strawberry notch encodes a conserved nuclear protein that functions downstream of *Notch* and regulates gene expression along the developing wing margin of *Drosophila*

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The dorsal/ventral (D/V) boundary functions as an organizer in the growth and patterning of the *Drosophila* wing disc and gives rise to the wing margin in the adult fly. Here we show that *strawberry notch* (*sno*) is a downstream component of the Notch signaling pathway and is important for the specification of this organizer. *sno* encodes a novel nuclear protein conserved in *C. elegans*, mouse, and humans. Mutations in wing margin genes interact dominantly with *sno* and loss of *sno* function results in loss of expression of *wingless*, *vestigial*, *cut*, and *E(spl)-m8* at the D/V boundary. In regulating these genes, *sno* functions in close cooperation with *Suppressor of Hairless* and *Hairless*. Finally, *sno* has no role in lateral inhibition suggesting that it may contribute to the specificity between lateral and inductive *Notch* signaling pathways.

[Key Words: *sno*; Notch pathway; wing margin; *Drosophila* development]

Received January 24, 1997; revised version accepted March 25, 1997.

The *Notch* (*N*) signaling pathway has been implicated in cell communication events necessary for proper cell fate decisions in many different developmental systems (Artavanis-Tsakonas et al. 1995). The genetic requirement of Notch signaling in developmental decisions was first described in the *Drosophila* embryo where the central nervous system arises as the selective choice of a bipotential group of cells to become neuroblasts (Lehmann et al. 1983). In mutants in which the Notch signal is compromised, the process of lateral inhibition, essential for making the proper developmental choice, does not occur, resulting in abnormalities in cell fate specification and an overcommitment to neural cell type at the expense of epidermal cells. Similar hypertrophy phenotypes are also seen in *Notch* mutants during mesoderm and endoderm development and during oogenesis, demonstrating that Notch function in lateral inhibition is not unique to neurogenesis (Corbin et al. 1991; Ruohola et al. 1991; Hartenstein et al. 1992; Xu et al. 1992). Recent studies have suggested an additional role of Notch in cell fate specification that involves an inductive rather than a lateral inhibitory mechanism. The best

studied example of this type of signaling is in the developing wing disc of *Drosophila*, where the proper expression of genes responsible for the development of the margin requires the activation of the Notch signal (Couso et al. 1995; Diaz-Benjumea and Cohen 1995; Kim et al. 1995; Doherty et al. 1996).

A number of genes have been identified as members of the Notch signaling pathway based on their mutant phenotype and their genetic and molecular interaction with the Notch receptor, and have been collectively referred to as the *Notch* group of genes (Artavanis-Tsakonas et al. 1995). These include *Serrate* (*Ser*) and *Delta* (*DI*), which encode ligands for Notch (Kopczynski et al. 1988; Fleming et al. 1990; Thomas et al. 1991), *Suppressor of Hairless* [*Su(H)*], *Hairless* (*H*), *mastermind* (*mam*), and *neuralized* (*neu*), which encode nuclear components of the Notch signaling pathway (Smoller et al. 1990; Boulianne et al. 1991; Bang and Posakony 1992; Schweisguth and Posakony 1992), and *deltex* (*dx*), which encodes a cytoplasmic protein (Busseau et al. 1994). The exact events following activation of Notch are not yet fully understood; however, studies in *Drosophila* cell lines suggest that *Su(H)* is sequestered in the cytoplasm by binding to the intracellular ankyrin repeats of Notch, and migrates into the nucleus only on the activation of Notch by its ligand Delta (Fortini and Artavanis-Tsakonas 1994). In

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this model, upon Notch signaling, Dx may function to displace Su(H), which then migrates to the nucleus (Die-derich et al. 1994; Matsuno et al. 1995). In the nucleus, Su(H) is known to positively regulate transcription of the *Enhancer of split* complex [*E(spl)-C*] (Jennings et al. 1994; Heitzler et al. 1996), which encodes a series of basic helix-loop-helix (bHLH) type of transcription factors that negatively regulate the pro-neural *achaete-scute* complex (AS-C) genes, therefore promoting epidermal cell fate (Jennings et al. 1994; Kramatschek and Campos-Ortega 1994; Oellers et al. 1994; Singson et al. 1994; Nakao and Campos-Ortega 1996). The AS-C and *E(spl)-C* genes appear to participate in a dynamic feedback regulatory loop that responds to input from Notch (Campos-Ortega 1993; Wilkinson et al. 1994; Chitnis et al. 1995; Heitzler et al. 1996). This feedback regulation constitutes a critical component of lateral signaling events mediated by Notch.

The Notch pathway also has a crucial role in the development of the *Drosophila* wing. Recent experimental results implicate both Serrate and Delta as ligands for Notch in the establishment of the Dorsal-ventral (D/V) boundary in the developing wing imaginal disc. The localized activation of Notch at the developing wing margin leads to the D/V boundary-specific expression of *wingless* (*wg*), *vestigial* (*vg*), *cut* (*ct*) and members of the *E(spl)* complex (Diaz-Benjumea and Cohen 1995; Kim et al. 1995; de Celis et al. 1996; Doherty et al. 1996). These genes have a crucial role in regulating growth and pattern formation along the boundary. For example, *vg* is a master regulator of genes responsible for the assumption of wing identity and loss of *vg* function results in complete loss of wing development (Williams et al. 1991; Kim et al. 1996). Similarly, *Wg* is required along the D/V boundary for the growth and patterning of the presumptive wing margin (Phillips and Whittle 1993; Diaz-Benjumea and Cohen 1995). Mutations in *ct* and *E(spl)* also result in a loss of wing margin formation (Jack and DeLotto 1992; de Celis et al. 1996), but the exact function of these two genes in this system is not completely clear. In genetic assays, *wg*, *vg*, and *ct* interact with members of the Notch pathway, further confirming that the patterning of the margin involves the regulation of these genes through the activation of Notch (Rabinow and Birchler 1990; Jack and DeLotto 1992; Hing et al. 1994; Couso and Martinez-Arias 1994).

Genetic and phenotypic analyses have shown that mutations in *strawberry notch* (*sno*) share common phenotypes with *Notch* in the developing leg, eye, ovary, and wing of the fly (Coyle-Thompson and Banerjee 1993). *sno* is essential for embryonic and pupal viability, but adult phenotypes can be examined using temperature sensitive alleles. Mutations in *sno* both enhance loss-of-function *Notch* phenotypes, and suppress gain-of-function *Notch* mutants, in a tissue-specific manner. Moreover, mutations in *Notch* group genes, such as *Ser*, *dx*, and *E(spl)*, dominantly enhance *sno* phenotypes. Together, these data imply that the level of *Notch* signaling is compromised in *sno* mutants and that *sno* is a positive regulatory component of the Notch signaling pathway.

Results

Positional cloning

Recombination mapping placed the *sno* locus between *furrowed* (*fw*) and *wavy* (*wy*) in the 11 D/E region of the X chromosome (Lefevre and Peterson 1972). Using deficiencies for the region (Fig. 1A), *sno* was mapped between the breakpoints of Df(1)wy^{54c} and Df(1)GP1. A genomic walk bridging the wy^{54c} and GP1 breakpoints was completed, starting with a pre-existing walk in the region (Wehrli et al. 1993). Recombination mapping with respect to polymorphic markers showed that at least part of the *sno* gene lay within a 60-kb interval in the middle of the walk (Fig. 1A). Additionally, genetic screens to obtain more alleles of *sno* yielded a new allele, *sno*⁹³ⁱ, which showed a deletion of ~2 kb mapping to the clone λ85. Fragments from this genomic clone were used to screen a 4- to 8-hr embryonic cDNA library (Brown and Kafatos 1988), and a 5.7-kb cDNA clone, 1a, which mapped to the genomic region deleted in *sno*⁹³ⁱ, was isolated. Transformation of *sno* mutant flies using an *hsp70-1a* construct or a cosmid including this genomic region rescued all *sno* phenotypes, including lethality.

The 5.7-kb *sno* cDNA contains 319 nucleotides of 5' and 780 nucleotides of 3' untranslated regions and a single long open reading frame (ORF) whose conceptual translation results in a 1547-amino-acid protein (Fig. 1B). The Sno amino-acid sequence shows no significant similarities to genes characterized previously. However, two putative nuclear localization signals and two acidic regions are found, suggesting a possible role for Sno in transcriptional activation (Ptashne and Gann 1993).

The Sno protein sequence shows a high degree of similarity to a translated product derived from a *Caenorhabditis elegans* cosmid sequence (Fig. 1C). The best overlap is in two blocks with residues 318–721 and 997–1205 of the *Drosophila* sequence. These two regions show 66% and 71% identity, respectively, to the *C. elegans* sequence. Mapping between the two blocks of similarity in the *C. elegans* sequence, is a highly acidic region, corresponding to the second acidic region in the *Drosophila* sequence. This further suggests that the acidic residues are important for Sno function.

A portion of the *Drosophila* Sno protein is also conserved in two independent incomplete human cDNAs derived from fetal cochlear epithelial and retinal cDNA libraries. The *Drosophila* and human proteins were found to be 75% identical and 88% similar over 300 amino acids (Fig. 1C). Also, an incomplete cDNA from a mouse embryonic library shows high levels of identity to a portion of *Drosophila* Sno (63% identity and 73% similarity, Fig. 1C). Although no other information is yet available on the genes that are similar to Sno in *C. elegans*, mouse, and humans, the similarities are striking and suggest a conservation of function for this gene across species.

Sno is a nuclear protein

Immunohistochemical staining with an antibody de-

rived against an amino-terminal peptide of Sno showed that it is expressed in the nuclei of many different tissues. This is seen most easily in Malpighian tubules that contain large nuclei (Fig. 2A). Salivary glands show no detectable expression of Sno (Fig. 2B), but when Sno is ectopically expressed using an *hsp70-sno* cDNA construct, its subcellular localization is found to be nuclear

Figure 2. Subcellular localization and expression pattern of the Sno protein. (A) Malpighian tubules stained with Sno antibody show intense nuclear staining. (B) Wild-type salivary glands show no expression of the Sno protein. (C) Upon application of heat shock to larvae carrying a single copy of the *hsp70-sno* cDNA transgene, strong ectopic expression of Sno is seen in salivary gland cell nuclei. (D) Syncytial blastoderm (stage 2). Sno expression is associated with nuclei (arrow). (E) Cellular blastoderm (stage 5). Sno is expressed in all the nuclei (arrow) of the blastoderm. (F) Gastrulation (stage 8). Sno is expressed in all of the nuclei with higher levels of expression seen in the ventral midline cells (arrow). (G) At stage 16, high levels of Sno expression are restricted to the central nervous system (arrow). (H) Sno expression is ubiquitous in third-instar leg discs, with higher levels of expression in the presumptive tarsal segments (arrow). (I) In the third-instar wing disc, Sno is seen in all cells. The arrow marks the D/V boundary. (J) In the third-instar eye disc, Sno expression is ubiquitous. (K) In the adult ovary, Sno expression is restricted to the follicle cells (arrows).

(Fig. 2C). Sno was not detected in mesodermal and endodermal tissues; therefore the expression of Sno is not as widespread as Notch, which is found in all three germ layers in the embryo (Fehon et al. 1991).

In the embryo, Sno is first detected in nuclei at the syncytial blastoderm stage (Fig. 2D). This expression is evident before the onset of zygotic transcription. Because no Sno protein is detected in oocytes, this early expression represents the translation of maternally derived *sno* mRNA. In the cellular blastoderm (stage 5), all nuclei of the embryo, except for the pole cells, appear to contain Sno (Fig. 2E). At gastrulation, Sno is seen transiently in the mesodermal precursors but shuts off as soon as these cells invaginate inside the embryo (data not shown). At stage 8, when the formation of the midline precursor cells depends on Notch signaling (Menne and Klamt 1994), we observe higher levels of Sno in the midline precursor cells than in the surrounding epidermal cells (Fig. 2F). Between stages 11 and 14, Sno is seen uniformly throughout the epidermis, and finally at around stage 16, high level of expression is restricted to the central nervous system (Fig. 2G).

Sno is expressed in all of the larval imaginal discs. In the leg disc, Sno expression is ubiquitous, although the central tarsal region shows higher levels of expression as compared with that seen in surrounding cells (Fig. 2H). All cells in wing (Fig. 2I) and eye (Figure 2J) discs also show high levels of Sno. During oogenesis, Sno protein is detected in follicle cell nuclei, but not in nurse cell or oocyte nuclei (Fig. 2K). The expression of Sno during oogenesis, embryonic and larval development in the wing, leg, eye, and ovary are all consistent with the *sno* phenotypes revealed by the temperature-sensitive alleles (Coyle-Thompson and Banerjee 1993).

sno function is needed for the expression of wing margin genes

Because *sno* seems to function as a member of the *Notch* group of genes, we asked whether *sno* is required for the expression of the wing margin genes, *wg*, *vg*, *ct*, and *E(spl)-m8* along the D/V boundary using reporter lines to assay their expression. Temperature sensitive *sno*^{71E3} mutants were pulsed at 30°C for 36 hr during the wandering third-instar-larval stage and the expression of the various genes along the D/V boundary was monitored. The *wg* enhancer trap line P2041, in which the expression of the β -galactosidase gene is under the control of the endogenous *wg* enhancer, was used to assay *wg* expression (Fig. 3A,C). When *sno*^{71E3} larvae were pulsed at the nonpermissive temperature, a dramatic reduction of *wg-lacZ* expression, compared with that seen in wild type, was observed along the presumptive margin (Fig. 3B,D). This observed loss of *wg* expression was specific to the wing margin, as expression of *wg* in other regions of the wing disc as well as in other imaginal discs was unaffected by the loss of *sno* function. The expression of *vg*, *ct*, and *E(spl)-m8* was monitored using flies bearing the corresponding D/V boundary enhancer fused to the *lacZ* gene (Jack et al. 1991; Williams et al. 1994; Leu-

Figure 3. Expression of wing margin genes at the D/V boundary is dependent on *sno*. (A) Wildtype expression of *wg* monitored using the enhancer trap line p2041. *Wg* is expressed along the wing margin (arrow) and in the notum. (B) *wg* expression in *sno*^{71E3} heat-pulsed larvae. The expression of *wg-lacZ* in the margin is reduced dramatically (arrow) but the expression in the notum is unaffected. (C) Wild-type expression of *wg-lacZ* at the margin shown at higher magnification. (D) *wg-lacZ* expression at the wing margin in heat-pulsed *sno*^{71E3} larvae (higher magnification) showing that cells at the margin are not missing, rather *Wg* expression in them is reduced severely. (E) Wild-type expression of a *vg*-D/V enhancer-*lacZ* line. Expression is observed along the D/V boundary (arrow) and also along the A/P boundary in the wing pouch. (F) Expression of *vg*-D/V enhancer-*lacZ* reporter in heat-pulsed *sno*^{71E3} background. The expression along the D/V boundary is reduced severely. (G) Wild-type expression of

a *lacZ* reporter fused to the wing margin enhancer of *ct*. Expression is observed along the D/V boundary (arrow). (H) Expression of *ct* reporter line in heat-pulsed *sno*^{71E3} background. Expression along the D/V boundary is completely eliminated. (I) Wild-type expression of *E(spl)m8-lacZ*. Expression is observed along the D/V boundary (arrow) and in proneural clusters. (J) *E(spl)m8-lacZ* expression in a heat-pulsed *sno*^{71E3} background. Expression along the D/V boundary is lost whereas expression in proneural clusters is unaffected. (K) Wing disc from *ptc-Gal4; UAS-Ser* larva. Ectopic outgrowth of the wing disc in the ventral compartment (arrows) is attributable to the ectopic expression of *Ser* along the A/P boundary. (L) Wing disc from *sno*^{71E1/Y; ptc-Gal4; UAS-Ser larva. Note the suppression of ectopic growth (arrow).}

courtois and Schweisguth 1995). These constructs all respond to the D/V signal in the wing disc, showing margin-specific β -galactosidase expression (Fig. 3E,G,I). When *sno*^{71E3} larvae containing these reporter constructs were pulsed at the nonpermissive temperature, a loss in reporter expression for *vg*, *ct*, and *E(spl)m8* along the D/V boundary was observed, whereas the expression of these reporters in other regions of the wing disc was unaffected (Fig. 3F,H,J). In some instances β -galactosidase expression is reduced dramatically, but not completely eliminated, perhaps attributable to the fact that when functioning independently of the rest of the gene these enhancers give unusually strong expression, and because the β -galactosidase product is very stable. Unlike the wing margin genes, the expression of *Sno* itself is ubiquitous throughout the wing disc (Fig. 2I) and is not dependent on Notch function (data not shown).

To establish that the effects of *Sno* are not upstream of Notch, we made use of the Gal4-UAS system to activate the Notch pathway ectopically along the anterior/posterior (A/P) boundary using a *ptc-Gal4/UAS-Ser* combination (Brand and Perrimon 1993; Speicher et al. 1994). This results in misexpression of *Ser* along the A/P boundary and causes an ectopic outgrowth of the wing pouch in the ventral compartment (Fig. 3K; Speicher et al. 1994). We reasoned that if *Sno* functions upstream of Notch, then inactivation of *sno* using a temperature-sensitive allele should have no effect on this ectopic out-

growth. In fact, when *sno*^{71E1}, a strong hypomorphic temperature-sensitive allele, was introduced into the *Ptc-Gal4/UAS-Ser* background and pulsed for 12 hr at 30°C, the ectopic outgrowth in the ventral compartment was suppressed entirely (Fig. 3L). In this experimental protocol, *Ser* expression is under the control of a heterologous promoter; therefore, the suppression of ectopic wing growth on loss of *Sno* indicates that *Sno* functions downstream of *Ser*. Because *Ser* has been shown to bind directly to Notch at the cell membrane, and *Sno* is a nuclear protein, this result also suggests that *Sno* functions downstream of Notch. This conclusion is consistent with our earlier genetic data that had suggested that *sno* functions downstream of *Notch* (Coyle-Thompson and Banerjee 1993).

Mutations in wing margin genes dominantly interact with *sno*

The function of *Sno* in regulating the expression of several fate determining genes along the wing margin suggested that mutations in these genes should interact prominently with *sno*. Flies bearing the temperature-sensitive allele *sno*^{71E3} show a modest loss of wing margin tissue when raised at 23°C (Fig. 4E). When such flies were also made heterozygous for a single copy loss of *wg*, extensive loss of wing margin tissue was observed suggesting a dominant synergistic interaction between *wg*

Figure 4. Genetic interactions between *sno* and mutations in wing margin genes. Adult wings from flies of various genotypes are shown. (A) Wild type. (B) *wg^{ts}/+*. These wings are identical to wild type. (C) *vg¹/+*. These wings are wild type. (D) *ct^{53d}/Y*. Note the slight notching of the wing margin. (E) *sno^{71E3}/Y* at 23°C. At this temperature a slight notching of the wing margin is seen. (F) *sno^{71E3}/Y; wg^{ts}/+* at 23°C. The wing margin phenotype of *sno^{71E3}* is dominantly enhanced by a single copy loss of *wg*. (G) *sno^{71E3}/Y; vg¹/+* at 23°C. Dominant enhancement of wing margin defects of *sno^{71E3}* is caused by a single copy loss of *vg*. (H) *sno^{71E3}ct^{53d}/Y* at 23°C. Note the synergistic interaction between *sno^{71E3}* and *ct^{53d}* wing margin phenotypes.

and *sno* (Fig. 4F). In similar experiments, *sno^{71E3}* flies with a single copy loss of *vg* also resulted in a dominant enhancement of wing margin defects and exhibited extensive loss of wing margin tissue (Fig. 4G). Finally, a genetic combination of *ct^{53d}*, a weak allele of *ct*, with *sno^{71E3}* shows extensive loss of wing margin tissue, suggesting a synergistic interaction between *sno* and *ct* (Fig. 4H). These results are reminiscent of the interaction of *Notch* with *wg*, *vg*, and *ct* (Rabinow and Birchler 1990; Jack and DeLotto 1992; Couso and Martinez-Arias 1994; Hing et al. 1994) and further establish that *sno*, like *Notch*, has a crucial role in the establishment of D/V boundary fate by participating in a common genetic pathway that regulates wing margin-specific genes. In addition to the wing margin defects, *sno* mutants also exhibit thickening of wing veins (Coyle-Thompson and Banerjee 1993; Fig. 4E). This is likely to be a secondary consequence of defective wing pouch development caused by improper D/V boundary specification. This same phenotype can also be seen in some of the other D/V boundary genes such as *vg* and *Ser* (Fleming et al. 1990; Williams et al. 1991).

sno genetically interacts with nuclear components of Notch signaling

Two nuclear proteins identified previously that function downstream of Notch have been shown to regulate margin-specific genes. These are *Su(H)* and *Hairless*. *Su(H)* is a sequence specific DNA binding factor that helps transduce the Notch signal from the membrane to the nucleus (Fortini and Artavanis-Tsakonas 1994), whereas *Hairless* is a nuclear factor that binds *Su(H)*, preventing

it from binding DNA (Brou et al. 1994). Because our results show that *Sno* is also a nuclear component downstream of Notch, we looked for genetic interactions of *sno* with *Su(H)* and *Hairless*. A single copy loss of *Hairless*, achieved using the *H^{E31}* allele, is able to suppress *sno* wing margin defects (Fig. 5C), suggesting that *Hairless* and *sno* have closely related antagonistic activities downstream of the Notch pathway. An equally strong but opposite effect was seen with *Su(H)*. At 23°C, the double mutant *sno^{71E3}/Y; Su(H)^{Ar9}/+* flies die at pupal stages. At 20°C, which is a fully permissive temperature for *sno^{71E3}*, the *sno^{71E3}/Y; Su(H)^{Ar9}/+* double mutant flies do eclose, but show severe defects in the wing margin (Fig. 5F). These results suggest that *sno* and *Su(H)* cooperate closely in the patterning of the wing. As *Sno* contains an apparent activation domain but not any obvious DNA-binding motif, and mammalian *Su(H)* is known to bind proteins that are important for transcriptional activation (Heish and Hayward 1995), it was reasonable to test if *Sno* and *Su(H)* interact directly. In co-immunoprecipitation assays, yeast two-hybrid systems, and in vitro GST-*Su(H)* fusion protein-binding assays, we were unable to detect any direct interaction between *Sno* and *Su(H)*. This may either mean that the two proteins work in parallel or that they interact too weakly for their binding to be detected in our assays.

sno is specific to inductive Notch signaling

Notch function has been best described in pathways that cause lateral inhibition (Artavanis-Tsakonas and Simpson 1991; Campos-Ortega 1993). Our earlier studies had shown that embryos with null mutations in *sno* do not

Figure 5. Genetic interactions of *sno* with *Su(H)* and *Hairless*. Adult wings from flies of various genotypes are shown. (A) *sno*^{71E1}/Y at 20°C. This allele is stronger than *sno*^{71E3} and shows wing margin defects at this temperature. (B) *H*^{E31}/+. These wings are wild type. (C) *sno*^{71E1}/Y; *H*^{E31}/+ at 20°C. A complete suppression of the wing margin defects of *sno*^{71E1} is caused by a single copy loss of *Hairless*. (D) *sno*^{71E3}/Y at 20°C. At this temperature, these wings are essentially wild type. (E) *Su(H)*^{Ar9}/+. These wings are wild type. (F) *sno*^{71E3}/Y; *Su(H)*^{Ar9}/+ at 20°C. Extensive loss of the anterior margin tissue in the wing can be seen.

show neural hypertrophy phenotypes (Coyle-Thompson and Banerjee 1993). Also, temperature shift experiments using *sno*^{71E3} showed no hypertrophy of the embryonic nervous system. The interpretation of these results is complicated by the fact that there is considerable maternal contribution of Sno in the embryo and that we were unable to generate germ-line clones with the *sno* null mutation *sno*^{EF531} (Coyle-Thompson and Banerjee 1993). We therefore investigated the possible role of Sno in lateral signaling in three additional and separate processes.

Each microchaete sensillum on the adult notum arises during pupal development from a sensory organ precursor (SOP) cell (Hartenstein and Posakony 1989). A single SOP is specified from within a group of equipotential cells called the proneural equivalence group through Notch mediated lateral signaling. In the absence of Notch signaling, created by maintaining *N^{ts}* flies at 37°C for 12 hr during 0–2 hr of pupal development, multiple SOPs develop from each field of competent cells, giving rise to a lawn of microchaetes on the pupal notum (Fig. 6B; Hartenstein and Posakony 1990). Under identical conditions, *sno* flies did not show any overproduction of microchaetes, suggesting that lateral signaling during SOP specification is not affected in this mutant (Fig. 6C). Although this is a negative result, the temperature used

to inactivate the *sno* alleles is clearly nonpermissive for all other *sno* phenotypes, and in fact, causes lethality at late pupal stages.

In the wing disc, specification of a single SOP within each pro-neural cluster was monitored using the *neu-lacZ* enhancer trap line A101, which marks the SOP that gives rise to each sensory bristle organ (Fig. 6D; Huang et al. 1991). The absence of Notch function results in two phenotypes in the wing disc. Along the wing margin, loss of *Notch* function leads to a loss of Wg expression and a consequent loss of A101 expressing cells in the anterior of the wing (Fig. 6E; Phillips and Whittle 1993; Rulifson and Blair 1995). Elsewhere in the wing, absence of *Notch* function results in a lack of lateral signaling and produces a neurogenic phenotype, appearing as an expansion of A101 expressing clusters of cells (Fig. 6E; Rulifson and Blair 1995). In *sno*^{71E3} flies, pulsed at the non-permissive temperature, loss of A101 expression was observed at the wing margin attributable to the requirement of *sno* for Wg expression. However, no neurogenic defect was observed in nonmargin proneural cluster development, suggesting again that lateral signaling is unaffected in *sno* mutants (Fig. 6F).

In the eye disc, neurogenesis was monitored by staining with monoclonal antibody 22C10, which highlights developing neurons (Zipursky et al. 1984). During normal third-instar eye imaginal disc development, lateral signaling ensures regular spacing between differentiating photoreceptor clusters behind the morphogenetic furrow (Fig. 6G). Absence of *Notch* results in a lack of lateral signaling leading to neural hypertrophy behind the morphogenetic furrow (Fig. 6H; Cagan and Ready 1989). When *sno*^{71E3} larvae were heat-pulsed during ommatidial development and stained with mAb 22C10, no neural hypertrophy phenotype was observed, further supporting the view that *sno* does not function in lateral signaling (Fig. 6I). The strongest hypomorphic allele of *sno* (*sno*^{E1}) when placed over a deletion and raised at 30°C gives a phenotype that is equivalent to null. Using these conditions during wing, eye, and notum development, we obtained the same results as are shown in Figure 6 (data not shown).

Although the mutant conditions used here are sufficient to inactivate *sno* function in many tissues, one could argue that the threshold levels for lateral signals are lower than that for inductive signals. To rule out this possibility, we used a null allele of *sno*, *sno*^{EF531}, to generate somatic clones in the eye. The *sno*^{EF531}/*sno*^{EF531} clone does show one of the phenotypes ascribed previously to *sno*, that is, the recruitment of a mystery cell as an outer neuron (Fig. 6J; Coyle-Thompson and Banerjee 1993). This demonstrates that the clone is homozygous for the mutation. In contrast, the clone does not show hypertrophy of neural development as is seen with loss of *Notch* function (Cagan and Ready 1989). Taken together, the above results, obtained from three separate tissues, indicate that *sno* does not function in lateral signaling within pro-neural equivalence groups and demonstrate that Sno participates only in inductive Notch signaling events.

Figure 6. *sno* does not participate in lateral signaling of Notch. (A) Notum from a wild-type adult fly showing the regular arrangement of bristles. (B) Notum from heat pulsed *N^{ts}* fly during pupation showing a hypertrophy phenotype of bristle development. (C) Notum from *sno^{71E3}/Y* fly heat-pulsed during pupation. No hypertrophic bristle phenotype is seen. (D) *neu-lacZ* enhancer trap line A101 expression in wild-type wing imaginal disc. Expression is observed in the SOP of each of the proneural clusters. (E) A101 expression in wing disc from *N^{ts}* males pulsed for 36 hr. Loss of A101 expression was observed in anterior bristle precursors along the margin attributable to the dependence of Wg on the Notch signal. Additionally, there is an increase in the number of SOPs within the proneural clusters (arrow) attributable to loss of lateral signaling mediated by Notch. (F) A101 expression in heat pulsed *sno^{71E3}* wing disc. A loss of expression of A101 is seen along the anterior bristle precursors at wing margin. However, proneural clusters outside the wing margin are unaffected. (G) Eye disc from wild-type larvae stained with monoclonal antibody 22C10. Note the regular organization of photoreceptor clusters behind the morphogenetic furrow (arrow). (H) Eye disc from heat-pulsed *N^{ts}* larva stained with the 22C10 antibody. Neural hypertrophy causing enlarged size of clusters is seen behind the furrow (arrow). (I) Eye disc from heat pulsed *sno^{71E3}/Y* larva stained with 22C10 antibody. No neural hypertrophy is evident behind the furrow. (J) Section through a *sno^{EF531}* clone in an adult eye. Note the mystery cell conversion phenotype in some mutant ommatidia (arrows). However, no neural hypertrophy is evident despite complete loss of *sno* function.

Discussion

The establishment of the D/V boundary in the wing imaginal disc results from a series of hierarchical steps (Williams et al. 1993). An early event in this process is the activation of *apterous*, a LIM domain containing transcription factor in the dorsal compartment of the wing disc (Cohen et al. 1992). *Apterous*, in turn, regulates the expression of *Fringe*, a boundary recognition molecule in the dorsal compartment (Irvine and Weischaus 1994). Apposition of *Fringe* expressing (dorsal) and *Fringe* nonexpressing (ventral) cells causes a local up-regulation of *Ser* and the activation of Notch at the ventral edge of the boundary (Kim et al. 1995). Similarly, *Delta* is required for the activation of Notch on the dorsal aspect of the boundary (Doherty et al. 1996). Therefore, activation of Notch occurs as a result of the asymmetric activity of its ligands on either side of the boundary, giving rise to the symmetrical expression of critical wing margin genes along the presumptive wing margin. These margin genes include *wg*, *vg*, *ct*, and *E(Spl)-m8* (Couso et al. 1994, 1995; Diaz-Benjumea and Cohen 1995; Doherty et al. 1996). The expression of these genes

along the D/V boundary is essential for the cells along the margin to function as a classical organizer (Spemann 1938) for the normal outgrowth of the wing imaginal disc. In this report we have shown that *sno* is a critical component of the Notch pathway that participates in the proper specification of this organizer.

Previous genetic analysis of *sno* has suggested that this gene is likely to encode a positive component of the Notch signaling pathway (Coyle-Thompson and Banerjee 1993). This conclusion was primarily based on the fact that mutations in genes belonging to the Notch group interact strongly with *sno*. The genetic and molecular analysis presented here confirms our earlier prediction and suggests that *Sno* functions downstream of Notch in controlling the expression of genes that respond to the localized signaling processes initiated by Notch. In the wing disc, the primary role of *sno* is to control the expression of the margin-specific genes *wg*, *vg*, *ct*, and *E(spl)-m8* along the D/V boundary. This conclusion is consistent with the genetic analysis showing that mutations in these wing margin genes enhance *sno* mutant phenotypes. Based on its nuclear localization, *Sno* would be expected to function in concert with *Su(H)*,

and as expected, *Su(H)* mutants dominantly enhance *sno* wing margin phenotypes. Additionally, *Su(H)* mutants are similar to *sno* in having a small wing pouch and in lacking Vg expression along the D/V boundary (Couso et al. 1995). It is of particular significance that Kim et al. (1996) have shown that the isolated 750-bp D/V boundary enhancer of *vg* contains a single *Su(H)*-binding site, which when mutated, eliminates Vg expression along the D/V boundary. In this report we have shown that transcription initiated by that same enhancer element is also dependent on functional Sno protein. Whereas Sno does not contain any known DNA binding motifs, it does contain acidic stretches of amino acids resembling transcriptional activation domains. Therefore, it is possible that Sno binds to a second DNA-binding protein to activate transcription from this enhancer. We have been unable to demonstrate that this second protein is *Su(H)*, but our results have not completely ruled out this possibility. The expression of the m8 transcript of *E(spl)* also depends on functional *Su(H)*-binding sites within its enhancer (Bailey and Posakony 1995; Leucourtis and Schweisguth 1995). Again, our results show that the same enhancer also requires functional Sno protein for its activation along the D/V boundary. In all respects, *Su(H)* and *sno* give rise to similar phenotypes during wing development and the observed synergy between *sno* and *Su(H)* is likely to translate into a close cooperation between them at the molecular level.

Inductive vs. lateral signaling of Notch

The developmental consequences of Notch function are dependent on the context in which the signaling takes place. For example, in the nervous system, Notch functions in lateral signaling between members of the proneural equivalence group to restrict the expression of neuronal fate to a single cell within the proneural cluster. In contrast, Notch signaling at the wing margin is inductive, in that activation of the signal in a particular cell is important for the assignment of the D/V boundary fate to that cell. Given that the ligand and the receptor molecules are the same for inductive and lateral signaling processes, it seems likely that components downstream of Notch are responsible for insuring the specificity of the signaling process.

The results of this study suggest the possibility that Sno is a downstream component that is specific to Notch inductive signaling events in *Drosophila*. Most mutants in the Notch signaling pathway, including *Notch*, *Dl*, *Su(H)*, and *E(spl)* are associated with a neurogenic or hypertrophy phenotype during nervous system development. *sno* mutants do not share this phenotype with these genes, and *sno* therefore seems to be a member of an emerging group of nonneurogenic Notch pathway genes. During eye development in the third-instar larva, and sensillum development in the pupa, *sno* mutants do not show the neural hypertrophy phenotypes that are seen with *Notch*. During wing development, lateral signaling and SOP specification seem to be perfectly operational in *sno* mutants, whereas the inductive events in

the neighboring margin are clearly affected. These results support the idea that *sno* is required in some, but not all, Notch signaling events. The selective activation of Sno in signal receiving cells may confer the specificity for inductive versus lateral signaling. Furthermore, these results imply that lateral and inductive Notch signaling may invoke separate sets of downstream genes and therefore have different genetic requirements. Like Sno, Numb, a negative regulator of Notch signaling, appears to function in a subset of Notch-mediated events. Numb function is needed for the Notch mediated specification of the MP2 lineage (Spana and Doe 1996). However, loss or overexpression of Numb during development does not alter Notch-mediated lateral signaling events (Rhyu et al. 1994; Jan and Jan 1995; Spana et al. 1995). Some genes, like *Su(H)*, may belong to a common pool of genes required for both types of signaling whereas other genes, like *sno*, may be specific for a single type of signaling event. A counterexample for Sno in this respect could be Neu, which may function specifically in lateral signaling as Sno does in inductive pathways, because Neu is not expressed along the D/V boundary and does not interact genetically with *sno* at the wing margin (Coyle-Thompson and Banerjee 1993). Therefore, the *Notch* pathway may function in different developmental contexts by co-opting different downstream components.

In *C. elegans*, two separate Notch receptor homologs, Lin-12 and Glp-1, function in separate developmental pathways (Austin and Kimble 1987). Glp-1 is involved in inductive signaling in the gonad and in the early embryo, whereas Lin-12 function is largely restricted to lateral signaling during many different stages of development (Greenwald et al. 1983). However, even in the worm, the specificity of the response of the two sets of receptors does not seem to depend on the receptors themselves, because they can be made to function interchangeably in inductive versus lateral signaling pathways (Fitzgerald et al. 1993). Therefore, in *C. elegans*, as in *Drosophila*, it is possible that a differential response to common signal transduction pathways might be brought about by downstream molecules similar to Sno.

Evolutionary conservation of Sno

The similarity of Sno to translated sequences from human, mouse, and *C. elegans* suggests that Sno is an evolutionarily conserved protein. This is particularly important because most of the other components of the Notch pathway have also been found to be structurally and functionally conserved from worms to mammals (Austin and Kimble 1989; Tax et al. 1994; Lindsell et al. 1995; Christensen et al. 1996). In vertebrates, the Notch signaling pathway has a crucial role in the patterning of the nervous system. For example, functional levels of Delta control neurogenesis in *Xenopus* (Chitnis et al. 1995), and inductive signaling via Notch is important in the developing spinal cord and hindbrain in chicken (Myat et al. 1996). In humans, truncations of Notch1 results in T-cell leukemia (Ellisen et al. 1991) and truncation of Notch3 causes an adult onset neurological abnormality

(Joutel et al. 1996). Finally, vertebrate limb development shows striking similarities with the development of the wing in flies. The apical ectodermal ridge (AER) is an organizer with similarities to the D/V boundary of the wing disc (Riddle et al. 1995). The dorsal ectoderm expresses Wnt7a (Parr and McMahon 1995), a homolog of *wg*, and the Notch ligands *Ser* and *Delta* are expressed in flanking cells much the same way as they do along the D/V boundary in flies (Myat et al. 1996; Shawber et al. 1996). Therefore, it seems clear that the Notch pathway has an important role in many different developmental decisions across species. One unifying characteristic of this pathway is the context dependence of its response. It seems possible that this context dependence results from downstream nuclear components such as Sno. It would therefore be interesting to investigate the relationship between Sno homologs identified in this study with Notch during vertebrate development.

Materials and methods

Molecular cloning

As an entry point for the genomic walk, we used a 47-kb λ clone walk from the region (kind gift of Marcel Wehrli; Wehrli et al. 1993). A new chromosomal walk was initiated from these clones using Tamkun λ and cosmid DNA libraries. For the isolation of the *sno* cDNA, ~250,000 clones were screened from a 4- to 8-hr embryonic cDNA library (Brown and Kafatos 1988). For transformation rescue, the *sno* cDNA was excised with *Hind*III and *Eco*RI, blunt ended with Klenow, and subcloned into the *Stu*I site of pCasper-*hsp70*. For further manipulation, the *sno* cDNA could then be released with *Eco*RI.

Transformation rescue

For transformation rescue, CsCl pure 500 ng/ μ l pCasper-Hsp70-*sno* 1a was mixed with 300 ng/ μ l pUChs π Δ2-3 helper plasmid and injected into *w*¹¹¹⁸ embryos using standard protocol (Rubin and Spradling 1982). Parallel transformations were done with cosmid 1-15, which includes the entire *sno* gene. For *hsp70-sno*, 1500–1700 embryos were injected and 12 *w*⁺ transgenic lines were recovered. For c1-15, 2200 embryos were injected and 12 transformants were recovered. Transformant males were crossed to *sno*/FM7c females and the sons were assayed for rescue. All c1-15 transformant lines rescued *sno* lethal and temperature-sensitive phenotypes. Two *hsp70-sno* transformant lines rescued lethality and temperature-sensitive phenotypes without heat shock. The remainder of the lines rescued with heat shock at 30°C. *hsp70-sno* flies have no phenotype on their own.

Sequencing and database search

Sequencing was done using the protocols and reagents of the USB Sequenase kit. The *sno* cDNA was sequenced on both strands and the sequence was compiled and edited using the Sequencher v2.1 program (Gene Codes Corporation). The first methionine in the reading frame is the start methionine, because antibodies raised against 15 amino acids immediately following this methionine could detect the endogenous Sno protein. In addition, the adjacent sequence matches the *Drosophila* consensus for translation starts (Cavener 1987). Database

searches were performed with the BLAST Network Service (Altschul et al. 1990).

α -Sno antibody

The 15-mer oligopeptide TSKKRKTLDDADDN corresponding to the Sno amino terminus was synthesized by the UCLA Peptide Synthesis Facility. The oligopeptide was sent to the Berkeley Antibody Company, where it was injected into rabbits to make antibodies. Bleeds were tested on Western blots. Sno antibody was affinity purified using the 15-mer oligopeptide on Bio-Rad Affi-Gel 10 and frozen at –70°C in PBS. On Western blots, affinity purified Sno antibody was used at a 1:500 dilution that detected a single band with a molecular weight of 140 kD, the predicted size of the Sno protein.

Immunocytochemistry

Imaginal discs were dissected in phosphate buffer and fixed in PLP with 4% paraformaldehyde for 20 min at room temperature. Incubation with Sno antibody was carried out overnight at 4°C at 1:250 dilution. Secondary antibody (Goat anti-Rabbit) was used at 1:1000 and the staining was visualized using DAB with nickel (Coyle-Thompson and Banerjee 1993).

Embryos were fixed in PEMS/heptane/4% formaldehyde for 10 min. Vitelline membrane was removed using methanol and embryos that were stained with anti-Sno antibody at 1:200 dilution (Ashburner 1989).

Detection of β -galactosidase activity

To detect β -galactosidase activity in imaginal discs, mature third-instar larvae were dissected in PBS, fixed in 4% glutaraldehyde for 20 min, washed in PBS for 10 min, and stained as described (Ashburner 1989). Discs were mounted in glycerol for microscopic analysis.

Phenotypic studies

Double mutants of *sno*^{71E3} with *vg*¹ (from Bloomington Stock center), *wg*^{1L114} (from A. Martinez-Arias, Cambridge University, UK), *ct*^{53d} (from J. Jack, University of Connecticut, Farmington), *Su(H)*^{Ar9}, and *H*^{E31} (from J. Posakony, University of California, San Diego) were raised at 23°C.

For the temperature-shift experiments, late second- and early third-instar larvae were collected and heat-pulsed at 30°C for 12, 24, or 36 hr. Wing discs were then dissected and stained with X-gal to reveal β -galactosidase reporter gene expression. Effects were observed with shorter shifts, but the strongest effects reported here were seen with the 36-hr shift. P-2041 *wg-lacZ* was obtained from the Bloomington Stock Center. The *vg-lacZ* transformant (gift of S. Carroll, University of Wisconsin, Madison) contains a 750-bp D/V boundary enhancer of the *vg* gene fused to *lacZ* (Williams et al. 1994). The *ct*^{HZ-2} reporter construct (gift from J. Jack) includes the wing margin enhancer of the *ct* gene fused to *lacZ* (Jack et al. 1991); *E(spl)-m8-lacZ* (from J. Campos-Ortega) contains a 2.61-Kb upstream sequence of *E(spl)-m8* fused to the *lacZ* gene (Kramatschek and Campos-Ortega 1994).

For temperature-shift experiments in the third-instar eye imaginal disc, early third-instar larvae were collected over 1 hr and incubated in a 32°C waterbath for 12 hr as in Cagan and Ready (1989). Eye discs were then dissected, fixed, and stained with mAb 22C10 to visualize neuronal differentiation (Zipursky et al. 1984).

For temperature-shift experiments during bristle develop-

ment, 0- to 2-hr white pre-pupae were collected, incubated in a 30°C waterbath for 12 hr, and then returned to 18°C for the remainder of pupal development (Hartenstein and Posakony 1990). After 4 to 5 days, pupae were dissected from their pupal cases and examined for the microchaete overproduction phenotype.

To test for *sno*^{71E1} suppression of *ptc-Gal4/UAS-Ser*-mediated ectopic outgrowth in the wing disc, *sno*^{71E1}/*FM7C* females were crossed to *UAS-Ser* males and larvae were pulsed for 12 hr at 30°C during the late second and early third-instar stage. Wing discs were dissected and analyzed directly for the suppression of ectopic growth of the wing pouch in the ventral compartment.

To generate somatic mosaic clones in the adult eye, *y w sn sno*^{71E3} *f car*, *y w sno*⁹³ⁱ, and *y w sno*^{EF531} chromosomes were used. *w*¹¹¹⁸ *P[w⁺]* 20D (from Bloomington Stock Center) males were crossed to *sno*/*FM7c* females. Mitotic recombination was induced in 12- to 36-hr embryos with X-rays at 1000 rads. Adults were screened for *w/w* eye patches. Mosaic fly eyes were embedded in plastic and sectioned as in Rogge et al. (1991). *w*¹¹¹⁸ controls gave a patch frequency of 4%, *y w sn sno*^{71E3} *f car* gave a patch frequency of 1%, *y w sno*⁹³ⁱ gave a patch frequency of 0.6%, and *y w sno*^{EF531} gave a patch frequency of only 0.1%. This low frequency for the *sno*^{EF531} chromosome was probably the cause for our earlier inability to generate germ-line clones (Coyle-Thompson and Banerjee 1993).

Acknowledgments

We acknowledge the participation of Cathy Coyle Thompson in early aspects of RFLP mapping and deletion analysis of the *sno* gene. We are grateful to S. Artavanis-Tsakonas for help and advice throughout this work and we also thank E. Verheyen for two-hybrid experiments with *sno* constructs. We thank M. Wehrli for providing the genomic walk, N. Brown and G.M. Rubin for the embryo and eye disc cDNA libraries, respectively, and J. Tamkun for *Drosophila* genomic libraries. We thank H. Bellen, S. Benzer, S. Carroll, J. Campos-Ortega, K. Irvine, J. Jack, E. Knust, A. Martinez-Arias, K. Matthews, K. Moses, J. Posakony, V. Rodrigues, and F. Schweisguth for fly stocks and antibodies. We thank S. Rundlett and R. Mann for help with sequence alignment, Lian Liu for help in sequencing, M. Ganiron for mutant screens, Amanda Pickup and Gail Shirley for helpful advice and J. Lengyel, V. Hartenstein, and members of our laboratory for comments on the manuscript. This work was supported by a National Science Foundation grant (no. IBN-9600391) to U.B.

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Note added in proof

The sequence data described in this paper have been submitted to GenBank under accession number U95760.

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Genes Dev. 1997, **11**:

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