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Notchless encodes a novel WD40-repeat-containing protein that modulates Notch signaling activity

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Signaling by Notch family receptors is involved in many cell-fate decisions during development. Several modifiers of Notch activity have been identified, suggesting that regulation of Notch signaling is complex. In a genetic screen for modifiers of Notch activity, we identified a gene encoding a novel WD40-repeat protein. The gene is called *Notchless*, because loss-of-function mutant alleles dominantly suppress the wing notching caused by certain *Notch* alleles. Reducing Notchless activity increases Notch activity. Overexpression of Notchless in *Xenopus* or *Drosophila* appears to have a dominant-negative effect in that it also increases Notch activity. Biochemical studies show that Notchless binds to the cytoplasmic domain of Notch, suggesting that it serves as a direct regulator of Notch signaling activity.

Keywords: signal transduction/*Drosophila*/*Xenopus*

Introduction

Signaling mediated by Notch-family receptors is involved in controlling the choice between alternative cell fates (reviewed in Artavanis-Tsakonas *et al.*, 1995; Gridley, 1997; Kimble and Simpson, 1997; Robey, 1997). In primary neurogenesis, Notch signaling directs cells to adopt an epidermal fate as opposed to the default state of neural differentiation (Heitzler and Simpson, 1991; Chitnis *et al.*, 1995; Henrique *et al.*, 1995). Later, in the peripheral nervous system, Notch signaling distinguishes between neural and accessory cell fate (Guo *et al.*, 1996). In some cases Notch is thought to have a permissive function, rendering cells insensitive to other signals which trigger differentiation (Fortini *et al.*, 1993). Notch signaling can also serve an instructive role, for example controlling mitotic division in the *Caenorhabditis elegans* germ line (reviewed in Kimble and Simpson, 1997), or establishing the dorsal–ventral boundary of the *Drosophila* wing imaginal disc by directing localized expression of *wingless*, *vestigial* and *cut* (Couso *et al.*, 1995; Diaz-Benjumea and Cohen, 1995; Kim *et al.*, 1995; Rulifson and Blair, 1995; Neumann and Cohen, 1996; de Celis and Bray, 1997; Micchelli *et al.*, 1997).

Notch encodes a large transmembrane protein which serves as a signal-transducing receptor for the EGF-repeat containing ligands of the Delta-Serrate-LAG2 family. Truncation of the extracellular domain of *Drosophila*, *Xenopus* or mouse Notch proteins generates ligand-independent, activated receptors that have constitutive signaling activity (Coffman *et al.*, 1993; Lieber *et al.*, 1993; Rebay *et al.*, 1993; Struhl *et al.*, 1993; Kopan *et al.*, 1994). When expressed without a transmembrane domain the intracellular portion of Notch concentrates in the nucleus (Lieber *et al.*, 1993; Struhl *et al.*, 1993). Expression of an extracellularly-truncated form of mouse Notch in cultured cells leads to spontaneous intracellular cleavage which allows the intracellular domain to localize to the nucleus, where it can activate transcription of Notch target genes together with CBF1 (Jarriault *et al.*, 1995; Kopan *et al.*, 1996; Schroeter *et al.*, 1998). CBF1 is the vertebrate homologue of Suppressor of Hairless, Su(H), a DNA binding protein required for Notch signal transduction (Fortini and Artavanis-Tsakonas, 1994; Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Together, this family of Notch binding proteins is called CSL for CBF1, Su(H) and LAG1.

Although *Drosophila* Notch cannot be detected in the nucleus under normal conditions *in vivo*, recent studies using Notch–GAL4 fusion proteins present strong evidence that cleavage of Notch liberates a fragment of the protein that can translocate to the nucleus and act there to regulate transcription of GAL4-dependent target genes (Struhl and Adachi, 1998). Another recent study has shown that mouse Notch cleavage can be stimulated by ligand binding in cell culture, leading to release of an intracellular fragment that binds to the CSL protein (Schroeter *et al.*, 1998). Using mutants that reduce ligand dependent proteolytic processing of Notch, Schroeter *et al.* (1998) have shown that the efficiency of processing correlates with the ability to stimulate Notch target gene expression. CSL binding may serve to target Notch to specific DNA sequences in the control regions of Notch-regulated target genes, such as the vertebrate *HES1* gene or the *vestigial* boundary enhancer (Jarriault *et al.*, 1995; Kim *et al.*, 1996; Schroeter *et al.*, 1998).

Several proteins have been identified as modifiers of the activity of Notch-family receptors. Deltex binds to the CDC10 repeats and positively regulates Notch (Diederich *et al.*, 1994; Matsuno *et al.*, 1995). Numb, Dishevelled and SEL-10 binding reduce Notch activity (Axelrod *et al.*, 1996; Frise *et al.*, 1996; Guo *et al.*, 1996; Hubbard *et al.*, 1997). Numb binds to the juxtamembrane and C-terminal regions of the Notch intracellular domain and inhibits Notch during specification of cell fates in the PNS (Guo *et al.*, 1996). Dishevelled binds to the C-terminal portion of the cytoplasmic domain of Notch and reduces Notch activity in mediating the choice between neural and epidermal cell fates (Axelrod *et al.*, 1996). *sel-10* was identified as a negative regulator of *lin-12* activity in *C.elegans*. *sel-10* encodes a protein with F-box and WD40

repeats that binds to the intracellular domain of Notch. Based on similarity to yeast CDC4, SEL-10 may be a component of a ubiquitin E3-ligase that targets Notch for degradation.

In this report we present genetic and molecular characterization of a new regulator of Notch signaling activity. The gene was identified in a screen for dominant modifiers of a *Notch* mutant phenotype in the *Drosophila* wing. The mutant dominantly suppresses the wing notching phenotype of *notchoid* mutations and so we call it *Notchless*. *Notchless* encodes a novel protein containing WD40-repeats that binds to the cytoplasmic domain of Notch. Notchless modifies Notch signaling activity in a variety of Notch-dependent signaling processes in *Drosophila* and *Xenopus* embryos.

Results

Genetic characterization of a novel modifier of Notch activity

notchoid^{1} (*nd*^{1}) is a viable mutant allele of *Notch* that causes scalloping of the wing (Figure 1C). The severity of the *nd*^{1} phenotype is sensitive to the level of activity at other loci encoding components of both the Notch and Wingless signaling pathways (Couso and Martinez Arias, 1994; Fortini and Artavanis-Tsakonas, 1994; Hing *et al.*, 1994). Thus *nd*^{1} provides a sensitized genetic background in which to screen for modifiers of Notch signaling activity. The BDGP collection of P-element induced lethal mutations (Spradling *et al.*, 1995) was screened for dominant modifiers of the *nd*^{1} phenotype. Several P-element induced mutants were found to enhance the severity of *nd*^{1} (not shown). One P-element induced mutant, l(2)k13714, was found that suppresses the scalloping of *nd*^{1} wings (Figure 1C and D). On the basis of its ability to dominantly suppress scalloping of the wing, we call the gene identified by the l(2)k13714 P-element *Notchless* (*Nle*).

To verify that the gene mutated by the P-element is responsible for the mutant phenotype we generated strains from which the original P-element was removed by transposase-mediated excision. These chromosomes differ from the original l(2)k13714 chromosome only by the lack of the P-element and fail to suppress the *nd*^{1} phenotype (data not shown). Although l(2)k13714 comes from a collection of P-elements that are supposed to be lethal mutations, we noted that homozygous mutant individuals are recovered in this stock. They are morphologically normal, though males are sterile.

The scalloping of *nd*^{1} mutant wings is thought to be caused by reduced Wingless activity because over-expression of Wingless can suppress the phenotype (Couso and Martinez Arias, 1994) and because further reducing *wingless* activity enhances the *nd*^{1} phenotype (Hing *et al.*, 1994). Removing one copy of the *Su(H)* gene enhances the severity of the *nd*^{1} phenotype and causes an obvious reduction of Wingless expression at the DV boundary [relative to the level in wild-type; compare Figure 1B with E; *nd*^{1} *Su(H)*/+]. Wingless is restored to wild-type levels and the loss of wing tissue is completely suppressed when the *Notchless* mutant is introduced in this background [Figure 1F; *nd*^{1} *Su(H)*/*Nle*]. *Notchless* also suppresses the phenotypes of *nd*^{fa} (Figure 1G and H) and *nd*^{2} (data not shown), indicating that the genetic interaction is not specific to one particular allele of *Notch*.

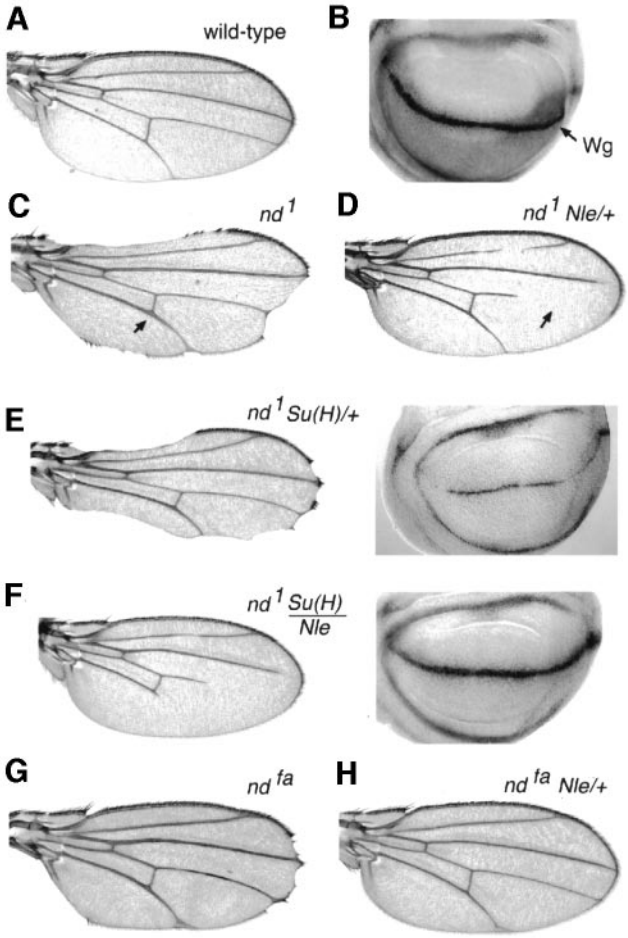


Fig. 1. Genetic interactions between *Notchless* and *notchoid*. (**A**) Cuticle preparation of a wild-type wing. (**B**) Wingless protein expression in a wild-type wing imaginal disc visualized by antibody staining. Only the wing pouch is shown. The arrow indicates the stripe of Wingless at the dorsal–ventral boundary. (**C**) Cuticle preparation of a *nd*^{1} wing (genotype *nd*^{1}/Y; note that *Notch* is on the X-chromosome so males carry only one copy of the gene). Note the notches of the wing (loss of tissue) and mild thickening of the wing veins (e.g. arrow). (**D**) *nd*^{1}*/Y; Nle*^{k13714}/+ wing. The notching of the wing is completely suppressed. Thickening of the veins is suppressed. Note that veins are interrupted or shortened in this genotype (e.g. arrow), suggesting overactivation of the Notch pathway. The same result was obtained using *nd*^{1} and the *Nle*$^{\Delta 8}$ allele and also using *nd*^{2}. (**E**) *nd*^{1}*/Y; Su(H)*^{AR9}/+. Removing one copy of the *Su(H)* gene enhances the severity of the notching of the wing. Wingless expression in a disc of the same genotype is shown at right. Wg is reduced and irregular at the dorsal–ventral boundary. (**F**) *nd*^{1}*/Y; Su(H)*^{AR9}*/Nle*^{k13714} wing. Removing one copy of *Nle* suppresses the notching of the *nd*^{1}*/Y; Su(H)*/+ wing and enhances the loss of veins. Wingless expression is restored to normal. (**G**) *nd*^{fa}*/Y* wing. (**H**) *nd*^{fa} */Y; Nle*^{k13714}/+ wing. The notching of the wing margin is completely suppressed. Veins are normal in this genotype. The same result was obtained using the *Nle*$^{\Delta 8}$ allele.

The scalloping phenotype of *nd* alleles is thought to be due to reduced Notch function. Notch signaling through Su(H) is required to induce Wingless at the wing margin (Couso *et al.*, 1995; Diaz-Benjumea and Cohen, 1995; Rulifson and Blair, 1995; Neumann and Cohen, 1996). Reducing *Su(H)* gene dosage enhances the *nd*^{1} phenotype. Introducing one copy of the *Notchless* mutant restores Wingless expression in the *nd*^{1} *Su(H)*/+ background. This

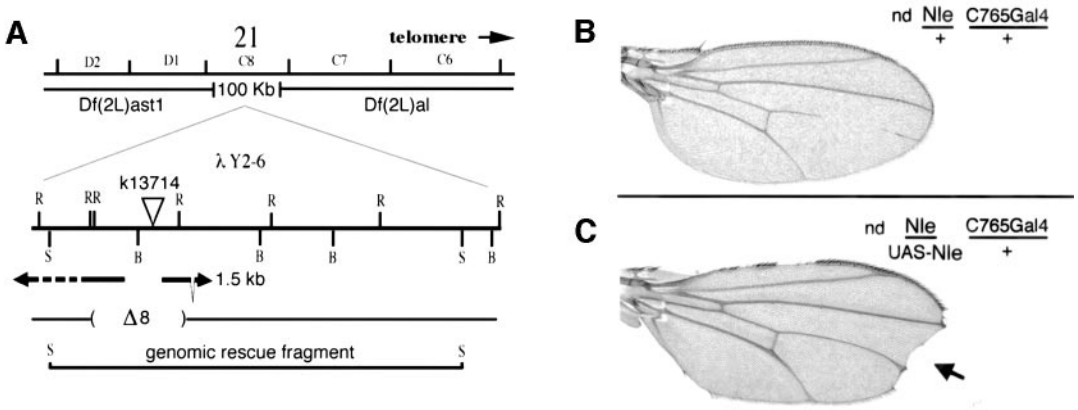


Fig. 2. Cloning the *Notchless* gene. (**A**) schematic representation of the *Nle* locus. The l(2)k13714 P-element was mapped to 21C8 in the interval between Df(2)al and Df(2)ast[1]. A chromosomal walk of ~100 kb (kindly provided by M.Noll) spans this interval. The P-element is inserted in a 3.3 kb *Eco*RI fragment of phage Y2-6 (map positions of *Eco*RI, *Bam*HI and *Sal*I sites are indicated). Transcription units were identified on both sides of the P-element by sequence analysis (indicated by arrows below). The 5′ ends of both transcripts are located close to the P-element. Genomic rescue fragment indicates the ~15 kb *Sal*I fragment. A transgene containing this fragment restores *Nle* activity (i.e. reverts suppression of the nd^1 phenotype by the *Nle* mutant; data not shown). This result excludes the transcript depicted at left as a candidate to encode Nle because it is only partially contained within the rescue fragment. *In situ* hybridization showed uniform low level expression of the *Nle* transcript in imaginal discs (not shown). Δ8 indicates the deletion generated by imprecise excision of the K13714 P-element. Quantitation of Southern blots indicates that the 3.3 kb *Eco*RI fragment is entirely deleted in Δ8 (not shown). The *Nle* gene and the adjacent transcription unit are disrupted. The end points of the deletion have not been mapped. It is likely that other genes are affected. (**B**) *Notchless* phenotype (suppressed nd^1 phenotype) produced when one copy of *Nle* is mutated in a nd^1 fly. In this example the fly also carried the GAL4 driver-line C765 on the third chromosome. (**C**) Wing from a fly of the genotype as in (B), which also carried a UAS-Nle transgene on the second chromosome. Placing the 1.5 kb transcript under C765-GAL4 regulation restores the nd^1 phenotype (arrow).

suggests that reducing Notchless activity increases Notch activity at the DV boundary of the wing disc.

Cloning the Notchless gene

The P-element insertion in l(2)k13714 was mapped to cytological position 21C7-8 by the BDGP (Flybase), between the breakpoints of two large deletions Df(2L)al and Df(2L)ast[1] (Figure 2A). Neither of these deletions acts as a dominant suppressor of nd^1 (data not shown), suggesting that the *Notchless* gene lies in the interval between them. DNA flanking the l(2)k13714 P-element was cloned by plasmid rescue and hybridized to a chromosomal walk spanning the 100 kb between the deletion breakpoints. The rescued DNA hybridized to a 3.3 kb *Eco*RI fragment of λ phage Y2-6. Sequencing of the genomic flank identified transcription units on both sides of the P-element insertion (Figure 2A). The 1.5 kb transcript was identified as the *Notchless* gene by two criteria: (i) the 15 kb *Sal*I fragment of phage Y2-6 was able to restore *Notchless* activity when introduced into a nd^1 *Nle/+* mutant background (data not shown). The transgene contains all of the 1.5 kb transcription unit but only part of the other transcription unit; and (ii) expression of the 1.5 kb cDNA under GAL4 control restores full *Nle* activity. nd^1*; Nle/+* mutant flies carrying a GAL4 driver show the suppressed nd^1 phenotype (Figure 2B). The wing notching phenotype is restored when the 1.5 kb transcript is expressed in the wing disc under GAL4 control in the nd^1*; Nle/+* mutant (compare Figure 2B with C). Thus, increasing the amount of *Notchless* product using GAL4 counteracts the effects of the *Nle* mutant and alleviates the suppression of the nd^1 mutant phenotype. This indicates that the *Nle* mutant phenotype is due to reduced gene activity.

The P-element insertion that causes the mutation is located 310 bp 5′ to the start of the *Nle* open reading frame. It is therefore likely that the P-element mutant reduces the level of *Nle* expression. To obtain a deletion that removes the *Nle* locus, we identified mutants generated by mobilization of the P-element. An excision mutant named $Nle^{\Delta 8}$ deletes sequences on both sides of the insertion (Figure 2A). To determine whether the $Nle^{\Delta 8}$ deletion allele would produce a stronger increase in Notch activity than the l(2)k13714 P-element insertion mutant, we first examined suppression of the nd^1 phenotype. We observed no difference in the extent of suppression of nd^1 (data not shown). The $Nle^{\Delta 8}$ deletion is embryonic lethal when homozygous, but deletes at least one additional transcription unit. Bearing in mind that any phenotypes produced by the deletion could be attributed to its being mutated in more than one gene, we examined homozygous $Nle^{\Delta 8}$ embryos and clones of $Nle^{\Delta 8}$ mutant cells for neurogenic phenotypes. No difference was detected between mutant and wild-type embryos in the developing PNS and CNS, visualized by 22C10 antibody (data not shown). Likewise, we did not observe any bristle pattern abnormality in the notum or wing of homozygous $Nle^{\Delta 8}$ mutant clones (data not shown). Flies heterozygous for the P-element insertion and the $Nle^{\Delta 8}$ deletion are viable, morphologically normal and male sterile, like the homozygous P-element mutant. Together these observations suggest that the original P-element mutant may be a null allele of *Nle*. The lethality caused by the $Nle^{\Delta 8}$ deletion is likely to be due to another gene.

Notchless enhances the effects of mutants that increase Notch activity

Certain *Abruptex* alleles of *Notch* have been classified as mutations that increase Notch activity. Their phenotypes are enhanced by increasing the level of wild-type *Notch* gene product and are suppressed by reducing it (de Celis and Garcia-Bellido, 1994; Brennan *et al.*, 1997). Like

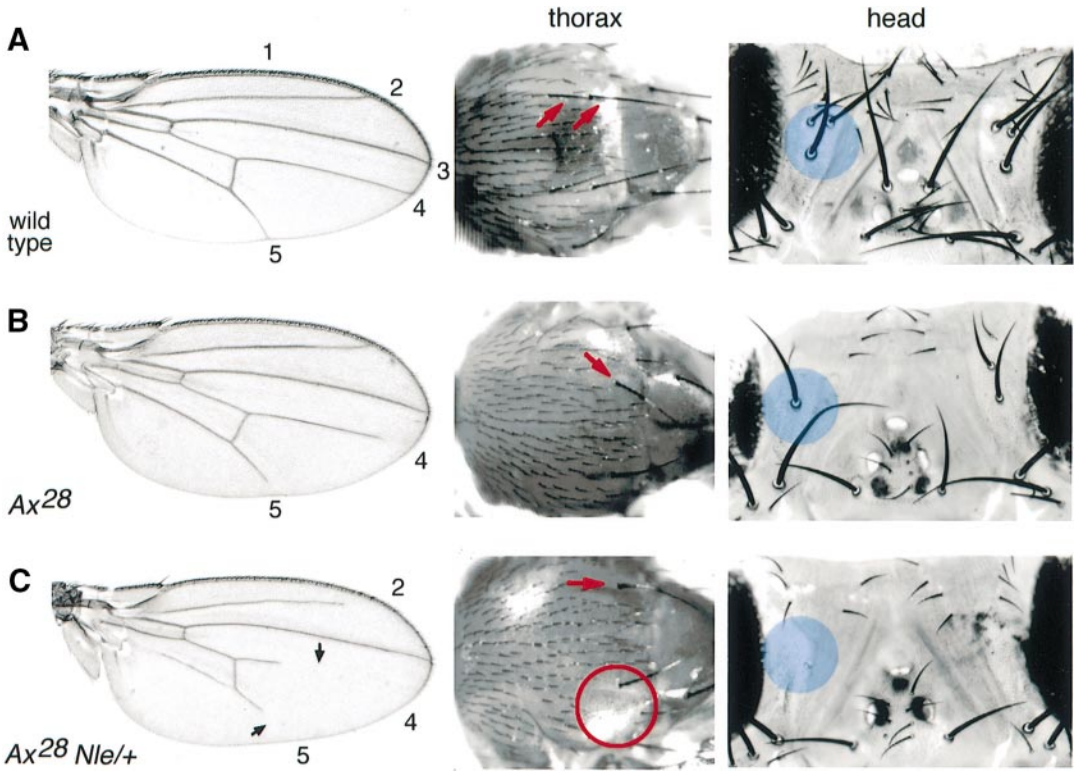


Fig. 3. *Notchless* enhances *Abruptex* mutant phenotypes. (**A**) Wild-type wing, thorax and head cuticles. Veins 1–5 are numbered. The red arrows in the central panel indicate two of the large bristles on the thorax. The blue shading in the right panel indicates the cluster of three orbital bristles above the eye. (**B**) *Abruptex*^{28} mutant wing, thorax and head cuticles. Note that veins 4 and 5 are incomplete and do not extend to the wing margin. The number of large bristles is reduced in the thorax (red arrow). Only one or two orbital bristles are found in the head. (**C**) *Abruptex*^{28} *Nle*$^{\Delta 8}$/+ mutant wing thorax and head cuticles. The loss of veins is more severe in the wing (arrows). Note also the extensive loss of small bristles in the thorax (red outline). Orbital bristles are absent in the head (blue shading). The same results were obtained using the *Nle*^{k13714} allele.

other gain-of-function *Abruptex* alleles, *Ax*^{28} flies show reduced numbers of some bristles on the head and thorax, as well as shortening of wing veins (Figure 3A and B). These phenotypes are made more severe by introducing an extra copy of the wild-type *Notch* gene (data not shown). They are also enhanced by removing one copy of the *Notchless* gene (Figure 3C). The shortening of the wing veins is more pronounced in *Ax*^{28} *Nle*/+ flies (arrows). *Ax*^{28} *Nle*/+ flies show increased loss of both small bristles in the thorax (note the large bare patch outlined in red in Figure 3C) and of large bristles in the head compared with *Ax*^{28} flies. Blue shading on the head indicates the cluster of orbital bristles. There are three in wild-type flies, one or two in *Ax*^{28} flies and none in *Ax*^{28} *Nle*/+ flies. Thus removing one copy of *Nle* enhances the severity of the phenotypes caused by increased Notch activity in *Ax*^{28} flies.

We observed that wing veins are reduced in mutant combinations involving *nd*^{1} and *Nle*/+ (Figure 1D and F). Similar results were obtained with *nd*^{2} (data not shown). This phenotype is likely to reflect increased Notch activity. Matsuno *et al.* (1995) have observed loss of wing veins in *nd*^{1} heterozygous flies (which are themselves morphologically normal) when a low level of the activated form of Notch is expressed under heat-shock control. Together, these observations suggest that the *nd*^{1} mutation shows an abnormal increase in Notch activity in wing vein formation. By analogy to the effects of expressing the activated form of Notch (Matsuno *et al.*, 1995), it is probable that the effect of the *Nle* mutation is to further increase the aberrant Notch activity in the *nd*^{1} mutation. We note that these results appear to be at odds with the observation that the *nd*^{1} mutation reduces Notch function at the wing margin (Figure 1). This suggests that the *nd*^{1} mutation behaves as a loss-of-function allele in one context and as a gain-of-function allele in another (see Discussion). Note that *nd*^{fa} shows only the phenotypes thought to be due to reduced Notch activity, loss of wing margin and vein thickening, and that these phenotypes are suppressed by removing one copy of *Nle* (Figure 1G and H).

Notchless opposes deltex function

Deltex is thought to function as a positive regulator of Notch activity (Diederich *et al.*, 1994; Matsuno *et al.*, 1995). *deltex* mutant flies show a phenotype resembling a reduction of Notch activity: nicking of the distal region of the wing blade and thickening of the wing veins (Figure 4A). Removing one copy of *Notchless* restores the *deltex* mutant wing to normal (Figure 4B). Thus the effects of reducing *deltex* activity can be compensated for by simultaneously reducing *Notchless* activity. Likewise, removing one copy of *Notchless* enhances the effects of overexpressing Deltex using a heat-shock *deltex* transgene (Matsuno *et al.*, 1995). Under conditions where Deltex overexpression produces no visible abnormality in an otherwise wild-type wing (Figure 4C), it causes loss of veins in a *Nle*/+ background (Figure 4D, arrow). This resembles the effects of increasing Notch activity in *Abruptex* mutants. These results suggest that Deltex and Notchless act in opposite directions as modifiers of Notch

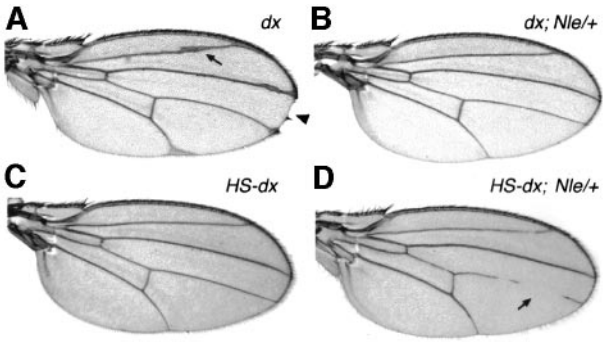


Fig. 4. Genetic interactions between *deltex* and *Notchless*. (**A**) *deltex*^{1} mutant wing. Note the slight notching of the wing tip (arrowhead) and the thickened veins (e.g. arrow). (**B**) *deltex*^{1} *Nle*$^{\Delta 8}$/+ mutant wing. Wing shape and vein pattern are completely restored to normal. The same result was obtained using the *Nle*^{k13714} allele. (**C**) Heat-shock Deltex overexpression under mild conditions produces no phenotype in an otherwise wild-type wing (see also Matsuno *et al.*, 1995). Two 1 h treatments at 37°C were given between 0 and 24 h after pupation. (**D**) Comparable heat-shock Deltex treatment causes loss of veins in a *Nle*$^{\Delta 8}$/+ wing (arrow). The same result was obtained using the *Nle*^{k13714} allele.

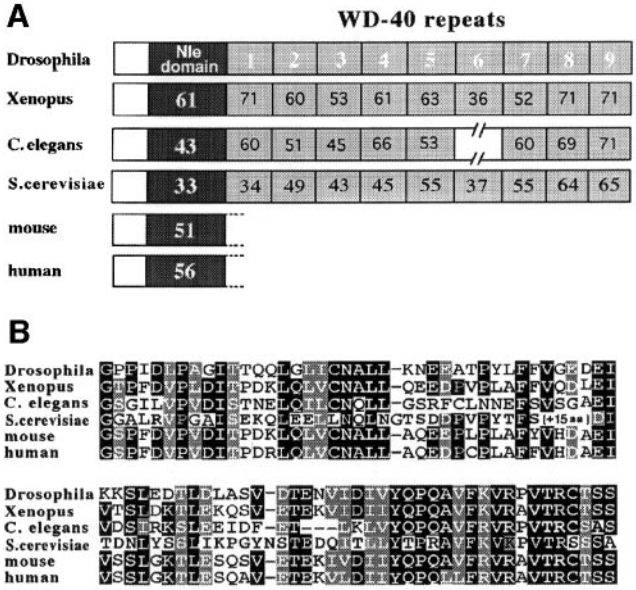


Fig. 5. Molecular features of Notchless protein. (**A**) Schematic representation of Notchless protein and its orthologues. The conserved Nle domain is indicated in dark gray. WD40 repeats are numbered 1–9 (white numbers). Percent identity to the *Drosophila* protein are indicated for the Nle domain and for individual WD40 repeats. DDBJ/EMBL/GenBank accession Nos for the sequences are *Drosophila* Nle (AJ012588); *Xenopus* Nle (AF069737); mouse EST (AA396500); Human EST (AA341327); *S.cerevisiae* (1351791); *C.elegans* sequence was compiled from multiple clones (C48486, D70156, C35601 and M89091) and has a gap in the sixth WD40 repeat. (**B**) Comparison of Nle domains. Sequence identity is highlighted in black, similarity in gray. Similarities are not highlighted if shared by fewer than four proteins. Dashes indicate gaps introduced to accommodate extra residues in the yeast protein. '+15 aa' indicates a larger insertion. As Notch homologues have not been reported in yeast, it is possible that the yeast Nle protein has a different function, reflected in the more divergent structure of this domain.

activity in wing development. *Nle* also shows genetic interaction with the Notch pathway genes *Su(H)* and *groucho*, but not with *Serrate*, *Delta*, *Hairless* or *strawberry Notch* (data not shown).

Notchless encodes a novel WD40-repeat-containing protein

The predicted Notchless protein has a novel highly conserved N-terminal domain followed by nine WD40 repeats (Figure 5A). The WD40 repeat is found in a wide variety of proteins of diverse function and is thought to be a protein interaction domain (reviewed in Neer *et al.*, 1994). Typically WD40 proteins contain seven repeats. Structure analysis of β-transducin suggests that these form a propeller-like structure and that seven repeats can pack to make a flat cylinder (Neer and Smith, 1996). Notchless is unusual in that it appears to contain nine WD40 repeats. Repeats 5 and 6, though recognizable as WD motifs, appear quite divergent in that they lack particular signature residues of the WD40 repeat (not shown).

BLAST searches using the N-terminal sequence (before the first WD repeat) identified closely related sequences in yeast, *C.elegans*, man and mouse. In all cases the N-terminal domain is followed by WD repeats. The human and mouse ESTs extend far enough to show the start of the first WD repeat. Degenerate PCR using primers directed against conserved sequences in the N-terminal domain of the mouse and human proteins was used to isolate a *Xenopus Nle* cDNA. The *Xenopus* protein also contains nine WD repeats with strong similarity to the *Drosophila* and *C.elegans* proteins. We note that particular WD40 repeats are more similar between species than they are to other WD40 repeats in the protein of the same species. Together, this suggests that these proteins represent true orthologues. Database searches suggest that there may only be one member of this gene family in *C.elegans*, mouse and human.

Sequence comparison indicates that the degree of conservation in the N-terminal domain is quite high among the different family members (Figure 5B). In the 80 amino acid region corresponding to residues 27–106 of Notchless, sequence identity ranges from 33% between *Drosophila* and *Saccharomyces cerevisiae* to 61% between *Drosophila* and *Xenopus* proteins. Particular residues are identical in all species examined, suggesting that they may be important for domain structure. We propose that this be called the Nle domain.

The *sel-10* gene of *C.elegans* encodes a WD40-repeat-containing protein that modifies *lin-12* function (*lin-12* is a *Notch* homologue; Hubbard *et al.*, 1997). Although SEL-10 and Notchless both contain WD40 repeats, they are not orthologues. Notchless has nine WD40 repeats rather than the seven repeats found in SEL-10, and does not contain the F-box that characterizes SEL-10 as a CDC4-related protein. SEL-10 does not share the conserved Nle domain in the N-terminus of Notchless. A different *C.elegans* predicted protein appears to be the orthologue of Notchless (Figure 5B).

Notchless expression in Xenopus

The *Xenopus Notchless* gene (*XNle*) is maternally transcribed and expression remains relatively constant during the early stages of embryonic development without obvious signs of localization. Elevated levels arise at the end of gastrulation and are maintained during neurulation and organogenesis (Figure 6A). Localized expression is observed in two lateral domains adjacent to the rostral

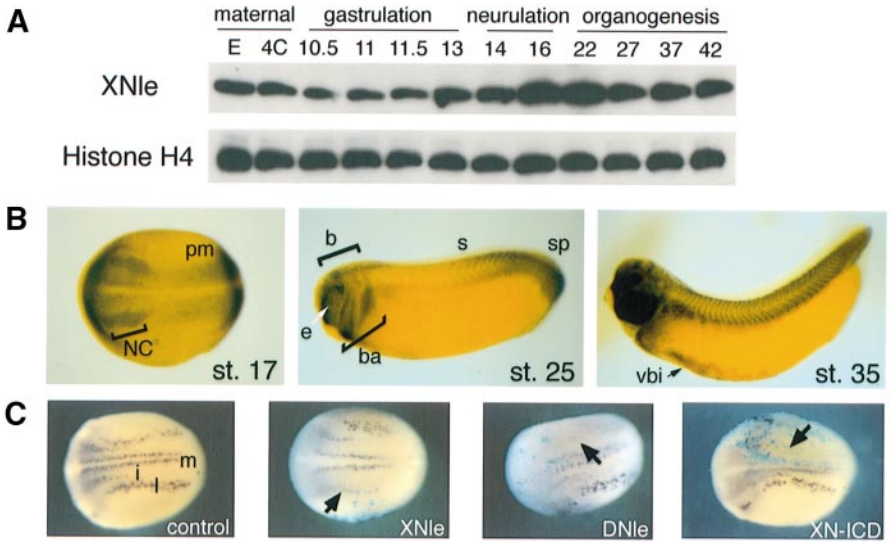


Fig. 6. Expression of *Xenopus Notchless* during embryonic development and phenotypic effects of Notchless overexpression on formation of primary neurons. (**A**) Temporal expression of *XNle*. Total RNA isolated from the indicated stages of development was analyzed by RT–PCR analysis for expression of *XNle* and *Histone H4* (loading control). E, egg; 4C, 4 cell stage; all other lanes are labeled with stage numbers according to Nieuwkoop and Faber (1956). (**B**) Spatial expression of *XNle*. Whole-mount *in situ* hybridization was used to visualize expression of XNle at neural plate stage (st. 17), tailbud stage (st. 25) and tadpole stage (st. 35). Expression patterns are described in the text. NC, neural crest; pm, paraxial mesoderm; b, brain; e, eye; ba, branchial arches; s, somites; sp, segmental plate; vbi, ventral blood islands. (**C**) Phenotypic consequence of overexpression of *XNle*, *DNle* and an activated form of *Xenopus NotchI* (XN-ICD) on primary neurogenesis. LacZ RNA was co-injected to mark the injected side. Control embryo: l, i and m denote lateral, intermediate and medial rows, respectively, of β-tubulin expressing primary neurons. Note the reduction in the number of primary neurons on the injected side in embryos injected with *XNle*, *DNle* or *XN-ICD*. Arrows indicate the absence of lateral and intermediate neurons in XNle and DNle injected embryos and all neurons in *XN-ICD*-injected embryo. In (B) and (C) anterior is to the left.

neural plate, which correspond to the premigratory neural crest cells, and in a region at the anterior end of the neural plate, which corresponds to placodal precursors (Figure 6B). There is also increased expression in the involuting paraxial mesoderm and in two patches lateral to the closing slit blastopore, through which future somitic cells involute. During subsequent stages expression is evident in the somites and unsegmented paraxial mesoderm, the segmental plate. High levels are also seen in the head region; in the branchial arches, eyes and different regions of the developing brain (Figure 6B, st. 25). Later on, expression is also found in two patches on the ventral site of the embryo, the ventral blood islands which generate the hematopoietic precursors of the early embryo (Figure 6B, st. 35). The pattern of *XNle* expression resembles that of other components of the Notch pathway, including *Delta* and *Kuzbanian* (Chitnis *et al.*, 1995; Pan and Rubin, 1997). These expression domains correspond to regions where Notch signaling has been implicated in cell fate specification events (Coffman *et al.*, 1993; Chitnis *et al.*, 1995; Jen *et al.*, 1997).

Overexpressing Notchless increases Notch activity

Based on the finding that reducing Nle activity increases Notch activity in *Drosophila* (Figures 1–4), we anticipated that overexpression of Nle would reduce Notch activity. To test this proposal we made use of the *Xenopus* neuronal specification assay (Chitnis *et al.*, 1995). Notch signaling is involved in controlling the choice between neural and epidermal fate. Overexpression of activated forms of Notch reduces the number of cells adopting neural fate in *Xenopus* (Chitnis *et al.*, 1995). Conversely, reduction of Notch activity would be expected to increase the number of cells adopting neural fate, as in *Notch* mutant embryos in *Drosophila* (Campos-Ortega and Jan, 1991). Surprisingly, we observed that overexpression of XNle and of *Drosophila* Nle reduces the number of neurons, as in the activated Notch control (Figure 6C). Although high levels of *Nle* RNA were injected, we did not observe any sign of other developmental defects: gastrulation and subsequent morphogenesis proceeded normally.

This unexpected finding led us to test whether overexpression of Nle in *Drosophila* would have a comparable effect on neural-fate specification. Expression of activated Notch reduces thoracic bristle formation (Rebay *et al.*, 1993; Struhl *et al.*, 1993). *UAS-Nle* was expressed in the notum under control of apterous-GAL4. The number of small bristles was reduced in flies expressing *UAS-Nle* compared with *apterous-GAL4* alone (Figure 7A). Although the reduction is not large in magnitude, it is statistically significant ($P < 0.00001$). To verify that this effect is due to increased Notch activity, we asked whether Nle overexpression would enhance the severity of *Abruptex* phenotypes (see Figure 3). *Abruptex*^{28} shows a reduction in bristle number; increased Nle expression further reduces the number of bristles in an *Abruptex*^{28} background. We also note that increased Nle expression increases the vein loss caused by *Abruptex*^{28} (Figure 7B). These results indicate that increased Nle expression enhances the severity of two different *Abruptex*^{28} phenotypes that have been attributed to increased Notch activity. Thus overexpression of Nle increases Notch activity in both *Xenopus* and *Drosophila*.

Notchless protein binds to the intracellular domain of Notch

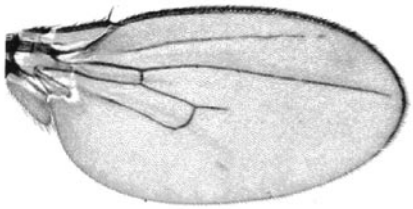
To determine whether Nle might regulate Notch through direct protein interaction we carried out GST pull-down

A

microcheate/notum

genotype	mean ± SE (n)	t-test
ap-Gal4/+	214 ± 2 (17)	
ap-Gal4/UAS-Nle	196 ± 3 (17)	p< 0.00001
Ax^{28}/Y; ap-Gal4/+	176 ± 3.2 (12)	
Ax^{28}/Y; ap-Gal4/UAS-Nle	143 ± 3.8 (14)	p< 0.000001

B



Ax^{28}/Y; C765/UAS-Nle

Fig. 7. Overexpression of Notchless increases Notch activity in *Drosophila*. (**A**) The number of small bristles on the thorax was counted in flies of the indicated genotypes. Wild-type flies have 260 small bristles per thorax on average (Brennan *et al.*, 1997). This number is reduced in Apterous-GAL4/+ flies. Overexpression of Nle further reduces the number of bristles. The number of small bristles is reduced in *Abruptex*^{28} mutants due to an increase in Notch activity. Overexpression of Nle in the *Abruptex* background further reduces the number of bristles. (**B**) Overexpression of Nle in the *Abruptex*^{28} background shows a stronger reduction of wing veins than in the *Abruptex*^{28} background alone (compare with Figure 3B). The C765 GAL4 driver by itself has no effect on *Abruptex*^{28} phenotype (not shown).

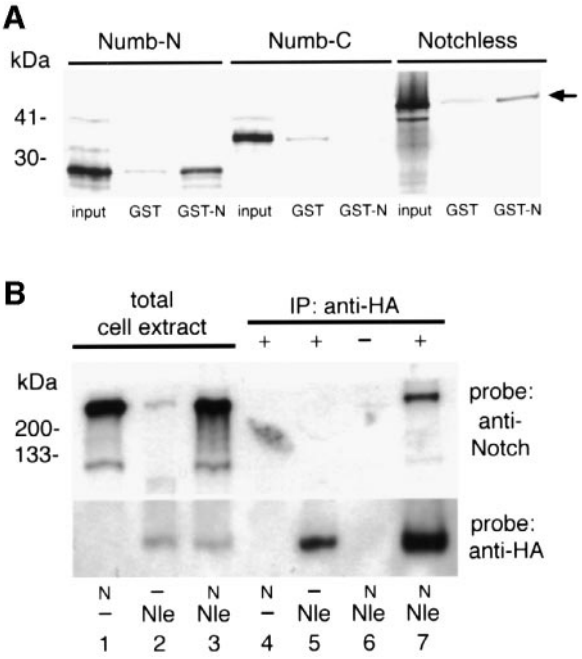


Fig. 8. Notchless binds Notch *in vitro*. (**A**) Binding of Nle, Numb-N and Numb-C to GST-Notch-ICD and GST control proteins. N-terminal and C-terminal fragments of Numb were used as controls for binding to the intracellular domain of Notch (Guo *et al.*, 1996). Input lanes show one-tenth of the input to the binding reaction. GST-N indicates GST–Notch-ICD beads; GST indicates GST beads. Numb-N and Nle (arrow) bind to GST-N beads more strongly than to GST control beads. Coomassie Blue staining of the gel (not shown) showed that there was significantly more protein bound to the GST control beads than to the GST-N beads. (**B**) Immunoprecipitation of Notch and Nle expressed in S2 cells. Upper panel, blot probed with mouse monoclonal anti-Notch (9C6). Lower panel, the same blot probed subsequently with mouse anti-HA. Lanes 1–3, total cell lysates from S2 cells expressing (1) Notch, (2) HA-Nle or (3) both proteins. Note that low levels of endogenous Notch are seen in the Nle-expressing cells (lane 2). Lanes 4–7: immunoprecipitates from cells expressing (4)Notch, (5) HA-Notchless or (6 and 7) both proteins. '+' indicates immunoprecipitated with rabbit anti-HA and protein A beads. '–' indicates control precipitation with protein A beads alone. Immunoprecipitation of HA-Nle co-precipitates Notch in cells that overexpress both proteins (lane 7). Neither protein is recovered in control precipitations lacking the anti-HA (lane 6) or in which HA-Nle was not expressed (lane 4). Immunoprecipitation in cells transfected with HA-Nle alone did not detectably co-precipitate Notch (lane 5), although endogenous Notch can be detected in cells not transfected with the inducible Notch expression construct (lane 2). Recovery of HA-Nle was lower in the reaction in lane 5 than in lane 7.

and immunoprecipitation assays. *In vitro* binding was tested using [^{35}S]methionine-labeled test proteins and the intracellular domain of Notch expressed in bacteria as a GST-fusion protein (Guo *et al.*, 1996). The N-terminal domain of Numb has been shown to bind to Notch and was used as a positive control for specific binding (Guo *et al.*, 1996; Figure 8A). The C-terminal domain of Numb does not bind Notch and was used as a negative control. GST control beads show weak non-specific binding to all three proteins, but this is well below the level of specific binding observed with Numb-N and Notchless (Figure 8A).

In vivo interaction between Notchless and Notch in *Drosophila* S2 cells was tested by immunoprecipitation. Expression of full-length Notch and hemagglutinin (HA)-tagged Notchless proteins was monitored by immunoblotting of total cell extracts (Figure 8B, lanes 1–3). Extracts from induced and uninduced cells were immunoprecipitated using antibody to the HA-tag, and a blot of the gel was probed with a monoclonal antibody directed against the intracellular part of Notch and reprobed subsequently with anti-HA to visualize the immunoprecipitated HA-Notchless. Notch protein immunoprecipitates with HA-Notchless from cells expressing both proteins (Figure 8B, lane 7). No precipitation was observed in controls lacking HA-Nle or anti-HA (Figure 8B, lanes 4 and 6). Together these results indicate that Notchless binds directly to the intracellular domain of Notch.

Discussion

Possible functions of Notchless

We have presented genetic and biochemical evidence that *Notchless* encodes a novel modifier of Notch activity. Notchless protein binds to the intracellular domain of Notch, and like Numb, Deltex, Dishevelled and SEL-10 modifies Notch activity when assayed *in vivo*.

Recent evidence suggest that Notch signaling depends on proteolytic cleavage to release the intracellular domain of Notch so that it can translocate to the nucleus with Su(H) (Schroeter *et al.*, 1998; Struhl and Adachi, 1998). The requirement for Notch cleavage suggests a possible mechanism for inhibition of Notch by Numb. Numb is localized to the cell cortex (Rhyu *et al.*, 1994; Knoblich *et al.*, 1995); thus, it is possible that Numb might inhibit Notch activity by tethering the intracellular domain of

Notch to the membrane. We have not been able to determine directly whether Notchless could act similarly, because antibodies to monitor the subcellular localization of the endogenous Notchless protein are not available. An epitope-tagged version of Nle protein expressed under GAL4 control does not show any obvious subcellular localization (data not shown). However, this observation must be interpreted with caution since overexpression of the Nle protein could obscure subcellular localization (e.g. Notch in S2 cells; see Fortini and Artavanis-Tsakonas, 1994).

A different mechanism seems likely for SEL-10, which resembles yeast CDC4 (Hubbard *et al.*, 1997). CDC4 targets specific cell-cycle proteins for ubiquitin-dependent proteolytic degradation (Bai *et al.*, 1996). SEL-10 may help to reduce LIN-12 activity by ensuring rapid turnover of activated receptor, whether at the membrane or in the nucleus. Notchless lacks the F-box that characterizes SEL-10 as a possible component of ubiquitin E3-ligase, and is unlikely to act by a similar mechanism.

How might Notchless act to reduce Notch activity? Genetic interactions suggest a possible link between *Notchless* and *deltex*. *deltex* mutants resemble weak *Notch* mutants, suggesting that Deltex helps to increase Notch activity (Matsuno *et al.*, 1995). Deltex protein binds to the CDC10/Ankyrin repeats in the ICN1 domain of Notch, but does not bind to the ICN2 domain (Diederich *et al.*, 1994; Matsuno *et al.*, 1995). Experiments using the yeast two-hybrid system showed that Nle expressed as an activator fusion protein binds to the ICN2 domain of Notch, but not to ICN1 (data not shown; ICN1 and ICN2 were expressed as LEXA DNA-binding-domain fusion proteins). This suggests that Notchless is likely to oppose Deltex function indirectly through an opposing activity on Notch, and not by direct competition for binding. Little is known about Deltex function, except that overexpression of Deltex can liberate Su(H) to translocate to the nucleus under conditions where Su(H) is artificially retained in the cytoplasm by binding to overexpressed Notch (Fortini and Artavanis-Tsakonas, 1994). It is possible that the balance between Deltex and Notchless activities in some way modulates processing of Notch.

Similar effects of increased and decreased Nle activity

The function of Notchless appears to be to reduce Notch activity. Mutants that reduce or remove Nle expression increase Notch activity in several different assays. Increased Nle expression in *Xenopus* or in *Drosophila* also leads to increased Notch activity and prevents cells from adopting neural fate. The effects of Nle overexpression appear to be conserved in that it is specific to the Notch pathway in both *Drosophila* and *Xenopus*. We suggest that Nle functions as a modulator to keep Notch activity levels in balance. *Nle* mutants show increased Notch activity but are viable even as homozygotes, indicating that the level of overactivation is not so severe as to be lethal. In this regard, Nle functions like Deltex, which modulates the level of Notch activity, but which is not absolutely required for Notch to function.

The observation that increasing or decreasing Nle has a similar effect on Notch activity raises the possibility that Nle forms a complex with proteins in addition to Notch. If the function of Nle is to bring other components together in a complex and if the level of any component other than Nle is limiting, it is possible that overexpression of Nle could reduce formation of the active complex by sequestering the limiting component(s) into incomplete or inactive complexes. This is easiest to imagine in a complex with several components, but it is also possible in tetramers of two components if a 1:1 stoichiometry is important for activity. Many other explanations could be proposed to explain the dominant-negative behavior of the overexpressed protein. It is worth noting that a similar phenomenon has been reported for Notch itself. Overexpression of wild-type Notch produces a phenotype of thickened veins which resembles that of reducing Notch or Delta activity. This is thought to occur by sequestration of Delta in cells overexpressing Notch, which reduces the ability of these cells to signal productively.

notchoid mutations

The wing scalloping phenotype of *notchoid* alleles is due to reduced wingless activity at the wing margin (Couso *et al.*, 1994; Hing *et al.*, 1994). Notch activity is required to induce Wg expression at the margin (Couso *et al.*, 1995; Diaz-Benjumea and Cohen, 1995; Rulifson and Blair, 1995), thus the *nd* defect appears to be due to a reduction of Notch activity. We have noted an apparently contradictory increase of Notch activity associated with nd^1 and nd^2 in the context of vein specification. As outlined above, the effects of the *Nle* mutation on the nd^1 and nd^2 mutations in this context are comparable to the effects of weak expression of the activated form of Notch (Matsuno *et al.*, 1995). When *Nle* was made homozygous in a nd^1 mutant we observed ectopic expression of Wg in the wing pouch, suggesting ectopic activation of Notch (data not shown). This was not observed in *Nle* homozygous discs without the nd^1 mutation. We note that the increase in Notch activity is not observed with nd^{fa} suggesting that it may reflect a particular feature of nd^1 and nd^2 alleles. nd^1 was reported to be due to a point mutation (Xu *et al.*, 1990); however, subsequent reanalysis does not show any alteration in the coding sequence (S.Artavanis-Tsakonas, personal communication). Thus it appears that nd^1 may be a regulatory mutation. If this is the case it is possible that *Notch* expression is differentially altered in DV-boundary specification which occurs early and in specification of wing veins which occurs later in wing development.

Materials and methods

Drosophila strains

l(2)k13714 is from the BDGP P-element lethal collection. P-element excisions were generated by providing a chromosomal source of transposase activity. 105 w^- excision lines were isolated. One of these was not able to suppress the nd^1 phenotype and was therefore reverted to wild type. Others were analyzed for imprecise excision of the P-element by Southern blots. $Su(H)^{SF8}$ and $Su(H)^{AR9}$ are described in Schweisguth and Posakony (1992); Ax^{28} is described in de Celis and Garcia-Bellido (1994); $deltex^1$ and pCaSpeR hs-dx are described in Diederich *et al.* (1994) and Matsuno *et al.* (1995); and nd^1, nd^2, nd^{fa} and Dp(1;2)51b are described in Flybase (1992). w^{1118} was used as wild-type control for cuticle preparations. For heat-shock experiments, *pCaSpeR hs-dx/+* and *pCaSpeR hs-dx/Nle* pupae were heat-shocked twice for 1 h at 37°C between 0 and 24 h after pupariation.

Antibodies

Mouse monoclonal anti-Wg is described in Brook and Cohen (1996). Mouse monoclonal anti-Notch C17.9C6 is described in Fehon *et al.* (1990). Mouse (12CA5) anti-HA and rabbit (HA-11) anti-HA were obtained from Babco.

Cloning Nle cDNA

DNA flanking the P element was cloned by plasmid rescue using *Eco*RI digested genomic DNA. A 2.5 kb *Eco*RI–*Hin*dIII fragment (devoid of P-element sequences) was used to screen a chromosomal walk that was kindly provided by Markus Noll. The rescue fragment hybridized to a 3.3 kb *Eco*RI fragment. Sequencing of the 3.3 kb DNA fragment revealed the presence of open reading frames on both sides of the P-element insert but in opposite orientation. Genomic rescue suggested that the gene was encoded by the 1.5 kb transcript (to the right of the insert in Figure 2). A 1.1 kb *Eco*RI–*Cla*I fragment to the right of the insertion site containing part of the predicted transcription unit was used to screen a λgt10 eye disc cDNA library (kindly provided by G.Rubin). Six cDNA clones were isolated. One encodes a putative full-length *Nle* cDNA of 1.5 kb. The Nle ORF begins 7 bp from the 5′ end of this clone. UAS constructs expressing this cDNA have full Nle activity *in vivo*.

Constructs for rescue and expression

A 15 kb *Sal*I genomic fragment of phage Y2-6 was inserted into the *Xho*I site of the transformation vector pCaSpeR4. UAS-*Nle* was prepared by cloning the 1.5 kb *Nle* cDNA as a *Not*I–*Xho*I fragment into pUAST (Brand and Perrimon, 1993). An HA-tagged version of Nle was generated by introducing three copies of the HA epitope (YPYDVPDYA) immediately downstream of the first methionine residue. The *Bam*HI–*Asc*I fragment of pKS-Nle was replaced by a corresponding PCR fragment amplified using the following primers: 5′-CGGATCCAAAAAATGTATCCCTATGACGTCCCCGATTATGCCTACCCTTACGATGTACCTGACTACGCGTATCCGTACGACGTTCCGGACTATGCTCAGGAGACGGACACGGAGCAAGAGGCCACGCCACATACGATACAGGCGCGCCaa-3′ and 5′-TAAACGAGGCGCGCCTATCGTAT-3′. pMT-HA-*Nle* was generated by cloning HA-*Nle* as a *Bam*HI–*Sal*I fragment into the inducible expression vector pRmHa-3. pRmHa-3-*Notch* is described in Fehon *et al.* (1990).

Xenopus Notchless

XNle was isolated by PCR using the degenerate primers, F 5′-CGCAGAATTCCITTYGAYGTICCIGTIGAYAT-3′ and R 5′-GGTGCTCGAGCYTGIGGYTGRTAIATDATRTC-3′, designed against the conserved peptides PFDVPVDI and DIIYQPQ, respectively, found in the Nle domain of the vertebrate proteins identified as expressed sequence tags. Phage stock of a stage 30 library (Stratagene) was used as template to amplify a 200 bp fragment that spans the Nle domain. Five independent clones were sequenced and found to be identical. This fragment was used to screen the stage 30 library, which resulted in the isolation of 25 positive clones of which the longest of 2.2 kb was sequenced on both strands. Temporal expression was assayed by RT–PCR analysis as described previously (Bouwmeester *et al.*, 1996) using the following primer set that amplifies a XNle fragment of 135 bp; F 5′-CACCAGATAAACTGCAGTTAG-3′, R 5′-CTGTTTCAACTGATTGCTTCT-3′ (28 cycles). Spatial expression was analyzed by whole-mount *in situ* hybridization essentially as described previously (Bouwmeester *et al.*, 1996), using antisense RNA synthesized from pBS-*XNle* linearized with *Xho*I and transcribed with T3 polymerase. For injection purposes pCS2-*XNle* was constructed by subcloning of a 2.2 kb *Eco*RI fragment in the complementary site of pCS2+. Capped RNA was synthesized using pCS2-*XNle*, pCS2-*Drosophila Nle* (kindly provided by J.Wittbrodt) and pCS2-*NOTCHI-ICD* (kindly provided by C.Kintner) digested with *Not*I and transcribed with Sp6. Synthetic RNA (2.5–5 ng of *XNle* and *DNle* RNA, 100–200 pg *XN-ICD*) was injected into one blastomere of the 2-cell stage embryo. Embryos were harvested at early neurula stage (st. 13–15). β-galactosidase activity, a lineage marker for injections, was revealed using X-gal as substrate prior to whole mount *in situ*. Primary neurons were identified by β-tubulin staining. Antisense β-tubulin RNA was synthesized from pBS-β-tubulin digested with *Not*I and transcribed with T3 polymerase.

GST-fusion protein binding assay

GST-NICD was expressed in bacteria and purified as described in (Guo *et al.*, 1996). ^{35}S-labeled Numb-N (aa 1–224), Numb-C (aa 224–547) and full-length Nle were synthesized by *in vitro* transcription/translation using the TNT system (Promega). Binding reactions were carried out with 10 μl of labeled protein and 5 μl of GST or GST-NICD coupled beads in 400 μl of phosphate-buffered saline (PBS) 0.1% NP-40 for 1 h at room temperature. The beads were washed six times in PBS, proteins eluted in SDS-gel sample buffer, separated on 10% SDS–polyacrylamide gels and visualized by autoradiography.

Immunoprecipitation

Schneider S2 cells were grown at 25°C in Schneider's medium (Gibco-BRL) with 1% fetal calf serum and 1% gentamicin. Cells were harvested and transferred into 6-well 30 mm diameter tissue culture plates at 75% confluence. Each well was then rinsed 3 times with Schneider medium without serum and incubated with 10 μg of DNA in 500 μl of Schneider medium and 50 μl of Lipofectin (Gibco-BRL) for 6 h. Cells were incubated overnight in medium without Lipofectin. Expression was induced by adding $CuSO_4$ to 0.7 mM and incubating for 12 h. Cells were harvested and lysed by sonication in PBS, 50 mM NaCl, 5 mM EDTA, 5 mM DTT, 1% Triton X-100 containing protease inhibitors (1 mM PMSF, 5 μg/ml aprotinin and leupeptin). Cells debris was removed by 10 000 *g* centrifugation. Five-hundred microliters of extract (corresponding to 1×10^6 cells) was incubated with 3 μl of rabbit anti-HA antibody for 1 h at 4°C followed by 1 h at 4°C with 20 μl of a 50% slurry of protein A–Sepharose beads (Pharmacia). The beads were washed four times with lysis buffer, proteins eluted in SDS-gel sample buffer and run on a 6% SDS–polyacrylamide gel. The gel was electrophoretically transferred to Immobilon-P membrane (Millipore), blocked for 1 h at room temperature in 5% dry milk in TTBS (10 mM Tris pH 8.0, 150 mM NaCl, 0.2% Tween-20) and incubated overnight at 4°C with mouse-anti Notch (9C6; used at 1:2000) or mouse anti-HA (1:1000). The membrane was washed 3× 5 min in TTBS and incubated for 1 h with peroxidase-conjugated goat-anti-mouse IgG (Jackson Laboratories) diluted 1:5000 in TTBS. The blot was washed 3 times for 5 min in TTBS and developed using ECL reagents (Amersham).

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