

# SNARE-Dependent Signaling at the *Drosophila* Wing Margin

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The wing of *Drosophila melanogaster* has long been used as a model system to characterize intermolecular interactions important in development. Implicit in our understanding of developmental processes is the proper trafficking and sorting of signaling molecules, although the precise mechanisms that regulate membrane trafficking in a developmental context are not well studied. We have therefore chosen the *Drosophila* wing to assess the importance of SNARE-dependent membrane trafficking during development. N-Ethylmaleimide-sensitive fusion protein (NSF) is a key component of the membrane-trafficking machinery and we constructed a mutant form of NSF whose expression we directed to the developing wing margin. This resulted in a notched-wing phenotype, the severity of which was enhanced when combined with mutants of VAMP/Synaptobrevin or Syntaxin, indicating that it results from impaired membrane trafficking. Importantly, we find that the phenotype is also enhanced by mutations in genes for *wingless* and components of the *Notch* signaling pathway, suggesting that these signaling pathways were disrupted. Finally, we used this phenotype to conduct a screen for interacting genes, uncovering two Notch pathway components that had not previously been linked to wing development. We conclude that SNARE-mediated membrane trafficking is an important component of wing margin development and that dosage-sensitive developmental pathways will act as a sensitive reporter of partial membrane-trafficking disruption. © 2001 Academic Press

**Key Words:** NSF; VAMP; Synaptobrevin; Syntaxin; Notch; Wingless.

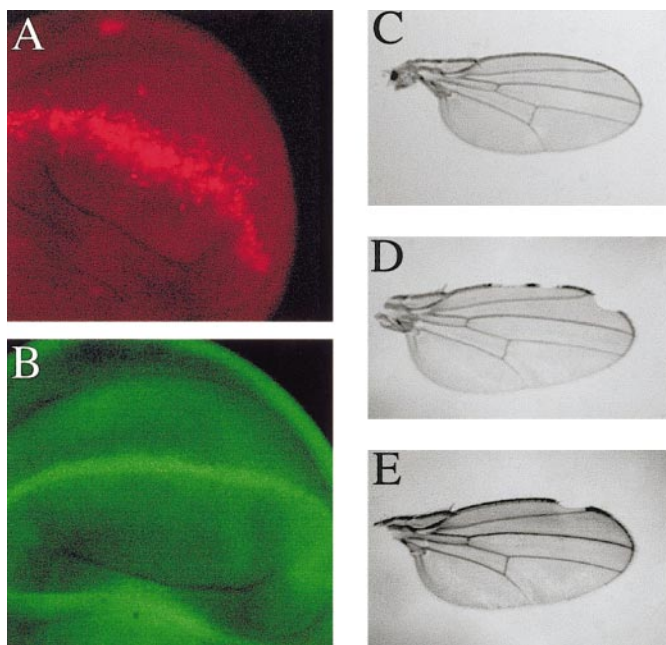
## INTRODUCTION

SNARE (soluble NSF attachment protein receptors) protein-dependent membrane trafficking has been the subject of intense study in recent years. The Syntaxin, VAMP, and SNAP-25 families of proteins are proposed to target and fuse transport vesicles with specific membrane compartments (McNew *et al.*, 2000; Sollner *et al.*, 1993; Weber *et al.*, 1998). One of the identifying characteristics of SNARE proteins is their ability to form tight, SDS-resistant, ternary complexes. The SNARE complex is a parallel four-helix bundle with one helix contributed by each of Syntaxin and VAMP and two contributed by SNAP-25 (Sutton *et al.*, 1998). The formation of a *trans*-membrane complex, with VAMP on the transport vesicle and Syntaxin and SNAP-25

on the target membrane, is thought to lead to the fusion of the two membranes, resulting in a *cis*-membrane complex.

It follows that the *cis*-residing protein complexes need to be broken apart to make those proteins available for further *trans*-complex formation. This complex breakdown occurs under the action of N-ethylmaleimide-sensitive fusion protein (NSF) (Malhotra *et al.*, 1988), an ATPase. NSF contains two nucleotide binding domains and demonstrable ATPase activity. Structural analyses have shown that NSF forms a hexamer *in vivo* (Hanson *et al.*, 1997). NSF is a homolog of the yeast gene SEC18 (Wilson *et al.*, 1989) and analysis of SEC18 function also revealed its requirement for intracellular membrane transport (Eakle *et al.*, 1988). Banerjee *et al.* (1996) showed that NSF-dependent ATP hydrolysis is required to disassemble SNARE complexes, although it is not required for the fusion step. Thus the role of NSF in vesicular transport appears to be primarily one of priming vesicles for fusion and dissociation of SNARE complexes to permit their recycling. In *Drosophila* there are two homologs of NSF: dNSF1 and dNSF2 (Boulianne and Trimble,

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**FIG. 1.** Wing phenotype caused by expression of  $dNSF2^{E/Q}$ . (A) Expression of C96-Gal4 in third instar imaginal wing disc visualized using UAS-lacZ as reporter. The genotype of this disc is  $dNSF2^{E/Q}C96/UAS-lacZ^{mhc}$ . The expression pattern overlaps, but is broader than, Wingless expression shown in (B). (C) Oregon R wing, (D, E)  $dNSF2^{E/Q}C96/+$  wings. The wings shown in D and E are examples of strongly and weakly affected wings, respectively.

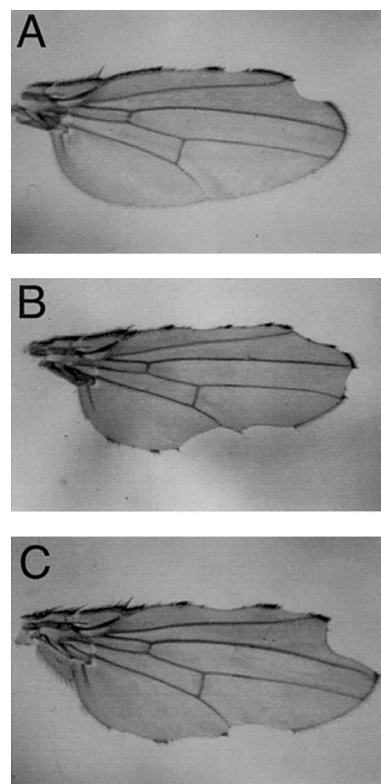
1995; Ordway et al., 1994).  $dNSF1$  is the gene product of *comatose* (Pallanck et al., 1995) and is primarily found in neurons, whereas  $dNSF2$ , in addition to being neuronally expressed, is broadly expressed within imaginal discs, salivary glands, and the ring gland (Boulianne and Trimble, 1995). Thus,  $dNSF2$  is the most likely isoform to contribute to intracellular trafficking in nonneuronal tissue.

Despite their proposed role in most intracellular trafficking events, *in vivo* studies of SNARE proteins have concentrated on two main systems: the budding yeast and calcium-triggered exocytosis in neurons. Relatively little attention has been given to other *in vivo* contexts in which the SNARE proteins are likely to have important roles. For example, in signaling pathways it is self-evident that transmembrane receptors and ligands need to be delivered to the plasma membrane, although few studies have been devoted to specifically studying the role of SNARE proteins in this process and their potential influence on the strength of intracellular signaling.

In the fruit fly *Drosophila melanogaster* there is a long history in developmental biology and many of the signaling pathways that control development of the organism are well known. In parallel, *Drosophila* SNARE proteins have also been intensively studied, mostly in the context of synaptic transmission at the neuromuscular junction. The complete sequencing of the *Drosophila* genome has re-

vealed all the members of the SNARE protein families and in general there is about one-third the number of fly genes compared to that estimated for mammalian species. Correspondingly, it appears that some *Drosophila* SNARE proteins are used in very different contexts. For example, Syntaxin 1A appears to be necessary both for cellularization of the early embryo and for neural synaptic transmission (Burgess et al., 1997; Schulze et al., 1995).

One of the most thoroughly studied developmental pathways in *Drosophila* is the development of the wing margin. These studies have revealed that the specification and establishment of the wing margin involves complex interaction between the Notch and Wingless signaling pathways (reviewed in Bray, 1998; Panin and Irvine, 1998). The early expression of the homeodomain protein Apterous establishes dorsal/ventral (D/V) polarity of the wing disc and initiates expression of Serrate, a Notch ligand. Serrate acts as a dorsal-to-ventral signal, to initiate the spatially restricted activation of the transmembrane receptor Notch (de Celis et al., 1996). The second Notch ligand, Delta, is expressed on both sides of the D/V border but is required only on the ventral side of the border (de Celis et al., 1996). Together these three molecules are believed to form a positive-feedback loop that enhances Notch activity at the D/V border and downregulates it outside this zone (de Celis



**FIG. 2.** Genetic interaction of  $dNSF2^{E/Q}C96$  with mutations in SNARE proteins. (A)  $dNSF2^{E/Q}C96/+$ , the same wing as in Fig. 1D, (B)  $syb^{K07703}/+; dNSF2^{E/Q}C96/+$ , (C)  $dNSF2^{E/Q}C96/syx^{L371}$ .

and Bray, 1997). Notch is further required for expression of *wingless*, *cut*, *vestigial*, and *achaete*, genes that are important for wing development and sense organ formation along the wing margin (Kim *et al.*, 1995, 1996; Neumann and Cohen, 1996; Rulifson and Blair, 1995). Given that Notch and its ligands are transmembrane proteins and that Wingless is a secreted protein, it is clear that membrane trafficking will be important to this signaling cascade.

To investigate the role of SNARE proteins within a defined developmental process, we took advantage of the key role of NSF in membrane-transport processes. Specifically, we expressed a dominant negative form of dNSF2 in wing imaginal discs and show that this disrupts proper wing margin formation. This phenotype is enhanced in *trans*-heterozygous combinations of mutant alleles of the SNARE proteins *syntaxin* or *synaptobrevin*, further supporting a role for SNAREs in this process. Using genetic and immunocytochemical analysis we show that this phenotype can be attributed to a failure in the signaling pathways that normally govern wing margin development. Thus, SNARE-dependent transport mechanisms are critical to wing formation and their manipulation may provide new insights into the mechanisms controlling developmentally important signaling pathways.

## MATERIALS AND METHODS

### Fly Stocks

*Drosophila* were raised on standard cornmeal medium at 25°C.

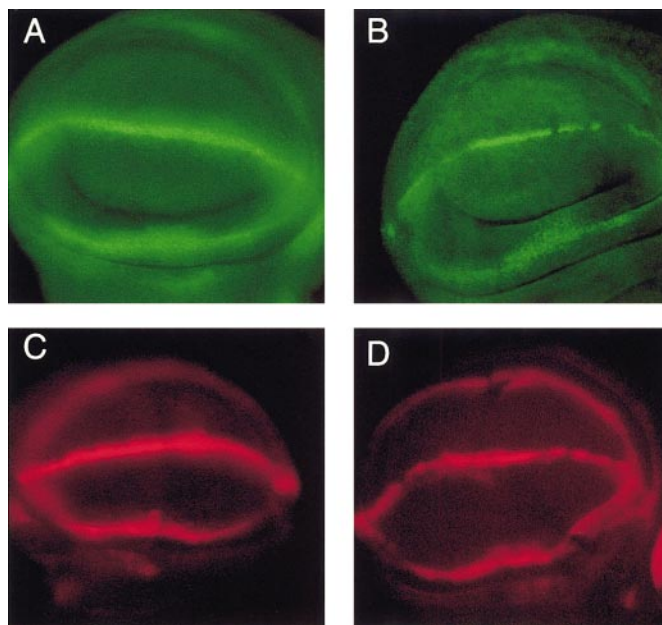
*UAS-dNSF2<sup>E/Q</sup>* is a UAS transgenic line containing the dNSF2 open reading frame with a glutamate-to-glutamine amino acid substitution at position 326 (amino acid position according to Boulianne and Trimble, 1995). We generated several independent insertion lines; here we report data from one representative line, *UAS-dNSF2<sup>EΔ2</sup>*. *UAS-dNSF2<sup>WT</sup>* is a transgenic line carrying the dNSF2 wild-type gene. Other UAS lines used were *UAS-N<sup>icd</sup>*, *UAS-N<sup>full length</sup>* (gift of P. Simpson), *UAS-DI<sup>36-1</sup>*, *UAS-Ser* (gift of E. Giniger), and *UAS-lacZ<sup>nuc</sup>*.

*C96-Gal4* is a third-chromosome Gal4-enhancer trap line (Gustafson and Boulianne, 1996) that is expressed strongly in a wide stripe along the wing margin. *dNSF2<sup>E/Q</sup>C96/TM3*, *Sb Ser* is a line with a recombinant third chromosome that has both *UAS-dNSF2<sup>E/Q</sup>* and *C96-Gal4*.

The following alleles, described in Lindsley and Zimm (1992) or Flybase (<http://flybase.bio.indiana.edu>), were used: *DI<sup>7</sup>*, *wg<sup>1-17</sup>*, *Ser<sup>RX82</sup>*, *porc<sup>pb16</sup>*, *spi<sup>1</sup>*, *bib<sup>1</sup>*, *syb<sup>k07703</sup>*, *syx<sup>Δ229</sup>*, and *syx<sup>06737</sup>*. Other alleles of *syntaxin* used were *syx<sup>L266</sup>*, a severe hypomorph, and *syx<sup>L371</sup>*, a null allele (Burgess *et al.*, 1997).

The following lacZ reporter lines used were: *wg-lacZ* (Perrimon *et al.*, 1991), *neu<sup>A101</sup>-lacZ* (Boulianne *et al.*, 1991), and *vg<sup>BE</sup>-lacZ* (Williams *et al.*, 1993).

Deficiency lines uncovering most of the *Drosophila* genome ("the deficiency kit") were obtained from the Bloomington Stock Centre. Each of the Df lines was crossed to *dNSF2<sup>E/Q</sup>C96/TM3*, *Sb Ser* and the wings of progeny that lacked marked balancer chromosomes were compared to *dNSF2<sup>E/Q</sup>C96/+* and scored for enhancement or suppression of the wing margin phenotype. The wings illustrated in the figures are representative examples from each genotype.



**FIG. 3.** Wingless pattern and expression in mutant wing discs. (A) In control discs Wingless protein appears as a continuous stripe across the disc, whereas in mutant discs (B) it appears in a patchy narrow pattern. The interrupted pattern is also observed for Wg expression using *Wg-lacZ* when control (C) and mutant (D) discs are compared.

### Production of a dNSF2 Point Mutant

To generate a glutamate-to-glutamine amino acid substitution in dNSF2 we PCR-amplified a fragment of the cloned dNSF2 (Boulianne and Trimble, 1995) from pBluescript SK+ using T3 as the 5' primer and AAT GGC GTC GAT CTG GTC GAA GAT GAT as the 3' primer. This fragment was then used as the 5' primer in a second PCR reaction with T7 as the 3' primer, to amplify the entire dNSF2 cDNA containing the point mutation using *Xba*I-cut dNSF2 in pBluescript SK+ as the template DNA. This DNA fragment was then used as the template DNA in a third reaction, in which T3 and the PCR product from the first reaction were 5' primers and T7 was the 3' primer. The DNA from this reaction was cut with *Xba*I and *Kpn*I, subcloned into pBluescript SK+ and DNA-sequenced, to confirm that the E-to-Q substitution was the sole mutation. pUAS constructs were made by subcloning an 800-bp *Not*I/*Kpn*I fragment from dNSF2<sup>WT</sup> and a 1.7-kb *Kpn*I/*Xba*I fragment from dNSF2<sup>E/Q</sup> into the *Not*I/*Xba*I sites of the pUAS vector (Brand and Perrimon, 1993). Transgenic flies carrying dNSF2<sup>WT</sup> or dNSF2<sup>E/Q</sup> UAS constructs were created using standard methods (Rubin and Spradling, 1982).

### ATPase Assay

dNSF2<sup>WT</sup> and dNSF2<sup>E/Q</sup> constructs were subcloned into the pGEX-3X expression vectors and transformed into BSI72 cells. Protein expression was induced with 0.1 mM IPTG for 3 h. The cells were disrupted with a French press and the proteins were extracted with 1% Triton X-100 in PBS plus 5 mM EDTA and 0.3 mM PMSF, and mixed with glutathione beads. The beads were then

packed into a column and washed with PBS with 1% Triton X-100. The protein was then eluted with a buffer containing 10 mM glutathione, 50 mM Tris (pH 7), 100 mM NaCl, and 0.5 mM ATP. The eluate was passed over a G-25 column into ATPase buffer (containing 25 mM Tris, pH 9; 0.2 mM ATP; 0.1 mM KCl; 0.5 mM DTT; 0.65 mM  $\beta$ -mercaptoethanol; 1 mM  $MgCl_2$ ; 10% glycerol) on ice. For controls with *N*-ethylmaleimide (NEM), the proteins were incubated with 2.5 mM NEM for 30 min on ice prior to the determination of ATPase activity. Following the addition of 5  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP to 1  $\mu$ g/20  $\mu$ L of NSF the samples were transferred to 37°C and incubated for 10 min. Samples from the reaction were taken and the reaction was stopped with a final concentration of 5 mM EDTA. The nucleotides were separated by thin-plate chromatography in developing buffer containing 0.7 M LiCl and 1 M acetic acid. The radioactive signals were obtained with a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and the signal intensity was then determined with ImageQuant software. For each protein sample, ATPase activity was determined in duplicate. NEM-sensitive ATPase activity was determined by subtracting the ATPase activity remaining after NEM treatment from the total ATPase activity.

### Immunocytochemistry

Imaginal discs were obtained from wandering third-instar larvae originating from the cross of *dNSF2<sup>E/Q</sup>C96/TM3*  $\times$  *Ore-R*. Thus, discs of the genotypes *dNSF2<sup>E/Q</sup>C96/+* and *TM3/+* were processed simultaneously in the same tube, the latter of which served as controls. After dissection in cold PBS, the discs were fixed for 20 min in 4% formaldehyde in PBS at room temperature. They were then washed for 30 min in PBT (PBS with 0.1% Triton X-100), blocked with 1% normal goat serum in PBT and then incubated with the primary antibody at the indicated dilution overnight at 4°C. The discs were then washed for 30 min in PBT, incubated with 1:500 dilution of FITC goat anti-rabbit or Texas Red goat anti-mouse secondary antibody (Jackson Laboratories, Bar Harbor, ME) at room temperature for 2 h, washed in PBT for 0.5–1 h, then mounted and cleared in 70% glycerol containing 2% 1,4-diazabicyclo(2.2.2)octane (DABCO; Sigma, St. Louis, MO).

Mouse monoclonal anti-wg (1:500) and purified rabbit anti-wg antisera (1:500) was provided by S. Cohen, mouse monoclonal anti-cut (1:200) was from K. Blochlinger, mouse monoclonal anti-ac (1:5) was from S. Carroll, mouse monoclonal anti-Delta (1:1) was from M. Muskavitch, and mouse monoclonal anti- $\beta$ -galactosidase (1:1000) was purchased from Promega (Madison, WI). Images were obtained with a Nikon Optiphot 2 microscope and CCD camera or Leica confocal microscope. Confocal signals from control and mutant discs were obtained at the same gain, black level, and pinhole settings. Images were processed with Adobe Photoshop. The genotype of the third-instar wing discs in all images is *dNSF2<sup>E/Q</sup>C96/+*, unless otherwise noted.

Adult wings were dissected and placed on glass slides in a drop of isopropanol and then mounted in a mixture of Canada balsam and methylsalicylate (Sigma).

## RESULTS

To investigate the function of SNARE-dependent transport mechanisms in *Drosophila* we constructed point mutants in the ATP-binding region of the D1 domain of dNSF2. Each nucleotide-binding subdomain of NSF contains consensus ATP-binding domains known as the

Walker A and Walker B motifs (Wilson *et al.*, 1989). The DEAD box of the Walker B motif is conserved in a large number of ATP-dependent enzymes and was first identified in RNA helicases that use ATP hydrolysis to unwind RNA prior to translation. This motif binds the  $Mg^{2+}$  ion that coordinates the phosphates of ATP for hydrolysis. In RNA helicases replacement of the glutamate residue within the modified DEAD box (DEID) eliminates ATP hydrolysis without affecting ATP binding (Pause and Sonenberg, 1992). In their study of mammalian NSF Whiteheart *et al.* (1994) demonstrated in CHO cell-free extracts that a similar substitution within that protein's DEID box, E329Q, reduces ATPase activity and NSF-dependent Golgi transport activity. NSF has been shown to form hexamers and, when mixed with wild-type protein NSF<sup>E329Q</sup>, forms hexamers that also lack ATPase activity, leading to a dominant negative effect. *Drosophila* NSF2 shows 59% overall amino acid identity with CHO NSF and nearly 100% conservation within the ATP-binding p-loop and DEID box of the D1 domain (Boulianne and Trimble, 1995). Thus the structural and functional properties of the dNSF2 ATPase domains are very likely to be identical to those previously defined in RNA helicases and mammalian NSF, and mutation of the glutamate residue with the *Drosophila* DEID box motif should also impair the ATPase activity of the protein.

We therefore created a dNSF2<sup>E/Q</sup> construct with a glutamate-to-glutamine substitution at position 326 of the dNSF2 D1 domain as described under Materials and Methods. In two separate ATPase assays we found that the NEM-sensitive ATPase activity of dNSF2<sup>E/Q</sup> was 47.5 and 57.1% that of dNSF2<sup>WT</sup>. The mean ATPase activity was 15.2 nmol Pi/ $\mu$ g/h for the wild-type protein and 7.8 nmol Pi/ $\mu$ g/h for the mutant protein. The remaining ATPase activity in dNSF2<sup>E/Q</sup> may be attributable to the second ATPase site within the D2 domain of the protein.

To express the mutant dNSF2 we created transgenic flies carrying *UAS-dNSF2<sup>E/Q</sup>* and *UAS-dNSF2<sup>WT</sup>* constructs for use in the Gal4-UAS expression system. *C96-Gal4* is expressed in developing wing discs in a pattern that is similar to, though slightly broader than, wing margin proteins such as Wingless (Figs. 1A and 1B; see also Gustafson and Boulianne, 1994). When *UAS-dNSF2<sup>E/Q</sup>* is driven by *C96-Gal4* we observed loss of wing margin (Figs. 1C–1E). The expression of dNSF2<sup>WT</sup> did not cause any visible phenotype (data not shown), indicating that simple overexpression of dNSF2 in the wing margin is not a cause of the phenotype.

Our observation that dNSF2<sup>E/Q</sup> causes loss of wing margin implies that SNARE-dependent transport is important for wing margin formation. To test this further we used mutant alleles of *synaptobrevin* and *syntaxin*, two well-characterized SNARE proteins, to determine whether they would enhance the wing phenotype (Fig. 2). Indeed, all *trans*-heterozygous combinations of *dNSF2<sup>E/Q</sup>C96* with *synaptobrevin* or *syntaxin* loss-of-function alleles enhanced the wing margin phenotype, thus providing further evidence of the involvement of SNARE proteins in wing margin development.

The wing phenotype we observe is similar to that observed with mutant alleles of Notch and Wingless signaling pathway genes. To determine whether components of these pathways could be contributing to the *dNSF2<sup>E/Q</sup>C96* wing phenotype we first examined the protein pattern of Wingless in third-instar imaginal wing discs and observed a striking effect on the distribution of Wingless. In control discs Wingless appears as a three- to four-cell-wide stripe across the wing disc, whereas in discs expressing the mutant *dNSF2* Wingless appears very narrow and patchy (Figs. 3A and 3B). We then examined Wg expression using a *Wg-lacZ* reporter construct and found an incomplete pattern of Wingless expression (Figs. 3C and 3D), as was observed for the Wingless protein.

Because Wg is a secreted protein we next examined Wg at higher magnification using confocal microscopy to determine directly whether Wg secretion was impaired (Fig. 4). In control discs there is punctate Wg staining, indicative of Wg secretion, in the tissue surrounding the narrow stripe of wing margin cells. In the regions of the mutant discs that were immunoreactive for Wg we also saw punctate staining surrounding the positive cells. However, the Wg signal was much stronger in those cells and confocal sectioning of the cells revealed the accumulation of Wg at the apical region of the wing margin cells. These data indicate that mutant *dNSF2<sup>E/Q</sup>* impairs, but does not eliminate, wingless secretion.

Because Wingless expression is impaired and its activation is under the control of Notch signaling, we next examined the distribution patterns of other proteins involved in the Notch pathway (Fig. 5). We first examined Notch protein distribution directly using a monoclonal antibody that recognizes the extracellular domain of Notch (gift of S. Artavanis-Tsakonas). At low magnification there is no major difference between mutant and control samples, with the antibody labeling the cell membranes in the wing pouch. However, at higher magnification, in addition to the membrane staining we also observed immunoreactive puncta within the cells of the mutant wing disc that were not readily observed in the control discs. These puncta likely represent improperly sorted Notch proteins.

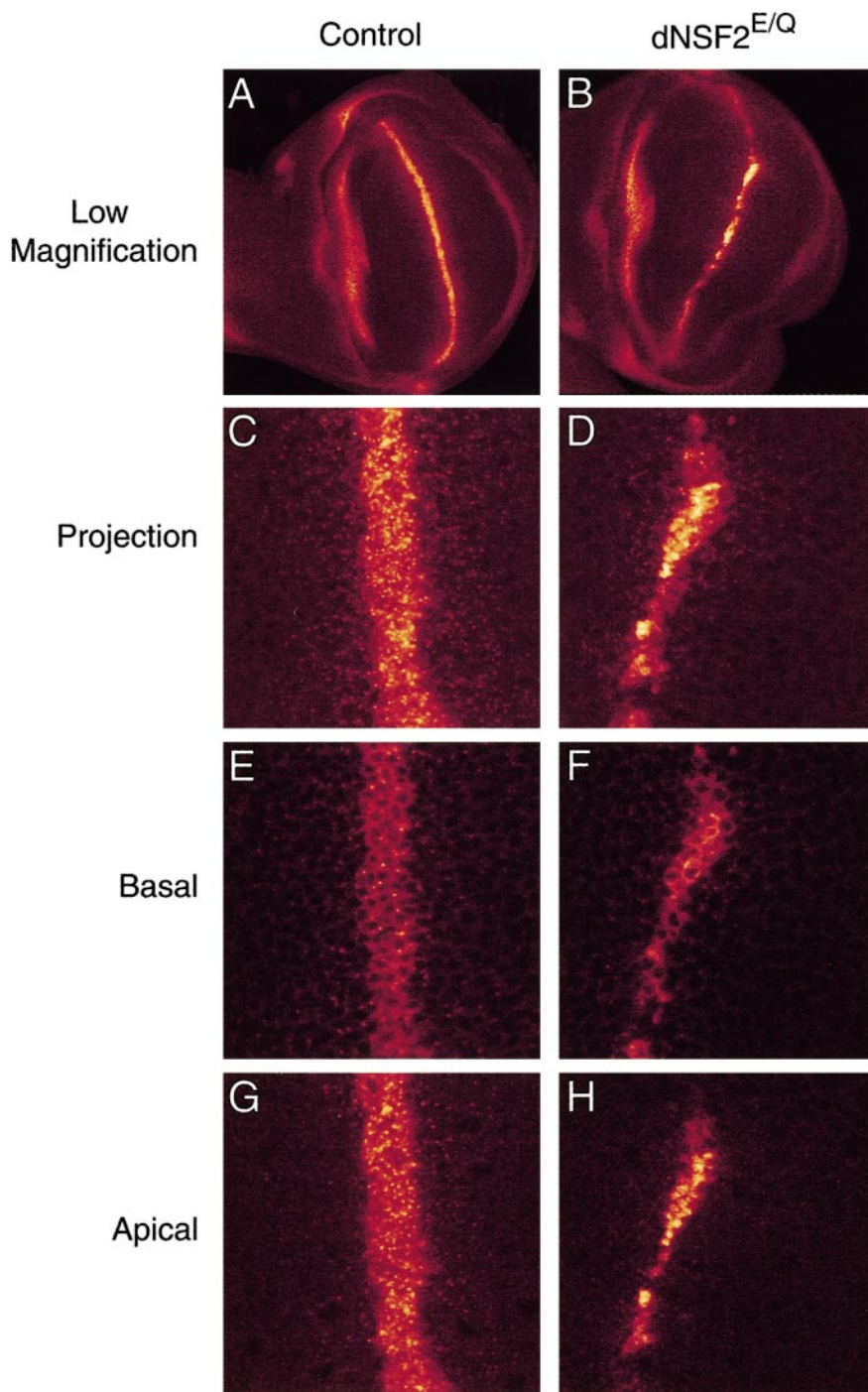
We next examined the distribution of Cut, Delta, and Achaete, genes that are downstream of Notch activation in the wing margin signaling pathway, and we found all of these markers were disrupted in *dNSF2<sup>E/Q</sup>C96* larval wing discs. Cut is normally found in a pattern that overlaps with Wg along the presumptive wing margin (Fig. 6A; see also Blochlinger *et al.*, 1993), whereas in the mutant discs it appeared in a broken pattern (Fig. 6B) similar to that of Wg. Delta is normally expressed in two parallel bands along the D/V boundary (Fig. 6C; see also Kooh *et al.*, 1993) and this pattern is thought to be the result of the downregulation of Delta in boundary cells by Cut and the upregulation of Delta in flanking cells by Wingless (de Celis and Bray, 1997). In *dNSF2<sup>E/Q</sup>C96* wing discs the expression of Delta is reduced and the two parallel bands appear to be collapsed into a single band along the boundary (Fig. 6D). Achaete is normally expressed in two broad bands parallel to the D/V

boundary in the anterior compartment of the wing disc defining a proneural cluster (Fig. 6E; see also Romani *et al.*, 1989). In the *dNSF2<sup>E/Q</sup>C96* discs this pattern is severely disrupted: the number of Achaete-expressing cells is reduced and there is complete absence of Achaete in some areas (Fig. 6F).

We found a similar pattern of disruption when we used *lacZ* reporter constructs to examine the expression of *neuralized* and *vestigial*, two other genes in the Notch pathway. *neu<sup>A101</sup>-lacZ* is normally detected in sensory organ precursors (SOPs) located in two rows of single cells parallel to the D/V boundary in the anterior compartment of late third-instar wing discs (Fig. 6G). In the mutant discs this pattern is disrupted and lacking in some areas along the wing margin, while SOPs elsewhere in the disc are unaffected (Fig. 6H). Similarly, *vg<sup>BE</sup>-lacZ* expression was disrupted. In wild-type discs it is seen in the D/V and anterior/posterior (A/P) boundaries, whereas in the mutant discs the expression in the D/V boundary is disrupted (Figs. 6I and 6J). Interestingly, expression in the A/P boundary remains, although the C96-Gal4 expression pattern overlaps this region. Taken together these results demonstrate that *dNSF2<sup>E/Q</sup>* affects the distribution and expression of several downstream components of the Notch signaling pathway.

To confirm the effect of *dNSF2<sup>E/Q</sup>* on Notch signaling we examined loss-of-function alleles of several genes in the Notch and Wingless pathways for their ability to enhance the adult wing phenotype caused by *dNSF2<sup>E/Q</sup>* expression. In that Notch signaling is known to be highly sensitive to haploinsufficiency of interacting gene products, we reasoned that these loss-of-function alleles should show genetic interaction. We tested two alleles of *Notch* and one each of *Delta*, *Serrate*, *wingless*, and *fringe* and found that they all enhanced the wing phenotype in *trans-heterozygous* combination with *dNSF2<sup>E/Q</sup>C96* (Fig. 7). The severity of the phenotype produced by each allele was similar, although *Df(1)N8*, a null allele of *Notch*, did produce a more severe phenotype than did *N<sup>nd-3</sup>* (not shown), a hypomorphic allele. With the exception of *Df(1)N8* (Fig. 7B, inset), none of these mutants produces a wing-nicking phenotype when examined alone as heterozygotes. Thus, the enhancement of the adult wing phenotype by mutants in the Notch pathway supports the conclusion that *dNSF2<sup>E/Q</sup>* expression causes a defect in wing margin signaling pathways.

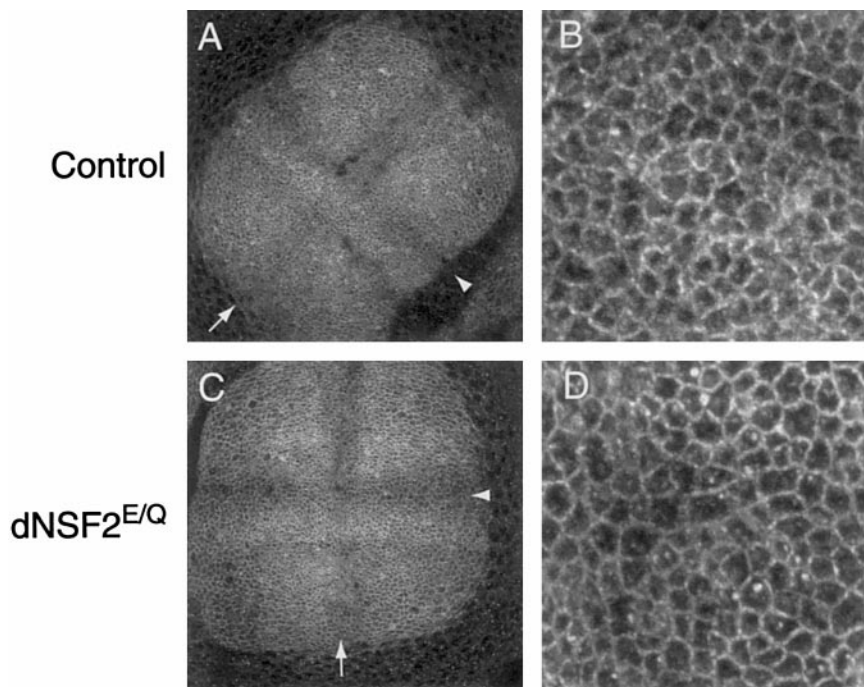
Finally, we tested the ability of UAS-constructs of Notch, Delta, and Serrate to rescue the wing phenotype generated by *dNSF2<sup>E/Q</sup>C96* (Figs. 8A–8D). Complete rescue could be obtained with both Notch and Delta constructs. Serrate generally appeared to rescue less well than did the other constructs because minor nicks in the distal wing persisted. Furthermore, no rescue effect was seen when crosses were made to *UAS-lacZ* lines (not shown), indicating that competition for Gal4 protein was not responsible for rescue of the phenotype. The observation that UAS-Notch and UAS-Delta could completely rescue the *dNSF2<sup>E/Q</sup>* wing phenotype further indicates that the mutation affects intracellu-



**FIG. 4.** Wingless secretion in mutant wing discs. (A, B) Low-magnification images of Wingless immunoreactivity reveal that the normal Wingless stripe across the imaginal disc is narrow and patchy in the mutants. (C, D) Higher-magnification images revealed punctate staining outside of the Wg stripe indicative of Wg secretion. (E-H) Confocal sections of the Wg containing cells revealed that Wg protein is labeled very intensely at the apical part of the cell in the mutant discs compared to that in the control discs.

lar transport and does not create a cell-lethal phenotype because cell lethality should not be rescued by Notch or Delta.

Having established that  $dNSF2^{E/Q}$  disrupts signaling at the wing margin in a SNARE-dependent manner, and that we could easily detect enhancement of the phenotype



**FIG. 5.** Notch subcellular localization. (A, C) Low-magnification images of wing discs labeled with anti-Notch<sup>EC</sup> revealed staining of the cell membranes of the wing disc. (B, D) Upon examination at higher magnification intracellular punctate Notch staining was readily observed in the mutant discs, whereas the control discs showed very little intracellular staining. Arrows in A and C point to the dorsal/ventral (D/V) boundary, whereas the arrowheads point to the anterior/posterior (A/P) boundary. The high-magnification images were taken near the intersection of the D/V and A/P boundaries.

attributed to haploinsufficiency of known genes, we sought to determine whether the wings of the *dNSF2<sup>E/Q</sup>C96* flies could be used as a sensitized background to find novel genes involved in wing margin formation. To this end we conducted a small-scale screen for enhancers and suppressors of the phenotype.

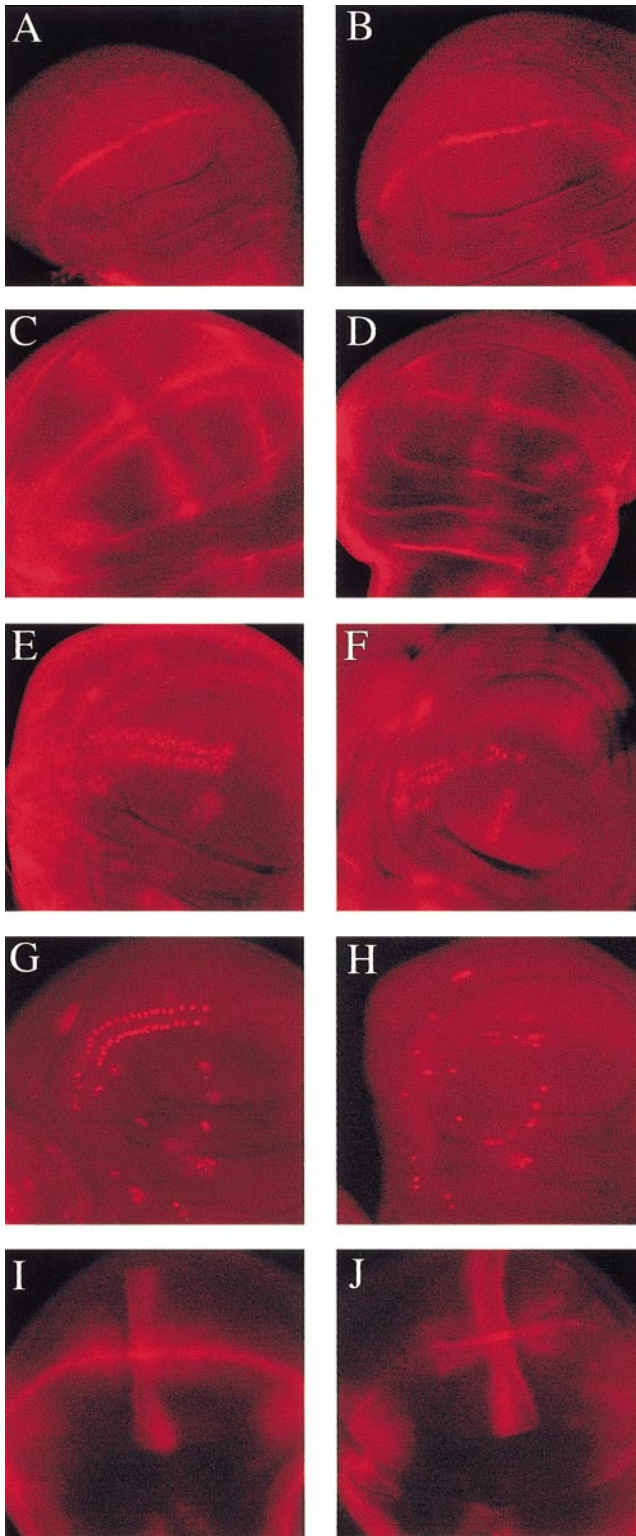
In the first set of experiments we used specific alleles of two genes, *big brain* and *porcupine*, that have been shown to be important in Notch and Wingless signaling in other developmental contexts but were not previously known to be important for wing margin development. In the *dNSF2<sup>E/Q</sup>C96* background we found that both mutant alleles of these genes enhanced the *dNSF2<sup>E/Q</sup>C96* wing margin phenotype (Figs. 4G and 4H). This result is the first report of the involvement of these two genes in wing margin development and suggests that *dNSF2<sup>E/Q</sup>C96* wings provide an ideal sensitized background for conducting forward genetic screens to identify novel genes involved in wing margin development.

In the second set of experiments we tested for genetic interactions with deficiencies that uncover most of the *Drosophila* genome. Of the deficiencies we tested, we identified 33 interacting lines that enhanced or suppressed the wing margin phenotype (Table 1). At present we have not determined which of the genes uncovered by these deficiencies led to the interacting phenotype. Indeed, be-

cause components of both the Notch and Wg signaling pathway and membrane trafficking genes may be involved, more than one gene within each deficiency may be affected. The further characterization of these loci may reveal novel components of the SNARE or Notch and Wg signaling pathways.

## DISCUSSION

Despite extensive study of the function of SNARE proteins, their role in mediating developmentally important processes is poorly understood. In *Drosophila* the presence of dNSF2 in imaginal discs and the maternal contribution of Syntaxin and Synaptobrevin indicates that those proteins have an important role in development. Burgess *et al.* (1997) used germ-line mosaics of hypomorphic *syntaxin* alleles to show that Syntaxin is necessary for cellularization of the early blastoderm. Schulze *et al.* (1995) found that certain combinations of *syntaxin* alleles gave rough eye- and wing-nicking phenotypes, indicating a potential role in eye and wing development. However, given the possible cell lethality caused by homozygous mutations of *syntaxin* (Burgess *et al.*, 1997; Schulze and Bellen, 1996), it is unclear whether the main effect in that study was on a developmental pathway or cell viability.

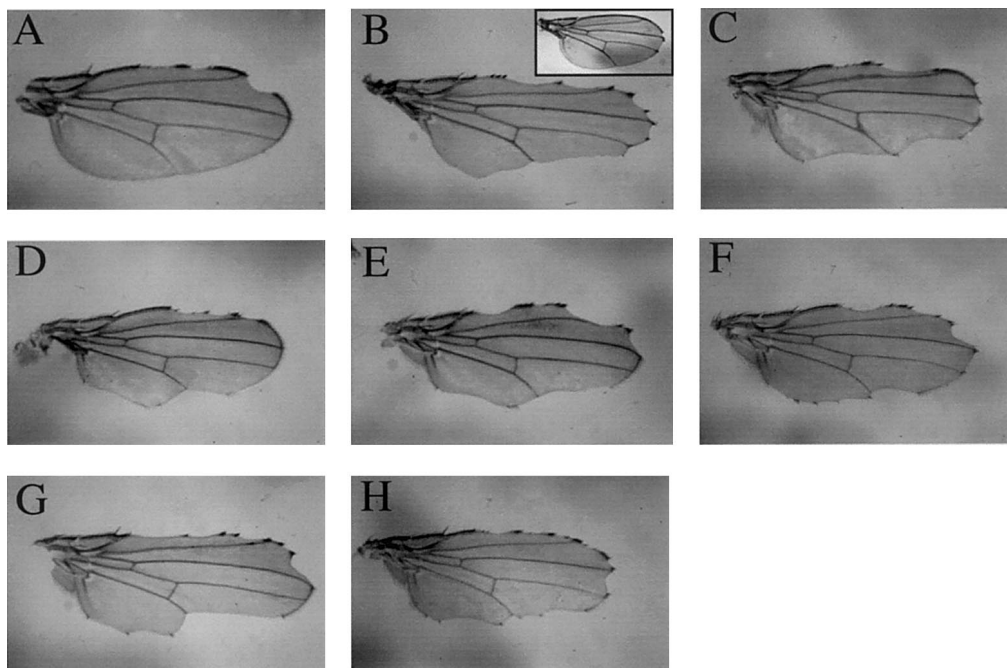


**FIG. 6.** Notch pathway markers disrupted in mutant wing discs. Control (A, C, E, G, I) and *dNSF2<sup>E/Q</sup>C96/+* (B, D, F, H, J) wing discs stained with anti-Cut (A, B), anti-Delta (C, D), anti-Achaete (E, F), anti-lacZ *neuralized<sup>A101</sup>-lacZ* (G, H), *vestigial<sup>BE</sup>-lacZ* (I, J). All markers are disrupted in the mutant discs.

In this investigation we examined the role of SNARE-dependent transport mechanisms during development by expressing a dominant negative form of dNSF2 at the developing *Drosophila* wing margin. In view of current membrane-trafficking models, we expect that expressing dNSF2<sup>E/Q</sup> will impair the ability of NSF to dissociate *cis*-SNARE complexes, making fewer SNARE proteins available for functional *trans*-membrane complex formation and thus reducing intracellular transport. When we expressed dNSF2<sup>E/Q</sup> within a narrow band of cells in the developing wing disc, we observed loss of wing margin and disruption of protein patterns in the wing disc. We further showed that this is not a cell-lethal phenotype because it can be rescued by UAS-Notch and UAS-Delta constructs, indicating that the cells are viable. We also showed that single-copy mutations of both *syntaxin* and *synaptobrevin* can enhance the wing phenotype, providing solid evidence that these SNARE proteins are important in wing margin formation. This implies that the mutant NSF must suppress but not block all membrane traffic. Indeed, we observed mislocalization of some, but not all, Wingless and Notch immunoreactive signals. Because wing margin development is particularly sensitive to gene dosage we were able to detect sublethal disruptions in membrane trafficking.

The disruption of molecular markers, such as Wg, Delta, Achaete, Cut, Vestigial, and Neuralized, indicates that the dNSF2<sup>E/Q</sup> wing phenotype we observed is the result of impaired signaling at the developing wing margin. This is consistent with data presented in other studies that manipulated the signaling pathway directly. For example, reduction of Notch activity with *N<sup>ts</sup>* alleles can lead to reduced and patchy Wingless expression (Diaz-Benjumea and Cohen, 1995). Wingless and Cut expression is also reduced and patchy in Notch mutant wing discs (de Celis *et al.*, 1996; Micchelli *et al.*, 1997; Rulifson and Blair, 1995). Stripes of Delta and Serrate that normally flank the D/V boundary collapse into a single stripe along the margin in *N<sup>ts</sup>* alleles exposed to restrictive temperature (de Celis and Bray, 1997). In our *dNSF2<sup>E/Q</sup>C96* wing discs we observe changes in Wingless, Cut, and Delta patterns that are similar to those that occur when Notch activity is directly manipulated; therefore, it seems that dNSF2<sup>E/Q</sup> expression phenocopies genetic mutants of Notch.

Because the Notch and Wingless signaling pathways are so intertwined in controlling wing margin development it is difficult to determine whether the dNSF2 mutants cause a primary defect in one or the other of these proteins, although it seems likely that there are parallel effects on both. Our experiments show not only a direct impairment of Wingless trafficking but also that Wg-lacZ expression is disrupted. The latter suggests that an upstream activator of Wingless expression is impaired (although this could be Wingless itself) (Rulifson *et al.*, 1996). We find that Notch subcellular localization is disrupted and that a Wg-independent target of Notch signaling, the *vestigial* boundary enhancer, is also disrupted. Because this *vestigial* enhancer element is thought to be under the sole control of



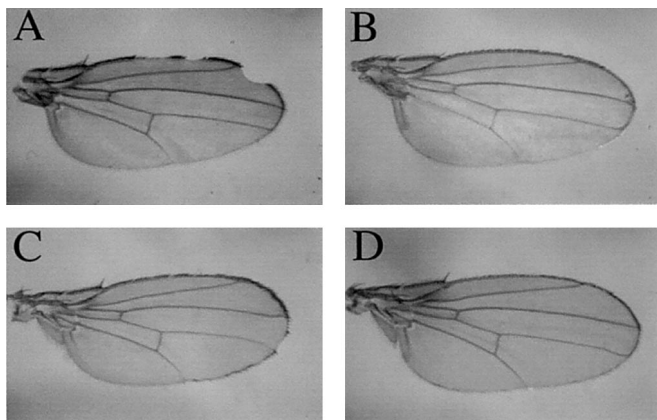
**FIG. 7.** Genetic interaction of  $dNSF2^{E/Q}$  with Notch and Wingless pathway alleles. (A)  $dNSF2^{E/Q}C96/+$ , the same as in Fig. 1D, (B)  $Df(1)N8/+; dNSF2^{E/Q}C96/+$ , inset  $Df(1)N8/+$ , (C)  $Dl^1/dNSF2^{E/Q}C96$ , (D)  $Ser^{RX82}/dNSF2^{E/Q}C96$ , (E)  $wg^{1-17}/+; dNSF2^{E/Q}C96/+$ , (F)  $frg^{52}/dNSF2^{E/Q}C96$ , (G)  $porc^{pb16}/+; dNSF2^{E/Q}C96/+$ , (H)  $bib^1/+; dNSF2^{E/Q}C96/+$ .

Notch (Neumann and Cohen, 1996) this supports the idea that  $dNSF2^{E/Q}$  has a direct effect on Notch signaling. Thus our data point to direct effects on both Wg and Notch. Moreover, because these molecules are at the top of the hierarchy controlling signaling at the wing margin this provides the likely explanation for the disruption of downstream targets of these genes.

A targeted screen using the  $dNSF2^{E/Q}C96$  wing as a

sensitized background allowed us to easily identify enhancement of the wing phenotype with several known members of the Notch signaling pathway; large-scale screens using this genotype could reveal new members of the pathway. In this study we identified two genes, *porc* and *bib*, not previously known to be involved in wing margin formation. Porcupine is a molecule thought to be important for Wingless secretion, in that Wingless is observed to accumulate in Wingless-secreting cells in a *porcupine* mutant background (van den Heuvel *et al.*, 1993). Our finding that the adult wing phenotype is enhanced by *porc* mutants supports this role and indicates that *porc* is important in multiple tissue types. Our immunocytochemistry revealed the accumulation of Wg in wing margin cells and our finding that *porc* mutants enhance the wing margin phenotype supports the idea that Wg secretion is compromised by  $dNSF2^{E/Q}$  wing margin expression.

It was previously proposed that *bib* modulates Notch signaling (Doherty *et al.*, 1997), although the mechanistic role this protein plays remains unclear. Doherty *et al.* (1997) did not identify a wing margin defect when *bib*<sup>-</sup> clones were generated in wing imaginal discs, even though they do report *bib* to be expressed specifically in the wing margin. In the present study we found that  $dNSF2^{E/Q}$  expression leads to a reduction in Notch signaling and we did find an enhancement by *bib*<sup>1</sup> alleles. Clonal analysis of *bib* in a *Notch* hypomorphic background may yield a similar result. Thus it seems the relative levels of Notch



**FIG. 8.** Rescue of mutant wing phenotype. (A)  $dNSF2^{E/Q}C96/+$ , the same as in Fig. 1D, (B)  $UAS-Notch/+; dNSF2^{E/Q}C96/+$ , (C)  $UAS-Serrate/+; dNSF2^{E/Q}C96/+$ , (D)  $dNSF2^{E/Q}C96/UAS-Delta$ .

**TABLE 1**  
Deficiencies That Show Genetic Interaction with *dNSF2<sup>E/Q</sup>C96*

Bloomington stock number	Breakpoints	Phenotypic effect
X chromosome		
Df(1)G4e <sup>1</sup> H24i <sup>R</sup> , f <sup>1</sup>	5E3-8;6B	Suppressed
Df(1)ct-J4, In(1)dl-49, f <sup>1</sup>	7A2-3;7C1	Suppressed
Df(1)ct4b1, y <sup>1</sup>	7B2-4;7C3-4	Slight suppression
Df(1)JA26	11A1-1;11D-E	Enhanced
Df(1)N12, ras <sup>1</sup> v <sup>1</sup>	11D1-2;11F1-2	Lethal
Second chromosome		
Df(2L)net-PMF	21A1;27B7-8	Suppressed
Df(2L)C144, dpp <sup>d-ho</sup> ed <sup>1</sup>	23A1-2;23C3-5	Suppressed
Df(2L)Mdh, cn <sup>1</sup>	30D-F;31F	Slight suppression
Df(2L)J39	31C-D;32D-E	Slight suppression
Df(2L)prd1.7, b <sup>1</sup> Adh <sup>mz</sup> pr <sup>1</sup> cn <sup>1</sup> sca <sup>1</sup>	33B2-3;34A1-2	Enhanced
Df(2L)TW50, cn1	36E4-F1;38A6-7	Suppressed
Df(2L)TW84, 1(2)74i <sup>1</sup> , Tft <sup>1</sup> Lar <sup>TW844</sup>	37F5-38A1;39D3-E1	Suppressed
Df(2R)nap9	42A1-2;42E6-F1	Enhanced
Df(2R)Pcl7B	54E8-F1;55B9-C1	Suppressed
Df(2R)Pcl11B, al <sup>1</sup> dpov <sup>1</sup> b <sup>1</sup> pr <sup>1</sup>	54F6-55A1;55C1-3	Suppressed
Df(2R)Pu-D17, cn <sup>1</sup> bw1 sp <sup>1</sup>	57B4;58B	Slight suppression
Df(2R)X58-7, pr <sup>1</sup> cn <sup>1</sup>	58A1-2;58E4-10	Suppressed
Df(2R)X58-12	58D1-2;59A	Suppressed
Df(2R)Px2	60C5-6;60D9-10	Suppressed
Third chromosome		
Df(3L)h-i22, hi <sup>22</sup> Ki <sup>1</sup> roe <sup>1</sup> p <sup>p</sup>	66D10-11;66E1-2	Enhanced
Df(3L)29A6, kni <sup>n1-1</sup> p <sup>p</sup>	66F5;67B1	Enhanced
Df(3L)lxd6	67E1-2;68C2	Lethal
Df(3L)vin2, ru <sup>1</sup> h <sup>1</sup> gl <sup>2</sup> e <sup>4</sup> ca <sup>1</sup>	67F2-3;68D6	Suppressed
Df(3L)Ly, mwh <sup>1</sup> Ly <sup>1</sup>	70A2-3;70A5-6	Suppressed
Df(3L)81k19	73A3;74F	Enhanced
Df(3L)W10, ru <sup>1</sup> h <sup>1</sup> Sb <sup>shd-2</sup>	75A6-7;57C1-2	Suppressed
Df(3L)31A	78A;78E	Enhanced
Df(3R)6-7	82D3-8;82F	Enhanced
Df(3R)p712, red <sup>1</sup> e <sup>1</sup>	84D4-6;85B6	Enhanced
Df(3R)by10, red <sup>1</sup> e <sup>1</sup>	85D8-12;85E7-F1	Enhanced
Df(3R)M-Kx1	86C1;87B1-5	Suppressed
Df(3R)ry615	87B11-13;87E8-11	Suppressed
Df(3R)TI-P, e <sup>1</sup> ca <sup>1</sup>	97A;98A-2	Enhanced

signaling may be critical to the role of *bib* and our data are consistent with the idea that *bib* serves as a positive modulator of Notch signaling in wing margin formation as it does in neuroectoderm determination.

Using available deficiencies that uncover most of the *Drosophila* genome we identified a number of lines that enhanced or suppressed the *dNSF2<sup>E/Q</sup>C96* wing phenotype. Among these lines some of the deficiencies uncovered clear candidate genes. For example Df(3R)TI-P, e<sup>1</sup> ca<sup>1</sup>, with breakpoints at cytological location 97A:98A1-2, enhanced our phenotype and likely disrupts Serrate. In other cases either there is no clear candidate or contradictory interactions

were found. This result may arise as a result of the uncertainty surrounding the chromosomal breakpoints and the genes that lie within the deficiencies. Alternatively, it may occur because each of the deficiencies removes many individual genes, some of which may have counterbalancing effects on the phenotype. Nevertheless, it is clear that the *dNSF2<sup>E/Q</sup>C96* wing provides a highly sensitized background and further study of the interacting loci are likely to reveal novel components of the intracellular trafficking pathway and the signaling pathways that control wing development.

The molecular and genetic interactions that regulate developmentally important signaling pathways are important for defining the final outcome of the signaling cascade. For example, previous studies identified several molecules, including Fringe, Big Brain, and Numb, that are proposed to influence Notch signals (Doherty *et al.*, 1997; Guo *et al.*, 1996; Panin *et al.*, 1997). Because the SNARE proteins interact with many protein partners, some of which are proposed to regulate their availability (e.g., Syntaxin's interaction with rop/nsec-1), our data indicate that regulation of SNARE-dependent transport steps may represent an additional mechanism by which signal transduction pathways can be modulated during development.

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