kuzbanian-mediated cleavage of *Drosophila* Notch

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Loss of Kuzbanian, a member of the ADAM family of metalloproteases, produces neurogenic phenotypes in *Drosophila*. It has been suggested that this results from a requirement for *kuzbanian*-mediated cleavage of the Notch ligand Delta. Using transgenic *Drosophila* expressing transmembrane Notch proteins, we show that *kuzbanian*, independent of any role in Delta processing, is required for the cleavage of Notch. We show that Kuzbanian can physically associate with Notch and that removal of *kuzbanian* activity by RNA-mediated interference in *Drosophila* tissue culture cells eliminates processing of ligand-independent transmembrane Notch molecules. Our data suggest that in *Drosophila*, *kuzbanian* can mediate S2 cleavage of Notch.

[*Key Words*: Notch; Kuzbanian; processing; *Drosophila*]

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Notch (N) is an ~3000-amino-acid transmembrane protein that is found in a wide variety of organisms ranging from *Caenorhabditis elegans* to humans. It is a receptor in a signal transduction pathway that mediates intercellular communication (for recent reviews, see Greenwald 1998; Artavanis-Tsakonas et al. 1999; Mumm and Kopan 2000). Upon binding its ligands, members of the DSL (Delta, Serrate, Lag2) family of transmembrane ligands, N is cleaved in its extracellular domain at a site 11 amino acids amino terminal to the transmembrane domain (Brou et al. 2000; Mumm et al. 2000). In vitro this S2 cleavage of mammalian N can be mediated by TNF-α converting enzyme (TACE; Brou et al. 2000), a member of the ADAM family of metalloproteases (for recent reviews, see Schlondorff and Blobel 1999; Primakoff and Myles 2000). Following S2 cleavage, N undergoes an intramembranous cleavage (S3) to release the soluble cytoplasmic domain, which, in conjunction with a member of the CSL (CBF1, Suppressor of Hairless, Lag1) family of DNA-binding proteins, enters the nucleus and activates transcription (Kidd et al. 1998; Lecourtois and Schweisguth 1998; Schroeter et al. 1998; Struhl and Adachi 1998). This S3 cleavage requires *Presenilin* (*Psn*) activity (De Strooper et al. 1999; Struhl and Greenwald 1999; Ye et al. 1999).

Mammalian N, but not *Drosophila* N, is also constitutively cleaved as part of its maturation process, in its extracellular domain at amino acid 1654, so that it is presented on the cell surface as a heterodimer (Blaumueller et al. 1997; Kidd et al., in prep.). This S1 cleavage was originally thought to be carried out by Kuzbanian (Pan and Rubin 1997), another member of the ADAM family, but has since been shown to be mediated by a furin-like enzyme (Logeat et al. 1998). More recently *kuzbanian* (*kuz*) has been shown to mediate the cleavage of Delta (Dl; Qi et al. 1999), yet in both *Drosophila* and *C. elegans*, *kuz* has been shown to be cell-autonomous, being required in the receiving cell (Rooke et al. 1996; Sotillos et al. 1997; Wen et al. 1997).

In this paper we show that Kuz can physically associate with N. This association led us to reexamine the role of *kuz* in the cleavage of N. We generated transgenic *Drosophila* expressing transmembrane N proteins that can act independently of *Dl* and assayed the function of these proteins in embryos, both phenotypically and biochemically. The function of a N protein whose activity is completely independent of *Dl* is almost completely dependent on *kuz*. Using RNA-mediated interference in *Drosophila* S2 cells, which do not express any known N ligands, we show that the cleavage of N proteins that can function independently of *Dl* requires *kuz*. This *kuz* activity acts upstream of *Psn* activity. Our data suggest that in *Drosophila*, *kuz* can mediate S2 cleavage of N.

Results

Kuzbanian can physically associate with Notch

We added 6 myc epitope tags to the carboxyl termini of both Kuz and a dominant-negative form of Kuz, lacking the protease domain Kuz^{DN} (Pan and Rubin 1997). Myc-tagged Kuz or Kuz^{DN} was coexpressed with N in S2 cells, and the extracts were immunoprecipitated with anti-myc antibodies. As can be seen in Figure 2A, lanes 2 and 3 (below), N is coimmunoprecipitated by anti-myc antibodies only when it is coexpressed along with Kuz or

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Kuz^{DN} (Fig. 2, cf. lanes 2 and 3 with lane 1; these cells were transfected with N alone). To address whether this interaction is direct, we generated in bacteria a GST–N fusion protein encoding amino acids 1623–1893 of N (BD in Fig. 1A). In vitro translated Suppressor of Hairless [Su(H)] and Kuz can be pulled down by this GST–N fusion (Fig. 2B, lanes 3,6), but in vitro translated human insulin receptor cannot (Fig. 2B, lane 9). None of the in vitro translation products associates with GST alone (Fig. 2B, lanes 2,5,8). Thus there is a direct interaction between Kuz and N.

Whereas Pan and Rubin originally proposed that *kuz* is responsible for cleavage of N (Pan and Rubin 1997), more recently it has been suggested that the phenotypes resulting from loss of *kuz* are attributable to its role in the processing of Dl, and no effect of the loss of *kuz* on N proteins was seen in flies (Qi et al. 1999) or in mammalian cells (Brou et al. 2000; Mumm et al. 2000). Because of the association we observed between N and Kuz, we reinvestigated the role of *kuz* in the cleavage of N. Because it has been proposed that Kuz cleaves Dl, we worked with N molecules that can function independently of Dl.

The N molecules we have used to analyze the role of *kuz* are depicted in Figure 1. All the N proteins are tagged at their carboxyl termini with the DNA-binding domain of LexA so that their cleavage can be monitored in vivo, and all the Western blots of embryonic extracts described below were probed with anti-LexA antibodies. N^{LexA}, $N^{IntraLexA}$, which encodes the soluble cytoplasmic domain of N, and $N^{\Delta LNrptsLexA}$ (LN^{LexA}; deleted for amino acids 1469–1625) have been described previously

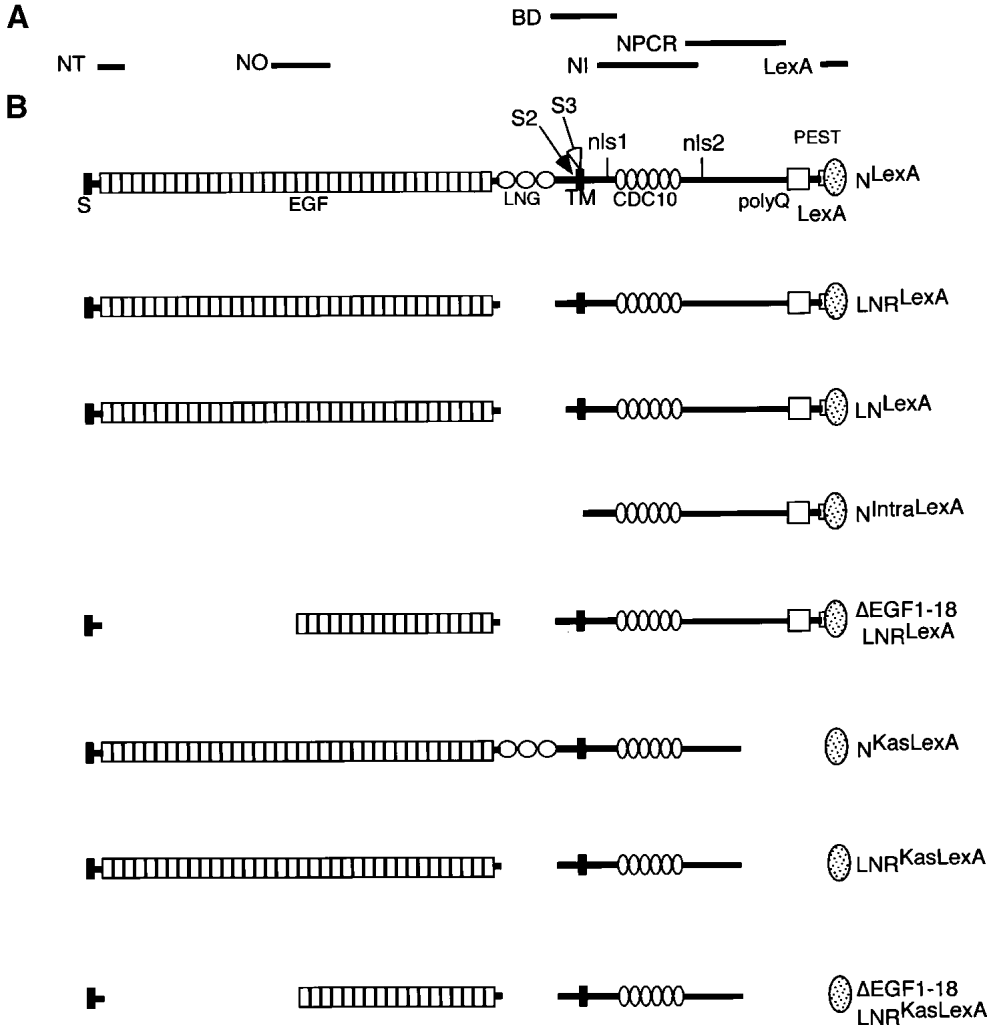

Figure 1. Diagram of N constructs and localization of epitopes recognized by antibodies. (*A*) The antibodies used in this work, above the regions of N used to generate them, and the region of N fused to GST (BD) used in the pull-down assays in Figure 2B. The top molecule in *B* is wild-type N tagged with the DNA-binding domain of LexA, N^{LexA}. (S) Signal sequence; (EGF) epidermal growth factor-like repeats; (LNG) Lin-12, N, Glp-1 repeats; (S2) location of TACE cleavage site in mammals; (S3) location of Psn-dependent cleavage site; (TM) transmembrane domain; (nls1, nls2) nuclear localization signals; (CDC10) cdc10 or ankyrin repeats; (polyQ) polymeric glutamines; (PEST) PEST sequence thought to be involved in protein stability. Shown beneath N^{LexA} are the various deletions used in this work.

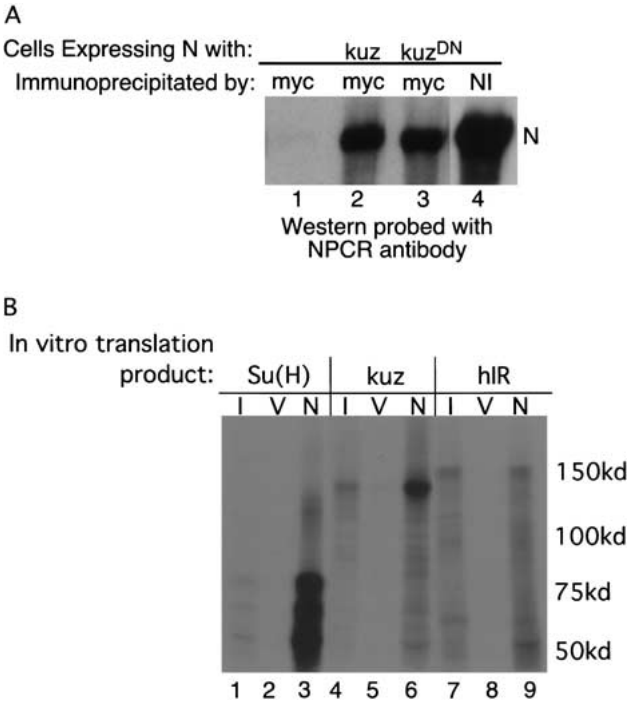

Figure 2. Kuz can associate with N. (*A*) S2 cells transfected with actin-driven *N* alone (lanes *1*,*4*) or with actin *N* plus actin-driven myc-tagged *kuz* (lane *2*) or kuz^{DN} (lane *3*) were immunoprecipitated with anti-myc (lanes *1*–*3*) or anti-NI (lane *4*) antibodies, and the Western probed with anti-NPCR antisera. See Figure 1A for location of the epitopes recognized by the antibodies. (*B*) Bacterially produced GST–N fusion protein encoding amino acids 1623–1893 of N (from the end of the LNG repeats to the start of the ankyrin repeats, BD in Fig. 1A) was used in pull-down assays of in vitro translated Su(H) (lane *3*), Kuz (lane *6*) and human insulin receptor (hIR, lane *9*) labeled with ^{35}S methionine. (I) 1% of the input in vitro translation product; (V) GST alone; (N) GST–N fusion.

(Lieber et al. 1993; Kidd et al. 1998). The deletion in $N^{\Delta LNRLexA}$ (LNR^{LexA}; deleted for amino acids 1482–1593) is a subset of $N^{\Delta LNrptsLexA}$ and encompasses just the LNG (Lin12/Notch/Glp1) repeats. $N^{\Delta EGF1-18\ and\ LNRLexA}$ is missing EGF repeats 1–18, as well as amino acids 1482–1593.

When expressed in S2 cells along with myc-tagged Kuz or Kuz^{DN}, both LN and LNR can be coimmunoprecipitated by anti-myc antibodies. We did not observe a difference in the degree of association of N, LN, or LNR with Kuz or with Kuz^{DN} (data not shown).

The strength of the antineurogenic phenotype correlates with both the levels of S3 cleavage and nuclear localization

In *Drosophila* embryos loss of members of the *N* signal transduction pathway causes overproduction of neuroblasts at the expense of epidermoblasts, resulting in hypertrophy of the embryonic central and peripheral nervous systems. Conversely, expression of gain-of-function N molecules generates antineurogenic phenotypes in which neuroblasts are underproduced, resulting in the loss of central and peripheral nervous system elements. Figure 3, A–E, shows the effect on the morphology of the embryonic central nervous system (CNS) of ectopically expressing N^{LexA}, LN^{LexA}, or LNR^{LexA} using *daughterless* (*da*) GAL4 as a driver. Although expression of N^{LexA} does not grossly affect the structure of the CNS (Fig. 3A), the expression of LN^{LexA} (Fig. 3B,C) or LNR^{LexA} (Fig. 3D,E) produces gaps in the architecture of the CNS characteristic of an antineurogenic phenotype (Lieber et al. 1993). However, the antineurogenic phenotype produced by LNR^{LexA} (Fig. 3D,E) is stronger than that produced by LN^{LexA} (Fig. 3B,C). It is possible that this difference is due to the insertion sites of the transgenes, but we think this is unlikely for two reasons. First, we immunoprecipitated extracts of embryos expressing N^{LexA}, LN^{LexA}, and LNR^{LexA} with anti-Su(H) antibodies and probed the Western with anti-LexA antibodies. We could then control for the expression level of the transgenes by determining the ratio of S3-cleaved N bound to Su(H) to uncleaved N associated with Su(H). An example is shown in Figure 3F. Although the ratio of S3-cleaved N to N ($N^{pp114LexA}$ or $N^{p100LexA}$:N^{LexA}) is approximately the same in extracts of embryos expressing N^{LexA} and LN^{LexA}, the ratio is at least 7-fold greater in embryos expressing LNR^{LexA}. (Six separate experiments were quantitated.) Second, as can be seen in Figure 3, G–I, when transiently expressed in *Drosophila* S2 cells, the cytoplasmic domain of LNR (Fig. 3I) unlike that of N (Fig. 3G) and LN (Fig. 3H) can readily be detected in nuclei. Using anti-LexA antibodies, we were able to detect the cytoplasmic domain of LexA-tagged LNR in the nuclei of embryos as well (Fig. 3K). Thus, the strength of the antineurogenic phenotype correlates with both the levels of S3 cleavage and nuclear localization. This suggests that the function of a N molecule as assayed phenotypically in different genetic backgrounds can be correlated with the activity of this N molecule as assayed biochemically and provides a basis for us to interpret the effects of loss of elements of the *N* pathway on N function.

LN and LNR are Delta- and kuzbanian-independent but Delta- and kuzbanian- responsive

We then assayed the function of the mutated N proteins in *Dl* and *kuz* backgrounds. Because *Dl* is required in the germ line for proper development of follicle cells (López-Schier and St. Johnston 2001), we were unable to generate embryos that were maternally *Dl* null, and our experiments were therefore carried out in embryos that were zygotic *Dl* nulls. As can be seen in Figure 4, A and B, both LN and LNR can function independently of *Dl*, although in neither case was the antineurogenic phenotype produced in a *Dl* background as strong as that produced in a wild-type background (Fig. 4, cf. 4A with 3B and 4B with 3D). This contrasts with what we observe with $N^{IntraLexA}$, where the antineurogenic phenotypes

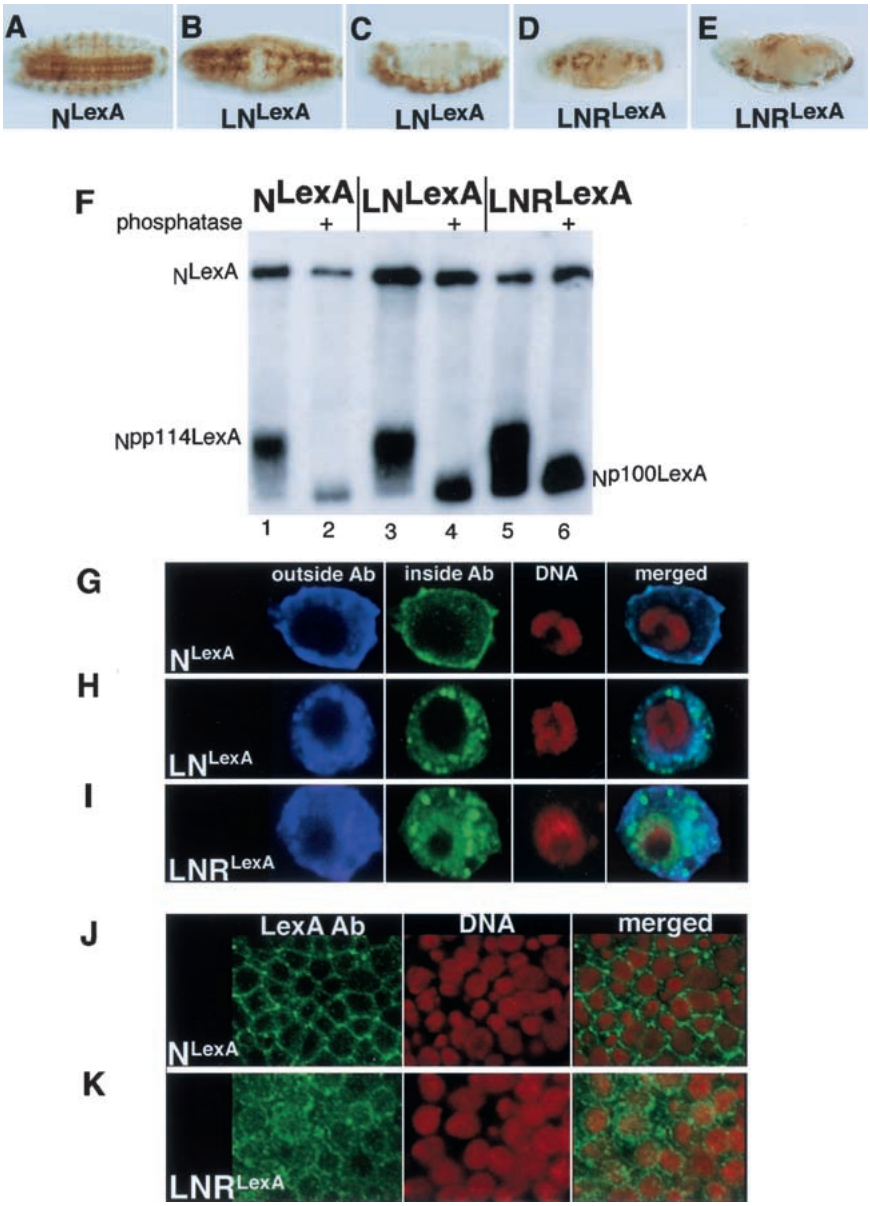

Figure 3. A comparison of the activities of LN^{LexA} and LNR^{LexA}. (*A–E*) The effect that expressing N^{LexA} (*A*), LN^{LexA} (*B,C*), or LNR^{LexA} (*D,E*) under control of *daughterless* (*da*) *GAL4* (a ubiquitous driver) has on the nervous system of wild-type embryos. (*A,B,D*) Ventral views; (*C,E*) lateral views. The embryos were stained with an anti-horseradish peroxidase (HRP) antibody that reacts with the nervous system. (*F*) Western blot showing increased production of $N^{pp114LexA}$ in embryos expressing LNR^{LexA}. Extracts of embryos expressing N^{LexA} (lanes *1,2*), LN^{LexA} (lanes *3,4*), or LNR^{LexA} (lanes *5,6*) under the control of *daGAL4* were immunoprecipitated with anti-Su(H) antisera, and the Western was reacted with anti-LexA antisera. The immunoprecipitates in lanes *2*, *4*, and *6* were treated with phosphatase prior to electrophoresis. N^{LexA} is the full-length N protein that coimmunoprecipitates with Su(H); $N^{pp114LexA}$ is the phosphorylated cleaved cytoplasmic domain that associates with Su(H); and $N^{p100LexA}$ is the phosphatased cytoplasmic domain that associates with Su(H) (Kidd et al. 1998). (*G–I*) The increase in nuclear entry of the cytoplasmic domain derived from LNR^{LexA}. S2 cells were transfected with UAS constructs encoding N^{LexA}, LN^{LexA}, or LNR^{LexA}, and a plasmid encoding *heat shock* (*hs*) *GAL4* at a ratio of 1:10. Cells were heat-shocked for 30 min and allowed to recover for 2 h prior to fixation. They were then reacted with the NT antibody, which reacts with the extracellular domain; the NPCR antibody, which reacts with the intracellular domain; and sytox green to label the DNA. See Figure 1A for location of the epitopes recognized by the antibodies. (*J,K*) embryos expressing N^{LexA} (*J*) or LNR^{LexA} (*K*) under control of *daGal4* were reacted with anti-LexA antibody to detect the N protein derived from the transgene and propidium iodide, which reacts with the DNA.

produced in *Dl*$^+$ (Fig. 4C) and *Dl*$^-$ (Fig. 4D) backgrounds are comparable. This indicates that although LN^{LexA} and LNR^{LexA} do not require Delta for function, they do respond to Delta and have greater activity when Delta is present. The *Dl* independence but *Dl* responsiveness of LN^{LexA} and LNR^{LexA} is also apparent when we compare the ratios of S3-cleaved N bound to Su(H) to uncleaved N associated with Su(H) ($N^{pp114LexA}$:N^{LexA}) in extracts of *Dl*$^+$ and *Dl*$^-$ embryos (Fig. 4E). Almost no $N^{pp114LexA}$ is detectable in the *Dl* N^{LexA} extracts (Fig. 4E, lane 2) in accord with the neurogenic phenotype of *Dl* N^{LexA} embryos (data not shown). Given that the phenotypes of LN^{LexA} and LNR^{LexA} are *Dl*-independent, it is not unexpected that N^{pp114} associated with Su(H) can still be detected in *Dl* LN^{LexA} (Fig. 4E, lane 4) and *Dl* LNR^{LexA} (Fig. 4E, lane 6) extracts, and this is further confirmation that the gain-of-function phenotypes observed are due to the unregulated production of the soluble cytoplasmic domain. However, although LN^{LexA} and LNR^{LexA} are ligand-independent, they are ligand-responsive. In a *Dl* background the ratio of N^{pp114}:N in LN^{LexA} and LNR^{LexA} extracts is reduced by 40%–70% compared with that found in a wild-type background (three separate experiments were quantitated). This would account for the weaker antineurogenic phenotype produced by these proteins in the absence of *Dl*. We show below that these deleted N proteins do, indeed, have ligand-independent activity and that the residual activity we see in *Dl* zygotic nulls is not solely due to maternally contributed Dl.

We then assayed the phenotypes produced by LN^{LexA} and LNR^{LexA} in embryos that were maternally and zy-

gotically *kuz* null. As can be seen in Figure 5A, using an antibody that reacts with nervous tissue, embryos lacking *kuz* show hypertrophy of nervous system elements resulting in the lack of an organized CNS (cf. Figs. 5A and 3A). Both LN^{LexA} (Fig. 5C) and LNR^{LexA} (Fig. 5D) can function independently of Kuz, as can be seen by the suppression of the neural hypertrophy and the presence of an identifiable CNS. But as we observe in *Dl* embryos, the phenotypes produced in *kuz*$^-$ embryos are weaker than those produced in *kuz*$^+$ embryos (cf. Fig. 5C with 3B and 5D with 3D). Although there is suppression of the neurogenic phenotype in the case of LN^{LexA}, no antineurogenic phenotype is manifest, and the antineurogenic phenotype of LNR^{LexA} is weaker in a *kuz* background. Again, this contrasts with what we observe with $N^{IntraLexA}$, which produces comparable phenotypes in *kuz*$^+$ and *kuz*$^-$ backgrounds (cf. Fig. 5E with 4C). Therefore, both LN^{LexA} and LNR^{LexA} are *kuz*-responsive. Surprisingly, although expression of N^{LexA} cannot suppress the *Dl* neurogenic phenotype (data not shown), it is able to suppress the *kuz* neurogenic phenotype (Fig. 5B). In fact, the size of the nervous system is smaller than in *kuz* LNR^{LexA} embryos (Fig. 5, cf. B with D).

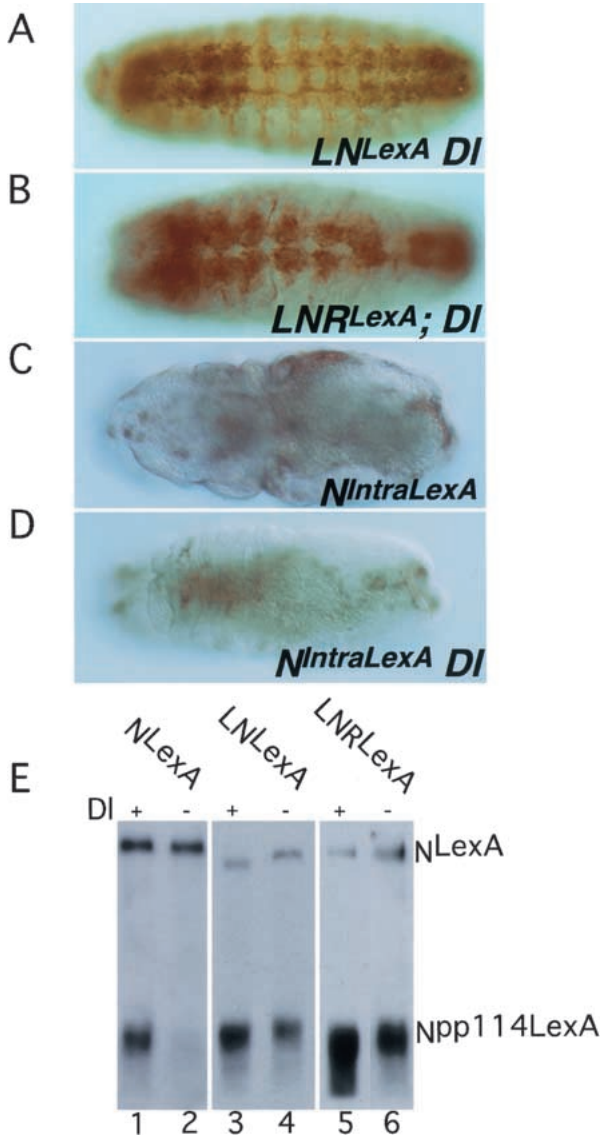

Figure 4. Signaling from LN^{LexA} and LNR^{LexA} is decreased in the absence of *Dl*. (*A–D*) Embryos expressing LN^{LexA} (*A*), LNR^{LexA} (*B*), and $N^{IntraLexA}$ (*C*,*D*) under control of *daGAL4*, reacted with anti-HRP antibody. The embryo in *C* is *Dl*$^+$, the other three are zygotic *Dl* nulls. The same transgenic lines were used in wild-type (Fig. 3B–E; Fig. 4C) and *Dl* backgrounds. Of the three antineurogenic N proteins, $N^{IntraLexA}$ produces the strongest antineurogenic phenotype in a wild-type background. (*E*) Extracts of embryos expressing N^{LexA} (lanes *1*,*2*), LN^{LexA} (lanes *3*,*4*), and LNR^{LexA} (lanes *5*,*6*) under control of *daGAL4*, in either wild-type (lanes *1*,*3*,*5*) or zygotic *Dl* backgrounds (lanes *2*,*4*,*6*), were immunoprecipitated with anti-Su(H) antibody and the Western reacted with anti-LexA antibody. To ensure that the protein being characterized is derived from *Dl* embryos, both the *N* transgenes and *daGAL4* were recombined onto *Dl* chromosomes.

We next asked what effect the loss of Kuz has on S3 cleavage as measured by coimmunoprecipitation of $N^{pp114LexA}$ with Su(H). As was the case in *Dl* embryos, the ratio of N^{pp114}:N in LN^{LexA} and LNR^{LexA} extracts is reduced by 50%–70% in *kuz*$^-$ embryos compared with in *kuz*$^+$ embryos (Fig. 5F, lanes 3–6). The loss of Kuz is therefore affecting the phenotypes produced by LN^{LexA} and LNR^{LexA} by reducing the amount of S3-cleaved N. Although expression of N^{LexA} is able to suppress the *kuz* neurogenic phenotype, there is a drastic reduction in the amount of N^{pp114} associated with Su(H) (Fig. 5F, lanes 1,2). As we have shown above that the strength of the antineurogenic phenotype correlates with the level of S3 cleavage, this suggests the phenotypic suppression is not caused by the canonical *Dl/Su(H)* pathway. We discuss this curious result below.

Delta *and* kuzbanian *embryos differ in the cleavage products immunoprecipitated by anti-N antibodies*

We are able to identify the band that corresponds to S3-cleaved N, the cytoplasmic form of N that associates with Su(H), in phosphatased anti-N immunoprecipitations of embryonic extracts based on its comigration with $N^{p100LexA}$, the phosphatased soluble form of N that coimmunoprecipitates with Su(H) (Fig. 6A, lanes 1,7), and based on its localization in the soluble fraction when embryonic extracts are fractionated (Fig. 6B, cf. lanes 2 and 3, 5 and 6, 8 and 9). In LNR^{LexA} extracts, S3-cleaved N is also found in nuclei (Fig. 6C, lanes 5,6). We are able to identify the band that corresponds to S2-cleaved N in phosphatased anti-N immunoprecipitations based on its accumulation in embryos that lack Psn (Fig. 6D, lane 4) and based on its localization in membranes (Fig. 6B, cf. lanes 2 and 3, 5 and 6, 8 and 9). Both S3 and S2 products are phosphorylated, although the level of phosphorylation of S2 is much lower than that of S3. (Compare the large shift in mobility upon phosphatase treatment of S3-cleaved N evident in anti-Su(H) immunoprecipitations of Figure 3F with the slight shift in mobility upon phosphastase treatment of S2-cleaved N in anti-N im-

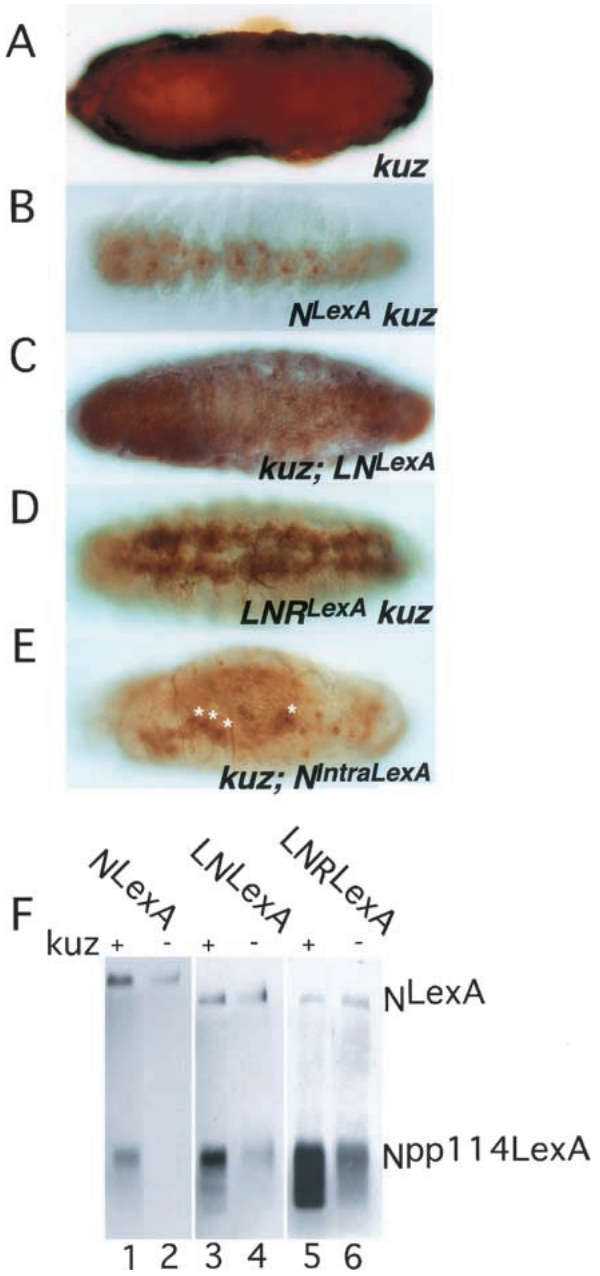

Figure 5. Signaling from LN^{LexA} and LNR^{LexA} is decreased in the absence of Kuz. All the embryos (*A–E*) were reacted with anti-HRP antibody. (*A*) Ventral view of an embryo that is maternally and zygotically *kuz* null. (*B–E*) The effect of expressing in a maternal and zygotic *kuz* background N^{LexA} (*B*), LN^{LexA} (*C*), LNR^{LexA} (*D*), and $N^{IntraLexA}$ (*E*). All the N proteins were expressed under control of *daGAL4*. The white stars in *E* point out the remnants of the nervous system in *kuz;* $N^{IntraLexA}$ embryos. The same transgenic lines were used in wild-type (Fig. 3A–E; Fig. 4C) and *kuz* backgrounds. (*F*) Extracts of embryos expressing N^{LexA} (lanes *1*,*2*), LN^{LexA} (lanes *3*,*4*), and LNR^{LexA} (lanes *5*,*6*) under control of *daGAL4*, in either wild-type (lanes *1*,*3*,*5*) or maternal and zygotic *kuz* backgrounds (lanes *2*,*4*,*6*) were immunoprecipitated with anti-Su(H) antibody and the Western reacted with anti-LexA antibody. To ensure that the protein being characterized is derived from *kuz* embryos, the *N* transgenes were recombined onto *kuz* chromosomes.

munoprecipitates of the membrane fraction in Figure 6C, lanes 1 and 2. This shift is more easily visible on shorter exposures.)

We can only detect S3-cleaved N in anti-N immunoprecipitates of embryonic extracts expressing LNR^{LexA} (Fig. 6A, lane 7), in accord with the increase in $N^{pp114LexA}$ that coimmunoprecipitates with Su(H). In anti-N immunoprecipitates of extracts of embryos expressing N^{LexA}, although we cannot detect S3-cleaved N, there is a band of ~97 kD that migrates more rapidly than S3-cleaved N (Fig. 6A, lanes 2,3, ▲), that does not associate with Su(H) (Fig. 6A, lane 1) but is dependent on *Psn*, as it is lacking in *Psn* embryos (Fig. 6D, cf. lanes 1 and 5 [WT] with lane 4 [*Psn*]). We will refer to this N cleavage product as N^{p97} and would like to reiterate that, although its production depends on *Psn*, it is not "S3" because it does not associate with Su(H). N^{p97} is also found to varying extents in anti-N immunopreciptates of extracts of embryos expressing LN^{LexA} (see, e.g., Fig. 6F, lane 3). S2-cleaved N can be detected in anti-N immunoprecipitates of embryos expressing each of the three N proteins (Fig. 6A).

Although loss of Delta or Kuzbanian in embryos expressing N^{LexA} causes a reduction in both S2-cleaved N as well as in N^{p97} (Fig. 6D, cf. lane 1 [WT] with lane 2 [*Dl*] and lane 3 [*kuz*]), the ratio of the two cleavage products differs in the two genotypes. In extracts of embryos lacking *Dl*, the ratio is qualitatively the same as that we observed in wild-type embryos (Fig. 6E, cf. lanes 2 and 5 [WT] with lanes 3 and 6 [*Dl*]), whereas in extracts of embryos lacking *kuz*, the level of $N^{p97}$is reduced to a greater extent (Fig. 6E, lane 7). In *kuz* extracts there is a protein the size of S2-cleaved N (S2*); however, it is present in much lower levels than is S2-cleaved N in *Psn* embryos (Fig. 6D), and it is not further processed to produce the *Psn*-dependent N^{p97}.

As we observed in *Psn* embryos expressing N^{LexA}, in extracts of *Psn* embryos expressing LN^{LexA} or LNR^{LexA}, there is an accumulation of S2-cleaved N (Fig. 6F, lane 6 [LN^{LexA}]; Fig. 6G, lane 6 [LNR^{LexA}]). There is also a great reduction in the level of full-length N (data not shown). Expression of LNR^{LexA} does not suppress the neurogenic phenotype of *Psn* embryos (data not shown), in accord with the lack of S3-cleaved N found in the anti-N immunoprecipitates (Fig. 6G, cf. lanes 3 and 6). Whereas in extracts of *Dl* embryos expressing LN^{LexA} or LNR^{LexA}, the ratio of cleavage products found in anti-N immunoprecipitates is qualitatively the same as in extracts of wild-type embryos expressing LN^{LexA} or LNR^{LexA} (Fig. 6F, lanes 3 and 4 [LN^{LexA}]; Fig. 6G, lanes 3 and 4 [LNR^{LexA}]), in extracts of *kuz* embryos the *Psn*-dependent cleavage products are not present (Fig. 6F, lane 5 [LN^{LexA}]; Fig. 6G, lane 5 [LNR^{LexA}]). Again, as we observed in extracts of *kuz* embryos expressing N^{LexA}, in *kuz* embryos expressing LN^{LexA} or LNR^{LexA}, there is a protein the size of S2-cleaved N; however, it is much less abundant than is S2-cleaved N in *Psn* embryos, and it is not further processed to produce *Psn*-dependent cleavage products.

The data we have presented in this section indicate

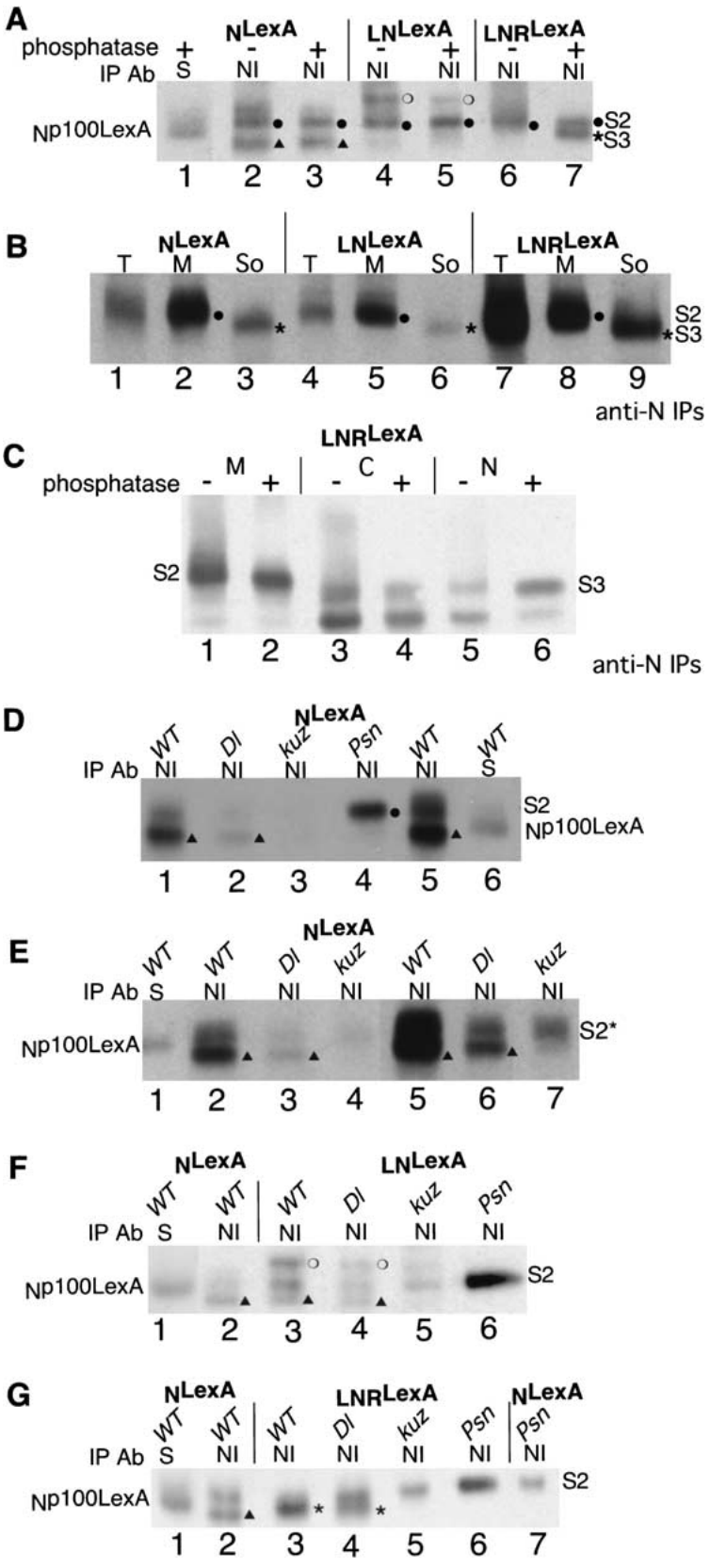

Figure 6. Anti-N immunoprecipitates of extracts from *Dl* and *kuz* embryos differ. (*A*) Extracts of embryos expressing N^{LexA} (lanes *2*,*3*), LN^{LexA} (lanes *4*,*5*), and LNR^{LexA} (lanes *6*,*7*), under the control of *daGAL4* were immunoprecipitated with anti-NI antibody (see Fig. 1A) and the Western reacted with anti-LexA antibody. Extracts of embryos expressing N^{LexA} were also immunoprecipitated with anti-Su(H) antibody (lane *1*). The immunoprecipitates in lanes *1*, *3*, *5*, and *7* were treated with phosphatase prior to electrophoresis. $N^{p100LexA}$ is the phosphatased cytoplasmic domain that interacts with Su(H) (Kidd et al. 1998). (S2 ●) Migration of S2-cleaved N; (S3 *) migration of S3-cleaved N; (S) Su(H); (▲ in lanes *2*,*3*) an ~97-kD cleavage product present in NI immunoprecipitates of extracts expressing N^{LexA} that does not associate with Su(H), but is dependent on *Psn* (see text); (○ in lanes *5*,*6*) an ~108-kD cleavage product found in NI immunoprecipitates of LN^{LexA}. (*B*) Extracts of embryos expressing N^{LexA} (lanes *1*–*3*), LN^{LexA} (lanes *4*–*6*), and LNR^{LexA} (lanes *7*–*9*), under the control of *daGAL4*, which had been fractionated into membrane and soluble fractions, were immunoprecipitated with anti-NI antibody and the Western reacted with anti-LexA antibody. (T) Unfractionated extract; (M) membrane fraction; (So) soluble fraction. All immunoprecipitates were phosphatased prior to electrophoresis. (*C*) Extracts of embryos expressing LNR^{LexA} under the control of *daGAL4* were fractionated into membrane, cytoplasmic, and nuclear fractions prior to immunoprecipitation with anti-NI antibody. The immunoprecipitates in lanes *2*, *4*, and *6* were phosphatased. The Western was reacted with anti-LexA antibody. (M) Membrane; (C) cytoplasm; (N) nuclear. The smear representing phosphorylated S3 in the nuclear fraction (lane *5*) is more easily visible on longer exposures. (*D*,*E*) Extracts of embryos expressing N^{LexA} were immunoprecipitated with anti-NI antibody (*D*, lanes 1–5; *E*, lanes 2–7) and the Western reacted with anti-LexA antibody. The extracts in lanes *1* and *5* of *D* and lanes *2* and *5* of *E* were from wild-type embryos (WT). The extracts in lane *2* of *D* and lanes *3* and *6* of *E* were from zygotic *Dl* embryos. The extracts in lane *3* of *D* and lanes *4* and *7* of *E* were from maternal and zygotic *kuz* embryos. The extract in lane *4* of *D* was from maternal and zygotic *Psn* embryos. (*D*, lane *6*; *E*, lane *1*) Extracts of N^{LexA} embryos were also immunoprecipitated with anti-Su(H) antibody (S). The immunoprecipitates in lanes *4* and *5* of *D* were derived from embryos expressing N^{LexA} under the control of *armadillo* (*arm*) *GAL4*. All the other immunoprecipitates were derived from embryos expressing N^{LexA} under the control of *daGAL4*. (▲ in *D*, lanes *1*,*2*,*5*; *E*, lanes *2*,*3*,*5*,*6*) The N^{LexA} derived protein that is dependent on *Psn* but does not associate with Su(H). (*E*, lanes *5*–*7*) A longer exposure of lanes *2*–*4*. (S2* in *E*) The N cleavage product the size of S2-cleaved N that is found in *kuz* extracts but is not processed further (see text). (*F*,*G*) Comparisons of the anti-NI immunoprecipitates of WT (lane *3*), *Dl* (lane *4*), *kuz* (lane *5*), and *Psn* (lane *6*) embryos expressing LN^{LexA} (*F*) and LNR^{LexA} (*G*). In lanes *1* and *2* extracts of embryos expressing N^{LexA} were immunoprecipitated with anti-Su(H) antibody (S) and anti-NI antibody respectively. (*G*, lane *7*) extracts of *Psn* embryos expressing N^{LexA} were immunoprecipitated with anti-NI antibody. (*G**) S3-cleaved N. All the immunoprecipitates in *D*–*G* were phosphatased prior to electrophoresis. To ensure that the protein being characterized is derived from *Dl* embryos, both the *N* transgenes and *daGAL4* were recombined onto *Dl* chromosomes and to ensure that the protein being characterized is derived from *kuz* and *Psn* embryos, the *N* transgenes were recombined onto *kuz* and *Psn* chromosomes, respectively. All the immunoprecipitates within each panel (*A*–*G*) were electrophoresed on the same gel. In some of the panels different exposures of the lanes were used to generate the figure. Only the regions where the S2 and S3 cleavage products migrate are shown.

that loss of Kuzbanian affects the cleavage of Notch differently than does the loss of Delta. Loss of *kuz* results in the specific loss of *Psn*-dependent cleavage products. A protein the size of S2-cleaved N is still present. Although this might appear to indicate that *kuz* is responsible for S3 cleavage of N, data are presented below showing that *kuz* is actually responsible for S2 cleavage, and that the protein the size of S2-cleaved N that is found in *kuz* embryos can be generated by TACE, another ADAM family member. This TACE-generated product is not efficiently processed to produce S3-cleaved N.

Gain-of-function Notch molecules that cannot signal via Delta *are dependent on* kuzbanian *for their activity*

That there is a difference in the cleavage products found in anti-N immunoprecipitates of *Dl* and *kuz* embryos expressing N^{LexA}, LN^{LexA}, or LNR^{LexA} suggests that *kuz* is not functioning merely to produce a soluble form of Delta that can then act as a ligand for Notch. If this were, indeed, the case, there should still be a requirement for *kuz* for the function of *Dl*-independent gain-of-function N molecules even in the absence of *Dl*. We tested this in two ways.

First, we generated transgenic flies that express N protein deleted for EGF repeats 1–18 as well as the LNG repeats ($N^{\Delta EGF1-18\ LNRLexA}$; see Fig. 1B). We and others have shown that N molecules deleted for EGF repeats 11 and 12 cannot transduce a *Dl*-dependent signal (Lieber et al. 1993; Lawrence et al. 2000). Expression of $N^{\Delta EGF1-18\ LNRLexA}$ in a wild-type background produces an antineurogenic phenotype (Fig. 7A). Unlike the results observed with LN^{LexA} and LNR^{LexA}, loss of *Dl* did not significantly reduce the severity of this phenotype (Fig. 7, cf. A with B). Nor did the loss of *Dl* decrease the

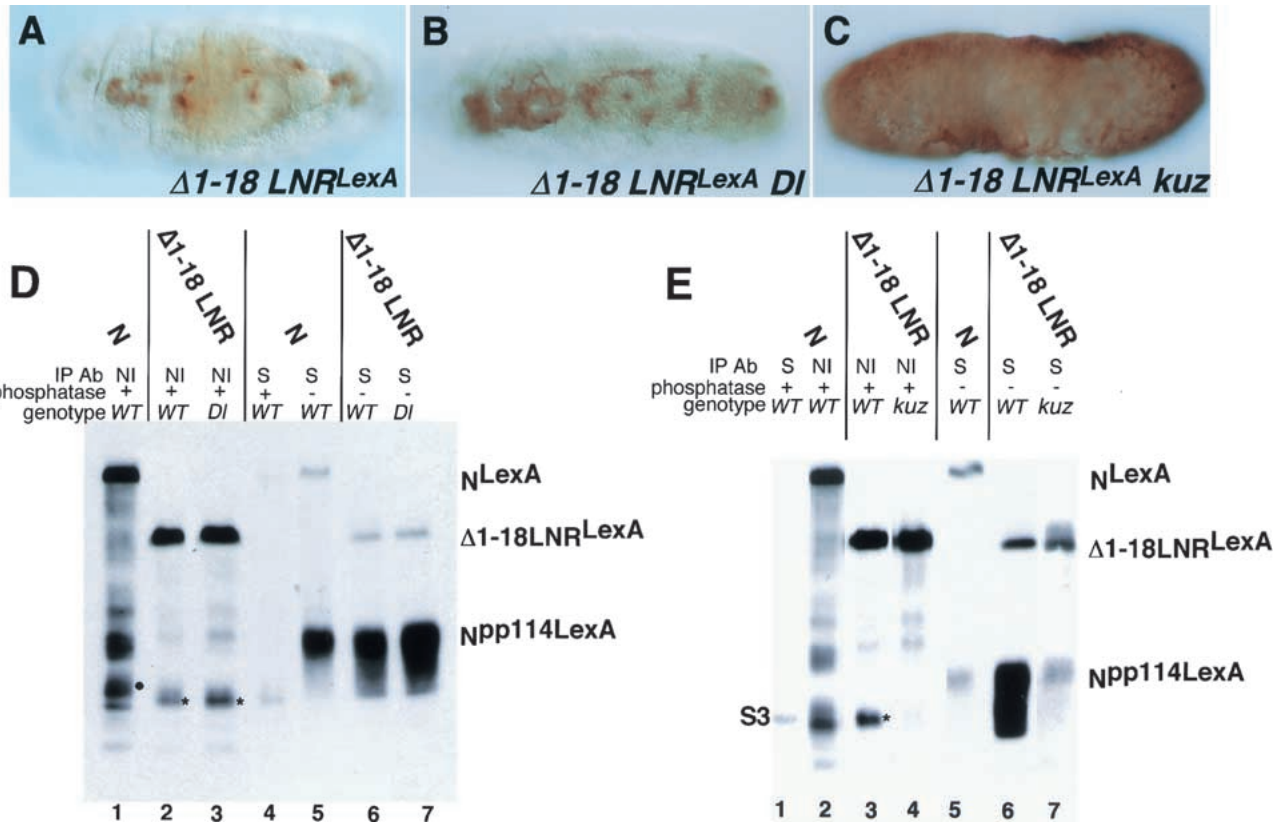

Figure 7. A *Dl*-independent N protein requires *kuz* for activity. (*A–C*) Ventral views of anti-HRP stains of embryos expressing ΔEGF 1–18 LNRLexA under control of *daGAL4*, in wild-type (*A*), *Dl* (*B*), or *kuz* (*C*) backgrounds. (*D*) Extracts of embryos expressing ΔEGF 1–18 LNRLexA under control of *daGAL4* were immunoprecipitated with anti-NI antibody (lanes *2*,*3*) or anti-Su(H) antibody (lanes *6*,*7*). The extracts in lanes *2* and *6* are from wild-type embryos, and the extracts in lanes *3* and *7* are from *Dl* embryos. (Lane *1*) An anti-NI immunoprecipitate of wild-type embryos expressing N^{LexA}; (lanes *4*,*5*) anti-Su(H) immunoprecipitates of embryos expressing N^{LexA}. The immunoprecipitates in lanes *1–4* were treated with phosphatase. (⋆, lanes *2*,*3*) S3-cleaved N; (●, lane *1*) S2-cleaved N. (*E*) Extracts of embryos expressing ΔEGF 1–18 LNRLexA under control of *daGAL4* were immunoprecipitated with anti-NI antibody (lanes *3*,*4*) or anti-Su(H) antibody (lanes *6*,*7*). The extracts in lanes *3* and *6* are from wild-type embryos, and the extracts in lanes *4* and *7* are from *kuz* embryos. (Lane *2*) An anti-NI immunoprecipitate of wild-type embryos expressing N^{LexA}; (lanes *1*,*5*) anti-Su(H) immunoprecipitates of embryos expressing N^{LexA}. The immunoprecipitates in lanes *1–4* were treated with phosphatase. (⋆, lane *3*) S3-cleaved N. The Westerns in both *D* and *E* were reacted with anti-LexA antisera.

level of $N^{pp114LexA}$ derived from $N^{\Delta EGF1-18\ LNRLexA}$ that associates with Su(H) (Fig. 7D, lanes 6,7; two experiments were quantitated). Thus none of the function or processing of $N^{\Delta EGF1-18\ LNRLexA}$ seems to be due to *Dl*.

Although the function of $N^{\Delta EGF1-18\ LNRLexA}$ is independent of *Dl*, production of an antineurogenic phenotype by $N^{\Delta EGF1-18\ LNRLexA}$ is completely dependent on the presence of *kuz*. There is no suppression of the *kuz* neurogenic phenotype by expression of $N^{\Delta EGF1-18\ LNRLexA}$ (Fig. 7C). This is unlike the result observed when LN^{LexA} or LNR^{LexA} was expressed in a *kuz* background (Fig. 5C,D). This discrepancy will be discussed in a later section. In accord with the inability of $N^{\Delta EGF1-18\ LNRLexA}$ to suppress the *kuz* neurogenic phenotype, there is a drastic reduction in the level of $N^{pp114LexA}$ that associates with Su(H), 85%–95% (Fig. 7E, lanes 6,7; two experiments were quantitated). Again, the decrease in the level of $N^{pp114LexA}$ derived from $N^{\Delta EGF1-18\ LNR^{LexA}}$ that associates with Su(H) is much greater than we observed with LN^{LexA} or LNR^{LexA} in the absence of *kuz* (Fig. 5F). The decrease in processing of $N^{\Delta EGF1-18\ LNR^{LexA}}$ in the absence of *kuz* is also apparent in anti-N immunoprecipitates of *kuz* extracts expressing $N^{\Delta EGF1-18\ LNR^{LexA}}$ (Fig. 7E, lanes 3,4).

The data presented in this section show that *kuz* has a role in the cleavage of N independent of any role it may have in the cleavage of Delta. As was the case with *kuz* embryos expressing N^{LexA}, LN^{LexA}, or LNR^{LexA}, the loss of *kuz* is manifested in anti-N immunoprecipitates of extracts expressing $N^{\Delta EGF1-18\ LNR^{LexA}}$ by the loss of S3-cleaved N. In the next section we present data showing that *kuz* is actually responsible for S2 cleavage. In contrast to *kuz* embryos expressing N^{LexA}, LN^{LexA}, or LNR^{LexA}, there is very little accumulation of a protein the size of S2-cleaved N in *kuz* embryos expressing $N^{\Delta EGF1-18\ LNR^{LexA}}$. Below we present data suggesting that this is because TACE cleaves this substrate less efficiently.

Removal of kuzbanian *activity by RNA-mediated interference in S2 cells abolishes cleavage of a transmembrane gain-of-function Notch molecule*

The second way we asked whether *kuz* has a *Dl*-independent role in N processing was to use RNA-mediated interference (RNAi) in S2 cells to remove *kuz* activity. S2 cells do not express any known N ligands (Fehon et al. 1990; Rebay et al. 1991), and RNAi has been shown to be an effective method for removing gene function in S2 cells (Clemens et al. 2000). For these experiments we deleted 381 amino acids from the carboxyl terminus of N, in order to achieve greater separation between S2 and S3 cleavage products. ($N^{KasLexA}$ and $LNR^{KasLexA}$ are shown in Fig. 1B.) As we observed in embryos expressing LNR^{LexA}, anti-N immunoprecipitates of S2 cells expressing $LNR^{KasLexA}$ contain S3-cleaved N that comigrates with $N^{p100KasLexA}$, the phosphatased soluble domain of $LNR^{KasLexA}$ that associates with Su(H) (Fig. 8A, lanes 1,3). This S3-cleaved N is not visible to any great extent in anti-N immunoprecipitates of S2 cells expressing $N^{KasLexA}$ (Fig. 8A, lane 2). As we observed in anti-N immunoprecipitates of extracts of *Psn* embryos expressing LNR^{LexA} (Fig. 6G, lane 6), cleavage of $LNR^{KasLexA}$ is blocked at S2 in cells in which RNAi has been used to remove *Psn* function (Fig. 8A, lane 7). Thus both S2 and S3 cleavages of $LNR^{KasLexA}$ can occur in S2 cells.

When *kuz* double-stranded RNA is used to remove *kuz* function, no processing of $LNR^{KasLexA}$ is observed (Fig. 8A, lane 4). Neither *Drosophila TACE* nor *Drosophila ADAM 10* double-stranded RNA has any affect on S3 cleavage (Fig. 8A, lanes 5,6). Therefore, in the absence of *Dl*, the cleavage of $LNR^{KasLexA}$ is dependent on *kuz*. This *kuz*-dependent cleavage acts upstream of *Psn* activity, as we do not observe S2-cleaved N in cells in which the function of both *kuz* and *Psn* has been removed by RNAi (Fig. 8A, lane 10). In contrast, removal of *TACE* or *ADAM10* function along with that of *Psn* results in accumulation of S2-cleaved N (Fig. 8A, lanes 11,12), indicating that in S2 cells neither *TACE* nor *ADAM10* is required for S2 cleavage of $LNR^{KasLexA}$. In fact, by Northern blot analysis, we could not detect any *TACE* or *ADAM10* RNA in S2 cells, although using the same probes, we were able to detect *TACE* and *ADAM10* in embryo RNA, and we could detect *kuz* and *Psn* mRNAs on Northerns of S2 cell RNA (data not shown). We then asked whether *kuz* RNAi would block cleavage of wild-type N when S2 cells expressing wild-type N were aggregated with S2 cells expressing Dl. We found that double-stranded *kuz* RNA added to the N-expressing cells blocks the production of N^{pp114}, the phosphorylated soluble cytoplasmic domain that associates with Su(H), but double-stranded *TACE* RNA has no effect (data not shown).

Brou et al. (2000) have reported that TACE mediates S2 cleavage of mammalian N. We therefore asked whether exogenous TACE could complement the loss of *kuz* activity generated by RNAi in S2 cells expressing $LNR^{KasLexA}$. As can be seen in Figure 8B, lane 7, although the production of S3-cleaved N is restored upon cotransfection of an actin *TACE* construct, the amount of S3 product compared with a product that migrates at the size of S2-cleaved N is substantially lower than in cells with endogenous *kuz* (Fig. 8B, cf. lanes 7 and 3). This pattern of N cleavage products is similar to the pattern we observe in *kuz* embryos (see Fig. 6G, lane 5), and suggests that although TACE is able to cleave N at a position close to that cleaved by Kuz, this product is not efficiently cleaved further to produce S3-cleaved N.

In vivo the function of $N^{\Delta EGF1-18\ LNR^{LexA}}$ is completely dependent on *kuz* activity. As we observed for $LNR^{KasLexA}$, treatment of S2 cells with double-stranded *kuz* RNA abolishes the processing of $N^{\Delta 1-18\ LNR^{KasLexA}}$ (Fig. 8B, lane 13), and treatment with double-stranded *Psn* RNA results in the accumulation of S2-cleaved N (Fig. 8B, lane 15). We therefore asked if exogenous TACE could restore the production of S3-cleaved N derived from $N^{\Delta EGF1-18\ LNR^{KasLexA}}$ in S2 cells that had been treated with double-stranded *kuz* RNA. We found that hardly any S3 product was generated (Fig. 8B, lane 14), accounting for the in vivo *kuz* dependence.

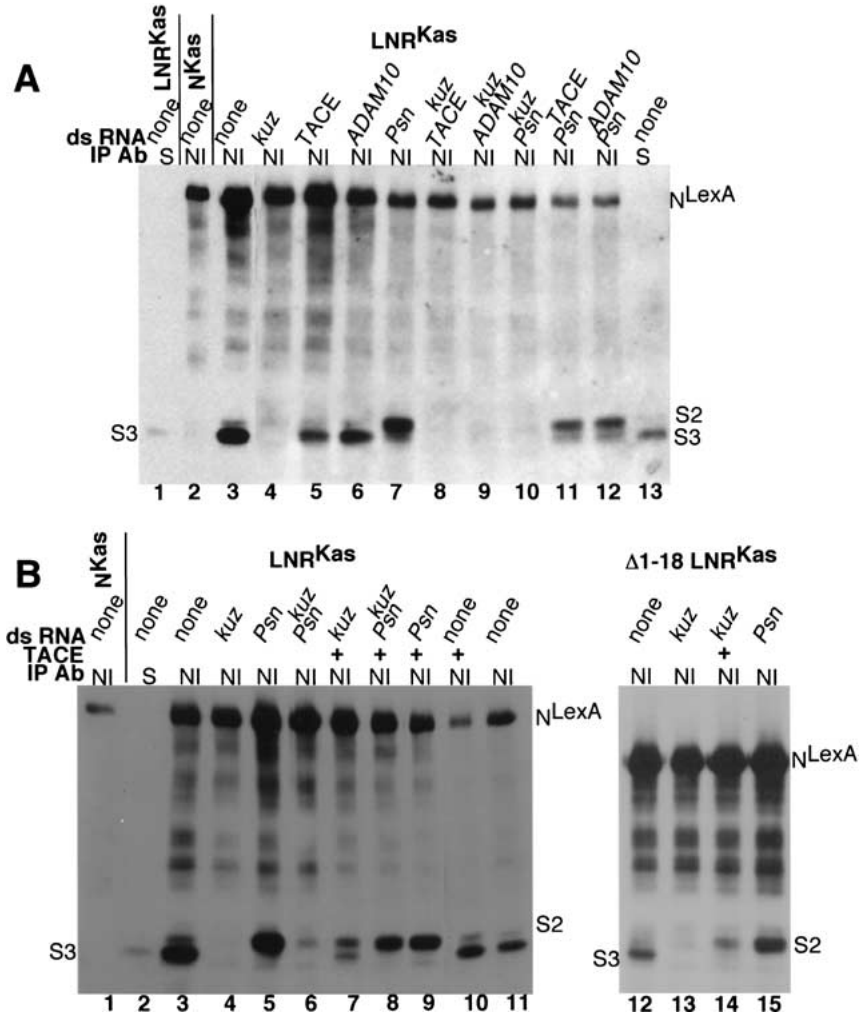

Figure 8. Kuzbanian promotes the cleavage of Notch. (*A*, lanes *3–12*) are anti-NI immunoprecipitates of S2 cells expressing heat-shock-induced $LNR^{KasLexA}$ (see Fig. 1B) that have been treated with the indicated double-stranded (ds) RNAs. (Lane *2*) An anti-NI immunoprecipitate of S2 cells expressing heat-shock-induced $N^{KasLexA}$ (see Fig. 1B); (lanes *1,13*) anti-Su(H) immunoprecipitates of S2 cells expressing $LNR^{KasLexA}$. (*B*, lanes *3–15*) anti-NI immunoprecipitates of S2 cells expressing heat-shock-induced $LNR^{KasLexA}$ (lanes *3–11*) or ΔEGF 1–18 $LNR^{KasLexA}$ (lanes *12–15*; see Fig. 1B) that have been treated with the indicated double-stranded (ds) RNAs. The cells in lanes *7–10* and *14* were cotransfected with an actin-driven *TACE* construct. (Lane *1*) An anti-NI immunoprecipitate of S2 cells expressing heat-shock-induced $N^{KasLexA}$; (lane *2*) an anti-Su(H) immunoprecipitate of S2 cells expressing $LNR^{KasLexA}$. All the immunoprecipitates were phosphatased prior to electrophoresis, and the Westerns were reacted with anti-LexA antisera. (S2, S3) Locations of S2 and S3 cleavage products.

Discussion

Pan and Rubin (1997) originally proposed that Kuz cleaves Notch. This proposal is in accord with the cell-autonomous requirement for *kuz* both in *Drosophila* and in *C. elegans* (Rooke et al. 1996; Sotillos et al. 1997; Wen et al. 1997). More recently it has been suggested that the phenotypes resulting from loss of *kuz* are attributable to its role in the processing of Dl (Qi et al. 1999), although in that case the requirement for *kuz* would be non-cell-autonomous. We have shown that Kuz can associate with N (Fig. 2), and that removal of *kuz* activity from S2 cells, which do not express Dl, blocks the processing of ligand-independent gain-of-function N molecules (Fig. 8). In vivo, a gain-of-function N molecule that is completely *Dl*-independent displays an absolute requirement for *kuz* (Fig. 7). Our results show that *kuz* can mediate the cleavage of N, and are therefore in agreement with Pan and Rubin's original proposal. However, whereas Pan and Rubin proposed that *kuz* mediates S1 cleavage, our data suggest that *kuz* is responsible for S2 cleavage.

The role of *kuz/ADAM10* in the *N* pathway in vertebrates is uncertain. It has been shown that expression of a dominant-negative form of mouse Kuz causes the overproduction of neurons in *Xenopus* (Pan and Rubin 1997) and inhibits Delta-1-like-induced transactivation of a *HES-1* reporter in HeLa cells expressing Notch-1 (Jarriault et al. 1998). Yet both S2 and S3 cleavages of a ligand-independent N protein occur in cells derived from *kuz* mice (Mumm et al. 2000). Whereas mammals have a single *kuz/ADAM10* gene, *Drosophila* has two, *kuz*, the focus of this work, and another *ADAM10* homolog. Perhaps *Drosophila kuz* has evolved a function distinct from that of *ADAM10*.

Although both LN^{LexA} and LNR^{LexA} function in the absence of the ligand Dl, the activity of LNR^{LexA} is greater than that of LN^{LexA}. This difference is not caused by an enhanced affinity of Kuz for LNR^{LexA}, as both associate equally with Kuz in S2 cells (data not shown). This suggests that the difference in activity of these gain-of-function N molecules results from an enhanced ability of LNR^{LexA} to be cleaved by Kuz. In fact, the interaction of LN and LNR with Kuz is no greater than that of wild-type N, and the association of Kuz with any of the N molecules occurs in the absence of Dl (Fig. 2; data not shown). In this regard, the association of Kuz with N is

like that of Kuz with ephrin-A2, which forms a stable complex with Kuz prior to Eph receptor binding. The binding of clustered Eph receptors to the Kuz–ephrin-A2 complex activates Kuz and triggers ephrin-A2 cleavage (Hattori et al. 2000). Likewise, the binding of Dl to the Kuz–N complex could activate Kuz and trigger N cleavage.

A curious result is the difference in Kuz dependence of LNR^{LexA} and ΔEGF1–18 LNRLexA (for $N^{\Delta EGF1-18\ LNRLexA}$) in vivo. We offer two explanations for this observation. The first takes into account the difference in Dl responsiveness between Δ1–18 LNRLexA and LNR^{LexA}. LNR^{LexA}, which has reduced activity in the absence of Dl, still retains some function in the absence of Kuz (Figs. 4 and 5), whereas Δ1–18 LNRLexA, which is completely Dl-independent, cannot function in the absence of kuz (Fig. 7). One possible explanation for the discrepancy is that there are two pathways that mediate N cleavage and function in embryos. One pathway requires Dl but is independent of Kuz, and the other pathway requires Kuz but is independent of Dl. LNR^{LexA} can operate in both pathways, so that upon removal of either Dl or Kuz, LNR^{LexA} still functions via the alternative remaining pathway. Δ1–18 LNRLexA can only operate in the *kuz* pathway, so that upon removal of kuz it is nonfunctional. There is, however, no strong evidence pointing to a *Dl*-independent *N* pathway involving cleavage in embryos, and given the requirement for *Dl* in the germ line for the differentiation of follicle cells (López-Schier and St. Johnston 2001), the generation of embryos that are maternally *Dl* null is not straightforward.

We therefore favor the hypothesis that the difference in *kuz*-dependence of LNR^{LexA} and Δ1–18 LNR^{LexA} in vivo is owing to their differing abilities to be cleaved by TACE. Brou et al. (2000) have shown that TACE, another member of the ADAM family of metalloproteases, mediates S2 cleavage of mammalian N in vitro. *Drosophila* S2 cells do not contain any detectable *TACE* RNA (data not shown), and exogenous TACE only poorly complemented the RNAi-mediated loss of *kuz* activity (Fig. 8B, lane 7). The restoration of some S3 product upon expression of TACE is in accord with the residual activity of LNR^{LexA} in *kuz* embryos (Fig. 5), and in vitro TACE and N do interact, albeit less well than do Kuz and N (data not shown). In the absence of a *TACE* mutant, we are unable to say for certain whether *kuz* and *TACE* have redundant functions, if another member of the ADAM family is responsible for the residual S3 cleavage, or if the residual in vivo activity is caused by the expression of LNR^{LexA} from a heterologous promoter. Hardly any S3 product was generated from Δ1–18 LNR^{LexA} by exogenous TACE in S2 cells that had been treated with *kuz* double-stranded RNA (Fig. 8B, lane 14), accounting for the in vivo Kuz-dependence. It is not clear why the ability of exogenous TACE to produce S3-cleaved N differs between LNR^{LexA} and Δ1–18 LNRLexA; however, in S2 cells, even in the presence of endogenous Kuz, Δ1–18 LNR^{LexA} is not cleaved as well as is LNR^{LexA} (Fig. 8B; data not shown), suggesting that perhaps differences in the secondary structure of the molecules account for their differing responses to TACE.

The pattern of cleavage products generated by expression of TACE in kuz^{-} S2 cells also provides an explanation for the in vivo biochemical data we present in Figure 6, which had seemed to suggest that *kuz* is responsible for S3 cleavage. It is intriguing that both in *kuz* embryos (Fig. 6) and in *TACE*-complemented kuz^{-} S2 cells (Fig. 8) there is an accumulation of a protein the size of S2-cleaved N, which is not efficiently cleaved further to produce S3-cleaved N. We propose that although TACE can cleave N at juxtamembrane sites, a large fraction of this cleavage is occurring at a site close to but distinct from the S2 site that allows for efficient S3 cleavage. This suggests that cleavage of N at any juxtamembrane site is not immediately followed by efficient S3 cleavage.

A surprising result was the suppression of the *kuz* neurogenic phenotype by expression of N^{LexA} (Fig. 5B). In fact, the size of the nervous system in N^{LexA} *kuz* embryos is smaller than in LNR^{LexA} *kuz* embryos (Fig. 5, cf. B [N^{LexA} *kuz*] with D [LNR^{LexA} *kuz*]). As *kuz* embryos are neurogenic, the suppression must result from the overexpression of N. This, along with the fact that the suppression does not involve the association of the cytoplasmic domain of N with Su(H) (Fig. 5F), suggests that in the absence of *kuz*, overexpressed N is competitively interacting with a protein required for neurogenesis. Wild-type N accumulates to a higher steady-state level on the cell surface than does LN or LNR (Fig. 3G–I; data not shown). It has been shown that expression of the extracellular domain of N can disrupt the establishment of proneural clusters in the developing wing disc (Brennan et al. 1999).

In summary, we have shown that in flies S2 cleavage can be mediated by *kuz*. This contrasts with mammalian data that suggest S2 cleavage occurs via TACE. The discrepancy might be owing to mechanistic differences between flies and mammals as has also been shown for S1 cleavage (Kidd et al., in prep.).

Materials and methods

Constructs

All constructs were generated by standard methods. *kuz* and kuz^{DN} DNAs used for the S2 cell experiments were from D.J. Pan (Pan and Rubin 1997). The *kuz* DNA used for in vitro translation was from EST clone ID SD03071. The *hIR* DNA was from C.R. Kahn. The *Psn* DNA was from I. Livne-Bar (Boulianne et al. 1997). RT-PCR, using primers based on the then preliminary *Drosophila* genome project, was used to amplify a segment of *Drosophila TACE* DNA (FlyBase accession no. CG7908), which was then used to screen a *Drosophila* cDNA library. RT-PCR, using primers based on the preliminary *Drosophila* genome project, was used to amplify a segment of *Drosophila ADAM10* DNA (FlyBase accession no. CG1964).

Antibodies

All the anti-N antibodies and the Su(H) antibody have been described previously (Kidd et al. 1989, 1998; Lieber et al. 1993). Anti-myc antibody was from Calbiochem or Sigma. The anti-LexA antibody was generated in mice against amino acids 1–87 of LexA.

Flies

daGAL4 (*daG32*) flies were from E. Knust (Wodarz et al. 1995). *arm* GAL4 files were from J.P. Vincent (Sanson et al. 1996). The *Dl* allele used, Dl^{x}, is described by FlyBase (1998). $kuz^{e29\text{-}4}$ *FRT* flies (Rooke et al. 1996) were from D.J. Pan. The *Psn* allele used was PS^{C1}, and *hs FLP*; PS^{C1} *FRT* flies were from G. Struhl (Struhl and Greenwald 1999).

For the biochemical experiments, to ensure that the protein being characterized is derived from *Dl* embryos, both the *N* transgenes and *daGAL4* were recombined onto *Dl* chromosomes. To ensure that the protein being characterized is derived from *kuz* embryos, the *N* transgenes were recombined onto *kuz* chromosomes, and to ensure that the protein being characterized is derived from *Psn* embryos, the *N* transgenes were recombined onto *Psn* chromosomes.

Immunocytyochemistry and immunofluorescence

Immunocytochemistry and immunofluorescence were carried out as described previously (Kidd et al. 1998). For the immunofluorescence experiments on S2 cells shown in Figure 3G–I, Cy5-coupled anti-rabbit IgG was used to detect the outside antibody and Texas Red-coupled anti-mouse IgG was used to detect the inside antibody. For the immunofluorescence experiments on embryos shown in Figure 3, J and K, biotinylated anti-mouse IgG and FITC-coupled streptavidin were used to detect the anti-LexA antibody. All secondary antibodies were from Jackson.

Immunoprecipitations and pull-down assays

With the exception of the experiments presented in Figure 6, B and C, detergent lysis used to prepare the embryonic and S2 cell extracts and immunoprecipitations were carried out as described previously (Kidd et al. 1998). The fractionation presented in Figure 6B was carried out on extracts prepared by isotonic lysis as described (Kidd et al. 1998), with the addition of 5 mM phenanthroline and the removal of molybadate. The fractionation presented in Figure 6C was carried out on extracts prepared by hypotonic lysis, as used to generate extracts for gel shifts (Kidd et al. 1998), with the addition of 5 mM phenanthroline. The nuclei were isolated by pelleting through a sucrose cushion as described by Rio et al. (1986). The preparation of extracts by hypotonic lysis tends to result in the production of some additional cleavage products that are not seen when extracts are prepared by detergent lysis.

GST fusion proteins were produced in the bacterial strain pLysS. After induction and growth for 3 h, proteins were extracted, bound to glutathione beads, and extensively washed in 20 mM Tris (pH 7.6), 1 M NaCl, 1 mM EDTA, 1% Triton X-100, and 2% sarkosyl. After the final wash the beads were equilibrated and stored in 10 mM HEPES (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, 1 mg/mL BSA, and protease inhibitors. *Su(H)*, *kuz*, and *hIR* were transcribed and translated in vitro using the Promega TNT system. Subsequent steps were all at 4°C or on ice. In vitro translation products were first precleared for 2 to 3 h in 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Triton X-100, 5 mM $CaCl_2$, and 1 mg/mL BSA with GST coupled beads. The supernatants were incubated overnight with either GST or GST–N fusion beads, which were then washed 3× in 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Triton X-100; 2× with 50 mM HEPES (pH 7.6), 250 mM NaCl, 0.1% Triton X-100; and 1× with 10 mM HEPES (pH 7.6), 150 mM NaCl, 0.1% Triton X-100. Proteins were eluted by boiling in Laemmli gel loading buffer.

Scanned autoradiographs were quantitated on a Macintosh computer with the public domain NIH image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

RNAi experiments

PCR using T7 primers was used to amplify *kuz*, *TACE*, *ADAM10*, and *Psn* DNAs. The following primers were used: *kuz*, for Figure 8A, 5′ primer, CATTGTATTCGTATCGAT; 3′ primer, GAATGTTGTTGTCGACGA; for Figure 8B to avoid any *TACE* homologous sequences, 5′ primer, ATGCAACGT CATCCCAAT; 3′ primer, ATAAACGATATTTCGGCG; *TACE*, 5′ primer, CAAGGACGATGTGGTGCAC; 3′ primer, GTAGATCTTGTGCACCCGATC; *ADAM10*, 5′ primer, CTC CAGGTCCTGTTCCTC; 3′ primer, CGAAAATCATCGTTG TAC; *Psn*, 5′ primer, ATGGCTGCTGTCAATCTC; 3′ primer, CAGAGGTCCCTGCCAATG. Double-stranded RNA was generated using a T7 Megascript Kit (Ambion). RNAi was carried out as described by Clemens et al. (2000). Cells were transfected using calcium phosphate 1 d after addition of the double-stranded RNA. The following day the cells were washed and double-stranded RNA was added again. The cells were heat-shocked and lysed the next day.

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References

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. 1999. Notch signaling: Cell fate control and signal integration in development. *Science* **284:** 770–776.

Blaumueller, C.M., Qi, H., Zagouras, P., and Artavanis-Tsakonas, S. 1997. Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell* **90:** 281–291.

Boulianne, G., Livne-Bar, I., Humphreys, J., Liang, Y., Lin, C., Rogaev, E., and St George-Hyslop, P. 1997. Cloning and characterization of the *Drosophila* presenilin homologue. *Neuroreport* **8:** 1025–1029.

Brennan, K., Tateson, R., Lieber, T., Couso, J.P., Zecchini, V., and Martinez Arias, A. 1999. The *Abruptex* mutations of *Notch* disrupt the establishment of proneural clusters in *Drosophila*. *Dev. Biol.* **216:** 230–242.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J.R., Cumano, A., Roux, P., Black, R.A., and Israël, A. 2000. A novel proteolytic cleavage involved in Notch signaling: The role of the disintegrin-metalloprotease TACE. *Mol. Cell* **5:** 207–216.

Clemens, J.C., Worby, C.A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B.A., and Dixon, J.E. 2000. Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci.* **97:** 6499–503.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts,

K., Mumm, J. S., Schroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., et al. 1999. A presenilin-1-dependent γ-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398:** 518–522.

Fehon, R.G., Kooh, P.J., Rebay, I., Regan, C.L., Xu, T., Muskavitch, M.A.T., and Artavanis-Tsakonas, S. 1990. Molecular interactions between the protein products of the neurogenic loci *Notch* and *Delta*, two EGF-homologous genes in *Drosophila*. *Cell* **61:** 523–534.

FlyBase. 1998. FlyBase—A *Drosophila* database. *Nucleic Acid Res.* **26:** 85–88. http://flybase.bio.indiana.edu/

Greenwald, I. 1998. LIN-12/Notch signaling: Lessons from worms and flies. *Genes & Dev.* **12:** 1751–1762.

Hattori, M., Osterfield, M., and Flanagan, J.G. 2000. Regulated cleavage of a contact-mediated axon repellent. *Science* **289:** 1360–1365.

Jarriault, S., Le Bail, O., Hirsinger, E., Pourquie, O., Logeat, F., Strong, C.F., Brou, C., Seidah, N.G., and Israël, A. 1998. Delta-1 activation of Notch-1 signaling results in HES-1 transactivation. *Mol. Cell Biol.* **18:** 7423–7431.

Kidd, S., Baylies, M.K., Gasic, G.P., and Young, M.W. 1989. Structure and distribution of the Notch protein in developing *Drosophila*. *Genes & Dev.* **3:** 1113–1129.

Kidd, S., Lieber, T., and Young, M.W. 1998. Ligand-induced cleavage and regulation of nuclear entry of Notch in *Drosophila melanogaster* embryos. *Genes & Dev.* **12:** 3728–3740.

Lawrence, N., Klein, T., Brennan, K., and Martinez Arias, A. 2000. Structural requirements for Notch signalling with delta and serrate during the development and patterning of the wing disc of *Drosophila*. *Development* **127:** 3185–3195.

Lecourtois, M. and Schweisguth, F. 1998. Indirect evidence for Delta-dependent intracellular processing of Notch in *Drosophila* embryos. *Curr. Biol.* **8:** 771–774.

Lieber, T., Kidd, S., Alcamo, E., Corbin, V., and Young, M.W. 1993. Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. *Genes & Dev.* **7:** 1949–1965.

Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N.G., and Israël, A. 1998. The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc. Natl. Acad. Sci.* **95:** 8108–8112.

López-Schier, H. and St. Johnston, D. 2001. Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during *Drosophila* oogenesis. *Genes & Dev.* **15:** 1393–1405.

Mumm, J.S. and Kopan, R. 2000. Notch signaling: From the outside in. *Dev. Biol.* **228:** 151–165.

Mumm, J.S., Schroeter, E.H., Saxena, M.T., Griesemer, A., Tian, X., Pan, D.J., Ray, W.J., and Kopan, R. 2000. A ligand-induced extracellular cleavage regulates γ-secretase-like proteolytic activation of Notch1. *Mol. Cell* **5:** 197–206.

Pan, D. and Rubin, G.M. 1997. Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during *Drosophila* and vertebrate neurogenesis. *Cell* **90:** 271–280.

Primakoff, P. and Myles, D.G. 2000. The ADAM gene family: Surface proteins with adhesion and protease activity. *Trends Genet.* **16:** 83–87.

Qi, H., Rand, M.D., Wu, X., Sestan, N., Wang, W., Rakic, P., Xu, T., and Artavanis-Tsakonas, S. 1999. Processing of the Notch ligand Delta by the metalloprotease Kuzbanian. *Science* **283:** 91–94.

Rebay, I., Fleming, R.J., Fehon, R.G., Cherbas, L., Cherbas, P., and Artavanis-Tsakonas, S. 1991. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: Implications for Notch as a multifunctional receptor. *Cell* **67:** 687–699.

Rio, D.C., Laski, F.A., and Rubin, G.M. 1986. Identification and immunochemical analysis of biologically active *Drosophila P* element transposase. *Cell* **44:** 21–32.

Rooke, J., Pan, D., Xu, T., and Rubin, G.M. 1996. KUZ, a conserved metalloprotease-disintegrin protein with two roles in *Drosophila* neurogenesis. *Science* **273:** 1227–1231.

Sanson, B., White, P., and Vincent, J.P. 1996. Uncoupling cadherin-based adhesion from wingless signalling in *Drosophila*. *Nature* **383:** 627–630.

Schlondorff, J. and Blobel, C.P. 1999. Metalloprotease-disintegrins: Modular proteins capable of promoting cell–cell interactions and triggering signals by protein-ectodomain shedding. *J. Cell Sci.* **112:** 3603–3617.

Schroeter, E.H., Kisslinger, J.A., and Kopan, R. 1998. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393:** 382–386.

Sotillos, S., Roch, F., and Campuzano, S. 1997. The metalloprotease-disintegrin Kuzbanian participates in Notch activation during growth and patterning of *Drosophila* imaginal discs. *Development* **124:** 4769–4779.

Struhl, G. and Adachi, A. 1998. Nuclear access and action of Notch in vivo. *Cell* **93:** 649–660.

Struhl, G. and Greenwald, I. 1999. Presenilin is required for activity and nuclear access of Notch in *Drosophila*. *Nature* **398:** 522–525.

Wen, C., Metzstein, M.M., and Greenwald, I. 1997. SUP-17, a *Caenorhabditis elegans* ADAM protein related to *Drosophila* KUZBANIAN, and its role in LIN-12/NOTCH signalling. *Development* **124:** 4759–4767.

Wodarz, A., Hinz, U., Engelbert, M., and Knust, E. 1995. Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. *Cell* **82:** 67–76.

Ye, Y., Lukinova, N., and Fortini, M.E. 1999. Neurogenic phenotypes and altered Notch processing in *Drosophila* Presenilin mutants. *Nature* **398:** 525–529.