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A Widespread and Early Requirement for a Novel Notch Function during *Drosophila* Embryogenesis

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The *Notch* pathway plays a key role in the formation of many tissues and cell types in Metazoans. We recently showed that *Notch* acts in two pathways to determine muscle precursor fates. The first is the "standard" *Notch* pathway, in which Delta activates the Notch receptor, which then translocates into the nucleus in conjunction with Su(H) to reprogram transcription patterns and bring about changes in cell fates. The second pathway is poorly defined, but known to be independent of the ligands and downstream effectors of the standard pathway. The standard pathway is required in many different developmental contexts and we wondered if there was also a general requirement for the novel pathway. Here we show that the novel *Notch* pathway is required for the development of each of five examined cell types. These results indicate that the novel pathway is a widespread and fundamental component of *Notch* function. We further show that both *Notch* pathways operate in the differentiation of the same cell types. In such cases, the novel pathway acts first and appears to set up or limit the size of equivalence groups. The standard pathway then acts within the equivalence groups to limit individual cell fates.

Key Words: **organogenesis; lateral inhibition; cell signaling; cell differentiation; neurogenic genes; cell fates; neurons.**

INTRODUCTION

Intercellular signaling is vital for the patterning of all multicellular organisms. *Notch* proteins function as receptors for intercellular signals during the differentiation and patterning of diverse cell types throughout the animal kingdom. The *Notch* pathway is best known for its role in lateral inhibition or specification, i.e., the process of signaling between developmentally equivalent cells (see Artavanis-Tsakonas *et al.*, 1999; Greenwald, 1998; for reviews). For example, during the formation of the *Drosophila* larval central nervous system (CNS), ectodermal cells are organized into small groups of cells, called equivalence groups. Within the equivalence groups, the cells compete to become neuroblasts by signaling to each other via the ligand, Delta, which binds to the Notch receptor on adjacent cells. In response, Notch is cleaved to release an activated, intracellular domain (Logeat *et al.*, 1998; Struhl and Adachi, 1998; Lecourtis and Schwiesguth, 1998), which apparently moves into the nucleus with an associated protein, Suppressor of Hairless (Su(H)) (Fortini and Artavanis-Tsakonas, 1994; Struhl and Adachi, 1998; Lecourtis and Schweisguth, 1998). The Notch–Su(H) complex activates the transcription of several transcription factors, including those encoded within the *Enhancer of split* complex (*E(spl)*) (Lecourtis and Schweisguth, 1995; Bailey and Posakony, 1995). The *E(spl)* proteins then repress the transcription of proneural genes, i.e., genes that encode bHLH transcription factors and promote neural development. Since the proneural genes activate *Delta* expression, the down-regulation of proneural genes reduces the signaling capacity of the cell (Heitzler *et al.*, 1996). Thus slight differences in the signaling intensity of neighboring cells are amplified and fixed such that cells with high levels of activated Notch stop signaling and choose a secondary, epidermal fate, while cells with low levels of activated Notch continue to signal and choose the primary, neural fate. In embryos that are defective for one or more components of the *Notch* pathway, all cells within the equivalence group differentiate as neuroblasts. Although this pathway is best understood in the ectoderm, it is used in many developmental contexts, including mesoderm (Corbin *et al.*, 1991; Ruohola *et al*, 1991; Bate *et al.*, 1993) and endoderm (Hartenstein *et al.*, 1992), to single out cells within equivalence groups.

Notch is also activated by inductive signals sent from one type of cell to another. For example, during the formation of

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the *Drosophila* wing, cells within the ventral and dorsal compartments signal to *Notch*-expressing cells along the wing margin. In response these cells begin to express the morphogen wingless and differentiate into an organizing center that patterns the entire wing (Diaz-Benjumea and Cohen, 1995; Doherty *et al*, 1996; Neumann and Cohen, 1996). The ventral cells signal via the Delta ligand, while the dorsal cells signal via a structurally similar ligand, Serrate. In response Notch activates the expression of downstream genes, including *wingless*. In this process, unlike in lateral inhibition, *Notch* acts through *Su(H)*, but independent of *E(spl)* (de Celis *et al.*, 1996).

Another example of inductive signaling through *Notch* occurs during sibling cell fate decisions in the neuroectoderm. For example, the dMP2 and vMP2 interneurons are born from the asymmetric division of the MP2 precursor. One feature of this division is the segregation of the membrane-associated protein numb specifically to the dMP2 cell. Nearby cells send an inductive Delta signal that apparently binds to Notch on both the vMP2 and the dMP2. The vMP2 cell responds by turning on downstream genes, including *sanpodo* and *mastermind* (Spana and Doe, 1996; Skeath and Doe, 1998; Schuldt *et al*, 1999). In contrast, the dMP2 sister cell does not respond because numb binds to the intracellular domain of Notch and antagonizes its signaling (Spana and Doe, 1996; Guo *et al.*, 1996). In *numb* mutant embryos, both cells respond to the Delta signal and become vMP2 cells (Spana and Doe, 1996), while in *N* and *Dl* mutant embryos, both cells become dMP2s (Skeath and Doe, 1998). Although *numb* and *sanpodo* are critical components of the *Notch* pathway during sibling cell fate determination, neither gene's activity is required during lateral inhibition (Skeath and Doe, 1998). These data, taken together with the fact that *N* acts independent of *E(spl)* during wing margin formation, indicate that the *Notch* pathway branches downstream of *Su(H)*.

Although *Notch* signaling appears to depend on *Su(H)* during lateral inhibition and inductive events, *Notch* can act independent of both *Su(H)* and *E(spl)* in certain developmental contexts. For example, transfection with activated *Notch* genes prevents cultured mouse myoblasts from differentiating in a manner that is independent of both *Su(H)* and *E(spl)* (Shawber *et al.*, 1996). More recently we showed that *Notch* acts in a new pathway that is independent of both *Su(H)* and *E(spl)* and its known ligands, *Delta* and *Serrate*, during the determination of embryonic muscle precursors from the *Drosophila* mesoderm (Rusconi and Corbin, 1998). In embryos that lack both maternal and zygotic *Notch* products (called holonull embryos for simplicity) more mesodermal cells differentiate as muscle precursors than in embryos holonull for any other gene of the known *Notch* pathway. Our findings indicated that *Notch* acts in two ways to determine muscle precursor fates. The first is the "standard" pathway. Although the standard pathway can branch downstream of Su(H), the transduction of the Delta or Serrate signal to Notch and then to Su(H) appears to be the same during the inductive and lateral inhibition processes. The second way in which *Notch* acts is through a novel pathway that is independent of the ligands and downstream effectors of the standard *Notch* pathway. Since the standard *Notch* pathway is required in a wide variety of embryonic tissues, we were curious to see if the novel pathway is required in these same tissues or if it is specific to somatic mesoderm. Here we report that holonull *Notch* embryos show more severe defects in all examined tissues than embryos holonull for other genes of the standard *Notch* pathway. Furthermore, we show that *Notch* is required earlier in various neural lineages than other components of the standard *Notch* pathway, i.e., both pathways act in the same cells or lineages, but at different times. The earlier *Notch* pathway appears to set up or limit the size or potential of equivalence groups, whereas the standard pathway acts later, within the equivalence groups, to select out individual cell fates.

MATERIALS AND METHODS

Fly Stocks and Generation of Holonull Embryos

Holonull embryos (embryos with no maternal or zygotic gene product) were generated as described in Rusconi and Corbin (1998) using the FRT and ovo^{D1}FRT fly stocks discussed there and in Chou and Perrimon (1992, 1993). *Ore*^{R} was the wild-type stock. The mutant, null alleles were as follows: N^{55e11}, N^{XK11}, Dl^{X}, Dl^{M2}, Su(H)^{SF8}, E(spl)^{RB251}, E(spl)^{R1}, neu^{IF65}, and neu^{IL119}. Two null alleles were looked at for each gene except Su(H), in which case only one was examined. In all cases, the two alleles gave identical results. Also, only one double mutation, Dl^{rev10} Ser^{RX82}, was used to examine the effects of removing both known *Notch* ligands. For consistency and simplicity, we show only the N^{XK11}, Dl^{M2}, Su(H)^{SF8}, E(spl)^{RB251}, and neu^{IF65} in the figures.

Antibody Staining of Embryos

Antibody staining was carried out as described in Rusconi and Corbin (1998). The rabbit anti-fushi tarazu (α-ftz) and rabbit anti-engrailed (α-en) antibodies were kindly provided by Bruce Dietrich and Steve DiNardo, respectively. The mouse anti-fasciclin III antibody (α-fasIII) and mouse anti-even-skipped (α-eve) were obtained from the Developmental Studies Hybridoma Bank at Johns Hopkins University and from Manfred Frasch, respectively. The secondary goat α-mouse and goat α-rabbit antibodies were from Vector Laboratories. Embryos stained with anti-eve were equilibrated in 80% glycerol/1× PBS, dissected, and flattened as described by Patel (1994).

In Situ Hybridization of Embryos

Whole-mount *in situ* hybridization of embryos was carried out as described in Rusconi and Corbin (1998) using the Genius DNA labeling and detection kit (Boehringer Mannheim). The *single-minded* (*sim*) probe was a full-length cDNA (Nambu *et al.*, 1991) kindly provided by Steve Crews.

TABLE 1
Mutant Alleles Used to Make Holonull Embryos

Allele	Type of mutation	Reference	Received from
N^{55e11}	Amorph, 2.6-kb insertion associated with first exon and premature termination of *N* transcripts	Kidd *et al.*, 1983	Bloomington Stock Center
N^{XK11}	Hypomorph, mutation is outside coding region of gene	Brennan *et al.*, 1997	G. Struhl
Dl^{x}	Amorph, extreme	Craymer, 1980	M. W. Young
Dl^{M2}	Amorph, deficiency	Alton *et al.*, 1988	K. Fechtel
neu^{9L119}	Strong loss-of-function	A. Laughon, pers. comm.	A. Laughon
neu^{IF65}	Amorph	Lehmann *et al.*, 1983	Bloomington Stock Center
$E(spl)^{RB251}$	Amorph, deficiency for entire complex	Knust *et al.*, 1987	J. Campos-Ortega
$E(spl)^{R1}$	Amorph, deficiency for entire complex	de Celis *et al.*, 1991	M. W. Young
$Su(H)^{SF8}$	Amorph	Schweisguth and Posakony, 1992	F. Schwiesguth
Dl^{rev10}, Ser^{RX82}	Amorph	Micchelli *et al.*, 1997	G. Struhl
Ser^{RX82}	Amorph	Thomas *et al.*, 1991	E. Knust

RESULTS

We recently reported that *Notch* acts in a previously unrecognized pathway during the determination of larval somatic muscle precursors (Rusconi and Corbin, 1998). To see if this new pathway is also required outside the somatic mesoderm, we compared the development of several different tissues in zygotic null and holonull embryos for various genes of the standard *Notch* pathway. The alleles used were known nulls (amorphs) or strong loss-of-function mutations (see Table 1). To control for potential differences in genetic backgrounds, two independently derived alleles were examined for each gene with the exception of Su(H), for which only one known amorphic allele was used. The choice of cell types was based on the availability of specific markers for following their development and, for the neural cells, the fact that their lineages are well described.

The Central Nervous System

In embryos mutant for any one of the standard *Notch* pathway genes, including *Notch* (*N*), *Delta* (*Dl*), *neuralized* (*neu*), *mastermind* (*mam*), *E(spl),* and *Su(H),* too many neuroblasts develop at the expense of epidermoblasts (Lehmann *et al.*, 1983; Lecourtois and Schweisguth, 1995). For example, in zygotic mutants, all of the five to seven cells in a proneural equivalence group become neuroblasts, as visualized by the high continued expression of proneural genes (Skeath and Carroll, 1992; Ruiz-Gomez and Ghysen, 1993; Martin-Bermudo *et al.*, 1995). Because the overall hypertrophy of the nervous system is difficult to quantitate, we compared the phenotypes of particular neural lineages in holonull embryos by using specific molecular markers (Broadus *et al.*, 1995).

The midline precursor cells, the MP2s (described above), are among the earliest neuronal cells to emerge from the ectodermal layer (Doe, 1992). The MP2s are marked by expression of *fushi tarazu* (*ftz*) protein, which they begin to express as they delaminate from the overlying epithelium and continue to express as they migrate and divide to give rise to daughter neurons, vMP2 and dMP2 (Doe *et al.*, 1988). In wild-type embryos there are two MP2s per segment, one on either side of the midline (blue stain, Fig. 1, top left) between the stripes of engrailed-expressing cells (brown stain). In contrast, in *Su(H)* and *Dl* holonull embryos and in *Notch* zygotic null embryos there are ~15 ftz-expressing MP2-like cells on either side of the midline in (Fig.1, bottom, and data not shown). These data indicate that the standard *Notch* pathway not only determines the fates of the dMP2 and vMP2 daughter cells (Skeath and Doe, 1998), but also helps to single out the MP2 mother cell from surrounding cells. In *Notch* holonull embryos, there are about twice as many MP2 cells as seen in the other holonull and zygotic null embryos, ~30 MP2s per hemisegment (Fig. 1, top right). The extra MP2 clusters extend farther laterally than seen in the other mutant embryos. These results show that *Notch* has a function outside the standard pathway during MP2 determination, as it affects more cell fate decisions than do the other genes of the standard *Notch* pathway.

To study whether *Notch* also has a novel effect on cell lineages in which the standard pathway works by induction, we examined the determination of sibling cell fates within the RP2 lineage. In wild-type embryos the NB4-2 neuroblast divides asymmetrically to give rise to a ganglion mother cell, GMC4-2A, and a neuroblast, NB4-2A (Chu-LaGraff *et al.*, 1995; diagrammed in Fig. 2F). GMC4-2a also divides asymmetrically to give rise to the RP2 and RP2sib neurons (Chu-LaGraff *et al.*, 1995). These sibling cells differentially respond to an inductive Dl signal because only the RP2 cell harbors the numb protein. Consequently, RP2 does not respond to the signal, while the RP2sib cell does respond and adopts the sib fate. As expected, in zygotic mutants for *Notch* pathway genes, e.g., *mam*, *Dl,* and *N*

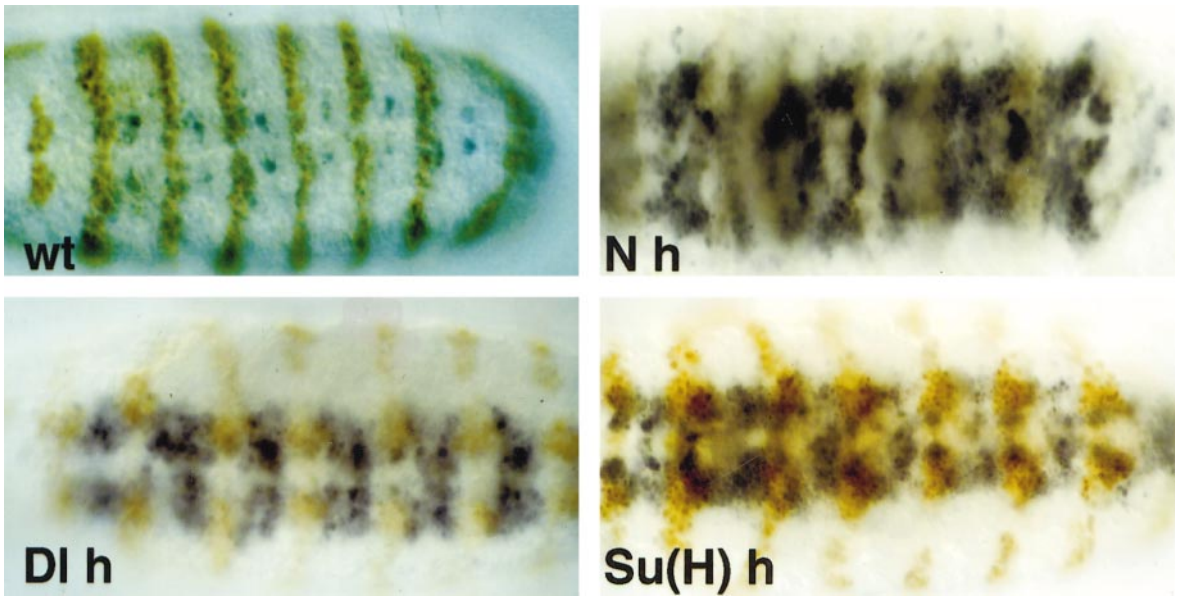


FIG. 1. A novel *Notch* activity is required for the determination of MP2 cells within the central nervous system. Ventral views are shown of whole-mount embryos (anterior to the left) double stained for engrailed protein (en, red/brown color), which marks the posterior compartment of each segment (DiNardo *et al.*, 1985; Karr *et al.*, 1985), and ftz (blue), which marks the MP2 cells. In wild-type embryos (top left) a single MP2 neuroblast (blue) is located midway between the two en stripes (brown) on either side of the midline in each hemisegment. In *Su(H)* and *Delta* (*Dl*) holonull embryos (bottom) as well as *Notch* (*N*) zygotic null embryos (data not shown) there are extra MP2s, ~15 per hemisegment. Doubly mutant *Delta, Ser* holonull embryos are indistinguishable from singly mutant *Delta* holonull embryos, indicating that *Serrate* does not activate *Notch* in this context. In *Notch* holonull embryos (top right) there are approximately twice as many MP2 cells as in the other holonull embryos. ftz expression appears to extend more laterally in the *Notch* holonull embryos than in the other mutant embryos. Although the strong ftz staining somewhat obscures the brown en staining in the *N* holonull embryo shown, we have not observed defects in the en pattern or segmentation defects in any *N* holonull embryos.

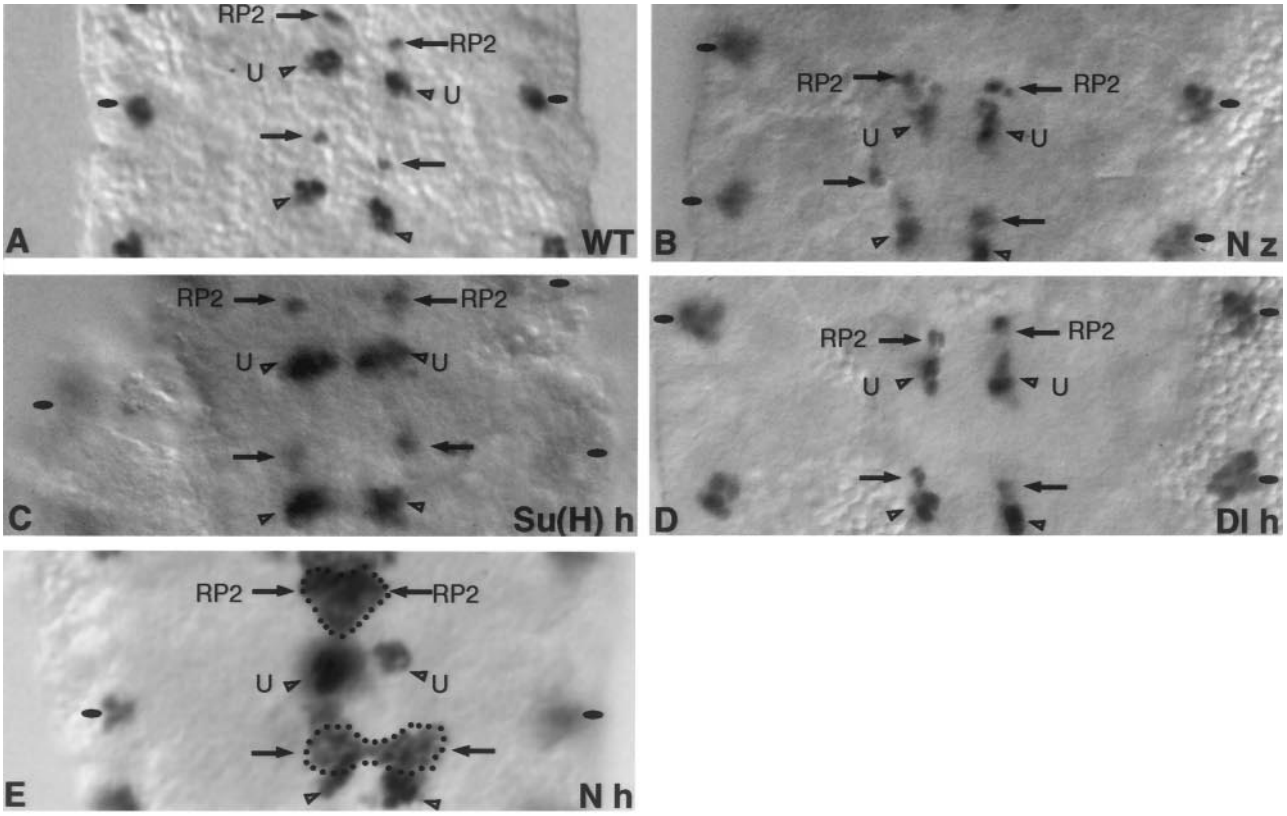
mutants (Skeath and Doe, 1998; also compare Fig. 2A to 2B), and in *Su(H)* and *Dl* holonull mutants (Figs. 2C and 2D), both daughter cells become RP2s as judged by the expression of the eve marker. In *Notch* holonull embryos, however, clusters of 10 to 15 eve-expressing RP2-like cells are found in place of the normal single RP2. These clusters often merge across the midline into a single large cluster of 20 to 30 cells, apparently displacing or replacing the cell types that normally occupy the midline. The origin of these extra cells is unclear (Figs. 2G–2I; see Discussion). Nonetheless, their presence and quantity show clearly that *Notch* plays a role in the RP2 lineage that the other components of the standard pathway do not.

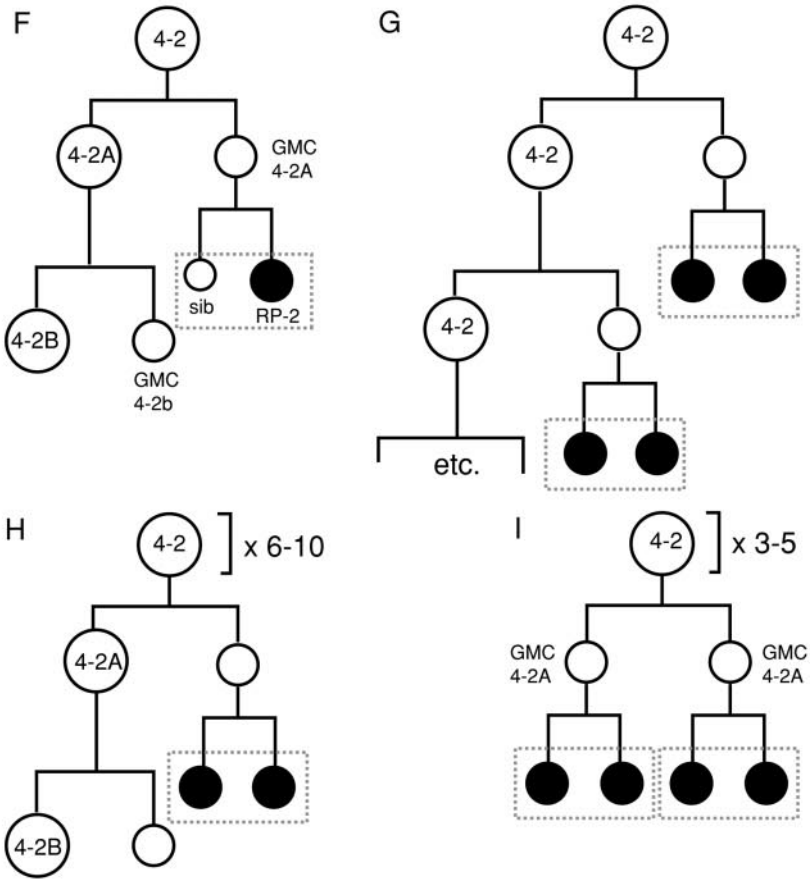
The same gene that marks RP2 neurons, i.e., eve, is also expressed in three U neurons and fleetingly in their three sibling neurons, the Usibs (Skeath and Doe, 1998, and Fig. 2A, arrowheads). In zygotic *mam*, *Delta*, and *Notch* mutants (Skeath and Doe, 1998; Fig. 2B) and in *Su(H)* and *Delta* holonull embryos (Figs. 2C and 2D) approximately 6 cells retain expression of eve, which is consistent with the idea that that the U neurons are duplicated at the expense of the Usibs. In *Notch* holonull embryos the number of U neurons varies between 6 and 10 cells (Fig. 2E). Although this phenotype is more variable than that seen with the RP2s or MP2s, it is consistent with the idea that *Notch* plays a more widespread role in the determination of neural cell fates than other components of the standard *Notch* pathway.

While all the standard *Notch* pathway genes are important in the examined neural lineages, the *Notch* gene itself appears to be doubly important. The phenotypes indicate that *Notch* functions in two ways to limit the numbers of these cells. Although other interpretations are possible, the simplest interpretation is that the standard pathway and a novel pathway act within the same cells, but at different times. For example, the novel pathway might act early to prevent too many cells from becoming NB4-2 cells (Figs. 2F–2I), while the standard pathway might act later to prevent too many RP2sib cells from becoming RP2s (see also Discussion).

The Mesectoderm and Visceral Mesoderm

The mesectoderm arises from two single-cell-wide stripes of cells that separate the ectoderm and mesoderm primordia. By stage 10 (Fig. 3A), the mesectodermal stripes have met along the midline and appear as a continuous two-cell-wide stripe that extends the length of the germ band as visualized by expression of the *single-minded* (*sim*) gene (Nambu *et al.*, 1991). Previous studies showed that *Notch* and other genes of the standard Notch pathway are involved





in the determination of the mesectoderm (Menne and Klambt, 1994; Martin-Bermudo *et al.*, 1995). In *Notch, neu, E(spl),* and *Delta* zygotic null embryos (data not shown) and *neu, E(spl), Su(H),* and *Delta* holonull embryos (Figs. 3B–3E), the mesectodermal stripe is broken by several small gaps as visualized by expression of *sim* mRNA (arrowheads). For example, along the dorsal side of these embryos there are generally two to four breaks in the stripe and each break removes two to three cells (Figs. 3B–3E). Thus the standard *Notch* signaling pathway is needed for cells to assume a mesectodermal fate. As seen in the neural lineages, the *Notch* holonull embryos have a more severe phenotype than other holonull embryos. Many more breaks occur in the stripe and each break removes at least four to five cells and often more (Figs. 3F and 3G). Frequently more mesectodermal cells are missing than present. The more severe phenotype of the *Notch* holonull embryos indicates that *Notch* acts both within the context of the standard pathway and independent of it during the determination of mesectodermal cells.

A similar reduction is seen in the number of cells that adopt a visceral mesoderm cell fate in the mutant embryos. In wild-type, stage 12 embryos the visceral mesoderm consists of two stripes of columnar cells which extend the length of the germ band, on either side of the gut, as visualized by antibodies to fasIII (Bate and Martinez-Arias, 1993; Fig. 4A). In embryos holonull for *Su(H), Delta,* or *Notch,* the column of cells is broken by gaps (Figs. 4B–4D), which are more frequent and longer in the *Notch* holonull embryos (Fig. 4D).

These results indicate that, relative to the other genes of the standard *Notch* pathway, the *Notch* gene itself is needed in more cells or at more stages during the development of the mesectoderm and visceral mesoderm. Curiously the decision to become mesectoderm or visceral mesoderm must not absolutely require *Notch* function since some of these cells remain in the holonull embryos. Furthermore those that do remain retain at least some of their cell-specific properties. For example, the remaining visceral mesoderm cells continue to express fasIII and retain aspects of their columnar morphology (Figs. 4D and 4E). Nonetheless, the more severe visceral mesoderm and mesectodermal phenotypes of the *Notch* holonull embryos indicate that when both *Notch* functions are defective more cells choose inappropriate fates than when only the standard *Notch* pathway is defective.

Serrate Is Not a Likely Activator for Notch in Early Embryos

A second ligand, Serrate, activates *Notch* during adult wing formation (Doherty *et al.*, 1996; Diaz-Benjumea and Cohen, 1995) and is also required late in embryogenesis for the normal patterning of the larval mouth hooks and denticle belts (Wiellette and McGinnis, 1999). *Serrate* mRNA and protein are detected in the fore- and hindgut, epidermis, tracheal system, salivary glands, and brains of stage 11 embryos (Thomas *et al.*, 1991). To see if Serrate signaling contributes to the determination of the MP2s, mesectoderm, or visceral mesoderm we examined these tissues in *Delta, Serrate* double holonull embryos. In all cases the phenotypes were indistinguishable from those of singly mutant *Delta* holonull embryos (data not shown). These results indicate that Serrate is not involved in the determination of these cell types or, as we showed previously, larval muscle precursors (Rusconi and Corbin, 1998).

DISCUSSION

We recently showed that *Notch* has a role in muscle precursor determination beyond its well-established role in the standard *Notch* pathway (Rusconi and Corbin, 1998). Here we show that *Notch* has a similar role in the determination of five additional cell types and suggest that this role is to limit the developmental potentials of cells before they choose their ultimate fates through lateral inhibition or a *Notch*-mediated induction.

FIG. 2. The novel *Notch* activity is required before the standard *Dl-to-N-to-Su(H)* pathway determines sibling cell fates in the NB4-2 lineage. The photographs show two consecutive segments of late stage 11 embryos stained with eve antibody (anterior up). After staining, the embryos were dissected and flattened to reveal more of the forming CNS in the same focal plane (Patel, 1994). In each hemisegment of wild type embryos (A) eve is expressed in a single RP2 neuron (arrows). eve is not expressed in the adjacent RP2 sib. Other eve-expressing cells are also marked, including the U neurons (arrowheads) and the cardioblasts (black ovals). In *Notch* zygotic null embryos (B) and in *Su(H)* (C) and *Dl* (D) holonull embryos there are two RP2s per hemisegment (see also Skeath and Doe, 1998). Occasionally, a third RP2-like cell is present in *Dl* holonull embryos, but the origins of this extra cell are unknown. In marked contrast to the other mutant embryos, *Notch* holonull embryos (E) have 10 to 15 RP2s per hemisegment and the RP2 cells often form into a single large cluster that merges across the midline. The clusters may merge across the midline because mesectodermal cells are lost, as discussed under Results and in the legend to Fig. 3. The number of extra RP2 cells is somewhat variable between different *Notch* holonull embryos as well as between different hemisegments within the same embryo, as seen in the two segments in E. Despite this variability, there are always at least threefold more RP2 cells present in the *Notch* holonulls than in any of the other zygotic null or holonull embryos. F shows the cell lineage of the 4-2 neuroblast in wild-type embryos as determined by Chu-LaGraff *et al.* (1995). The cell names are shown within or to the side of each cell. The RP2 cells are shown in black. Boxed cells are affected by the standard *Notch* pathway, such that both cells become RP2 cells in embryos with defects in the pathway (Skeath and Doe, 1998). (G, H, and I) Possible models for the origins of the extra RP2 cells in *Notch* holonull embryos. See Results for more details.

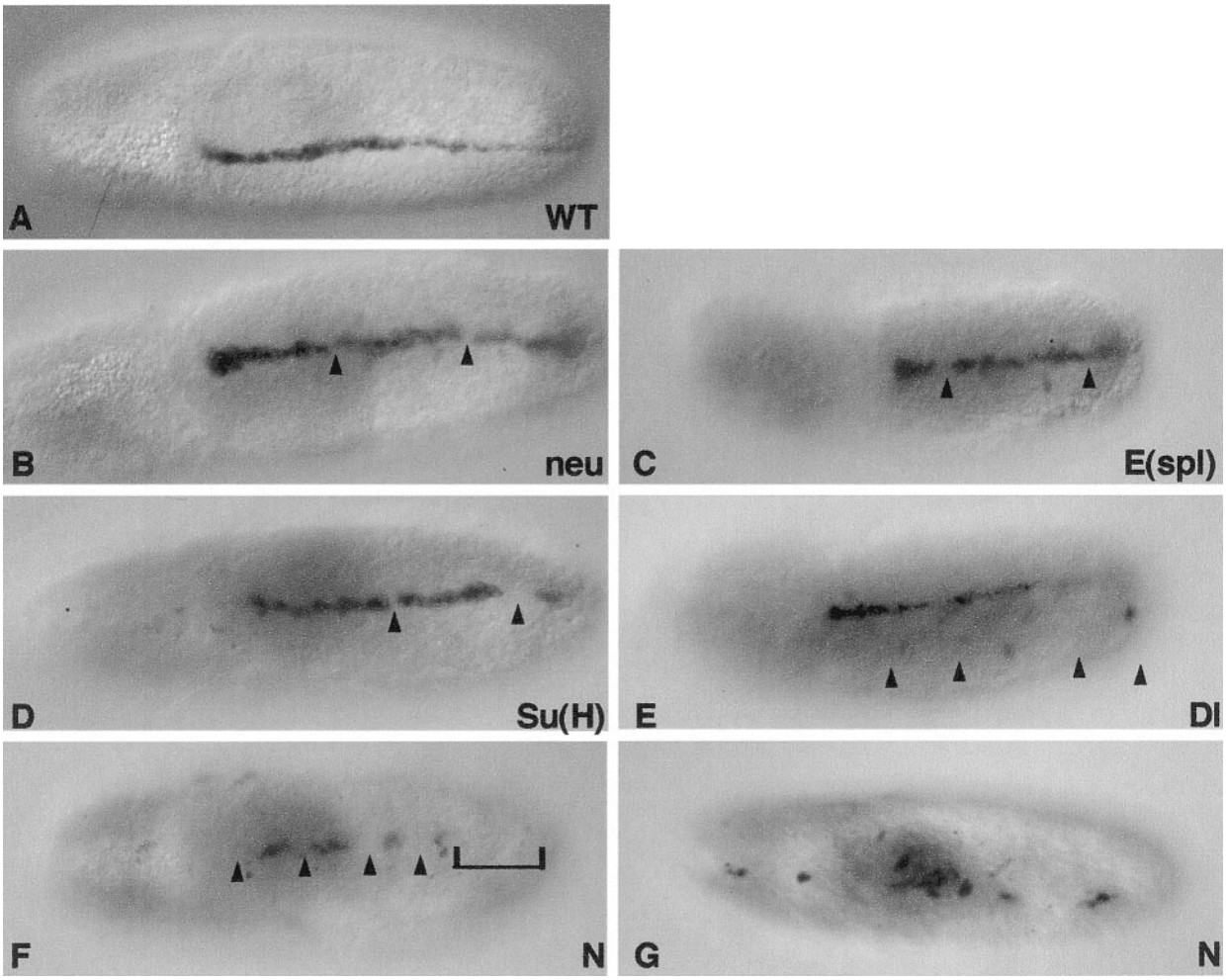


FIG. 3. The novel *Notch* activity is required for enough cells to adopt mesectoderm fates. The photographs in A–F show dorsal views of whole-mount embryos (anterior to the left) at stage 11 treated with an *in situ* probe to *sim* mRNA (blue). G shows a ventral view of the same embryo shown in F. In wild-type embryos (A) *sim* is expressed in the mesectoderm which forms a two-cell-wide stripe that extends the length of the embryo. In *neu*, *E(spl)*, *Su(H)*, and *Dl* holonull embryos (B–E) mesectodermal cells are lost, which causes discontinuities in the stripe of *sim* staining (arrowheads). These results are similar to those previously reported for zygotic nulls (Martin-Bermudo *et al.*, 1995), but less severe. These differences may be attributed to the fact that we looked at endogenous *sim* mRNA in stage 11 embryos, while Martin-Bermudo *et al.* (1995) looked at protein expressed from a *sim-lacZ* fusion gene in stage 8 embryos. F and G show a dorsal and ventral view, respectively, of the same *N* holonull embryo. The phenotype is much more severe. There are several short regions that lack mesectodermal cells (arrowheads in F) and often a large portion of the germ band completely lacks mesectodermal cells (bracket in F). The region devoid of *sim*-expressing cells on the dorsal side extends around much of the ventral side (G), where only a few small clusters of mesectodermal cells remain. Curiously, there is no apparent pattern to the placement of the mesectodermal cells that remain.

The Novel Notch Function Is Required in Multiple Cell Fate Decisions

Our previous work demonstrated that more cells adopt a muscle precursor fate when *Notch* is defective than can be accounted for by simply removing the function of the standard *Notch* pathway (Corbin and Rusconi, 1998). Here, we report that *Notch* affects three neuronal cell types in the same way that it affects muscle precursors, namely, it prevents too many cells from adopting these fates. At the same time, the novel *Notch* activity affects visceral mesoderm and the mesectoderm in the opposite way: it is required for cells to adopt those fates. This gain of some cell types and loss of others in *Notch* holonulls is not surprising since *Notch*, in its lateral inhibition function, is required for cells to decide between alternate cell fates. The key point is that in all tissues, the phenotype is more severe

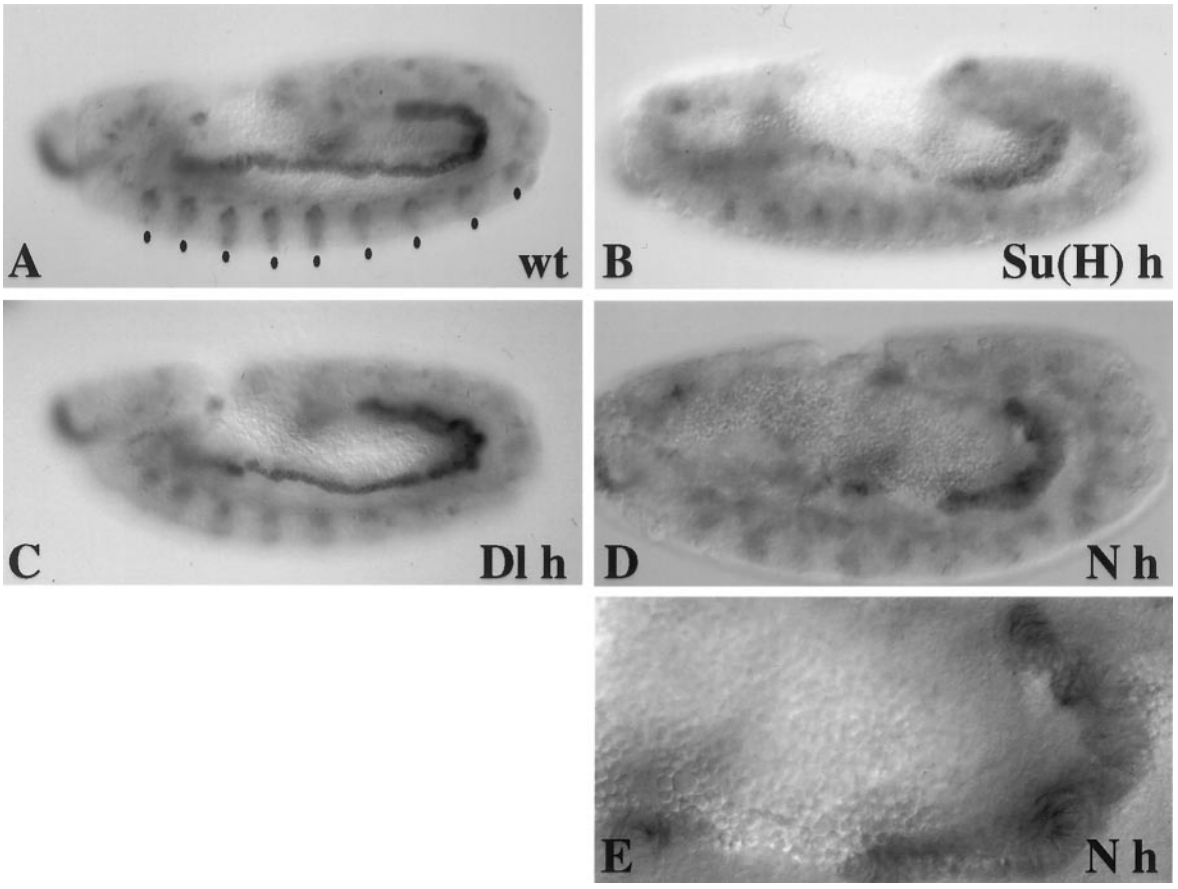


FIG. 4. The novel *Notch* activity is required for enough cells to become visceral mesoderm cells. The photographs show lateral views of whole-mount stage 12 embryos (anterior to the left) stained with an antibody to fasIII (red/brown), which is expressed in the visceral mesoderm. In wild-type embryos (A) the visceral mesoderm appears as a stripe of columnar cells just lateral to the presumptive gut. In addition, fasIII stains some neural cells in a segmentally repeated fashion (marked by black dots). Many visceral mesodermal cells are missing in *Su(H)* and *Dl* holonull embryos (B and C). Even more visceral mesodermal cells are missing in *Notch* holonull embryos (D, E), and often large sections of the visceral mesoderm are missing. For example, most of the anterior and posterior parts of the visceral mesoderm are missing in the embryo shown in D and E, leaving only the central part intact. The cells that remain retain both fasIII expression as well as some of their columnar morphology, although the column often puckers in on itself on one side when neighboring cells are missing (enlarged in E).

when *Notch* is defective than when the ligands or downstream components of the standard pathway are defective. These results show that *Notch* has an additional, critical function in all of these tissues and suggest that this function promotes or represses cell fates in the same direction as it does in the lateral inhibition pathway. The similarity of the defects in the six different tissues suggests that *Notch* is involved in the same novel function in all of the tissues.

What Is the Role of the Novel Notch Function?

When we analyzed muscle precursors in *Notch* holonull embryos, the overall defects were so severe and there were so many extra cells that it was difficult to determine the origins of the extra cells (Rusconi and Corbin, 1998). Using the neural markers, we were able to analyze the nature of the phenotypic defects in more detail. Within the NB4-2 lineage, we found that at least six times more cells become RP2 cells in *Notch* holonulls than in *Delta* or *Su(H)* holonulls, i.e., when both *Notch* pathways were defective as opposed to just the standard pathway (Fig. 2; see also Skeath and Doe, 1998). The extra RP2-like cells could arise in a number of ways. First, in the absence of the novel *Notch* function, the NB4-2 cell might act as a stem cell and regenerate itself and the GMC4-2A cell at each division (Fig. 2G). The extra GMC4-2A cells would give rise to extra presumptive RP2 and RP2sib cells (enclosed in dotted boxes), which would all adopt the RP2 fate since the standard pathway is also disrupted. However, to generate all of the extra RP2-like cells in this way would require at least four extra rounds of cell division between late stage 9, when the original NB4-2 is born, and late stage 11, when we

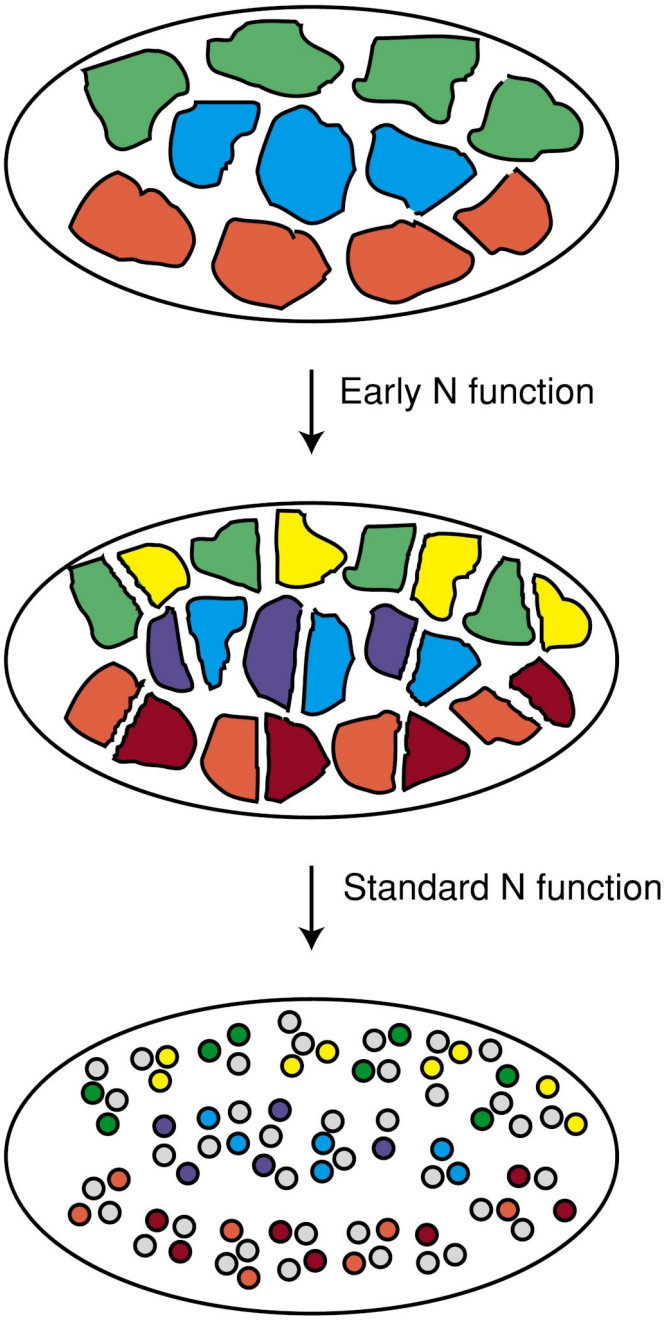


FIG. 5. Model for *Notch* function at two stages in cell determination. Three cartoons of embryos from the lateral view (dorsal on top; anterior to the left) during successive stages of development (top to bottom) are shown. For simplicity the tissues that normally would have invaginated in older embryos are shown in their original locations in the cartoons. Top: Before the early, novel *Notch* pathway is active, large groups of cells have similar developmental potentials based on their positions within the embryo, as depicted by the green, blue, and red clusters. The novel *Notch* function shunts many of these cells into equivalence groups with different developmental potentials (middle). For example, the novel pathway subdivides the light blue clusters in the top picture into the light blue and purple equivalence groups of the middle. It is as though the early *Notch* function facilitates alternative cell fate choices when cells are organizing into equivalence groups, just as the standard pathway does later within the equivalence groups. Although not shown, the early function may also determine individual cell fates, as in the case of the NB4-2 cell (see Discussion for more details). Bottom: Later in development, the standard *Notch* pathway facilitates lateral inhibition between cells within equivalence groups and allows other cells to respond to inductive cues, thus allowing cells to chose their final fates.

assayed for eve expression. A more likely possibility is that, in the absence of the novel *Notch* function, extra cells chose to become NB4-2 cells (Figs. 2H and 2I). Based on these data we suggest that the role of this novel pathway is to help cells choose their developmental potentials before they (or their progeny, in the case of the NB4-2 cell) choose a final fate via the action of the standard pathway. This idea is consistent with the loss of mesectodermal and visceral mesodermal cells in holonull embryos, since an increase in the number of cells that choose one developmental potential would cause a concomitant decrease in the number of cells with the alternate developmental potentials.

Another possibility is that the novel *Notch* activity limits cell division. This idea is supported by the observations that, in vertebrates, mutant forms of *Notch* have been implicated in T-cell leukemia and mouse mammary tumors (Ellison *et al.*, 1991; Robbins, *et al.*, 1992). While extra cell divisions might account for the increased number of certain cell types in *Notch* holonull embryos, they cannot account for the loss of other cell types. Since more visceral mesoderm and mesectodermal cells types are missing in *Notch* holonulls than in *Delta* or *Su(H)* holonulls, it seems more likely that the novel *Notch* pathway is involved in cell fate determination rather than proliferation. Of course, some cell fate decisions, e.g., the decision to become a neuroblast versus a terminally differentiated glial or support cell, would lead to more cell divisions in the embryo, but this would be a secondary rather than a direct consequence of the lack of *Notch* activity. A second reason for favoring the cell-determination model is that the *Notch* holonull phenotype is similar to that of the zygotic phenotype, albeit more severe. Embryos mutant for zygotic *Notch* function show increases in some cell types accompanied by decreases in other cell types, which are not dependent on extra cell divisions (Corbin *et al.*, 1991). Thus the simplest explanation of the holonull *Notch* phenotypes is that the same sorts of changes in cell ratios occur, but to a greater extent.

The phenotypic differences we observed in *Notch* holonull versus the other holonull embryos are consistent with a model recently proposed by Brennan *et al.* (1999). Based on their examination of muscle precursors in *Notch*, *Delta*, and *Su(H)* holonull embryos and embryos doubly mutant for these genes and *wg*, they proposed that *Notch*

functions twice. First *Notch* acts to repress groups of cells from acquiring muscle precursor potential, i.e., it limits the number of cells in the muscle precursor equivalence group. This function is sensitive to positional cues, as we previously suggested (Rusconi and Corbin, 1998), in that a localized wingless signal counteracts the repressive *Notch* function and allows some of the cells to form a muscle precursor equivalence group (Brennan *et al.*, 1999). Second, *Notch* acts again via the standard pathway, to prevent all but a few cells within the equivalence group from becoming muscle precursors.

Our data is consistent with the idea that the novel *Notch* pathway carries out a similar role in organizing many kinds of equivalence groups (Fig. 5, top). While the novel pathway would repress some developmental potentials, e.g., RP2 and muscle precursor potentials (depicted by the green-, light blue-, and orange-colored cell groups in Fig. 5, middle), it would promote others, e.g., visceral mesoderm and mesectodermal potential (depicted by the yellow, purple, and brown colors). The cartoon shows uniformly large equivalence groups for simplicity, but the equivalence groups defined by the novel pathway will probably vary in size. As demonstrated for the NB4-2 cell, the novel pathway alone may sometimes limit a particular developmental potential to a single cell per hemisegment rather than to a group of cells. One could think of such a singled-out cell as an "equivalence group of one." Later in development, the cells within these large and small equivalence groups choose their final cell fates, in many or perhaps all cases through lateral inhibition or induction mediated by the standard *Notch* pathway (bottom).

Despite the fundamental requirement of the novel *Notch* pathway, the mechanism by which the pathway functions and its components remain a mystery. It is likely that the intracellular domain of Notch is the active component of both *Notch* pathways, since the intracellular domain appears to completely rescue the neural hypertrophy of *Notch* holonull embryos (Struhl *et al.*, 1993). Further mapping of *Notch* functional domains should show whether these two activities can be separated and will be useful in identifying other components of the early pathway.

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