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The *Abruptex* domain of Notch regulates negative interactions between Notch, its ligands and Fringe

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SUMMARY

The Notch signalling pathway regulates cell fate choices during both vertebrate and invertebrate development. In the *Drosophila* wing disc, the activation of Notch by its ligands Delta and Serrate is required to make the dorsoventral boundary, where several genes, such as *wingless* and *cut*, are expressed in a 2- to 4-cell-wide domain. The interactions between Notch and its ligands are modulated by Fringe via a mechanism that may involve post-transcriptional modifications of Notch. The ligands themselves also help to restrict Notch activity to the dorsoventral boundary cells, because they antagonise the activation of the receptor in the cells where their expression is high. This function of the ligands is critical to establish the polarity of signalling, but very little is known about the mechanisms involved in the interactions between Notch and its ligands that result in suppression of Notch activity. The extracellular domain of Notch contains an array of 36 EGF repeats, two of which, repeats 11 and 12, are necessary for direct interactions between Notch with Delta and Serrate. We investigate here the function of a region of the Notch extracellular domain where several missense mutations, called *Abruptex*, are localised. These *Notch* alleles are characterised by phenotypes opposite to the loss of *Notch* function and also by complex complementation patterns. We find that, in *Abruptex* mutant discs, only the negative effects of the ligands and Fringe are affected, resulting in the failure to restrict the expression of *cut* and *wingless* to the dorsoventral boundary. We suggest that *Abruptex* alleles identify a domain in the Notch protein that mediates the interactions between Notch, its ligands and Fringe that result in suppression of Notch activity.

Key words: Wing development, Notch signalling, *Abruptex*, *fringe*

INTRODUCTION

Signalling by the transmembrane receptor Notch (N) influences cell fates in many developmental processes during invertebrate and vertebrate development (Artavanis-Tsakonas et al., 1995). Components of the Notch signalling pathway in *Drosophila* include the ligands Delta (Dl) and Serrate (Ser) and the intracellular transducer Suppressor of Hairless (Su(H)) (Nye and Kopan, 1995; Artavanis-Tsakonas et al., 1995). Current evidence indicates that the interaction between Notch and its ligands induces proteolytic processing of the receptor releasing an intracellular fragment, N^{intra} (Kopan et al., 1996; Struhl and Adachi, 1998; Lecourtois and Schweisguth, 1998). This fragment translocates to the nucleus and, through its interaction with the DNA-binding protein Su(H), promotes the transcription of Notch-target genes (Tamura et al., 1995; Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). The precise targets that are activated by Notch vary according to the cell type and stage of development but, in most cases, the primary consequences of activating Notch are mediated through the tissue-specific regulation of genes affecting cell differentiation and proliferation (Muskavitch, 1994; Artavanis-Tsakonas et al., 1995, 1999).

The activation of Notch is regulated both by the temporal and spatial distribution of the ligands and by the expression of proteins such as Fringe (Fng) that are able to modulate ligand-receptor interactions (Irvine and Vogt, 1997). This was first evident in the developing wing, where Notch activity results in the expression of genes such as *wingless* (*wg*) and *cut* (*ct*) in a narrow 2- to 4-cell-wide domain at the dorsoventral boundary (Blair, 1995). In this process, Fng influences the effectiveness of the interactions between Notch and its ligands by preventing Ser-mediated activation and potentiating Notch activation by Dl (Panin et al., 1997). The localised activation of Notch initially occurs because Apterous promotes the expression of both Ser and Fng in dorsal cells, while the inhibitory effect of Fng on Ser/Notch restricts Ser signalling primarily to ventral cells (Kim et al., 1995; Diaz-Benjumea and Cohen, 1995; Couso et al., 1995; de Celis et al., 1996; Fleming et al., 1997; Panin et al., 1997; Klein and Martinez-Arias, 1998). At the same time, the effect of Fng on Dl has the consequence that ventral Dl-expressing cells signal primarily to dorsal cells (de Celis et al., 1996; Doherty et al., 1996). A similar process occurs in the eye, where again the compartment-specific expression of *fng* allows localised activation of Notch at the eye dorsoventral boundary

(Domínguez and de Celis, 1998; Cho and Choi, 1998; Papayannopoulos et al., 1998).

Conventionally Dl and Ser are considered activating ligands of Notch and, in many instances, their elimination has non-autonomous effects on development that are characteristic of a membrane-associated ligand (Heitzler and Simpson, 1991). However, in the *Drosophila* wing and eye, both Notch ligands have also been shown to have cell-autonomous inhibitory effects on the activity of the receptor (Kim et al., 1995; Thomas et al., 1995; Doherty et al., 1996; Jonsson and Knust, 1996; de Celis and Bray, 1997; Hukriede et al., 1997; Klein et al., 1997; Micchelli et al., 1997). Thus, the elimination of both ligands in clones of cells in the wing can result in Notch activation within the clone, detected as ectopic *ct* expression, indicating that a normal function of Dl and Ser is to prevent Notch activation within the cells in which they are expressed (Micchelli et al., 1997). In addition, ectopic expression of Dl or Ser in groups of cells causes Notch activation only in the adjacent cells (de Celis and Bray, 1997). Consistent with the suggestion that the inhibitory activity of the ligands relies on interactions occurring between molecules within the same cell, the negative effects of ectopically expressed Ser can be alleviated by co-expression of full-length Notch (Doherty et al., 1996; Klein et al., 1997). The negative effect of the ligands could be instrumental in determining the polarity of Notch signalling: cells expressing higher levels of ligand would have reduced Notch responsiveness compared to adjacent cells with lower ligand levels and hence Notch would be more readily activated in the cells with relatively less ligand. The concept that the relative levels of Notch and Dl are important for signalling is also evident from the phenotypes caused by varying the dosage of these genes (de la Concha et al., 1988; de Celis and Garcia-Bellido, 1994 and see Fig. 1). Finally, Dl and Notch have been seen to co-localise on the surface of cultured cells, suggesting that they could interact in the plasma membrane (Fehon et al., 1990). However, the antagonistic interactions could be occurring anywhere within the cell and the functional domain of Notch involved in this process has not been characterised.

The Notch extracellular domain has 36 EGF repeats, two of which, EGF repeats 11 and 12, are necessary and sufficient to promote cell aggregation between cells expressing Dl and cells expressing Notch (Rebay et al., 1991; Lieber et al., 1992). The importance of these two repeats is also evident in vivo, as a single amino-acid substitution in EGF repeat 11 abolishes Notch activity (de Celis et al., 1993). A second functional domain in EGF-repeats 24-29 is identified by a class of *Notch* missense mutations called *Abruptex* (N^{Ax}) (Portin, 1975; Foster, 1975; Kelley et al., 1987; de Celis and Garcia-Bellido, 1994). These mutations all result in amino-acid substitutions in the repeats 24, 25, 27 and 29, and cause phenotypes opposite to those characteristic of reduced Notch levels (Kelley et al., 1987; de Celis and Garcia-Bellido, 1994). For example, in sensory organ formation and vein differentiation, N^{Ax} alleles are associated with the loss of these structures, whereas *Notch* loss-of-function alleles cause the formation of extra sensory organs and vein tissue. In addition, *wg* and *ct* are expressed broadly in wing discs mutant for some N^{Ax} alleles, suggesting that Notch activation is not restricted to the cells at the dorsoventral boundary in these mutant discs (de Celis et al., 1996). Because N^{Ax} alleles are point mutations that map in a restricted region of *Notch*, it is possible that the N^{Ax} domain mediates interactions between Notch and other protein/s that prevents Notch activity

We have characterised the effects of Notch ligands and Fng on the ectopic Notch activation at the wing pouch in several N^{Ax} backgrounds. Our results suggest that all N^{Ax} mutations have different degrees of insufficiency in a function of Notch that is required to limit the extent of Notch activation. The presence of ectopic Dl or Ser still causes some repression of Notch activity in N^{Ax} mutant backgrounds. However, the effectiveness of this repression is reduced in N^{Ax} compared to wild type. Finally, we find that ectopic Notch activation of N^{Ax} proteins is insensitive to the presence of Fng. Our results point to a critical role of the N^{Ax} domain in the interactions between Notch, Dl, Ser and Fng that function to restrict Notch activation, and suggest that inhibition of Notch by its ligands plays a major role in modulating Notch function, at least in the developmental processes affected by N^{Ax} mutations.

MATERIALS AND METHODS

Genetic strains

We have used the *Notch* gain-of-function alleles Ax^{M1}, Ax^{28} and Ax^{16172} (de Celis and Garcia-Bellido, 1994), the *Notch* null allele N^{55e11} (Kidd et al., 1983), the *Notch* duplication $Dp(1;2)w^{+51b7}$ (Lindsley and Zimm, 1992), the *Dl* lethal allele Dl^{M1} (de Celis et al., 1991a), the *Dl* duplication $Dp(3;3)bxd^{110}$ (Lindsley and Zimm, 1992), an *E(spl)mβ–CD2* reporter construct (de Celis et al., 1998), the UAS lines *UAS-Ser* (Speicher et al., 1994), $UAS\text{-}N^{intra}$ (de Celis and Bray, 1997), $UAS\text{-}N^{ecd}$, $UAS\text{-}N^{fl}$ (Klein et al., 1997), *UAS-Dl* (Doherty et al., 1996), *UAS-wg* and *UAS-fng* (Kim et al., 1995), and the Gal4 lines *Gal4-sal* (Speicher et al., 1994), *Gal4-1348* (de Celis, 1997) and *Gal4-756* (Gomez-Skarmeta et al., 1996). Crosses were done at 25°C, and larvae were grown at 17°C, 25°C and 29°C.

Generation of mosaics

Clones of cells expressing Gal4 were induced 48-72 hours after egg laying by 7 minutes heat shock at 37°C in flies of the following genotypes:

(1) *hsFLP1.22; P{abx/Ubx<FRTf⁺FRT>Gal4-lacZ}/UAS-X*

(2) Ax^{M1}*hsFLP1.22; P{abx/Ubx<FRTf⁺FRT>Gal4-lacZ}/UAS-X*

(3) Ax^{M1}*hsFLP1.22/*Ax^{16172}*; P{abx/Ubx<FRTf⁺FRT>Gal4-lacZ}/UAS-X*

(4) Ax^{16172}*hsFLP1.22/*Ax^{28}*; P{abx/Ubx<FRTf+>Gal4-lacZ}/UAS-X*.

UAS-X corresponds to *UAS-Dl*, *UAS-Ser* or *UAS-fng*. The flip out of the $FRTf^{+}FRT$ cassette results in the expression of a di-cistronic *Gal4-lacZ* mRNA under the control of the *abx/Ubx* promoter (de Celis and Bray, 1997). Clones were detected by expression of β-galactosidase and were analysed in third instar larvae. Lower levels of ectopic Dl expression (data not shown) were induced in *hsFLP1.22;P{Act5C<FRTy⁺FRT>Gal4} UAS-lacZ/UAS-Dl* larvae. In this experiment, the expression of Gal4 was driven by the *Act5C* promoter, and clones were also detected by expression of β-galactosidase (Ito et al., 1997).

Immunocytochemistry

We used rabbit anti-β-galactosidase (Cappel), mouse monoclonals anti-Delta (Fehon et al., 1991), anti-Ct (Blochinger et al., 1990), anti-Wg (Diaz-Benjumea and Cohen, 1995) and anti-Dll (Lecuit and Cohen, 1997), mouse anti-CD2 (Serotec), rat anti-Ser (Panin et al., 1997) and anti-Ap (a gift from J. Botas). Secondary antibodies were from Jackson Immunological Laboratories (used at 1/200 dilution).

RESULTS

Notch signalling requires correct relative levels of Dl and Notch

Dl and *Notch* are amongst the rare haplo-insufficient *Drosophila* genes, whose elimination in one copy results in a dominant mutant phenotype (Lindsley and Zimm, 1992). Surprisingly, however, the wing phenotypes caused by heterozygous deficiencies of *Dl* or *Notch* are different, as illustrated in Fig. 1. In *Dl* heterozygous flies, the veins L2 and L5 are irregularly broader than wild-type veins, an effect that is most obvious at the distal tips of these veins (Fig. 1A). In contrast, the thickened veins of *N* heterozygous flies have smooth borders, and only L3 and L5 veins are noticeably thicker than wild-type veins (Fig. 1B). In addition, *N*/+ wings have moderate scalloping of the wing margin, a phenotype that is never observed in *Dl* heterozygotes. These differences are paradoxical, because if *Dl* and *Notch* were only involved in interactions that result in activation of the receptor, reductions in the level of either the ligand or the receptor should cause the same phenotypic alteration. Furthermore, in flies where both *Dl* and *Notch* are heterozygous (N^{-}/+; Dl^{-}/+), the veins and wing margin are normal, instead of having a more abnormal phenotype as expected for combinations that reduce both ligand and receptor (data not shown). Conversely, if the copy number of *Notch* is increased (using $Notch^{+}$ duplications), the wings have phenotypes indistinguishable from those of *Dl* mutations (Fig. 1C,D). This phenotype becomes exaggerated when a copy of *Dl* is removed (in DpN^{+}/+; Dl^{-}/+ flies; Fig. 1E) and suppressed when an extra copy of Dl (DpN^{+}/+; $DpDl^{+}$/+) is present (data not shown). The phenotype of DpN^{+} flies is also observed when high levels of Notch are expressed ectopically using the Gal4 system (Fig. 1F), confirming that this phenotype is directly caused by an increase in the levels of Notch expression. These genetic data therefore led to the conclusion that it is the relative levels of *Dl* and *Notch* gene products that determines the efficiency of signalling (de la Concha et al., 1988; de Celis and Garcia-Bellido, 1994). One explanation for the paradoxical results from *N/Dl* dosage combinations is that, in addition to interactions between Dl and Notch that result in the activation of the receptor, there are other interactions between these proteins that suppress the activity of Notch. Here we have explored whether the N^{Ax} gain-of-function alleles of Notch identify a domain in the Notch receptor that mediates antagonistic interactions between Notch and its ligands.

Abruptex proteins fail to restrict Notch activity to the dorsoventral boundary

As a prelude to analysing the consequences of manipulating the expression of Notch ligands in N^{Ax} mutant backgrounds, we have characterised the expression of several markers in N^{Ax} mutant discs. First we examined the expression of *ct* and *wg*, which is restricted to the dorsal and ventral cells forming the dorsoventral boundary in wild-type wing discs, but expands into a broad territory in some N^{Ax} mutants (Baker, 1988; Jack et al., 1991; Blochinger et al., 1993; de Celis et al., 1996; Micchelli et al., 1997). To determine whether there is a relationship between the severity of the N^{Ax} phenotype and the extent of *ct* and *wg* expression, we analysed several different N^{Ax} alleles and allelic combinations. The weak viable alleles Ax^{28} and Ax^{16172} do not modify *ct* and *wg* expression (data not shown). However, several N^{Ax} allelic combinations gave rise to characteristic patterns of ectopic *ct* and *wg* expression (Fig. 2A-D). For example, a transheterozygous combination of the viable alleles Ax^{28} and Ax^{16172} results in a subtle broadening of the domain of *ct* expression, whereas either of the lethal alleles Ax^{M1} and Ax^{59d} in hemizygosity causes a moderate extension of *ct* expression into the dorsal compartment (Fig. 2A-C and data not shown). The strongest phenotypes are observed in transheterozygotes between the lethal allele Ax^{M1} and the viable allele Ax^{16172}, where almost all cells in the wing blade express the dorsoventral boundary markers (Fig. 2D). A similar

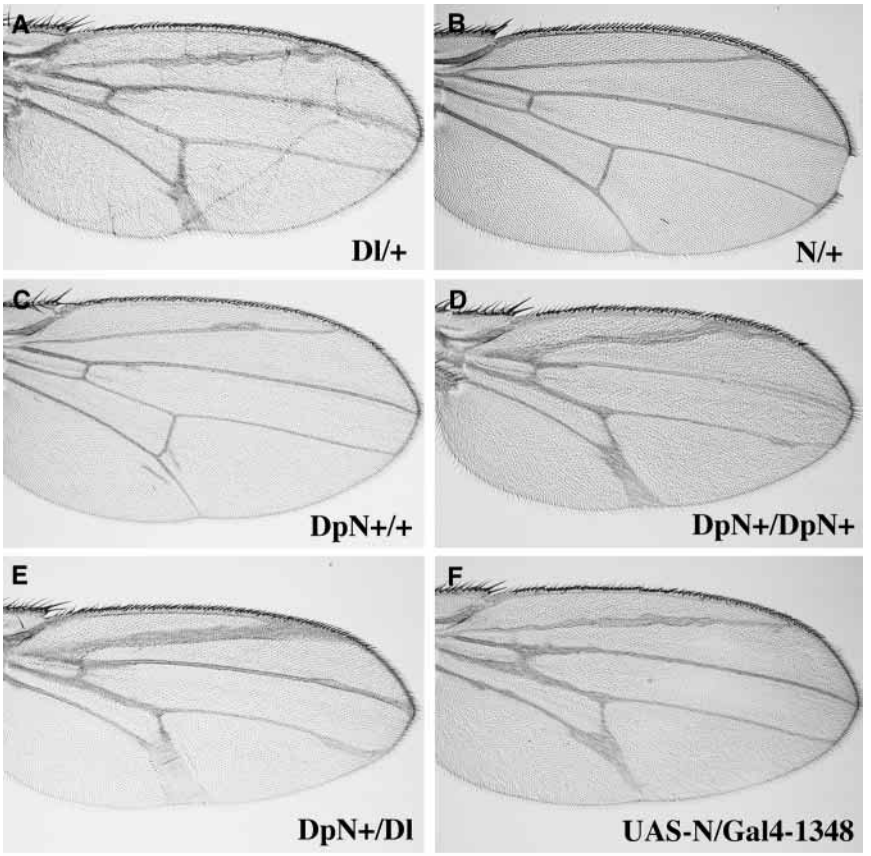

Fig. 1. Dose interactions between Dl and Notch. (A,B) Adult wing phenotypes of flies heterozygous for Dl^{M1} (Dl/+; A) and N^{55e11} (N/+; B). (C,D) Wing phenotype of flies carrying one (DpN^{+}/+; C) or two (DpN^{+}/DpN^{+}; D) extra copies of *Notch* ($Dp(1;2)w^{+51b7}$). Note the similarity in the vein pattern between *Dl* mutant wings and wings with two extra copies of *Notch*. (E) Wing phenotype of the combination DpN^{+}/+; *Dl*/+ showing an increase in the vein thickening phenotype. (F) Wing phenotype of ectopic expression of *Notch* in pupal wings using *Gal4-1348* as a driver.

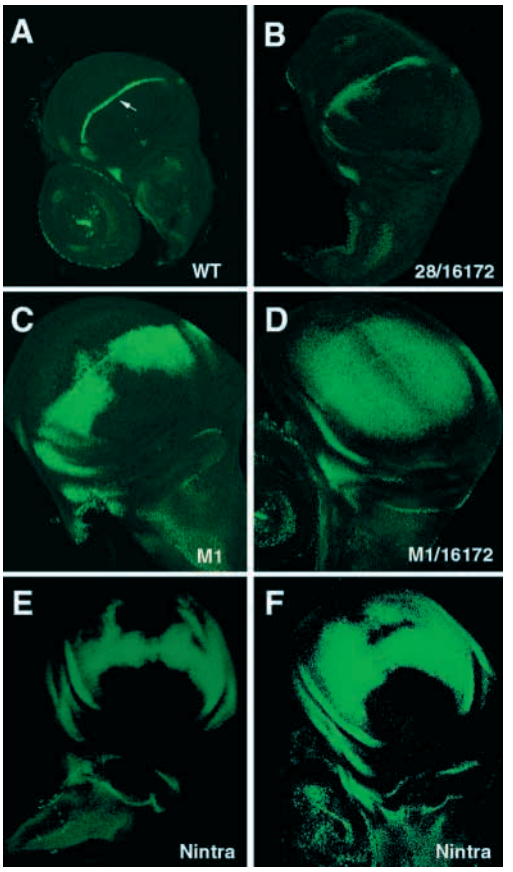

Fig. 2. Expression of *ct* in *Ax* mutant backgrounds. (A) Expression of Ct in wild-type discs is restricted to the dorsoventral boundary (arrow). (B-D) Expression of Ct in three different *Ax* allelic combinations Ax^{28}/Ax^{16172} (B), Ax^{M1} (C) and Ax^{16172}/Ax^{M1} (D), which cause different degrees of expansion in the *ct* domain. (E,F) Ectopic expression of Ct caused by generalised expression of a ligand-independent activated form of Notch (N^{intra}) in *UAS-N^{intra}/Gal4-756* discs.

domain of ectopic expression of *ct* and *wg*, localised close to the normal dorsoventral boundary, is observed when a constitutively activated fragment of Notch, N^{intra}, is expressed throughout the wing disc (Fig. 2E,F and data not shown). These results indicate that (1) the N^{Ax} phenotype is equivalent to that caused by inappropriate ectopic activity of Notch and (2) the sensitivity of *ct* to Notch activation is heterogenous in the wing pouch. There appears therefore to be a widespread but heterogenous potential to express *ct* and *wg*, which normally becomes restricted to the actual dorsoventral boundary by a mechanism requiring the integrity of the N^{Ax} domain of the Notch protein.

Secondly, we analysed the expression of several other genes that normally have restricted expression within the wing pouch including *apterous* (*ap*), *E(spl)mβ*, *Dl* and *Ser*, and the Wg target-genes *ventral veinless* (*vvl*) and *distalless* (*dll*). The expression of *ap* marks the dorsal compartment of the wing disc and the dorsoventral boundary forms where cells expressing *ap* confront cells that do not express *ap* (Cohen et al., 1992 Diaz-Benjumea and Cohen, 1993; Blair et al., 1994). In all N^{Ax} mutant backgrounds examined, Ap expression is normal, indicating that N^{Ax} alleles do not interfere with the initial specification of dorsal cells or the mechanism that prevents dorsal and ventral cells mixing (Fig. 3A,C and data not shown). The expression of Dl and Ser is heterogeneous in the wild-type wing pouch of late third instar discs, with maximal levels being present in the developing veins and in dorsal and ventral cells flanking the dorsoventral boundary (Fehon et al., 1991; Kooh et al., 1993; Speicher et al., 1994; Miccheli et al., 1997; de Celis and Bray, 1997; see Fig. 3E,H). In Ax^{M1}/Ax^{16172} and Ax^{M1} mutant discs, however, Dl is detected at homogeneous low levels throughout the wing pouch (Fig. 3I-K). The expression of *Ser* in Ax^{M1}/Ax^{16172} is also detected at homogeneous levels through the dorsal compartment and is also reduced in the veins (Fig. 3E-G). Similar changes are observed in the expression of a *E(spl)mβ*–CD2 fusion gene, which responds to Notch activity in the wing pouch. This is normally expressed at higher levels in intervein territories and at the dorsoventral boundary but in Ax^{M1}/Ax^{16172} discs, the heterogeneity in the expression of *E(spl)mβ* is lost and the level of expression appears to be homogeneously high (Fig. 3A-D).

Because there are some genetic interactions between *wg* and N^{Ax} mutations (Couso and Martinez-Arias, 1994; Hing et al., 1994), we also monitored the expression of two targets of Wg to see whether their response correlated with the domain of Wg in the normal way. In wild-type wing discs, *vvl* is repressed by Wg at the dorsoventral boundary and in the adjacent dorsal and ventral cells (de Celis et al., 1995; Fig. 3M,N) and, in all N^{Ax} mutant backgrounds, examined the domain of *vvl* repression correlates with the extended domain of *wg* expression (Fig. 3H,O). Similarly, expression of Dll, which is a positive target of Wg signalling, is extended in N^{Ax} mutant discs (Zecca et al., 1996; Fig. 3M,P). In addition, Wg-expressing clones are able to induce expression of Dll in Ax^{M1} mutant discs, in a similar manner to wild-type discs (see below). From these experiments, we conclude that, in N^{Ax} mutant backgrounds, a variable number of wing pouch cells acquire most characteristics typical of dorsoventral boundary cells, even though other parameters such as dorsoventral specification and Wg signalling are not affected.

To confirm that the ectopic activation of *ct* observed in N^{Ax} mutant backgrounds requires continuous Notch activity, rather than a single event early in the specification of the dorsoventral boundary, we made use of the temperature sensitivity associated with N^{Ax} lethal alleles (Portin and Sirem, 1976, Portin, 1977; de Celis and Garcia-Bellido, 1994). These alleles have typical N^{Ax} phenotypes at 17°C and at 25°C, but at 29°C behave as *N* loss-of-function lethal alleles. When N^{Ax} mutant larvae are shifted to the restrictive temperature in the third larval instar the expression of *ct* is lost from the dorsoventral boundary (Fig. 4B,D). In the same discs, clones of cells expressing Fng or Dl fail to induce *ct* expression (Fig. 4A,D and see below). These data indicate that Notch activity is required to maintain *ct* expression, both at the normal dorsoventral boundary and in the novel places where *ct* is induced by Notch ligands. Wg signalling appears normal in N^{Ax} mutant discs grown at the restrictive temperature, as *wg*-expressing clones are still able to activate *dll* and suppress *vvl* effectively (see below).

Effects of ectopic Dl and Ser on the activity of Abruptex proteins

We used the ectopic expression of *ct* observed around the

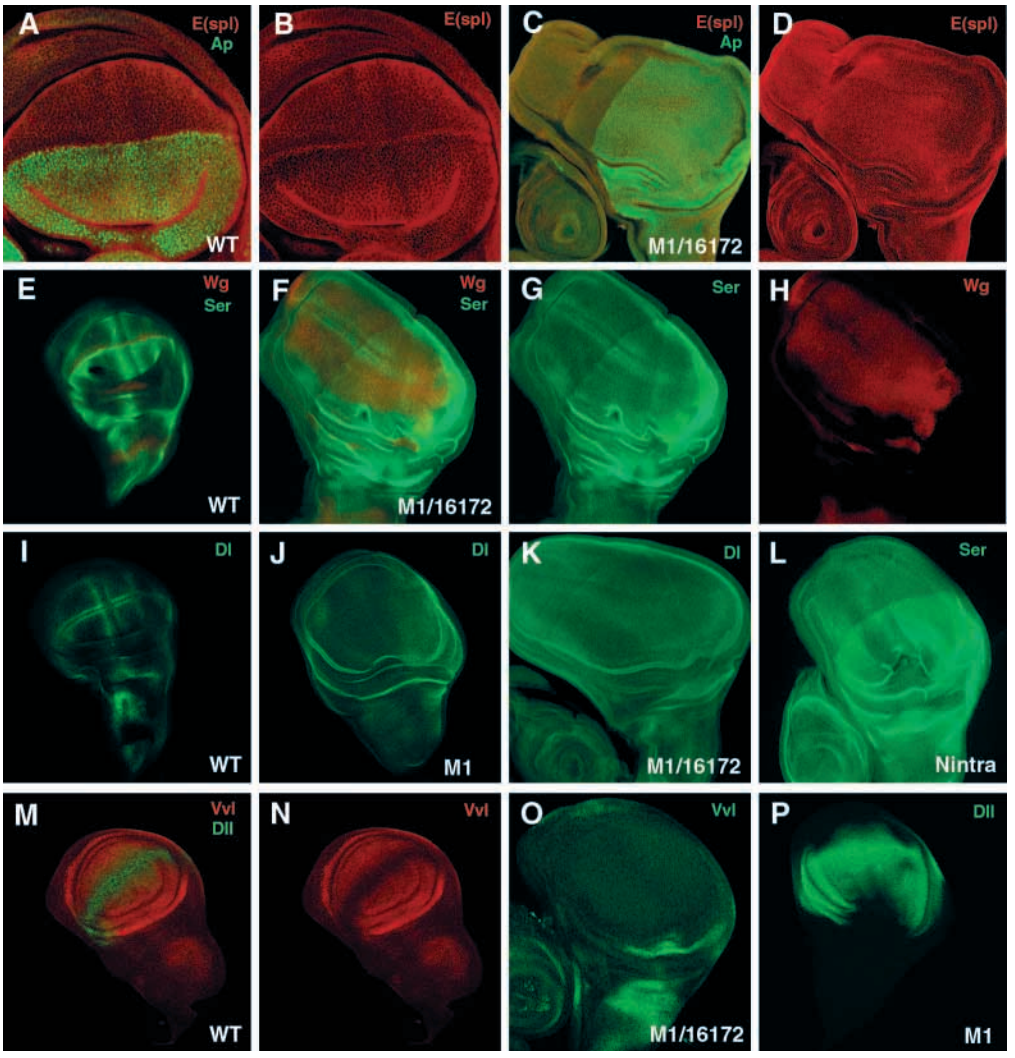

Fig. 3. Expression of dorsoventral markers and of Notch and Wingless targets in N^{Ax} discs. (A) Expression of Apterous (Ap, green) and E(spl)mβ–CD2 (E(spl), red) in wild-type discs. (B) The corresponding red channel. (C) Expression of Apterous (Ap, green) and E(spl)mβ–CD2 in Ax^{16172}/Ax^{M1} discs. The corresponding red channel is shown in D. (E) Expression of Ser (Ser, green) and Wg (Wg, red) in wild-type discs. (F-H) Expression of Ser (Ser, green in F and G) and Wg (Wg, red in F and H) in Ax^{16172}/Ax^{M1} discs. (I) Expression of Dl in a wild-type wing disc. (J,K) Expression of Dl in Ax^{M1} (J) and Ax^{16172}/Ax^{M1} (K) mutant discs. (L) Expression of Ser in *UAS-N^{intra}/Gal4-756* discs (Nintra). (M,N) Expression of Dll (Dll, green) and Vvl (Vvl, red in M and N) in wild-type wing disc. (O) Expression of Vvl (Vvl, green) in Ax^{16172}/Ax^{M1} disc. (P) Expression of Dll (Dll, green) in Ax^{M1} disc. The magnification is the same in C-P and double in A,B.

dorsoventral boundary in N^{Ax} mutant discs as an assay for the negative effects of Dl and Ser on Notch activity. In wild-type discs, the expression of high levels of Dl or Ser in clones of cells is always associated with repression of *ct* expression within the clone (Fig. 5A-C). This is similar to the phenotype observed when a dominant negative version of Notch (Notch^{ecd}) is misexpressed (Fig. 5F). Interestingly, the dominant negative effects of Dl are reduced when lower levels of Dl are ectopically expressed using a different Gal4 driver (Fig. 5D), indicating that these effects are very sensitive to the levels of ectopic Dl expression. Dl-expressing clones in dorsal regions and Ser-expressing clones in ventral regions also lead to *ct* being expressed in the cells adjacent to the clone (Fig. 5C,D). If Notch is simultaneously expressed in the same cells, Ct is detected in the Notch/Dl-expressing cells, as well as in the adjacent cells (Fig. 5E), indicating that the negative effects of the ligands are suppressed by increased levels of the receptor. Therefore, if N^{Ax} mutations perturb the negative interactions between Notch and its ligands, Ct should be detected within cells expressing high levels of Dl in a similar manner to the combined Dl/Notch and Ser/Notch ectopic expression clones (Jonsson and Knust, 1996; Doherty et al., 1996; Klein et al., 1997; this work). We therefore compared the effects of ectopically expressing Dl and Ser in three different N^{Ax} backgrounds. In the weak N^{Ax} heteroallelic combination Ax^{28}/Ax^{16172} and in Ax^{M1} hemizygous males, both Ser- and Dl-expressing cells are capable of suppressing *ct* cell autonomously, although a fraction of Ser and Dl clones fail to suppress *ct* expression in Ax^{M1} and Ax^{28}/Ax^{16172} discs, respectively (Table 1; Fig. 6A,B,D,E). In the strongest N^{Ax} heteroallelic combination (Ax^{16172}/Ax^{M1}), the suppression of *ct* expression by Dl is reduced so that a large fraction of clones exhibit *ct* expression (Table 1; Fig. 6C,F).

The partial failure of Dl and Ser to suppress *ct* expression in N^{Ax} backgrounds suggests that N^{Ax} mutant proteins have a reduced capacity to be antagonised by Dl and Ser. However, in these experiments, the levels of Gal4-driven Dl and Ser are very high compared to the endogenous expression (Fig. 5B). Therefore, we also analysed the effects of Dl and Ser on N^{Ax} activity in two different experimental situations where the levels of ectopic Dl and Ser are lower, first using the *spalt* Gal4 line (*Gal4-sal*) in combination with UAS-Dl and UAS-Ser, and second using the ectopic Dl and Ser expression induced in Wg-expressing clones. In wild-type discs, the combinations between *UAS-Dl* and *UAS-Ser* with *Gal4-sal* causes both inhibition of *ct* at the dorsoventral boundary and induction of *ct* in cells adjacent to the *sal* domain in the ventral (*Gal4-sal/UAS-Ser*; Fig. 7D) or dorsal (*Gal4-sal/UAS-Dl*; Fig. 7A)

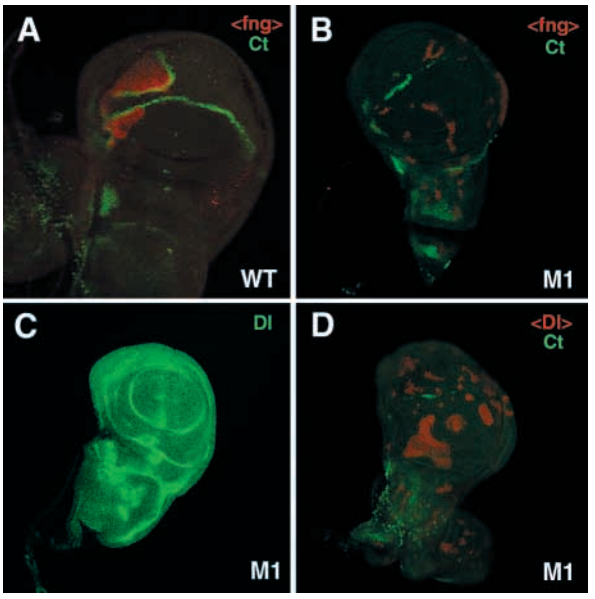

Fig. 4. Temperature sensitivity of *Ax* lethal alleles. (A) Ectopic expression of Ct (Ct, green) in the ventral compartment associated with a clone of Fng-expressing cells detected with anti-β-galactosidase (<fng>, red). (B) Partial suppression of Ct expression at the normal dorsoventral boundary in an Ax^{M1} disc grown at the restrictive temperature (29°C). Clones of cells expressing Fng (<fng>, red) do not induce ectopic Ct expression. (C) Expression of Dl in an Ax^{M1} disc grown at the restrictive temperature. (D) Failure of Dl-expressing clones (<Dl>, red) to induce Ct expression (Ct, green) in an Ax^{M1} mutant discs grown at the restrictive temperature.

Table 1. Number of clones of cells expressing Delta (<Dl>), Serrate (<Ser>), Fringe (<fng>) or Wingless (<wg>) induced in different N^{Ax} backgrounds (*26/16172*, *M1* and *16172/M1*)

	Dorsal			Ventral		
	+	–	+/–	+	–	+/–
28/16172						
<Dl>	29	0	12	35	0	0
<Ser>	15	0	0	18	0	0
<fng>	2	4	6	3	2	4
M1						
<Dl>	33	0	3	26	0	0
<Ser>	10	3	9	8	5	9
<fng>	0	20	0	0	18	0
<wg>	0	30	3	0	12	2
M1/16172						
<Dl>	6	9	3	6	3	1
<Ser>	11	4	0	6	2	3
<fng>	0	27	0	0	17	1

+, *ct* expression suppressed.
–, *ct* expression not suppressed.
+/–, partial suppression.
Dorsal and ventral indicates the dorsal and ventral wing compartments, respectively.

compartments. When Dl is similarly misexpressed in Ax^{M1} discs, however, *ct* is present throughout the entire dorsal domain of ectopic Dl expression (Fig. 7B). Similar effects are seen in the strong heteroallelic combination (Ax^{M1}/Ax^{16172}); ectopic Dl does not inhibit *ct* expression in the dorsal compartment although it can still inhibit it in the ventral compartment (Fig. 7C). In contrast to Dl, *Gal4-sal*-driven

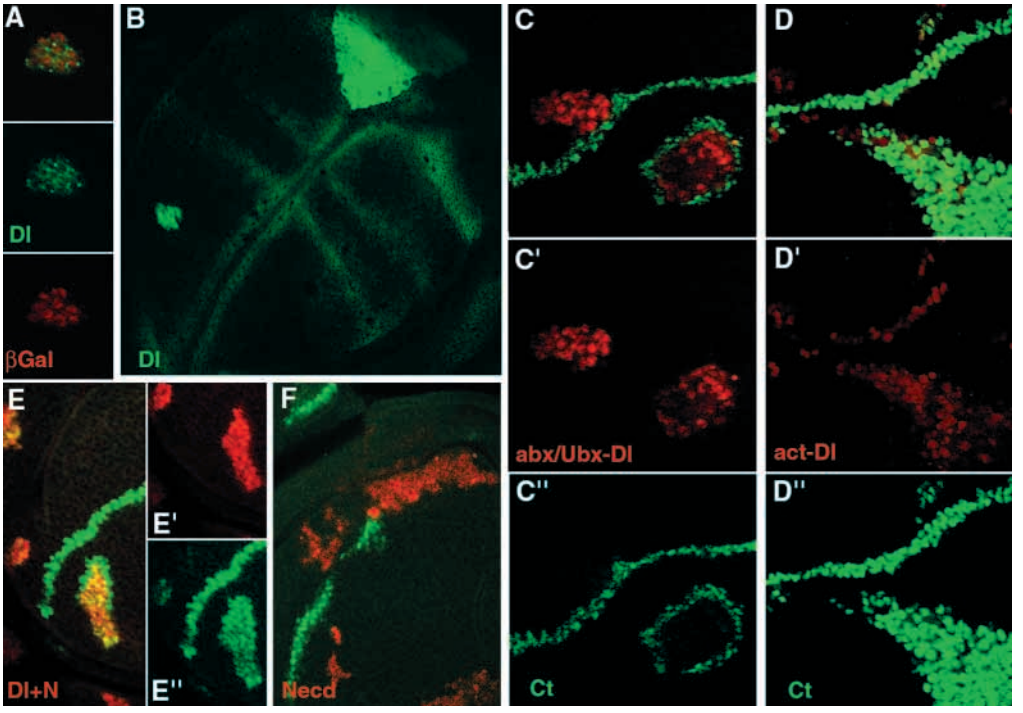

Fig. 5. Activation of *ct* in Dl-expressing clones. (A) Co-expression of β-galactosidase (βGal, red) and Dl (Dl, green) in a clone localised in the wing blade induced in larvae of genotype *hsFLP1.22; P{abx/Ubx<FRTf⁺FRT>Gal4-lacZ}/UAS-Dl*. (B) Expression of Dl (green) in the normal pattern (light green) and in two clones of Dl-expressing cells induced as in A (dark green spots). Note the difference in levels of expression between the endogenous Dl and its expression in the clones. (C) Induction of Ct (green, C″) in the dorsal compartment in Dl-expressing clones (red, C′) induced in *hsFLP1.22; P{abx/Ubx<FRTf⁺FRT>Gal4-lacZ}/UAS-Dl* larvae. (D) Induction of Ct (green, D″) in Dl-expressing clones (red, D′) induced in *hsFLP1.22;act5C<FRTy⁺FRT>Gal4 UAS-lacZ/UAS-Dl* larvae. (E) Induction of Ct (green, E″) in clones expressing Dl and Notch (red, E′) induced in *hsFLP1.22; P{abx/Ubx<FRTf⁺FRT>Gal4-lacZ}/UAS-Dl UAS-N* larvae. Note the difference with C. (F) Suppression of Ct (green) in N^{ecd}-expressing clones (red) induced in *hsFLP1.22; P{abx/Ubx<FRTf⁺FRT>Gal4-lacZ}/UAS-*N^{ecd} larvae.

ectopic expression of Ser still retains the capacity to suppress *ct* expression in both the dorsal and ventral compartments in Ax^{M1}/Ax^{16172}, although it appears less inhibitory in Ax^{M1} discs (Fig. 7E,F).

Clones of Wg-expressing cells cause autonomous ectopic expression of Dl and Ser (Micchelli et al., 1997; de Celis and Bray, 1997) and, in wild-type discs, we also observed induction of *ct* in ventral cells abutting the clones (Fig. 8A). When Wg-expressing clones are induced in Ax^{M1} heterozygous discs Ct is detected in cells adjacent to the clones in the dorsal and ventral compartments, but not within the clones (Fig. 8B). These results indicate that the levels of ectopic Dl and Ser induced by Wg are sufficient to cause both the dominant effects of the ligands and the activation of Notch. In Ax^{M1} hemizygotes, *ct* induction occurs both in cells adjacent to the Wg-expressing clone and in many cells within the clone, indicating that the levels of ectopic Dl and Ser induced by Wg are insufficient to inhibit the activity of N^{Ax} mutant proteins (Fig. 8C,D). In the same N^{Ax} mutant discs, ectopic expression of *wg* is still able to activate and suppress the Wg target genes *dll* and *vvl*, respectively, both at 25°C and at 29°C (Fig. 8E-H).

From these experiments, we conclude that N^{Ax} mutations impair the part of Notch that is involved in mediating the negative effects of the ligands on Notch. This is affected to different degrees, depending on the N^{Ax} mutation, but all of the alleles can still be inhibited at the highest levels of ectopic ligand expression.

Effects of *fng* on the activity of N^{Ax} proteins

In wild-type discs, the response of Notch to Dl and Ser is affected by the presence of Fng, which is expressed in dorsal cells (Irvine and Wieschaus, 1994). Since the domain of Fng expression corresponds to the region where Dl loses its capacity to antagonise Notch in N^{Ax} mutants, we analysed whether N^{Ax} mutations have an altered sensitivity to Fng by comparing the consequences of ectopic *fng* expression in wild-type and N^{Ax} discs. As with the ectopic ligand expression, clones of cells expressing *fng* that cross the dorsoventral boundary inhibit expression of *ct* except at the clone borders (Fig. 9B). When the Fng-expressing clones lie in the ventral compartment, *ct* is induced in the cells at the boundary of the clone, with the result that *ct* is detected in neighboring fng^{+} and fng^{-} cells (Fig. 9A,B). The ability of Fng to prevent *ct* expression is reduced when Fng-expressing clones are induced in N^{Ax} mutant backgrounds. In the weaker combination Ax^{28}/Ax^{16172}, the expression of *ct* is still highest at clone boudaries, but significant expression is detected within the clone (Table 1; Fig. 9D). In the more severe mutants, Ax^{M1} and Ax^{16172}/Ax^{M1}, the Fng-expressing cells have little or no inhibitory effect on *ct*, and there are high levels of Ct throughout the clone (Table 1; Fig. 9E,F). Similar effects are seen when *fng* misexpression is driven by *Gal4-sal*. Normally this causes an inhibition of *ct* expression at the dorsoventral boundary, in N^{Ax} mutant discs, however, Ct is detected throughout most of the domain of ectopic Fng-expression (Fig. 9C).

If the N^{Ax} domain is significant in the interactions between Notch and Fng, the N^{Ax} mutations should modify phenotypes caused by alterations in *fng* expression. In the allele fng^{D4}, *fng* is expressed throughout the wing pouch, causing severe scalloping of the wing margin (Irvine and Wieschaus, 1994; Fig. 10C). This correlates with the loss of *ct* and *wg* expression

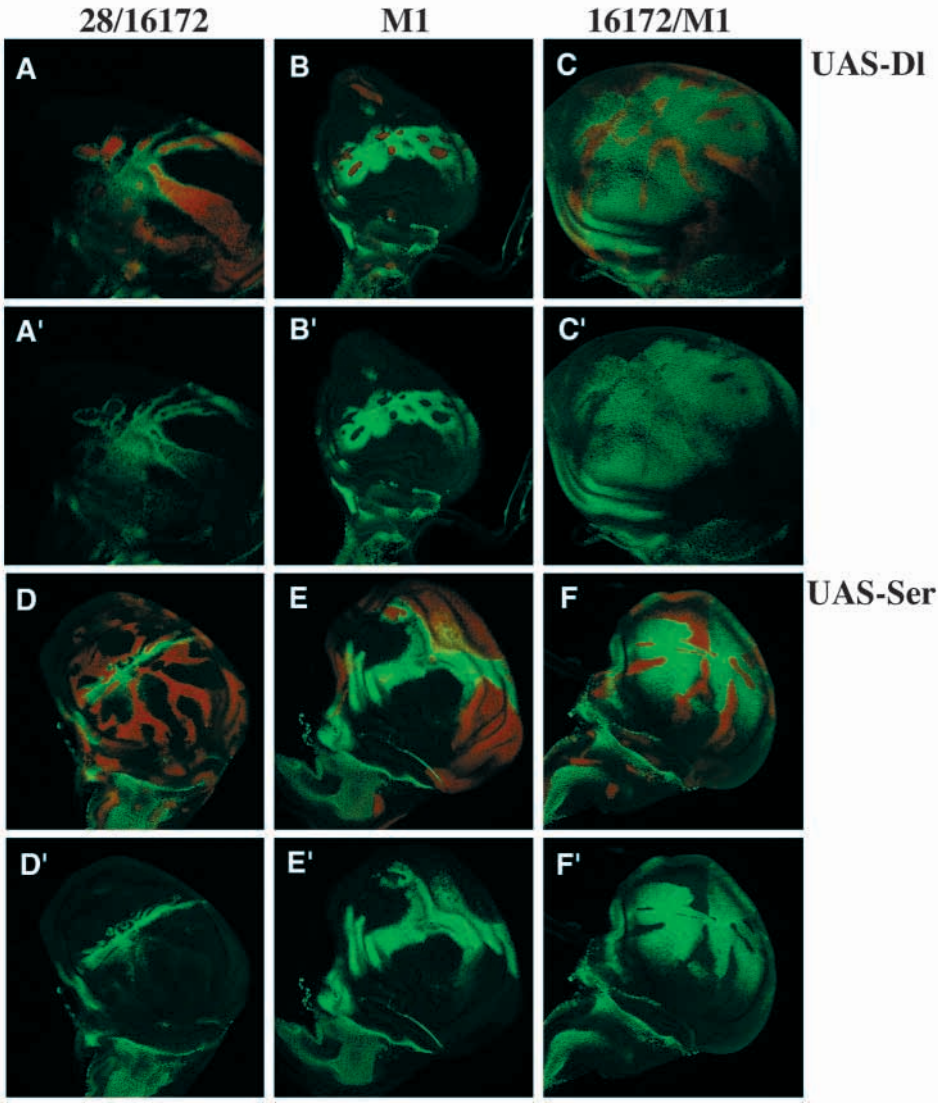

Fig. 6. Effects of clones of Dl- and Ser-expressing cells on Ct expression in N^{Ax} mutant backgrounds. (A-C) Clones of Dl-expressing cells (red) induced in Ax^{28}/Ax^{16172} (A), Ax^{M1} (B) and Ax^{16172}/Ax^{M1} (C) mutant discs. Ct expression is in green and the corresponding green channels are shown below (A′,B′,C′). (D-F) Clones of Ser-expressing cells (red) induced in Ax^{28}/Ax^{16172} (D), Ax^{M1} (E) and Ax^{16172}/Ax^{M1} (F) mutant discs. Ct expression is in green and the corresponding green channels are shown below (D′,E′,F′).

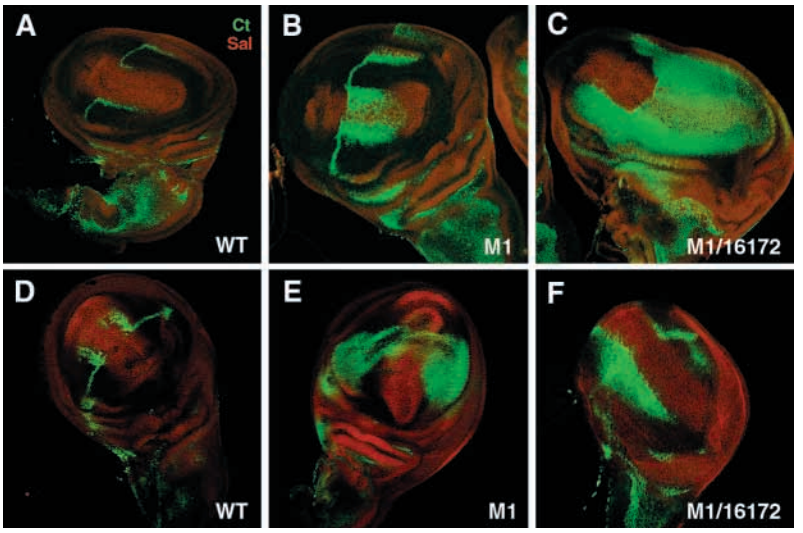

Fig. 7. Effects on Ct expression of ectopic Dl and Ser in the domain of expression of *spalt*. (A-C) *Gal4-sal/UAS-Dl* combination in (A) wild type, (B) Ax^{M1} and (C) Ax^{16172}/Ax^{M1}. Note the suppression of *ct* expression at the normal dorsoventral boundary when Dl is induced in wild-type discs, but not when Dl is induced in N^{Ax} backgrounds. Also note that in the dorsal compartment Ct is expressed throughout the domain of ectopic Dl expression. (D-F) *Gal4-sal/UAS-Ser* combination in (D) wild type, (E) Ax^{M1} and (F) Ax^{16172}/Ax^{M1}. The expression of Spalt is in red and the expression of Ct in green.

at the dorsoventral boundary and the expansion of *vvl* expression (Fig. 10A and data not shown). In Ax^{M1} heterozygous flies, the phenotype of fng^{D4} is reduced both at the level of wing scalloping and the expression of dorsoventral boundary markers (Ax^{M1}/+; fng^{D4}/+, Fig. 10D and data not shown). In hemizygous Ax^{M1} males, both the expression of *ct* and *vvl* and the adult phenotype are similar to that typical of Ax^{M1} (Fig. 10B,E). Taken together, these results suggest that N^{Ax} proteins are also deficient in some activity related to the capability of Fng to restrict Notch activity.

DISCUSSION

Wing development requires the activation of Notch at the confrontation between dorsal and ventral cells in the early wing disc (Diaz-Benjumea and Cohen, 1993, 1995; Couso et al., 1995; de Celis et al., 1996; Doherty et al., 1996). The consequences of this localised Notch activation include the transcription of genes such as *vestigial*, *wg* and *ct*, which mediate some of the effects of Notch on growth and patterning (Blair, 1995). In addition, Notch activity also participates in the lineage segregation between dorsal and ventral cells (Micchelli and Blair, 1999; Rauskolb et al., 1999). Several mechanisms contribute to the establishment of a localised domain of Notch activity at the dorsoventral boundary. They include the asymmetrical distribution of Fng, the localised expression of the ligands and the negative inhibition of Notch activity by the ligands Ser and Dl in cells flanking the dorsoventral boundary (Irvine and Vogt, 1997). The N^{Ax} alleles of *Notch* result in many more cells acquiring the characteristics of boundary cells, in that they transcribe the genes *ct* and *wg* (de Celis et al., 1996 and this work). The expansion of *ct* and *wg* that we detect correlates with the severity of the allele, suggesting that these mutations perturb to different extents a domain of Notch that is normally involved in restricting its activation. Furthermore, as the N^{Ax} mutations modify the consequences of ectopic ligand and *fng* expression, the affected region of the

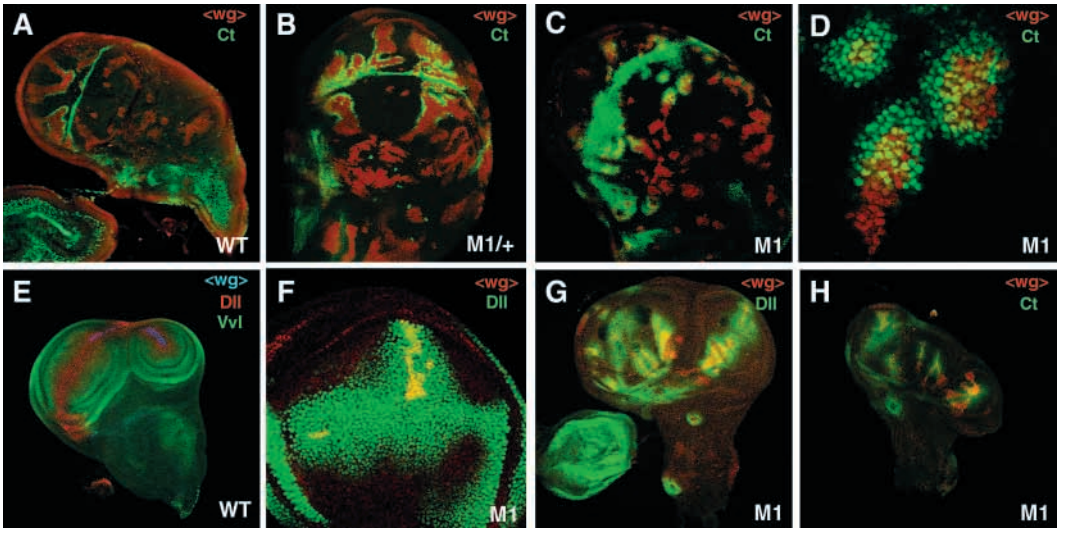

Fig. 8. Effects of Wg-expressing clones on Ct expression. (A) Induction of Ct (Ct, green) adjacent to Wg-expressing clones (<wg>, red) in the ventral but not in the dorsal wing compartment of wild-type discs. (B) Induction of Ct (Ct, green) adjacent to Wg-expressing clones (<wg>, red) in the ventral and dorsal wing compartments of Ax^{M} /+ discs. (C,D) Expression of Ct in Wg-expressing clones induced in Ax^{M} discs is present also within the clones. (D) High magnification picture of three dorsal clones shown in C. (E) Induction of Dll (red) and suppression of Vvl (green) expression caused by Wg-expressing clones (blue) in Ax^{M} /+ discs. (F,G) Induction of Dll (green) by wg-expressing clones (red) in Ax^{M} discs grown at 25°C (F) and 29°C (G,H).

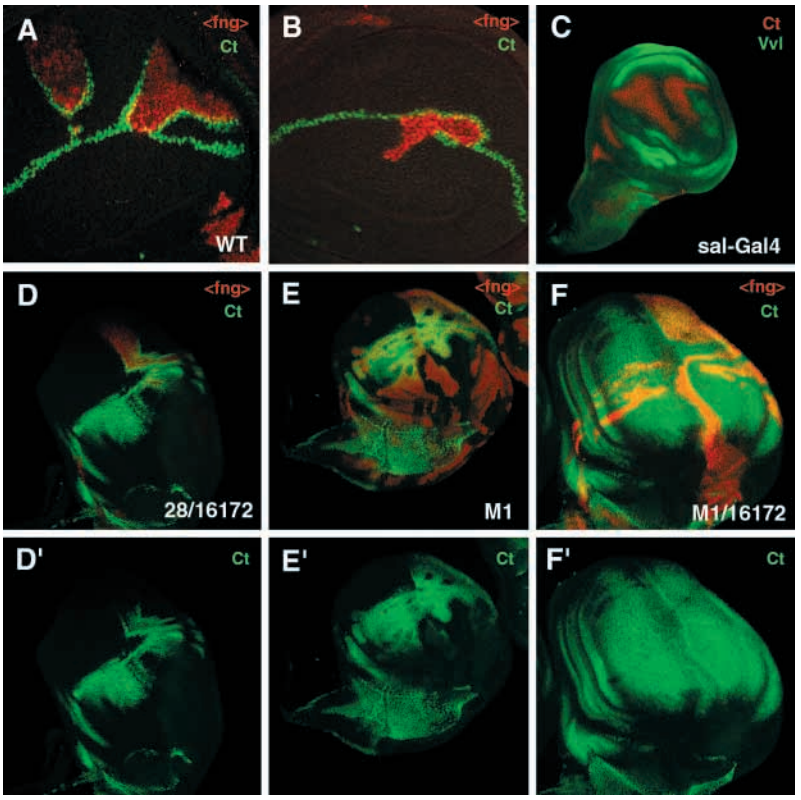

Fig. 9. Effects of *fng* on Ct expression in N^{Ax} mutant discs. (A,B) Induction of Ct (Ct, green) by Fng-expressing clones (<fng>, red) in the ventral compartment (A) and suppression of Ct at the dorsoventral boundary by a Fng-expressing clone that included the dorsoventral boundary. (C) Expression of Ct (red) and Vvl (green) in *sal-Gal4/+; UAS-fng/+* flies. (D-F) Expression of Ct (green) in Fng-expressing clones induced in (D) Ax^{28}/Ax^{16172}, (E) Ax^{M1} and (F) Ax^{16172}/Ax^{M1}. The corresponding green channels are shown below as D′, E′ and F′, respectively.

extracellular domain is likely to be mediating negative interactions with the ligands and may be a target for modification by Fng.

Expression of *ct* and *wg* in cells flanking the normal boundary cells also occurs in clones of cells that lack both *Dl* and *Ser* (Micchelli et al., 1997). This demonstrated that the ligands have a cell-autonomous inhibitory effect on Notch activation and it is consistent both with the results of varying the relative doses of Notch and Delta, and with the results obtained from misexpressing Notch ligands (Kim et al., 1995; Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Doherty et al., 1996; Thomas et al., 1995; Jonsson and Knust, 1996; de Celis and Bray, 1996, Klein et al., 1997). Since the effect of N^{Ax} alleles is similar to the removal of Dl and Ser, it is likely that the negative effects of the ligands and the domains identified by N^{Ax} mutations are related. Our results support this hypothesis, particularly with respect to Dl: the negative effects observed when Dl is ectopically expressed are abrogated by strong N^{Ax} mutations. This is most dramatic in the heteroalleleic combination Ax^{16172}/Ax^{M1}, where ectopic Dl loses its ability to perturb expression of the boundary marker *ct* in the dorsal compartment. Although the modifications to the behavior of Ser are less dramatic, nevertheless, in some N^{Ax} alleles, the dominant negative effects of Ser are also compromised.

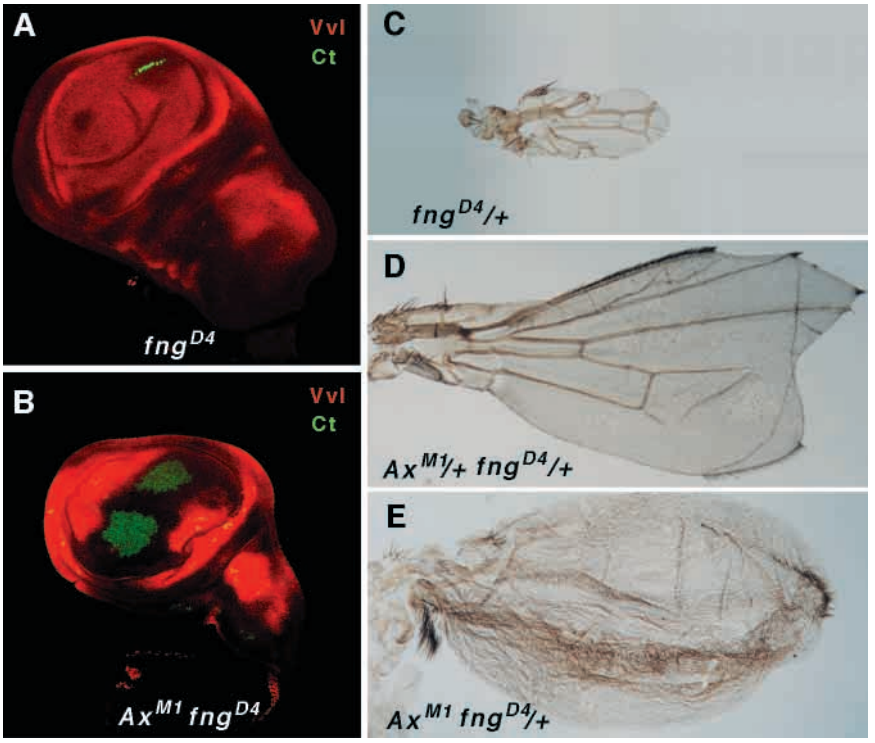

Fig. 10. Rescue of the fng^{D4} allele in Ax^{M1} mutants. (A) Expression of Ct (green) and Vvl (red) in fng^{D4} heterozygous discs. (B) Expression of Ct (green) and Vvl (red) in Ax^{M1}; $fng^{D4}/+$ discs. (C) Wing phenotype of $fng^{D4}/+$. (D,E) Rescue of the $fng^{D4}/+$ phenotype in Ax^{M1} (D) heterozygous and (E) hemizygous wings.

The activity of N^{Ax} proteins requires interactions with the ligands, at least during sensory organ and vein development (Heitzler and Simpson, 1993; de Celis and Garcia-Bellido, 1994). It has been suggested that the basis for the *Ax* phenotypes is an increased sensitivity to Notch ligands by N^{Ax} proteins (Heitzler and Simpson, 1993). Based on this interpretation, the increased sensitivity to positive interactions between the ligands and N^{Ax} proteins would mask a normal level of negative interactions. However, when combined with a Notch deficiency (N^{Ax}/N^{-}), all strong *Ax* alleles demonstrate an insufficiency of Notch function (de Celis and Garcia-Bellido, 1994) showing that these proteins actually have less than normal Notch activity. This is consistent with the observation that the binding in vitro between N^{Ax} and Dl proteins is reduced, suggesting that N^{Ax} have less than normal affinity for the ligands (Lieber et al., 1992). Therefore, although our data are compatible with the hypothesis that N^{Ax} alleles have a greater capability to be activated by the ligands Dl and Ser, we favour the interpretation that the N^{Ax} proteins have a reduced susceptibility to the negative effects of the ligands. It is also likely that a reduction in negative interactions between N^{Ax} and the ligands results in an apparent increase of the sensitivity of N^{Ax} proteins to activation by the same ligands. In the context of the dorsoventral boundary, this would explain why there is spreading of Notch activity, as assayed by boundary markers, in N^{Ax} mutants. Increased Notch activity caused by a failure of negative interactions also explains the results of mosaic experiments, where N^{Ax} mutant cells behave as though they have a greater activity of Notch than neighboring wild-type cells (de Celis et al., 1991b; Heitzler and Simpson, 1993).

The failure of negative inhibition could also explain other N^{Ax} phenotypes, such as loss of wing veins and bristles (Portin, 1975; Foster, 1975, Heitzler and Simpson, 1993; de Celis and Garcia-Bellido, 1994). In the case of the wing veins, the cells that form the vein itself contain the highest levels of Dl and, in these cells, Notch is not activated (de Celis et al., 1997; Huppert et al., 1997). The negative effects of Dl in the vein cells could be one of the mechanisms that permit the selection of the stripe of vein-forming cells from the provein field. A similar mechanism could operate during selection of sensory precursors; the accumulation of Dl by a precursor cell would render any Notch on its surface less capable of responding to Dl on the surrounding cells. If, as we propose, N^{Ax} mutations perturb the negative effects of the ligands, they would compromise this mechanism for silencing Notch in the vein and sensory organ precursors, thereby increasing the probability that Notch would be activated in these cells, thus suppressing vein and sensory organ precursor fates respectively. This interpretation implies that negative interactions between Notch and its ligands regulate Notch activity in many developmental processes in addition to the establishment of developmental boundaries.

Two other proteins that have been associated with Notch function are Wg and Fng (Couso and Martinez-Arias, 1994; Hing et al., 1994; Fleming et al., 1997; Panin et al., 1997; Klein and Martinez-Arias, 1998). In our assays, no difference in the activity of Wg is detected in N^{Ax} discs, based on the expression of Wg-target genes such as *vvl* and *dll*. Furthermore, Wg signalling also appears to function normally when Notch activity is reduced in N^{Ax} lethal alleles grown at the restrictive temperature. The activity of Fng is, however, modified by N^{Ax} mutations, both in ectopic expression experiments and in combinations between N^{Ax} and *fng* alleles. In wild-type discs, misexpression of *fng* inhibits expression of *ct* at the dorsoventral boundary and results in the expression of boundary-cell markers at the novel confrontation between ventral cells expressing and not expressing *fng*. However, in N^{Ax} discs, there is little or no suppression of *ct* expression by ectopic *fng*, and *ct* is activated both within and surrounding *fng*-expressing cells. Furthermore, N^{Ax} mutations modify the phenotypes produced by fng^{D4}, an allele of *fng* that results in expression of *fng* throughout the wing pouch (Irvine and Wieschaus, 1994), restoring the expression of boundary markers in these discs. Taken together, these results suggest that N^{Ax} mutations alter the sensitivity of Notch to Fng, in particular they alter the sensitivity to the inhibitory actions of Fng. The amino-acid sequence of Fng indicates that it could be a glycosyltransferase (Yuan et al., 1997). Since N^{Ax} mutations affect the extracellular domain of Notch (Kelley et al., 1987), the fact that the N^{Ax} alleles have altered behavior with respect to Fng suggests that the mutated domain could be a target for Fng-mediated glycosylation. If the N^{Ax} mutations perturb glycosylation of Notch by Fng, this might explain why they only affect the activity of Notch in the imaginal discs and not in the early embryo, since *fng* is only required at later stages of development. N^{Ax} alleles also affect several processes, such as sensory organ development and vein cell differentiation, that do not seem to require *fng* activity (J. F. dC., unpublished results). This indicates that the N^{Ax} domain also affects negative interactions between Notch with Dl and Ser independently of *fng* function.

The results shown here indicate that the N^{Ax} domain of Notch is only necessary to mediate the functions of Fng and the ligands that result in suppression of Notch activity. A comparison between the effects on Fng, Dl and Ser indicates that the interactions between these molecules and Notch are affected to different extents by N^{Ax} mutations. For example, although the dominant negative effects of Dl and Fng are dramatically reduced in N^{Ax} alleles, these mutations do not appear to compromise the potentiating effect of Fng on Dl activation, since there is still a strong bias towards Dl activity in the dorsal domain where Fng is present. Similarly high levels of ectopic Ser can efficiently suppress Notch activity in N^{Ax} backgrounds, even though the phenotype of N^{Ax} mutant discs indicates that N^{Ax} mutations perturb the dominant negative effects of Ser when it is expressed at normal levels. Each N^{Ax} allele has a characteristic strength that is reflected in their phenotypes and in the extent of ectopic *ct* activation. Furthermore, heteroallelic combinations between N^{Ax} alleles often result in synergistic phenotypes, a phenomenon called negative complementation (Portin, 1975; Foster, 1975). This suggests that the correct conformation of the N^{Ax} domain in Notch multimers is critical for the efficiency of the interactions between Notch, its ligands and Fng that determine suppression of Notch activity.

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