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The Snail repressor positions Notch signaling in the *Drosophila* embryo

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SUMMARY

The maternal Dorsal nuclear gradient initiates the differentiation of the mesoderm, neurogenic ectoderm and dorsal ectoderm in the precellular *Drosophila* embryo. Each tissue is subsequently subdivided into multiple cell types during gastrulation. We have investigated the formation of the mesectoderm within the ventral-most region of the neurogenic ectoderm. Previous studies suggest that the Dorsal gradient works in concert with Notch signaling to specify the mesectoderm through the activation of the regulatory gene *sim* within single lines of cells that straddle the presumptive mesoderm. This model was confirmed by misexpressing a constitutively activated form of the Notch receptor, Notch^{IC}, in transgenic embryos using the *eve stripe2* enhancer. The Notch^{IC} stripe induces ectopic expression of *sim* in the neurogenic ectoderm where there are low levels of the Dorsal gradient. *sim* is not activated in the ventral mesoderm, due to inhibition by the localized zinc-finger Snail repressor, which is selectively expressed in the ventral mesoderm. Additional studies suggest that the Snail repressor can also stimulate Notch signaling. A *stripe2-snail* transgene appears to induce Notch signaling in 'naïve' embryos that contain low uniform levels of Dorsal. We suggest that these dual activities of Snail, repression of Notch target genes and stimulation of Notch signaling, help define precise lines of *sim* expression within the neurogenic ectoderm.

Key words: *Drosophila*, Notch, Dorsal, Snail

INTRODUCTION

Dorsal is a maternal regulatory protein that is distributed in a broad dorsoventral gradient in the precellular *Drosophila* embryo (reviewed by Drier and Steward, 1997). It initiates the differentiation of the mesoderm, neurogenic ectoderm and dorsal ectoderm by regulating a variety of target genes in a concentration-dependent manner (reviewed by Rusch and Levine, 1996). Two lines of cells that straddle the presumptive mesoderm express the regulatory gene *sim* and ultimately form derivatives of the mesectoderm at the ventral midline of advance-stage embryos (Crews et al., 1988; Nambu et al., 1990; Nambu et al., 1991). Perhaps as little as a twofold difference in the levels of Dorsal determines whether a naïve embryonic cell adopts a mesodermal or mesectodermal fate (Gonzalez-Crespo and Levine, 1993; Ip et al., 1992; Kosman et al., 1991). We have investigated the basis for this precise regulatory switch in cell fate.

Previous studies suggest that *sim* responds directly to the Dorsal gradient through high-affinity Dorsal-binding sites in the 5′ *cis*-regulatory region (Kasai et al., 1998). In principle, high and intermediate levels of Dorsal can activate *sim* in the presumptive mesoderm and mesectoderm, but high levels of Dorsal also lead to the activation of the Snail repressor in the ventral mesoderm (Gonzalez-Crespo and Levine, 1993; Ip et al., 1992). Snail represses *sim* in the mesoderm, and thereby restricts expression to lateral regions that form the mesectoderm (Kasai et al., 1992; Kasai et al., 1998; Nibu et al., 1998). Twist-binding sites in the *sim* 5′ regulatory region might work in concert with Dorsal to activate gene expression (Kasai et al., 1998). Dorsal-Twist synergy has been implicated in the formation of the sharp lateral borders of the *snail* expression pattern that define the boundary between the mesoderm and mesectoderm (Ip et al., 1992). The Dorsal and Twist gradients extend several cell diameters beyond this boundary, yet *sim* is activated in only a single line of cells (Kosman et al., 1991). Recent studies suggest that Notch signaling helps restrict *sim* expression to the mesectoderm (Hartenstein et al., 1992; Martin-Bermudo et al., 1995; Menne and Klambt, 1994; Morel and Schweisguth, 2000).

The activation of the Notch receptor triggers the conversion of the Su(H) transcription factor from a repressor into an activator (reviewed by Artavanis-Tsakonas et al., 1999; Bray, 1998; Kadesch, 2000; Mumm and Kopan, 2000). Su(H) is maternally expressed and uniformly distributed throughout the early embryo (Lecourtois and Schweisguth, 1995). It is initially associated with a co-repressor complex consisting of Hairless (H) and possibly dCtBP (Bang and Posakony, 1992; Bray and Furriols, 2001; Morel et al., 2001). Upon signaling, the Notch intracellular domain (Notch^{IC}) enters the nucleus and interacts with Su(H) (Kidd et al., 1998; Rebay et al., 1993; Struhl and Adachi, 1998; Struhl et al., 1993). The resulting Su(H)-Notch^{IC} complex functions as a transcriptional activator (Bailey and Posakony, 1995; Fortini and Artavanis-Tsakonas, 1994; Lecourtois and Schweisguth, 1995). It has been suggested that Su(H)-H represses *sim* in the neurogenic ectoderm, but

activation of the Notch receptor in the presumptive mesectoderm permits *sim* expression, owing to disruption of the Su(H)-H repressor complex (Morel and Schweisguth, 2000). We have investigated the basis for localized Notch signaling in the mesectoderm.

A constitutively activated form of the Notch receptor, Notch^{IC} (Struhl et al., 1993), was placed under the control of the *even-skipped* (*eve*) stripe 2 enhancer. This *stripe2-Notch^{IC}* transgene induces ectopic activation of *sim* and *m8*. The latter gene is a member of the Enhancer of split [E(spl)] complex that encodes Notch-responsive HES-family transcriptional repressors, which inhibit neurogenesis through the silencing of *achaete-scute* proneural genes (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Nakao and Campos-Ortega, 1996; Nellesen et al., 1999). Both the *sim* and *m8* 5′ *cis*-regulatory regions contain optimal, Su(H) binding sites (Morel and Schweisguth, 2000; Nellesen et al., 1999). Nonetheless, the *stripe2-Notch^{IC}* transgene differentially regulates the two genes. It induces a stripe of *m8* expression, but causes a 'pyramid' pattern of ectopic *sim* staining that corresponds to the spatial intersection between Notch signaling and the Dorsal gradient. Ectopic activation of *sim* and *m8* is inhibited in the ventral mesoderm by the Snail repressor. However, Snail also appears to stimulate Notch signaling. When introduced into mutant embryos that contain low, uniform levels of Dorsal, a *stripe2-snail* transgene activates *sim* and *m8* expression. These results suggest that Snail functions both to generate a Notch signal and repress Notch target genes, thereby restricting mesectodermal fate to a precise line of cells. We discuss the basis for this dual activity of the Snail repressor and consider other cases where Snail and Snail-related repressors might localize Notch signaling.

MATERIALS AND METHODS

In situ hybridization

Embryos from wild-type, mutant, and transgenic lines were collected, fixed and then hybridized with dioxygenin-UTP labeled antisense RNA probes as previously described (Jiang et al., 1991). The *snail*, *sim* and *T3* cDNAs used to produce these probes were previously described (Gonzalez-Crespo and Levine, 1993; Ip et al., 1992; Kosman et al., 1991). The *m8* cDNA used to generate antisense RNA probe was a gift from S. Bray. The *Delta* cDNA was kindly provided by E. Lai.

P-element transformation vectors

The construction of the *hsp83-Toll^{10B}-bcd* 3′UTR has been previously described (Huang et al., 1997). For the construction of the *stripe2-Notch^{IC}* transformation vector, a genomic fragment containing the intracellular domain of Notch (a gift from G. Struhl) (Struhl et al., 1993) was placed under the control of the *eve* stripe 2 enhancer by cloning it into the *Asc*I site of a modified pCasPeR injection vector. This injection vector contains two tandem copies of an augmented stripe 2 enhancer upstream of a frt-stop-frt cassette (Kosman and Small, 1997). The *stripe2-Notch^{IC}* transformation vector was then injected into *yw* embryos as previously described (Kosman and Small, 1997). The construction of the *stripe2-snail*, *stripe2-snail/hairy* and *stripe2-snailM1M2* has been described previously (Nibu et al., 1998). Transgenic females carrying the *stripe2-snail* and *stripe2-Notch^{IC}* transgenes were mated with males homozygous for the yeast Flp recombinase under the control of a sperm-specific tubulin promoter. F_1 males containing both the transgene and the Flp recombinase were selected for subsequent matings. The F_2 progeny derived from these males have ectopic *snail* or *Notch^{IC}* expression that is due to the rearrangement of the frt-stop-frt cassette.

Fly strains

The *Toll^{rm9}* and *Toll^{rm10}* mutations cause constitutive, low levels of Dorsal nuclear transport in affected embryos (Anderson et al., 1985). *Toll^{rm9}/Toll^{rm10}* females were obtained by mating *Toll^{rm9}/TM3, Sb, Ser* males with *Toll^{rm10}/TM3, Sb* females. Non-*Sb*, non-*Ser* F_1 females were collected and mated with *yw*, flipped *stripe2-snail*, or flipped *stripe2-Notch^{IC}* males. Embryos from this cross were then collected for in situ hybridization. All crosses and collections were conducted at 25°C.

The *gd^7* allele was used to generate *gd$^-$/gd$^-$* females (Konrad et al., 1988), which were mated with *yw*, flipped *stripe2-snail* or flipped *stripe2-Notch^{IC}* males. Embryos from this cross were collected and fixed for in situ hybridization. All crosses and collections were conducted at 25°C.

RESULTS

Notch signaling activates *m8* and *sim* expression

Previous studies have indicated a role for Notch signaling in the regulation of *sim* expression (Hartenstein et al., 1992; Martin-Bermudo et al., 1995; Menne and Klambt, 1994). Removal of maternal *Notch$^+$* gene activity results in a loss of *sim* expression, while overexpression of a *UAS-Notch^{IC}* transgene with ubiquitous GAL4 driver lines expands the *sim* pattern (Morel and Schweisguth, 2000). The importance of Notch signaling for mesectodermal specification was confirmed using a *stripe2-Notch^{IC}* transgene that produces a localized source of Notch signaling in the early embryo (Figs 1-3).

The *eve* stripe 2 enhancer directs early expression of *Notch^{IC}* at the boundary between the presumptive head and thorax. Expression is initially detected by the onset of nuclear cleavage cycle 14 (Fig. 1A) and persists during gastrulation (data not shown). In situ hybridization assays also detect the endogenous *Notch* RNA, which is distributed throughout basal regions of the cytoplasm. The *stripe2-Notch^{IC}* transgene induces an ectopic stripe of *m8* expression (Fig. 1B). Staining might be initially asymmetric, but the stripe becomes uniformly intense in lateral and dorsal regions by the completion of cellularization (Fig. 1B and data not shown). However, the strong expression of *Notch^{IC}* in ventral regions (Fig. 1A) is not sufficient to induce *m8* expression, probably due to repression by Snail as *m8* expression expands into ventral regions of *snail$^-$/snail$^-$* mutant embryos (data not shown).

The *stripe2-Notch^{IC}* transgene also induces expression of *sim* (Fig. 1C), and, like *m8*, ectopic expression is excluded from the ventral mesoderm. However, unlike *m8*, *sim* is not activated in the dorsal-most regions, but is restricted to a pyramid pattern in ventrolateral regions. This pyramid is detected before the expression of the endogenous pattern (Fig. 2A), and might reflect a requirement for both Notch signaling and Dorsal + Twist activators in the regulation of *sim* expression. Occasionally, *stripe2-Notch^{IC}* induces *sim* expression in dorsal regions during gastrulation, although staining is stronger in ventral regions containing the Dorsal and Twist activators (Fig. 2B). This 'Notch-only' *sim* activation may depend on high levels of Notch signaling, as it is not seen in transgenic lines that express low levels of *Notch^{IC}*.

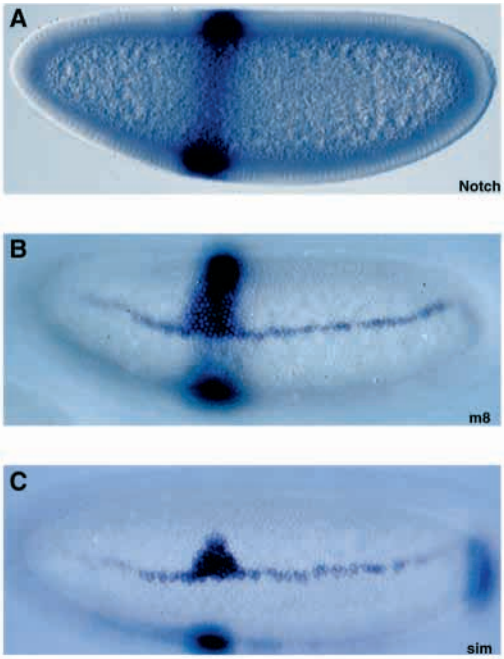

Fig. 1. Notch^{IC} induces ectopic activation of *sim* and *m8*. Cellularizing embryos express a *stripe2-Notch^{IC}* transgene and are oriented with dorsal upwards and anterior towards the left. Transgenic embryos were hybridized with digoxigenin-labeled *Notch* (A), *m8* (B) and *sim* (C) antisense RNA probes, and stained to visualize the indicated gene expression patterns. (A) The *Notch* probe detects an ectopic stripe of *Notch^{IC}* expression. General staining of the wild-type *Notch* RNA is detected throughout the basal cytoplasm. (B) The *m8* gene exhibits endogenous lateral lines of expression in the mesectoderm, as well as an ectopic stripe. (C) Ectopic *sim* expression is restricted to a pyramid pattern in the neurogenic ectoderm.

Ectopic *sim* expression persists in ventrolateral regions, the presumptive neurogenic ectoderm, during gastrulation and germ band elongation (Fig. 2C). Marker genes that are expressed in the CNS exhibit gaps in the vicinity of this ectopic *sim* pattern (Fig. 2D), which may reflect a transformation of neurogenic ectoderm into mesectoderm. The persistence of ectopic *sim* expression in the ventral nerve cord is probably the result of Sim autoregulation (Morel and Schweisguth, 2000; Nambu et al., 1991).

Differential regulation of *m8* and *sim*

The differential response of *m8* and *sim* to the *stripe2-Notch^{IC}* transgene might reflect the difference between a 'hard-wired' target gene (*m8*) that is activated primarily by Notch signaling, and a conditional target gene (*sim*) that is jointly regulated by Notch and the Dorsal gradient. This issue was examined by comparing the ability of two separate *stripe2-Notch^{IC}* transgenic lines to induce ectopic expression of *sim* and *m8* in mutant backgrounds. One of the lines directs strong expression of *stripe2-Notch^{IC}*, while the other directs much lower levels of *Notch^{IC}* expression based on in situ hybridization assays (data not shown). Each line was introduced into mutant embryos derived from *Toll^{rm9}/Toll^{rm10}* females. Owing to the mutant Toll receptor, these embryos contain low, uniform levels of Dorsal that are insufficient to activate *twist* or *snail*. Neither *sim* nor *m8* expression is detected in central regions of *Toll^{rm9}/Toll^{rm10}* mutant embryos, though there is staining at the anterior and posterior poles (Fig. 3A; Fig. 5G,J). Introduction of the strong *stripe2-Notch^{IC}* transgene into this mutant background induces strong expression of *sim* (Fig. 3B), whereas the weaker line leads to low levels of expression (Fig. 3C). However, both *stripe2-Notch^{IC}* lines are capable of driving strong ectopic expression of *m8* (data not shown). The absence of the Snail repressor probably accounts for the uniform induction of *sim* and *m8* expression across the dorsoventral axis. These results also suggest that Notch signaling is sufficient to activate *sim* and *m8* in the absence of Twist.

To determine if Notch signaling was sufficient to activate *sim* or *m8* in the absence of both Twist and Dorsal, the *stripe2-Notch^{IC}* lines were crossed into mutant embryos derived from *gd^{-}/gd^{-}* females. These embryos fail to process the Spätzle ligand, and there is a block in Dorsal nuclear transport (Drier and Steward, 1997). As a result, there is no expression of *twist*, *snail* or *sim* in central regions (data not shown, Fig. 3D). However, mutant embryos exhibit weak, broad expression of *m8*, probably owing to the derepression of the dorsal ectoderm pattern (Fig. 3G); *m8* is normally expressed both in the mesectoderm and the dorsal ectoderm of wild-type embryos

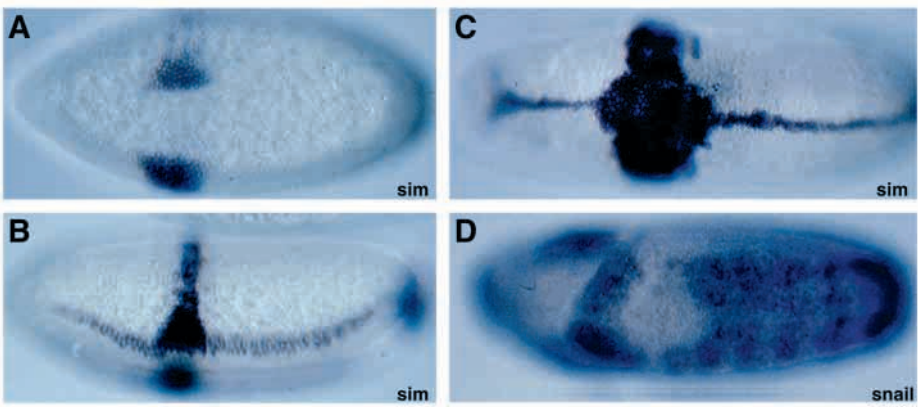

Fig. 2. Notch^{IC} induces cell fate changes in the neurogenic ectoderm. Transgenic embryos express the *stripe2-Notch^{IC}* fusion gene and were hybridized with *sim* or *snail* antisense RNA probes. (A) The transgene induces the ectopic *sim* pyramid pattern prior to the onset of the endogenous pattern in the mesectoderm. (B) The ectopic *sim* pattern is maintained during gastrulation. By this stage *sim* expression extends into dorsal regions. The endogenous expression pattern is also observed. (C) After gastrulation, the two lines of *sim* expression converge at the ventral midline to form the mesectoderm. There is also a broad domain of ectopic staining that probably arises from the ectopic pyramid seen in younger embryos. Ectopic *sim* expression leads to a loss of identifiable neurons, as judged by the *snail* expression pattern (D). (D) The *snail* expression pattern is altered in delaminating neuroblasts during germ band elongation. There is a gap in the presumptive ventral nerve cord that coincides with the ectopic *sim* expression pattern (see C).

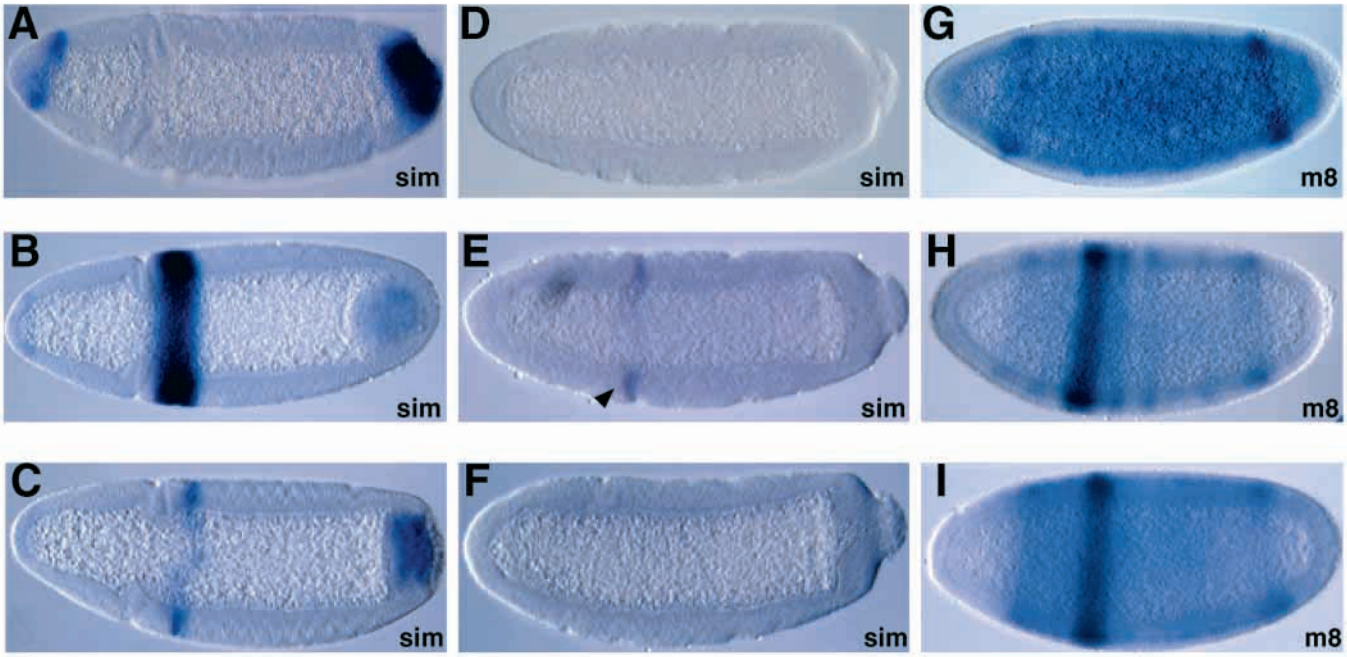

Fig. 3. Symmetric stripes of *sim* and *m8* are induced in embryos lacking Dorsal gradients. Embryos were collected from *Toll^{rm9}/Toll^{rm10}* (A-C) or *gd^{-}/gd^{-}* females (D-I). The former mutants contain low, uniform levels of Dorsal in all nuclei. The latter embryos completely lack nuclear Dorsal protein. Different *stripe2-Notch^{IC}* transgenes were introduced into the mutant backgrounds, and stained with either a digoxigenin-labeled *sim* (A-F) or *m8* probe (G-I). (A-C) *Toll^{rm9}/Toll^{rm10}* embryos. (A) *sim* expression is restricted to the termini of mutant embryos that lack a *stripe2-Notch^{IC}* transgene. (B) A strong stripe of *sim* staining is induced by a strongly expressed *stripe2-Notch^{IC}* transgene. (C) A faint *sim* stripe is induced by the weakly expressed *stripe2-Notch^{IC}* transgene. These mutant embryos lack *snail* expression (Fig. 4E), which might explain why the ectopic *sim* stripes are symmetrically expressed in dorsal and ventral regions. (D-F) *gd^{-}/gd^{-}* embryos. (D) *sim* expression is essentially absent in mutant embryos lacking a *stripe2-Notch^{IC}* transgene. A weak *sim* stripe (see arrowhead) is induced by the strongly expressed *stripe2-Notch^{IC}* transgene (E), whereas the weakly expressed transgene fails to induce *sim* expression (F). (G-I) *gd^{-}/gd^{-}* embryos. There is general, weak expression of the *m8* gene throughout mutant embryos (G). This staining might be due to the derepression of the staining pattern that is normally restricted to the dorsal ectoderm. Both the strong (H) and weak (I) *stripe2-Notch^{IC}* transgenes induce stripes of *m8* expression.

(Wech et al., 1999) (Fig. 5A). In this background, only the strong *stripe2-Notch^{IC}* transgene induces weak expression of *sim* (Fig. 3E), while the weaker line fails to induce any expression whatsoever (Fig. 3F), suggesting that Dorsal is necessary for *sim* expression. By contrast, both *stripe2-Notch^{IC}* lines induce strong stripes of *m8* expression in mutant embryos (Fig. 3H,I).

Snail regulates *sim* and *m8* expression

stripe2-Notch^{IC} transgenes fail to induce *m8* and *sim* expression in ventral regions of wild-type embryos (Fig. 1B,C; Fig. 2A,B), but cause uniform expression in mutant embryos lacking Snail (Fig. 3B,H). Similarly, both *sim* and *m8* are derepressed in ventral regions of *snail^{-}/snail^{-}* mutant embryos (Hemavathy et al., 1997) (data not shown). These results are consistent with earlier models suggesting that the Snail repressor forms the ventral border of the *sim* expression pattern (Gonzalez-Crespo and Levine, 1993; Kasai et al., 1992; Kosman et al., 1991; Nibu et al., 1998; Rusch and Levine, 1996). To test this idea, Snail was misexpressed in transgenic embryos by placing the *snail* coding sequence under the control of the *eve* stripe 2 enhancer (Figs 4, 5).

snail is normally expressed in the ventral mesoderm, but exhibits an ectopic stripe in transgenic embryos carrying a *stripe2-snail* fusion gene (Fig. 4A,B). This ectopic stripe represses several target genes that are expressed in the neurogenic ectoderm, including *rhomboid* and *Brinker*. An example of ectopic repression is shown for *sog* (Fig. 4C,D). There is a gap in the pattern that corresponds to the location of the ectopic Snail stripe (Fig. 4C,D). A mutant form of Snail that lacks the two dCtBP co-repressor interaction motifs (PxDLSxK and PxDLSxR) fails to repress *sog* (data not shown) (see Nibu et al., 1998).

The *stripe2-snail* transgene causes complex alterations in the *sim* and *m8* expression patterns. There is an initial gap in the early *m8* pattern (Fig. 5B), followed by ectopic staining in the neurogenic ectoderm (Fig. 5C). The ectopic ventrolateral staining persists in advanced-stage embryos and is associated with a gap in the developing ventral nerve cord (data not shown). The *stripe2-snail* transgene causes the same type of alteration in the *sim* expression pattern. There is an initial gap in the pattern (Fig. 5E), but in older embryos ectopic expression is detected in one or two cells in the neurogenic ectoderm (Fig. 5F). These alterations in *sim* and *m8* depend upon the ability of Snail to function as a transcriptional repressor, as neither pattern is altered when the dCtBP interaction motifs are removed from Snail (data not shown).

The preceding results suggest that Snail both represses and activates *sim* and *m8* expression. Additional evidence for this dual activity was obtained by crossing the *stripe2-snail* transgene into mutant embryos derived from *Toll^{rm9}/Toll^{rm10}* females. The uniform, low levels of Dorsal that are present in

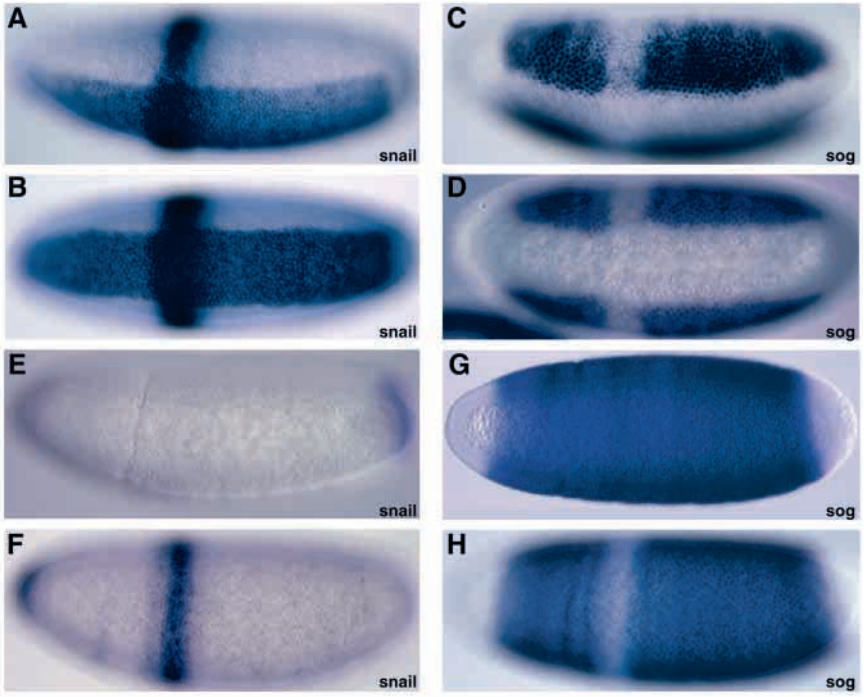

Fig. 4. Snail represses *sog* expression. Wild-type (A-D) and *Toll^{rm9}/Toll^{rm10}* mutant (E-H) embryos were stained with either a *snail* (A,B,E,F) or *sog* (C,D,G,H) hybridization probe. The embryos in A-D and F,H contain a *stripe2-snail* transgene. (A,B) Lateral and ventral views of wild-type embryos that contain a *stripe2-snail* transgene. An ectopic stripe of *snail* expression is detected in addition to the normal pattern in the ventral mesoderm. (C,D) Lateral and ventral views of wild-type embryos expressing the *stripe2-snail* transgene. There are gaps in the *sog* expression pattern within the lateral, neurogenic ectoderm near the position of the Snail stripe. (E,F) *Toll^{rm9}/Toll^{rm10}* mutant embryos that lack (E) or contain (F) a *stripe2-snail* transgene. Mutant embryos that lack the transgene exhibit residual snail staining at the poles (E). The *stripe2-snail* transgene provides the sole source of *snail* expression in middle body regions (F). This stripe is transiently expressed and rapidly disappears in older embryos (data not shown). (G,H) *sog* staining patterns in *Toll^{rm9}/Toll^{rm10}* mutant embryos that either lack (G) or express (H) a *stripe2-snail* transgene. In mutant embryos lacking the transgene, *sog* is uniformly expressed along the anteroposterior axis, with the exception of the extreme termini (G). The *stripe2-snail* transgene creates a gap in the *sog* pattern (H).

mutant embryos fail to activate *snail* expression (Fig. 4E), so that the *stripe2-snail* transgene encodes the sole source of the Snail repressor (Fig. 4F). Though unable to induce *snail* expression, the low levels of Dorsal present in the mutant embryos are sufficient to induce nearly ubiquitous expression of *sog* (Fig. 4G). When introduced into this mutant background, *stripe2-snail* is still capable of repressing *sog* (Fig. 4H). Mutant embryos that lack the *stripe2-snail* transgene do not exhibit either *m8* (Fig. 5G) or *sim* (Fig. 5J) expression in middle body regions, although there is residual staining at the anterior and posterior poles. The *stripe2-snail* transgene leads to ectopic induction of *m8* (Fig. 5H) and *sim* (Fig. 5K) expression. In both cases, staining is detected in the vicinity of the *eve* stripe 2 pattern, but expression is not uniform. Instead, both genes, especially *sim*, exhibit patchy 'salt and pepper' staining patterns (Fig. 5H,K).

The induction of *sim* and *m8* expression depends on the ability of Snail to function as a transcriptional repressor. Mutant proteins that lack the dCtBP interaction motifs weakly activate *m8* and altogether fail to activate *sim* in *Toll^{rm9}/Toll^{rm10}* mutants (data not shown). Conversely, a *stripe2-snail/hairy* transgene that contains the Hairy repression domain continues to induce *sim* and *m8* in mutant embryos (Fig. 5I,L).

Snail represses potential regulators of Notch signaling

It is possible that the *stripe2-snail* transgene establishes a domain of Notch signaling by repressing regulators of the Notch pathway. One candidate is the Notch ligand Delta, which is broadly expressed in lateral and dorsal regions of cellularizing and gastrulating embryos (Fig. 6A). There is little or no expression in the ventral mesoderm, probably owing to repression by Snail, as the *Delta* pattern expands into ventral regions of *sna^{-}/sna^{-}* mutant embryos (data not shown). The *stripe2-snail* transgene causes a subtle attenuation in the Delta expression pattern (Fig. 6B, compare with 6A). There is reduced staining in the vicinity of *stripe2-snail*, particularly in one or two cells straddling the presumptive mesoderm/mesectoderm boundary (arrowhead, Fig. 6B). It is conceivable that this slight reduction in *Delta* expression helps trigger Notch signaling (see Discussion).

The activation of Notch leads to the induction of *E(spl)* genes such as *m8*, which encode transcriptional repressors that block the expression of proneural genes in the Achaete-Scute complex (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Nakao and Campos-Ortega, 1996; Nellesen et al., 1999). *T3*, or *lethal of scute*, is normally expressed in a series of lateral stripes in the neurogenic ectoderm of wild-type embryos (Kosman et al., 1991). *T3* stripes are expressed throughout mutant embryos derived from *Toll^{rm9}/Toll^{rm10}* females (Fig. 6C). The *stripe2-snail* transgene creates a gap in this staining pattern (Fig. 6D), which might help define a zone of Notch signaling, as Achaete-Scute activators can inhibit Notch target genes (Heitzler et al., 1996).

snail is initially expressed in a relatively broad pattern that extends into ventral regions of the presumptive neurogenic ectoderm. This pattern is refined during cellularization, and the final borders coincide with the boundary between the presumptive mesoderm and mesectoderm (data not shown). The refinement process is also observed in transgenic embryos that contain an ectopic anterior-posterior Dorsal nuclear gradient (Fig. 7A-C). Before nuclear cleavage 14, the *snail* expression pattern exhibits a 'fuzzy' border (Fig. 7A). This border is refined by the completion of cellularization (Fig. 7B), and *sim* expression is detected shortly thereafter (Fig. 7C).

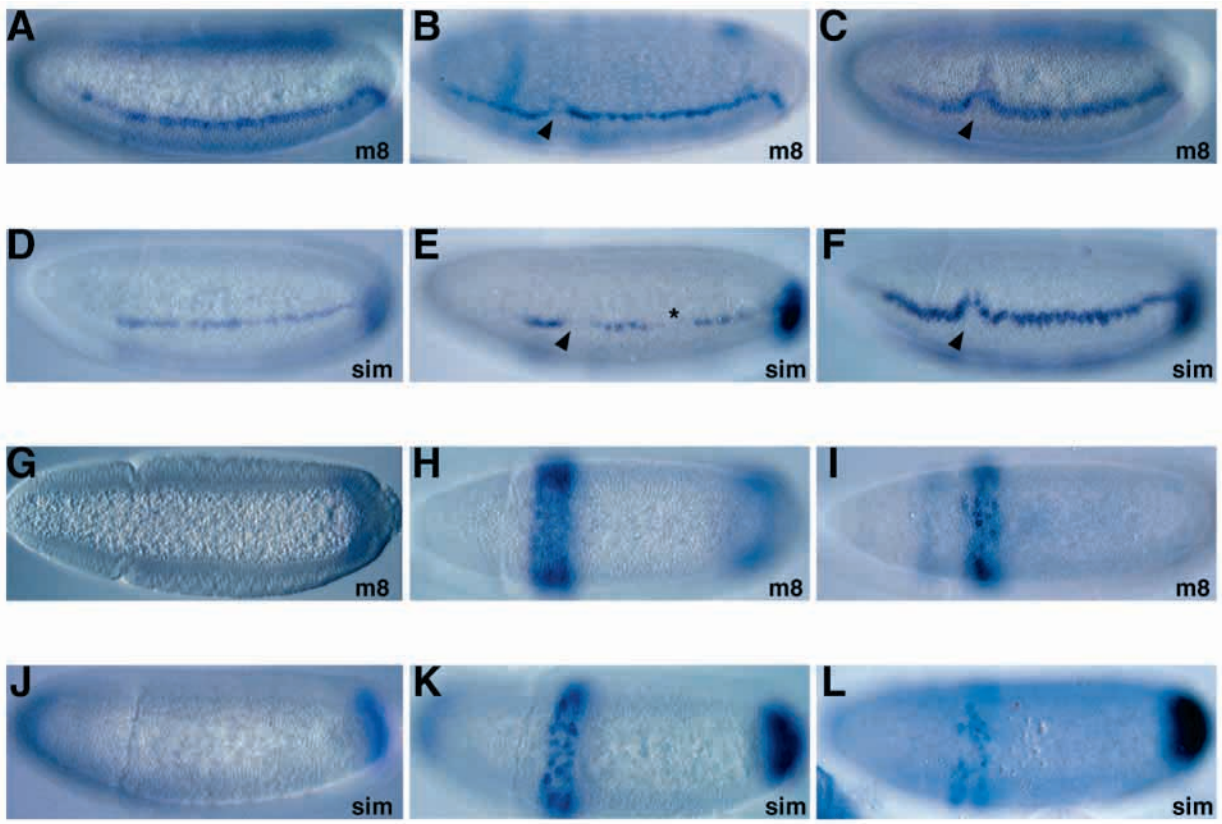

Fig. 5. The *stripe2-snail* transgene induces complex changes in the *m8* and *sim* expression patterns. Wild-type (A-F) and $Toll^{rm9}/Toll^{rm10}$ (G-L) were stained with *m8* (A-C;G-I) or *sim* (D-F;J-L) hybridization probes. (A-C) Lateral views of wild-type embryos that either lack (A) or carry (B,C) the *stripe2-snail* transgene. In early embryos (just after cellularization), the transgene creates a gap in the normal lateral lines within the presumptive mesectoderm (arrowhead, B; compare with A). In older embryos (gastrulation) there is both a gap in the pattern and ectopic staining in lateral regions (arrowhead, C). (D-F). Lateral views of wild-type embryos that either lack (D) or carry (E,F) the *stripe2-snail* transgene. There is a gap in the pattern in early embryos (arrowhead in E; compare with D), but the top of the gap is filled to produce a continuous bump of staining in older embryos (arrowhead, F). The asterisk indicates a gap in the *sim* pattern that is due to normal discontinuities in the initial *sim* pattern, not to the *stripe2-snail* transgene. (G-I) Mutant embryos obtained from $Toll^{rm9}/Toll^{rm10}$ females. There is no *m8* expression in middle body regions of mutant embryos (G), although there is expression at the posterior pole. Mutants that express the *stripe2-snail* transgene exhibit broad stripes of *m8* staining (H). Mutants that express the *stripe2-snail/hairy* transgene also exhibit ectopic *m8* staining (I). (J-L) Mutant embryos derived from $Toll^{rm9}/Toll^{rm10}$ embryos. *sim* expression is restricted to the termini of mutant embryos lacking the *stripe2-snail* transgene (J). By contrast, patchy stripes of *sim* expression are observed both in mutants that contain the *stripe2-snail* transgene (K) and the *stripe2-snail/hairy* transgene (L).

Perhaps the early *snail* refinement process serves to control the temporal onset of *sim* expression. When broad, the Snail repressor keeps *sim* off, but after refinement *sim* can be activated in the domain where *snail* was transiently expressed.

DISCUSSION

This study provides further evidence that Notch signaling is essential for the formation of the mesectoderm at the boundary between the mesoderm and neurogenic ectoderm. Two different Notch target genes were examined: *m8* expression appears to depend almost exclusively on Notch signaling, whereas *sim* is a conditional Notch target gene that is activated only in cells containing Dorsal. Evidence is presented that Snail functions as both a repressor and an indirect activator of Notch signaling. In particular, a transient stripe of the Snail repressor creates a domain of Notch signaling in apolar embryos that contain low, uniform levels of Dorsal. We discuss how Snail induces Notch signaling and also represses Notch target genes, and thereby specifies localized lines of *sim* and *m8* expression in the mesectoderm.

Competition between the Snail repressor and Notch signaling produce sharp stripes

A crucial finding of this study is that a *stripe2-snail* transgene induces ectopic expression of *m8* and *sim* in both wild-type and $Toll^{rm9}/Toll^{rm10}$ mutant embryos, suggesting that the Snail repressor is actually playing a positive role in Notch signaling. Importantly, this stimulatory activity depends on the ability of Snail to function as a transcriptional repressor. Mutant forms of the *stripe2-snail* transgene that contain single amino acid substitutions in the two repression domains (PxDLSxK and PxDLSxR) fail to induce *sim* and *m8* expression in either wild-type or $Toll^{rm9}/Toll^{rm10}$ mutant embryos (data not shown). By contrast, a *stripe2-snail/hairy* transgene that contains the Hairy

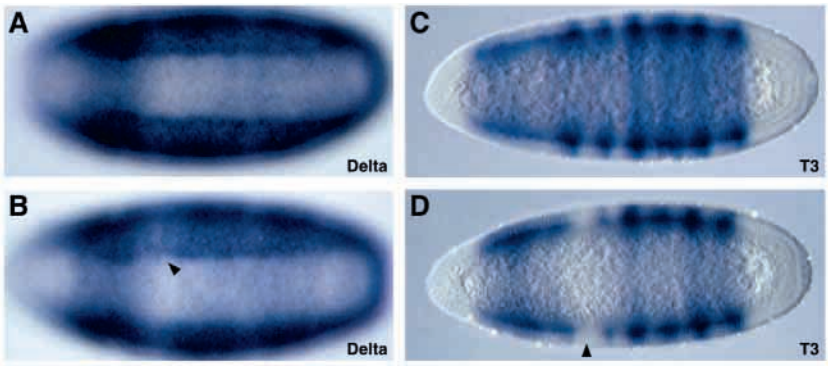

Fig. 6. Snail represses potential inhibitors of Notch signaling. Wild-type (A,B) and *Toll^{rm9}/Toll^{rm10}* (C,D) mutant embryos were stained with either a *Delta* (A,B) or *T3* hybridization probe (C,D). Embryos have completed cellularization and are oriented with anterior to the left. (A,B) *Delta* is expressed in lateral and dorsal regions of wild-type embryos (A). Staining is excluded from the ventral mesoderm, possibly by the Snail repressor as the *Delta* pattern is derepressed in *sna^{-}/sna^{-}* mutant embryos (not shown). The *stripe2-snail* transgene (B) causes a slight weakening of the normal Delta pattern (arrowhead). (C,D) *T3* is expressed in a series of stripes along the entire dorsoventral axis of *Toll^{rm9}/Toll^{rm10}* mutant embryos (C). The *stripe2-snail* transgene (D) creates a gap in the T3 expression pattern (arrowhead).

repression domain continues to activate both *sim* and *m8* in mutant embryos (see Fig. 5I, L).

The localized Snail repressor restricts Notch signaling to the mesectoderm of early embryos, presumably by directly repressing Notch target genes. Indeed, the *sim* 5′ regulatory region contains a series of high-affinity Snail repressor sites (Kasai et al., 1992). It is conceivable that Snail restricts Notch signaling in other developmental processes. For example, after its transient expression in the ventral mesoderm of early embryos, *snail* is reactivated in delaminating neuroblasts at the completion of germ band elongation (see Fig. 2D). At this stage, Notch signaling subdivides the neurogenic ectoderm into neurons and ventral epidermis. Notch is selectively activated in epidermal cells, where it induces the expression of E(spl) repressors that silence Achaete-Scute proneural genes (Bailey and Posakony, 1995). The localized expression of the Snail repressor in delaminating neuroblasts might help ensure neuronal differentiation by inhibiting Notch-specific target genes. Removal of *snail* along with two related linked zinc-finger repressors (Worniu and Escargot) leads to a reduction in the number of CNS neuroblasts (Ashraf et al., 1999; Cai et al., 2001).

Snail as a gradient repressor

We propose that Snail functions as a gradient repressor to restrict Notch signaling (summarized in Fig. 7D). In precellular embryos, the initial *snail* expression pattern is broad and extends into the future mesectoderm. During cellularization, the pattern is refined and *snail* expression is lost in the mesectoderm and restricted to the mesoderm. The early, broad

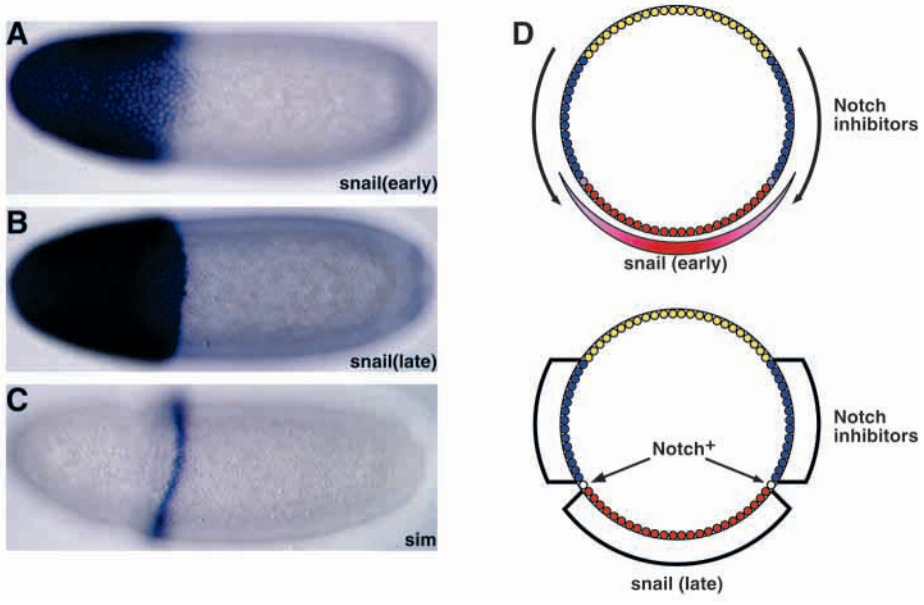

Fig. 7. A Snail repressor gradient helps localize Notch signaling. Embryos were collected from females that contain an *hsp83-Toll^{10b}-bcd* transgene and thereby express a broad anterior-posterior Dorsal nuclear gradient. These embryos were derived from *gd^{-}/gd^{-}* females, and therefore lack the normal dorsoventral Dorsal gradient. Mutants were stained with either a *snail* (A,B) or *sim* (C) hybridization probe. (A,B) *snail* staining pattern in precellular (A) and cellularized (B) embryos. *snail* is activated by high levels of the ectopic anteroposterior Dorsal nuclear gradient in anterior regions of mutant embryos. The *snail* pattern is initially broad and fuzzy (A), but refines during cellularization (B) and exhibits the very sharp border seen for the normal *snail* pattern at the boundary between the mesoderm and mesectoderm. (C) *sim* expression is not detected until the onset of gastrulation. Staining is detected in cells that reside just posterior of the sharp *snail* expression pattern. These *sim*-positive cells exhibited weak, transient *snail* expression at earlier stages (A). (D) A model for the positioning of Notch signaling by the Snail repressor. The top and bottom circles represent cross-sections through precellular (top) and cellularized (bottom) embryos. *snail* is initially expressed in a broad pattern in ventral and ventrolateral regions that encompass the presumptive mesoderm and mesectoderm. At this early stage Snail might repress a number of inhibitors of Notch signaling, such as *Delta* and *T3*. At later stages, the snail expression pattern is refined and restricted to the mesoderm. Notch signaling is activated in the cells that transiently expressed the Snail repressor.

snail pattern might create a broad domain of potential Notch signaling by repressing components of the Notch pathway, such as Delta and T3. After cellularization, Notch signaling is blocked in the presumptive mesoderm by sustained, high levels of the Snail repressor. However, Notch can be activated in the mesectoderm because of the loss of Notch inhibitors repressed by transient expression of the Snail repressor. According to this model, the dynamic *snail* expression pattern determines both the timing and limits of Notch signaling.

The results obtained in $Toll^{rm9}/Toll^{rm10}$ mutant embryos can be interpreted in the context of this Snail gradient model. The *stripe2-snail* transgene produces transient expression of the Snail repressor when compared with the endogenous gene. Consequently, the *snail* stripe creates an early zone of potential Notch signaling in $Toll^{rm9}/Toll^{rm10}$ by repressing Delta, T3, and other components of the pathway (Fig. 6). Perhaps the initially intense expression of the *stripe2-snail* transgene inhibits the activation of *m8* and *sim*, but these genes are activated as expression from the transgene diminishes. Previous studies lend support to the idea that low levels of Snail can repress some target genes such as T3, while failing to repress others (Hemavathy et al., 1997).

We do not wish to imply that repression by a Snail gradient is the sole basis for positioning Notch signaling. Previous studies suggest that expression of neurogenic genes such as *neuralized* are also important for the restricted expression of *sim* and *m8* within the mesectoderm (Hartenstein et al., 1992; Martin-Bermudo et al., 1995). Perhaps Neuralized and Snail act separately to establish precise lines of Notch signaling.

Differential regulation of Notch target genes

Notch, like other signaling pathways, is not dedicated to a particular developmental process (Artavanis-Tsakonas et al., 1999). While first identified as an agent of neurogenesis, it has been shown to play a role in the dorsoventral patterning of the wing imaginal disk, and the specification of the R7 photoreceptor cell in the adult eye (Cooper and Bray, 2000). We have provided additional evidence that Notch signaling specifies the mesectoderm at the ventral border of the neurogenic ectoderm in the early embryo. The regulation of *sim* may provide insights into how the Notch signaling cassette can perform so many disparate functions.

The analysis of $Toll^{rm9}/Toll^{rm10}$ embryos suggests that Dorsal functions synergistically with Notch signaling to activate *sim* expression. A *stripe2-*$Notch^{IC}$ transgene induces strong *sim* expression in these embryos, even though they contain low levels of Dorsal and lack Twist. However, the same transgene barely activates *sim* when crossed into embryos that lack both Dorsal and Twist. By contrast, *m8* is strongly expressed in these mutants, indicating *m8* is primarily activated by Su(H)-$Notch^{IC}$ and does not require Dorsal (Bailey and Posakony, 1995; Kramatschek and Campos-Ortega, 1994; Lecourtois and Schweisguth, 1995; Nellesen et al., 1999).

Perhaps the low levels of Dorsal present in the presumptive mesectoderm are not sufficient to activate *sim*. Instead, activation might rely on protein-protein interactions between Dorsal and the Su(H)-$Notch^{IC}$ complex within the *sim* 5′ *cis*-regulatory region. *sim* contains a number of optimal Su(H) recognition sequences (Morel and Schweisguth, 2000; Nellesen et al., 1999); these might help recruit Dorsal to adjacent sites. By contrast, the *stripe2-*$Notch^{IC}$ transgene appears to be sufficient to activate *m8*, even though it contains fewer optimal Su(H) binding sites than the *sim* 5′ *cis*-regulatory region (Morel and Schweisguth, 2000; Nellesen et al., 1999). Perhaps *m8* is 'poised' for activation by ubiquitous bHLH activators that are maternally expressed and present throughout early embryos (e.g. Daughterless and Scute). Notch signaling might trigger expression upon binding of the Su(H)-$Notch^{IC}$ complex. By relying on ubiquitous bHLH 'co-factors', Notch signaling may be sufficient to activate *m8* in diverse cellular contexts. Accordingly, the differential regulation of *sim* and *m8* by Notch signaling is combinatorial and depends on the distribution of distinct co-factors.

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REFERENCES

Anderson, K. V., Jurgens, G. and Nusslein-Volhard, C. (1985). Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product. *Cell* **42**, 779-789.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Ashraf, S. I., Hu, X., Roote, J. and Ip, Y. T. (1999). The mesoderm determinant snail collaborates with related zinc-finger proteins to control Drosophila neurogenesis. *EMBO J.* **18**, 6426-6438.

Bailey, A. M. and Posakony, J. W. (1995). Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. *Genes Dev.* **9**, 2609-2622.

Bang, A. G. and Posakony, J. W. (1992). The Drosophila gene Hairless encodes a novel basic protein that controls alternative cell fates in adult sensory organ development. *Genes Dev.* **6**, 1752-1769.

Bray, S. (1998). Notch signalling in Drosophila: three ways to use a pathway. *Semin. Cell Dev. Biol.* **9**, 591-597.

Bray, S. and Furriols, M. (2001). Notch pathway: making sense of suppressor of hairless. *Curr. Biol.* **11**, R217-R221.

Cai, Y., Chia, W. and Yang, X. (2001). A family of snail-related zinc finger proteins regulates two distinct and parallel mechanisms that mediate Drosophila neuroblast asymmetric divisions. *EMBO J.* **20**, 1704-1714.

Cooper, M. T. and Bray, S. J. (2000). R7 photoreceptor specification requires Notch activity. *Curr. Biol.* **10**, 1507-1510.

Crews, S. T., Thomas, J. B. and Goodman, C. S. (1988). The Drosophila single-minded gene encodes a nuclear protein with sequence similarity to the per gene product. *Cell* **52**, 143-151.

Drier, E. A. and Steward, R. (1997). The dorsoventral signal transduction pathway and the Rel-like transcription factors in Drosophila. *Semin. Cancer Biol.* **8**, 83-92.

Fortini, M. E. and Artavanis-Tsakonas, S. (1994). The suppressor of hairless protein participates in notch receptor signaling. *Cell* **79**, 273-282.

Gonzalez-Crespo, S. and Levine, M. (1993). Interactions between dorsal and helix-loop-helix proteins initiate the differentiation of the embryonic mesoderm and neuroectoderm in Drosophila. *Genes Dev.* **7**, 1703-1713.

Hartenstein, A. Y., Rugendorff, A., Tepass, U. and Hartenstein, V. (1992). The function of the neurogenic genes during epithelial development in the Drosophila embryo. *Development* **116**, 1203-1220.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. and Simpson, P. (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. *Development* **122**, 161-171.

Hemavathy, K., Meng, X. and Ip, Y. T. (1997). Differential regulation of gastrulation and neuroectodermal gene expression by Snail in the Drosophila embryo. *Development* **124**, 3683-3691.

Huang, A. M., Rusch, J. and Levine, M. (1997). An anteroposterior Dorsal gradient in the Drosophila embryo. *Genes Dev.* **11**, 1963-1973.

Ip, Y. T., Park, R. E., Kosman, D., Yazdanbakhsh, K. and Levine, M. (1992). dorsal-twist interactions establish snail expression in the presumptive mesoderm of the Drosophila embryo. *Genes Dev.* **6**, 1518-1530.

Jiang, J., Kosman, D., Ip, Y. T. and Levine, M. (1991). The dorsal morphogen gradient regulates the mesoderm determinant twist in early Drosophila embryos. *Genes Dev.* **5**, 1881-1891.

Kadesch, T. (2000). Notch signaling: a dance of proteins changing partners. *Exp. Cell Res.* **260**, 1-8.

Kasai, Y., Nambu, J. R., Lieberman, P. M. and Crews, S. T. (1992). Dorsal-ventral patterning in Drosophila: DNA binding of snail protein to the single-minded gene. *Proc. Natl. Acad. Sci. USA* **89**, 3414-3418.

Kasai, Y., Stahl, S. and Crews, S. (1998). Specification of the Drosophila CNS midline cell lineage: direct control of single-minded transcription by dorsal/ventral patterning genes. *Gene Exp.* **7**, 171-189.

Kidd, S., Lieber, T. and Young, M. W. (1998). Ligand-induced cleavage and regulation of nuclear entry of Notch in Drosophila melanogaster embryos. *Genes Dev.* **12**, 3728-3740.

Klein, T. and Arias, A. M. (1999). The vestigial gene product provides a molecular context for the interpretation of signals during the development of the wing in Drosophila. *Development* **126**, 913-925.

Konrad, K. D., Goralski, T. J. and Mahowald, A. P. (1988). Developmental genetics of the gastrulation defective locus in Drosophila melanogaster. *Dev. Biol.* **127**, 133-142.

Kosman, D. and Small, S. (1997). Concentration-dependent patterning by an ectopic expression domain of the Drosophila gap gene knirps. *Development* **124**, 1343-1354.

Kosman, D., Ip, Y. T., Levine, M. and Arora, K. (1991). Establishment of the mesoderm-neuroectoderm boundary in the Drosophila embryo. *Science* **254**, 118-122.

Kramatschek, B. and Campos-Ortega, J. A. (1994). Neuroectodermal transcription of the Drosophila neurogenic genes E(spl) and HLH-m5 is regulated by proneural genes. *Development* **120**, 815-826.

Lecourtois, M. and Schweisguth, F. (1995). The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. *Genes Dev.* **9**, 2598-2608.

Martin-Bermudo, M. D., Carmena, A. and Jimenez, F. (1995). Neurogenic genes control gene expression at the transcriptional level in early neurogenesis and in mesectoderm specification. *Development* **121**, 219-224.

Menne, T. V. and Klambt, C. (1994). The formation of commissures in the Drosophila CNS depends on the midline cells and on the Notch gene. *Development* **120**, 123-133.

Morel, V., Lecourtois, M., Massiani, O., Maier, D., Preiss, A. and Schweisguth, F. (2001). Transcriptional repression by suppressor of hairless involves the binding of a hairless-dCtBP complex in Drosophila. *Curr. Biol.* **11**, 789-792.

Morel, V. and Schweisguth, F. (2000). Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the Drosophila embryo. *Genes Dev.* **14**, 377-388.

Mumm, J. S. and Kopan, R. (2000). Notch signaling: from the outside in. *Dev. Biol.* **228**, 151-165.

Nakao, K. and Campos-Ortega, J. A. (1996). Persistent expression of genes of the enhancer of split complex suppresses neural development in Drosophila. *Neuron* **16**, 275-286.

Nambu, J. R., Franks, R. G., Hu, S. and Crews, S. T. (1990). The single-minded gene of Drosophila is required for the expression of genes important for the development of CNS midline cells. *Cell* **63**, 63-75.

Nambu, J. R., Lewis, J. O., Wharton, K. A., Jr and Crews, S. T. (1991). The Drosophila single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* **67**, 1157-1167.

Nellesen, D. T., Lai, E. C. and Posakony, J. W. (1999). Discrete enhancer elements mediate selective responsiveness of enhancer of split complex genes to common transcriptional activators. *Dev. Biol.* **213**, 33-53.

Nibu, Y., Zhang, H., Bajor, E., Barolo, S., Small, S. and Levine, M. (1998). dCtBP mediates transcriptional repression by Knirps, Kruppel and Snail in the Drosophila embryo. *EMBO J.* **17**, 7009-7020.

Rebay, I., Fehon, R. G. and Artavanis-Tsakonas, S. (1993). Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. *Cell* **74**, 319-329.

Rusch, J. and Levine, M. (1996). Threshold responses to the dorsal regulatory gradient and the subdivision of primary tissue territories in the Drosophila embryo. *Curr. Opin. Genet. Dev.* **6**, 416-423.

Struhl, G. and Adachi, A. (1998). Nuclear access and action of notch in vivo. *Cell* **93**, 649-660.

Struhl, G., Fitzgerald, K. and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell* **74**, 331-345.

Wech, I., Bray, S., Delidakis, C. and Preiss, A. (1999). Distinct expression patterns of different enhancer of split bHLH genes during embryogenesis of Drosophila melanogaster. *Dev. Genes Evol.* **209**, 370-375.