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SHORT COMMUNICATION

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kuzbanian is required cell autonomously during Notch signalling in the *Drosophila* wing

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Abstract The *kuzbanian* gene encodes a metalloprotease of the ADAM family that is involved in *Notch* signalling. However, its precise role is a matter of controversy. While original reports concluded that *kuz* is required on the receiving side of the Notch signalling pathway, a more recent report suggests that Kuz is required on the signal-emitting side for the generation of an active secreted form of the ligand Delta. In this scenario, *kuz* should act cell non-autonomously. A third possibility is that Kuz is required on the signal-emitting as well as the receiving side. Here I present the clonal analysis of *kuz* in *Drosophila* wing. The results show that Kuz acts on the receiving side of the pathway and is not required for Delta signalling. This further confirms the hypothesis that Kuz is required for the release of the intracellular domain of Notch that transduces the signal to the nucleus. The presented results complement recent data that indicate that Kuz can perform the S2 proteolytic cleavage of the Notch receptor that is required for its activation.

Keywords Notch signalling · *kuzbanian* · Wing development · Delta · Cell autonomy

Introduction

The Notch pathway is an evolutionary conserved signalling pathway that is involved in many developmental processes (Mumm and Kopan 2000). It serves to mediate the communication between immediately neighbouring cells. In *Drosophila*, two ligands for the pathway exist and are encoded by the *Delta* (*Dl*) and *Serrate* (*Ser*) genes. Both activate the Notch (N) receptor. Several lines of evidence from vertebrate and invertebrate systems suggest that the activation of the Notch receptor requires three proteolytic events, S1–3. S1 is constitutive, occurs in the Golgi, and creates the mature receptor, consisting of the extracellular domain rejoined with the rest that is inserted in the membrane and has only a small extracellular part (Logeat et al. 1998). The two following cleavages are elicited upon binding of a ligand to the receptor. S2 occurs in the small extracellular remnant of the membrane-inserted part (Brou et al. 2000; Mumm et al. 2000) and is ligand dependent. It is probably performed by a member of the ADAM/TACE family of metalloproteases (Brou et al. 2000; Mumm et al. 2000). A third cleavage, S3, releases the intracellular domain (Nintra). This cleavage occurs within the cell membrane through the *Presinilin* (*Psn*) proteases (Brou et al. 2000; Mumm et al. 2000; Schroeter et al. 1998; Struhl and Greenwald 1999; Ye et al. 1999). The released Nintra is transported into the nucleus, where it associates with a transcription factor of the CSL family, Suppressor of Hairless [Su(H)], to control expression of target genes (Morel and Schweisguth 2000). Recent work in *Caenorhabditis elegans* and *Drosophila* has revealed that a member of the ADAM/TACE family, ADAM 10, is required for signal transduction through the Notch pathway (Pan and Rubin 1997; Sotillos et al. 1997; Wen et al. 1997). The *Drosophila* homologue of ADAM 10 is encoded by the gene *kuzbanian* (*kuz*) (Pan and Rubin 1997). Loss of *kuz* causes phenotypes that are characteristic for genes involved in Notch signalling (Pan and Rubin 1997; Sotillos et al. 1997). Furthermore, expression of the activated intracellular form of Notch bypasses the requirement of Kuz, suggesting that it is required for the activation of the Notch receptor or further upstream in the pathway (Sotillos et al. 1997). However, controversy arose about the specific role of Kuz during Notch signalling. Kuz/ADAM10 was the obvious candidate to perform the S2 cleavage, but experiments performed in vitro and vertebrate cell culture failed to support this idea (Brou et al. 2000; Mumm et al. 2000). In contrast, a more recent report indicates that, in *Drosophila*, Kuz can perform S2 cleavage of certain variants of Notch that lack a region of the extra-

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cellular domain, the lin 12 region (Lieber et al. 2002). Moreover, whereas the initial reports suggested that *kuz* acts cell autonomously (Sotillos et al. 1997; Wen et al. 1997) and therefore would be required on the receiving end of the pathway, a recent report suggested that it is required for the activation of Dl and therefore should act cell non-autonomously (Qi et al. 1999). This conclusion was based on cell culture experiments and genetic interaction studies, which showed that *kuz* interacts phenotypically with *Dl*, but not significantly with *Notch*. The determination of the autonomic behaviour of *kuz* is, however, an important indication of its function during *Notch* signalling.

Here I have determined the cell autonomic behaviour of *kuz* during Notch signalling in the *Drosophila* wing. I found that *kuz* behaves strictly cell autonomously. I further have analysed a situation where a Dl signalling cell could be unambiguously discriminated from the signal-receiving cell and found that Kuz is not required for Dl to signal. Altogether, the presented results suggest that Kuz is required to generate the active intracellular form of Notch that transduces the signal to the nucleus.

Materials and methods

The *kuz*^{ES24} FRT 40A stock was a gift of Yanxia Li and is described by Li and Baker (2001); UAS*Dl* and UAS Nintra are described by Klein and Martinez-Arias (1998). The Gbe+Su(H)m8 stock was a gift of S. Bray and is described by Furriols and Bray (2001). Clones were induced with a UAS Flp construct () activated by *dpp*Gal4 and *vg*Gal4. The FRT 40A GFP chromosome was a gift from S. Lüschnig.

Antibody staining was performed according to standard protocols. The fluorescein isothiocyanate- and Texas red-conjugated secondary antibodies were purchased from Jackson Immuno Research. The anti-Wg antibody was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA 52242, USA).

Results and discussion

During *Drosophila* wing development, the Notch pathway is required to activate the expression of genes along the dorso-ventral compartment boundary (D/V boundary) that orchestrate the growth and patterning of the developing wing primordium. Examples of these genes are *vestigial* (*vg*) and *wingless* (*wg*) (Fig. 1). The expression of target genes is restricted to a stripe of cells on each side of the D/V boundary and is initiated through Ser signalling from dorsal to ventral cells at the boundary and Dl signalling from ventral to dorsal cells. Here I have used the expression of *wg* and a lacZ reporter construct, Gbe+Su(H)m8, which has been shown to be directly controlled by Su(H), as a readout of Notch activity (Furriols and Bray 2001). The activity of Gbe+Su(H)m8 is suppressed by Su(H) alone, but activated if Su(H) associates with Nintra.

In a first experiment, I induced *kuz* mutant clones at the D/V boundary of the wing and looked for the expression of *wg* and the activity of the Gbe+Su(H)m8 construct in the mutant cells (Fig. 1). If *kuz* acts cell non-autonomously, mutant cells in the middle of the clone should lose the expression of Notch target genes. In contrast, mutant cells at the clone boundary should still express the target genes, since they have a functional Notch receptor that can receive the signal emitted by its wild type neighbours. I found that all mutant cells at the D/V boundary had lost the expression of *wg* and the activity of the Gbe+Su(H)m8 reporter (Fig. 1). Even at the boundary of the clones, the expression of the markers was lost in mutant cells, but unaffected in adjacent wild type cells. A particularly good example is shown in Fig. 1A-C. The *arrow* points to a single *kuz*-positive cell at the D/V boundary that expresses *wg*, whereas all mutant neighbours have lost its expression. This behaviour suggests that *kuz* is required cell autonomously during Notch signalling.

Although these results clearly indicate that *kuz* is required on the signal-receiving side of the Notch pathway, they do not rule out the possibility that *kuz* is additionally required on the signal-emitting side, especially for Dl signalling, as has been shown recently by Qi et al. (1999). This possibility cannot be tested at the D/V boundary, since Notch activation is required to maintain the expression of Dl and Ser. Therefore, loss of Notch activity would lead to the loss of ligand expression, and consequently to all Notch activity. Furthermore, due to the involvement of both of the ligands in Notch signalling at the D/V boundary, it is difficult to test a possible dependence of Dl on *kuz* there. To determine whether Kuz is required for Dl to signal, one would ideally like to have a situation where the signalling cell is separated from the signal-receiving cell. Such a situation can be created by expressing Dl ectopically with the help of the Gal4 system. Expression of UAS *Dl* with *dpp*Gal4 results in a broad stripe of ectopic expression of *Dl* along the anterior side of the A/P compartment boundary (A/P boundary) (Fig. 2A, B). Since Dl activates the Notch pathway, *wg* is expressed throughout the *dpp* expression domain and in a row of cells located along the posterior side of the A/P boundary (Fig. 2A, B, highlighted by the *arrows*). Since *dpp*Gal4 and therefore Dl is not expressed in posterior cells, *wg* expressed in these cells is induced by Dl-expressing cells signalling from the anterior side of the boundary (Fig. 2C). Thus, at the A/P boundary, the signalling cell can be unambiguously discriminated from the signal-receiving cell. Furthermore, cell clones do not cross the A/P compartment boundary. Hence, it is possible to remove *kuz* function from each side of the boundary separately by induction of *kuz* mutant clones. If *kuz* function is required for Dl to signal, cells at the anterior side of the A/P boundary that have lost *kuz* function should not be able to activate the Notch pathway in cells on the other side of the boundary. Consequently these cells should lose the expression of *wg*. As shown in Fig. 2D–G, this is not the case. Anterior cells that have

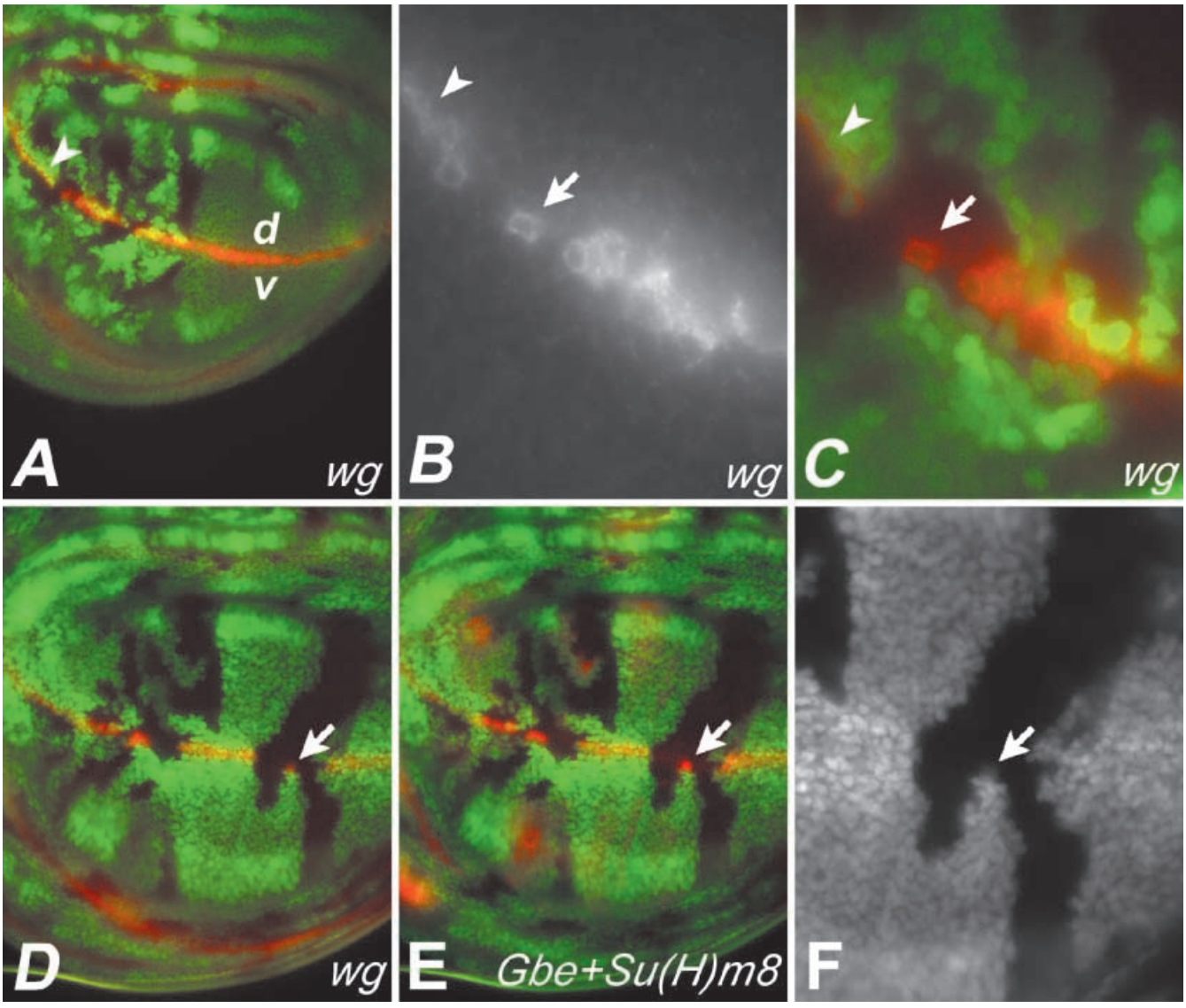
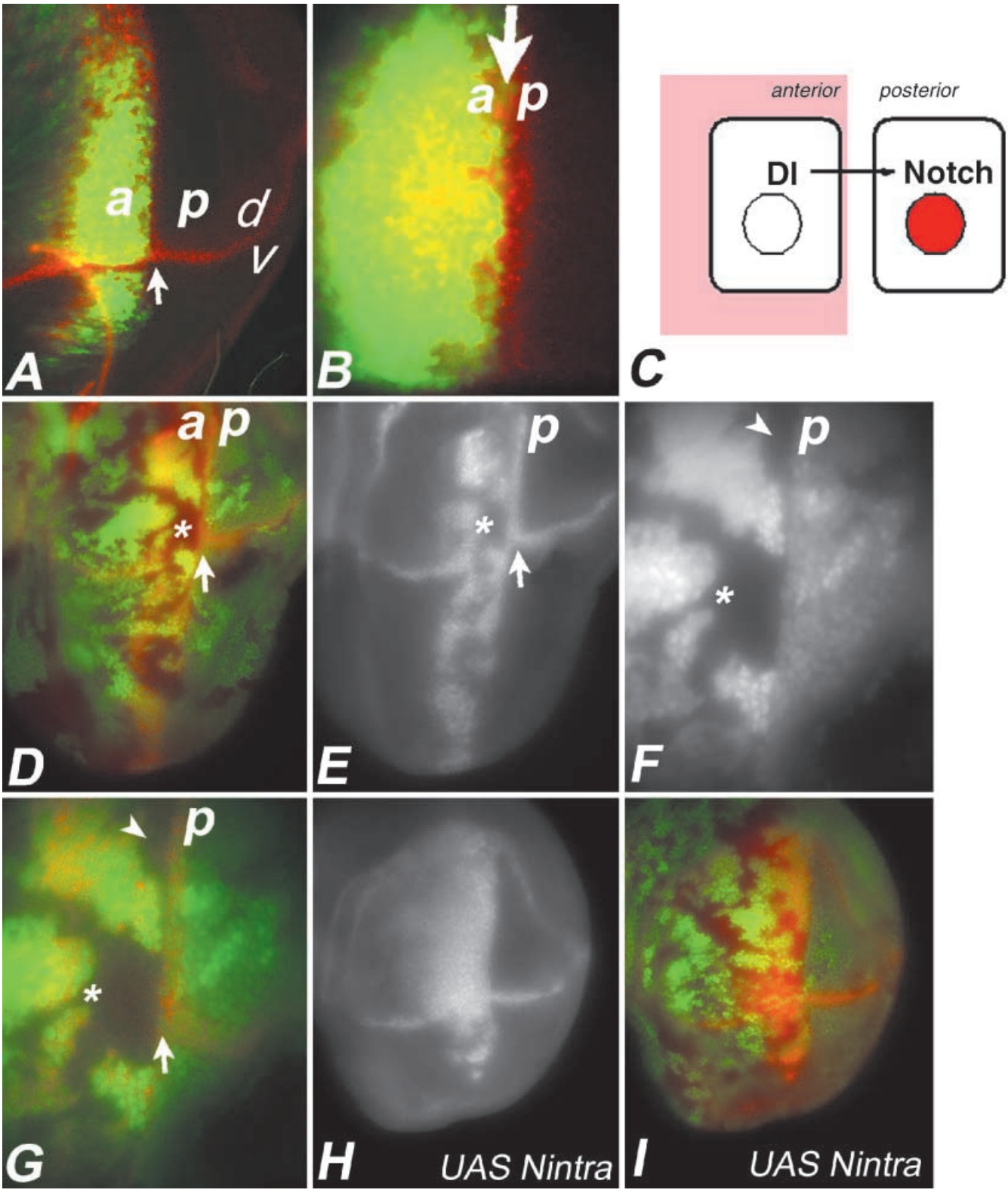


Fig. 1A–E Clonal analysis of *kuz*^{ES24} at the dorsoventral boundary (D/V boundary) of the wing disc. In all pictures, *kuz* mutant territories are marked by the absence of the green fluorescence of GFP. The clones were induced by an UAS Flp construct activated with *dpp*Gal4 (**A–C**) or *vg*Gal4 (**C–E**). **A–C** Wing imaginal disc stained with anti-Wg antibody (*red*). wg is expressed along the D/V boundary and its expression is induced by the Notch pathway. The wing disc in **D** is stained with an anti-β-galactosidase (βGal) antibody (*red*) to reveal the activity of the Gbe+Su(H)m8 lacZ reporter construct. **A** Overview of a wing imaginal disc bearing *kuz*^{ES24}mutant clones. All mutant cells at the D/V boundary have lost expression of Wg, even if located adjacent to wild type cells. **B, C** Magnification of the region highlighted by the *arrow* in **A**. **B** Wg staining. The *arrows* in **C, B** point to a single cell expressing Wg. This cell contains *kuz* activity, whereas all other cells around are *kuz* negative and have lost Wg expression. **D–F** Anti-Wg anti-βGal double staining of a wing disc bearing *kuz* mutant clones. Again expression of Wg (*red* in **D**) as well as the activity of the Gbe+Su(H)m8 reporter (*red* in **E**) is lost in all cells that are mutant for *kuz*, even if they are adjacent to wild type cells. The *arrow* points to a few *kuz*-positive cells at the D/V boundary surrounded by *kuz* mutant cells. Only these cells express Wg and βGal, indicating that *kuz* function is required for signal reception (**F**). Altogether the results indicate that *kuz* acts cell autonomously

lost *kuz* function can still induce expression of *wg* in posterior cells at the A/P boundary. This indicates that at least under these experimental conditions *kuz* is not required in the signal-emitting cell for Dl to signal.

Furthermore, all cells of *kuz* mutant clones that are located in the *dpp*Gal4 expression domain lose expression of *wg*, even at the clone boundaries. Hence, the Notch pathway cannot be activated in mutant cells, even if the *kuz*-positive neighbours express high levels of Dl. This observation further confirms that *kuz* is acting cell autonomously (Fig. 2D–F). As expected from earlier experiments (Sotillos et al. 1997), loss of *kuz* does not influence the induction of *wg* expression through ectopic expression of the activated from of Notch (Nintra) with *dpp*Gal4 (Fig. 2H, I).

Altogether, the presented results clearly show that Kuz is required in the signal-receiving cell, but not in the signal-emitting cell during wing development. Hence, Kuz is probably involved in the generation of the active intracellular form of Notch. This reopens the possibility that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Kuz is involved in the S2 cleavage. Although experiments with *kuz*-deficient mouse cells indicate that S2 cleavage can occur in the absence of Kuz (Mumm et al. 2000), it is possible that functionally redundant proteases can cleave Notch in the absence of Kuz in these cells. Alternatively Kuz might be involved in another unknown proteolytic event that is required for the activation of the Notch ligand. The results presented here support the conclusion of a recent paper by Lieber et al. (2002), which reports that Kuz can perform S2 cleavage on Notch of truncated molecules during *Drosophila* embryogenesis. They also extend the conclusions of this work by providing evidence that Kuz is involved in S2 cleavage of the normal Notch molecule during wing development. In contrast, the results do not support the conclusion of Qui et al. (1999) that Kuz is required to generate a soluble active form of Dl. In fact another recent publication investigating the role of a soluble form of the mouse homologue of Dl, Dll 1, reports that this form acts as a antagonist of Dl signalling and that the intracellular domain of Dl is required for processing of Notch2 (Shimizu et al. 2002). However, there is still the possibility that in other developmental situations, such as during neurogenesis, Kuz is required for Dl signalling. Alternatively, Kuz might be required for the inactivation of Dl through cleavage and release of the extracellular domain.

◀ **Fig. 2A–I** Kuz is not required in the Dl signalling cell. In all pictures anti-Wg antibody staining is shown in *red*. *kuz* mutant clones (in **D–I**) are induced through UAS Flp activated by *dpp*Gal4 and marked by the absence of the green GFP marker. Arrows in **A**, **B**, **D**, **E**. and **G** point to the A/P boundary. **A, B** Activation of UAS *Dl* by *dpp*Gal4 results in the expression of Dl in a broad band-like domain along the anterior side of the anteroposterior compartment boundary (A/P boundary) as revealed by the co-expression of UAS GFP (*green*). This leads to the activation of Wg expression throughout the *dpp* domain and, although Dl is restricted to anterior cells, *wg* expression is also activated in cells on the posterior side of the boundary, as highlighted at the magnification shown in **B**. Since cells never cross the compartment boundary, the expression of Wg in posterior cells of the boundary must be induced by the Dl-expressing cells on the anterior side of the boundary. Therefore, at the boundary, it is possible to unambiguously discriminate between signal-sending (anterior) and receiving (posterior) cells. This is schematically summarized in **C**: anterior cells signal across the compartment boundary to posterior cells to activate the Notch pathway and *wg* expression. This signalling across the compartment boundary is restricted to the dorsal side (d) of the wing, since *dpp*Gal4 is only weakly expressed in the ventral side (v) until the middle of third larval instar. **D–G** *kuz* mutant clones induced in a wing disc that express UAS *Dl* with *dpp*Gal4. **D, E** Overview. **F, G** Magnification of the region highlighted with the asterisk in **D–E**. **F**The GFP channel reveals that the clones highlighted by the *asterisk* and *arrowhead* are located at the A/P boundary and form a straight smooth border along the A/P boundary. **G** Although these cells do not have *kuz* function, they are able to signal via Dl across the A/P boundary to activate Wg expression in posterior cells. This result shows that Kuz is not required for Dl to activate Notch during wing development. All mutant cells at the boundary around the clones have lost the expression of wg, although they are located adjacent to *kuz*-positive cells that strongly express Dl. This further confirms that *kuz* acts cell autonomously. **H, I** Kuz is not required for the induction of wg expression through Nintra. **H** Expression of UAS Nintra with *dpp*Gal4 results in the activation of the expression of Wg. This induction is not abolished in *kuz* mutant clones (**I**). Hence, the function of *kuz* is required before Nintra is created in the signal-receiving cell. The data shown here suggest that Kuz is involved in the generation of the Nintra fragment that transduces the signal to the nucleus. *a* Anterior; *p* posterior

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