Development 135, 2445-2454 (2008) doi:10.1242/dev.018101

LvNumb works synergistically with Notch signaling to specify non-skeletal mesoderm cells in the sea urchin embryo

Ryan C. Range*, Thomas D. Glenn, Esther Miranda and David R. McClay†

Activation of the Notch signaling pathway segregates the non-skeletogenic mesoderm (NSM) from the endomesoderm during sea urchin embryo development. Subsequently, Notch signaling helps specify the four subpopulations of NSM, and influences endoderm specification. To gain further insight into how the Notch signaling pathway is regulated during these cell specification events, we identified a sea urchin homologue of Numb (LvNumb). Previous work in other model systems showed that Numb functions as a Notch signaling pathway antagonist, possibly by mediating the endocytosis of other key Notch interacting proteins. In this study, we show that the vegetal endomesoderm expresses *lvnumb* during the blastula and gastrula stages, and that the protein is localized to the presumptive NSM. Injections of *lvnumb* mRNA and antisense morpholinos demonstrate that LvNumb is necessary for the specification of mesodermal cell types, including pigment cells, blastocoelar cells and muscle cells. Functional analysis of the N-terminal PTB domain and the C-terminal PRR domain of LvNumb shows that the PTB domain, but not the PRR domain, is sufficient to recapitulate the demonstrable function of full-length LvNumb. Experiments show that LvNumb requires an active Notch signal to function during NSM specification and that LvNumb functions in the cells responding to Delta and not in the cells presenting the Delta ligand. Furthermore, injection of mRNA encoding the intracellular domain of Notch rescues the LvNumb morpholino phenotype, suggesting that the constitutive intracellular Notch signal overcomes, or bypasses, the absence of Numb during NSM specification.

KEY WORDS: Numb, Notch, Delta, Sea urchin, Endomesoderm specification

INTRODUCTION

The Notch pathway was identified in *Drosophila* and it was shown that in a process called lateral inhibition that the Delta ligand activates Notch in the surrounding cells, thereby inhibiting them from taking on the fate of the Delta-expressing cell (Artavanis-Tsakonas et al., 1999; Baker, 2000). Since that initial discovery, Notch signaling has been found to contribute to development of many organisms. In the sea urchin, Notch signaling initiates specification of the non-skeletogenic mesoderm (NSM) from the surrounding endomesoderm (Sherwood and McClay, 1999). Initially, the endomesoderm cells express the Notch receptor, and those closest to the vegetal pole receive a Delta signal from micromeres (Sweet et al., 2002). Those cells in direct contact with the Delta-expressing cells become NSM, and those cells not making direct contact remain endomesoderm. This first signal initiates specification of NSM cells that become pigment and blastocoelar cells, between the seventh to ninth cleavage. Later, Notch signaling again contributes to the further subdivision of the endomesoderm, this time initiating specification of muscle cells and ceolomic pouch cells (McClay et al., 2000; Sherwood and McClay, 1997; Sherwood and McClay, 1999; Sweet et al., 2002). Thus, the sea urchin embryo uses Delta-Notch signaling to specify all of the NSM cell fates.

Both the Delta ligand and Notch receptor rely on a number of important modifiers to influence the outcome of the signal. One of these modifiers is Fringe, which is necessary for the Notch reception of the Delta signal (Irvine and Wieschaus, 1994; Panin et al., 1997; Peterson and McClay, 2005). Another modifier is Numb: a membrane-localized intracellular protein that antagonizes Notch signaling in many contexts (Cayouette and Raff, 2002). The most detailed studies of Numb/Notch interactions examined their involvement in asymmetric cell divisions in the *Drosophila* peripheral nervous system, central nervous system and mesoderm, all of which rely on Delta-Notch signals (Han and Bodmer, 2003; Le Borgne and Schweisguth, 2003b; Ruiz Gomez and Bate, 1997; Skeath and Doe, 1998). A model developed from these studies suggests that Notch and Delta segregate equally from a progenitor cell into both daughter cells, whereas Numb segregates into only one of the daughter cells. The Delta ligand signals to both cells, but activation of Notch target genes only occur in one cell. In the other cell, Numb blocks the Notch signal by an unresolved mechanism. Thus, Numb is modeled to be the molecular cue causing the asymmetry between the daughter cells (Jan and Jan, 1998; Posakony, 1994). This antagonistic relationship between Numb and Notch also occurs during vertebrate neurogenesis and myogenesis, and has been implicated in breast cancer (Cayouette and Raff, 2002; Pece et al., 2004).

Biochemical studies suggest that Numb has three protein-protein binding domains that have the potential to influence its function. Numb binds to the Notch intracellular domain via its N-terminal phosphotyrosine binding domain (PTB domain). This domain is thought to act as a scaffolding domain that targets proteins to the intracellular domain of the Notch receptor (Guo et al., 1996; Li et al., 1997; Rice et al., 2001). Numb also has a C-terminal proline rich region (PRR), which has an affinity for the SH3 domains of SRC-family tyrosine kinases, suggesting a link between Numb and tyrosine-kinase-mediated signaling pathways (Verdi et al., 1996). Finally, an EH domain, which is located within the PRR domain, interacts with a network of proteins involved in endocytosis, actin remodeling and intracellular transduction of signals (Confalonieri and Di Fiore, 2002; Santolini et al., 2000). Thus, Numb may influence Notch signaling by bringing multiple proteins to the Notch receptor.

Department of Biology, Duke University, Durham, NC 27708, USA

*Present address: Biologie du Développement, UMR 7009 CNRS Universite de Paris VI, Observatoire Océanologique, 06230 Villefranche-sur-Mer, France
†Author for correspondence (e-mail: dmcclay@duke.edu)

Accepted 23 May 2008

Because Numb has been demonstrated to have important roles in the regulation of Notch signaling in many diverse contexts, we isolated the first echinoderm Numb homologue, LvNumb, and examined its role as a regulator of Notch signaling in the sea urchin. Surprisingly, our results indicate that LvNumb is not a negative regulator of Notch signal as expected; instead, it mediates specification of NSM in the sea urchin embryo, and is required for full NSM specification.

MATERIALS AND METHODS

Animals

L. variegatus sea urchins were obtained from either Florida or the Duke University Marine Laboratory in Beaufort, NC. The gametes were harvested, fertilized and cultured at 23°C as described (Hardin et al., 1992).

Phylogenetic analysis

Phylogenetic trees were created using the maximum likelihood method with PhyML using the WAG substitution model (http://atgc.lirmm.fr/phyml/) (Guindon et al., 2005).

A consensus tree with a 50% cut-off value was derived from a 250 bootstrap analysis using Mega 3.1 (http://www.megasoftware.net/). Numbers above bootstraps represent posterior possibilities calculated from this consensus.

Cell isolation and transplantation

Micromeres were removed at the 16-cell stage by hand using a small glass pipette as previously described (McClay, 2000). Embryos were halved by incubation of 60-120 cell stage embryos in Ca^{2+}-free seawater and separation of the halves through the animal and vegetal poles. A half from a control embryo injected with the green fluorescent stain CFDA-SE (carboxyfluorescein diacetate succinimidyl ester) (Invitrogen) was combined with a half from a morpholino-injected embryo of the same stage.

Cloning a LvNumb fragment

LvNumb was isolated during a search of the sea urchin genome for components of the Notch signaling pathway. Exact primers were designed against a small region of *S. purpuratus* DNA corresponding to Numb and used to amplify *SpNumb* from midgastrula cDNA via PCR. The amplified, 105 bp product was cloned into the pGEMT vector (Promega) and sequenced bi-directionally (Duke Sequencing Core). Clones were identified as PCR products of *SpNumb* by BLAST search. *SpNumb* was used to probe a *L. variegatus* cDNA library macroarrayed on filters.

Northern analysis

Total RNA was isolated from embryos with Trizol. RNA (10 μg) from each developmental stage was loaded onto a 1% agarose formaldehyde gel, fractioned by electrophoresis and blotted onto Nylon membrane using Turboblot (Schleicher and Schuell) and hybridized with a *LvNumb* fragment lacking the PTB domain. Blots were given two 5 minute washes with 6×SSPE, 0.5% SDS at room temperature, one 45-minute wash with 1×SSPE, 0.1% SDS at 37°C, and one 45 minute wash with 1×SSPE, 0.1% SDS at 50°C. The blot was placed on film for 72 hours at –80°C with an intensity screen.

Generation of LvNumb constructs

A *LvNumb* clone was generated by splicing partial clones into a pCS2 expression vector. The 5′UTR and first 1300 bp of the open reading frame were cloned from the macroarray screens. The remaining sequence was cloned by 3′RACE. The pCS2 vector has a 5′UTR that provides an excellent translation start site for mRNA constructs in the sea urchin (Sweet et al., 2002). pCS-2 constructs containing the sequence of LvNumb, the PTB domain of LvNumb and the PRR domain of Numb were generated by standard molecular biology techniques. All clones were verified by sequence analysis.

mRNA preparation and injection

LvnNumb-pCS2, *PTB domain-pCS2* and *PRR domain-pCS2* constructs were linearized with *Not*I and used as template to generate in vitro transcribed 5′ capped mRNA using the SP6 mMessage Machine kit (Ambion). mRNA concentrations were determined by spectrophotometry. Injections were carried out as described (Sherwood and McClay, 1999).

QPCR analysis

RNA from 25 *L. variegatus* embryos was isolated with Trizol (Invitrogen). The samples were treated with Dnase I (Ambion) and then reverse transcription reactions were performed using a Taqman Gold RT-PCR kit (Applied Biosystems). A LightCycler instrument and Fast Start SYBR Green PCR kit (Roche) were used for QPCR analysis based on manufacturers instructions. Primers used were ubiquitin (Ub) (Davidson et al., 2002a), and two sets of primers designed to LvNumb. The primer sets generated similar results. A pCS2 plasmid containing the *LvNumb* clone was used to determine the specificity and efficiency of each primer set. The data from each cDNA sample was normalized against ubiquitin mRNA. QPCR analysis was performed on three separate samples at least two times, and each reaction product was confirmed by gel electrophoresis. Ubiquitin was used as a standard to determine *LvNumb* transcript numbers.

Counts of SMC types

The number of SMC-derived cell types was examined in 50-55 hour pluteus larvae, as previously described (Sherwood and McClay, 1999).

Immunolocalization and image analysis

Embryos were fixed either in 2% paraformaldehyde for 10 minutes and then washed through methanol for 1 minute, or they were fixed in methanol for 2 minutes. The embryos were returned to SW plus 4% normal goat serum and immunochemistry was performed as described previously (Sherwood and McClay, 1999).

Morpholino injection

The LvNumb sequence was used to design two morpholino antisense oligonucleotides, which GeneTools produced. Sequences of morpholino oligonucleotides are: Numb 1, 5′-GTATAATACATGAGAAGAAGAC-CAC-3′; Numb 2: 5′-GAGAAGAAGACCACTGTTTATATCC-3′.

mRNA encoding a GFP reporter construct fused to the 5′UTR of LvNumb was co-injected with the with LvNumb morpholino to determine the effectiveness of the LvNumb1 morpholino in blocking target mRNA translation. In addition, a control morpholino was injected as a control against any nonspecific effects due to toxicity in the morpholino solution. The LvNumb1 morpholino was injected at 1.5 mM and the LvNumb2 morpholino at 0.5 mM in a solution containing 25% glycerol. The two morpholinos produced identical phenotypes, and were rescued by expression of Numb protein from mRNA not containing the sequence recognized by the morpholinos.

In situ hybridization

In situ hybridization was performed on staged embryos using a protocol adapted from Harland (1991). A *lvnumb* sequence lacking the PTB domain, but containing the 3′ polyA tail, was cloned into pGEMT-Easy and linearized with *Spe*I. The probe was synthesized with T7 RNA polymerase (New England Biolabs). Control *lvnumb* sense probe was used to verify the specificity of the anti-sense probe hybridization (data not shown). Anti-sense and sense probes were incubated for the same amount of time in each experiment.

RESULTS

Isolation of sea urchin LvNumb cDNA clones and deduced amino acid sequence

A 1300 bp fragment of *LvNumb* was subcloned from a *Lytechinus variegatus* midgastrula cDNA library. The final 557 bp of *LvNumb* were obtained using 3′ RACE. The LvNumb open reading frame contains 1857 bp with a predicted amino acid sequence length of 619. LvNumb contains the two major domains shared by all Numb proteins based upon sequence alignment with *Drosophila* Numb. These domains are the N-terminal phosphotyrosine binding (PTB) domain and the proline rich region (PRR). In addition, LvNumb contains an EH domain at the extreme C terminus (Fig. 1A).

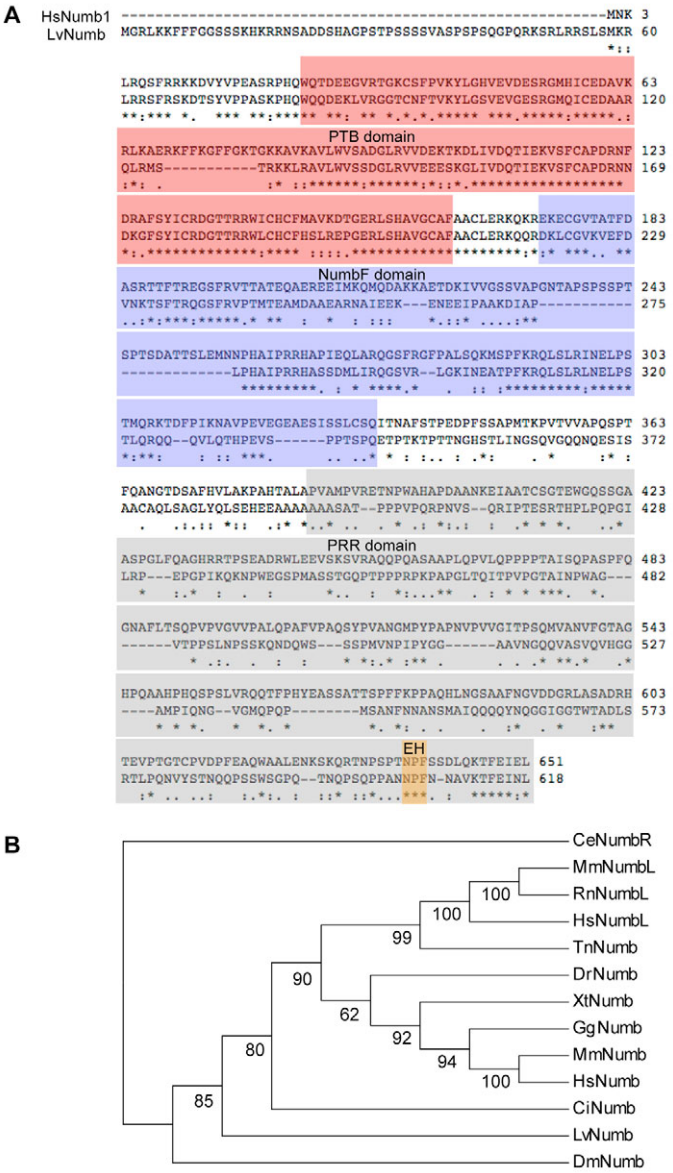

Fig. 1. Alignment of the predicted amino acid sequence of LvNumb and Human Numb1 proteins. (**A**) Identical residues are denoted by an asterisk and conserved by '.' or ':'. The four conserved domains are labeled. (**B**) A molecular phylogeny of the Numb and Numb-like family members. Abbreviations: Ce, *C. elegans*; Mm, mouse; Rn, rat; Hs, human; Tn, Tetraodon (puffer fish); Dr, zebrafish; Xt, *Xenopus tropocalis*; Gg, chicken; Ci, *Ciona*; Lv, *Lytechinus*; Dm, *Drosophila*.

Sequence comparisons between Human Numb and LvNumb domains indicated that the PTB and PRR domains share identities of 70% and 21%, respectively. This is similar to other Numb homologues, which generally show greater sequence similarity in the PTB domain than in the PRR domain.

To determine the relationship of LvNumb to other Numb and Numb-like orthologs, we performed a phylogenetic analysis (Fig. 1B). The tree shows that LvNumb is more closely related to its vertebrate homologs than to either its *Drosophila* or *C. elegans* homologues, from which it diverged evolutionarily. In the tree, sea urchin Numb clusters outside of the vertebrate Numb and Numb-like clusters. In addition, the sea urchin genome does not contain a Numb-like homologue. These findings suggest that LvNumb and vertebrate Numb homologues diverged from a common ancestral gene and that the two gene families, Numb and Numb-like, formed after the common ancestral split.

The LvNumb transcript is expressed during blastula and gastrula stages in the endomesoderm, and the protein is expressed within the presumptive non-skeletogenic mesoderm

Developmental northern analysis showed that a single *lvnumb* transcript accumulated at hatched blastula stage and remained expressed throughout development (Fig. 2A). The spatial location of *lvnumb* transcripts was determined by whole-mount in situ hybridization and whole-mount immunofluorescence with an antibody generated against LvNumb (Fig. 2B; see Fig. S1A in the supplementary material). In the egg and early cleavage stages, there was little to no accumulation of *lvnumb* transcripts (Fig. 2B, parts a,b). At the hatched blastula and mesenchyme blastula stages, *lvnumb* expression localized to the vegetal plate endomesoderm (Fig. 2B, parts c,d). From early- to mid-gastrula, *lvnumb* mRNA localized throughout the invaginating endoderm, with reduced expression in delaminating secondary mesenchyme cells (Fig. 2B, part e). Later in gastrulation, transcripts localized to regions corresponding to the foregut and the blastopore of the embryo, and *lvnumb* transcripts were reduced in the midgut (Fig. 2B, part f). A similar profile of expression was observed when embryos were stained with an antibody against the LvNumb protein. Following reception of the Delta ligand, Notch protein is removed from the plasma membrane of the NSM (Sherwood and McClay, 1997; Sherwood and McClay, 1999). At the vegetal pole in hatched blastula stage embryos, the Notch receptor is missing after signaling there, and Numb protein is present in the area vacated by the Notch receptor, with a small overlap on either side with the remaining surface Notch (Fig. 2C, parts a-c) (Sherwood and McClay, 1997; Sherwood and McClay, 1999). The spatial and temporal expression of LvNumb continues to be coincident with LvNotch expression throughout gastrulation (Fig. 2), indicating that the expression of LvNumb is appropriate for it to act as a modifier of Notch signaling during SMC specification. Notch signaling does not appear to activate *lvnumb* expression in the vegetal plate, as QPCR and whole-mount in situ hybridization analysis of embryos injected with either activated or dominant-negative forms of LvNotch show minimal changes in the expression levels of *lvnumb* (see Fig. S1D in the supplementary material).

LvNumb is necessary for non-skeletogenic mesoderm specification

Based on previous studies in *Drosophila* and vertebrates, we hypothesized that LvNumb would function as a negative regulator of Notch signaling in the sea urchin. Previous studies in the sea urchin showed that injecting mRNA encoding the intracellular domain of the LvNotch receptor (LvN^{ACT}) constitutively activated Notch signaling, causing an increase in all four NSM subtypes. Conversely, expression of a dominant-negative Notch (LvN^{NEG}) construct caused a decrease in all NSM subtypes (Sherwood and McClay, 1999). Therefore, manipulating LvNumb activity should give predictable phenotypes if sea urchin Numb functions as reported in other systems. Accordingly, we designed two antisense morpholino oligonucleotides to interfere with the translation of endogenous *lvnumb* and injected these into fertilized embryos. Both morpholinos produced the same phenotype. Embryos injected with

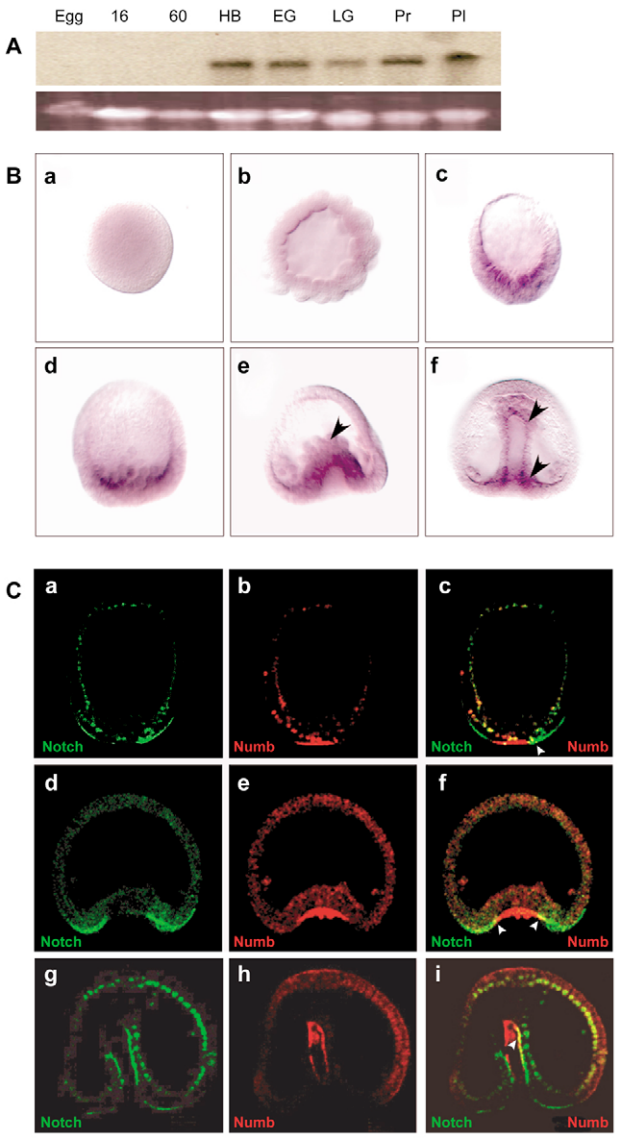

Fig. 2. *lvnumb* is transcribed beginning at hatched blastula and remains expressed until the end of embryogenesis in the endomesoderm. The protein is localized to the presumptive NSM. (**A**) Northern blot of LvNumb expression during early development (5 μg/lane). Ethidium bromide staining of the 18s rRNA bands used as a loading control. A single transcript accumulates beginning at hatched blastula stage and is expressed throughout the rest of development. 16, 16-cell stage; HB, hatched blastula; MB, mesenchyme blastula; EG, early gastrula; LG, late gastrula; PL, pluteus larvae. (**B**) *lvnumb* expression is upregulated at the vegetal plate beginning at hatched blastula stage. (a,b) *lvnumb* transcripts are not localized in the egg or 60-cell stage embryos. (c) Beginning at hatched blastula, *lvnumb* is upregulated in the vegetal plate (region of thickened epithelia). (d) *lvnumb* maintains high expression in the vegetal plate region of mesenchyme blastula embryos. (e) *lvnumb* is expressed in involuting endoderm, but not delaminating secondary mesenchyme cells in early gastrula embryos (arrowheads indicate SMCs). (f) By late gastrula, *lvnumb* is expressed primarily in the hindgut and the foregut (arrowheads), but is largely excluded from the midgut. (**C**) (a-c) In the blastula, LvNumb protein is apically localized to the presumptive NSM that has been cleared of LvNotch. LvNotch (a) and LvNumb (b) staining and merge (c) showing overlap at the edge of the NSM field (arrowhead). (d-f) Apical LvNumb expression expands with the presumptive NSM territory in early gastrula embryos. LvNotch (d) and LvNumb (e) staining and merge (f) showing overlap at the edge of the NSM field (arrowheads). (g-i) In gastrula embryos, the apical expression of LvNumb has expanded into the endoderm, significantly overlapping with dorsal LvNotch expression. (g) LvNotch staining. (h) LvNumb staining. (i) Merged image of LvNotch and LvNumb protein expression. Arrowhead indicates where LvNumb and LvNotch overlap.

LvNumb morpholino showed a large decrease in the number of pigment cells, blastocoelar cells and muscle cell fibers produced by the embryo (Fig. 3B,E,J) (Table 1), whereas embryos injected with the control morpholino showed no defects in these cell types (Fig. 3A,D,G) (Table 1). Overexpression of *lvnumb* mRNA did not increase the number of primary mesenchyme cells (Fig. 3K), but did increase the number of NSM (Fig. 3L), including pigment cells and blastocoelar cells (Fig. 3C,F) (Table 1). Thus, embryos injected with the LvNumb morpholino or *lvnumb* mRNA had similar phenotypes to embryos injected with LvN^{NEG} or LvN^{ACT} mRNA, respectively. This result was surprising because it indicated that LvNumb does not antagonize Notch signal mediated specification of the NSM in sea urchin development. Instead, LvNumb acts similarly to Notch, contradicting our hypothesis based on the previous detailed studies on Numb and Notch interactions in *Drosophila*.

At the hatched blastula stage, LvNumb is expressed in the vegetal plate region that contains both the presumptive NSM and the micromeres. The micromeres present the Delta ligand to the surrounding macromeres, initiating the first wave of Notch signaling. As Numb is thought to be an adapter protein involved in endocytic degradation, it was feasible that LvNumb had a function in the micromeres, possibly by endocytosing the Delta ligand (Le Borgne and Schweisguth, 2003a). To test this hypothesis, we removed micromeres from normal host embryos at the 16-cell stage and then transferred micromeres from embryos injected with LvNumb2 morpholino. Whole embryos injected with LvNumb2 morpholino lacked pigment cells (Fig. 4B), whereas the control host embryos that received micromeres bearing the LvNumb morpholino developed with pigment cells (Fig. 4D). Thus, the Numb morpholino in the micromeres does not affect the transfer of the Delta to the overlying macromere progeny.

Table 1. Perturbation of Numb alters NSM cell numbers

Cell type	Control	Control morpholino	Numb morpholino injection	Numb mRNA injection	PTB mRNA injection	PRR mRNA injection
Pigment cells	84.4±15.6 (30)	81.7±17.6 (17)	6.1±8.8 (30)	122.4±22.9 (29)	134.5±17.6 (30)	82.9±14.3 (15)
Blastocoelar cells	81.9±8.1 (9)	86.3±8.8 (4)	46.2±16.9 (10)	102.7±9.2 (9)	109.9±12.9 (10)	83.0±8.2 (5)
Muscle cells	14.4±2.3 (19)		5.4±2.1 (19)			

Shown are means±s.d. The total number of embryos counted is in parentheses.

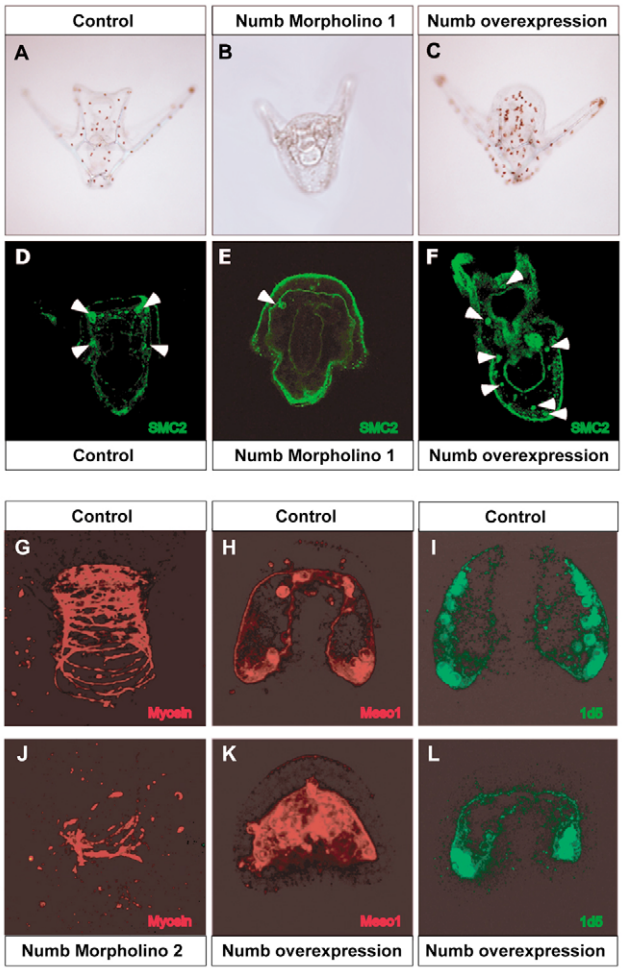

Fig. 3. Differentiation of primary and non-skeletogenic mesoderm cells after manipulation of the LvNumb levels. (**A-C**) Pigment cells. (**D-F**) Embryos stained with SMC2, which stains blastocoelar cells (indicated by arrowheads; there is background staining in the ectoderm and endoderm). (A,D) Control pluteus larvae show normal levels of pigment cells and blastocoelar cells. (B,E) Embryos injected with LvNumb1 morpholino (1.8 mM) have few pigment cells or blastocoelar cells. (C,F) Following injection with the *lvnumb* mRNA (0.5-1 pg/pl), there are increases in pigment cells and blastocoelar cells. (**G**) Control embryo stained with α-myosin heavy chain antibody showing esophageal muscle cell fibers. (**J**) The number of muscle cell fibers is reduced in embryos injected with LvNumb2 morpholino (0.5 mM). (**H**,**I**) Control embryos stained with the PMC marker 1d5 (H), and the general mesenchyme marker Meso1, which stains both NSM and PMCs (I). (**K**,**L**) Images of the same embryo injected with *lvnumb* mRNA showing that the large increase in mesenchyme is seen in L comes largely from an increase in NSM cells, not the PMCs (K).

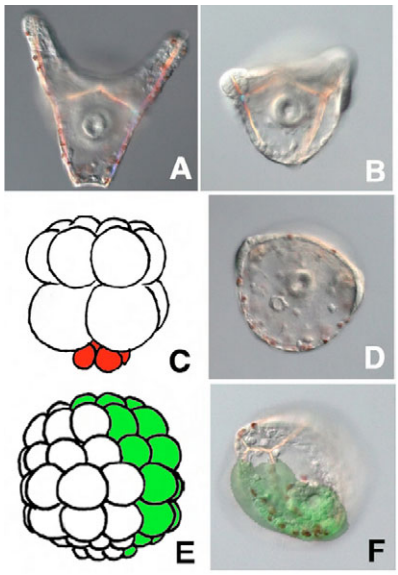

Fig. 4. Numb functions in Notch bearing cells that receive the Delta signal. (**A**) Control pluteus larva with pigment cells, a necessary consequence of presentation of the Delta ligand on micromeres to Notch on the receiving macromeres. (**B**) A pluteus larva with injected LvNumb2 morpholino. (**C**) The experiment in D. Micromeres bearing a LvNumb2 morpholino (red) were transplanted to a host micromereless embryo. (**D**) Numb morpholino in the micromeres does not affect the transfer of the Delta signal as the embryo has pigment cells. (**E**) Experiment in F. At the 60-cell stage, two embryos were cut in half from the animal to vegetal pole. A half bearing LvNumb2 morpholino was combined with a control half (green). (**F**) A fluorescent overlay shows that pigment cells produced by the control half embryo migrate into the LvNumb morpholino half embryo, indicating that if control pigment cells are provided to the Numb half they develop normally and migrate to the correct position in the ectoderm.

During the subdivision of the NSM subtypes, pigment cell specification requires the first Delta signal from the micromeres. Soon after invagination of the archenteron, these first NSM subtypes delaminate from the tip of the archenteron, migrate within the blastocoel and then intercalate between ectoderm cells (Gibson and Burke, 1985). It was possible that the ectoderm of embryos injected with the LvNumb2 morpholino compromised the pigment cells and prevented them from differentiating with pigment. To test this hypothesis, we halved normal 60-cell staged embryos and embryos injected with LvNumb2 morpholino and combined the two halves (Fig. 4E). Although embryos injected with Numb morpholino developed without pigment cells, the chimeric embryos had pigment cells from the control half, and some of those migrated into the ectoderm of the half injected with the LvNumb2 morpholino (Fig. 4F; green half). This result suggests that the ectoderm is not affected by the Numb morpholino and that the observed lack of pigment cells in embryos injected with the Numb morpholino is due to a reduction in NSM specification.

Deletion constructs show that the PTB domain is responsible for LvNumb activity

To develop a better understanding of the function of LvNumb in NSM specification, two deletion constructs were expressed: one construct containing only the PTB domain and the other construct containing the PRR and EH domains (Fig. 5H). We reasoned that expression of the PTB domain might interfere with the ability of endogenous LvNumb to interact with binding partners in a dominant-negative manner. In addition, we hypothesized that expression of the PRR domain might interfere with the ability of endogenous LvNumb to interact with the endocytic machinery (Nishimura et al., 2003). Instead, expression of the PTB domain increased the number of pigment cells and blastocoelar cells (Fig. 5B,E) (Table 1), whereas expression of the PRR domain construct had no effect on SMC specification (Fig. 5C,F). The PRR domain

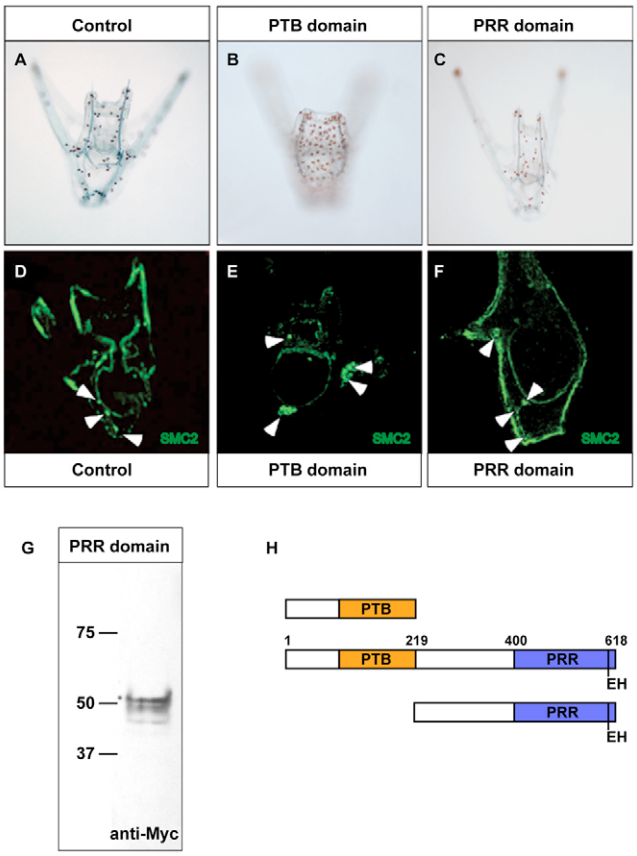

Fig. 5. Expression of the PTB domain, but not the PRR domain, affects non-skeletogenic mesoderm specification. (**A-C**) Pigment cells. (**D-F**) Pluteus larvae stained with SMC2. (A,D) Control embryos. (B,E) Injection of *ptb domain* mRNA (2 pg/pl) increases the formation of pigment cells and blastocoelar cells (arrowheads indicate large aggregations of blastocoelar cells). (C,F) Pigment cell and blastocoelar cell specification is unaffected by expression of Myc-PRR domain (3-6 pg/pl) (image shown) or untagged LvPRR domain (3-6 pg/pl) (data not shown). (**G**) Western blot showing expression of Myc-PRR domain protein in early gastrula-stage embryos. The protein is the predicted size of 52 kDa. (**H**) Schematics showing the LvNumb constructs used for experiments. The PTB domain, full-length LvNumb, the PRR domain and EH domains are indicated.

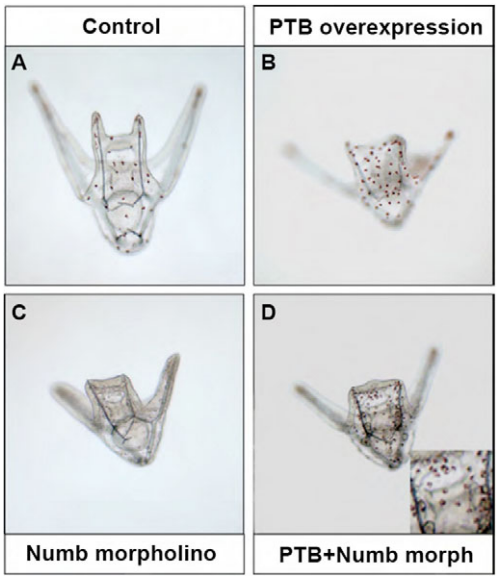

Fig. 6. Expression of the PTB domain rescues the LvNumb morpholino phenotype. (**A**) Embryos injected with control morpholino have normal levels of pigment cells. (**B**) Injection *ptb domain* mRNA (2 pg/pl) increases pigment cell specification. (**C**) Pluteus larvae injected with LvNumb morpholino (1.7 mM) showed pigment cells specification in less than 9% of the embryos examined. (**D**) Injection of *ptb domain* mRNA with LvNumb morpholino showed pigment cells specification in 77% of the embryos examined. Inset, high magnification of embryo in D.

protein was expressed and stable during NSM specification as seen by injecting embryos with Myc-PRR domain mRNA. Western blot analysis of samples taken at the early gastrula stage shows that the PRR domain was not degraded during the NSM specification window (Fig. 5G). Thus, expression of the PTB domain produced a phenotype that is opposite to the LvNumb morpholino phenotype. The phenotype was similar to both the LvNumb overexpression and the constitutively active Notch phenotypes, suggesting that the PTB region of LvNumb is the domain involved in NSM specification. The observation that the PRR domain has no effect on NSM specification suggests that the function of sea urchin LvNumb may not involve the endocytic machinery. Alternatively, the PRR domain may require the PTB to function in endocytosis.

To test whether the PTB domain is sufficient for the function of LvNumb, we attempted to rescue the LvNumb morpholino phenotype by injecting *ptb domain* mRNA. In three separate experiments, four sets of eggs were injected: one set was injected with a control morpholino (Fig. 6A), one set with *ptb domain* mRNA (Fig. 6B), one set with LvNumb morpholino (Fig. 6C) and one set was co-injected with LvNumb morpholino and *ptb domain* mRNA (Fig. 6D). Translation of the PTB domain mRNA was not affected by the LvNumb morpholino because it lacks the 5′UTR to which the LvNumb morpholino is directed. Embryos co-injected with LvNumb morpholino and *ptb domain* mRNA formed pigment cells in 77% of all co-injected embryos scored (n=34) (Fig. 6D), whereas embryos injected with the LvNumb morpholino alone formed pigment cells in fewer than 9% of the scored embryos (n=40). Thus, the PTB domain of LvNumb is sufficient to replace the function of LvNumb in pigment cell specification.

LvNumb requires the LvNotch signaling pathway to function during NSM specification

To test more directly the relationship between Numb and Notch, we designed a set of experiments to test whether the Notch and Numb pathways interact. We first asked whether overexpressing the PTB domain could rescue pigment cell specification in embryos expressing dominant-negative Notch, and therefore unable to respond to the Delta signal. LvN^{NEG} blocks Notch signaling by expressing an extracellular domain of Notch only, thereby binding Delta without an intracellular domain to transduce the signal. In three separate experiments, we injected one set of embryos with 25% glycerol (Fig. 7A), one set with the *ptb domain* mRNA (2 pg/pl) (Fig. 7B), another set injected with LvN^{NEG} mRNA (3 pg/pl) (Fig. 7C) and finally one set of embryos was co-injected with *ptb domain* mRNA (2 pg/pl) and LvN^{NEG} mRNA (3 pg/pl) (Fig. 7D). Embryos injected with dominant-negative Notch lacked pigment cells, whereas embryos injected with *ptb domain* mRNA showed an increase in pigment cells. The PTB domain had no effect on pigment cell formation when co-injected with dominant-negative Notch, as none of the double-injected embryos displayed

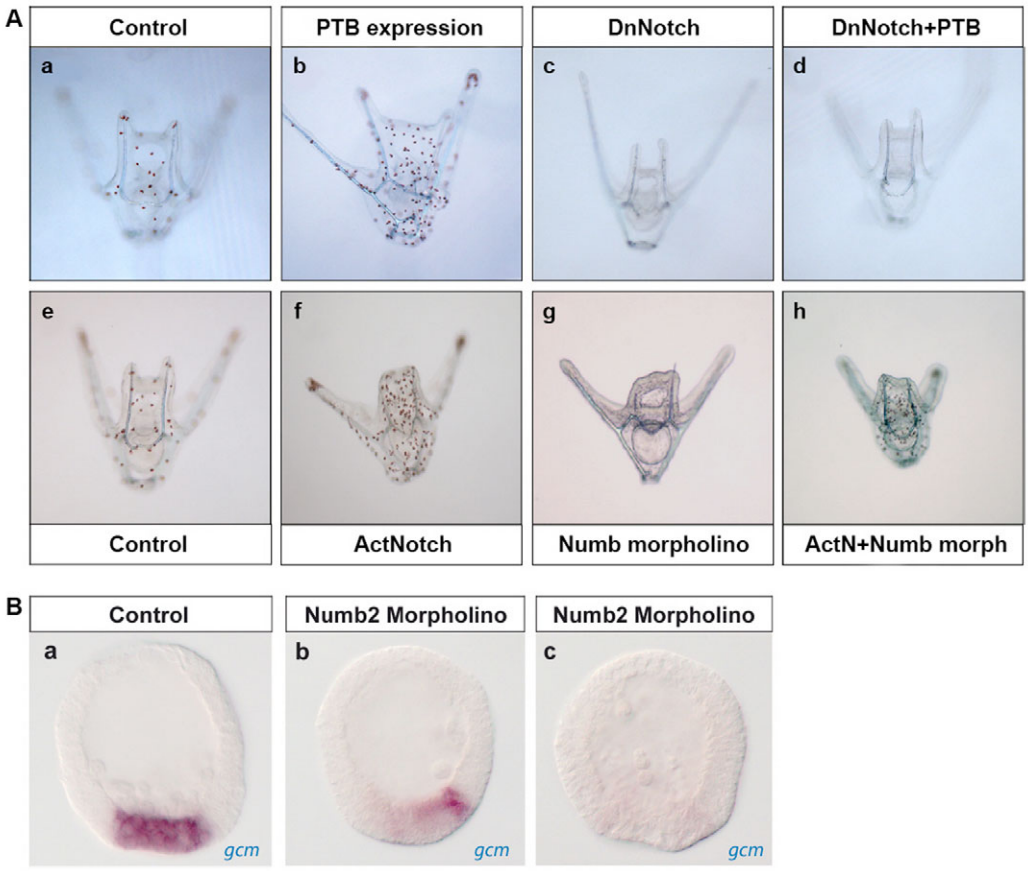

Fig. 7. LvNumb requires a functional Notch signal. High levels of Notch signaling override the requirement for LvNumb during NSM specification and LvNumb is necessary for the expression of the earliest known Notch target. (**A**) (a) Pluteus larvae injected with control morpholino have normal numbers of pigment cells. (b) Injection of either *ptb domain* mRNA (2 pg/pl) or (f) LvN^{ACT} mRNA (2 pg/pl) increases pigment cell specification. (c) Embryos injected with either LvN^{NEG} (3 pg/pl) or (g) LvNumb morpholino display a lack of pigment cells. (d) Injection of *ptb domain* mRNA in embryos expressing LvN^{NEG} does not rescue pigment cell specification. (h) Expression of LvN^{ACT} (2 pg/pl) rescues pigment cell specification in 86% of the embryos injected with the LvNumb morpholino (1.8 mM). (**B**) (a) A mesenchyme blastula stage embryo injected with control morpholino shows *gcm* expression in the NSM. (b,c) Embryos injected with LvNumb2 morpholino show reduced (b) or no expression of *gcm* (c).

pigment cells (n=40) (Fig. 7D). This result suggests that LvNumb requires activation of the Notch signal to function during pigment cell specification. We next asked whether expression of activated Notch could rescue pigment cell specification in embryos injected with LvNumb morpholino (Fig. 7E-H). Similar to the above experiments, we performed three separate experiments in which one set of embryos was injected with control morpholino (1.8 mM) (Fig. 7E), one set with LvN^{ACT} mRNA (2 pg/pl) (Fig. 7F), a third set with LvNumb morpholino (1.8 mM) (Fig. 7G) and finally a fourth set was injected with LvNumb morpholino and LvN^{ACT} mRNA (Fig. 7H). Embryos injected with activated Notch mRNA showed an increase in pigment cells, but fewer than 10% of embryos injected with LvNumb morpholino showed pigment cell specification (n=49). However, pigment cell formation occurred in more than 86% of the embryos co-injected with activated Notch mRNA and LvNumb morpholino (n=46) (Fig. 7H). Thus, expression of the Notch intracellular domain overcomes the lack of Numb expression, suggesting that a constitutively active intracellular Notch signal can overcome, or bypass, the absence of Numb during NSM specification.

Recently, Ransick et al. showed that Notch signaling directly activates Suppressor of Hairless, and this transcription factor directly targets the promoter of Glial Cells Missing (GCM), which is necessary for pigment cell specification during the first Delta/Notch signal between the seventh and ninth cleavage (Ransick and Davidson, 2006). Consistent with the observations described above, our molecular analysis showed that *gcm* expression is attenuated at early mesenchyme blastula stage in embryos injected with LvNumb2 morpholino (Fig. 7J-K). This result indicates that LvNumb is necessary in the macromeres for the activation of a direct target of Notch signaling at the time Delta-Notch is known to initiate specification of pigment cells, further strengthening support for the hypothesis that LvNumb and Notch work synergistically to specify non-skeletogenic mesenchyme.

DISCUSSION

LvNumb expression overlaps temporally and spatially in the area where LvNotch signaling is necessary for NSM specification

The results presented here strongly suggest that LvNumb is a crucial factor necessary for non-skeletogenic mesoderm specification, and the expression pattern and perturbations suggest that LvNumb acts as a positive modifier of Notch signaling. The first differential expression of LvNumb transcripts and protein occurs at the vegetal pole of blastula stage embryos coincident with the first Notch signal (Fig. 8A,B). The LvNumb protein is expressed at the apical membrane of the NSM in the area of Notch clearance, slightly

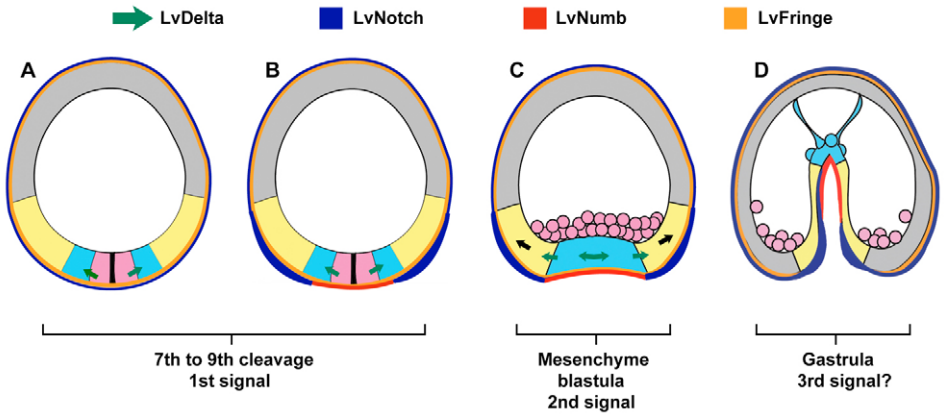

Fig. 8. Model for NSM specification in the sea urchin embryos. (**A**) During the first Notch signal, the blastula stage (seventh to ninth cleavage) embryo expresses LvNotch (blue) and Fringe (orange) on the membranes of all cells of the embryo. Micromeres (pink) express the LvDelta protein (green arrows) and signal to adjacent macromeres (light blue), specifying pigment cells and blastocoelar cells. LvFringe is necessary for Delta to activate the LvNotch receptor in the macromere lineage. (**B**) After LvNotch signals in the macromere lineage, the receptor is cleared from the presumptive NSM territory and presumptive endoderm cells begin to express high levels of apical Notch. The presumptive NSM expresses apical LvNumb (red), partially overlapping with LvNotch expression at the boundary of the territory. LvNumb works with LvNotch, either directly or synergistically, to specify pigment and blastocoelar cells at this time. (**C**) During the second Notch signal, the micromeres have ingressed as PMCs and no longer express Delta. Delta is expressed in the presumptive NSM where they signal to Notch to specify blastocoelar cells and muscle cells (double headed green arrow). Delta may also activate Notch signaling in neighboring cells (green arrows). Similar to the first signal, Delta requires LvFringe to activate Notch signaling. In addition, LvNumb is necessary for specification of blastocoelar cells and muscle cells in the NSM, and perhaps it is necessary in neighboring cells as well. Notch signaling also activates a secondary signal that is necessary for some aspect of endoderm specification (black arrows). (**D**) By gastrula stage, LvDelta is no longer expressed. However, LvNotch is apically expressed on the dorsal side of the archenteron and LvNumb is apically expressed in the NSM territory (light blue) and the archenteron. LvFringe is not expressed in the archenteron. We speculate that these expression patterns suggest a role for LvNotch and LvNumb in later aspects of NSM and gut specification. It is possible that an as yet unidentified Notch ligand, Serrate, which cannot activate LvNotch in the presence of LvFringe, activates LvNotch at this time.

overlapping with the area of active Notch signaling at the edge of the NSM field. This expression pattern is maintained until embryos reach early gastrulation (Fig. 8C). Thus, LvNumb is in the right place at the right time to influence Notch signaling during both sequential Notch signals that are necessary to specify the full complement of non-skeletogenic mesoderm subtypes.

The role of LvNotch and LvNumb in non-skeletogenic mesoderm specification

During *Drosophila* neurogenesis, myogenesis and apoptosis, Numb antagonizes Notch signaling during asymmetric cell division (Lundell et al., 2003; Ruiz Gomez and Bate, 1997; Skeath and Doe, 1998). However, whether Numb has a positive or negative impact on Notch signaling in vertebrate embryos is less clear. In fact, the function of Numb during vertebrate neurogenesis suggests that it may depend on the cellular context (Cayouette and Raff, 2002; Wakamatsu et al., 1999; Zhong et al., 2000; Zhong et al., 1997; Zilian et al., 2001). For example, in the developing chicken central nervous system, neural progenitor cells divide asymmetrically to produce one progenitor cell and one neural cell. Notch signaling is necessary in the apical cell to specify progenitor cell fate and chicken Numb (cNumb) asymmetrically localizes to the basal cell. In that context it appears that cNumb inhibits the Notch signal in the basal daughter cell, promoting neural cell fate (Wakamatsu et al., 1999). By contrast, mouse Numb (mNumb) is localized to the apical progenitor cell (the Notch signal-requiring cell) in the developing mouse central nervous system (Zhong et al., 1997). Moreover, mNotch knockouts and mNumb knockouts have remarkably similar phenotypes, suggesting that they are both necessary for progenitor cell fate in the apical cell (de la Pompa et al., 1997; Lutolf et al., 2002; Zhong et al., 2000). These results suggest that both mNumb and mNotch signaling may function as positive regulators of progenitor cell fate in this context, a result that suggests the sea urchin is not alone in using Numb as a positive regulator of Notch signaling.

NSM specification depends on LvNumb; however, it is unclear whether LvNumb acts by associating with the intracellular domain of the Notch receptor to facilitate its release from the membrane after γ-sequestrase activity, the transport of the Notch intracellular domain to the nucleus or by another mechanism. Several recent studies present evidence suggesting that Numb has functions independently of the Notch pathway. In cultured mouse neurons, Numb was shown to localize at the tip of growing axons where it is necessary for growth by way of its endocytosis of L1, a neuronal cell-adhesion molecule (Nishimura et al., 2003). In mouse neuroepithelial progenitor cells, Numb was shown to interact with several proteins at the adherens junctions, including E-cadherin, N-cadherin and catenins. These interactions appear to be necessary for the integrity of the neural epithelium, which is disrupted in Numb mutants (Rasin et al., 2007). In the sea urchin, LvNumb is expressed progressively in the presumptive NSM field where the Notch receptor is degraded from the plasma membrane, and LvNumb protein expression occurs at sites where Notch is actively transducing a Delta signal. Numb is then retained for a period of time in the sites where Notch has previously signaled. This expression pattern is consistent with a role in Notch signaling and perturbation studies reinforce this conclusion as the Numb morpholino knocks down expression of the earliest known Notch target, GCM. However, these data do not rule out a function independent of Notch. Interestingly, the Notch receptor is still somewhat cleared from the presumptive NSM in Numb morphants (see Fig. S1F-I in the supplementary material). Notch receptor clearance from the NSM has been shown to be a

consequence of Notch signaling (Sherwood and McClay, 1999; McClay et al., 2001), suggesting that some aspects of Notch signaling may still be intact in Numb morphants. Furthermore, expression of the Notch intracellular domain (NICD) rescues the Numb morpholino phenotype, suggesting that a high level of Notch signaling overcomes a LvNumb requirement. Nevertheless, overexpression of LvNumb, under conditions that normally augment Notch signaling, fails to rescue the dominant-negative Notch phenotype, suggesting that a Notch signal must be triggered for augmentation to occur. Thus, LvNumb works synergistically with the Notch signal in initiating NSM specification.

Endocytic-independent function of LvNumb

Many studies in *Drosophila* and vertebrates have linked the function of Numb to an association with the endocytic machinery: Numb has an EH domain that interacts with the endocytic machinery, Numb can interact with α-adaptin (a member of the A2 endocytic complex), Numb localizes to clathrin-coated pits, and it is associated with endocytic organelles (Berdnik et al., 2002; Jafar-Nejad et al., 2002). Furthermore, in a study focusing on Numb and L1 interactions in axon growth, the PTB domain and the PRR domain both acted as dominant-negative versions of Numb because they prevented endogenous Numb from connecting L1 to the endocytic pathway (Nishimura et al., 2003). Interestingly, however, in *Drosophila*, the elimination of the binding motifs for endocytic proteins does not affect the ability of Numb proteins to specify cell fates (Tang et al., 2005). Similarly in the sea urchin, the PTB domain of LvNumb rescues pigment cell specification in LvNumb morpholino-injected embryos, which contain little to no endogenous LvNumb, whereas expression of the PRR domain had no effect on NSM specification. As it is likely that the ability of Numb to interact with the endocytic machinery resides in the PRR domain, specifically in the EH domain (Salcini et al., 1997; Santolini et al., 2000; Smith et al., 2004), this result suggests that the function of LvNumb need not include interactions with the endocytic machinery. Rather, it appears that the function of LvNumb resides in its interactions with other proteins via the PTB domain. Indeed, as suggested by data shown in Fig. S1F-I in the supplementary material, in the presence of the Numb morpholino the Notch signal begins with Delta binding to Notch, and as a consequence the Notch extracellular domain is lost as normal (see Fig. S1H in the supplementary material). However, the signal transduction is not completed because the Numb morpholino causes a failure in the specification of pigment cells (see Fig. S1J in the supplementary material).

The present results raise important questions about the function of Numb, in particular its role as a Notch pathway agonist. It is possible that the function of Numb has changed during the course of evolution. Although LvNumb is still closely associated with Notch signaling in the sea urchin, the PTB domain may have changed to the point that it activates or protects the Notch intracellular domain on its way to the nucleus. Alternatively, LvNumb may bind other factors in cells via its PTB domain and as a consequence Notch signaling is positively affected. This is the second study to suggest that Numb has a function exclusive of endocytosis and that the PTB domain alone is able to specify cell fates, suggesting that this may be a conserved mechanism in both protostomes and deuterostomes. Thus, further studies are merited to identify the proteins that interact with the LvNumb domains. Identification of such factors will not only help clarify NSM specification in the sea urchin, but will have implications for Notch and Numb signaling in general.

We thank Dr Charles Ettensohn for the SMC2 antibody and Francois Lapraz for help with the phylogenetic analysis. We thank both past and present members of the McClay laboratory for their advice during the course of this work, particularly Dr Cyndi Bradham for critical comments on the manuscript, Dr Jenifer Croce for help with in situ hybridization and Dr Yu-Ping Yang for help with protein interaction assays. NIH grants 14483 and 61464 funded this work.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/14/2445/DC1

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