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The metalloprotease-disintegrin Kuzbanian participates in *Notch* activation during growth and patterning of *Drosophila* imaginal discs

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SUMMARY

The Notch transmembrane protein is the receptor of an evolutionary conserved pathway that mediates intercellular signalling leading to the specification of different cell types during development. In this pathway, many aspects of the signal transduction mechanism remain poorly understood, especially the role of proteolytic processing of Notch. We present genetic evidence indicating that the metalloprotease-disintegrin *kuzbanian* (J. Rooke, D. Pan, T. Xu and G. M. Rubin (1996) *Science* 273, 1227-1231) is a new component of the Notch signalling pathway and is involved in Notch activation. *kuzbanian* genetic mosaics demonstrate that, during neurogenesis, wing margin formation and vein width specification *kuzbanian* is autonomously required in the cell where Notch is activated. Genetic interactions between *kuzbanian* and different genes of the *Notch* pathway indicate that *kuzbanian* is required upstream of *Suppressor of Hairless*. Moreover, the requirement of *kuzbanian* for signalling by a ligand-dependent Abruptex receptor, but not by a constitutively activated form of Notch, suggests that *kuzbanian* is involved in the generation of a Notch functional receptor and/or in its activation. However, differences in the phenotypes of loss-of-function *Notch* and *kuzbanian* mutations suggest the existence of alternative Kuzbanian-independent mechanisms that generate Notch functional receptors.

Key words: *kuzbanian*, N pathway, imaginal disc, *Drosophila*, metalloprotease, disintegrin, *Notch*, patterning

INTRODUCTION

Development of multicellular organisms relies on the spatially and temporally restricted expression of genes that specify different cell fates. Local cell-cell interactions are paramount in this process. They occur either among initially equivalent cells, to help single out cells that acquire a fate different from that of their neigbours, or between already different cell populations (inductive interactions). In *Drosophila*, many of these interactions are mediated by *Notch* (*N*), a multiple-ligand transmembrane receptor that is activated during intercellular signalling (reviewed in Artavanis-Tsakonas et al., 1995). The components of the *N* signalling pathway and their respective functions were first analyzed in the context of neurogenesis (Campos-Ortega, 1993). Loss-of-function mutations at the genes of this signalling pathway cause hyperplasia of the nervous system and, accordingly, its components have been collectively termed 'neurogenic' genes. However, neural potential is conferred to cells by a different set of genes named proneural genes, prominent among which are the members of the *achaete-scute* complex, which encode transcription factors of the bHLH family (reviewed in Modolell, 1997). These genes are expressed in groups of cells, the proneural clusters (Cubas et al., 1991; Skeath and Carroll, 1991), but cellular interactions mediated by the N pathway restrict acquisition of the neural fate to only one or a few cells of each cluster. In this process, known as 'lateral inhibition' (Simpson, 1990), the transmembrane Delta (Dl) protein acts as a ligand that activates the N receptor (Heitzler and Simpson, 1991). This, with the help of the DNA-binding protein Suppressor of Hairless [Su(H)], whose activity is antagonized by the Hairless (H) nuclear protein, promotes the expression of several of the genes of the *Enhancer of split* complex [*E(spl)C*] (Brou et al., 1994; Fortini and Artavanis-Tsakonas, 1994; Bailey and Posakony, 1995; Jennings et al., 1995; Lecourtois and Schweisguth, 1995). *E(spl) m5, m7* and *m8,* which encode bHLH transcription factors, prevent cells of the proneural clusters from adopting the neural fate (Tata and Hartley, 1995; de Celis et al., 1996b; Heitzler et al., 1996; Nakao and Campos-Ortega, 1996). Similarly, during the formation of the wing veins, N activation leads to expression of *E(spl)mβ*, which in turn represses *veinlet/rhomboid* (*ve/rho*) transcription, therefore restricting the number of cells that become wing veins from a larger equivalent group (de Celis et al., 1997). In a different context, the growth of the wing disc and the formation of the wing margin depend on the transcriptional activation of the *vestigial*, *wingless* and *cut* genes by Su(H), subsequent to the activation of N by the ligands Serrate (Ser) and Dl at the dorsoventral boundary of the wing imaginal disc (Couso et al., 1995; Díaz-Benjumea and Cohen, 1995; de Celis et al., 1996a; Doherty et al., 1996; Kim et al., 1996).

Proteins homologues of N and other members of the N pathway have been found in vertebrates, where they apparently play similar roles in restricting cell fates, indicating the func-

tional conservation of this intercellular signalling pathway (Chitnis et al., 1995; de la Pompa et al., 1997). However, many aspects of the signal transduction mechanism remain poorly understood. This is especially the case for the role of the processed forms of N found in many organisms (Fehon et al., 1990; Zagouras et al., 1995). In vertebrates, it is postulated that a processed intracellular fragment of N is involved in transducing N activation to the nucleus. Thus, N intracellular domain is cleaved after receptor activation, translocates to the nucleus and binds Su(H) forming an active transcriptional complex (Jarriault et al., 1995; Kopan et al., 1996). In *Drosophila*, an alternative, non-exclusive model proposes that the intracellular domain of N participates in Su(H) retention in the cytoplasm. Notch activation leads to displacement of Su(H) by Deltex, thus enabling Su(H) to translocate to the nucleus where it activates target genes (Fortini and Artavanis-Tsakonas, 1994; Matsuno et al., 1995; Gho et al., 1996). The occurrence of truncated forms of N associated with several neoplasias (Ellisen et al., 1991) highlights the practical importance of a thorough analysis of this signalling pathway.

N is also processed in its extracellular domain (Kopan et al., 1996), but the significance of this observation was unclear. The results presented in this report suggest a requirement of N extracellular processing for N activation. We find that the metalloprotease-disintegrin *kuzbanian* (*kuz*) (Rooke et al., 1996) is a new component of the N signalling pathway. Our analysis of *kuz* genetic mosaics demonstrate that during neurogenesis, wing margin formation and vein width specification *kuz* is autonomously required in the cell where N is activated. Genetic interactions between *kuz* and *N*, *Ser*, *E(spl)m8* and *H* indicate that, in the N pathway, kuz is required upstream of *Su(H)* activation. Furthermore, the requirement of *kuz* for activity of a ligand-dependent Abruptex receptor, but not for the signalling by a constitutively activated form of N, suggest that *kuz* participates in the generation of a N functional receptor and/or in its activation. After the completion of this work, we have learned that a similar conclusion has been reached by Pan and Rubin (1997) who, in addition, have shown a Kuz-dependent proteolytic processing of Notch. However, the incomplete neurogenic transformation in *kuz* null mutations and the ability of *kuz* cells to proliferate normally suggest the existence of mechanisms other than Kuz-dependent proteolysis to generate N functional receptors.

MATERIALS AND METHODS

Drosophila strains

The strains used are described in the following references. *l(2)k01405*, *l(2)k01403* and *l(2)k11804* are P-lacW (*P [w⁺; lacZ]*) insertions (Török et al., 1993, Roch et al., 1997). These P insertions fail to complement the lethality of the kuz^{H143} allele (Fambrough et al., 1996) and of *l(2)34Da* mutation (Lindsley and Zimm, 1992). Their *lacZ* gene is not expressed in the imaginal discs. The *UAS-kuz* line is described in Fambrough et al. (1996); the gain-of-function alleles of *Notch*, Ax^{M3}, Ax^{16172} and the P[ry^+, *Hsp70-Notch(intra)*] line [*HS-N(i)* in the text] in de Celis and García-Bellido (1994a) and Struhl et al. (1993); the dominant allele $Serrate^D$ (Ser^D) in Thomas et al. (1991); the strong H^2 allele in Bang et al. (1995), and the enhancer-trap line P[ry^+; *lacZ*] *A101-IF3*, a marker for SMCs, in Huang et al. (1991). The *UAS-E(spl)m8* and *UAS-B4* lines were generated by subcloning a 0.72 kb *Dra*I fragment of *E(spl)m8* genomic DNA and a 5.6 kb *Bam*HI fragment of *B4* cDNA, respectively, in the pUAST vector (Brand and Perrimon, 1993). The resulting pUAST-*E(spl)m8* and pUAST-*B4* plasmids were used to transform w^{1118} embryos (Ashburner, 1989). Other stocks are described in Lindsley and Zimm (1992). Homozygous *kuz* and *H* larvae were identified by the absence of the *Tb* marker of the *T(2,3)SM6aTM6B CyO Hu Tb* balancer chromosome. Phenotypes were determined in flies raised at 25°C.

Reversion mutagenesis

The *l(2)k01405* P-lacW insertion was mobilized as described (Ashburner, 1989). Out of 57 events identified by loss of w^+ expression, 50 were phenotypically wild type and corresponded to precise excisions of the P element and 7 showed phenotypic defects. Two lines ($kuz^{1405Rev4}$ and $kuz^{1405Rev23}$) were fully viable, whereas $kuz^{1405Rev11}$ only reached the pharate adult stage. These strains carry partially deleted copies of the original P-lacW transposon. $kuz^{1405Rev11}$ must retain some kuz function since the scalloping phenotype of $kuz^{1405Rev11}/kuz^{1405Rev4}$ is milder than that of $kuz^{1405}/kuz^{1405Rev4}$ flies (see Fig. 4B,E).

Clonal analysis

Mitotic recombination was induced by X-ray irradiation as described (de Celis et al., 1997). Larvae derived from crossing kuz^{1405}/CyO or kuz^{H143}/Cyo males with f^{36a}*; M(2)Z P[f⁺]30B/CyO* females were irradiated at 24-48, 48-72 and 72-96 hours after egg laying (AEL), corresponding to first, second and early third larval stages, respectively. Mitotic recombination proximal to the f^+ insertion produces homozygous mutant cells labelled with *f* and *Minute*⁺. Twin spot analysis was performed by irradiating f^{36a}*;* kuz^{1405}*/P[f⁺] 30B ck pr pwn* larvae.

Clones of *kuz* homozygous cells were also generated by FLP/FRT recombination (Xu and Rubin, 1993). Larvae from the cross of *w;* kuz^{1405} *P[w⁺; hs-neo; FRT]40A/CyO* males with *f hsFLP; P[f⁺]30B ck P[w⁺; hs-neo; FRT]40A/CyO* females were aged 24-48, 48-72 and 72-96 hours AEL and incubated for 1 hour at 37°C. Male flies were scored for the presence of *f*-marked kuz^{1405} clones and *crinkled* (*ck*)-marked wild-type twin clones. Homozygous kuz^{11804} cell clones were similarly generated.

Phenotypic rescue of *kuz* mutations

kuz^{1405}*;T(2,3)SM6aTM6B;GAL4 C-765* females were crossed with either kuz^{1405}*; T(2,3)SM6aTM6B; UAS-kuz or* kuz^{1405}*; T(2,3)SM6aTM6B; UAS-B4* or kuz^{1405}*; T(2,3)SM6aTM6B; UAS-E(spl)m8* males and the progeny was raised at 25°C.

In situ hybridization and immunocytochemistry

In situ hybridization to whole-mount embryos and imaginal discs with digoxigenin-labelled cDNA probes and staining with anti-sc (a gift from S. Carroll), anti-β-galactosidase (Promega) and mAb22C10 (a gift from L. García-Alonso) antibodies were performed as described (Cubas et al., 1991; Hartenstein and Posakony, 1990).

Molecular mapping of P-element insertions

Genomic DNA adjacent to the *kuz* insertions was obtained by plasmid rescue (Gómez-Skarmeta et al., 1996) and the insertion points were determined by DNA sequencing.

cDNA clones

B4 and *kuz* cDNA clones were retrieved from an imaginal disc cDNA library using as probes the genomic fragment obtained by plasmid rescue from kuz^{1405} or a genomic fragment located to the right to the insertion point of P-lacW in this line, respectively. One representative apparently full-length *B4* cDNA (5.7 kb) was sequenced (GenBank accession number AF022364). The location of some *B4* and *kuz* exons in the genomic DNA was determined by hybridization with fragments of the cDNAs and sequencing of genomic DNA.

DNA sequencing

cDNA subclones in pBluescriptKS (+) (Stratagene) were sequenced in an ABI 373 automatic sequencer using T7, T3 and custom-ordered synthetic oligonucleotide primers (Isogen). Sequences were assembled and analyzed with the University of Wisconsin GCG software packages (Devereux et al., 1984).

Other methods

Basic techniques of molecular biology were carried out as described (Sambrook et al., 1989).

RESULTS

Phenotypes of P-element insertion lines

The P-element insertion *l(2)k01405* was recovered in a mutagenesis experiment aimed at identifying second chromosome genes whose mutation led to larval/pupal lethality (Török et al., 1993; Roch et al., 1997). Late lethality is often associated with defects in the growth of the imaginal discs and, indeed, *l(2)k01405* larvae have imaginal discs of reduced size (Fig. 1E-G and not shown). Notice the specially underdeveloped wing pouch of the wing imaginal disc. *l(2)k01405* mutants develop to pharate adults that display severely reduced wings and halteres, small nota, shortened legs with fused tarsal segments, rough eyes and gross alterations in the pattern of sensory organs (SOs, Figs 2A, 8C and not shown) which range from the appearance of abnormal tufts of bristles or other SOs at places where, in the wild type, only one SO is present to patches of naked cuticle devoid of SOs (Fig. 2A). These defects are caused by the P-element insertion since wild-type revertants were recovered after mobilization of the transposon (Materials and methods).

The *l(2)k01405* insertion was mapped by in situ hybridization to chromosomal subdivision 34C (Roch et al., 1997). This was verified by the failure of *l(2)k01405* to complement the *Df(2L)b88h49* that uncovers the 34C5-34C7; 35A2 interval. Complementation tests showed *l(2)k01405* to be allelic to the *l(2)k01403*, *l(2)k07601* and *l(2)k11804* P-element insertions recently described as alleles of the *kuzbanian* (*kuz*) gene (Rooke et al., 1996). Imaginal discs from *l(2)k01403* and *l(2)k11804* are similar in size and shape to those from *l(2)k01405* larvae (Fig. 3C,E and not shown).

Molecular analysis

Over 20 kb of the genomic DNA in the vicinity of the

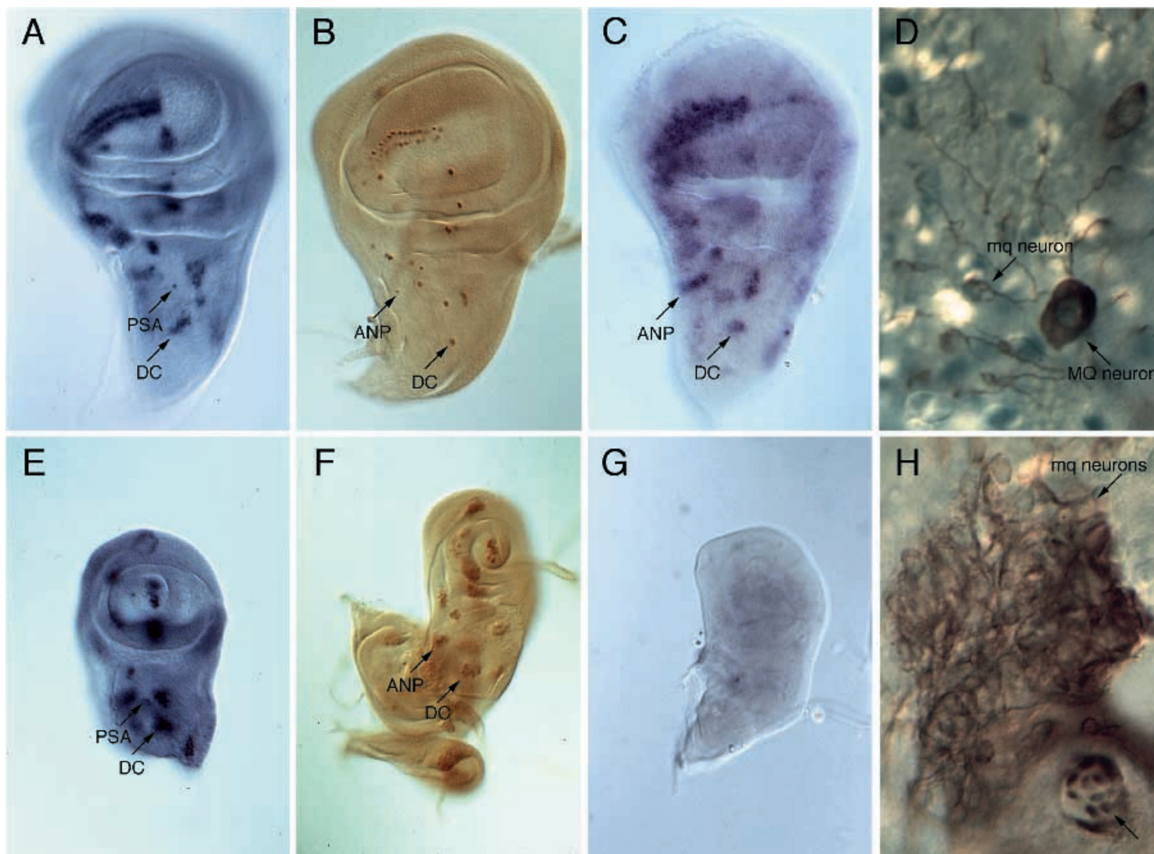

Fig. 1. Cellular basis of *kuz* neurogenic phenotype. Wild-type (A,C) and kuz^{1405} (E,G) imaginal wing discs showing expression of *sc*, as detected with anti-*sc* antibody (A,E) and of *E(spl)m8*, visualized by in situ hybridization (C,G). Note in E a cluster of *sc*-expressing cells at the position where only the precursor of the posterior supraalar (PSA) macrochaetae appears in A. DC, dorsocentral; ANP, anterior notopleural. (B,F) Pattern of SMCs in *A101-IF3* and kuz^{1405}*; A101-IF3* wing discs, respectively, as detected with anti-β-galactosidase antibody. Clusters of SMCs develop in kuz^{1405}; *A101-IF3* discs at positions where single SMCs are found in *A101-IF3* discs (arrows). (D,H) mAb22C10 stained whole mounts of 24-36 hours APF wild-type and kuz^{1405} nota, respectively. mq, microchaetae; MQ, macrochaetae; the neuron arrowed corresponds to one DC MQ; arrow in H (bottom) points to a tuft of shaft cells at the position of a DC macrochaeta. All discs are reproduced at the same magnification, with anterior to the left and ventral up.

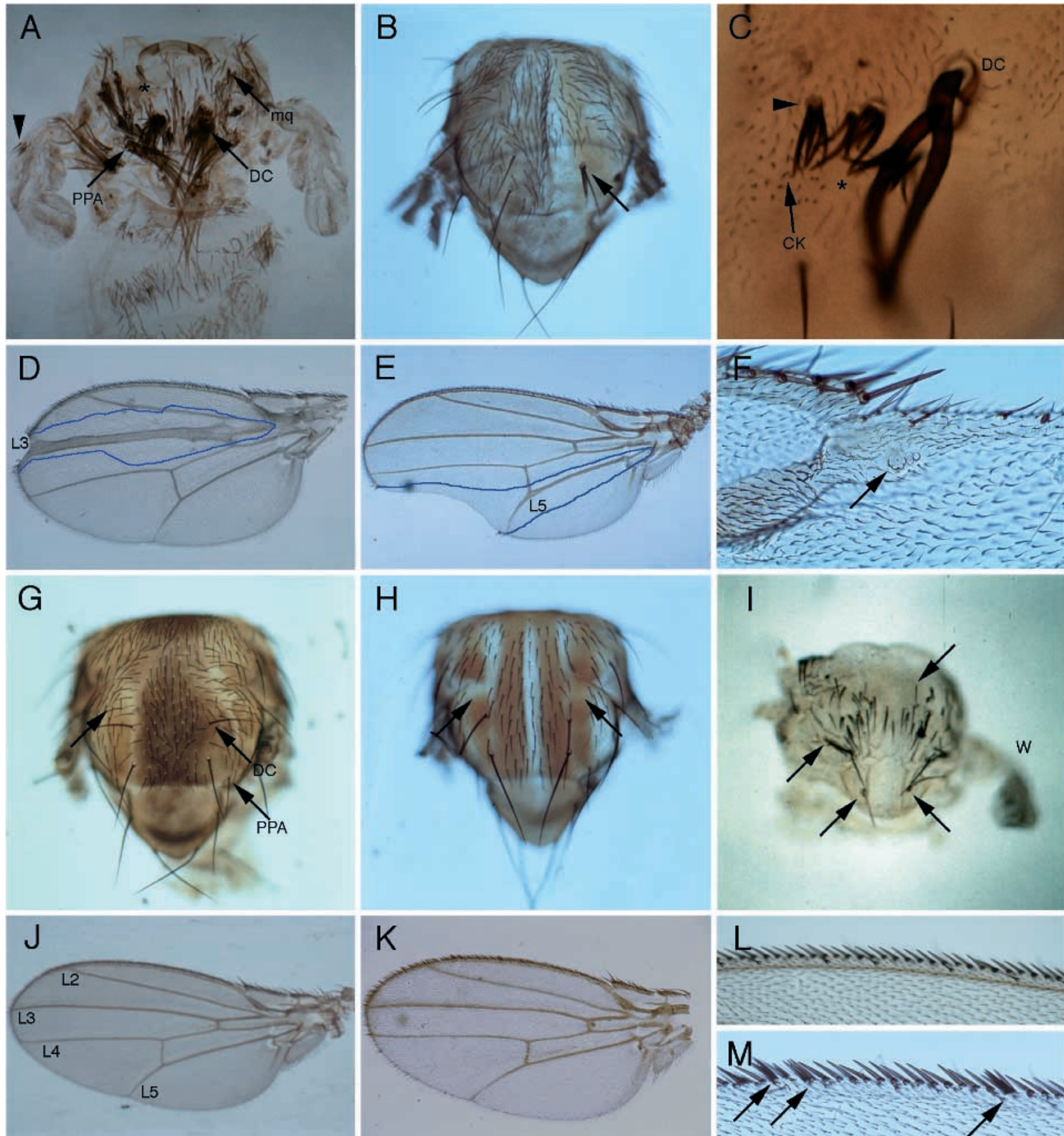

Fig. 2. Adult phenotype of *kuz* mutations and rescue of kuz^{1405} phenotype by *kuz* or *E(spl)m8* overexpression. (A) Notum and wings from a kuz^{1405} pharate adult. Arrows point to tufts of microchaetae (mq) and macrochaetae. Cuticle devoid of both macrochaetae and microchaetae is also observed (star). Arrowhead points to some bristles remaining at the proximal anterior wing margin. Wings are severely reduced and halteres are absent. (B) Female mosaic thorax showing a M^+kuz^{H143} clone induced at 48-72 hours AEL. Macrochaetae and microchaetae are missing; a tuft of macrochaetae (arrow) develops in the area devoid of bristles. (C) kuz^{11804} clone induced at 48-72 hours AEL. Note development of a tuft of *f;* kuz^{11804} microchaetae (arrowhead) and presence of naked cuticle (star) in an area adjacent to the wild-type *ck* territory. (D) Mosaic wing showing a dorsal *f;* M^+kuz^{H143} clone induced at 48-72 hours AEL. Limits of the clone are shown in blue. Note thickening of the L3 vein and a nick at the wing margin. (E,F) Dorsal *f*; M^+ kuz^{1405} clones induced by X-ray irradiation at 60-84 hours AEL. (E) Thickening of the L5 vein and wing margin nick are observed in association with the mutant territory (marked with a blue line). (F) A cluster of campaniform sensilla (arrow) develops at the position of one of the twin sensilla of the margin within a *f*; kuz^{1405} clone. (G) Wild-type notum. (H) Notum from a kuz^{1405}*; UAS-kuz/GAL4-C-765* pharate adult. Arrows point to regions where microchaetae are missing. (I) Notum and wing from a kuz^{1405}*; UAS-E(spl)m8/GAL4-C-765* pharate adult. Arrows point to recovered single SOs. The unrecovered small wing (w) is out of focus. (J,L) Wild-type wing and wing margin. (K,M) Wing and wing margin from a kuz^{1405}*; UAS-kuz/GAL4-C-765* individual. Arrows point to abnormal stout bristles. L2, L3, L4 and L5, longitudinal veins 2, 3, 4 and 5. In all figures (excepting F), wings are shown with anterior up and distal to the left.

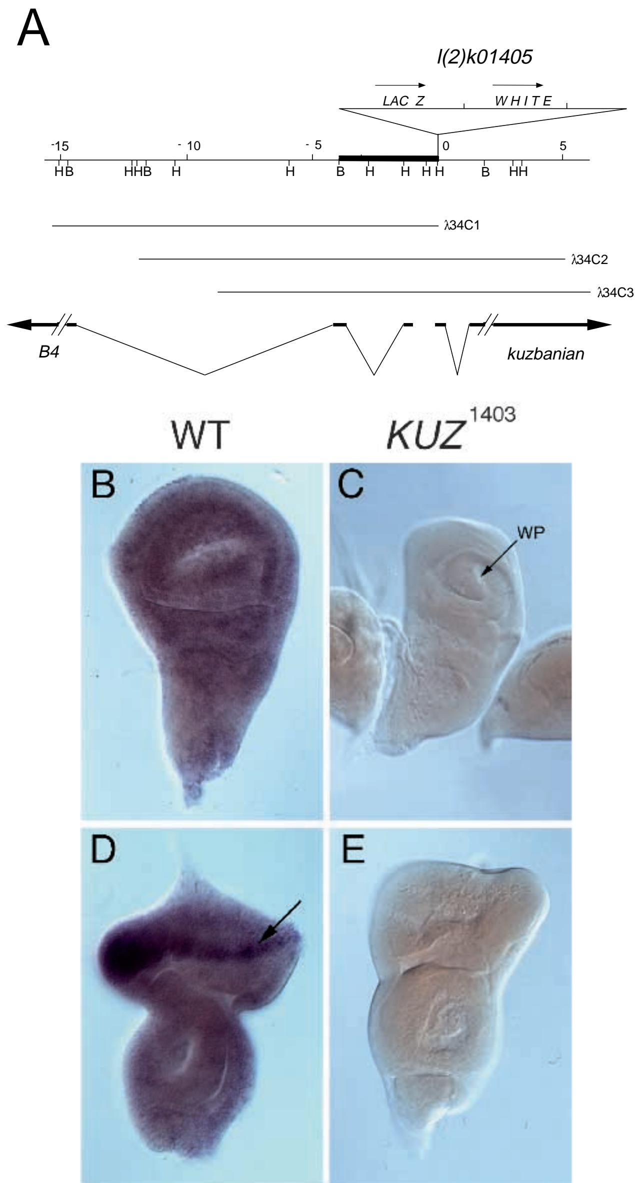

Fig. 3. Molecular organization of the 34 C region and expression of *kuz*. (A) Physical map of the genomic DNA containing the *B4* and *kuz* genes. The horizontal line shows part of the cloned genomic region. The black bar indicates the DNA recovered by plasmid rescue from the kuz^{1405} insertion. The genomic λ phages which cover the region are shown below. *Kuz* and *B4* transcription units extend further to the right and to the left, respectively, of the genomic region shown. B, *Bam*HI; H, *Hin*d III. (B-E) Expression of *kuz* in late third instar wild-type (B,D) and kuz^{1403} (C,E) imaginal discs visualized by in situ hybridization. B,C, wing discs; D,E, eye-antenna discs; wp, wing pouch. Arrow in D points to the morphogenetic furrow.

l(2)k01405 P-insertion point were cloned by plasmid rescue followed by screening of a *Drosophila* genomic library (Fig. 3A). Northern blot analyses and screening of a third instar imaginal disc cDNA library, using as probes several genomic fragments, identified two divergent transcription units (Fig. 3A). One of them corresponds to the *kuz* gene (Rooke et al., 1996), which encodes a metalloprotease-disintegrin protein of the conserved ADAM family (Wolfsberg at al., 1995). Both in

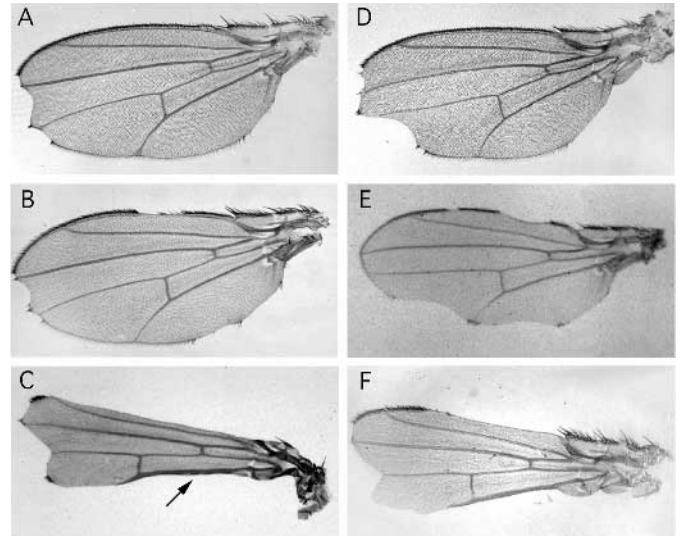

Fig. 4. *kuz* insufficiency increases *N* phenotypes. Wings of (A) $N^{55e11}/+$, (B) $kuz^{1405Rev11}/kuz^{1405Rev4}$, (C) $N^{55e11}/+; kuz^{1405Rev11}/kuz^{1405Rev4}$, (D) $Ser^D/+$, (E) $kuz^{1405}/kuz^{1405Rev4}$, (F) $kuz^{1405}/kuz^{1405Rev4}$; $Ser^D/+$. Arrow points to thickened L5 vein. All wings are reproduced at the same magnification.

embryos and imaginal discs, *kuz* expression is ubiquitous but is strongest in the condensed ventral chord (not shown and Fambrough et al., 1996), the morphogenetic furrow and the developing ommatidia of the eye-antenna disc (Fig. 3B,D).

The other transcription unit (named *B4*) gives rise to several transcripts ranging in size from 7.8 to 3.6 kb (not shown). The longest one encodes a novel 964 aminoacid protein with no significant similarity to known proteins. In embryos and in imaginal discs, *B4* is expressed ubiquitously and at increased levels in neural precursors (neuroblasts and sensory organ mother cells (SMCs), not shown).

The four P-insertions map within a very short stretch of DNA located within the untranslated leader sequences of *kuz*. *l(2)k01405, l(2)k01403* and *l(2)k11804* are inserted 69 bp downstream from the 5′ end of the longest *kuz* cDNA while *l(2)k07601* is inserted, in the opposite orientation, 48 bp downstream from that point. Transcription of *kuz* is undetectable by in situ hybridization in *l(2)k01405* and *l(2)k01403* imaginal discs, whereas that of B4 is only weakly affected (Fig. 3C,E and not shown). The 5′ end of *B4* cDNA is 892 bp from the *l(2)k01405* insertion point.

The above data suggested that *kuz* is the transcription unit affected by the P-insertions. To determine whether alteration of *B4* transcription also contributed to the mutant phenotype, we examined the rescuing ability of either *kuz* or *B4* using the GAL4/UAS system (Brand and Perrimon, 1993). A *UAS-kuz* transgene driven ubiquitously using the GAL4 line C-765 (Gómez-Skarmeta et al., 1996 and our data), rescues the development of wings, halteres, thorax, legs and eyes and restores an almost normal pattern of SOs (Fig. 2H,K,M and not shown). However, several notum microchaetae are missing and extra stout bristles occur on the anterior wing margin, some of them with abnormal morphology (Fig. 2H,M). This residual phenotype was never observed in kuz^+*;UAS-kuz/GAL4-C765* flies (not shown). The low viability of *l(2)k01405* flies is not rescued by *UAS-kuz.* In contrast, the ubiquitous expression of

UAS-B4 does not modify the abnormal morphology of *l(2)k01405* flies, although it does increase their viability. These results indicate that the *l(2)k01405* phenotype is mostly due to interference with *kuz* function. Accordingly, we will refer to it as kuz^{1405}.

kuz is a neurogenic gene

The phenotype of *kuz* pharate adults is similar, albeit not identical, to those associated with loss-of-function mutations of the neurogenic genes (Artavanis-Tsakonas et al., 1995). During SO development, N signalling limits SMC singling out in proneural clusters and, subsequently, helps implement the correct fates to the SMC descendants (Shellenbarger and Mohler, 1978; Hartenstein and Posakony, 1990; de Celis et al., 1991). Thus, in the absence of *N* or *Su(H)* function, many cells in each proneural cluster become SMCs and all four SMC descendants differentiate as neurons, which results in patches of naked cuticle (Heitzler and Simpson, 1991; Schweisguth, 1995; de Celis et al., 1996a,b). We observe these anomalies in kuz^{1405} wing discs that account for the generation in *kuz* clones of both patches of naked cuticle and tufts of SOs (Fig. 2). Thus, although the proneural clusters of the notum region are present at their normal locations (Fig. 1A,E), most or all of their cells accumulate high levels of AC/SC proteins and express the *neuralized* gene, two exclusive characteristics of SMCs (Cubas et al., 1991; Huang et al., 1991; Skeath and Carroll, 1991) (compare Fig. 1A,B with 1E,F). Moreover, immunostaining of kuz^{1405} pupal nota with mAb22C10 (Hartenstein and Posakony, 1990), revealed the presence of lawns of neurons at sites where only one or a few neurons are present in the wild type (Fig. 1D,H). This indicates that the abnormal regions of naked cuticle of kuz^{1405} pharate adults correspond to sites where an initial overproduction of SMCs is followed by the differentiation of all SMC descendants as neurons. These results indicate that *kuz* belongs to the neurogenic class of genes.

Cell autonomous requirements of *kuz* in SO and wing development

The function of *kuz* in the development and patterning of wing and notum was analyzed in mitotic recombination clones of cells homozygous for kuz^{1405}, kuz^{11804} and kuz^{H143} mutations. The three alleles have identical clonal phenotypes, which consist on the differentiation of tufts of SOs, patches of naked cuticle, thicker veins and deletions of wing material in the proximity of the wing margin. *kuz* clones were recovered all over the adult epidermis at the expected frequency and were of similar size to the control twin clones (not shown) indicating that *kuz* is dispensable for cell proliferation.

Clones of *kuz* cells induced before 96 hours AEL display simultaneously loss of any kind of SO (Fig. 2B,C) and the replacement of extant single SOs by tufts or groups of adjacent sensilla (Fig. 2B,C,F). We never observe tufts of SOs outside of SO wild-type positions suggesting that loss of *kuz* does not modify the distribution of neural potential within the disc (see above).

The analysis of twin clones show that clusters of *f kuz* SOs

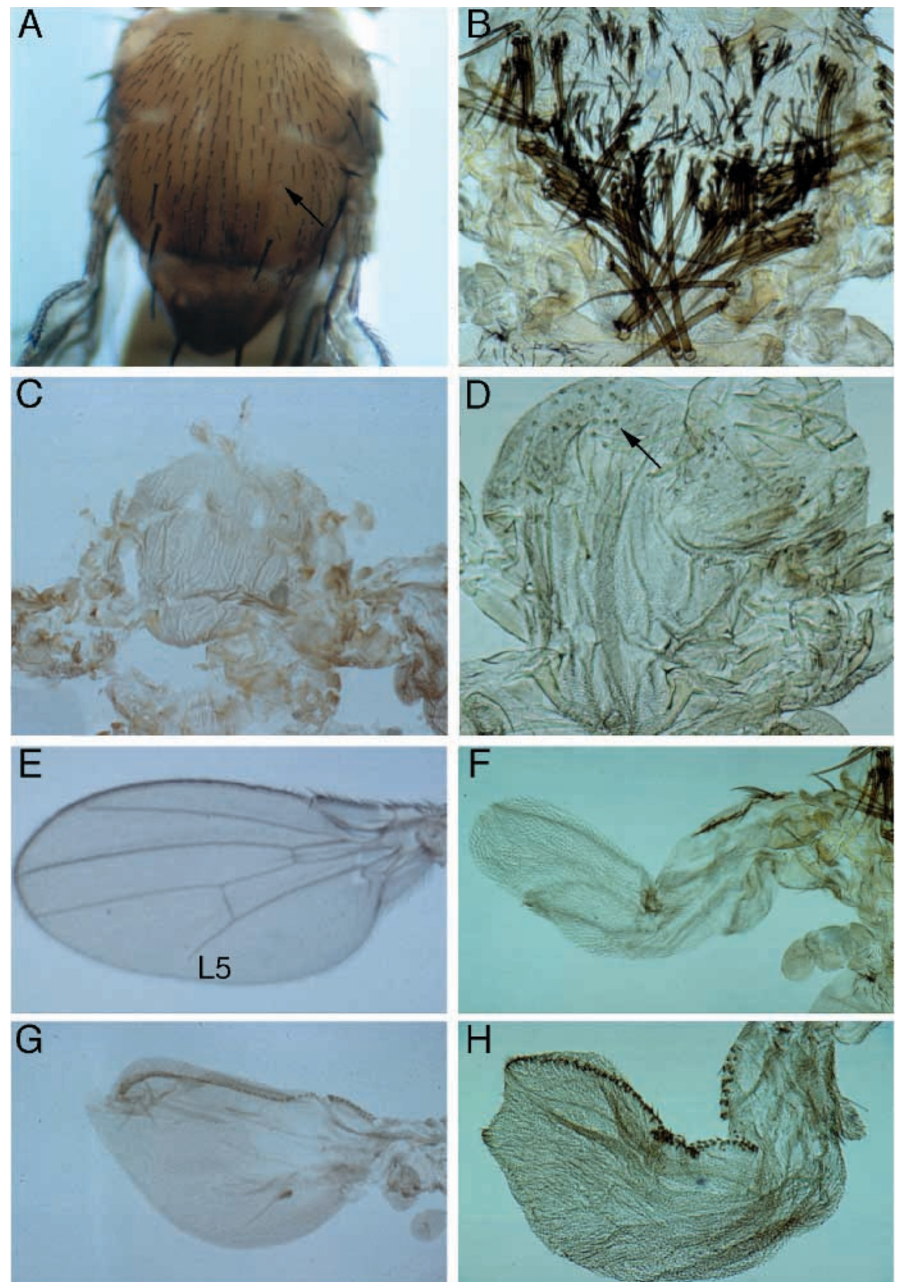

Fig. 5. Dose-sensitive interactions between *kuz* and *H*. Nota (A-D) and wings (E-H) from $H^2/+$ (A,E), H^2 (C,G), $kuz^{1405}; H^2/+$ (B,F) and $kuz^{1405}; H^2$ (D,H). Arrows in A and D point to bristles showing the double-socket phenotype. Note in H that $kuz^{1405}; H^2$ wings show notches at the wing margin.

and regions of naked cuticle are adjacent to *ck*-marked wild-type clones (Fig. 2C), which indicates that the mutant phenotype is not rescued by wild-type adjacent cells. Moreover, tufts of bristles are always formed by *f; kuz* mutant cells. These results show the cell autonomy of the *kuz* loss-of-function phenotype. However, microchaetae are frequently missing within the wild-type twin clone (Fig. 2C and not shown), a phenomenon also observed near *N* or *E(spl)* clones and attributed to excessive inhibitory signal originating from the extra SMCs of the mutant clone (Heitzler and Simpson, 1991; Heitzler et al., 1996).

Clusters of SOs may appear in the middle of the clone (Fig. 2F). This indicates that, contrary to the suggestion of Rooke et al. (1996), *kuz* mutant cells do not require wild-type *kuz* function provided by kuz^{+} neighbouring cells to follow the neural fate.

On the wing, *kuz* cells differentiate vein histotype autonomously but only when they are in close proximity to normal veins (Fig. 2D,E) indicating a failure in the lateral inhibition process restricting vein width. Moreover, *kuz* clones also affect wing growth and the formation of the wing margin. Thus, dorsal or ventral clones initiated during the first or second larval instar and abutting the wing margin induce nicks and loss of wing blade tissue (scalloping phenotype, Fig. 2D,E). The extent of missing tissue depends on the time of clone induction and ranges from small notches to loss of one entire compartment (not shown).

In summary, the phenotypes of *kuz* clones in vein differentiation, wing margin and SO formation, are largely similar to those associated with loss-of-function alleles of *N* and *Su(H)* (Heitzler and Simpson, 1991; de Celis and García-Bellido, 1994b; Schweisguth, 1995; de Celis et al., 1996a,b, 1997).

Interaction with Notch

A functional relationship between the Notch pathway and *kuz* function predicts a genetic interaction between *N* and *kuz* alleles. Indeed, kuz^{1405} and its partial revertants strongly potentiate the scalloping of the wing margin and thickening of the wing veins typical of $N^{55e11}/+$ flies (Fig. 4A-C), suggesting that *kuz* insufficiency reduces *N* activity.

N function is also decreased by the $Serrate^{D}$ (Ser^{D}) mutation, which apparently titrates active N by an excess of the Ser ligand (Thomas et al., 1991). In agreement with a reduced *N* function in *kuz* mutants, the scalloping phenotype of Ser^{D} is enhanced by $kuz^{1405}/kuz^{1405\text{-}Rev4}$ (Fig. 4D-F) just as the Ser^{D} phenotype is enhanced by different *N* alleles (Thomas et al., 1991). Taken together, the interactions between *kuz* and *Notch* mutations, the similarity of *kuz* and *N* phenotypes and the cell autonomy of *kuz* requirement indicate that *kuz* participates in the N pathway in the receptor cell. To place *kuz* function in this pathway, either in N activation or in N signalling subsequent to its activation, we examined the effects of reducing *kuz* activity in several genetics backgrounds.

Overexpression of *E(spl)m8* partially corrects the neurogenic phenotype of *kuz* larvae

N activation in the cells of proneural clusters leads to transcription of the *E(spl)m8* gene (Bailey and Posakony, 1995; Jennings et al., 1995; Lecourtois and Schweisguth, 1995). In kuz^{1405} discs, *E(spl)m8* expression is undetectable (Fig. 1G), indicating again that N signalling is impaired. Restoring *E(spl)m8* expression independently from the *N* pathway by means of a GAL4-activated *UAS-E(spl)m8* transgene, partially rescues *kuz* neurogenic phenotype (Fig. 2I) since many single SOs, rather than tuft of SOs, develop. In addition, many SOs failed to develop, an effect typical of *E(spl)m8* overexpression (Tata and Hartley, 1995; de Celis et al., 1996b; Nakao and Campos-Ortega, 1996 and S. S. unpublished) that is not suppressed by the *kuz* mutation. These data indicate that *kuz* functions upstream of *E(spl)m8* activation.

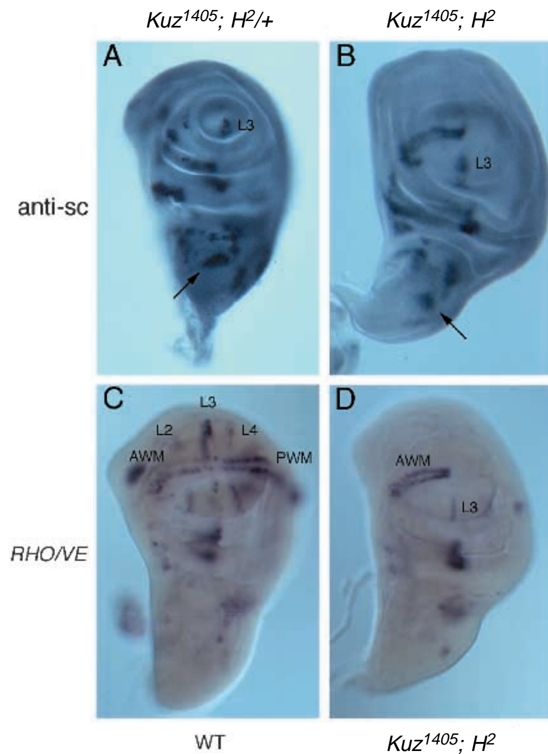

Fig. 6. Imaginal disc phenotypes of kuz^{1405}; H^{2} combinations Expression of *sc* (A,B) and *rho/ve* (C,D) in kuz^{1405}; $H^{2}/+$ (A), kuz^{1405}; H^{2} (B,D) and wild-type (C) wing discs. Note in D that only *rho/ve* expression in SMCs is maintained. L2, L3 L4, longitudinal veins; AWM, anterior wing margin; PWM, posterior wing margin.

kuz interacts with *H*

N signalling is mediated by *Su(H)*, a putative transcriptional activator, whose function is antagonized by the H nuclear protein (Brou et al., 1994). *Su(H)* imaginal discs are very similar to kuz^{1405} discs in size, shape and large number of SMCs in proneural clusters (Schweisguth and Posakony, 1992), suggesting that *kuz* mutation causes insufficiency of *Su(H)* function. If this were the case, *kuz* phenotypes should be particularly sensitive to changes in the level of the Su(H) competitor H. (Interaction between *kuz* and *Su(H)* was not tested directly due to their neighbouring chromosomal positions).

Indeed, the combination of kuz^{1405} with H^{2} normalizes the phenotype of both mutations. H^{2} mutants lack most SOs and wing veins (Fig. 5C,G) and have enlarged wing discs (Schweisguth and Posakony, 1994; Bang et al., 1995) while $H^{2}/+$ flies lack few SOs (some of them displaying the ‘double socket’ phenotype) and only lack the distal part of the L5 wing vein (Fig. 5A,E). kuz^{1405} largely increases the number of SOs

of H^2 and $H^2/+$ flies and all of them show the double socket phenotype (in kuz^{1405}; H^2, Fig. 5D,H) or a normal morphology (in kuz^{1405}; $H^2/+$, Fig. 5B). In contrast, the absence of veins and the wing disc overgrowth associated with H^2 are not normalized by *kuz* (Fig. 6B,D).

Conversely, the neurogenic phenotype of *kuz* and the reduced size of the wing discs and the resulting wings are gradually corrected with increasing *H* insufficiency (Figs 5B,D, 6A,B and 5 F,H, respectively). Since depletion of H results in increased free Su(H) (Brou et al., 1994; Bang et al., 1995), these findings suggest that *kuz* mutation decreases the amount of active Su(H) and, therefore, that *kuz* acts upstream of *Su(H)*.

N activation bypasses the requirement for *kuz*

We next analyzed the effect of reduced *kuz* activity in flies harbouring either of two forms of N protein, an Ax variant and a constitutively active fragment of N. The Ax^{M3} allele, a *N* allele that shows ligand-dependent hyperactivation (de Celis and García-Bellido, 1994a), has enlarged discs with reduced accumulation of AC/SC proteins in proneural clusters (Fig. 7E). Consequently, Ax^{M3} escapers have wings larger than normal and lack most SOs (Fig. 7A,C and de Celis et al., 1991). These effects depend on kuz^+ function. Thus, Ax^{M3}; $kuz^{1405\text{-}Rev11}/+$ imaginal discs are normal sized, with partially rescued AC/SC accumulation (Fig. 7F) and Ax^{M3}; $kuz^{1405\text{-}Rev11}$ imaginal discs are further reduced (morphologically similar to those of $kuz^{1405\text{-}Rev11}$) and with strongly staining proneural clusters (Fig. 7G). Accordingly, the resulting flies have reduced wings and SOs arranged in a pattern similar to that of $kuz^{1405\text{-}Rev11}$ flies, albeit with less bristles (Fig. 7B,D). The vein shortening of Ax^{16172} flies is also partially corrected in the Ax^{16172}; $kuz^{1405}/+$ combination (not shown).

The *HS-N(i)* transgene expresses a truncated N molecule, devoid of the extracellular domain, which displays constitutive *N* activity (Struhl et al., 1993). Signalling by N(i) is not impaired in kuz^{1405} flies since pharate kuz^{1405}; *HS-N(i)/+* flies have normal-sized eyes and notum and their legs, wings and halteres are substantially larger than those of kuz^{1405} flies (Fig. 8 and not shown). Moreover the loss of SOs associated to *N(i)* expression (Struhl et al., 1993) is not modified in *kuz* mutants (compare Figs 8A and 2A). Taken together, our results indicate that *kuz* is required for generating a functional N receptor and/or for N interaction with its ligands, but not for signalling downstream of the activated receptor.

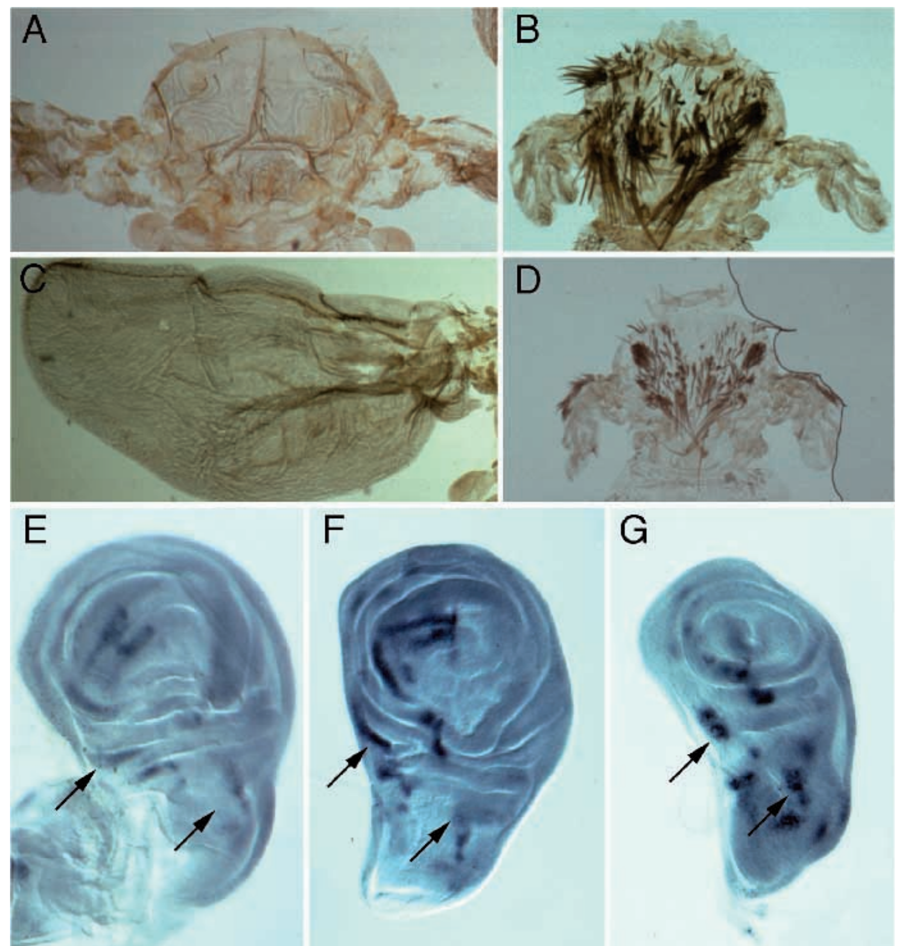

Fig. 7. The *Ax* phenotype requires *kuz* function. (A-D) Nota and wings from Ax^{M3} (A,C), $kuz^{1405\text{-}Rev11}$ (B) and Ax^{M3}; $kuz^{1405\text{-}Rev11}$ (D) males. (E-G) Expression of *sc* in wing imaginal discs from Ax^{M3} (E), Ax^{M3}; $kuz^{1405\text{-}Rev11}/+$ (F) and Ax^{M3}; $kuz^{1405\text{-}Rev11}$ (G) detected by staining with anti-*sc* antibody. Arrows point to equivalent proneural clusters.

DISCUSSION

The phenotype of *kuz* mutations indicates that *kuz* is required for all the *N*-mediated developmental processes that we have examined: neural versus epidermal cell fate decisions, imaginal disc growth, wing margin formation, vein differentiation and leg and eye development. The possibility of *kuz* functioning in a pathway parallel to that of *N* seems ruled out by the genetic interactions of *kuz* with several components of the *N* pathway described in this work and in Pan and Rubin (1997). These results thus indicate that *kuz* is a new component of this intercellular signalling pathway.

kuz is autonomously required for reception of Notch-mediated intercellular signals

In *kuz* mutants, all or most cells of proneural clusters develop as SMCs, indicating a failure of lateral inhibition. We have investigated whether this failure is due to the inability of *kuz* cells to send or to receive the inhibitory signal. Clusters of SOs or patches of naked cuticle develop in *kuz* homozygous clones even when the mutant cells are adjacent to wild-type ones. This indicates that *kuz* cells do not respond to the inhibitory signal sent by wild-type neighbours. However, these mutant cells are able to signal to their neighbours, since they inhibit development of chaetae in the adjacent wild-type territory. Moreover,

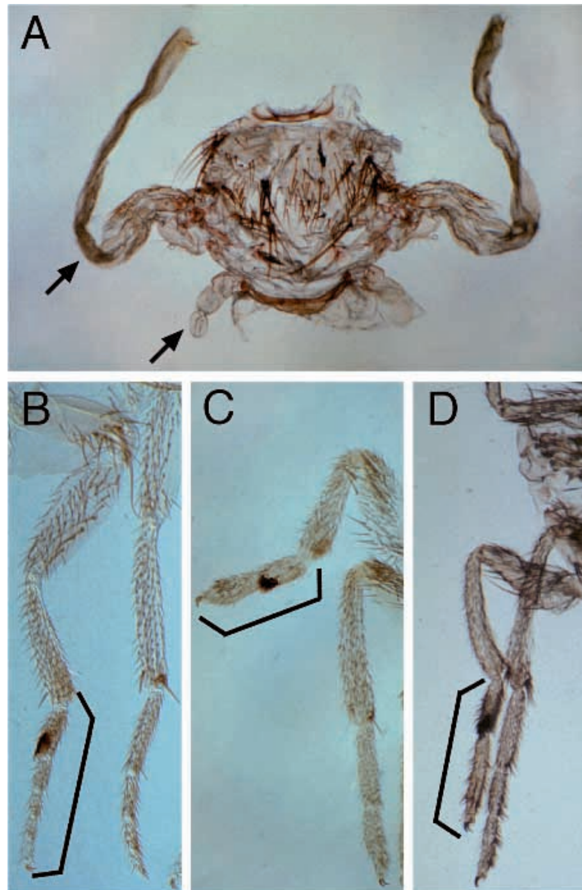

Fig. 8. Signalling downstream of activated N does not require *kuz* function. (A) Notum and wing of a kuz^{1405}; *HS-N(i)/+* pharate adult raised at 25°C. Arrows point to the partially rescued wing and haltere. Legs of wild-type (B), kuz^{1405} (C) and kuz^{1405}; *HS-N(i)/+* (D) flies. Brackets indicate the tarsal segments which are reduced in size and fused together in kuz^{1405} pharate adults and are partially rescued by basal *HS-N(i)* expression. Enhanced expression of *HS-N(i)*, by short incubations at 37°C, was lethal.

in the wing, *kuz* cells autonomously differentiate vein histotype and *kuz* clones cause wing margin nicks and loss of wing blade tissue regardless of whether they are dorsal or ventral, whereas clones of cells mutant for the N ligands *Dl* or *Ser* show this phenotype only when present in the ventral or dorsal surface, respectively (Díaz-Benjumea and Cohen, 1995; de Celis et al., 1996a; Doherty et al., 1996). These results indicate that, at least in neurogenesis and wing development, *kuz* function is required in the cell where Notch is activated.

kuz functions upstream of *E(spl)m8* and *Su(H)* activation

The neurogenic *kuz* phenotype can be rescued by an expression of *E(spl)m8* independent of the N pathway and by depletion of H (the Su(H) antagonist), indicating that *kuz* is required for N-dependent activation of *E(spl)m8* or *Su(H)* genes. On the contrary, *kuz* mutations seem not to interfere with the funtion of their products. Indeed, the development of fewer SOs in kuz^{1405}; *UAS-E(spl)m8/GAL4-C765* than in kuz^{1405} flies and the suppression of their tufts of bristles indicate that *E(spl)m8*, an antagonist of neurogenesis (Tata and Hartley, 1995; de Celis et al., 1996b; Heitzler et al., 1996; Nakao and Campos-Ortega, 1996), is functional in *kuz* flies. Similarly, the development of normally spaced SOs in kuz^{1405}, H^2 flies suggests the presence of active Su(H) product, sufficient to mediate lateral inhibition within proneural clusters. However, a partial suppression of the *Su(H)* neurogenic phenotype in double null mutant *Su(H); H* individuals (Schweisguth and Posakony, 1994) suggests that H may antagonize the function of other protein(s), in addition to Su(H), that restrict neurogenesis (Brou et al., 1994). Accordingly, the function of this unidentified protein(s) may also account for the normal spacing of SMCs in kuz^{1405}, H^2 discs. Still, the double sockets, the overgrown wing discs and the lack of wing veins of kuz^{1405}, H^2 flies, which are identical in H^2 individuals, also support the presence of active Su(H) in these flies. Moreover, wing disc overgrowth cannot be attributed to alternative proteins, putatively antagonized by H, since *Su(H); H* and *Su(H)* discs are similarly reduced in size (Schweisguth and Posakony, 1994).

Kuz is required for functional Notch molecules

The discussed data place the requirement for *kuz* function at the cell where the Notch receptor is activated and at a step previous to *Su(H)* activation. We have used two different gain-of-function N variants, a ligand-dependent *Ax* allele and a ligand-independent *N(i)* fragment, to further locate *kuz* function in the N pathway. *kuz* mutation reduces or eliminates the *Ax* phenotypes but it does not interfere with the effects of the overexpression of *N(i)* (our results and see also Pan and Rubin (1997). This suggests that *kuz* is required for N to interact with its ligands and/or for a subsequent step in the activation of N, previous to N signalling, mediated by the intracellular domain of N.

The sequence of the Kuz molecule indicates that it harbours a metalloprotease center located in the extracellular domain of the protein (Rooke et al., 1996). These data and the requirement of *kuz* for ligand-dependent N activation in *Ax* mutants suggest that Kuz is involved in processing the extracellular domain of N to generate a functional receptor. This seems to be the case as Pan and Rubin (1997) have demonstrated a Kuz-dependent cleavage of N (although the possibility of Kuz activating a proteolytic cascade has not been ruled out). This proteolysis, which probably occurs in the trans-Golgi network and between the EGF and Lin-12/Notch repeats (Blaumueller et al., 1997), is independent of the binding of Dl (Pan and Rubin, 1997) and generates two fragments, which remain tethered at the plasma membrane forming the functional receptor (Blaumueller et al., 1997; Pan and Rubin, 1997). Accordingly, N signalling can be envisaged as a cascade of N cleavage events. Kuz-dependent proteolysis would generate a functional receptor able to interact with Dl. A conformational change of N subsequent to Dl binding would make N a substrate for another unidentified protease, such that the intracellular domain of N would be released and translocated to the nucleus to regulate, as a dimer with Su(H), the expression of target genes (Jarriault et al., 1995; Kopan et al., 1996). Note that both Kuz-dependent cleavage and binding of Dl seem to be required for N activity since a variant of mammalian N that includes the extracellular Lin-12/Notch repeats but lacks the Dl-binding site is inactive (Kopan et al., 1996). This protein is processed in its extracellular domain and yields a fragment of a size compatible with that produced by Kuz-dependent cleavage. However, it is not cleaved in its intracellular domain.

Although Kuz is necessary for generating a N functional receptor, the phenotypes associated with loss of function of *N* or of *kuz* are not identical. Thus, whereas *N* cells have a reduced capacity to proliferate (de Celis and García-Bellido, 1994b) we find that *kuz* cells proliferate normally. Moreover, our results and those of Rooke et al. (1996), obtained with a different *kuz* null allele, show that *kuz* neurogenic phenotype is milder than those associated with loss of *N* or *Su(H)*. Thus, clones of cells homozygous for *N* or *Su(H)* null alleles only develop patches of naked cuticle, as all SMC descendants differentiate as neurons (Heitzler et al., 1991; Schweisguth, 1995; de Celis et al., 1996) while those of *kuz* cells produce both naked patches and tufts of SOs. This suggest that *kuz* cells have residual active Su(H), which allows part of the SMC progeny to develop as the epidermal component of SOs. Since Su(H) activation depends on the N signalling pathway (Artavanis-Tsakonas et al., 1995), these results suggest that there may be alternative ways other than Kuz-dependent proteolysis to generate a functional N receptor. Interestingly, the residual amount of Su(H) present in *kuz* flies appears to be increased by depletion of *H* and to become excessive for correct SMC daughter cell specification, wing disc growth and vein differentiation. In contrast, SMC singling out operates normally in these flies, which suggests a differential requirement for Su(H) in these processes.

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