the base, the relative change in collector current $(I_P - I_{AP})/I_{AP}$ can be greater than 1000%, depending on the scattering asymmetry $\lambda_\uparrow/\lambda_\downarrow$ and $W_{Co}/\lambda_\uparrow$ (Fig. 4A). In general, such large changes may not be necessary. The noise in the SVT is caused mainly by three sources: (i) (particle-related) shot noise from the forward-biased emitter barrier; (ii) ordinary thermal, or Johnson, noise in the base resistance; and (iii) shot noise in the collector barrier. These fluctuations produce white noise at the terminals of the SVT. Because the thermal noise of electrons at E_F does not affect hot-electron transport, and the base thermal noise voltage does not influence the hot-electron I_C (the emitter is driven by a current source), the thermal noise is not found in I_C. Hence, the collector noise current i_c is pure shot noise $i_c = (2qI_c df)^{1/2}$ as in ordinary (Schottky) diodes (*10*). For this reason, the SNR increases with I_C and the absolute change in collector current $I_P - I_{AP}$ is a more useful parameter for sensor applications than the relative change $(I_P - I_{AP})/I_{AP}$. Because the output current decreases with $W_{Co}/\lambda_\uparrow$ but the relative change increases with $W_{Co}/\lambda_\uparrow$ and $\lambda_\uparrow/\lambda_\downarrow$, an optimum for the SNR is found by plotting $I_P - I_{AP}$ versus $W_{Co}/\lambda_\uparrow$ (Fig. 4B). Here,

$$(I_P - I_{AP})_N = \frac{I_P - I_{AP}}{\frac{1}{2}I_E T_{SI}} = T_P - T_{AP}$$

is the normalized collector current difference between parallel and antiparallel magnetizations resulting from the two-channel model. We compare the SVT with a GMR film-measured CIP on an SNR basis by assuming a device area 1 μm by 1 μm, a bandwidth *df* of 100 MHz, $\lambda_\uparrow/\lambda_\downarrow = 10$, $W_{Co}/\lambda_\uparrow = 1 \Rightarrow (I_P - I_{AP})_N \approx 0.4$, $I_E = 1$ mA, and $T_{SI} = 0.05$ (loss is lower when a thinner Pt film is used, for example). For the SVT, the shot noise results in an SNR = $20 \log(I_c/i_c) = 58$ dB. Addition of a typical low-noise broadband (100 MHz) current (transimpedance) amplifier to this signal adds 1 pA/Hz$^{1/2}$ noise, resulting in a noise level of $i_{tot} = (i_c^2 + i_a^2)^{1/2}$ or SNR = 57 dB. Clearly, a large collector current is desired, which can be obtained by optimizing $I_P - I_{AP}$ through $W_{Co}/\lambda_\uparrow$ (type of metal and number of interfaces) and optimizing $\lambda_\uparrow/\lambda_\downarrow$ (spin-valve quality) and T_{SI} (Schottky barrier quality and nonmagnetic layer thickness). In epitaxial structures, spin-dependent resonance effects in the base might further enhance $I_P - I_{AP}$. In cases where the input noise current of the amplifier limits the SNR of the SVT or when local amplification is desired, a major enhancement may be achieved by avalanche multiplication of the collector current. Application of a larger V_{CB} bias generates electron-hole pairs in the collector depletion layer, and multiplication of I_C may be as large as 100. The noise current of the SVT is also multiplied with this factor, therefore its SNR stays the same, but because the signal is much greater, the SNR of the system will be enhanced. A comparable 20-ohm GMR film with 1-mV effective output voltage (*11*) has a Johnson noise of $u_n = (4kTRdf)^{1/2} \approx 6$ μV and an SNR of 45 dB. A typical hard disk amplifier adds 0.55 nV/Hz$^{1/2}$ or 5.5 μV of noise, resulting in 42 dB overall. In spite of the larger SNR of the SVT, the power dissipated in the SVT structure is greater (≈1 mW). The maximum current through the GMR film is determined by electromigration, whereas the maximum current in the SVT is determined by heating. Analysis of this factor for specific sensor designs is required for a realistic comparison. Its intrinsic diode characteristics make selection transistors per storage cell redundant and make the SVT attractive for MRAM development.

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Boundary Formation in *Drosophila* Wing: Notch Activity Attenuated by the POU Protein Nubbin

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Cell interactions mediated by Notch-family receptors have been implicated in the specification of tissue boundaries in vertebrate and insect development. Although Notch ligands are often widely expressed, tightly localized activation of Notch is critical for the formation of sharp boundaries. Evidence is presented here that the POU domain protein Nubbin contributes to the formation of a sharp dorsoventral boundary in the *Drosophila* wing. Nubbin represses Notch-dependent target genes and sets a threshold for Notch activity that defines the spatial domain of boundary-specific gene expression.

Spatially localized activation of Notch is required for specification of the dorsoventral (DV) boundary of the *Drosophila* wing (*1–5*). Notch signaling has also been implicated in establishing tissue boundaries in somite formation, in neurogenesis, and at the DV boundary of the vertebrate limb (*6–8*). The tight localization of Notch activity in these systems contrasts with the broad distribution of Notch ligands. The problem of spatially limiting Notch activation is partially solved through activity of *fringe* genes (*9*), which modulate the sensitivity of Notch for its ligands and contribute to spatially limiting Notch activity (*8, 10*). Certain features of the abnormal wings in flies mutant for the *nubbin* gene suggested a possible role for Nubbin protein in spatially limiting Notch activity at the DV boundary of the wing (*11, 12*). The *nubbin* gene encodes a POU domain protein that is expressed in the developing wing primordium (*11*) (Fig. 1A).

The row of sensory bristles that makes up the wing margin is disorganized in *nubbin* mutant wings (*11*), suggesting a defect in Wingless or Notch activity. In preparations where the wing margin is viewed edge on, this disorganization reflects a broadening of the region where bristles form (Fig. 1, B and C). Margin bristles are normally specified in cells very close to the DV boundary, reflecting a requirement for high levels of Wingless signaling activity (*13*). The broadening of the margin suggests that Wingless might be ectopically expressed in *nubbin* mutant wing discs. Wingless is normally expressed in a stripe of two to three cells straddling the DV boundary (Fig. 1D). In *nubbin* mutant discs this stripe is widened considerably (Fig. 1E). Expression of the Notch targets *vestigial* and *cut* is similarly expanded at the DV boundary

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in *nubbin* mutants (Fig. 1, F and G) (*14*).

To determine whether the effect on bristle specification is a direct consequence of removing *nubbin* activity, we generated clones of *nubbin* mutant cells in a wild-type background. Ectopic wing margin bristles were found in *nubbin* mutant clones located near the endogenous wing margin (Fig. 2A) (*11, 15*). The *nubbin* mutant clones showed ectopic expression of *neuralized-lacZ*, a molecular marker for precursors of the sensory neurons that innervate the bristles (Fig. 2B). Ectopic bristle precursors were usually confined to the clone, but in rare instances they arose in adjacent wild-type cells (*14*). Specification of wing margin sense organs and induction of *neuralized-lacZ* are known to depend on localized expression of Wingless at the DV boundary (*13*). Clones of cells that are simultaneously mutant for *wingless* and *nubbin* do not show ectopic *neuralized-lacZ* staining (Fig. 2C), suggesting that ectopic bristle specification in *nubbin* mutant cells is due to ectopic Wingless activity. The *nubbin* mutant clones misexpressed *wingless* and *vestigial* (Fig. 2, D and F). The largely autonomous effect of *nubbin* mutant clones on bristle specification may be due to the relatively low levels of ectopic Wg protein expressed in *nubbin* mutant clones (*14*). Together with the results on *cut* expression (Fig. 1), these observations suggest that Notch target genes are transcriptionally up-regulated in *nubbin* mutant cells near the DV boundary.

To test whether ectopic activation of these genes in *nubbin* mutant clones directly depends on Notch signaling activity, we generated clones of cells that were simultaneously mutant for *nubbin* and *Suppressor of Hairless* [*Su(H)*]. *Su(H)* encodes a DNA-binding protein that mediates transcriptional activation of Notch target genes (*16*). *Su(H)* is autonomously required for the expression of *wingless*, *vestigial*, and *cut* at the DV boundary (*4*) and binds directly to the *vestigial* DV boundary enhancer (*17*). Clones of cells mutant for both *nubbin* and *Su(H)* do not ectopically activate *wingless* (Fig. 2E), demonstrating that ectopic expression of *wingless* in *nubbin* mutant cells depends on activity of the Notch pathway. To confirm that Nubbin acts down-

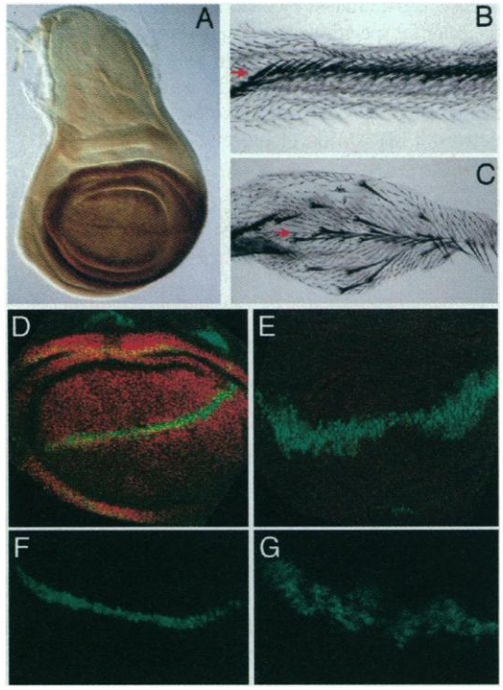

Fig. 1. Ectopic activation of Notch target genes in *nubbin* mutant wings. **(A)** Nubbin protein expression (brown) in a mature third-instar wing imaginal disc visualized by histochemical staining with mouse anti-Nubbin (*27*). **(B)** Cuticle preparation of a wild-type wing margin (detail of a region in the anterior compartment). The dorsal and ventral surfaces of the wing were peeled apart and flattened so the margin is viewed from the edge. The dorsal surface to the top. Orderly rows of wing-margin bristles line the dorsal and ventral sides of the DV boundary of the wing (arrow). **(C)** Cuticle of a *nubbin*^{1} wing margin prepared as in (B). Bristles are found at a distance from the DV boundary (arrow). The DV identity of bristles is ambiguous in the *nubbin*^{1} wing margin, possibly because clones fail to respect the DV lineage restriction in *nubbin*^{1} wings (*12*). **(D)** Wild-type and **(E)** *nubbin*^{1} mutant wing discs labeled for Nubbin protein (red) and for a *wingless-lacZ* reporter gene [green, visualized with anti–β-Gal. **(F)** Wild-type and **(G)** *nubbin*^{1} mutant wing discs labeled for a *cut-lacZ* reporter gene.

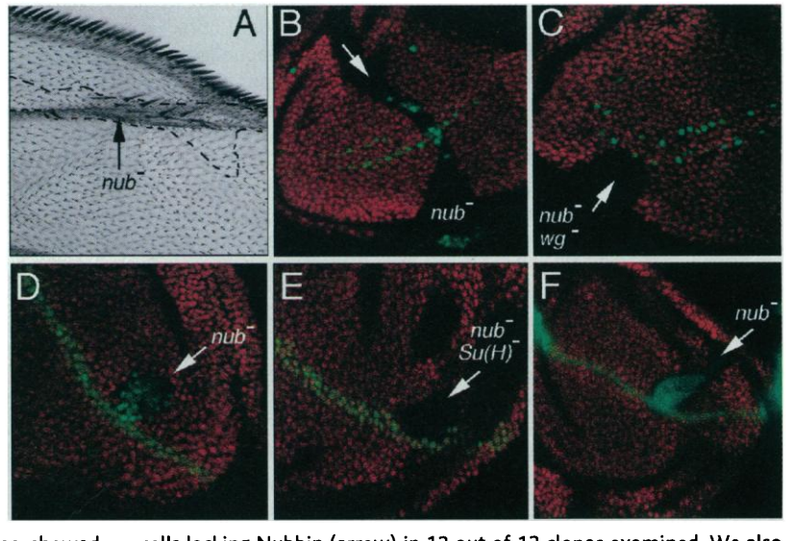

Fig. 2. Cell-autonomous effects of *nubbin* mutant clones. **(A)** *nubbin*^{1} mutant clone in the adult wing (*15*). Of 26 clones examined, 20 showed ectopic bristles distant from the wing margin (arrow). We observed one adult clone that showed ectopic bristle differentiation immediately outside of the clone border (*14*). All clones caused buckling of the wing surface, caused by formation of some ectopic wing-vein material and possibly mild overgrowth. Consequently, the field of wing hairs is not all in the same focal plane. The *nubbin*^{1} mutant cells (outlined) are marked by loss of a *forked*$^{+}$ transgene on the wild-type chromosome. The *forked* marker labels bristles and wing hairs. **(B to F)** *nubbin*^{1} mutant clones in the wing disc were visualized by the absence of Nubbin protein (red). Expression of the lacZ reporter genes, *neuralized-lacZ*, *wingless-lacZ*, and *vestigial-lacZ*, was visualized with anti–β-Gal (green). Overlap of the two signals appears yellow. Clones were induced in mid–second-instar larvae except as indicated. All discs are shown with dorsal to the top and anterior to the left. **(B)** *neuralized-lacZ* in a wing disc carrying a *nubbin*^{1} mutant clone (*nub*$^{-}$). Ectopic expression of *neuralized-lacZ* (arrow) was observed in 7 out of 10 *nubbin* mutant clones examined. Clones were induced in first-instar larvae to get large clones that cross the DV boundary. Two of 10 examples showed *neuralized-lacZ* expression in wild-type cells adjacent to the clone (*14*). (C) *neuralized-lacZ* expression in a wing disc carrying a *wingless*^{CX4} *nubbin*^{1} double-mutant clone (*nub*$^{-}$ *wg*$^{-}$) (*15*). The clone is ventral anterior and abuts the row of *neuralized-lacZ*–expressing cells. The anterior compartment of this disc is slightly distorted so that the row of *neuralized-lacZ*–expressing cells bends more sharply than usual. *neuralized-lacZ* was not misexpressed in 7 out of 7 clones examined (arrow). (D) *wingless-lacZ* expression in a wing disc carrying a *nubbin*^{1} mutant clone. *wingless-lacZ* was ectopically expressed in cells lacking Nubbin (arrow) in 13 out of 13 clones examined. We also found that 15 out of 17 clones examined misexpress Wingless protein (*14*). Ectopic *wingless* expression was also observed in clones that do not touch the DV boundary. (E) *wingless-lacZ* expression in a wing disc carrying a *nubbin*^{1} *Su(H)* double-mutant clone (*nub*$^{-}$ *Su(H)*$^{-}$; *15*). *wingless-lacZ* is not expressed in 11 out of 11 *nubbin*^{1} *Su(H)* double-mutant clones examined. (F) Expression of the *vestigial* DV boundary enhancer in a wing disc carrying a *nubbin*^{1} mutant clone. Ectopic expression of *vestigial-lacZ* in cells lacking Nubbin (arrow) was observed in 7 out of 11 clones examined.

stream of Notch, we tested whether overexpression of Nubbin could suppress the effects of a ligand-independent form of Notch (Notch[intra]) (*18*). Expression of Notch[intra] causes ectopic Wingless expression in the wing disc (*1*) (Fig. 3A). When Nubbin is coexpressed with Notch[intra], ectopic Wingless expression is strongly reduced (Fig. 3B). Together, these observations suggest that Nubbin may act as a direct repressor of Notch-dependent target gene expression (*19*). These findings argue that the effects of Nubbin are unlikely to be mediated by indirect effects on expression of Notch ligands (*20*).

Ectopic expression of *wingless* and *vestigial* in *nubbin* mutant clones indicates that the Notch signaling pathway is active in cells at a considerable distance from the wing margin. Notch is activated by Delta and Serrate. The broad distribution of both ligands in the developing wing disc poses a problem in limiting high-level activation of the Notch pathway to cells near the DV boundary. This is partly solved by modulating the sensitivity of Notch for Serrate and Delta through Fringe activity (*9, 10*). Serrate is expressed only in dorsal cells at the time when boundary-specific expression of *wingless* and *vestigial* is induced; nonetheless, Serrate activates Notch in ventral cells. Dorsal cells, which express both Fringe and Serrate, are refractory to Serrate at this stage (*1, 2, 5*). Delta is expressed both dorsally and ventrally, but appears to activate Notch mainly in dorsal cells (*3, 5, 21*). Although Fringe modulates the sensitivity of Notch to Serrate and Delta, our findings indicate that Fringe is not sufficient to limit high-level Notch activity to cells near the wing margin. In the absence of Nubbin, Notch targets are activated in cells at a distance from the boundary. We can estimate the range over which Notch activity is sufficient to induce boundary-specific genes by examining where *nubbin*^{1} mutant clones induce ectopic expression of *wingless* or *vestigial*. Clones located more than 10 cell diameters from the DV boundary do not induce target genes (as seen in 20 out of 20 examples) (*14*).

These observations suggest that Notch is activated in a broad region centered on the DV boundary and that Nubbin antagonizes the ability of Notch to induce its target genes. If so, overexpression of Nubbin should interfere with endogenous expression of Notch targets at the DV boundary. Large Nubbin-expressing clones (*22*) cell-autonomously reduce Wingless expression when they cross the DV boundary (Fig. 4). Thus Nubbin appears to act as a repressor that competes with a Notch-dependent activation signal to determine the amount of target gene expression.

To examine whether *nubbin* might directly regulate Notch target genes, we tested Nubbin protein binding to the *vestigial* boundary enhancer (*23*). In deoxyribonuclease I footprinting experiments, Nubbin bound to a cluster of four sites between residues 260 and 400, as well as to two weaker sites (Fig. 5) (*24*). The cluster of Nubbin binding sites is well separated from the single Su(H) binding site required for Notch-dependent activation of this enhancer (residues 100 to 108) (*17*) (diagram in Fig. 5B). To determine whether Nubbin mediates repression of the boundary enhancer through these binding sites, we compared the expression of a lacZ reporter gene under control of the wild-type enhancer with that under control of a mutant enhancer from which the central two Nubbin binding sites had been deleted. We observed considerable broadening of the stripe of reporter gene expression in the mutant enhancer (Fig. 5C) (*24*).

Nubbin and Notch appear to play opposing roles in the regulation of boundary-specific genes. Nubbin acts as a general repressor of *wingless*, *vestigial*, and other Notch targets in the wing primordium. Activation of the Notch signaling pathway to high levels at the DV boundary provides sufficient stimulation to override repression by Nubbin. The idea that Nubbin sets a threshold level for Notch activity is supported by observations that overexpression of Nubbin can prevent Notch from activating Wingless at the DV boundary.

Tight regulation of Notch signaling is necessary for normal wing development. We have shown that Notch is activated in a broad region of the wing at levels sufficient to induce boundary-specific target genes and that Nubbin appears to limit the effective width of this domain so as to create a sharp boundary. Members of the *fringe* gene family also contribute to limiting Notch activity to cells near the DV boundary (*2, 8*). At later stages of wing development a third mechanism comes into play. Wingless limits its own expression to the DV boundary, possibly

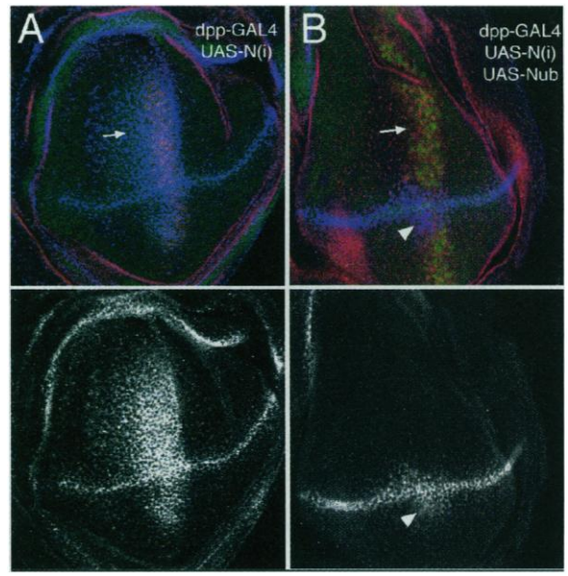

Fig. 3. Nubbin acts downstream of Notch. Wing imaginal discs were simultaneously labeled for Notch protein (red), Nubbin protein (green), and Wingless protein (blue) (*19*). Wg expression is shown separately below (in black and white). (**A**) Disc of genotype dpp-GAL4/UAS Notch(intra). Notch(intra) expression was driven by dpp-GAL4 in a stripe along the AP compartment boundary (perpendicular to the endogenous stripe of Wg expression along the DV boundary). Notch(intra) induced a high level of ectopic Wg expression (arrow; compare with the narrower endogenous Wg domain). The normal Nubbin expression domain is shown in green. (**B**) Disc of genotype UAS-Nub/+; dpp-GAL4/UAS Notch(intra). Both Nubbin and Notch(intra) are expressed in the dpp-GAL4 stripe. Ectopic Wg expression is prevented in the cells where a high level of Nubbin is expressed (strong green label, arrow) together with notch(intra). A small area of ectopic Wg expression is apparent near the DV boundary where Nubbin is not overexpressed (arrowhead).

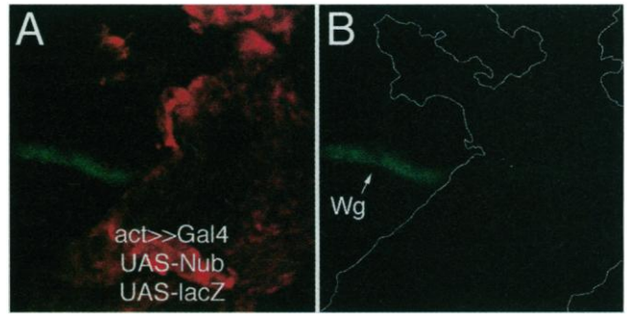

Fig. 4. Nubbin overexpression represses Wingless expression at the DV boundary. (**A**) Portion of a wing imaginal disc with a clone of cells overexpressing Nubbin under Gal4 control (*22*). The clone expresses GAL4 under control of the Act5C promoter (act ≫ Gal4) and directs expression of both β-Gal (UAS-lacZ) and Nubbin (UAS-Nub). The disc was labeled with anti–β-Gal to mark the clone (red) and with anti Wingless (green). Large Nubbin-expressing clones reduce Wingless expression (nine out of nine clones examined). Small Nubbin-expressing clones, induced later in development, show a weaker effect (*14*). (**B**) Same disc as in (**A**), but showing Wingless expression alone. The clone is outlined (white).

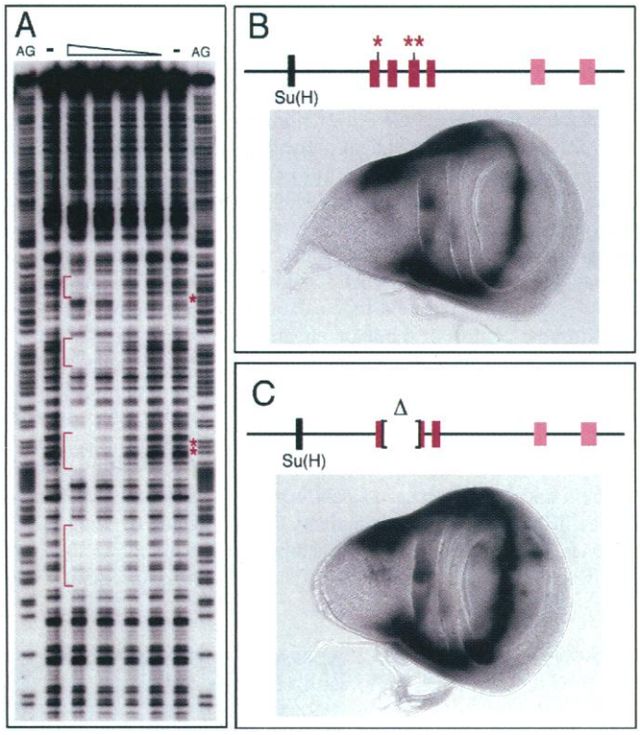

Fig. 5. Nubbin represses the *vestigial* boundary enhancer. (**A**) deoxyribonuclease I footprinting of the vestigial boundary enhancer with bacterially expressed Nubbin protein. The probe was asymmetrically end-labeled by end repair at a unique Age I site. AG lanes: A+G chemical cleavage sequencing reaction. (−) No added protein. Nubbin protein was diluted 1:10, 1:30, 1:90, and 1:120 (left to right). Four strong binding sites are indicated by red brackets. The positions of these sites are indicated as red boxes in (B) and (C). The uppermost binding site is located toward the left end of the enhancer, as depicted in the maps in (B) and (C). Protected sequences (sites from left to right in B): TTATACAAGCCGC, TTATGTAAGTAACC, TTTTGCATGCCCAT, and CCGCCTGGATATTGCGC. POU protein binding sites do not have a simple consensus (*28*). The asterisks indicate the positions of the restriction sites used to delete binding sites in (C). (**B**) Expression of a *lacZ* reporter gene by the modified wild-type vestigial enhancer (*24*), visualized by X-Gal staining for β-Gal activity. The map shows the positions of the four strong Nubbin binding sites (red boxes). Two weaker Nubbin sites are indicated by pink boxes. The single Su(H) site is indicated by a black box. The Sac II site is indicated by one asterisk, the Sph I site by two asterisks. (**C**) Two Nubbin binding sites were removed from the boundary enhancer by deleting sequences between the Sac II and Sph I sites (*24*). The deleted region (Δ) is indicated by brackets. To permit direct comparison, we prepared and processed in parallel samples expressing the wild-type and mutant enhancers.

by modulating Notch activity (*25*) or by modulating the late expression of Notch ligands (*26*).

Three distinct mechanisms are used to spatially limit Notch activity in boundary formation in the *Drosophila* wing. The complex, multilevel control of Notch signaling highlights both the importance of tightly regulating Notch activity to allow precise boundary formation and the difficulty in achieving the necessary precision of regulation. Notch signaling and members of the *fringe* gene family have been implicated in boundary formation in vertebrate development (*7, 8*). It will be of interest to determine whether a factor similar to Nubbin functions to restrict Notch signaling in vertebrate boundary determination.

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12. Analysis of *nubbin* mutant phenotypes suggests several distinct functions unrelated to Notch. Nubbin activity in the wing hinge is required to support proximal-distal outgrowth of the wing. This effect is nonautonomous. Other phenotypes reported by Cifuentes and Garcia-Bellido (*11*) include reduced proliferation of *nubbin* clones, loss of the anterior-posterior (AP) and DV lineage restrictions, and distal to proximal transformation of the wing margin. These phenotypes do not relate in an obvious way to Notch functions [J. F. de Celis and A. Garcia-Bellido, *Mech. Dev.* **46**, 109 (1994) and (*1–4*)] and will not be considered further in this report.
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19. dpp-GAL4 was used to direct expression of UAS-Notch[intra] or UAS-Notch[intra] and UAS-Nubbin (parental genotypes: dppGAL4/TM6B × UAS-Nub/+; UAS-Notch[intra]/+). Larvae were triply labeled with rat antibody to Notch (anti-Notch) [R. G. Fehon *et al.*, *Cell* **61**, 523 (1990)], mouse anti-Nubbin (*27*), and rabbit anti-Wingless [P. Bhanot *et al.*, *Nature* **382**, 225 (1996)] to verify the genotype and assess the effects of Nubbin on Notch[intra] induction of Wg expression. Preliminary results suggest that genetic interactions occur between UAS-Nubbin and UAS-Notch[intra] in other developmental contexts (*14*). Further work will be required to determine whether this reflects a conserved regulatory relationship outside the developing wing.
20. We considered the possibility that the effects of *nubbin* could be mediated indirectly by effects on expression of Notch ligands. Delta is up-regulated in *nubbin* clones (*14*). Delta and Serrate can induce *wingless* expression but do so nonautonomously (*1, 4, 5, 21, 25*). If the effects of *nubbin* clones were mediated by increased expression of *Delta* or *Serrate*, we would expect *wingless* to be expressed in cells adjacent to the clone as well as in *nubbin* mutant cells. However, *nubbin* clones induce Wingless and *vestigial* strictly cell autonomously. Nubbin overexpression also argues against an indirect effect mediated by Delta and Serrate. If the effects were due to reduced expression of Notch ligands, Wingless expression should be rescued at the edge of the clone of Nubbin-expressing cells (as observed in Serrate or Delta mutant clones) (*26*), but it is not (Fig. 4).
21. The DV asymmetry in the action of Delta and Serrate reflects the function of these ligands when boundary-specific gene expression is initiated. Later, in third instar, Serrate and Fringe are also expressed on both sides of the DV boundary, and the refinement of the expression domains of boundary-specific genes depends on a more complex interplay between Wingless, Delta, and Serrate signaling [J. F. de Celis and S. Bray, *Development* **124**, 3241 (1997); *25, 26*].
22. Clones of cells overexpressing Nubbin were produced by using a combination of the flip-out and Gal4 systems (F. Pignoni and S. L. Zipursky, *ibid.*, p. 271) in larvae of genotype Actin>CD2>Gal4; UAS-Nubbin; UAS-lacZ. Clones in which the flip-out cassette is excised will express GAL4 and direct expression of both Nubbin and β-Gal, which are under UAS control. Mouse anti-Wg has been described [W. J. Brook and S. M. Cohen, *Science* **273**, 1373 (1996)].
23. J. A. Williams, S. W. Paddock, K. Vorwerk, S. B. Carroll, *Nature* **368**, 299 (1994).
24. Bacterially expressed Nubbin protein was recovered as a pellet of insoluble inclusion bodies and solubilized in 2 M guanidine isothiocyanate in phosphate-buffered saline (PBS). Footprinting reactions were carried out as described [P. Rorth and D. Montell, *Genes Dev.* **6**, 2299 (1992)]. Nubbin protein was diluted with a solution of 50% glycerol and 1% bovine serum albumin in PBS to 1:10, 1:30, 1:90, and 1:270. We incubated 1 μl of each dilution with 10 fmol of end-labeled DNA per 20 μl of footprinting reaction. To produce vestigial enhancer mutants, we modified the wild-type intron 2 enhancer (*2*) by polymerase chain reaction to introduce an Xho I site at the 5′ end and cloned this into Casper–HS43–AUG–β-Gal cut with Xho I–Eco RI. Expression of the modified enhancer is indistinguishable from the original

version (2). Nubbin-binding sites were deleted by cutting the modified enhancer plasmid at unique Sac II and Sph I sites and re-ligating after end repair.
25. E. J. Rulifson, C. A. Micchelli, J. D. Axelrod, N. Perrimon, S. S. Blair, *Nature* **384**, 72 (1996).
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28. W. Herr and M. A. Cleary, *Genes Dev.* **9**, 1679 (1995).

Our sites 1 to 3 are similar to other POU sites in having the sequence TT(A/T)T(A/G)A(A/T). Despite their lack of a more extensive consensus sequence, these three sites show near perfect sequence conservation in the corresponding enhancer from *D. virilis* (*23*). We suggest that these sequences are conserved because they represent functionally important binding sites for Nubbin and possibly other proteins.

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Uncoupling of Nonreceptor Tyrosine Kinases from PLC-γ1 in an SLP-76–Deficient T Cell

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Activation of nonreceptor protein tyrosine kinases (PTKs) is essential for T cell receptor (TCR) responsiveness; however, the function of individual PTK substrates is often uncertain. A mutant T cell line was isolated that lacked expression of SLP-76 (SH2 domain–containing leukocyte protein of 76 kilodaltons), a hematopoietically expressed adaptor protein and PTK substrate. SLP-76 was not required for TCR-induced tyrosine phosphorylation of most proteins, but was required for optimal tyrosine phosphorylation and activation of phospholipase C-γ1 (PLC-γ1), as well as Ras pathway activation. TCR-inducible gene expression was dependent on SLP-76. Thus, coupling of TCR-regulated PTKs to downstream signaling pathways requires SLP-76.

The T cell antigen receptor (TCR) is coupled to downstream signaling events by nonreceptor protein tyrosine kinases (PTKs) (*1*). The TCR-induced PTKs Lck and ZAP-70 trigger calcium-dependent and Ras-dependent signaling pathways (*2*). TCR-induced calcium flux depends on tyrosine phosphorylation and activation of phospholipase C-γ1 (PLC-γ1), leading to increases in inositol phosphates and intracellular calcium (*3*). Activation of Ras is thought to result from recruitment of a GRB2-SOS complex to the membrane. After TCR stimulation, a membrane-bound adaptor protein, LAT(pp36) (linker for activation of T cells), is heavily tyrosine phosphorylated and subsequently binds GRB2 and PLC-γ1 through their SH2 domains (*4–6*). LAT, therefore, may link PTK activity to activation of both the Ras and calcium pathways.

SLP-76, like LAT, is a GRB2-binding adaptor protein that is tyrosine phosphorylated after TCR stimulation (*7*). SLP-76 comprises a COOH-terminal SH2 domain, a central proline-rich region that binds to the GRB2 SH3 domains, and multiple NH_2-terminal tyrosine phosphorylation sites, which mediate TCR-inducible association of SLP-76 with Vav, a guanine nucleotide exchange factor for Rho-family guanosine triphosphatases (*7–10*). Overexpression of SLP-76 augments TCR-induced transcriptional responses (*8, 9, 11, 12*). However, the mechanism by which overexpression of SLP-76 augments TCR signaling is not understood, and the function of endogenous SLP-76 has been difficult to address.

An SLP-76–deficient T cell, J14, was isolated while screening Jurkat T cell subclones for TCR-inducible expression of the CD69 activation marker (Fig. 1A) (*13*). CD69 is induced in a Ras-dependent manner following stimulation with anti-TCR or with phorbol myristate acetate (PMA) (*14*). Clone J14 lacked TCR-inducible expression of CD69, despite normal TCR expression and normal PMA-induced up-regulation of CD69 (Fig. 1A). This phenotype suggested that the clone is defective at a proximal step in the TCR pathway leading to Ras activation.

Initial characterization of J14 revealed that it lacked SLP-76 protein (Fig. 1B). Numerous other signaling proteins examined were present, including Vav, ZAP-70, PLC-γ1, Erk2, Cbl, Pak1, LAT, Itk, and Nck [(*15*) and below]. Whereas SLP-76 was detected in anti–SLP-76 immune complexes prepared from Jurkat cells, it was undetected in immune complexes prepared from 125 times more J14 cells (*15*).

To determine the significance of the SLP-76 defect, we compared TCR signaling in SLP-76- and vector-transfected subclones of J14 (*16*). SLP-76 transfectants were selected that retained TCR surface expression (*15*) and expressed SLP-76 at wild-type levels. Vector transfectants did not express SLP-76 (Fig. 1B). TCR-induced CD69 expression was restored by expression of SLP-76 (Fig. 1A), showing that the signaling defect of J14 is attributable to the lack of SLP-76.

Northern (RNA) blot analysis revealed that SLP-76 transcripts were reduced in the J14 mutant cells and restored in the SLP-76–reconstituted cells, correlating with expression of the protein (Fig. 1C, top panel). Southern (DNA) blot analysis did not reveal differences in the genomic structure of SLP-76 in J14 cells compared to Jurkat (*15*).

Whereas CD69 induction depends only on the Ras pathway (*14*), TCR-dependent activation of interleukin-2 (IL-2) transcription requires both the TCR-induced Ras and calcium pathways, as well as additional signals, which can be provided by PMA or by the CD28 costimulatory receptor (*17*). After stimulation with anti-TCR and PMA, Jurkat cells up-regulated expression of an IL-2–luciferase reporter, whereas J14 cells did not (Fig. 1D). The J14 subclones stably transfected with SLP-76 responded normally (Fig. 1D). Thus, all of the proteins required for transcriptional activation of IL-2 by the TCR are present in J14, except SLP-76.

In Jurkat T cells, Raji B lymphoblastoid cells plus the superantigen, staphylococcal enterotoxin D (SED), can be used to activate IL-2 transcription. This cell-cell interaction, in which the complex of molecular and associated superantigen major histocompatibility complex stimulates the TCR while other ligands expressed on Raji cells provide costimulation, is a close approximation of in vivo stimulation of a T cell by an antigen-presenting cell (APC). SLP-76–deficient J14 cells did not respond to Raji plus SED, whereas the response was restored in SLP-76–reconstituted cells (Fig. 1D). Thus, SLP-76 is essential for TCR responsiveness to a physiological stimulus delivered during a T cell–APC interaction.

On the basis of the CD69 defect, we hypothesized that TCR-mediated activation of the Ras pathway was defective in J14. Consistent with this interpretation, TCR-mediated, Ras-dependent phosphorylation of Erk2 was substantially reduced in J14 cells relative to Jurkat (Fig. 2A). In contrast, PMA-induced Erk2 activation was equivalent in both cell types (Fig. 2A), and an SLP-76–reconstituted

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