

LvNotch signaling mediates secondary mesenchyme specification in the sea urchin embryo

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Accepted 1 February; published on WWW 17 February 1999

SUMMARY

Cell-cell interactions are thought to regulate the differential specification of secondary mesenchyme cells (SMCs) and endoderm in the sea urchin embryo. The molecular bases of these interactions, however, are unknown. We have previously shown that the sea urchin homologue of the LIN-12/Notch receptor, LvNotch, displays dynamic patterns of expression within both the presumptive SMCs and endoderm during the blastula stage, the time at which these two cell types are thought to be differentially specified (Sherwood, D. R. and McClay, D. R. (1997) *Development* 124, 3363-3374). The LIN-12/Notch signaling pathway has been shown to mediate the segregation of numerous cell types in both invertebrate and vertebrate embryos. To directly examine whether LvNotch signaling has a role in the differential specification of SMCs and endoderm, we have overexpressed activated and dominant negative forms of LvNotch during early sea urchin development. We show that activation of LvNotch signaling increases SMC specification, while loss or

reduction of LvNotch signaling eliminates or significantly decreases SMC specification. Furthermore, results from a mosaic analysis of LvNotch function as well as endogenous LvNotch expression strongly suggest that LvNotch signaling acts autonomously within the presumptive SMCs to mediate SMC specification. Finally, we demonstrate that the expansion of SMCs seen with activation of LvNotch signaling comes at the expense of presumptive endoderm cells, while loss of SMC specification results in the endoderm expanding into territory where SMCs usually arise. Taken together, these results offer compelling evidence that LvNotch signaling directly specifies the SMC fate, and that this signaling is critical for the differential specification of SMCs and endoderm in the sea urchin embryo.

Key words: Sea urchin, LIN-12/Notch signaling, LvNotch, Mesoderm, Specification

INTRODUCTION

Classical embryological studies have revealed that cell-cell interactions play important roles in cell fate specification throughout sea urchin development (reviewed in Davidson et al., 1998; Etensohn, 1992). The molecular basis of these interactions, however, remains largely unknown. One region of the embryo in which cellular interactions appear to play a critical role is in the diversification of cell types that emerge from the vegetal plate of the mesenchyme blastula embryo (Davidson, 1993; McClay and Logan, 1996). At this stage, the presumptive secondary mesenchyme cells (SMCs) are situated as a contiguous patch of epithelial cells at the center of the vegetal plate and are surrounded by a ring of presumptive endoderm cells (Ruffins and Etensohn, 1996). Following gastrulation, the SMCs give rise to the majority of mesodermal cell types in the pluteus larvae, while the endoderm forms the larval gut.

One fundamental segregation that must occur in the vegetal plate is the differential specification of SMCs and endoderm. Cell lineage analyses have demonstrated that presumptive SMCs become clonally distinct from neighboring presumptive

endoderm cells at some time after the 120-cell stage (late cleavage) and before the late blastula stage (Horstadius, 1973; Ruffins and Etensohn, 1996; Logan and McClay 1997), suggesting that cell fate restrictive events occur between the presumptive SMCs and endoderm during the blastula stage. Furthermore, experiments have shown that microsurgical removal of the presumptive SMCs from the center of the vegetal plate results in a regulative replacement of most SMC-derived cell types by neighboring presumptive endoderm cells (McClay and Logan, 1996). This regulative behavior implies that cell-cell interactions are crucial for the differential specification of SMCs and endoderm.

The evolutionarily conserved LIN-12/Notch intercellular signaling pathway has been shown to play an essential role in the segregation of a diverse array of cell types in both invertebrate and vertebrate embryos (e.g. Tepass and Hartenstein, 1995; Chitnis et al., 1995; Greenwald et al., 1983; reviewed in Kimble and Simpson, 1997; Gridely, 1997). One of the fundamental components of this pathway are the LIN-12/Notch receptors, which are single-spanning transmembrane proteins (reviewed in Artavanis-Tsakonas et al., 1995; Kimble and Simpson, 1997; Greenwald, 1998). We have previously

reported that the sea urchin homologue of the LIN-12/Notch receptor, LvNotch, displays a dynamic expression pattern within both presumptive SMCs and endoderm cells during the blastula stage (Sherwood and McClay, 1997). LvNotch is expressed uniformly in cleavage-stage embryos, but becomes specifically lost in cells at the vegetal pole of the early blastula embryo where the future SMCs are located. At the end of the blastula stage, LvNotch is expressed at high levels on the apical surface of presumptive endodermal cells, while the presumptive SMCs located at the center of the vegetal plate continue to lack LvNotch expression. The dynamic expression pattern of LvNotch, and known function of LIN-12/Notch signaling in mediating the segregation of distinct cell types, suggested that LvNotch signaling could have a role in the differential specification of SMCs and endoderm in the sea urchin embryo.

In this paper, we have directly tested the function of LvNotch signaling in the segregation of SMCs and endoderm by examining the phenotypic and molecular consequences of overexpressing activated and dominant negative forms of LvNotch in sea urchin embryos. We first show that activation of LvNotch signaling increases SMCs, while loss or reduction of LvNotch signaling eliminates or dramatically reduces SMC specification. An examination of endogenous LvNotch expression and results from a mosaic analysis of LvNotch function suggest that LvNotch signaling acts within the presumptive SMCs to specify the SMC fate in the early blastula embryo. Furthermore, marker and morphological analyses reveal that the expansion of SMCs seen with activation of LvNotch signaling comes at the expense of presumptive endoderm cells, while loss of SMC specification results in the endoderm extending vegetally into territory which usually gives rise to SMCs. Based on this evidence, we propose that LvNotch signaling functions within the presumptive SMCs to mediate SMC specification, and that this signaling plays a crucial role in the differential specification of SMCs and endoderm in the sea urchin embryo.

MATERIALS AND METHODS

Animals

Sea urchins (*Lytechinus variegatus*) were obtained from Tracy Andacht (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.

LvNotch constructs

All *LvNotch* constructs were cloned into pBluescript II SK– plasmid (Stratagene) and oriented so that sense mRNA would be made using the T3 promoter. All constructs were initiated with a PCR-amplified fragment encompassing the 5' UTR and DNA encoding the first 5 or 6 amino acids (aa) of *SpOtx* (Gan et al., 1995), which has been shown to effectively drive translation in the sea urchin (Mao et al., 1996). *LvN^{full}* was assembled by ligating previously described *LvNotch* cDNA clones (Sherwood and McClay, 1997) and *LvN^{neg}* was derived from *LvN^{full}* by deleting the DNA sequence encoding aa 1759–2516 (Sherwood and McClay, 1997). Two inframe *Clal* sites that removed DNA sequence encoding aa 296–1129 of *LvN^{neg}* were used to generate *LvN^{neg}ΔEGF7-29*. *LvN^{act}*, aa 1758–2531, was assembled by ligation of previously described *LvNotch* cDNA clones (Sherwood and McClay, 1997). *LvN^{act}ΔANK5* was derived from *LvN^{act}* using two inframe *PstI* sites that removed DNA sequence encoding aa 1990–2003. PCR

amplifications were performed using Deep Vent (New England Biolabs) and all PCR-amplified products were sequenced after ligation to ensure fidelity of amplification.

mRNA preparation and injection

All *LvNotch* DNA constructs were linearized with *XhoI* and 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 as described (Mao et al., 1996) and resuspended in ddH₂O. The concentration of mRNA was estimated by using OD₂₆₀ and OD₂₈₀ readings, and compared to known concentrations of RNA by gel electrophoresis. Eggs were prepared and injected as described (Mao et al., 1996). Survival of embryos was 90–100%. *LvN^{full}* was not injected at >0.3 μg/μl, as the large size of the *LvN^{full}* transcript (≈8.0 kb) caused this mRNA to precipitate and clog the injection needle at high concentrations.

Western analysis

Protein extracts of known numbers of embryos were prepared by mouth pipetting counted embryos into Eppendorf tubes, spinning these tubes at 16,000 RCF for 2 minutes, aspirating off the supernatant by mouth pipet under a dissecting microscope, and then adding 20 μl of SDS lysis buffer (100 mM Tris, pH 6.8; 4% SDS; 20% glycerol; 1 mM PMSF). Extracts were then triturated, boiled for 5 minutes and stored at –70°C until use. Samples were run on 3–15% gradient gels or 10% gels, transferred to nitrocellulose and probed with either LvNotch intracellular domain-directed rabbit α-ANK polyclonal antibody (pAb) or extracellular domain-directed guinea pig α-Bam1 pAb (as in Sherwood and McClay, 1997). Blots were processed and developed as described (Sherwood and McClay, 1997).

Antibody production, immunolocalization and image analysis

An affinity-purified guinea pig pAb specific to the intracellular domain of LvNotch (aa 2243–2440; referred to as Rael B) was generated using the pGex system as described previously (Sherwood and McClay, 1997) and used for all intracellular-directed whole-mount immunolocalizations. Whole-mount analysis with extracellular-directed pAbs to LvNotch employed either mouse or guinea pig α-Bam1 pAbs as described (Sherwood and McClay, 1997). The fixation and staining procedures for endogenous LvNotch with α-LvNotch pAbs, esophageal muscle cells with rabbit α-myosin heavy chain pAb (Wessel et al., 1990), PMCs with 1d5 mAb (Hardin et al., 1992) and SMCs and PMCs with Meso1 mAb (Wessel and McClay, 1985), was as described (Sherwood and McClay, 1997). Fixation and staining procedures for viewing double-labeled coelomic pouch cells for α-LvNotch pAb/YoPro (Molecular Probes) and for mosaic analysis of SMC specification using α-SMC1 mAb (generous gift of C. A. Etensohn)/guinea pig α-LvNotch pAb/guinea pig α-LvGadherin pAb (Miller and McClay, 1997) was as described in Sherwood and McClay (1997) with the following exceptions: embryos were fixed with freshly prepared room temperature 2% paraformaldehyde in artificial sea water for 10 minutes, followed by brief (≈1 minute) permeabilization in 100% methanol. In addition, YoPro (5 μm final concentration) was added during the first wash after incubation with the secondary antibody. A Zeiss confocal laser scanning microscope was employed to acquire all immunofluorescent images shown. Double labeling was achieved using either simultaneous or sequential confocal sectioning and images were overlaid using Adobe Photoshop 3.0.5.

SMC and PMC cell counts

The number of SMC-derived cell types was examined in 60–65 hour pluteus larvae. Pigment and blastocoel cells were counted using DIC optics as previously described (Etensohn and Ruffins, 1993). Coelomic pouch cells were quantified by optically sectioning larvae

double labeled for nuclear YoPro and endogenous LvNotch. The number of lateral esophageal muscle fibers was examined by optically sectioning larvae stained for α -myosin heavy chain pAb and rendering two-dimensional projections of the sections. PMCs were counted by optically sectioning embryos stained with the PMC-specific mAb 1d5 (Hardin et al., 1992). To ensure that nonspecific alterations in cleavage divisions (occasionally observed after mRNA injection) did not delay PMC emergence, only embryos that cleaved correctly through the 16-cell stage were selected for PMC cell counts.

RESULTS

Perturbation of LvNotch signaling in the sea urchin embryo

Numerous studies have shown that overexpression of the intracellular domain of LIN-12/Notch receptors results in a constitutively activated form of the receptor (reviewed in Weinmaster, 1997). Furthermore, experiments in *Drosophila* have suggested that overexpression of Notch lacking the majority of the intracellular domain results in a dominant negative protein (Rebay et al., 1993). Using these insights, an activated (*LvN^{act}*) and a dominant negative (*LvN^{neg}*) form of

the sea urchin *LvNotch* receptor were generated (Fig. 1A). To directly examine the functions of LvNotch during early sea urchin development, these constructs were transcribed into mRNA and introduced into embryos via injection into single-cell zygotes. As controls for overexpression of *LvN^{act}* and *LvN^{neg}*, a construct encoding a protein similar to *LvN^{act}*, but lacking 14 amino acids at the beginning of ankyrin (ANK) repeat 5 (*LvN^{act} Δ ANK5*) and a construct encoding a protein similar to *LvN^{neg}*, but missing EGF-like repeats 7 through 29 (*LvN^{neg} Δ EGF7-29*), were also generated (Fig. 1A). The integrity of the ANK repeats and the EGF-like repeats between 7 and 29 have been shown to be critical for Notch signaling in *Drosophila* (Lieber et al., 1993; Rebay et al., 1993). Therefore, these control constructs should not alter LvNotch signaling. A full-length *LvNotch* (*LvN^{full}*) construct was also made to examine the consequences of overexpressing the intact receptor (Fig. 1A).

LvNotch constructs are expressed, processed and localized normally

To understand the relationship of possible phenotypes to altered LvNotch signaling, the levels, duration and localization

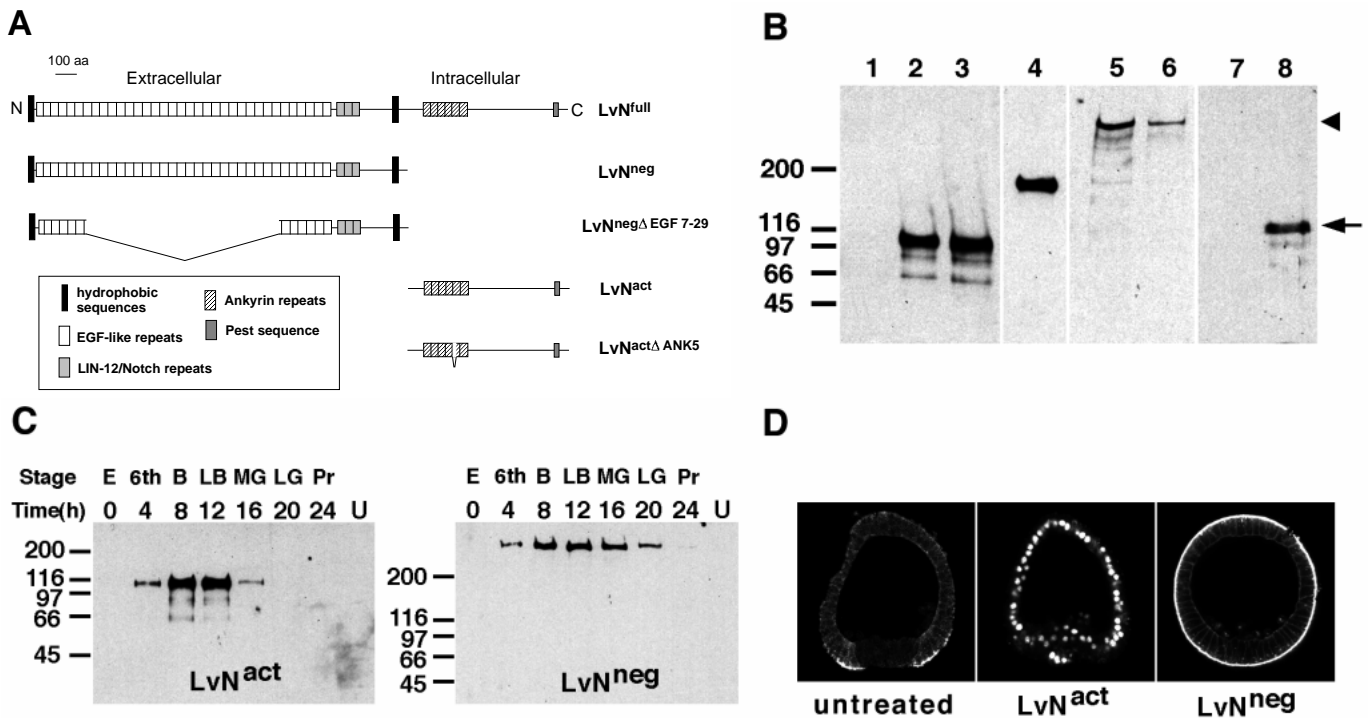


Fig. 1. Expression of LvNotch constructs. (A) Schematic diagram of LvNotch constructs. (B) Western analysis of protein extracts (30 embryos/lane) collected from mRNA-injected and uninjected blastula-stage embryos (8–12 hours of development). Lanes: (1) uninjected, (2) *LvN^{act}*-injected, (3) *LvN^{act} Δ ANK5*-injected, (4) *LvN^{neg} Δ EGF7-29*-injected, (5) *LvN^{neg}*-injected, (6) *LvN^{full}*-injected, (7) *LvN^{neg}*-injected, (8) *LvN^{full}*-injected. Antibodies used: lanes 1–3,7,8, α -LvNotch intracellular domain-directed pAb; lanes 4–6, α -LvNotch extracellular domain-directed pAb. Arrowhead indicates the extracellular fragment of overexpressed full-length LvNotch in lane 6, and the predominantly intracellular fragment of full-length LvNotch in lane 8. Positions of molecular mass markers (kDa) are indicated with dashes on the left side of the figure. (C) Developmental western analysis of *LvN^{act}*- and *LvN^{neg}*-injected embryos (30 embryos/lane) using intracellular- and extracellular-directed α -LvNotch pAb, respectively. Three independent trials for each mRNA showed similar results. Abbreviations: E, egg; 6th, 6th cleavage; B, blastula; LB, late blastula; MG, mid-gastrula; LG, late gastrula; Pr, prism; U, untreated gastrula stage. (D) Confocal analysis of untreated, *LvN^{act}*- and *LvN^{neg}*-injected embryos (12 hours of development) viewed along animal-vegetal axis. Endogenous LvNotch is expressed at high levels apically in the presumptive endoderm in untreated embryos. Intracellular-directed α -LvNotch pAb shows that the intracellular fragment *LvN^{act}* localized predominantly to nuclei (as did *LvN^{act} Δ ANK5*; data not shown), while extracellular-directed α -LvNotch pAb demonstrates that *LvN^{neg}* localized predominantly to the apical membranes of cells (as did *LvN^{full}* and *LvN^{neg} Δ EGF7-29*; data not shown). Endogenous LvNotch was not visible in injected embryos with the confocal parameters used.

of proteins expressed from *LvNotch* constructs were examined. For these studies, 3.0-6.0 pg/zygote of mRNA was injected for all constructs except for *LvN^{full}*, which was injected at the highest possible concentration of 1.0-3.0 pg/zygote (see Methods). Protein immunoblot studies were carried out using protein extracts collected from 30 mRNA-injected blastula-stage embryos (8-12 hours of development) for each construct. The level of endogenous *LvNotch* protein in 30 embryos was found to be slightly below the detection limit of α -*LvNotch* pAbs for endogenous *LvNotch* and overexpression of these constructs did not result in a detectable increase in endogenous *LvNotch* expression (see Fig. 1B,C; data not shown). Thus, this quantity of protein extract revealed only the expression of *LvNotch* protein arising from injected mRNA. This analysis demonstrated that all constructs were translated into appropriately sized proteins, and that *LvN^{full}* was correctly processed into an extracellular and a predominantly intracellular fragment, which are thought to remain associated at the plasma membrane by a reductive-sensitive linkage (Fig. 1B; Blaumueller et al., 1997).

A developmental western blot of *LvN^{act}*- and *LvN^{neg}*-injected embryos revealed that these constructs were expressed by 4 hours of development, peaked in expression between 8 and 12 hours after injection (blastula stage), then declined in abundance and were undetectable by 20-24 hours of development (late gastrula/prism stage; Fig. 1C). The *LvN^{neg}* protein was consistently expressed approximately 4 hours longer than *LvN^{act}* (Fig. 1C). Densitometry analysis showed that peak levels of expression for both *LvN^{act}* and *LvN^{neg}* were approximately 15-fold higher than endogenous *LvNotch* in uninjected embryos (data not shown).

Staining of embryos revealed that *LvN^{act}* and *LvN^{act} Δ ANK5* localized to the nucleus (Fig. 1D), consistent with the localization of intracellular LIN-12/Notch fragments in *Drosophila* and vertebrates, and with the proposed role of this fragment in regulating transcription (reviewed in Chan and Jan, 1998). *LvN^{neg}*, *LvN^{neg} Δ EGF7-29* and *LvN^{full}* localized predominantly to the apical surface of cells in blastula-stage embryos, demonstrating that these proteins were trafficked to the plasma membrane (Fig. 1D).

Alterations in *LvNotch* signaling affect secondary mesenchyme formation during gastrulation

To understand possible functions of *LvNotch* signaling in SMC and endoderm specification, we first examined the emergence of these distinct cell types during gastrulation in mRNA-injected embryos. Gastrulation begins with the early ingression of primary mesenchyme cells (PMCs; the skeletogenic mesenchyme) during the mid-blastula stage. Later, the SMCs and endoderm become morphologically distinct during the gastrula stage when the vegetal plate buckles inward to form the archenteron. The SMCs delaminate from the tip of the archenteron, while the remaining archenteron cells give rise to the endoderm (see Fig. 2A).

Overexpression of activated and full-length *LvNotch*

Embryos injected with high levels (3.0-6.0 pg) of *LvN^{act}* underwent normal gastrulation through the end of the blastula stage (mesenchyme blastula); the PMCs ingressed and migrated within the blastocoel appropriately. At about the time that the vegetal plate would normally begin to invaginate and

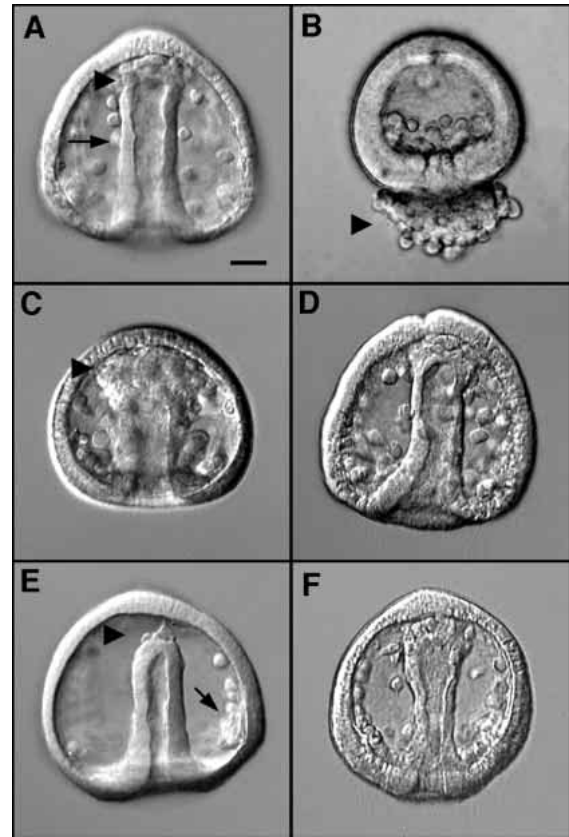


Fig. 2. Injection of *LvN^{act}* and *LvN^{neg}* affects SMC formation during gastrulation. (A) Untreated late gastrula embryo with SMCs (arrowhead) delaminating at tip of the archenteron (arrow). (B) Early gastrula embryo injected with *LvN^{act}* (3.0-6.0 pg) shortly after undergoing mesenchymal extrusion (arrowhead). (C) Late gastrula embryo injected with a lower concentration of *LvN^{act}* (0.3-1.0 pg) has an increased number of SMCs (arrowhead) at the tip of the archenteron. (D) Normal appearing late gastrula embryo injected with *LvN^{act} Δ ANK5* (3.0-6.0 pg). (E) Late gastrula embryo injected with *LvN^{neg}* (3.0-6.0 pg) displays a loss of SMCs (arrowhead) at the tip of the archenteron. PMCs (arrow) are localized normally in ventrolateral cluster. (F) Normal appearing late gastrula embryo injected with *LvN^{neg} Δ EGF7-29* (3.0-6.0 pg). Bar, 25 μ m.

form the archenteron (early gastrula stage), however, most *LvN^{act}*-injected embryos extruded a large number of mesenchymal cells from the center of the vegetal plate (Figs 2B, 3). Immunostaining with the PMC-specific mAb 1d5, and PMC and SMC-recognizing mAb Meso1 (Fig. 4), revealed that the vast majority of extruded cells were SMCs (Fig. 4D,F). Remarkably, an archenteron eventually invaginated in most embryos that underwent SMC extrusion. Extruded SMCs usually broke off or were taken inside the archenteron and later expelled through the larval gut. Injection of lower doses of *LvN^{act}* decreased both the severity and number of embryos that underwent SMC extrusion. In these embryos, there was an increase in the number of SMCs at the tip of the archenteron during gastrulation, and the archenteron often appeared shorter and slightly broader than normal (Figs 2C, 3). Embryos injected with high levels of *LvN^{act} Δ ANK5* mRNA gastrulated and developed normally (Figs 2D, 3), showing that the phenotypes observed were caused by altered *LvNotch* signaling rather than

RNA toxicity or protein overexpression. Injection with 1.0-3.0 pg of *LvN^{full}* mRNA also led to an increase in SMC formation in a significant number of embryos (Fig. 3), demonstrating that overexpression of full-length LvNotch can cause overactivation of LvNotch signaling.

Overexpression of dominant negative LvNotch

Embryos injected with high levels (3.0-6.0 pg) of *LvN^{neg}* also underwent normal development through the late blastula stage. During the mid- to late gastrula stage, however, a phenotype opposite to *LvN^{act}*-injected embryos was observed: there was a loss or dramatic decrease in the number of SMCs at the tip of the archenteron and within the blastocoel (compare Fig. 2E with 2A and C). The only mesenchymal cells consistently in the blastocoel were the PMCs, which were not reduced in number compared with untreated controls ($P > 0.05$; Table 1; Fig. 2E). Injection of lower doses of *LvN^{neg}* led to a graded decrease in the penetrance and severity of the phenotype (Fig. 3). Injection of the control mRNA *LvN^{neg}ΔEGF7-29* did not affect gastrulation or later development (Figs 2F, 3), strongly suggesting that the phenotype observed in *LvN^{neg}*-injected embryos was a result of a loss or decrease in endogenous LvNotch signaling.

LvNotch signaling is required for the normal specification of all SMC-derived cell types

The most striking phenotype observed in embryos injected with *LvNotch* constructs was the perturbation in the formation of SMCs. We therefore undertook a detailed analysis of the role of LvNotch signaling in SMC specification.

The SMCs give rise to four characterized cell types: pigment, blastocoelar, circumesophageal muscle and coelomic pouch cells (reviewed in Ettensohn, 1992). To determine if all SMC-derived cell types were affected by altered *LvNotch* signaling, the number of these cells were quantified in late stage pluteus larvae (60-65 hours of development) that developed from embryos injected with *LvN^{neg}* and *LvN^{act}* mRNA (see Fig. 5). This advanced larval stage was chosen to allow for full differentiation of all SMC-derived cell types (Ettensohn and Ruffins, 1993).

To examine the effects of increased LvNotch signaling, we injected moderate levels of *LvN^{act}* (0.3-1.0 pg/zygote). These levels of *LvN^{act}* avoided the severe extrusion of SMCs that resulted from higher doses of *LvN^{act}*. To decrease LvNotch signaling, we injected high levels of *LvN^{neg}* (3.0-6.0 pg/zygote). Injection of *LvN^{act}* and *LvN^{neg}* led to significant alterations in the number of most SMC-derived cell types compared with uninjected controls (Table 1). For example, injection of *LvN^{neg}* caused an approximately 60% decrease in the number of muscle cell fibers, blastocoelar cells and coelomic pouch cells and essentially eliminated the specification of pigment cells ($P < 0.05$, Table 1; Fig. 5C-G). Furthermore, injection of *LvN^{act}* resulted in a 17% increase in the number of muscle fibers and an approximately 45% increase in pigment and blastocoelar cells. Injection of *LvN^{act}*, however, did not significantly increase the number of coelomic pouch cells (Table 1). The reasons for a lack of increase in coelomic pouch cell number are unclear, but may reflect tight regulatory control over the number

Table 1. Influence of LvNotch signaling on PMCs and SMC-derived cell types

Cell type	Number of cells (no. of embryos counted)		
	Control uninjected (n)	<i>LvN^{act}</i> -injected ^a (n)	<i>LvN^{neg}</i> -injected ^b (n)
PMCs ^c	63.0±1.7 (15)	63.9±0.7 (15)	65.5±1.6 (15)
SMC-derived ^d			
Pigment	97.3±3.3 (40)	139.0±5.0 (34)*	1.4±0.7 (40)*
Blastocoelar	24.9±1.3 (21)	36.7±1.9 (16)*	9.8±1.6 (21)*
Coelomic pouch	63.9±1.8 (14)	67.4±2.1 (14)	23.9±3.1 (14)*
Muscle	6.9±0.2 (30)	8.1±0.4 (23)*	2.8±0.5 (30)*

^aInjected with 0.3-1.0 pg mRNA/zygote.

^bInjected with 3.0-6.0 pg mRNA/zygote.

^cPMCs were counted during the mid-gastrula stage (approximately 16 hours in development).

^dSMCs were counted during the pluteus larval stage (between 60 and 65 hours in development).

Note: muscle cells were not counted directly, but rather the number of lateral muscle fibers was scored (see Fig. 5C).

*Means ± s.e.m. are significantly different in number compared with the untreated control ($P < 0.05$; by ANOVA).

For each treatment, three or more independent experiments were performed on approximately equal numbers of embryos and the data pooled.

of this cell type in pluteus larvae. Nevertheless, the loss or reduction of all SMC-derived cell types after injection with *LvN^{neg}*, and largely opposite phenotypes after injection with *LvN^{act}*, demonstrate that LvNotch signaling plays a critical role in the normal specification of all SMC-derived cell types.

Endogenous LvNotch is found in vesicles in presumptive mesoderm cells near the time SMCs are thought to be specified

Fate-mapping studies have shown that the SMCs are not distinctly specified until sometime after the 120-cell stage

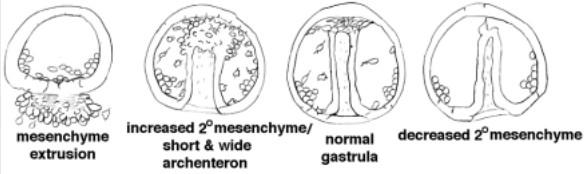
Construct/ concentration injected (pg)				
	mesenchyme extrusion	increased 2 nd mesenchyme/ short & wide archenteron	normal gastrula	decreased 2 nd mesenchyme
<i>LvN^{act}</i>				
3.0-6.0	137 (93%)	10 (7%)	0	0
1.0-3.0	42 (87%)	6 (13%)	0	0
0.3-1.0	33 (33%)	63 (63%)	4 (4%)	0
0.10-0.25	0	26 (55%)	21 (45%)	0
<i>LvN^{act}ΔANK5</i>				
3.0-6.0	0	0	50 (100%)	0
<i>LvN^{neg}</i>				
3.0-6.0	0	0	0	82 (100%)
1.0-3.0	0	0	1 (2%)	44 (98%)
0.6-1.0	0	0	5 (7%)	67 (93%)
0.10-0.25	0	0	52 (73%)	19 (27%)
<i>LvN^{neg}ΔEGF7-29</i>				
3.0-6.0	0	0	60 (100%)	0
<i>LvN^{full}</i>				
1.0-3.0	3 (4%)	20 (25%)	57 (71%)	0

Fig. 3. Gastrulation phenotypes elicited by injection of different forms and concentrations of *LvNotch*. The percentage of embryos analyzed is indicated in parentheses next to the number of embryos.

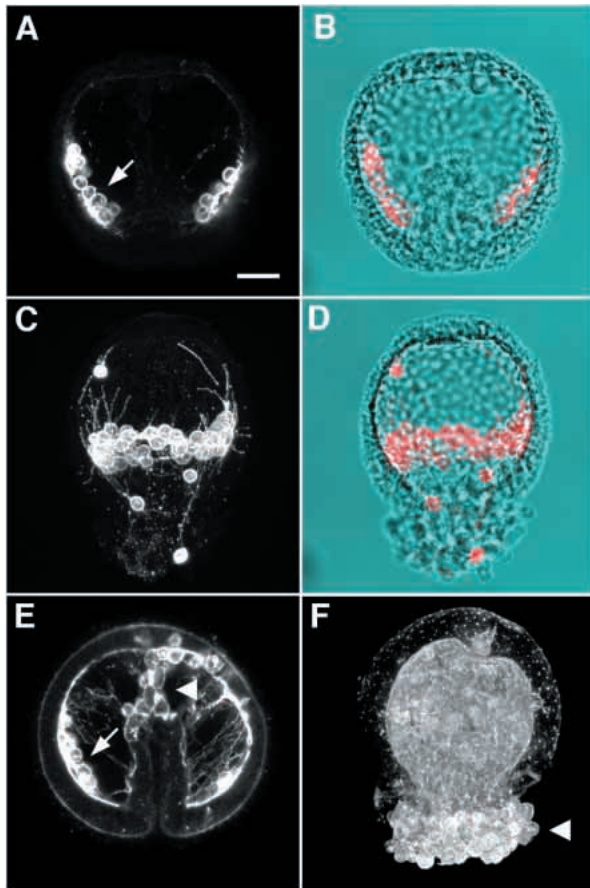
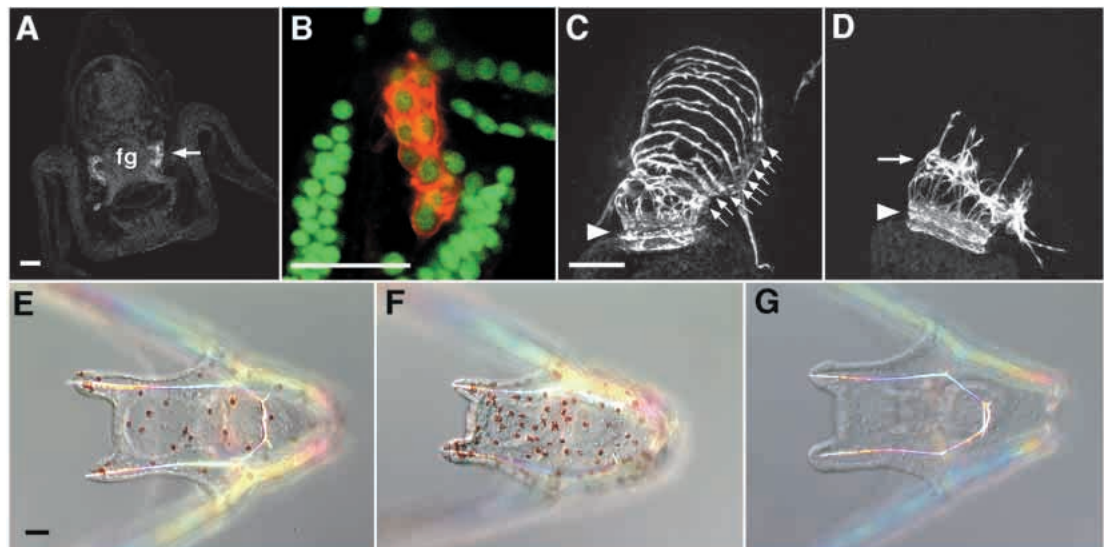


Fig. 4. Overexpression of high levels of *LvN^{act}* (3.0-6.0 pg) results in SMC extrusion during gastrulation. Gastrula embryos viewed along the animal-vegetal axis and stained with the PMC-specific mAb 1d5 (A-D) or the PMC- and SMC-specific mAb Meso 1 (E,F). (A) A single confocal section of an untreated embryo showing a cluster of PMCs (arrow) near the vegetal pole. (B) DIC overlay with A, PMCs shown in red. (C) Two-dimensional projection of stacked images of gastrula embryo injected with *LvN^{act}* shows that most PMCs are in a characteristic ring-like pattern. (D) DIC overlay with (C) demonstrates that only a few PMCs (red) have been extruded. (E) A single confocal section of an untreated embryo stained with Meso 1 shows SMCs at the tip of the archenteron (arrowhead) and PMCs near the vegetal pole (arrow). (F) Two-dimensional projection of stacked images of gastrula embryo injected with *LvN^{act}* demonstrates that all extruded cells stain positively (arrowhead) for Meso1. The absence of 1d5 staining in the vast majority of extruded cells and the staining of all extruded cells with Meso1 indicates that the extruded cells are SMCs. Projected images with Meso1 mAb show staining throughout the blastocoel because of expression along the basal side of ectodermal cells. Bar, 25 μ m.

(approximately 5 hours of development; Horstadius, 1973; Logan and McClay, 1997). An early indicator of SMC specification is the initial expression of a sea urchin homologue of *Brachyury*, whose mRNA is first detected at the hatched blastula stage (9 hours of development; Harada et al., 1995). These data suggest that the SMCs are specified at some time during the early to mid-blastula stage (between 5 and 9 hours of development). We have previously reported that *LvNotch* is expressed uniformly during cleavage stages but that, during the early blastula stage, *LvNotch* expression is specifically lost at the vegetal pole (the location of the presumptive PMCs, SMCs

Fig. 5. Analysis of SMC-derived cell types in late stage larvae (60-65 hours of development). (A) A larva viewed along the oral surface and stained for endogenous *LvNotch*, which is expressed at high levels in the coelomic pouch cells (arrow) situated along the foregut (fg). (B) A confocal image showing a single coelomic pouch stained with α -*LvNotch* pAb (red) and the DNA dye YoPro (green). Adjacent to the coelomic pouch, the nuclei correspond to the oral ectoderm tissue (left) and the foregut (right). This double-staining procedure allowed the number of coelomic pouch cells to be determined (see Materials and methods). (C) A two-dimensional projection of the esophageal muscle (arrows), and the endoderm derived sphincter located between the foregut and midgut (arrowhead) stained with α -myosin heavy chain pAb in an untreated larva. Because cell fusions make individual muscle cells difficult to count, we scored the number of fully differentiated lateral muscle fibers (arrows) as an indication of muscle cell specification. (D) Larvae injected with *LvN^{neg}*, had fewer lateral muscle fibers (arrow), but the endoderm-derived sphincter was unaltered (arrowhead). (E-G) The dark-red pigment cells were counted using DIC optics. Larvae from embryos injected with *LvN^{act}* (F) have a greater number of pigment cells than untreated larvae (E), while injection of *LvN^{neg}* eliminated pigment cell specification (G). Blastocoelar cells were also counted using DIC optics (see Materials and methods). Bars, 25 μ m.



These data suggest that the SMCs are specified at some time during the early to mid-blastula stage (between 5 and 9 hours of development). We have previously reported that *LvNotch* is expressed uniformly during cleavage stages but that, during the early blastula stage, *LvNotch* expression is specifically lost at the vegetal pole (the location of the presumptive PMCs, SMCs

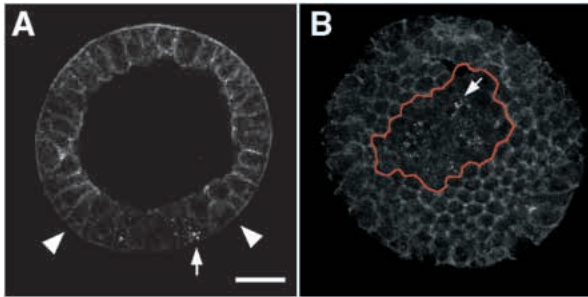


Fig. 6. Endogenous LvNotch is found in vesicles in the presumptive SMCs. Early blastula embryos (7 hours of development) were stained for endogenous LvNotch and viewed along the animal-vegetal axis (A) and along the vegetal pole (B). LvNotch is found in apically localized vesicles (arrows) present throughout the vegetal pole (site of the presumptive PMCs, SMCs and a small portion of the endoderm) at the time that LvNotch expression is being lost from the membranes of these cells: demarcated with arrowheads in A and outlined in red in B. Bar, 25 μ m.

and a portion of the endoderm; Sherwood and McClay, 1997). Interestingly, a careful examination of this loss of membrane LvNotch revealed that it was accompanied by the appearance of apically localized vesicles containing LvNotch (Fig. 6A,B). These vesicles were present throughout the vegetal pole of the embryo between 6 and 8 hours of development as membrane-localized LvNotch was being lost. Consistent with a potential signaling function for LvNotch within this region, similar vesicular structures have been observed in both *Drosophila* and *C. elegans* within and near cells that actively signal with LIN-12/Notch receptors (Fehon et al., 1991; Kooh et al., 1993; Crittenden et al., 1994; Henderson et al., 1994). Thus, LvNotch is expressed and found in vesicles in a spatial and temporal pattern consistent with a direct role in SMC specification.

Mosaic analysis of LvNotch function in SMC specification

To test the function of LvNotch signaling within the presumptive SMCs, a mosaic analysis of LvNotch function was performed by injecting *LvN^{neg}* into one cell at the 2-cell stage. Because the SMC precursors are distributed radially around the animal-vegetal axis and the first cleavage plane divides the embryo along the animal-vegetal axis, this injection scheme placed *LvN^{neg}* into approximately half of the presumptive SMCs in blastula-stage embryos (Fig. 7A; Horstadius, 1973; Cameron et al., 1987; Ruffins and Ettensohn, 1996). SMC specification was assayed at the mesenchyme blastula stage using the mAb 2.8f2 (SMC1), which stains an apically localized intracellular epitope in the majority of SMC precursors at this time (C. A. Ettensohn, personal communication). To address the potential nonautonomous and autonomous function of LvNotch signaling in SMC specification, we quantified the number of SMC1-staining cells at the border of *LvN^{neg}* expression. Furthermore, the total numbers of SMC1-positive cells were scored for cells lacking or expressing *LvN^{neg}*. As controls for this experiment, *LvN^{neg}ΔEGF7-29* was similarly injected and scored, and uninjected embryos were also evaluated.

Counts of SMC1-staining cells revealed that control injections of *LvN^{neg}ΔEGF7-29* had no effect on SMC

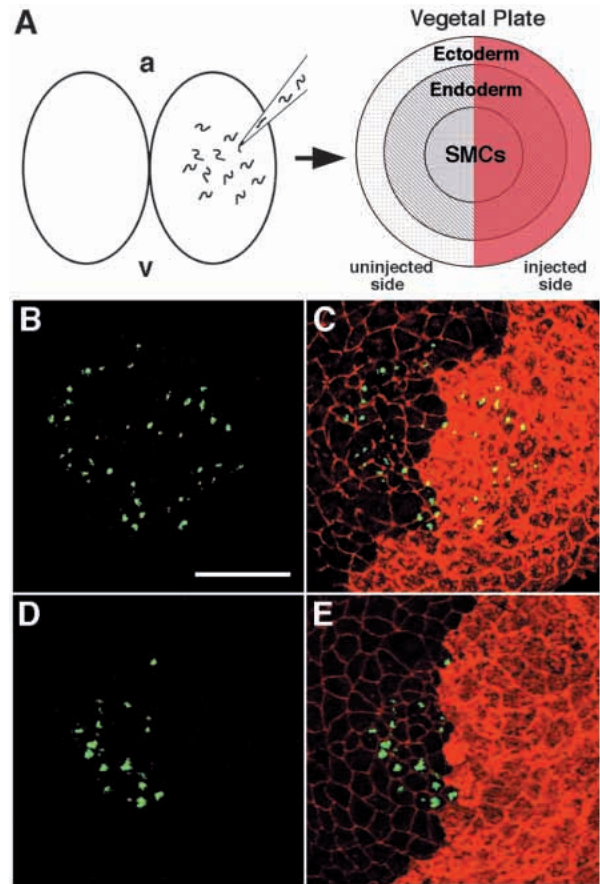


Fig. 7. LvNotch signaling appears to function cell autonomously in SMC specification. (A) *LvN^{neg}* or *LvN^{neg}ΔEGF7-29* was injected into one cell at the 2-cell stage (left), placing the mRNA into roughly half of the presumptive SMCs in the vegetal plate of mesenchyme blastula embryos (right, see fate map of Ruffins and Ettensohn, 1996). (B-E) Both *LvN^{neg}* and *LvN^{neg}ΔEGF7-29* were expressed along the apical surface of cells (red) at high levels in excess of endogenous LvNotch, which was not observed with the detection parameters used. Uninjected cells were outlined using α -LvGcadherin pAb (red), which localizes to apical adherens junctions, and SMCs were identified with α -SMC1 mAb (green). SMC1 staining in the vegetal pole (B) overlaid with *LvN^{neg}ΔEGF7-29* and LvGcadherin staining (C) shows that SMC specification was not affected by overexpression of *LvN^{neg}ΔEGF7-29*. (The slight difference in the number of SMC1-staining cells on both halves of the vegetal plate shown in B was often seen, and was a largely a result of the natural variability in SMC1 staining.) In contrast, SMC1 staining at vegetal pole (D) overlaid with *LvN^{neg}* and LvGcadherin staining (E) demonstrates that *LvN^{neg}* eliminates SMC specification in cells expressing this protein, but does not effect SMC specification in neighboring cells lacking *LvN^{neg}*. Bar, 25 μ m.

specification in cells expressing or lacking this protein compared with uninjected embryos (Table 2; Fig. 7B,C). Injection of *LvN^{neg}*, however, essentially eliminated SMC specification in cells expressing *LvN^{neg}*, but did not affect the specification of SMCs in uninjected cells bordering *LvN^{neg}* expression nor the total number of SMCs specified on the uninjected side of the vegetal plate (Table 2; Fig. 7D,E). The elimination of SMC specification in cells expressing *LvN^{neg}*, and unaltered specification of neighboring SMCs, strongly

Table 2. Mosaic analysis of LvNotch signaling and SMC specification in the mesenchyme blastula stage vegetal plate

Treatment ^a	n	Number of SMC1-staining cells			
		Uninjected side of VP	mRNA injected side of VP	Bordering on injected side of VP ^b	Total in VP
<i>LvN^{meg}</i>	18	23.8±1.6	1.1±0.3*	4.9±0.4	24.9±1.6*
<i>LvN^{meg}ΔEGF7-29</i>	18	24.5±1.7	26.7±1.4	5.1±0.3	51.1±2.4
Uninjected	17	25.2±0.8 ^c	25.2±0.8 ^c	NA	50.4±1.7

^aEach treatment was performed on embryos from the same batch of eggs. Similar results were quantitated in two additional batches of fertilized eggs involving a smaller number of total embryos (Fig. 7B-D is from one of these separate batches).

^bSMC1-staining cells on the uninjected side of the VP directly adjacent to cells on the injected side were scored in this column.

^cExpected number of SMC1-staining cells in half of the vegetal plate.

**LvN^{meg}* treatment means ± s.e.m. followed by an asterisk are significantly different in number compared with the respective control(s) within a column ($P < 0.05$; by ANOVA).

Abbreviations: VP, vegetal plate; NA, not applicable.

suggests that LvNotch signaling is acting autonomously within presumptive SMCs to mediate SMC specification.

LvNotch signaling in the differential specification of SMCs and endoderm

To investigate the relationship of *LvNotch* signaling in SMC specification and neighboring endoderm formation, we first examined the establishment of endoderm-derived guts in plutei that developed from *LvN^{act}*- and *LvN^{meg}*-injected embryos. Approximately 80% ($n=158/197$) of larvae from embryos injected with *LvN^{act}* (3.0-6.0 pg/zygote) had guts that were significantly smaller than those of untreated controls (Fig. 8A,B) and often had stunted arms (a phenotype that we are currently exploring). The remaining 20% of embryos had either small, incompletely partitioned guts or no apparent gut tissue. Injection of *LvN^{meg}* (3.0-6.0 pg/zygote) did not affect gut formation in the vast majority of larvae examined (96%; $n=248/265$; Fig. 8C), and only occasionally resulted in a reduction or loss of the foregut (4%; $n=17/265$; data not shown). Thus, activation of LvNotch signaling appears to result in a loss of endoderm, while inhibition of LvNotch signaling has little effect on endoderm formation.

To gain further insights into the relationship of LvNotch signaling in SMC and endoderm formation, we examined the expression pattern of endogenous LvNotch, the earliest known marker that distinguishes presumptive SMCs and endoderm cells, in *LvN^{act}*- and *LvN^{meg}*-injected embryos (Sherwood and McClay, 1997). In the late mesenchyme blastula stage, endogenous LvNotch is expressed at high levels on the apical

surface of presumptive endoderm cells bordering the presumptive SMCs, which specifically lack LvNotch expression (Fig. 9A). Injection of *LvN^{act}* (3.0-6.0 pg/zygote) led to a large increase in the volume at the vegetal pole that lacked endogenous LvNotch expression, and a reduction in the amount of apical LvNotch in all cases examined (Fig. 9B,D; $n=30/30$). These results suggest that activation of LvNotch signaling increases the number of SMCs at the expense of presumptive endoderm cells, and is consistent with the smaller guts present in larvae injected with *LvN^{act}*. Injection of *LvN^{meg}* (3.0-6.0 pg/zygote) resulted in an opposite change; endogenous apical LvNotch extended more vegetally in all cases ($n=25/25$), and stretched continuously along vegetal pole in the majority of embryos ($n=21/25$; Fig. 9C,D). These results imply that the loss of SMC specification results in the endoderm extending vegetally into cells that usually give rise to SMCs. The above changes in endogenous LvNotch localization, together with the larval phenotypes, offer compelling evidence that LvNotch signaling is required for the differential specification of SMCs and endoderm.

DISCUSSION

LvNotch signaling is essential for normal secondary mesenchyme specification

We have examined the function of the sea urchin LIN-12/Notch receptor during early development by overexpressing activated and dominant negative forms of this protein. These

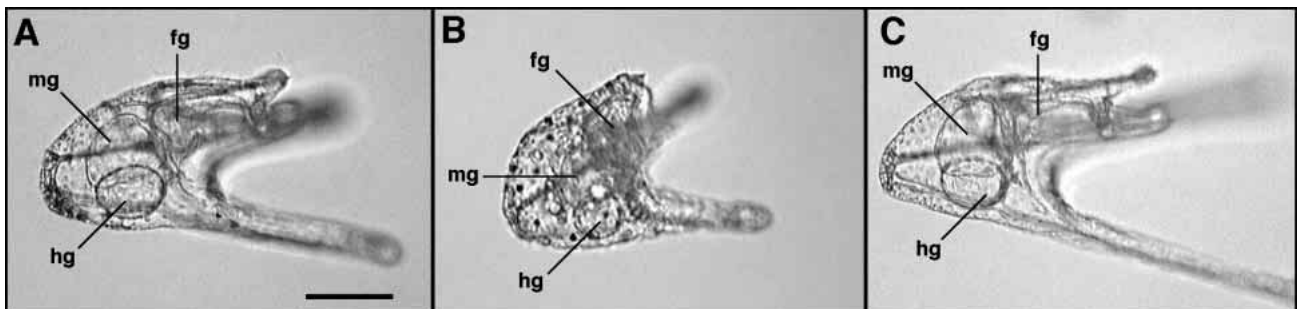


Fig. 8. Analysis of gut formation in late stage larvae (60-65 hours of development). (A-C) Lateral views of larvae from untreated and injected embryos. (A) Untreated larva displaying normal three-part gut. (B) Larva from *LvN^{act}* (3.0-6.0 pg)-injected embryo has a smaller three-part gut and stunted arms. (C) Larva from embryo injected with *LvN^{meg}* (3.0-6.0 pg) developed a normal-appearing three-part gut. Abbreviations: fg, foregut; mg, midgut; hg, hindgut. Bar, 100µm.

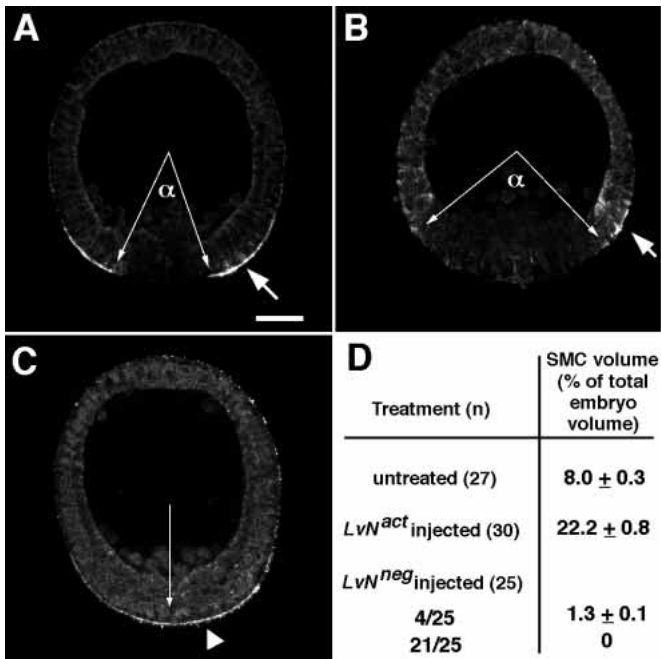


Fig. 9. Relationship of SMC and endoderm specification in *LvN^{act}*- and *LvN^{neg}*-injected mesenchyme blastula embryos. (A) Confocal section along the animal-vegetal axis stained for endogenous LvNotch. Endogenous apical LvNotch expression marks the presumptive endoderm (arrow). These cells border the SMCs at the center of the vegetal plate, which lacks detectable LvNotch expression (outlined by angle α). (B) Embryos injected with *LvN^{act}* (3.0-6.0 pg) and stained for only endogenous LvNotch using an extracellular domain-directed α -LvNotch pAb show a large increase in SMC territory (angle α) at the expense of endoderm (arrow). (C) Conversely, embryos injected with *LvN^{neg}* (3.0-6.0 pg) and stained for only endogenous LvNotch using an intracellular domain-directed α -LvNotch pAb demonstrate that the endoderm (arrowhead) now extends down to the vegetal pole into territory that would usually form SMCs. (D) The volume of the embryo occupied by the SMCs (\pm SEM) in untreated, *LvN^{act}*-injected and *LvN^{neg}*-injected embryos. The SMC volume was calculated using the angle α and the equation volume = $0.5 (1 - \cos\alpha/2)$ (see Reynolds et al., 1992). Bar, 25 μ m.

experiments demonstrate that LvNotch signaling plays a critical role in the specification of SMCs, a population of mesenchyme that gives rise to the majority of mesoderm-derived cell types in the sea urchin larvae. Overexpression of a dominant negative form of LvNotch (*LvN^{neg}*) during early development eliminated pigment cell specification and dramatically reduced all other SMC-derived cell types in late stage pluteus larvae, while overexpression of an activated form of LvNotch (*LvN^{act}*) resulted in an increase in most SMC-derived cell types. Furthermore, two presumptive SMC markers in mesenchyme blastula embryos, the SMC1 antigen and endogenous LvNotch, revealed that *LvN^{neg}* injections eliminated SMC specification in most mesenchyme blastula embryos. Instead, the endoderm extended vegetally into the cells that would usually form SMCs.

Given that injection of *LvN^{neg}* appeared to abolish the specification of SMCs prior to the mesenchyme blastula stage, it may seem surprising that any SMC-derived cell types formed

in pluteus larvae after injection of *LvN^{neg}*. Previous studies, however, have shown that the cells of the archenteron are highly regulative. Microsurgical removal of the SMCs throughout gastrulation results in endoderm switching fate to replace a portion of most SMC-derived cell types, with the exception of pigment cells (McClay and Logan, 1996). This recovery of SMC-derived cell types in surgically altered embryos is strikingly similar to the formation of SMCs in *LvN^{neg}*-injected embryos: although the SMCs were initially eliminated, a portion of most SMC-derived cell types were eventually formed in pluteus larvae, with the similar exception of pigment cells. Based on these observations, we propose that the SMCs that formed after injection of *LvN^{neg}* resulted from the conversion of endoderm cells to SMCs.

It is possible that this conversion of endoderm to SMCs is also regulated by LvNotch signaling. We have previously shown that removal of the SMCs results in a rapid downregulation of endogenous LvNotch expression in the endoderm cells that switch fate to replace SMCs (Sherwood and McClay, 1997). This is similar to what appears to occur to endogenous LvNotch expression in presumptive SMCs during normal SMC specification in the early blastula embryo (see Discussion below). Furthermore, *LvN^{neg}* expression declines during the late gastrula stage (Fig. 1C), which would permit the functioning of endogenous LvNotch signaling within the endoderm to replace missing SMCs. The development of techniques to extend the timing of *LvN^{neg}* expression should allow us to directly test this possible regulative function for LvNotch signaling in the future.

The function and timing of LvNotch signaling in secondary mesenchyme specification

To perform a mosaic analysis of LvNotch function in SMC specification, we injected *LvN^{neg}* into one cell at the 2-cell stage, which placed *LvN^{neg}* into half of the SMC precursors in the vegetal plate of the blastula embryo (see Fig. 7A). It is important to note that this injection scheme also placed *LvN^{neg}* into half of the presumptive endoderm, ectoderm and primary mesenchyme cells. If LvNotch signaling were regulating SMC specification in these other cells by controlling the release of a diffusible signal, we would likely have seen nonautonomous effects on SMC specification. Instead, we observed a sharp boundary of SMC specification that correlated precisely with the cells expressing *LvN^{neg}*; SMC specification was eliminated in vegetal plate cells expressing *LvN^{neg}*, but was unaltered in neighboring uninjected cells on the other half of the vegetal plate. These data thus support the hypothesis that LvNotch signaling functions autonomously within presumptive SMCs to mediate SMC specification.

The notion that LvNotch signaling functions autonomously within the presumptive SMCs is further strengthened by the localization of endogenous LvNotch. In the early blastula embryo, LvNotch is found in apically localized vesicles in and near the presumptive SMCs. Supporting a signaling role of LvNotch in these cells, similar patterns of vesiculation of *Drosophila* Notch and the *C. elegans* LIN-12/Notch receptor GLP-1 have been observed within and near cells that actively signal with these receptors (Fehon et al., 1991; Kooh et al., 1993; Crittenden et al., 1994). The functional significance of the vesiculation of LIN-12/Notch receptors in signaling is not well understood. In *Drosophila* and *C. elegans*, a subset of

Notch and GLP-1 intracellular vesicles co-localize with the ligands for these receptors *in vivo* (Kooch et al., 1993; Henderson et al., 1994), and it has been suggested that these vesicles may be important for either the activation or inactivation of the pathway (Henderson et al., 1994; Klueg et al., 1998). Notably, LvNotch vesiculation accompanies both the loss of this receptor from cell membranes in the presumptive SMCs, which require LvNotch signaling for their specification, and is seen in the PMCs and a small portion of the presumptive endoderm – cells that do not require LvNotch signaling for their specification. Thus, one possibility is that this vesiculation represents a broad and rapid mechanism to downregulate LvNotch in the vegetal pole after LvNotch signaling functions to specify SMC fate. Such a mechanism may prevent the misspecification of other cells at the vegetal pole to an SMC fate. Consistent with vesiculation being triggered by LvNotch signaling, we have observed that overexpression of LvN^{act} increases the region that undergoes vesiculation and loss of membrane LvNotch at the vegetal pole in the early blastula embryo (D. R. S., unpublished observation; evidenced by the expanded SMC territory lacking endogenous LvNotch in late mesenchyme embryos in Fig. 9B,D). Overexpression of full-length LvNotch (LvN^{full}) may thus increase SMC specification by interfering with the normal downregulation of the receptor. A second possibility is that LvNotch vesiculation represents active LvNotch signaling throughout a broad vegetal region in the embryo. Specificity to LvNotch signaling could arise from factors present within the presumptive SMCs that make these cells responsive to LvNotch signaling. Consistent with the existence of such vegetal factors, isolated animal halves containing high levels of LvN^{act} never form pigment cells, and usually do not develop other SMC-derived cell types (D. R. S., unpublished observation). Given that overexpression of LvN^{full} and LvN^{act} cause presumptive endoderm cells to be specified as SMCs, it is possible that factors that confer responsiveness to LvNotch signaling are present in lower abundance in presumptive endoderm cells and that elevated levels of LvNotch signaling cause these cells to be specified as SMCs. Clearly, the isolation and localization of ligands for LvNotch, as well as vegetal factors allowing responsiveness to LvNotch signaling, will be crucial in resolving the functional significance of LvNotch vesiculation.

The finding that LvNotch signaling appears to directly mediate SMC specification has further implications for the timing of LvNotch signaling. Because the expression of LvNotch is lost within the presumptive SMCs after the early blastula stage (by 6–8 hours of development), this suggests that the SMCs must be specified by LvNotch signaling prior to or during this time. Given that vesiculation of LIN-12/Notch proteins often accompanies active signaling with these receptors, we favor the idea that LvNotch signaling acts to specify the SMC fate sometime during the early blastula stage, shortly before or during LvNotch vesiculation.

LvNotch signaling and the differential specification of secondary mesenchyme and endoderm

Previous studies have shown that several cell-cell interactions, as well as a cell-intrinsically regulated GSK3 β -kinase/ β -catenin signaling pathway, are required during cleavage stages for the initial specification of both SMC and endodermal fate

at the vegetal pole (Horstadius, 1973; Henry et al., 1989; Ransick and Davidson, 1995; Emily-Fenouil et al., 1998; Wikramanayake et al., 1998; Logan et al., 1998). A final important finding from our study is that the specification of SMCs by LvNotch signaling appears to ultimately mediate the segregation of SMC and endoderm fate. Injection of LvN^{act} resulted in an increase in SMCs at the expense of endoderm, while injection of LvN^{neg} abolished the specification of SMCs and resulted in an extension of endoderm tissue vegetally into cells that would usually form SMCs. Remarkably, despite the fact that endogenous LvNotch is expressed at high levels apically in presumptive endoderm cells, overexpression of LvN^{neg} had no effects on the establishment of a complete endoderm in nearly all larvae examined (94%). Previous cell-dissociation experiments have demonstrated that cellular interactions that commit the presumptive endoderm cells to an endoderm fate occur during the early mesenchyme blastula stage (Chen and Wessel, 1996). Western analysis revealed that LvN^{neg} protein was expressed at high levels throughout the blastula stage (Fig. 1C), indicating that LvNotch signaling is not strictly required for the specification or commitment to an endoderm fate. Endogenous LvNotch is expressed in the endoderm throughout gastrulation (Sherwood and McClay, 1997) and LvN^{neg} expression did decline in the late gastrula stage (Fig. 1C). Thus it is possible that endogenous LvNotch signaling may play a later role in the patterning of the endoderm, which was not revealed by these experiments.

The findings that LvNotch signaling plays a crucial role in SMC specification, and that this signaling facilitates the segregation of SMC and endoderm fate, opens an important door into understanding the molecular mechanisms of SMC specification and vegetal patterning in the sea urchin embryo. Further studies on the regulation and function of LvNotch signaling in the sea urchin promise to reveal additional insights into endomesodermal patterning, as well as general cellular and molecular mechanisms underlying LIN-12/Notch signaling.

We are indebted to Chai-An Mao, Athula Wikramanayake and William Klein for training and suggestions on mRNA injection, and are very grateful to Charles Etensohn for communication of results prior to publication. In addition, we thank Charles Etensohn and Gary Wessel for the generous gifts of antibodies, and Nina Tang Sherwood, Rick Fehon and members of the McClay laboratory for invaluable advice throughout this work. This research was supported by an NIH graduate training grant and Sigma Xi grants to D. R. S., and NIH grant HD14483 to D. R. M.

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