. Opin. Genet. Dev.* **9**, 434-441.
- Johnston, L. A. and Edgar, B. A. (1998). Wingless and Notch regulate cell-cycle arrest in the developing *Drosophila* wing. *Nature* **394**, 82-84.
- Khaner, O. and Wilt, F. (1991). Interactions of different vegetal cells with mesomeres during early stages of sea urchin development. *Development* **112**, 881-890.
- Kimble, J. and Simpson, P. (1997). The LIN-12/Notch signaling pathway and its regulation. *Annu. Rev. Cell Dev. Biol.* **13**, 333-61.
- Logan, C. Y. and McClay, D. R. (1997). The allocation of early blastomeres to the ectoderm and endoderm is variable in the sea urchin embryo. *Development* **124**, 2213-2223.
- Logan, C. Y. and McClay, D. R. (1998). The lineages that give rise to the endoderm and mesoderm in the sea urchin embryo. In *Cell Fate and Lineage Determination* (ed. S. Moody), pp. 41-58. New York: Academic Press.
- Logan, C. Y., Miller, J. R., Ferkowicz, M. J. and McClay, D. R. (1999). Nuclear β -catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development* **126**, 345-357.
- Mao, C. A., Wikramanayake, A. H., Gan, L., Chuang, C. K., Summers, R. G. and Klein, W. H. (1996). Altering cell fates in sea urchin embryos by overexpressing SpOtx, an orthodenticle-related protein. *Development* **122**, 1489-98.
- McClay, D. R., Peterson, R., Range, R., Winter-Vann, A. and Ferkowicz, M. (2000). A micromere induction signal is activated by β -catenin and acts through Notch to initiate specification of secondary mesenchyme cells in the sea urchin embryo. *Development* **127**, 5113-5122.
- Micchelli, C. A., Rulifson, E. J. and Blair, S. S. (1997). The function and regulation of cut expression on the wing margin of *Drosophila*: Notch, Wingless and a dominant negative role for Delta and Serrate. *Development* **124**, 1485-1495.
- Neumann, C. J. and Cohen, S. M. (1996). A hierarchy of cross-regulation involving Notch, wingless, vestigial and cut organizes the dorsal/ventral axis of the *Drosophila* wing. *Development* **122**, 3477-3485.
- Qi, H., Rand, M. D., Wu, X., Sestan, N., Wang, W., Rakic, P., Xu, T. and Artavanis-Tsakonas, S. (1999). Processing of the Notch ligand Delta by the metalloprotease Kuzbanian. *Science* **283**, 91-94.
- Ransick, A. and Davidson, E. H. (1993). A complete second gut induced by transplanted micromeres in the sea urchin embryo. *Science* **259**, 1134-1138.
- Ransick, A. and Davidson, E. H. (1995). Micromeres are required for normal vegetal plate specification in sea urchin embryos. *Development* **121**, 3215-3222.
- Ransick, A. and Davidson, E. H. (1998). Late specification of veg1 lineages to endodermal fate in the sea urchin embryo. *Dev. Biol.* **195**, 38-48.
- Rawls, A., Wilson-Rawls, J. and Olson, E. N. (2000). Genetic regulation of somite formation. *Curr. Top. Dev. Biol.* **47**, 131-154.
- Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* **67**, 687-699.
- Reynolds, S. D., Angerer, L. M., Palis, J., Nasir, A. and Angerer, R. C. (1992). Early mRNAs, spatially restricted along the animal-vegetal axis of sea urchin embryos, include one encoding a protein related to tolloid and BMP-1. *Development* **114**, 769-786.
- Ruffins, S. W. and Ettensohn, C. A. (1996). A fate map of the vegetal plate of the sea urchin (*Lytechinus variegatus*) mesenchyme blastula. *Development* **122**, 253-263.
- Rulifson, E. J. and Blair, S. S. (1995). Notch regulates wingless expression and is not required for reception of the paracrine wingless signal during wing margin neurogenesis in *Drosophila*. *Development* **121**, 2813-2824.
- Sherwood, D. R. and McClay, D. R. (1997). Identification and localization of a sea urchin Notch homologue: insights into vegetal plate regionalization and Notch receptor regulation. *Development* **124**, 3363-3374.
- Sherwood, D. R. and McClay, D. R. (1999). LvNotch signaling mediates secondary mesenchyme specification in the sea urchin embryo. *Development* **126**, 1703-1713.
- Sweet, H. C., Hodor, P. G. and Ettensohn, C. A. (1999). The role of micromere signaling in Notch activation and mesoderm specification during sea urchin embryogenesis. *Development* **126**, 5255-5265.
- Vonica, A., Weng, W., Gumbiner, B. G. and Venuti, J. M. (2000). Tcf is the nuclear effector of the β -catenin signal that patterns the sea urchin animal-vegetal axis. *Dev. Biol.* **217**, 230-243.
- Walker, L., Lynch, M., Silverman, S., Fraser, J., Boulter, J., Weinmaster, G. and Gasson, J. C. (1999). The Notch/Jagged pathway inhibits proliferation of human hematopoietic progenitors in vitro. *Stem Cells* **17**, 162-171.
- Wesley, C. S. (1999). Notch and Wingless regulate expression of cuticle patterning genes. *Mol. Cell. Biol.* **19**, 5743-5758.
- Wessel, G. M. and McClay, D. R. (1985). Sequential expression of germ-layer specific molecules in the sea urchin embryo. **111**, 451-463.
- Wessel, G. M. and Wikramanayake, A. (1999). How to grow a gut: ontogeny of the endoderm in the sea urchin embryo. *BioEssays* **21**, 459-471.
- Wikramanayake, A. H., Huang, L. and Klein, W. (1998). β -Catenin is essential for patterning the maternally specified animal-vegetal axis in the sea urchin embryo. *Proc. Natl. Acad. Sci. USA* **95**, 9343-9348.
- Wodarz, A. and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.