Subdividing the embryo: A role for Notch signaling during germ layer patterning in *Xenopus laevis*

Stacey P. Contakos, Carolyn M. Gaydos, Emily C. Pfeil, Kelly A. McLaughlin*

Department of Biology, Tufts University, 120 Dana Hall, 163 Packard Avenue, Medford, MA 02155, USA

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Abstract

The development of all vertebrate embryos requires the establishment of a three-dimensional coordinate system in order to pattern embryonic structures and create the complex shape of the adult organism. During the process of gastrulation, the three primary germ layers are created under the guidance of numerous signaling pathways, allowing cells to communicate during development. Cell–cell communication, mediated by receptors of the Notch family, has been shown to be involved in mediating diverse cellular behaviors during development and has been implicated in the regulation of cell fate decisions in both vertebrate and invertebrate organisms. In order to investigate a role for Notch signaling during boundary formation between the mesoderm and endoderm during gastrulation, we manipulated Notch signaling in gastrula stage embryos and examined gene expression in resultant tissues and organs. Our findings demonstrate a much broader role for Notch signaling during germ layer determination than previously reported in a vertebrate organism. Activation of the Notch pathway, specifically in gastrula stage embryos, results in a dramatic decrease in the expression of genes necessary to create many different types of mesodermal tissues while causing a dramatic expansion of endodermal tissue markers. Conversely, temporally controlled suppression of this pathway results in a loss of endodermal cell types and an expansion of molecular markers of mesoderm. Thus, our data are consistent with and significantly extend the implications of prior observations suggesting roles for Notch signaling during germ layer formation and establish an evolutionarily conserved role for Notch signaling in mediating mesoderm–endoderm boundaries during early vertebrate development.

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Introduction

Once fertilized, the developing embryo must spatially and temporally coordinate a multitude of cellular behaviors such as proliferation, migration, apoptosis, and morphogenesis in order to create a properly patterned multicellular organism. Pattern formation is made possible through molecular mechanisms of cell–cell communication, which allow cells to influence each other’s fate and behavior. Vertebrate embryos rely extensively upon inductive interactions to diversify their various cell types. Cells can undergo changes either autonomously or by interaction with their neighbors during development. Interestingly, these signals can either stimulate or inhibit a given process, and the same signals are used repeatedly to produce distinct effects in different developmental contexts, even within the same organism.

Gastrulation marks the onset of changes in cell behaviors that begin to shape an individual. During this complex process, the embryo becomes a multilayered entity, with an outer layer of ectoderm, an inner layer of endoderm, and an intermediate layer of mesoderm. Within the patterned embryo, all tissues are composed of cells from one or more of the three original germ layers. Once formed, the cells of these three initial layers assemble into the organ and tissue rudiments and, ultimately, the functional organs and tissues which comprise the adult.

The rearrangement of cells during gastrulation not only allows new cell–cell signaling to occur between cells that were previously juxtaposed, but also culminates in the formation of the three primary germ layers (for examples see, De Robertis et al., 1994; Beetschen, 2001; Locascio and Nieto, 2001; Lu et
al., 2001; Keller et al., 2003). Once created, these germ layers must be further subdivided to create organ fields from which functional organs and tissues arise. The patterning that occurs during this early period of development is important for proper boundary formation between the germ layers. While there is an overall understanding of numerous signaling pathways that contribute to formation of the three germ layers, to date, the mechanisms which act to segregate these layers and the molecules used to pattern embryonic organ fields remain poorly understood. Characterizing the molecular events that underlie the specification and patterning of the three germ layers, as well as the subsequent creation of the primary organ fields, is critical for understanding how complex structures are formed during embryogenesis.

In recent years, Notch signaling has been shown to mediate a wide array of cell fate decisions in both invertebrates and vertebrates (for examples see: Artavanis-Tsakonas et al., 1999; Weinmaster, 1997, 1998; Brennan and Gardner, 2002; Harper et al., 2003; Schweisguth, 2004; Lai, 2004). Genetically identified in Drosophila melanogaster almost 100 years ago, Notch-like proteins and other members of the Notch signaling pathway have now been identified in both invertebrate and vertebrate species ranging from Caenorhabditis elegans (LIN-12 and GLP-1) to humans, highlighting the considerable conservation of this signaling pathway (Morgan, 1917; Coffman et al., 1990; Weinmaster et al., 1991, 1992; Reaume et al., 1992; Bierkamp and Camos-Ortega, 1993; Lardelli et al., 1994; Myat et al., 1996; Uyttendaele et al., 1996; Westin and Lardelli, 1997).

In the canonical pathway, the Notch receptor, a 300-kDa transmembrane protein, functions both at the cell surface to receive extracellular signals from one of its ligands (members of the Delta or Serrate/Jagged gene families) and in the nucleus to regulate gene expression (as reviewed in Artavanis-Tsakonas et al., 1999; Weinmaster, 1998; Brennan and Gardner, 2002; Harper et al., 2003; Schweisguth, 2004; Lai, 2004). Interaction of either single-pass transmembrane ligand Delta or Serrate (Jagged in vertebrates, LAG-2 and APX-1 in C. elegans) with the Notch receptor induces cleavage within the cytoplasmic domain of the protein allowing for the nuclear translocation of the intracellular domain (ICD). Along with the transcription factor Suppressor of Hairless [Su(H)] (also known as CBF-1/ RBPJks), the presence of ICD and Su(H) in the nucleus activates downstream genes that include members of the Hairy and Enhancer of Split complex [E/(spl)] (also known as HES in vertebrates) family of proteins that encode repressive basic helix–loop–helix (bHLH) transcription factors. These in turn suppress expression of genes encoding activating bHLH factors such as members of the Achaete–Scute (AS) and Atonal families (also known as ASH and ATH families).

In Drosophila, Notch signaling has been found to mediate cell fate decisions in tissues arising from all three original germ layers (Corbin et al., 1991; Hartenstein et al., 1992; Han and Bodmer, 2003). Similarly, Notch signaling in vertebrates influences differentiation of many tissues including: neural ectoderm, somites, T-lymphocytes, heart, and kidney (Austin et al., 1995; Chitnis and Kintner, 1996; Conlon et al., 1995; De la Pompa et al., 1997; Dorsky et al., 1997; Jen et al., 1997, 1999; McLaughlin et al., 2000; Robey et al., 1996; Rones et al., 2000; Wettstein et al., 1997). Although different combinations of ligand, receptor, and downstream genes are involved in each of these systems, all involve the initial expression of Notch and its ligands throughout a field of equivalently specified cells. Resolution of this pattern such that ligand and receptor become expressed preferentially in different cells establishes the basis for the intercellular signaling that eventually subdivides a tissue field into distinct cell types (as reviewed in Artavanis-Tsakonas et al., 1999).

Earlier studies have led to the notion that Notch signaling can function to both inhibit differentiation and maintain multipotent cells in a precursor state (Austin et al., 1995; Dorsky et al., 1995; Wang et al., 1998; Le Roux et al., 2003). In this model, subsequent release from Notch signaling is envisaged to permit the precursor to differentiate. Changes in local differentiation cues or a shift in competence of the precursor populations can result in secondary, or even tertiary, fates arising with successive rounds of release from Notch signaling. Such a model may account for cell fate decisions taking place during the patterning of early organ fields.

Numerous studies have shown that Notch signaling is essential for cell fate decisions involving later patterning and morphogenesis of organs and tissues, including mesodermally derived organs such as the heart and kidney, establishing a clear role for Notch signaling during organ formation (Rones et al., 2000; McLaughlin et al., 2000). Interestingly, many laboratories have observed the expression of Notch and related family members in all three germ layers during much earlier stages of development in both vertebrate and invertebrate systems (Coffman et al., 1990; Appel et al., 1999; Jen et al., 1997, 1999; Caprioli et al., 2001; Lamar et al., 2001; Latimer et al., 2002; Li et al., 2003; Lopez et al., 2003, 2005).

The identification and subsequent characterization of a sea urchin Notch orthologue, LvNotch, suggested a new role for Notch signaling during early cell fate decisions used to establish germ layers during early embryogenesis (Sherwood and McClay, 1997, 1999; Sherwood and McClay, 2001). Subsequent studies in Xenopus and zebrafish have demonstrated a role for Notch signaling in the patterning of three midline structures: floorplate, notochord, and hypochord (Appel et al., 1999, 2002; Lopez et al., 2003, 2005). For example, based on their work in Xenopus, Lopez and colleagues proposed that Notch signaling mediates a binary cell fate choice during early development, resulting in the segregation between floorplate and notochord precursors (Lopez et al., 2003). Of particular interest, although not all of the experiments conducted allowed for temporal regulation of Notch signaling, they clearly demonstrated that activation of Notch signaling in gastrula stage embryos decreased expression of two notochord patterning genes, Xbra and chd, while suppression of Notch signaling produced the opposite effect (Lopez et al., 2003). Several studies examining midline cell fate specification in zebrafish have also suggested a role for Notch signaling during the formation of the floorplate, notochord, and hypochord (Appel et al., 1999; Latimer et al.,
2002). Interestingly, although these experiments did not manipulate Notch activity in a temporally regulated fashion, the activation of Delta–Notch signaling markedly induced a subset of midline precursors to develop into hypochord tissue (Latimer et al., 2002). However, given that the lineage of the hypochord as derived from an endodermal or mesodermal precursor population is currently unclear, the implication of these studies is uncertain (for examples see: Loefberg and Collazo, 1997; Alexander et al., 1999; Warga and Nusslein-Volhard, 1999; Cleaver et al., 2000; Eriksson and Lofberg, 2000). These results demonstrated that Notch activity during fish development influences hypochord development, but whether this indicates a role for Notch during early endoderm or mesoderm formation remains to be determined. More recent work in zebrafish has provided further evidence suggestive of a role for Notch signaling during early embryogenesis, and in particular, during endoderm patterning (Kikuchi et al., 2004; see Results and discussion).

These experiments performed in several different model systems indicate a function for Notch signaling during the early subdivision of the midline structures in vertebrates. Although intriguing, it remained uncertain whether the previously observed effects were specific to the patterning of midline structures (floorplate, notochord, hypochord) or whether there was a more global role for Notch activity in the subdivision of germ layers during early embryogenesis. We now provide evidence demonstrating a much broader role for Notch signaling in germ layer determination than previously reported. More specifically, our results demonstrate a more general role for Notch signaling in mediating the subdivision between the mesoderm and endoderm in a vertebrate system during gastrulation. These results expand the previously described roles for Notch signaling during either early development, or later during organogenesis, and further demonstrate that Notch signaling is used repeatedly during both early and late stages of development.

In order to investigate the role of Notch signaling specifically during the subdivision of the germ layers beginning at gastrulation and to avoid potential confounding results from perturbing Notch activity prematurely, we used inducible reagents that allowed for the manipulation of Notch signaling pathway in a temporally regulated fashion. Using this system, we were able to examine the role of Notch signaling during the subdivision of the early embryo, specifically during the formation of endoderm and mesoderm germ layers. Our findings demonstrate that activation or suppression of Notch signaling during gastrula stages leads to considerable modification of the expression of markers of both mesodermal and endodermal cell types. In particular, activation of Notch signaling leads to an increase in expression of many endodermal tissue markers and a decrease in expression of markers of many different mesodermal cell types (i.e. paraxial, lateral plate, intermediate). The opposite result was observed when Notch signaling was suppressed during gastrulation. These findings are consistent with and extend the evidence obtained from earlier studies and indicate a much broader role than previously reported for Notch signaling in mediating early cell fate choices necessary to form the boundary between the developing endoderm and mesoderm during early vertebrate development.

Materials and methods

Embryo manipulations

*Xenopus laevis* embryos were fertilized in vitro and subsequently dejellied with 2% cysteine (pH 7.8) (Kay and Peng, 1991). Embryos were reared at 14–20°C in 0.1× MMR (Marc’s Modified Ringer’s solution) and staged according to Nieuwkoop and Faber (1994). Capped mRNA encoding inducible constructs capable of activating Notch signaling [GRSu(H)VP16, GRNotchICD] or suppressing Notch signaling [GRSu(H)DBM] was synthesized in vitro using the mMessage Machine kit (Ambion). 500–800 pg of mRNA was injected into one blastomere at the 4–8 cell stage along with 100 pg of nuclear β-galactosidase mRNA as a lineage tracer. The inducible constructs injected were as follows: GRSu(H)VP16, GRNotchICD, GRSu(H)DBM (see Rones et al., 2000). The GRSu(H)VP16 construct consists of the Su(H) transcription factor fused in frame to a VP16 activating domain and the ligand-binding domain of the human glucocorticoid receptor (GR). The GRSu(H)DBM construct consists of the Su(H) transcription factor containing a point mutation in the DNA-binding domain, which acts as a dominant negative to suppress Notch signaling, fused to the ligand-binding domain of the human glucocorticoid receptor (GR). The GRNotchICD construct was also used to activate Notch signaling and consists of the intracellular domain of the receptor fused to the ligand-binding domain of the human glucocorticoid receptor (GR). Injected embryos were cultured in 0.1× MMR until they reached the desired stages. Induction of GR-fused constructs was by addition of the synthetic glucocorticoid dexamethasone (10 μM) (stock: 10 mM in EtOH) to the culture medium. Dexamethasone containing culture medium was changed daily when embryos were cultured longer than 24 h. At appropriate stages (N.F. stages 13–41), embryos were fixed in MEMFA and processed for either in situ hybridization with digoxigenin-coupled cRNA probes or for immunohistochemistry using antibodies described below.

In situ hybridization

Prior to in situ hybridization, embryos were fixed for 15 min at room temperature in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde), rinsed in 1× PBS, and incubated at 37°C with the substrate magenta-gal to allow for the detection of β-galactosidase activity (Sigma magenta-gal; pink color). After lineage detection, embryos were rinsed in 1× PBS and post-fixed in MEMFA. Embryos were hybridized with digoxigenin-labeled antisense RNA probes as described in Harland (1991). Briefly, hybridizations were performed on whole embryos, and chromogenic detection of digoxigenin-labeled cRNA probes was performed using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer) to deposit a dark blue BCIP/NBT precipitate that contrasts the pink color of the nuclear β-galactosidase precipitate (lineage label). Probes used included: endodermin (Sasai et al., 1996), fibrinogen (Pastori et al., 1990), vito (Costa et al., 2003), lim-1 (Taira et al., 1994), nkx2.5 (Tonissen et al., 1994), bax (Horb and Thomsen, 1999), Xbra (Smith et al., 1991), pax8 (Carroll and Vize, 1999), sox17α (Hudson et al., 1997), MHCα (Logan and Mohun, 1993), and Tfl (Drysdale et al., 1994). After color precipitate formed, embryos were post-fixed for 1 h in MEMFA and stored in methanol at −20°C. Phenotypes were documented using a Qimaging camera (Retiga-1300 CCD-color) in conjunction with computer/imaging software (Openlab).

Immunohistochemistry

Injected embryos were fixed at appropriate stages for 15 min in MEMFA and assayed for β-galactosidase activity (Sigma magenta-gal; pink color), post-fixed in MEMFA, and subsequently examined for mesoderm patterning using one of the following: the somite-specific antibody 12/101 (Kintner and Brockes, 1984; Developmental Hybridoma Bank), cardiac troponin T (TnT; Developmental Hybridoma Bank), differentiated pronephric duct marker
Over the years, much research has been directed at understanding how early embryonic tissues are created and subsequently patterned. All three original germ layers give rise to a broad spectrum of tissues and organs. For example, the vertebrate mesoderm is the source of diverse organs and systems including the heart, kidney, skeletal muscle, skeleton, and blood, whereas cells of the endoderm differentiate into the epithelial lining of the embryonic gut and eventually give rise to the gastrointestinal tract, the respiratory tract, and associated organs including the liver and pancreas. Because of its central importance in the body plan of the vertebrate organism, investigators have strived to understand how complex cellular and molecular events control germ layer formation and patterning. Amphibian embryos, which are amenable to experimental manipulation, were used in this study to investigate the role of the Notch signaling pathway during the subdivision and patterning of the early germ layers during vertebrate embryogenesis.

In order to investigate the consequences of Notch signaling during the subdivision of the gastrulating embryo, cleavage stage embryos were injected with mRNA constructs that either activated or suppressed Notch signaling. Because Notch signaling is used during a multitude of developmental stages, we employed an experimental strategy that allowed for temporal control of the perturbation of signaling specifically during the time germ layers are patterned. The use of temporally regulated, inducible constructs facilitated our examination of the role of Notch signaling during germ layer formation without affecting any earlier developmental steps in which Notch functions (for examples of early effects, see Coffman et al., 1993; Appel et al., 1999; Latimer et al., 2002; Kikuchi et al., 2004; Raya et al., 2004). Activated and dominant negative forms of the transcription factor Su(H) and the intracellular domain (ICD) of the Notch receptor were fused to the human glucocorticoid receptor ligand-binding domain (see Materials and methods). The GR fusion allows transcription factors to be maintained in inactive complexes until the glucocorticoid dexamethasone (DEX) is added to the culture medium (for examples, see Kolm and Sive, 1995; Rones et al., 2000; Rones et al., 2002; Wawersik et al., 2005).

By allowing signaling to be manipulated at a time consistent with the subdivision of the germ layers, this approach circumvented any confounding effects that early (pre-gastrula stage) constitutive alteration of Notch signaling could cause (as demonstrated in Coffman et al., 1993; Appel et al., 1999; Latimer et al., 2002; Kikuchi et al., 2004; Raya et al., 2004). For our experiments, temporally controlled activation of these inducible constructs was accomplished via the addition of the synthetic glucocorticoid, dexamethasone (DEX), to the culture medium between stages 10 and 12 (gastrula stages). As described below in more detail, perturbation of Notch signaling during gastrulation resulted in an alteration of normal gene expression of molecular markers of both endodermal and mesodermal derivatives (summarized in Fig. 4 and Table 1).

**Results and discussion**

Activation of Notch signaling during gastrula stages via the transcription factor Su(H) produces opposite effects on mesoderm and endoderm gene expression

To examine the effects of activating Notch signaling in gastrula stage embryos, we injected mRNA encoding a temporally inducible form of the transcription factor Su(H). Embryos were injected into one dorsovegetal or ventrovegetal blastomere at the 8-cell stage with mRNA encoding GRSu(H)-VP16 plus mRNA encoding the lineage tracer β-galactosidase. Injected embryos were cultured in the absence of glucocorticoid (uninduced) until stages 10–12 at which time Notch signaling was activated via the addition of dexamethasone to the culture medium. Resultant tadpoles were examined for modifications of endogenous markers of either mesoderm or endoderm development by in situ hybridization or immunohistochemistry. Embryos were injected on one side only; thus, the opposite side served as an internal negative control. For all experiments, injected-sibling embryos cultured in the absence of the inducing agent (no DEX) were also examined and exhibited no detectable changes in gene expression. Lastly, there was no detectable change in gene expression in uninjected embryos cultured in

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<th>Marker</th>
<th>DEX stage 10</th>
<th>DEX stage 14</th>
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<td></td>
<td>Increase %</td>
<td>Decrease %</td>
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<tr>
<td><strong>Activated Notch</strong></td>
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<tr>
<td>lim-1</td>
<td>0</td>
<td>79</td>
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<tr>
<td>pax-8</td>
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<td>60</td>
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<tr>
<td>nkk2.5</td>
<td>1</td>
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<td>edd</td>
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<th>Suppressed Notch</th>
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<tr>
<td>lim-1</td>
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Changes for each marker were scored in individual embryos by comparison between the injected and uninjected side of each embryo. Gene expression was examined at the following stages: **lim-1**: stages 16–17, **pax-8**: stages 17–18, **nkk2.5**: stages 20–23, and **edd**: stages 19–20. Injected embryos were scored according to the variation of each marker observed on the injected side in comparison to the uninjected side (increase, decrease, or no change). Embryos were injected on one side of the embryo as described in Materials and methods with either GRSu(H)VP16 or GRSu(H)VP16 mRNA and left untreated until either stage 10 or stage 14 when 10 μM dexamethasone was added to the cultures. Percent values represent the mean average from duplicate experiments. *n* indicates the total number of embryos analyzed. Untreated control-sibling embryos were compared to uninjected untreated (no DEX) and uninjected treated (DEX only) embryos, and no differences in expression were observed (data not shown).
dexamethasone, indicating that the hormone alone does not alter normal gene expression patterns during the stages examined (data not shown).

Activation of Notch signaling during gastrula stages with GRSu(H)VP16 resulted in a decrease or complete ablation of expression of markers of mesodermal cell types on the injected side of the embryos when compared to the uninjected control side (Figs. 1, 4). Specifically, a decrease in expression of the pronephric marker lim-1 and the cardiac markers nkx2.5 and tbx5 was observed using in situ hybridization (Fig. 1). Although lim-1 expression decreased most dramatically in the posterior portion of the lim-1 positive field, anterior expression could also be ablated if injected mRNA was targeted to this region. On the GRSu(H)VP16-injected side of DEX-treated embryos, a decrease of lim-1 expression was observed in 78% of injected embryos (n = 273; Figs. 1K, L). A considerable reduction in expression of heart markers, nkx2.5 (63% of injected embryos, n = 173) and tbx5 (61% of injected embryos, n = 171), was also observed on the injected side of DEX-treated embryos, most strikingly in the lateral region of the heart field (see arrows Figs. 1G, I). Since expression of markers of both lateral plate and intermediate mesoderm cell types decreased following activation of Notch signaling during gastrulation, we examined a molecular marker of the paraxial mesoderm using the somite-specific antibody, 12/101 (Kintner and Brookes, 1984). As observed with the markers of cell types of the intermediate and lateral plate mesoderm, the activation of Notch signaling via the transcription factor Su(H) resulted in a reduction of the expression of the paraxial mesodermal marker 12/101 on the injected side of the embryo when compared to the uninjected control side (54% of injected embryos, n = 123; Figs. 1M, N).

In sharp contrast to these effects of Notch signaling on mesodermal cell types, activation of Notch signaling with GRSu(H)VP16 during gastrulation resulted in a notable increase in expression of molecular markers of endodermal derivatives (Figs. 1, 4). We observed an increase or expansion of expression of the endodermal tissue markers endodermin, fibrinogen, and vito on the injected side of embryos when compared to the uninjected control side (Figs. 1A–F). Specifically, increased endodermin expression was observed in 72% of injected DEX-treated embryos (n = 172), and an increase in fibrinogen expression was observed in 64% of injected DEX-treated embryos (n = 264). No change in gene expression was observed in control sibling embryos (injected, no-DEX-treated or uninjected, DEX-treated embryos; data not shown). Because vito transcripts are detected at the midline of early embryos in the region of the developing ventral midgut (Costa et al., 2003), for this endodermal marker, we compared our experimental

Fig. 1. Activation of Notch signaling using GRSu(H)VP16 increases expression of markers of endodermal cell types and decreases expression of markers of mesodermal cell types. (A–F) Increased expression of endodermal tissue markers (fibrinogen, endodermin, and vito) was observed in embryos injected with mRNAs encoding GRSu(H)VP16 as described in Materials and methods. Activation of the Notch pathway in gastrula stage embryos resulted in an increase of gene expression of the endodermal tissue marker, fibrinogen, on the injected side of embryos (A) when compared to the uninjected control side of embryos (B). (C–D) Similar effects were observed in GRSu(H)VP16-injected embryos examined for another endodermal tissue marker endodermin. Expansion of the normal region of expression of the early ventral midgut endodermal marker, vito, was observed in GRSu(H)VP16-injected (DEX) embryos (E) when compared to the sibling-injected (no DEX) control stage-matched embryos (F). (G–N) In sharp contrast, expression of mesodermal tissue markers tbx5 (G–H), nkx2.5 (I–J), lim-1 (K–L), and 12/101 (M–N) decreased or was completely ablated on the injected side of embryos when compared to the uninjected control sides of embryos (or no DEX control embryos, data not shown). In all experiments, mRNAs encoding β-galactosidase were co-injected as a lineage tracer (pink). Embryos were induced with dexamethasone at stages 10–12 and cultured until fixation at stages 22–33. Arrows indicate the injected side of embryos. Most embryos are oriented laterally except panels I–J that include a ventral image shown in inset and ventral views of embryos shown in panels E and F. The white line denotes the midline of the embryo.
embryos to injected, stage-matched, sibling embryos not exposed to our construct-inducing agent dexamethasone (no DEX). Similar to what we observed with the other endodermal markers examined, increased expression of \textit{vito} transcripts was observed in 58% of GRSu(H)VP16-injected (DEX, \( n = 38 \), Fig. 1E) experimental embryos examined when compared to control embryos (no DEX 12% embryos \( n = 24 \), Fig. 1F).

These experiments revealed that activation of Notch signaling during gastrula stages results in the reduction of expression of molecular markers of many different mesodermal cell types and the expansion of expression of molecular markers of endodermally derived tissues. In these experiments, aberrant gene expression in injected embryos was assayed at stages 22–35, and the elimination or expansion of the various molecular markers examined persisted over a prolonged period of development. This, as well as additional experiments demonstrating that the abnormal gene expression observed persists throughout the late stages of organ formation, provides strong evidence that the differentiation of these mesodermally derived organ fields was prevented, and not merely delayed, by activated Notch signaling.

\textit{Activation of Notch signaling during gastrula stages via the Notch intracellular domain (ICD) also decreases expression of mesodermal markers and increases expression of endodermal derivatives}

The experiments described above demonstrate that activation of Notch signaling in gastrula stage embryos through the transcription factor Su(H) alters normal mesoderm and endoderm tissue patterning. To confirm that this observation was due to activated Notch signaling and not due to a Notch-independent function for Su(H), Notch signaling was also perturbed in embryos using an active Notch-1 receptor. Like the Su(H) constructs described above, the intracellular domain of \textit{Xenopus} Notch-1 was fused to the glucocorticoid receptor ligand-binding domain (GR) to create an inducible reagent. Similar to the activated GRSu(H)VP16 construct, GRNotchICD functions downstream of ligand binding and has previously been utilized to constitutively activate Notch signaling in a temporally controlled manner (Wettstein et al., 1997; McLaughlin et al., 2000; Rones et al., 2002; Lopez et al., 2003).

We performed parallel experiments in which GRNotchICD was injected into cleavage stage embryos. As before, dexamethasone was added to the culture medium at stages 10–12 to induce the construct and activate Notch signaling. Consistent with the results obtained following activation of Notch signaling by GRSu(H)VP16, activation of Notch signaling using GRNotchICD resulted in a decrease in expression of molecular markers of mesodermally derived tissues. Specifically, the expression of the pronephric tissue marker, \( \text{lim-1} \), decreased in 58\% \( (n = 170) \) of injected embryos on the injected side when compared to the uninjected control side of the embryo (Figs. 2I–J). Expression of early cardiac mesoderm tissue markers, \( \text{nkx2.5} \) and \( \text{tbx5} \), also decreased on the injected side of the embryo \( (\text{nkx2.5} 53\% \text{ of injected embryos}, n = 188; \text{and tbx5} 59\% \text{ of injected embryos}, n = 145) \) when compared to
Expression of the paraxial tissue marker 12/101 in the developing somites decreased in 52% (n = 121) of injected embryos on the injected side of the embryo (Figs. 2K–L). Similar to the results observed and described following activation of Notch signaling using GRSu(H)VP16, activation of Notch signaling using GRNotchICD resulted in an increase in expression of the endodermal cell markers fibrinogen (63% of injected embryos, n = 182) and endodermin (67% of injected embryos, n = 210) in injected embryos (Figs. 2A–D). Thus, activation of Notch signaling using either GRSu(H)VP16 or GRNotchICD produced similar alterations in germ layer patterning. Specifically, activation of Notch signaling resulted in an expansion of endodermal cell types and a concomitant decrease in expression of markers of mesodermal cell types.

Suppression of Notch signaling during gastrula stages alters mesoderm and endoderm gene expression conversely to activation of Notch signaling

The consequences of suppressing endogenous Notch signaling during gastrulation were examined by using a dominant negative form of the transcription factor Su(H). The GRSu(H)DBM was made by fusing the human glucocorticoid receptor ligand-binding domain in frame to a construct harboring a point mutation in the DNA-binding domain of Xenopus Su(H) (Wettstein et al., 1997). Using this construct, we observed that suppression of Notch signaling during gastrulation resulted in an opposite effect on expression of genes encoding markers of either mesoderm or endoderm derivatives. As before, cleavage stage embryos were injected into one blastomere with mRNA encoding GRSu(H)DBM and mRNA encoding the lineage tracer β-galactosidase and cultured in the absence of dexamethasone (uninduced) until stages 10–12 when glucocorticoid was added to the culture medium to induce the injected constructs and suppress Notch signaling. At the desired stages of development, resultant tadpoles were examined for alterations in expression of endogenous markers of either mesoderm or endoderm formation by in situ hybridization or immunohistochemistry. As before, changes in gene expression were compared to the uninjected side of the same embryo.

Unlike the effects observed following activation of Notch signaling, the suppression of endogenous Notch signaling increased the expression of all mesodermal markers examined. For example, this increase in mesodermal gene expression is clearly visible on the GRSu(H)DBM-injected side of embryos using the cardiac marker nkx2.5 (62% of injected embryos, n = 380; Figs. 31–J and 4). Similar increases in expression were observed when additional mesoderm markers were examined including: pronephric marker lim-1 (69% of injected embryos, n = 213), cardiac marker tbx5 (65% of injected embryos, n =...
208), and somite marker 12/101 (49% of injected embryos, \( n = 125 \); Figs. 3G–N and 4).

In contrast to the effects of activation of Notch signaling during gastrulation, the suppression of Notch signaling resulted in a decrease in gene expression of the endoderm tissue markers, endodermin, fibrinogen, and \( vito \) (Figs. 3, 4). In fact, suppression of Notch signaling resulted in a decrease in endodermal gene expression on the injected side of the embryo compared to the uninjected control side in 58% (fibrinogen, \( n = 177 \)) and 55% (endodermin, \( n = 253 \)) of GRSu(H)DBM-injected embryos. As described earlier, the midline early gut marker \( vito \) was also examined in early embryos in which Notch signaling was suppressed during gastrulation. Consistent with what was seen with the other endodermal markers examined, a striking decrease in the expression of \( vito \) was observed in DEX-induced GRSu(H)DBM-injected embryos (54% of embryos with an increase of gene expression, \( n = 76 \); Fig. 3E) when compared to sibling GRSu(H)DBM-injected embryos (no DEX) control embryos (0% of embryos with an increase of expression, \( n = 22 \); Fig. 1F). The results from the experiments exemplified in Figs. 1–3 are summarized in Fig. 4.

**A role for Notch signaling in the establishment and subsequent maintenance of germ layer identities in the early embryo**

Numerous studies have suggested a role for Notch signaling during the subdivision of a precursor cell population that gives rise to three midline structures in zebrafish: notochord, floorplate, and hypochord (Appel et al., 1999; Latimer et al., 2002). Time-course experiments examining the effects of activated Notch signaling in gastrula stage *Xenopus* embryos provided additional evidence that Notch signaling is needed to subdivide notochord and floorplate lineages during gastrulation (Lopez et al., 2003). However, a subsequent study in chick demonstrated a different later function of Notch signaling in floorplate development, well after chick floorplate specification has occurred, suggesting an additional role for Notch signaling in floorplate maintenance (Le Roux et al., 2003).

The results presented in this manuscript are consistent with both of the two previously established roles for Notch signaling: 1) Notch signaling is involved in mediating the early subdivision of germ layers, and 2) Notch signaling is necessary for the maintenance of tissues after the original specification has occurred. The inducible constructs used in our studies permitted modulation of Notch signaling in a temporally regulated manner, specifically at the beginning of gastrulation. However, since these GR-inducible constructs continue to perturb Notch signaling once they are induced, it is possible that the observed alterations in expression of markers of mesodermal and endodermal lineages reflect a role for Notch signaling in the maintenance of these two germ layers rather than the subdivision of tissues prior to the completion of gastrulation. The former maintenance role for Notch signaling would be analogous to that observed by Le Roux and colleagues in chick floorplate development (Le Roux et al., 2003). The later cell fate choice role for Notch signaling would
be similar to the mechanism proposed during the formation of the amphibian and zebrafish midline structures (Appel et al., 1999; Latimer et al., 2002; Lopez et al., 2003, 2005).

To try and distinguish a role for Notch signaling during gastrulation from a later role in tissue maintenance, experiments were conducted where Notch signaling was manipulated beginning at two different stages of development. In this set of experiments, embryos were injected with mRNA constructs that either activated or suppressed Notch signaling and induced via the addition of hormone at two windows of development: either the onset of gastrulation (stage 10) or after the completion of gastrulation (stage 14). Changes in gene expression of numerous mesodermal markers (nkx2.5, lim-1, pax-8, Xbra) and molecular markers of early endodermal cells (endodermin, fibrinogen, sox17α) were examined in much earlier stages than analyzed in previous experiments (Figs. 1–4), in particular, early neurula through tailbud stage embryos (stages 13–23) (Table 1, Fig. 5, and data not shown).

Similar to the results described in Figs. 1–4, activation of Notch signaling [GRSu(H)VP16] in the early induction group (induced at stage 10) resulted in decreased expression of mesodermal cell types on the injected side of embryos examined (reduced: nkx2.5 63%, n = 47; lim-1 79%, n = 52 [Figs. 5A–B]; pax-8 60%, n = 53) and an increase in expression of markers of endodermal cell types (increased: endodermin 70% of injected embryos, n = 33; Figs. 5C–D) (Table 1 and Fig. 5). However, unlike what was observed in the early induction group, the embryos in which Notch signaling was manipulated after the completion of gastrulation (stage 14) demonstrated very different expression patterns. Following these later manipulations of Notch signaling, nkx2.5 expression was not decreased, as found in the earlier experiments, but instead the injected side of embryos appeared indistinguishable from the control side of hormone-treated embryos (unchanged: nkx2.5 82% of injected embryos, n = 24). The two different effects on nkx2.5 expression observed raise the possibility that, once nkx2.5 expression has already been established, it is no longer susceptible to perturbation via signaling through Notch.

To further explore a later role for Notch signaling in the maintenance of cell types in neurula stage embryos, we examined two markers of early intermediate mesoderm that demarcate the pronephric anlage, as well as an endoderm precursor gene. Interestingly, in contrast to what was observed in gastrula-induced embryos (stage 10), activation of Notch signaling in post-gastrula embryos resulted in a slight increase of expression of two markers of mesoderm formation (lim-1 56% of injected embryos, n = 32; pax-8 48% of injected embryos, n = 33) and a decrease in expression of the endoderm marker endodermin (59% of injected embryos, n = 22). These intriguing results indicate distinct roles for Notch signaling during two different developmental windows (either during gastrulation or after gastrulation) (summarized in Table 1).

To further test this hypothesis, we analyzed the consequences of blocking Notch signaling using GRSu(H)DBM and, as described above, induced injected embryos at either gastrula (stage 10) or post-gastrula (stage 14) stages. As shown in Figs. 1–4, blocking Notch signaling during gastrulation resulted in an increase of mesodermal cell types and a decrease of endodermal tissue (Table 1, Fig. 5, and data not shown). However, no change in the expression of the mesodermal marker, nkx2.5, was observed in GRSu(H)DBM-injected embryos when constructs were induced at stage 14 (no change: 89% of injected embryos, n = 31). As summarized in Table 1, this was not the case when constructs were induced earlier (stage 10) where expression of nkx2.5 increased on the injected side of 64% of GRSu(H)DBM-injected embryos. Other markers of mesodermal precursors, lim-1 and pax-8, resulted in 50% (n = 28) decreased expression on the injected side of the embryos, while endodermin expression was increased (74% of injected embryos, n = 31).

Table 1 summarizes results that demonstrate different effects on mesodermal and endodermal gene expression depending on whether Notch signaling is perturbed during or after gastrulation. These results provide additional evidence for a separate and distinct role for Notch signaling during the subdivision of cell types in gastrula stage embryos. Because we also observed aberrant mesoderm and endoderm gene expression patterns in post-gastrula-induced embryos, it remains possible that Notch signaling has a second role during tissue maintenance, as was observed in the developing chick floorplate (Le Roux et al., 2003). However, further experimentation is required to more fully investigate whether Notch signaling plays a subsequent role in the maintenance of germ layers post-gastrulation or if in fact the results described above are merely reflective of

![Fig. 5. Prior to morphogenesis, perturbation of Notch signaling during gastrulation modulates endodermal and mesodermal cell types (A–D). All embryos are oriented dorsally. Embryos were injected on one side as described in Materials and methods with mRNA encoding either GRSu(H)VP16 or GRSu(H)DBM, left untreated until stage 10, and then induced with dexamethasone. In all experiments, mRNAs encoding β-galactosidase were co-injected as a lineage tracer (pink). Embryos were induced with dexamethasone at stage 10 and cultured until fixation at stages 16–17 (for mesoderm marker lim-1) or stages 19–20 (for endoderm marker endodermin [edd]). Arrows indicate the injected side of embryos. (A) Ablation of expression of lim-1 was observed in embryos injected with mRNAs encoding GRSu(H)VP16. (B) In sharp contrast, increased expression of lim-1 was observed in GRSu(H)DBM-injected embryos. (C) Activation of the Notch pathway in gastrula stage embryos resulted in an increase of gene expression of the endodermal tissue marker endodermin (edd). (D) The opposite effect was observed in GRSu(H)DBM-injected embryos where a decrease of edd expression was detected.](image-url)
previously described roles for Notch signaling during later stages of organ formation (see Rones et al., 2000; McLaughlin et al., 2000).

The effects of modulations of Notch signaling on subsequent stages of mesodermal and endodermal tissue formation and morphogenesis

Our results demonstrate that experimental manipulation of Notch signaling during gastrulation results in improper apportioning of mesodermal and endodermal cell types that persist at least from early neurula through tailbud stages. Since a multitude of organs and tissues arise from these two original germ layers, their ordered subdivision is likely to be critical for proper later morphogenesis of the organism. However, an unlikely possibility is that the aberrant expression patterns observed following activation or suppression of Notch signaling were merely due to a delay of differentiation of the cell types examined. To address this possibility, we examined the consequences of manipulating Notch signaling inappropriately during gastrulation by examining later characteristics and morphology of organ formation.

For these studies, cleavage stage embryos were injected with mRNAs encoding the lineage tracer β-galactosidase and mRNAs encoding either GRSu(H)VP16 or GRSu(H)DBM, induced with dexamethasone at stages 10–12, fixed at stages 35–45, and assayed for β-galactosidase activity using the substrate magenta-gal (pink color). Surviving embryos containing positive cells for the lineage marker were examined for late molecular markers of differentiation as well as organ morphology. Although the morphology of injected embryos was fairly normal through late tailbud stages (see Figs. 1–5), experimental manipulation of Notch signaling during gastrula stages resulted in multiple gross malformations during later development (tadpole stages). For example, activation of Notch signaling caused reduced head size, shortened body axis, and severe edemas located primarily in the trunk region (Fig. 6 and data not shown). These severe defects prevented detailed analysis of late morphology of many mesodermally or endodermally derived organs. Nevertheless, in a number of embryos where Notch had been perturbed, the phenotype of the developing gut tract could still be examined. During early developmental stages, the endoderm and mesoderm tissue markers described above are expressed on both the left side and the right side of the embryo, allowing the uninjected side of the embryo to serve as an internal control for these experiments. However, since the differentiated gut is formed at the midline of the embryo, sibling embryos that were injected but not

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**Fig. 6.** Activation and suppression of Notch signaling result in morphological anomalies of the gut and perturbation of a molecular marker of pronephric duct cells. (A–C) Ventral views of embryos injected with inducible mRNAs encoding dominant negative Su(H) [GRSu(H)DBM] and induced at stage 10 to suppress Notch signaling. Injected tadpoles displayed miscoiling of the gut (B, C) in comparison to the normal coiling pattern observed in injected embryos cultured in the absence of DEX (no DEX) (A). (D–E) Ventral views with lateral inset images of embryos injected with mRNA encoding Su(H) [GRSu(H)VP16] and induced at stage 10 to activate Notch signaling. Panel E illustrates gut malformation observed in embryos exposed to DEX compared to normal phenotypes observed in injected no DEX sibling control embryos (D). (F–G) Lateral image of embryo injected with mRNA encoding Su(H) [GRSu(H)VP16] and induced at stage 10 to activate Notch signaling. Activation of Notch signaling substantially decreased expression of a late marker for pronephric duct cells 4A6 (F). Left bottom panels show schematic tracing of the observed 4A6 expression pattern. Right bottom panel shows a magnification of boxed areas.
exposed to dexamethasone (no DEX) were used as controls for the experiments described below. As shown in Fig. 6, activation of Notch signaling via Su(H) resulted in severe gut malformations in 70% of surviving injected tadpole stage embryos. In comparison, abnormal gut phenotypes were observed in only 17% of injected-no-DEX sibling control embryos (Figs. 6D–E; DEX n = 104, no DEX n = 102). In contrast, when Notch signaling was suppressed during gastrulation, overall embryo morphology was less distorted. However, defects in gut coiling including failure to coil and aberrant coiling were observed in 81% (n = 31) of the embryos examined when compared to the sibling-injected no DEX (33% of injected embryos, n = 30) control embryos (Figs. 6A–C). Since earlier markers of endoderm formation were increased when Notch was activated and decreased when Notch signaling was suppressed, our observations of morphological malformations later in organogenesis provide further support that perturbation of Notch signaling during gastrulation alters the allocation of mesoderm and endoderm cell types.

Given that early markers of paraxial, lateral plate, and intermediate mesoderm were not expressed properly in tailbud stage embryos following perturbation of Notch signaling during gastrulation, we wanted to examine later patterning of mesodermally derived organs. For these experiments, we examined several molecular markers of differentiated tissues (pronephric duct [4A6]; pronephric tubules [3G8]; cardiac markers TnI, MHCx, troponin T [CT-3]; and somite marker [12/101]) (Fig. 6 and data not shown). For example, when Notch signaling was activated using GRSu(H)VP16 during gastrula stages, embryos displayed a reduction or complete ablation of pronephric duct protein expression on the injected side of the embryo when compared to the control uninjected side (Figs. 6F–G). As discussed in the previous sections, the somite marker 12/101 was also reduced in embryos in which Notch signaling was activated (Figs. 1, 2). Unfortunately, because we are unable to deactivate our constructs once they are activated at gastrula stages, it is difficult to determine whether these observed late pronephric, heart, and somite phenotypes were a direct result of activated Notch signaling or a secondary consequence resulting from prolonged activation of Notch signaling.

Summary

Our results suggest that Notch signaling plays an important role in early patterning of the embryo during gastrulation and organogenesis. These findings provide strong evidence that Notch/Su(H) signaling during gastrulation (stages 10–12) helps to regulate the formation of the endoderm and mesoderm germ layers. This is illustrated by the increase in markers of multiple mesodermal cell types and decrease in endodermal tissue markers observed when Notch/Su(H) signaling is suppressed in gastrula stage embryos. Further proof is provided by the demonstration that Notch/Su(H) activation leads to an expansion of markers of endodermal tissues at the expense of mesodermal cell types (as described in the model presented in Fig. 7).

Members of the Notch signaling family of proteins have been shown to be involved in mediating some of the earliest developmental events in many different organisms (Appel et al., 1999; Sherwood and McClay, 2001; Raya et al., 2004). Previous work conducted in sea urchins by Sherwood and McClay (1997, 1999, 2001) provided initial evidence for a role of Notch/Su(H) signaling in endoderm–mesoderm boundary formation in an invertebrate system. These observations were extended into vertebrates when a role for Notch signaling was demonstrated in the early patterning of three specific midline structures: floorplate, notochord, and hypochord (Appel et al., 1999; Latimer et al., 2002; Lopez et al., 2003, 2005). It has recently been shown that the expression of several markers of zebrafish endoderm cell types decreases when Notch signaling is activated during some of the earliest stages of endoderm precursor formation (Kikuchi et al., 2004). Although, at first glance, our results may seem inconsistent with those obtained by Kikuchi and colleagues, significant differences in experimental methodology make it difficult to directly compare the two sets of results. In particular, unlike in our experiments where Notch was specifically activated during late gastrula stages, activation of Notch signaling in the zebrafish experiments was not under temporal control. Thus, Notch signaling in the Kikuchi experiments was likely perturbed prior to gastrulation.

Fig. 7. Proposed model for role of Notch signaling during the subdivision of mesoderm and endoderm germ layers during gastrulation. (A) Normal levels of Notch signaling allow for appropriate amounts of endoderm and mesoderm tissue formation. (B) Ectopic activation of Notch signaling during gastrulation results in an increase of endodermal cell types and a decrease in mesodermal cell types. (C) The opposite effect is observed when Notch signaling is suppressed, where mesoderm cell types increase at the expense of endodermal tissues.
In an attempt to address the differences between the results of Kikuchi et al. and our results, we examined endoderm formation in embryos in which our injected constructs were induced with DEX earlier (stage 8, blastula stage) than the experiments described throughout this manuscript. Similar to the results obtained in fish, we observed a reduction or ablation of expression of endodermal markers in embryos in which Notch signaling was activated during earlier blastula stages (data not shown). Taken together, these results suggest at least two distinct roles for Notch signaling during early development: a role for Notch signaling prior to gastrulation and a distinct and broad role for Notch signaling during gastrulation to subdivide the germ layers. The present manuscript addresses this distinct role for Notch signaling during gastrulation (Fig. 7).

Numerous studies have shown that perturbation of endogenous Notch signaling leads to changes in cell fate and/or the borders that normally distinguish tissue compartments. Thus, not surprisingly, mutations in components of the Notch signaling pathway have severe biological repercussions. Previously, it has been demonstrated that Notch receptors and ligands are often expressed for extended periods of time in a tissue, suggesting multiple roles within a single system. Nonetheless, the mechanisms regulating the repeated use of Notch signaling within a tissue remain unclear. Future work investigating downstream components of Notch signal transduction should provide further insights regarding the regulation of this complex signaling pathway. To date, several families of basic helix–loop–helix (bHLH) transcription factors have been identified which function downstream of Suppressor of Hairless [Su(H)] in the Notch pathway and are likely to be involved in mediating cell fate decisions (for review, see Davis and Turner, 2001). Hairy and Enhancer of Split [E(spl)] (HES in vertebrates) family members as well as members of the Hey family can be directly activated by the transcription factor Su(H) (Bailey and Posakony, 1995; Jarriault et al., 1998; Lecourtois and Schweisguth, 1995; Rones et al., 2002). However, Notch signaling at the level of bHLH transcription factors is more complicated than this linear model suggests. Since there are multiple members of each family expressed in overlapping patterns in the majority of tissues examined, only a subset of these genes are likely to be direct effectors of Notch signaling. For example, although the bHLH gene Xhe-1 is expressed in multiple mesodermally derived tissues during embryogenesis such as somites, embryonic kidney, and heart, perturbation of Notch signaling during gastrula stages resulted in a change of Xhe-1 expression in only a subset (i.e. pronephros and somites) of these tissues, while there was no detectable change in others (i.e. heart) (Rones et al., 2002).

Future research examining proteins presumed to act downstream of Su(H)/NotchICD will help elucidate how this complex pathway is able to mediate such a diverse number of cellular behaviors during embryonic development of organs and tissues.

Previous research which demonstrated that the Notch/Su(H) pathway functions during the later development of numerous organs (Chitnis and Kintner, 1996; Robey et al., 1996; Wetstein et al., 1997; Jen et al., 1999; McLaughlin et al., 2000; Rones et al., 2000; Schonhoff et al., 2004; Esni et al., 2004), combined with our data which provides evidence for an earlier broader role for Notch signaling in subdividing the mesoderm and endoderm during gastrulation, offers new insights into the role of Notch signaling during the process of vertebrate development. Since contingency plans are an important component of development, it is quite common that early embryonic fields of tissue contain more cells than ultimately necessary. Several studies have shown that one important role for Notch signaling is to prevent these “backup” cells from actually taking on specialized fates under normal developmental conditions. The inappropriate apportioning of the mesoderm and endoderm germ layers observed after perturbation of Notch signaling described above could be the result of a number of different Notch-mediated cell behaviors. For example, it is possible that activated Notch signaling during gastrula stages is causing a general block of cellular differentiation of mesodermal cell types. This type of role for Notch signaling has been suggested by numerous other studies and is consistent with the data described above where activated Notch signaling results in a decrease of all mesodermal cell types examined (for example, see Noziger et al., 1999).

Additionally, our lineage analysis is consistent with a differentiation delay model for Notch signaling rather than a binary cell fate choice mechanism. Lineage labeling analysis demonstrated that cells containing activated Notch signaling constructs could be detected in all three germ layers and were not excluded from the mesoderm. However, the β-gal-positive cells found in the developing mesoderm rarely co-expressed mesodermal differentiation markers (data not shown). Further investigation will be needed if the precise mechanism for how Notch is serving to subdivide the early embryo is to be determined.

In summary, it is important to remember that a general theme of developmental biology is that nature is frugal. Thus, proteins and signaling pathways are reused and must function in a context-dependent manner. Not surprisingly, multiple roles for Notch signaling have been demonstrated over the years. Therefore, it is imperative to remember that context-dependent utilization of Notch activity is an important consideration for understanding how organs are formed. Future work examining the mechanisms regulating Notch signaling throughout development will aid in our understanding of the complex cellular behaviors that lead to tissue specification and subsequent organ morphogenesis.

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