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Down-regulation of Notch target gene expression by Suppressor of deltex

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Abstract

In *Drosophila, Suppressor of deltex (Su(dx))* mutations display a wing vein gap phenotype resembling that of *Notch* gain of function alleles. The Su(dx) protein may therefore act as a negative regulator of Notch but its activity on actual Notch signalling levels has not been demonstrated. Here we show that Su(dx) does regulate the level of Notch signalling in vivo, upstream of Notch target genes and in different developmental contexts, including a previously unknown role in leg joint formation. Overexpression of Su(dx) was capable of blocking both the endogenous activity of Notch and the ectopic Notch signalling induced by the overexpression of Deltex, an intracellular Notch binding protein. In addition, using the conditional phenotype of the *Su(dx)^sp* allele, we show that loss of Su(dx) activity is rapidly followed by an up-regulation of *E(spl)mβ* expression, the immediate target of Notch signal activation during wing vein development. While *Su(dx)* adult wing vein phenotypes are quite mild, only affecting the distal tips of the veins, we show that the initial consequence of loss of *Su(dx)* activity is more severe than previously thought. Using a time-course experiment we show that the phenotype is buffered by feedback regulation illustrating how signalling networks can make development robust to perturbation.
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Keywords: Notch; Suppressor of deltex; *Drosophila*; Ubiquitin ligase

Introduction

The Notch receptor signalling pathway has multiple roles during development, including lateral inhibition, boundary formation, and lineage decisions (reviewed by Artavanis-Tsakonas et al., 1999; Baron et al., 2002; Bray 1998; Mumm and Kopan, 2000). The Notch signal involves an extracellular, ligand-dependent, cleavage of the receptor. A subsequent Presenilin-dependent cleavage then releases the intracellular domain from the membrane, allowing it to translocate to the nucleus, where it binds to the transcriptional regulator Suppressor of Hairless (Su(H)) and activates transcription of target genes such as those of the *Enhancer of split (E(spl))* gene complex (Kidd et al., 1998; Kopan et al., 1996; Lecourtois and Schweisguth, 1998; Schroeter et al., 1998). Other less well-characterised Su(H)-independent signals may also contribute to some functions of Notch (Brennan et al., 1999; Ramain et al. 2001; Wesley and Saez, 1999). Numerous proteins have been identified which regulate Notch, some through its covalent modification such as the glycosyl-transferase Fringe (Bruckner et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000), and others through direct binding to the Notch intracellular domain. The latter class includes the positive regulator Deltex (Matsuno et al., 1995) and the negative regulators Dishevelled and Numb (Axelrod et al., 1996; Guo et al., 1996). We have identified an additional gene, *Suppressor of deltex (Su(dx))*, which based on genetic analysis, we hypothesise may also regulate the Notch pathway (Cornell et al., 1999; Fostier et al., 1998). *Su(dx)* encodes a member of

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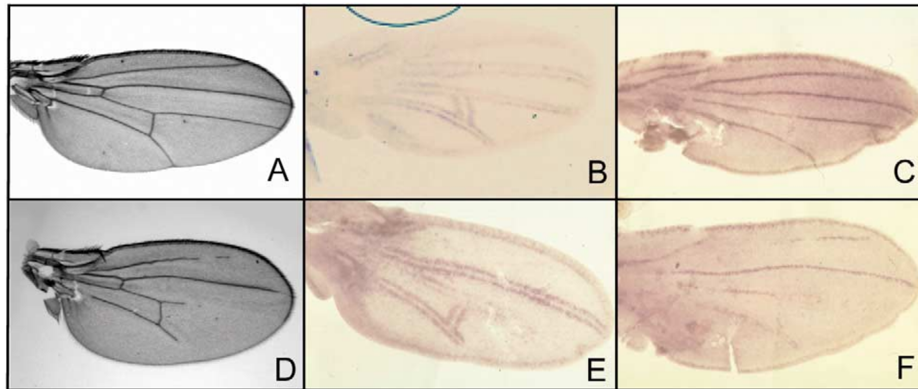

Fig. 1. The *Su(dx)* phenotype. (A) Wild-type wing. (B and C) Wild-type pupal wings in situ stained for *E(spl)mβ* and *rhomboid* expression, respectively. *E(spl)mβ* expression reflects Notch activation in the cells at the borders of the vein precursor territories where it down-regulates *rhomboid* expression. (D) *Su(dx)*^{sp} wing displaying wing vein gaps in the longitudinal veins. (E and F) *Su(dx)*^{sp} pupal wings, in situ stained for *E(spl)mβ* and *rhomboid* expression, respectively, showing gaps in the expression pattern of both genes corresponding to the adult phenotype. All flies were reared at 29°C.

the Nedd4 class of HECT domain proteins (Rotin et al., 2000). HECT domains from several proteins have been implicated in the protein ubiquitin labelling pathway (Huibregtse et al., 1995). We have shown that loss of function *Su(dx)* mutations display a wing vein gap phenotype which resembles that of a gain of function of Notch activity, for example, as found in the *Abruptex (Ax)* class of *Notch* alleles. In addition mutations that lead to elevated Notch pathway activity enhance the penetrance and severity of the *Su(dx)*^{sp} mutant phenotype. These data are however insufficient to conclude a direct role for *Su(dx)* in Notch pathway signalling and an activity of Su(dx) in vivo on actual Notch signalling levels has not yet been demonstrated. Such is the cross-talk between signalling pathways which leads to wing vein formation (de Celis et al., 1997; Sturtevant and Bier 1995) that it is possible that Su(dx) might have an indirect effect on Notch via another signalling pathway, act on a parallel signal, or act downstream of immediate Notch target genes.

To answer these questions, we have examined the in vivo expression of *Drosophila* genes which act as reporters of Notch signalling levels. We show that loss and gain of function of *Su(dx)* regulates Notch target gene expression in different developmental contexts, in a fashion which is consistent with a role as a negative regulator of the Notch pathway. Furthermore, we show that Su(dx) can block the Notch activation phenotype induced by the overexpression of the positive regulator, Deltex. Using the temperature sensitivity associated with the *Su(dx)*^{sp} vein gap phenotype, we show that an elevation of Notch signalling in the pupal wing is an early response resulting from the shift to a nonpermissive temperature. By following a time course of Notch target gene expression, we show that this early response is compensated for by feedback regulation which substantially moderates the final outcome of the adult *Su(dx)*^{sp} phenotype. Thus the development of the wing veins is robust to perturbation of Notch activity and this partially suppresses the consequences of *Su(dx)* mutation at the adult phenotypic level. The development of other tissues may be similarly resistant to perturbation induced by *Su(dx)* loss of function, masking wider roles for this gene. In support of this supposition we uncover, in an enhancing genetic background, a previously unknown role for *Su(dx)* in the formation of the leg joints. We also discuss allele-specific interactions of *Su(dx)* with two enhancing *Notch* alleles, and data which indicate that *Su(dx)* may have additional roles other than that of Notch down-regulation.

Materials and methods

Drosophila stocks

The *UAS::Su(dx), UAS::dx, Su(dx)*^{sp}, *notchoid*^{1} *(nd*^{1}*)*, and the vestigial boundary enhancer element *Lac*Z reporter (referred to as *vg*^{BE}*-LacZ*) fly stocks are as previously described (Fostier et al., 1998; Lindsley and Zimm, 1982; Matsuno et al., 2002; Williams et al., 1994). The *UAS::Su(dx)*$^{\Delta\text{-}HECT}$ construct is derived from a replacement of a *Sac*I fragment of plasmid 2bIa (Cornell et al., 1999) with a primer sequence carrying an in-frame stop codon. This removes the reading frame C-terminal to amino acid 628 of Su(dx), deleting the HECT domain, but leaving the N-terminal C2 domain and four WW domains intact. The *UAS::Su(dx)*$^{\Delta\text{-}HECT}$ construct was inserted into the Gal4 UAS expression vector pUAST (Brand and Perrimon, 1993) and transgenic lines were created by embryo injection using standard methodology. Expression of the *UAS::Su(dx)*$^{\Delta\text{-}HECT}$construct was tested by in situ hybridisation following crossing to Gal4 driver lines and was found to be efficiently expressed at levels similar to the full-length *UAS::Su(dx)* construct (data not shown).

In situ hybridisation

In situ hybridisation on late third instar discs or pupal wings was performed using digoxigenin-labelled antisense

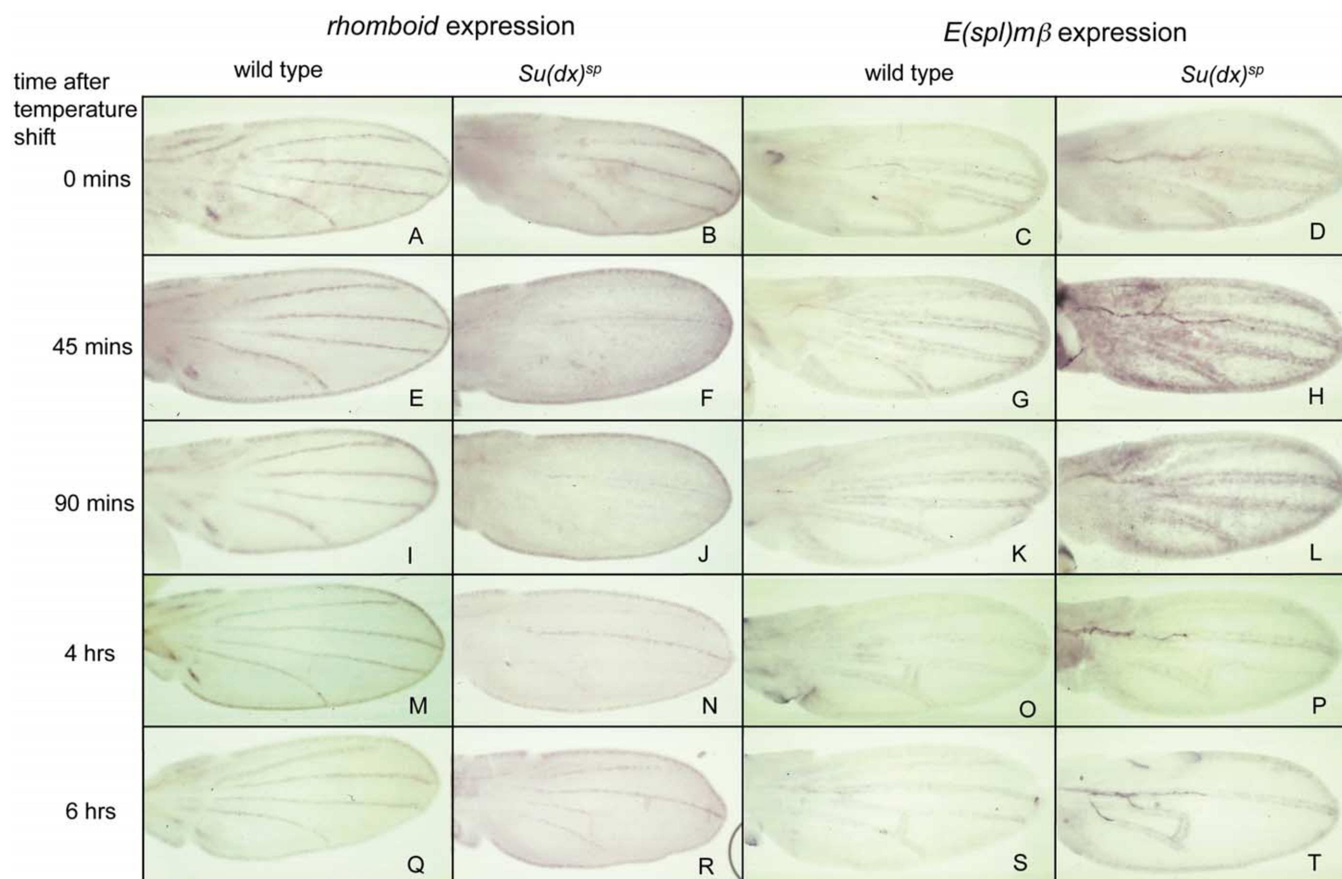

Fig. 2. *Su(dx)* loss of function in the pupal wing induces increased Notch signalling which is moderated in a subsequent time course. Larvae were cultured and allowed to pupate at 25°C. After 24 h APF pupae were either dissected and fixed for RNA in situ staining (A–D) or were transferred to 29°C for 45 min (E–H), 90 min (I–L), 4 h (M–P), or 6 h (Q–T) before fixing and staining. A, C, E, G, I, K, M, O, Q, S are wild-type; B, D, F, H, J, L, N, P, R, T are $Su(dx)^{sp}$. The consequences of $Su(dx)^{sp}$ mutation on Notch signalling were monitored by in situ staining of *rhomboid* (A, B, E, F, I, J, M, N, Q, R) and *E(spl)mβ* (C, D, G, H, K, L, O, P, S, T). In wild-type pupal wings the temperature shift has no discernable consequence on *rhomboid* or *E(spl)mβ* expression levels or pattern. In $Su(dx)^{sp}$ mutants 45 min after the shift to the nonpermissive temperature, *E(spl)mβ* expression is strongly elevated (H) compared to wild-type (G). In (H) *E(spl)mβ* expression can be seen to be elevated along the borders of the presumptive wing veins where it is normally expressed and also in the intervein regions and within the central provein territories. At the same time point *rhomboid* expression is reduced in the $Su(dx)^{sp}$ mutants (F) compared to wild-type (E). Elevated *E(spl)mβ* expression is maintained throughout the pupal wing up to 90 min following the time shift (compare L with K). At this time point *rhomboid* expression reaches a minimum in $Su(dx)^{sp}$ mutant pupal wings, being virtually undetectable in the longitudinal veins apart from some residual expression in L3 (J). Four hours following the temperature shift, *E(spl)mβ* expression across the whole pupal disc has declined back to wild-type levels (compare P and O) but with gaps in its expression pattern along the vein borders beginning to emerge. At the same time *rhomboid* expression is beginning to increase towards wild-type levels especially in the L3 vein (N). Six hours after the temperature shift, where expression of *E(spl)mβ* and *rhomboid* are detectable in the $Su(dx)^{sp}$ mutant wings, the level of expression resembles that found in wild-type wings. However gaps in the expression of both *E(spl)mβ* (T) and *rhomboid* (R) have appeared which correspond well with the positions of wing vein gaps observed in adult $Su(dx)^{sp}$ flies.

cRNA probes and staining with alkaline phosphatase conjugated anti-digoxigenin antibody as previously described (Cornell et al., 1999). For time-course experiments *Drosophila* progeny were cultured at 25°C until 24 h after puparium formation (APF) before vials were transferred to a water bath at 29°C for specific times before fixation. Digoxigenin-labelled cRNA probes were prepared using a cRNA labelling kit (Boehringer Mannheim) according to the manufacturer's instructions. For each experiment in situs were performed on mutant and wild-type pupae in the same tube for each time point, to avoid any variability of staining. To subsequently distinguish the different genotypes, one class was marked by the removal of the pupal heads. This procedure was found not to affect the efficiency of the in situ technique.

β-Galactosidase staining

Late third instar larvae were dissected and fixed for 20 min at room temperature (RT) in phosphate-buffered saline (PBS) + 0.5% glutaraldehyde. The discs were then washed in PBS + 0.2% Tween (PBTw) for 30 min at RT. Discs were stained at 37°C in staining solution {PBS + 0.3% Xgal + 5 mM $K_3[Fe^{III}(CN)_6]$ + 5 mM $K_4[Fe^{II}(CN)_6]$} for 20–30 min and the reaction was stopped by washes in PBTw. Discs were mounted in glycerol.

Immunological staining

Late third instar larvae were dissected and fixed for 20 min RT in PBS + 4% formaldehyde. The discs were then washed in PBTw for a minimum of 30 min at RT and blocked overnight at 4°C in permeabilisation solution (PBS + 0.2% Triton X + 0.2% Saponin + 0.3% deoxycholate). The mouse anti-Cut primary antibody (Hybridoma bank, University of Iowa) was then incubated at a dilution of 1:10 (in PBTw) for minimum of 4 h at RT and then washed for 1 h at RT in PBTw prior to incubation with the secondary antibody, anti-mouse horseradish peroxidase (Sigma), at a dilution of 1:100 in PBTw, for 90 min at RT. The antibody was washed in PBTw for 1 h at RT before the antibody conjugate was detected using a DAB substrate kit for peroxidase (Vector Laboratories). Discs were mounted in glycerol.

Results

Su(dx) regulates Notch target gene expression in pupal wing vein development

We have previously shown that *Su(dx)*^{sp} has a temperature-sensitive wing vein gap phenotype with rising penetrance when flies are reared at increasing temperatures. To determine whether Su(dx) activity affected actual Notch signalling levels, we investigated the effect of the *Su(dx)*^{sp} mutation on the expression of the immediate Notch target gene *E(spl)mβ*, which marks the borders of the developing vein territories. When *Su(dx)*^{sp} mutant flies were raised at the nonpermissive temperature of 29°C, gaps in the expression pattern of *E(spl)mβ* along the longitudinal veins were observed (Fig. 1), which corresponded to the positions of gaps in the wing veins observed in adult flies, i.e., the distal tips of longitudinal veins L4 and L5 were most frequently affected, L2 was variably affected, and gaps in the L3 vein were infrequent. This result was apparently in conflict with the prediction that *Su(dx)* loss of function causes elevated Notch activity, since the latter would be expected to result in increased *E(spl)mβ* expression. However it is possible that the loss of *E(spl)mβ* expression was the end result of a series of events that, on eliminating vein cell fate, resulted in loss of all vein-associated expression. For example, despite the fact that *E(spl)mβ* normally represses *rhomboid* expression, the latter is also lost in a similar pattern in the *Su(dx)*^{sp} mutant wings (Fig. 1). To investigate this further we used a temperature jump experiment to allow the time course of events to be followed, subsequent to transfer to the nonpermissive temperature. Fig. 2 shows the time course for the expression of *E(spl)mβ* after shifting to 29°C. The temperature jump did not perturb *E(spl)mβ* expression in wild-type pupae, but *E(spl)mβ* expression became elevated in the *Su(dx)*^{sp} pupal wings with maximal expression occurring around 45 min following the shift. The elevated *E(spl)mβ* expression was not restricted to the borders of the veins where Notch is normally activated but was also observed within the central provein region and in the intervein cells (Figs. 2H and 2L and Figs. 3A and 3B). We also investigated in a similar temperature jump experiment the expression pattern of *rhomboid* whose expression is down-regulated by *E(spl)mβ* activity (de Celis et al., 1997). Consistent with our observations of elevated *E(spl)mβ* expression in the *Su(dx)*^{sp} background, *rhomboid* expression decreased in the *Su(dx)*^{sp} pupal wings following the temperature shift. Maximal repression of rhomboid expression was achieved around 90 min after the temperature shift when it was not detectable, apart from in the presumptive L3 vein, which had a gapped expression pattern. Together, these data support a role of Su(dx) in the regulation of the Notch pathway, upstream of the activation of *E(spl)mβ* expression.

The Su(dx) phenotype is moderated during a developmental time course

When the time-course experiment was extended to include later time points, we found that the initially elevated *E(spl)mβ* expression levels were maintained for up to 2 h (data not shown), but then decreased to approach wild-type levels between 2 and 4 h (Fig. 2). Moreover gaps in the expression pattern emerged during this time and 6 h after shifting, the *E(spl)mβ* expression pattern was indistinguishable from that seen when *Su(dx)*^{sp} flies were maintained in continuous culture at 29°C, as in Fig. 1. Over the same time period of 2 to 4 h after the temperature shift, *rhomboid* expression levels began to increase with expression in the L3 vein being restored first, followed by the other longitudinal veins. However only the *rhomboid* expression in the L3 vein was restored completely to wild-type levels. In the other veins, *rhomboid* expression was not restored in the distal parts of L4 and L5 and gaps were also present along the L2 vein. As with *E(spl)mβ*, the expression pattern of *rhomboid,* 6 h after the temperature shift, was indistinguishable from that observed when *Su(dx)*^{sp} flies were maintained in continuous culture at 29°C (see Fig. 1). The adult wing phenotype in flies shifted under this regime was also indistinguishable from adults emerging after continuous 29°C culture (data not shown). These data indicate that the final adult phenotype in a *Su(dx)*^{sp} mutant is the result of a time course of events following the initial perturbation of Notch activity. The latter may involve feedback regulation which moderates the overall consequences of the removal of *Su(dx)* activity since the final phenotype is less severe than might be predicted from the expression patterns observed at earlier stages.

Su(dx) regulates Notch signal activation at the wing disc dorsal–ventral boundary

To determine whether Su(dx) was a regulator of Notch signalling levels only in the special context of wing vein development or whether it has a more general significance

in Notch signal regulation, we measured Notch activity in a different developmental context. For this purpose we examined a different signal reporter gene, namely *wingless* expression at the dorsal–ventral (D-V) boundary of the mid to late third instar imaginal wing disc (Rulifson and Blair, 1995). *Su(dx)* mutations alone have not been shown to have a wing margin phenotype, so we also investigated *wingless* expression in the background of *Notch* alleles that have been previously shown to enhance the *Su(dx)* wing vein phenotype, i.e., *notchoid^1* (*nd^1*) and *Abruptex*^{E2} (*Ax*^{E2}) (Fostier et al., 1998). *Su(dx)^{sp}* wing discs appeared wild-type for *wingless* expression at both 25 and 29°C (data not shown). However in a *nd^1* background, the mutant combination led to an ectopic expression of *wingless* exclusively on the ventral side of the D-V boundary of the wing imaginal disc (Figs. 3D and 3E). This was not an effect of the *nd^1* mutation alone since the latter results in reduced *wingless* expression, i.e., not only does *Su(dx)* rescue *nd^1*, but *nd^1* also enhances the Notch activation which results from *Su(dx)* loss of function. In contrast, in combination with the *Ax^{E2}* allele, the *Su(dx)^{sp}* mutation led to a broadening of the *wingless* expression on both the dorsal and the ventral sides of the D-V boundary (Figs. 3F and 3G).

Down-regulation of Notch target gene expression by ectopic Su(dx) expression

To investigate the consequences of ectopic expression of Su(dx) on Notch signalling levels, we monitored the expression in the third instar wing disc, of three genes, *wingless, cut*, and *vestigal,* whose expression has been shown to be driven by Notch in a Su(H)-dependent manner (Klein et al., 2000). In wild-type wing discs, Notch signalling drives *wingless* and *cut* expression along the D-V boundary. In contrast, *vestigal* is expressed throughout the wing pouch region of the disc, under control of the quadrant and boundary enhancer elements. The two enhancers can be dissected with appropriate reporter lines expressing β-galactosidase (Williams et al., 1994). The boundary enhancer element (*vg^{BE}-LacZ*) is under the direct control of Notch signalling along the D-V and anterior–posterior (A-P) boundaries. When *Su(dx)* cDNA was ectopically expressed along the A-P boundary of the imaginal wing disc using the Patched Gal4 driver (Ptc^{Gal4}), gaps in the expression of *wingless, cut,* and *vg^{BE}-LacZ* were observed corresponding to the intersection of the *patched* expression domain with the D-V boundary (Figs. 4C, 4E, and 4G). In the adult there is a corresponding notching of the wing margin (data not shown). *vg^{BE}-LacZ* expression along the A-P boundary was also suppressed (Fig. 4G). Interestingly Su(dx) overexpression also induced overgrowths of both the ventral and the dorsal wing pouch (Fig. 4C). This overgrowth phenotype is the contrary of what would be expected to result from a loss of Notch activity (Baonza and Garcia-Bellido, 2000) and may therefore reflect an activity of Su(dx) which is separate from its role in Notch signal down-regulation. Both the wing growth and the Notch down- regulatory effects of overexpressed Su(dx) were found to be dependent on the presence of the Su(dx) C-terminal HECT domain, because the expression of a construct lacking this domain did not elicit these responses (data not shown).

Su(dx) mutations show strong genetic interactions with the Notch regulatory gene *deltex.* Mutation of just one copy of *Su(dx)* is sufficient to rescue the vein thickening and wing margin loss induced by *deltex* alleles (Fostier et al., 1998). We therefore tested the interaction between the gain of function phenotypes of *Su(dx)* and *deltex.* As previously described (Matsuno et al., 2002), we found that Ptc^{Gal4} driven *deltex* expression in the wing disc activates Notch signalling along the A-P boundary in the ventral wing pouch resulting in ectopic *wingless* expression and an overgrowth of the ventral compartment of the wing disc (Fig. 4H). These phenotypes were reflected in the adult by the formation of an ectopic margin on an overgrown ventral surface of the wing (data not shown). Coexpression of Su(dx) blocked all of the Deltex-induced phenotypes (Fig. 4I). In contrast Deltex expression did not inhibit the Su(dx)-dependent down-regulation of Notch signalling at the D-V boundary (Fig. 4I) or prevent the corresponding notches in the adult wing margin (data not shown). Interestingly however Deltex expression did block the wing overgrowth phenotype of ectopic Su(dx). This demonstrated that the Su(dx)- dependent loss of Notch target gene expression was not an indirect consequence of the overgrowth and distortion of the wing pouch.

A role for Su(dx) in formation of the leg joints

The expression of *Su(dx)* using the Ptc^{Gal4} driver also led to strong leg phenotypes which resulted from complete or partial fusion of the leg tarsal segments due to repression of joint formation (Figs. 5E and 5F) and shortening of the leg. This is consistent with a negative regulatory role for Su(dx) on Notch signalling since similar phenotypes have been previously attributed to loss of Notch signalling in leg development (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). Interestingly these phenotypes were accompanied by duplication of the sex combs (Fig. 5E), a phenotype that has also been linked to reduction in Notch signalling (Mishra et al., 2001).

To establish whether there was a role for endogenous *Su(dx)* in leg development, we examined *Su(dx)* mutant flies. The adult legs of flies with the *Su(dx)^{sp}* mutation alone appeared wild type (data not shown). However since activity of *Su(dx)* in other tissues has been uncovered in a *nd^1* mutant background, we reexamined adult flies which were homozygous for both *nd^1* and *Su(dx)^{sp}*. A small amount of extra joint material in tarsal segment four can occasionally be seen in *nd^1* flies (Fig. 5G). This ectopic joint phenotype was strongly enhanced in the *nd^1, Su(dx)^{sp}* double-mutant combination, with ectopic joints occurring at high penetrance in both tarsal segments three and four (Figs. 5H and

5I). Interestingly these ectopic joints were found to be of reversed polarity and lay just proximal to the existing joints at the distal ends of the third and forth tarsal segments. These data demonstrate for the first time a role for *Su(dx)* in *Drosophila* leg development.

Discussion

Su(dx) negatively regulates Notch signalling in different developmental contexts

Su(dx) mutations interact genetically with *Notch;* however, a role for Su(dx) in modulating the actual level of Notch signalling itself has not previously been demonstrated. To begin to unravel the mechanism of action of Su(dx), it is an important prerequisite to establish whether Su(dx) acts on the Notch pathway itself, or whether the genetic interactions observed reflect an indirect, parallel, or downstream activity. The data presented in this article argue that Su(dx) can indeed negatively regulate Notch signalling, upstream of the immediate Notch target genes. First we showed, using the temperature sensitivity of the $Su(dx)^{sp}$ wing vein gap phenotype, that *Su(dx)* loss of function is rapidly followed by the up-regulation of *E(spl)mβ* expression in the pupal wing. Second we showed, in third instar wing imaginal discs, that in two enhancing genetic backgrounds, *Su(dx)* loss of function results in the up-regulation of *wingless,* another Notch target gene at the D-V boundary. Third Su(dx) overexpression in the wing imaginal disc was capable of down-regulating the Notch-dependent expression of three genes, *wingless* and *cut* at the D-V boundary, and the vg^{BE}-*LacZ* element at both the D-V and the A-P boundaries. These data show that Su(dx) is capable of down-regulating Notch in different developmental contexts and that its activity on Notch is not limited to the particular situation of wing vein development.

We also showed that Su(dx) was capable of blocking the stimulation of Notch signalling which was induced by the overexpression of Deltex, a regulatory protein which binds to the Notch intracellular domain. Thus our data suggest that the activity of Su(dx) lies upstream of the regulation of Notch target gene expression but downstream of, or at the level of, Deltex. This, together with the rapidity of the response of increased Notch signalling that we observed following Su(dx) loss of function, supports the hypothesis that Su(dx) acts directly on the Notch pathway. Our in vivo data are thus consistent with the in vitro observation that a related mammalian Nedd4 family protein, Itch, can promote the ubiquitination of the Notch1 intracellular domain (Qui et al., 2000).

Su(dx) shows allele-specific interactions with different Notch mutations

We examined the phenotype of $Su(dx)^{sp}$ in two different enhancing genetic backgrounds and obtained different con-

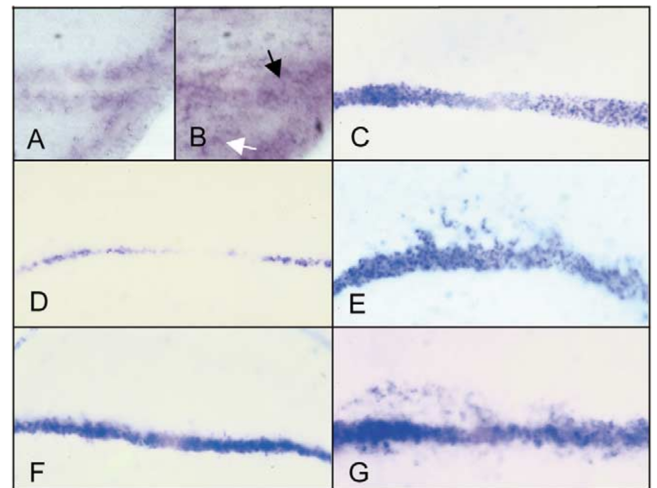

Fig. 3. *Su(dx)* regulates Notch signalling at the dorsal–ventral boundary of imaginal wing discs. (A) Region of wild-type pupal wing depicting the tip of vein L4 from a time-course experiment, 90 min after temperature shifting to 29°C, showing expression of *E(spl)mβ.* The typical tramline-like expression of *E(spl)mβ* results from Notch activation at the borders of the vein precursor cell territories. (B) Similar region of a $Su(dx)^{sp}$ pupal wing 90 min after temperature shift to 29°C. Elevated *E(spl)mβ* expression can be seen both within the central provein region (black arrow) and in the intervein cells (white arrow). (C) Notch signalling activates *wingless* expression (blue staining) along the D-V boundary in wild-type late third instar wing disc. Ventral compartment lies above the D-V boundary, dorsal below. $Su(dx)^{sp}$ discs also appear wild-type (data not shown). (D) D-V boundary of nd^1 allele of *Notch* showing thinning and gaps in expression of *wingless.* (E) D-V boundary of nd^1; $Su(dx)^{sp}$ wing disc. The double mutant combination rescues the nd^1 phenotype and induces ectopic *wingless* expression, on the ventral side of the D-V boundary. Note the sharp dorsal (lower) boundary of *wingless* expression. (F) D-V boundary of the weak Ax^{E2} allele of *Notch* resembles wild-type. (G) D-V boundary of Ax^{E2}; $Su(dx)^{sp}$ double mutant is diffuse with ectopic *wingless* expression observed on both Dorsal and Ventral sides. Wing discs in C, F, G were obtained from larvae reared at 25°C; discs shown in D, E were obtained from larvae reared at 29°C.

sequences on the spatial distribution of ectopic Notch activation at the wing disc D-V boundary, as monitored by *wingless* expression. We observed that ectopic *wingless* expression in nd^1; $Su(dx)^{sp}$ discs was restricted to the ventral side of the D-V boundary, but was found on both sides of this boundary in Ax^{E2}; $Su(dx)^{sp}$ discs. A similar ventral compartment-specific Notch activation is observed when *Serrate* is expressed along the anterior–posterior axis, while expression of constitutively active Notch intracellular domain does not show such a restriction (Doherty et al., 1996). This spatial restriction of the ectopic *Serrate*-induced response has been explained by the inhibitory effect of dorsally expressed Fringe, which represses Serrate-dependent but not Delta-dependent Notch activation through the glycosylation of the Notch extracellular domain (Bruckner et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000). Normally Serrate, which is expressed in the dorsal compartment, is only able to signal to Notch in adjacent ventral cells where Notch is not modified by Fringe. A possible interpretation of our data therefore is that the ectopic Notch

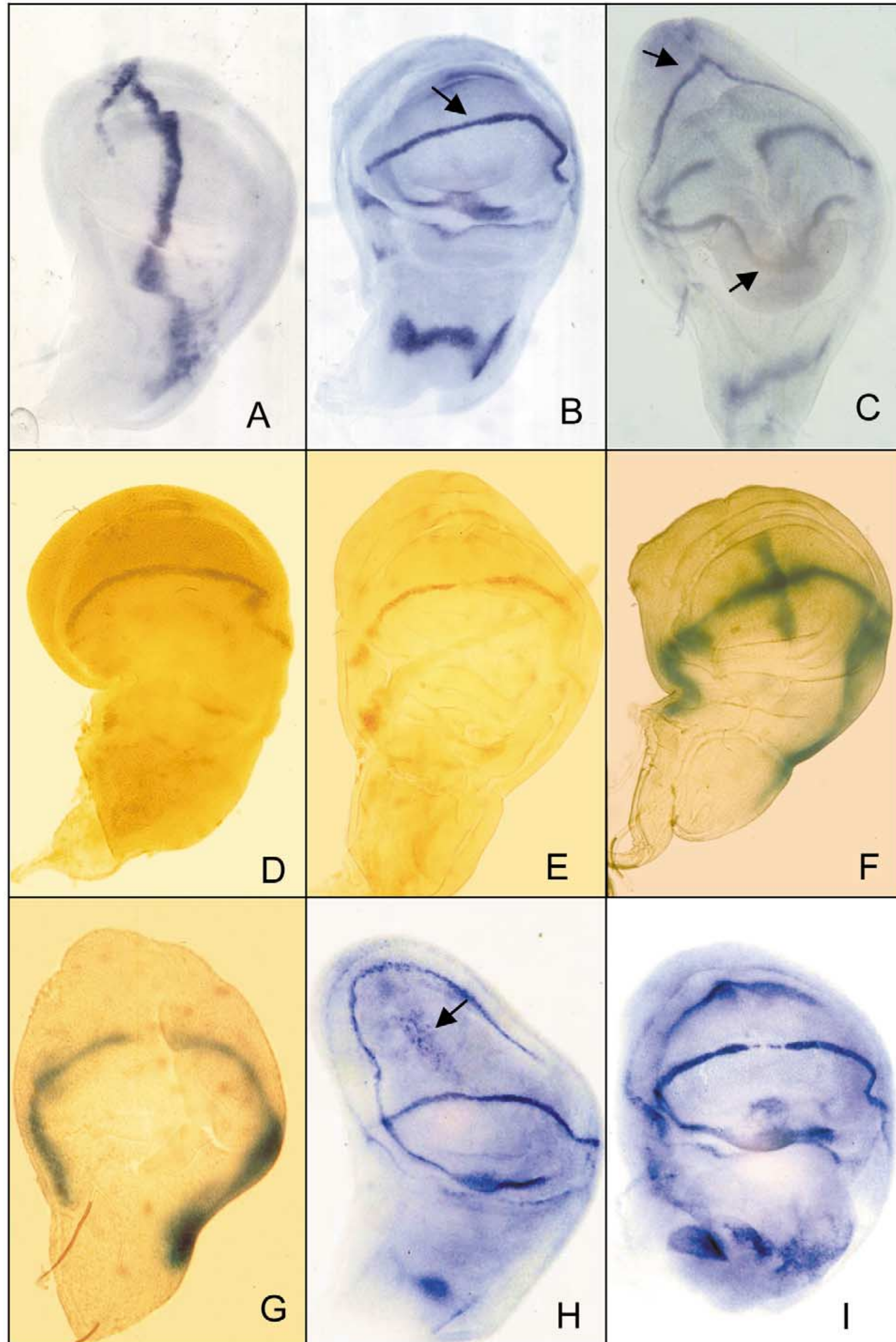

Fig. 4. Overexpression of Su(dx) down-regulates Notch signalling. All wing discs are late third instar, oriented with ventral up and anterior left, and derived from larvae raised at 29°C. (A) Expression pattern of Ptc^{Gal4} driver lies along the A-P boundary of late third instar wing disc. (B) Notch signalling activates *wingless* expression (blue staining) along the D-V boundary (arrow) in wild-type disc. (C) Expression of full-length Su(dx) protein usiing the $\mathrm{Ptc}^{\mathrm{Gal4}}$ driver results in a gap in the *wingless* expression pattern in the D-V boundary. Note also the enlargement of the wing disc which can easily be seen from the distortion of the ring of *wingless* expression that surrounds the wing pouch (arrows). (D) Wild-type disc immunostained for Cut protein, showing its expression along the D-V boundary. (E) $\mathrm{Ptc}^{\mathrm{Gal4}}$ driven expression of Su(dx) blocks *cut* expression giving a gap at the D-V boundary. (F) X-gal staining of β-galactosidase protein from a wild-type disc carrying the vg^{BE} *-LacZ* reporter construct. (G) $\mathrm{Ptc}^{\mathrm{Gal4}}$ driven expression of full-length Su(dx) blocks vg^{BE} *-LacZ* at the D-V and A-P boundaries. (H) Ectopic *wingless* expression (arrow) induced in ventral compartment by $\mathrm{Ptc}^{\mathrm{Gal4}}$ driven expression of Deltex. Note the enlarged ventral compartment. (I) Coexpression of Su(dx) and Deltex blocks the ectopic *wingless* expression induced by Deltex and endogenous *wingless* expression at the D-V boundary. The wing disc has a wild-type morphology. Both the ventral disc enlargement resulting from Deltex overexpression and the dorsal and ventral overgrowth resulting from Su(dx) expression are suppressed.

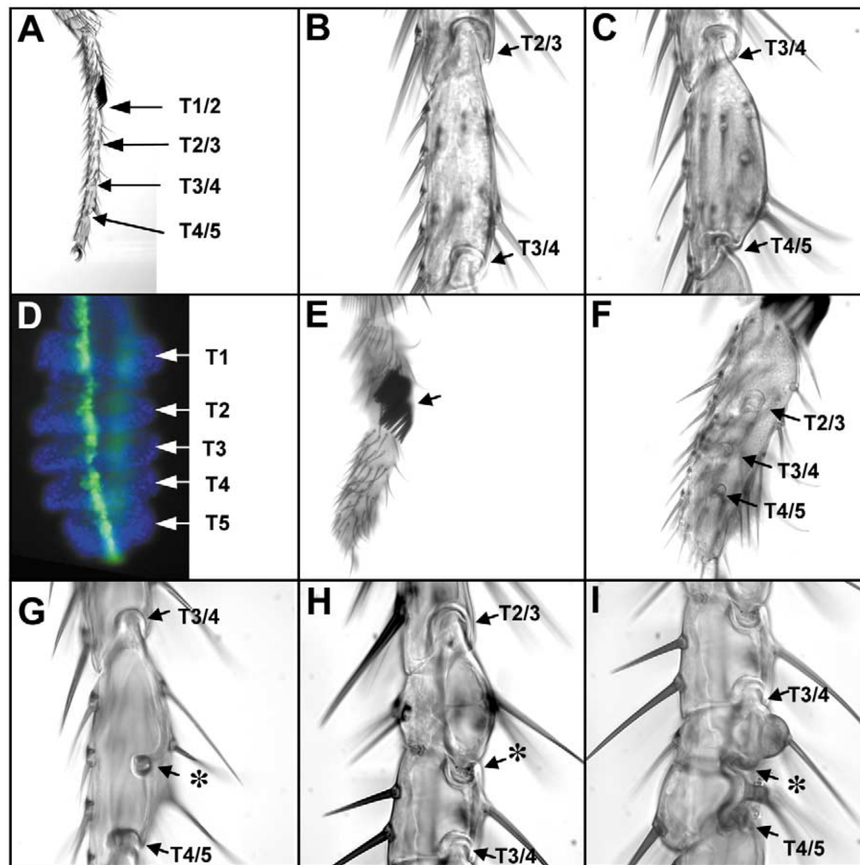

Fig. 5. Su(dx) regulates segmentation in the leg. (A) shows a wild-type male tarsus with the joint positions marked (for example, the boundary between tarsal segments one and two, labelled T1/2); (B, C) show wild-type tarsal segments three and four, respectively. (D) A dorsal view of the tarsal region of a wild-type early pupal leg disc expressing GFP (green) under the control of Ptc^{Gal4}, dapi-labelled nuclei (blue). (E, F) Overexpression of full-length Su(dx) protein driven by Ptc^{GAL4} at 25°C results in truncated tarsi and extra sex combs (arrow) (E is shown at twice the magnification of the wild-type in A). The joints are almost completely eliminated, although some residual joint material (arrows) can be seen at higher magnification (F) at locations where ectopic Ptc^{Gal4} driven Su(dx) expression is weaker. (G) nd^1/y legs showing some extra joint material (asterisk) in tarsal segment four but not in segment three (not shown). (H, I) $nd^1/y;Su(dx)^{sp}$ have extra, reversed polarity joints (asterisks) in tarsal segments three and four, respectively. Legs in G, H, I were obtained from flies raised at 18°C.

activation in the nd^1; $Su(dx)^{sp}$ combination is similarly blocked in the dorsal compartment by Fringe. If this is the case then it is unlikely that this interaction results in a constitutive activation of the Notch receptor since the latter would be independent of Fringe. The *Abruptex* class of mutations have been shown to make the Notch receptor less sensitive to the down-regulatory effect of Fringe (de Celis and Bray, 2000) and this may explain why the Ax^{E2}; $Su(dx)^{sp}$ combination, unlike the nd^1; $Su(dx)^{sp}$ combination, allows ectopic Notch activity on both sides of the D-V boundary.

Developmental buffering may mask the full extent of Su(dx) activity

Frequently in studies of the effects of mutations on gene expression, the patterns detected represent only the end point of the aberrant developmental history. Here we were able to exploit the temperature-sensitive nature of the $Su(dx)^{sp}$ wing vein gap phenotype to investigate the evolving expression pattern of two Notch regulated genes, *E(spl)mβ* and *rhomboid.* We showed that the shift to the nonpermissive temperature was closely followed by increased expression of the Notch target gene *E(spl)mβ* and a concomitant decrease in *rhomboid* expression in the wing vein precursor cells. The latter is expected because *rhomboid* expression is repressed by *E(spl)mβ* (de Celis et al., 1997). The initial elevation in *E(spl)mβ* expression level was found to be transient, peaking around 45 min. Subsequently *E(spl)mβ* expression levels were progressively reduced and were lost altogether from regions of the vein precursor territories that corresponded to positions of vein gaps found in the adult wings. In a previously proposed model of vein development (de Celis et al., 1997), *rhomboid* expression is necessary to activate EGF receptor signalling which in turn is required to maintain Notch signalling levels. A loss of *rhomboid* expression due to elevated Notch activation would therefore be predicted to cause a subse-

quent reduction in Notch activity via decreased EGF receptor signalling. In turn this would be predicted to lead to the derepression of *rhomboid* expression. In our time-course experiment we were able to follow the operation of this predicted feedback loop for the first time, and the oscillation in Notch signalling activity that we observed is in broad agreement with this model. However our data are not completely in agreement. We also see up-regulation of *E(spl)mβ* expression followed by moderation of the raised levels in intervein territories where *rhomboid* is not detectably expressed and presumably is therefore not involved in the feedback regulation in these cells. This implies that an additional uncharacterised means of feedback control might be in operation.

The implementation of the feedback loop makes *Drosophila* wing vein development relatively robust to perturbations of Notch activity. The final adult phenotype may depend on the kinetics of the feedback loop leading to the restoration of EGF receptor signal required to drive cells into the vein cell fate, compared to the kinetics of the process of commitment itself. The variable sensitivity of different parts of the wing veins could therefore be due to different times at which cells in different regions pass through a critical point at which they irreversibly commit to a vein cell or intervein cell fate. These data illustrate an important point that in a mutant background a cell can ultimately adopt a wild-type fate even though its developmental history is altered, providing the interacting signals produce a network which is robust enough to withstand and adjust for the perturbation. Such interacting networks could be important buffers for development against genetic variation in a population. We speculate that this robustness together with other forms of redundancy may help to mask wider activities of *Su(dx)* which are uncovered in different genetic backgrounds.

In the light of the above discussion it is interesting that our data have uncovered, in an enhancing nd^1 genetic background, a previously unknown function of Su(dx) in leg development. The resulting extra joint phenotype is consistent with a role for wild-type Su(dx) in down-regulating the Notch pathway. It is interesting that the extra joints observed in the tarsus of $nd;Su(dx)^{sp}$ are of reversed polarity. In the third instar and pupal leg *Serrate, Delta,* and *fringe* are expressed in largely overlapping domains proximal to the site of joint formation (Bishop et al., 1999; de Celis et al., 1998; Rauskolb, 2001; Rauskolb and Irvine, 1999). In the wild-type leg, the joints always form distal to the stripes of high *Serrate* and *Delta* expression and not proximal. However, in polarity mutants such as dsh^1, extra joints of reversed polarity are formed just proximal to the high levels of *Delta* and *Serrate* expression and are coincident with ectopic Notch activation (Bishop et al., 1999; Held et al., 1986). It was proposed that repressor elements possibly involving planar polarity signalling, together with Fringe, repress Notch activation proximal to the stripe of high *Serrate* and *Delta* expression, and the $nd^1; Su(dx)^{sp}$ combination may thus be able to overcome this inhibition. Overexpression of Su(dx) using the Ptc^{GAL4} driver, which drives along the A-P boundary of the leg disc, causes fusion of the tarsal joints and a shortening of the leg. This is again consistent with the role of Su(dx) being to inhibit Notch signalling as loss of function of *Notch, Serrate,* and *Delta* results in fusion between leg segments and reduced leg growth (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999).

Other roles for Su(dx)

Interestingly while Deltex expression did not block the Notch down-regulatory activity of Su(dx), it did inhibit the latter's wing overgrowth phenotype. This uncoupling of phenotypes suggests that Su(dx) has multiple activities. One activity down-regulates the Notch signal and thus blocks the ectopic wing margin and wing growth phenotype induced by Deltex overexpression. The overexpression of Deltex may in turn titrate Su(dx) away from a second activity responsible for a distinct wing overgrowth phenotype. This could explain how the coexpression of these two proteins fails to produce a wing overgrowth when the expression of each singly does result in an overgrowth phenotype.

Additional Su(dx) activities may also explain an unexpected interaction of *Su(dx)* with *daughterless* (Smith et al., 2002). Loss of function of *Su(dx)* enhanced the *daughterless* phenotypes during ovary development, similar to the enhancement of *daughterless* shown by loss of function mutations of *Notch.* This is in contrast to what would be predicted if the activity of Su(dx) in the ovary was a negative regulator of Notch and therefore supports the hypothesis that Su(dx) may have more than one role.

In conclusion, our data provide support for a direct role, in vivo, for Su(dx) in the regulation of the Notch pathway in different developmental contexts. The phenotype of Su(dx) may be moderated by the feedback activity of interlocking networks of signals and other means of developmental redundancy. Further analysis of interacting genetic backgrounds should reveal the full scope of Su(dx)-dependent functions which may also include additional activities beyond Notch down-regulation. Work is now in progress to elucidate the molecular interactions of Su(dx) and identify the direct targets of its activity, which are relevant to its roles in vivo.

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