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Involvement of a proline-rich motif and RING-H2 finger of Deltex in the regulation of Notch signaling

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SUMMARY

The Notch pathway is an evolutionarily conserved signaling mechanism that is essential for cell-cell interactions. The *Drosophila deltex* gene regulates Notch signaling in a positive manner, and its gene product physically interacts with the intracellular domain of Notch through its N-terminal domain. Deltex has two other domains that are presumably involved in protein-protein interactions: a proline-rich motif that binds to SH3-domains, and a RING-H2 finger motif. Using an overexpression assay, we have analyzed the functional involvement of these Deltex domains in Notch signaling. The N-terminal domain of Deltex that binds to the CDC10/Ankyrin repeats of the Notch intracellular domain was indispensable for the function of Deltex. A mutant form of Deltex that lacked the proline-rich motif behaved as a dominant-negative form. This dominant-negative Deltex inhibited Notch signaling upstream of an activated, nuclear form of Notch and downstream of full-length Notch, suggesting the dominant-negative Deltex might prevent the activation of the Notch receptor. We found that Deltex formed a homo-multimer, and mutations in the RING-H2 finger domain abolished this oligomerization. The same mutations in the RING-H2 finger motif of Deltex disrupted the function of Deltex in vivo. However, when the same mutant was fused to a heterologous dimerization domain (Glutathione-S-Transferase), the chimeric protein had normal Deltex activity. Therefore, oligomerization mediated by the RING-H2 finger motif is an integral step in the signaling function of Deltex.

Key words: Notch, Deltex, Cell-cell interaction, Wing formation, SH3-domain, RING-H2 finger, *Drosophila*

INTRODUCTION

Local cell-cell interactions are essential for the development of multicellular organisms. Notch signaling is involved in cell-cell communications that regulate a broad spectrum of cell-fate determinations in organisms ranging from the fly to mammals (reviewed by Artavanis-Tsakonas et al., 1995; Blaumueller and Artavanis-Tsakonas, 1997; Gridley, 1997; Kimble and Simpson, 1997; Weinmaster, 1998; Greenwald, 1998; Artavanis-Tsakonas et al., 1999; Kadesch, 2000).

In *Drosophila*, *Notch* encodes a 300 kDa single-pass transmembrane receptor (Artavanis-Tsakonas et al., 1983). The extracellular domain of Notch contains 36 epidermal growth factor (EGF)-like repeats and three Notch/Lin-12 repeats. In the intracellular domain of Notch, there are six CDC10/Ankyrin repeats and a PEST-like sequence. Delta and Serrate have been identified as transmembrane ligands for Notch (Vässin et al., 1987; Nye and Kopan, 1995). There is strong evidence supporting the idea that the ligand-dependent activation of Notch induces the proteolytic cleavage of Notch itself, so that the intracellular domain of Notch is released from the cell membrane and moves to the nucleus (Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). This cleavage has been shown to depend on the function of Presenilin and a γ-secretase-like proteinase (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999; Brou et al., 2000; Mumm et al., 2000). In the nucleus, the intracellular domain of Notch physically interacts with a transcription factor, Suppressor of Hairless [Su(H)], which functions as a suppressor of transcription when it is not complexed with the intracellular

domain of Notch (Fortini and Artavanis-Tsakonas, 1994; Honjo, 1996; Klein et al., 2000). The complex involving the Notch intracellular domain and Su(H) is an activator of transcription and binds to promoter elements that regulate the expression of the target genes of Notch signaling, such as *Enhancer of split* and *vestigial* (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Kim et al., 1996).

Although an increasing number of genes have been identified as components of the Notch pathway, the biochemical function of the *deltex* gene product remains elusive. *Drosophila deltex* encodes a cytoplasmic regulator of Notch, although its function may not be essential for signaling (Xu and Artavanis-Tsakonas, 1990; Gorman and Girton, 1992; Busseau et al., 1994). The N-terminal region of Deltex physically interacts with the CDC10/Ankyrin repeats of the Notch intracellular domain (Busseau et al., 1994; Matsuno et al., 1995). This interaction appears to be crucial for the function of Deltex (Matsuno et al., 1995). Two other Deltex domains, a proline-rich motif and a RING-H2 finger motif have been identified previously (Matsuno et al., 1995). In general, proline-rich motifs are known as binding sites for various SH3-domains (Cohen et al., 1995; Di Fiore et al., 1997; Pawson and Scott, 1997; Kay et al., 2000). Indeed, it has been shown that human GRB2, a SH3-domain-containing protein, binds to the human Deltex homolog and to *Drosophila* Deltex (Matsuno et al., 1998). RING-H2 finger motifs have also been shown to function in protein-protein interactions in various systems (Freemont, 1993; Freemont, 2000; Joazeiro and Weissman, 2000). These three motifs in Deltex, which are all presumably involved in protein-protein interactions, are conserved among the mammalian homologs of Deltex, suggesting that they have functional importance (Pampeno and Meruelo, 1996; Matsuno et al., 1998; Frolova and Beebe, 2000; Kishi et al., 2001).

Genetic analysis in *Drosophila* and biochemical studies involving mammalian Deltex and tissue culture cells support the idea that Deltex is a positive regulator of Notch signaling (Xu and Artavanis-Tsakonas, 1990; Diederich et al., 1994; Fortini and Artavanis-Tsakonas, 1994). It has been shown that human and mouse Deltex homologs have a similar activity to that of mammalian Notch1, suggesting mammalian Deltex regulates Notch signaling in a positive manner (Matsuno et al., 1998; Kishi et al., 2001). However, a study using a mammalian neural cell culture system derived from adult brain revealed that Deltex homologs could act as negative regulators of Notch signaling (Sestan et al., 1999). Thus, it is possible that the role played by Deltex in Notch signaling could be influenced dramatically by the developmental and cellular contexts.

In the present study, we have used molecular genetic approaches to investigate the role of Deltex motifs in the regulation of Notch signaling. A dominant-negative form of Deltex was generated and used in an epistatic analysis. The results showed that the dominant-negative form of Deltex acts on Notch signaling upstream of an active form of Notch and downstream of full-length Notch. We also showed that the RING-H2 finger motif of Deltex is involved in its multimerization. Experiments involving forced dimerization using a heterologous domain have suggested that the self-association of Deltex mediated by the RING-H2 finger motif is a crucial step in Deltex-dependent signaling.

MATERIALS AND METHODS

Creation of Deltex and Deltex mutant constructs

The amino acids of Deltex were numbered according to Busseau et al. (Busseau et al., 1994). A Chameleon double-stranded site-directed mutagenesis kit (Stratagene) was used to create the deletion and point mutation constructs of *deltex*. $Dx^{\Delta NBS}$ and $Dx^{\Delta PRM}$ lack a domain for binding to Notch (amino acids 46 to 204) (Matsuno et al., 1995) and the proline-rich motif (amino acids 475 to 483), respectively. Dx^{mRZF} has mutations in which two histidine residues (amino acids 570 and 573) have been replaced by alanine residues. The cDNA encoding Dx^{mRZF} was generated using the primer 5′CTGAGTCGCTGCC-AGGCTCTCATGGCTTTGCAGTGCCTCAAT3′. All junctions and point mutations were confirmed by sequencing. The $Dx^{\Delta NBS\text{-}\Delta PRM}$ construct was made by replacing the *Bgl*II fragment of $Dx^{\Delta PRM}$ cDNA with that of the $Dx^{\Delta NBS}$ cDNA. The $Dx^{\Delta NBS\text{-}mRZF}$ construct was made by replacing the *Xho*I fragment of the Dx^{mRZF} cDNA with that of the $Dx^{\Delta NBS}$ cDNA. The *Not*I-*Kpn*I fragments from all the constructs were subcloned into a P-element transformation vector, pUAST (Brand and Perrimon, 1993).

The constructs producing fusion proteins of GST with various Deltex derivatives were generated as follows. A cDNA of *S. japonicum* glutathione-S-transferase (GST) with an extra *Cla*I site was generated by a PCR with two primers, 5′TGACGG-ATATGTCCCCTATACTAGG3′ and 5′AATCGATTATTTTGGAGG-ATGGTC3′, using a pGEX vector (Amersham Pharmacia Biotech) as the template. The *deltex* cDNA fragment was amplified using two primers, 5′TCCAGGTCGTGCCTTCTTCGC3′ and 5′GGGG-ACATATCCGTCACGCCCAGG3′. The resulting two PCR fragments were used as the templates in a recombinant PCR and amplified with the following primers: 5′AATCGATTATTTTGGAGGATGGTC3′ and 5′TCCAGGTCGTGCCTTCTTCGC3′. The 3′-noncoding region of *deltex* cDNA with an extra *Cla*I site was amplified using the following primers: 5′AATCGATGGATTAGTTCCCTGTCC3′ and the M13 reverse primer. The junctions of the resulting constructs and point mutations were sequenced for confirmation. These *deltex* and GST cDNA fragments were ligated into the pUAST-Deltex constructs to replace the corresponding cDNA fragments, as described above.

Production of transgenic flies

The germline transformations and subsequent crosses were described previously (Sawamoto et al., 1994). In all experiments, several independent lines (~10) of each construct were established and examined. All crosses of UAS lines to *patched-GAL4* (*ptc-GAL4*) were performed at 18°C (Johnson et al., 1995).

Western blot analysis

Transformant lines were crossed to an *hs-GAL4* line (Brand and Perrimon, 1993). The resulting third-instar larvae were collected and heat shocked at 37°C twice for 1 hour, with a 1 hour 25°C interval between heat shocks. Larvae were homogenized in phosphate-buffered saline (PBS) (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.0) containing 1% SDS. The samples were boiled, and the protein concentration was determined using a bovine serum albumin (BSA) protein assay kit (Pierce). Protein samples were fractionated by SDS-PAGE on 7.5% or 10% acrylamide gels, transferred to Immobilon-P membranes (Millipore), and blocked. The protein blots were probed with rat anti-Deltex antibody (C645-17A) (Busseau et al., 1994) or rabbit anti-GST antibody (Santa Cruz biotechnology). The signal was detected using an HRP-conjugated secondary antibody (Cappel) and the ECL western blotting analysis system (Amersham Pharmacia Biotech).

Immunohistochemistry

Wing imaginal discs of the third-instar larvae were dissected in PBS and fixed in PLP (2% paraformaldehyde, 0.01 M $NaIO_4$, 0.075 M lysine, 0.037 M sodium phosphate, pH 7.2) (Tomlinson and Ready,

1987). Discs were washed in PBS-DT (0.3% sodium deoxycholate, 0.3% Triton X-100 in PBS) and incubated with the following primary antibodies: mouse anti-Wg (1:5) (van den Heuvel et al., 1989); rat anti-Deltex (1:25) (Busseau et al., 1994); mouse anti-Notch (1:5000) (Fehon et al., 1990); mouse anti-Delta (1:500) (Fehon et al., 1990); and rabbit anti-β-Galactosidase (1:500) (Cappel). After several washes in PBS-DT, the discs were incubated with fluorescently labeled secondary antibodies, rhodamine-conjugated goat anti-rat (Chemicon) and goat anti-mouse (Jackson Laboratories) antibodies, and FITC-conjugated goat anti-rabbit antibodies (Invitrogen), for 1-2 hours at room temperature, followed by washing in PBS-DT. The samples were mounted in 80% glycerol/PBS containing 1% N-propyl gallate.

Cell culture and in vitro binding assay

Drosophila S2 cells were cultured and transfected as described previously (Fehon et al., 1990; Diederich et al., 1994). To produce Deltex derivatives or GST fusion to Deltex derivatives, UAS constructs encoding Deltex derivatives and pWA-GAL4 were co-transfected. pWA-GAL4 expressed GAL4 protein under the control of an actin gene promoter. A total of 2 μg of DNA and 8 μl of Cellfectin reagent (Invitrogen) were mixed and added to cells in serum-free SFM medium (Invitrogen) and incubated for 4 hours, followed by incubation in a serum-containing medium for another 48 hours at 25°C. The cells were harvested and lysed in 200 μl TNE buffer (10 mM Tris-HCl pH 7.8, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 mM PMSF). After centrifugation at 18,000 ***g*** for 10 minutes at 4°C, the supernatant was incubated at 4°C for 1 hour with Glutathione-Sepharose 4B resin (Amersham Pharmacia Biotech), which was equilibrated with binding buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, 0.5 mg/ml BSA, 5 mM β-mercaptoethanol). The resin was washed five times in binding buffer, then incubated in elution buffer (10 mM glutathione, 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol) at room temperature for 20 minutes. Aliquots of the total lysates and the eluants from the Glutathione-Sepharose 4B resin were fractionated by 7.5% SDS-PAGE, and Deltex derivatives and the GST-Deltex derivative fusion proteins were detected on a western blot as described above, using an anti-Deltex antibody.

Rescue of *deltex* mutant by overexpression of Deltex derivatives

deltex^{24}*;hs-GAL4/TM3* was crossed to either *UAS-Dx*^{full} or *UAS-Dx*^{mRZF}*+GST*. Progeny were raised at 25°C, heat shocked at 37°C for 1 hour at the early pupae stage and then cultured at 25°C.

RESULTS

Notch signaling is required for the proper formation of the wing margin in *Drosophila* (for reviews, see Brook et al., 1996; Cohen, 1996; Irvine and Vogt, 1997). For example, partial loss of Notch activity results in the wing nicking phenotype for which Notch was named (Fig. 1C). Most *deltex* mutant alleles also yield a recessive wing-notch phenotype similar to that of *Notch* mutants, indicating the involvement of *deltex* function in wing margin development (Fig. 1B). Our previous study showed that the ectopic expression of Deltex results in the activation of Notch signaling (Matsuno et al., 1995). Because ectopic activation of Notch signaling in the wing pouch of the third-instar larval wing disc induces an ectopic wing margin-like structure that includes ectopic wing outgrowth and bristle formation (Diaz-Benjumea and Cohen, 1995; de Celis and Bray, 1997), we examined whether the overexpression of Deltex might induce a similar ectopic wing margin structure (Matsuno et al., 1995). Using the UAS/GAL4 system, we overexpressed the full-length Deltex protein under the control of *patched-GAL4* (*ptc-GAL4*) (Fig. 1D) (Brand and Perrimon, 1993; Johnson et al., 1995). As shown in Fig. 1D, in the wing discs of third-instar larvae from the *ptc-GAL4* line, the region expressing the GAL4 protein was located between two proveins (arrowheads in Fig. 1D), which corresponded to the longitudinal veins III and IV in the adult wing (arrowheads in Fig. 1A).

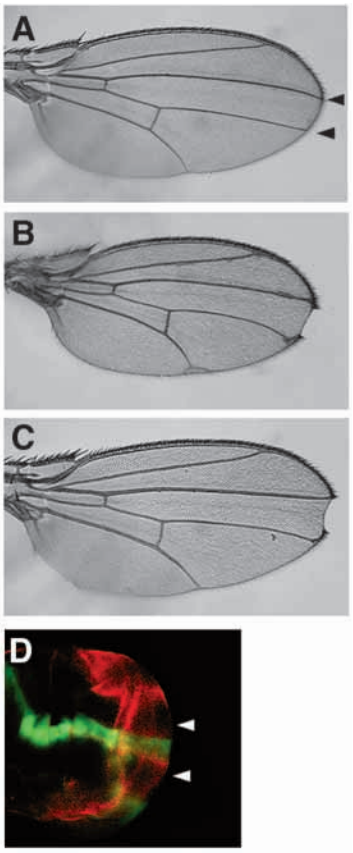

Fig. 1. *deltex* and *Notch* are required for the normal development of the wing margin. (A) Wild-type adult wing. The wing veins III and IV are indicated by arrowheads (see D). (B) *deltex/Y* wing. (C) *Notch*^{54l9}*/+* wing. (D) Protein expression from a UAS responder line under the control of the *ptc-GAL4* driver. *UAS-GFP* was crossed to *ptc-GAL4*, and wing discs of the third-instar larvae were stained with mouse anti-Delta antibody. GFP and Delta are shown in green and red, respectively. Presumptive cells of wing veins III and IV expressing Delta are indicated by arrowheads.

As a result of full-length Deltex (Dx^{full}) overexpression, an ectopic secondary wing margin-like structure was induced along the region expressing Deltex (see Fig. 3H). This secondary wing margin-like structure included ectopic outgrowth and ectopic bristle formation (see Fig. 3B,H). Taking advantage of this Deltex activity, we decided to analyze the function of three conserved motifs found in Deltex: (1) a domain that binds to the CDC10/Ankyrin repeats of the Notch intracellular domain, (2) a proline-rich motif, and (3) a RING-H2 finger motif (Fig. 2A). Constructs to produce mutant Deltex proteins were generated and introduced into flies by P-element-mediated transformation (Brand and Perrimon, 1993). The Deltex mutant proteins used in this study are shown schematically in Fig. 2A. The production of each Deltex derivative in vivo was confirmed by western blotting using an anti-Deltex antibody, and the molecular weights of these proteins were as expected (Fig. 2C). Judged by the relative intensity of the bands, similar amounts of each protein were produced (Fig. 2C).

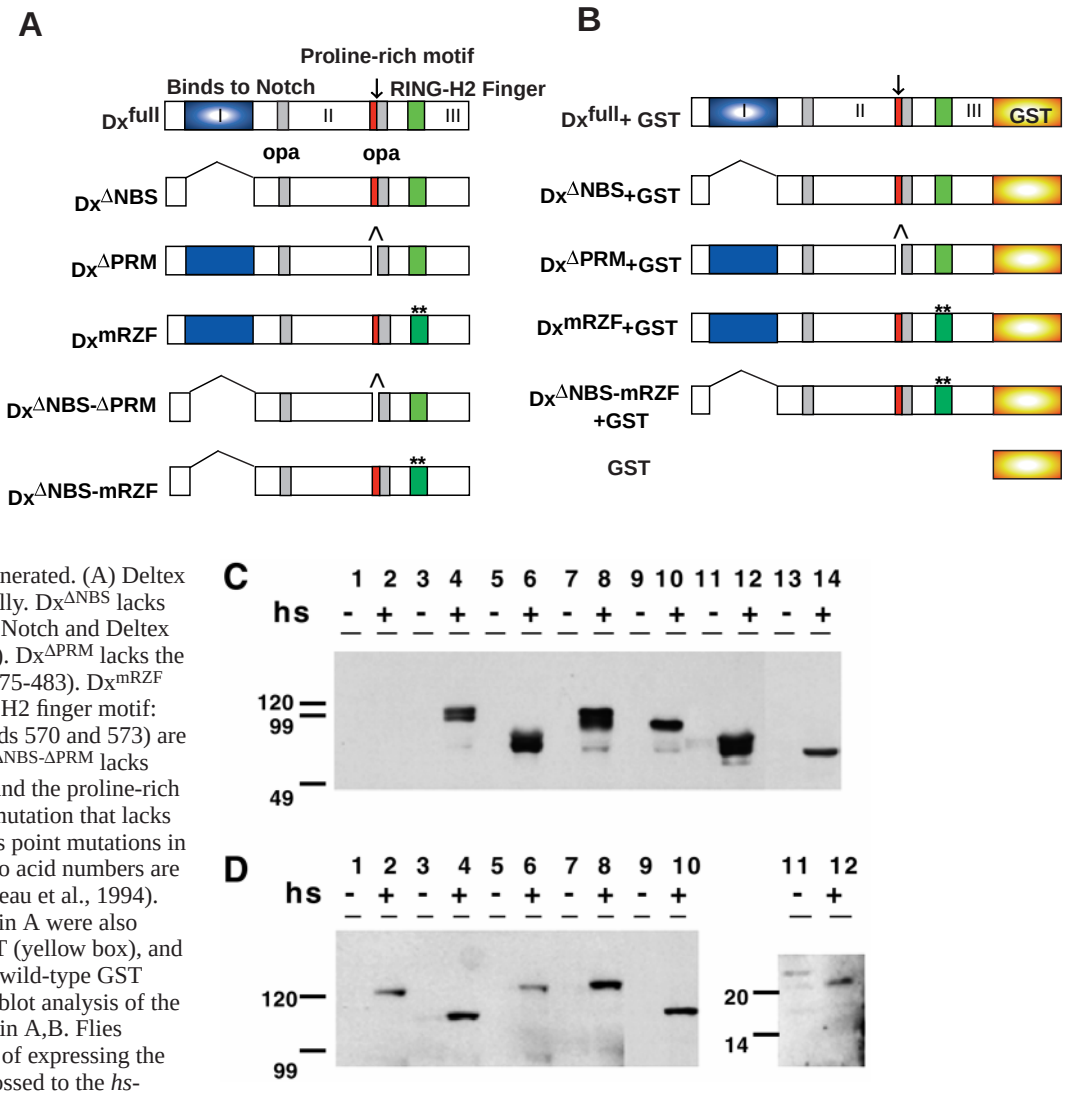

Fig. 2. Wild-type and mutant Deltex proteins used in this study. The Deltex protein is arbitrarily divided into three regions (domains I, II and III), based on the position of two OPA repeats, which separate the regions. The region binding to the Notch CDC10/Ankyrin repeats (blue box), a proline-rich motif that is a putative SH3-binding site (red box) and a RING-H2 finger motif (green box) are shown. The full-length Deltex protein consists of 737 amino acids (Busseau et al., 1994). All the cDNAs encoding the Deltex derivatives shown were inserted into the pUAST vector, and transgenic flies carrying these constructs were generated. (A) Deltex derivatives are shown schematically. $Dx^{\Delta NBS}$ lacks the domain capable of mediating Notch and Deltex interactions (amino acids 46-204). $Dx^{\Delta PRM}$ lacks the proline-rich motif (amino acids 475-483). Dx^{mRZF} has point mutations in the RING-H2 finger motif: two histidine residues (amino acids 570 and 573) are replaced by alanine residues. $Dx^{\Delta NBS\text{-}\Delta PRM}$ lacks both the binding sites for Notch and the proline-rich motif. $Dx^{\Delta NBS\text{-}mRZF}$ is a double mutation that lacks the binding site for Notch and has point mutations in the RING-H2 finger motif. Amino acid numbers are according to Busseau et al. (Busseau et al., 1994). (B) The Deltex derivatives listed in A were also made as fusion proteins with GST (yellow box), and are shown schematically. GST is wild-type GST used as a control. (C,D) Western blot analysis of the Deltex mutant derivatives shown in A,B. Flies carrying UAS constructs capable of expressing the Deltex derivatives (A,B) were crossed to the *hs-GAL4* line. Samples isolated before (shown by –) and after (shown by +) heat shock. (C) Lanes 1 and 2, Canton-S; lanes 3 and 4, Dx^{full}; lanes 5 and 6, $Dx^{\Delta NBS}$; lanes 7 and 8, $Dx^{\Delta PRM}$; lanes 9 and 10, Dx^{mRZF}; lanes 11 and 12, $Dx^{\Delta NBS\text{-}\Delta PRM}$; lanes 13 and 14, $Dx^{\Delta NBS\text{-}mRZF}$. The protein blot was probed with the anti-Deltex antibody. Molecular weight markers are shown in kDa. (D) Lanes1 and 2, Dx^{full}+GST; lanes 3 and 4, $Dx^{\Delta NBS}$+GST; lane 5 and 6, $Dx^{\Delta PRM}$+GST; lanes 7 and 8, Dx^{mRZF}+GST; lanes 9 and 10, $Dx^{\Delta NBS\text{-}mRZF}$+GST; lanes 11 and 12, GST alone. The protein blot was probed with anti-GST antibody. Molecular weight markers are shown in kDa.

All three motifs of Deltex are required for normal Deltex function

As mentioned above, overexpression of Dx^{full} under the control of *ptc-GAL4* induced a secondary wing margin-like structure (Fig. 3B,H). As shown in Fig. 3N, ectopic sensory organ precursor (SOP) cells (green and arrowhead) along the region expressing Dx^{full} protein (red) were formed in these flies. The induction of SOPs appeared to be non-cell-autonomous and occurred only in the ventral compartment (Fig. 3N). This observation provides further support to the interpretation that the overexpression of Dx^{full} leads to the development of a secondary wing margin-like structure. The endogenous Deltex protein could be detected as faint, ubiquitous staining throughout the entire wing discs of the late third-instar larvae (Fig. 3M, red). Using this overexpression system, we examined the activity of four different mutant forms of Deltex (Fig. 3C-R). A mutant Deltex protein lacking the domain that binds to the Notch CDC10/Ankyrin repeats ($Dx^{\Delta NBS}$) or carrying two amino acid substitutions in the RING-H2 finger motif (Dx^{mRZF}) failed to induce the secondary wing margin-like structure in the adult wing, although very slight effects were still observed occasionally (Fig. 3C,I,E,K). These findings were confirmed by the observation that ectopic SOPs were not formed in the wing discs expressing these two mutant proteins (Fig. 3O,Q). By contrast, the overexpression of a mutant Deltex lacking the nine amino acids (amino acids 475-483) that encompass the proline-rich motif ($Dx^{\Delta PRM}$) resulted in wing nicking (Fig. 3D,J). We noted that this phenotype resembled those of *deltex/Y* and *Notch/+* flies (Fig. 1).

Dominant-negative behavior of a mutant Deltex lacking the proline-rich motif

The wing-notch phenotype induced by the overexpression of $Dx^{\Delta PRM}$ suggested that $Dx^{\Delta PRM}$ might be a dominant-negative

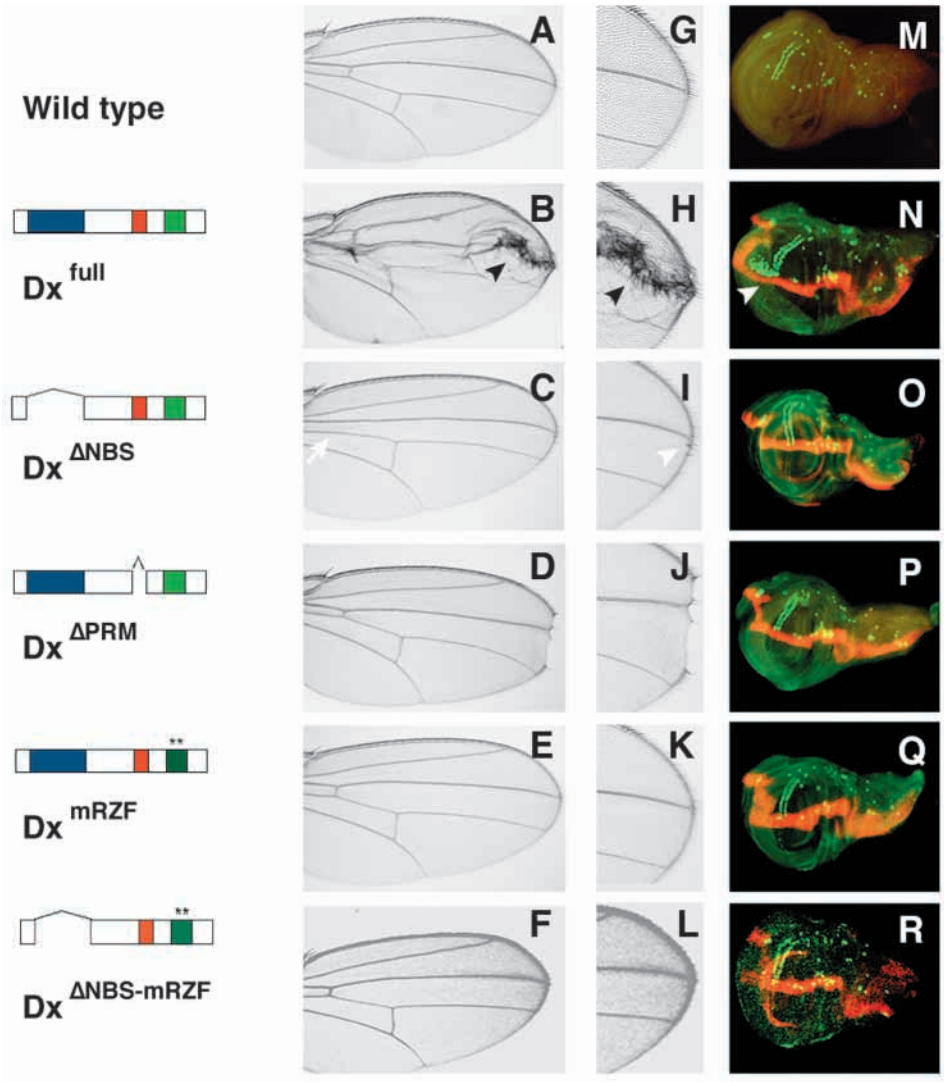

Fig. 3. Effects of overexpression of Deltex derivatives under the control of the *ptc-GAL4* driver. Transgenic flies carrying the various UAS constructs expressing the Deltex derivatives were crossed to *ptc-GAL4* or *ptc-GAL4/CyO; A101/TM6B*. (A-L) Adult wings and high magnifications of regions of wings. (M-R) The SOP cells in the third-instar wing discs are shown in green, and the Deltex derivatives are shown in red. An enhancer trap line, A101, was used to visualize the SOP cells. (A,G,M) Wild-type wing and disc. (B,H,N) Overexpression of Dx^{full}. Note a secondary wing margin-like structure (black arrowheads) and ectopic SOPs (white arrowhead). (C,I,O) Overexpression of $Dx^{\Delta NBS}$. Occasionally, a missing crossvein (white arrow) and a few extra bristles (white arrowhead) are observed. (D,J,P) Overexpression of $Dx^{\Delta PRM}$. Note the wing-notch phenotype. (E,K,Q) Overexpression of Dx^{mRZF}. (F,L,R) Overexpression of $Dx^{\Delta NBS\text{-}mRZF}$.

form of Deltex that inhibited Notch signaling during wing margin development. To test this hypothesis, we performed two different lines of experiments. First, $Dx^{\Delta PRM}$ and Dx^{full} were overexpressed simultaneously under the control of *ptc-GAL4*. We expected that $Dx^{\Delta PRM}$ and Dx^{full} would counteract each other's activity, if the $Dx^{\Delta PRM}$ was a dominant-negative protein. As described above, overexpression of Dx^{full} induced an ectopic wing margin-like structure (Fig. 3B,H), and under the same conditions, overexpression of $Dx^{\Delta PRM}$ resulted in the wing nick phenotype (Fig. 3D,J). However, as expected, the co-expression of $Dx^{\Delta PRM}$ and Dx^{full} did not have a substantial effect on the wing development, indicating these two proteins suppressed each other's activities (Fig. 4A,C). This result was consistent with the observation that $Dx^{\Delta PRM}$ suppressed the ectopic induction of SOPs by Dx^{full} (Fig. 4E).

Second, we examined the effect of $Dx^{\Delta PRM}$ overexpression on endogenous Notch activity. Fig. 4G shows the expression of the Wingless (Wg) protein in the wing discs of third-instar larvae. The expression of Wg along the boundary of the dorsal and ventral compartments has been shown to depend on the activation of Notch signaling (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; Axelrod et al., 1996; Doherty et al., 1996). As shown in Fig. 4H, the endogenous expression of Wg (green) in the dorsal/ventral compartment boundary was suppressed by the overexpression of $Dx^{\Delta PRM}$ (red and highlighted in the upper right of the panel).

The dominant-negative activity of $Dx^{\Delta PRM}$ appeared to require the Deltex domain for binding to the intracellular domain of Notch. A Deltex protein lacking two regions, the proline-rich motif and the domain binding to Notch ($Dx^{\Delta NBS\text{-}\Delta PRM}$ in Fig. 2A) did not result in the wing-notch phenotype (Fig. 4B,D,F) and failed to suppress the expression of Wg in the dorsal/ventral compartment boundary (Fig. 4I). This result suggested that the dominant-negative activity of $Dx^{\Delta PRM}$ requires interaction with the intracellular domain of Notch.

$Dx^{\Delta PRM}$ acts on Notch signaling upstream of an active form of Notch and downstream of full-length Notch

The results presented above are consistent with the idea that $Dx^{\Delta PRM}$ is a dominant-negative form of Deltex. We performed an epistatic analysis between $Dx^{\Delta PRM}$ and full-length Notch (N^{full}) or an activated form of Notch (N^{act}) (Fig.

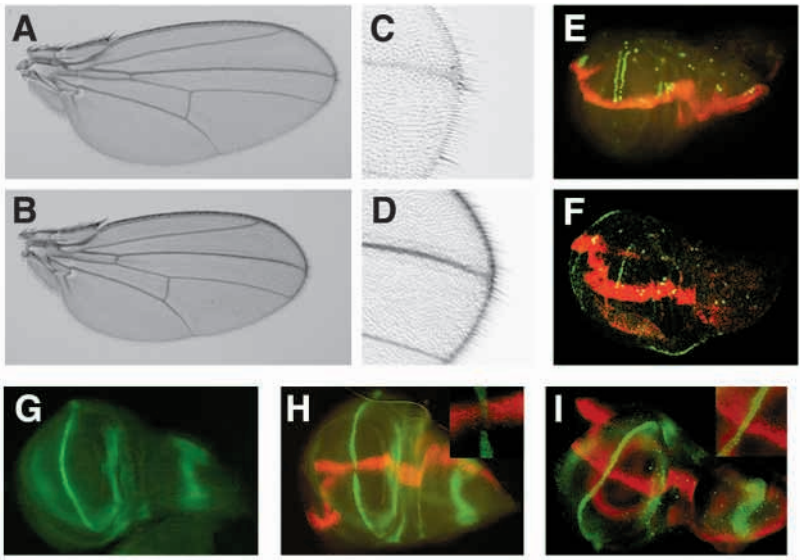

Fig. 4. Dominant-negative behavior of $Dx^{\Delta PRM}$. (A-D) Adult wings. (E,F) Wing discs of third-instar larvae. SOPs are shown in green, and Deltex derivatives are shown in red. (G-I) Wing discs of third-instar larvae. Wg and Deltex proteins are shown in green and red, respectively. (A,C,E) Co-expression of Dx^{full} and $Dx^{\Delta PRM}$. Note that phenotypes induced by either Dx^{full} or $Dx^{\Delta PRM}$ were suppressed by co-expression of both proteins (see Fig. 3B,D,H,J,N,P). (B,D,F) Overexpression of $Dx^{\Delta NBS\text{-}\Delta PRM}$ with the *ptc-Gal4* driver. Note that overexpression of $Dx^{\Delta NBS\text{-}\Delta PRM}$ did not result in the wing-notch phenotype. (G) Endogenous expression of Wg (green) was detected along the boundary of the dorsal/ventral compartments in the wild-type wing discs of third-instar larvae. (H) Overexpression of $Dx^{\Delta PRM}$. Endogenous expression of Wg (green) was suppressed in the cells expressing $Dx^{\Delta PRM}$ (red). A high-magnification photograph is shown at the top right. (I) Overexpression of $Dx^{\Delta NBS\text{-}\Delta PRM}$. Note that $Dx^{\Delta NBS\text{-}\Delta PRM}$ (red) did not suppress the Wg (green) expression. A high-magnification photograph is shown at the top right.

5A). First, $Dx^{\Delta PRM}$ was co-expressed with N^{act} under the control of the *ptc-GAL4* driver. As shown in Fig. 5B, the expression of N^{act} alone resulted in the formation of ectopic SOPs (Rebay et al., 1993; Struhl et al., 1993; Lyman and Yedvobnick, 1995). Ectopic SOPs are indicated by an arrowhead (Fig. 5B). As shown in Fig. 5C, the co-expression of $Dx^{\Delta PRM}$ did not substantially affect the ectopic SOP induction caused by the overexpression of N^{act} (compare with Fig. 5B). Similarly, the co-expression of $Dx^{\Delta PRM}$ did not cause any marked effect on the ectopic induction of Wg that was caused by overexpressed N^{act} (compare Fig. 5D with 5E). Although Dx^{full} induced ectopic SOPs only in the ventral compartment of the wing pouch, N^{act} induced SOPs and Wg expression in both the dorsal and ventral compartments (Fig. 3N, Fig. 5B,D). As shown in Fig. 5F, the overexpression of N^{full} resulted in the ectopic and non-cell-autonomous induction of Wg expression, which contrasted with the cell-autonomous induction of Wg by the overexpression of N^{act}. We found that co-expression of $Dx^{\Delta PRM}$ suppressed the induction of the ectopic Wg expression that was caused by the overexpressed N^{full} (compare Fig. 5F with 5G). Therefore, these results suggest that $Dx^{\Delta PRM}$ acted downstream of N^{full} and upstream of N^{act}.

Deltex signaling activity is regulated by oligomerization mediated by its RING-H2 finger

The above results demonstrate that the Deltex RING-H2 finger

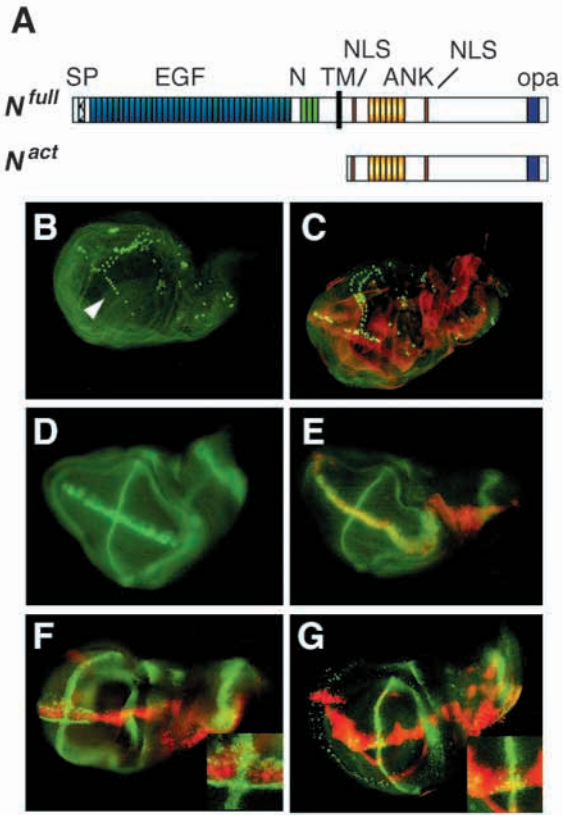

Fig. 5. The dominant-negative form of Deltex ($Dx^{\Delta PRM}$) suppressed Notch signaling downstream of the full-length Notch and upstream of an activated form of Notch. (A) Notch and its derivative. Protein motifs in Notch: SP, a signal peptide; EGF, 36 EGF-like repeats; N, 3 Notch/Lin-12 repeats; TM, the transmembrane domain; NLS, two nuclear localization signals; ANK, 6 CDC10/Ankyrin repeats; opa, polyglutamine repeat. The full-length Notch and an activated form of Notch are shown at the top and bottom of A, respectively. N^{act} is a truncated form that lacks the entire extracellular domain and the transmembrane domain. It functions as a constitutively active form of Notch. (B-G) UAS-N^{full} or UAS-N^{act} was expressed alone or co-expressed with UAS-$Dx^{\Delta PRM}$ under the control of the *ptc-GAL4* driver. Wing discs of third-instar larvae are shown. (B) Overexpression of N^{act}. SOPs are shown in green. Note that a row of ectopic SOP cells was formed (arrowhead). (C) Co-expression of N^{act} and $Dx^{\Delta PRM}$ (red). Ectopic formation of SOPs (green) was not suppressed. (D) Overexpression of N^{act} induced the ectopic expression of Wg (green) (Couso et al., 1994; Williams et al., 1994). (E) Co-expression of N^{act} and $Dx^{\Delta PRM}$ (red). Note that the ectopic Wg expression (green) remained essentially the same. (F) Overexpression of N^{full} (red) induced the ectopic Wg expression (green). (G) Co-expression of N^{full} and $Dx^{\Delta PRM}$ (red). Note that the ectopic expression of Wg (green) was suppressed.

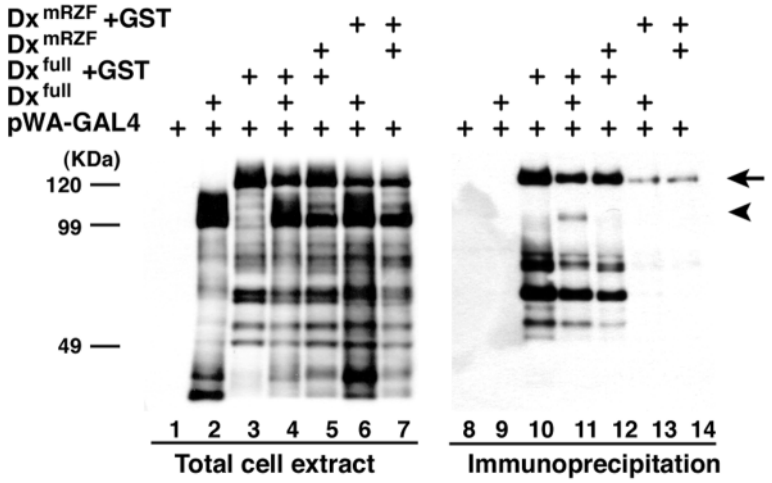

Fig. 6. Mutations in the RING-H2 finger motif of Deltex disrupted its homotypic interaction. UAS-Dx^{full}, -Dx^{mRZF}, -Dx^{full}+GST, and -Dx^{mRZF}+GST (see Fig. 2A,B) were expressed with pWA-GAL4 in *Drosophila* S2 cells. Deltex derivatives were detected by western blot with an anti-Deltex antibody. Lanes 1-7, the total cell extract that was added to the resin; lanes 8-14, eluants from the Glutathione-Sepharose 4B resin. Dx^{full} and Dx^{mRZF} are indicated by an arrowhead (~90 kDa). Dx^{full}+GST and Dx^{mRZF}+GST are indicated by an arrow (~120 kDa). Molecular weight markers are shown in kDa.

motif is essential for Deltex function. RING-H2 finger motifs have been shown to mediate various protein-protein interactions (Freemont, 1993; Freemont, 2000; Joazeiro and Weissman, 2000). Ste5, a yeast protein, and Deltex have similar RING-H2 finger motifs (Inouye et al., 1997). Ste5 is an essential component of the mitogen-activated protein kinase (MAPK) cascade in a yeast pheromone response pathway (for a review, see Madhani and Fink, 1998; Schaeffer and Weber, 1999). In response to pheromone, Ste5 binds through its RING-H2 finger motif to the free Gβγ complex, which is composed of Ste4 and Ste18 (Whiteway et al., 1995; Inouye et al., 1997; Feng et al., 1998). This interaction leads to the activation of the MAPK cascade (Feng et al., 1998). The interaction between Ste5 and Ste4 is a prerequisite for Ste5 to self-associate and to function as part of the signal-transduction pathway (Whiteway et al., 1995). Moreover, it has been shown that the RING-H2 finger motif of Ste5 is also required for this self-association (Inouye et al., 1997).

The homology between the RING-H2 finger motifs of Ste5 and Deltex raised the possibility that the Deltex RING-H2 finger motif might have a similar function to the RING-H2 finger motif in Ste5. To test this hypothesis, we first performed an in vitro binding experiment. Two chimeric forms of Deltex, a wild-type Deltex (Dx^{full}+GST) and a Deltex carrying mutations in the RING-H2 finger motif, Dx^{mRZF} (Dx^{mRZF}+GST), in which GST was fused to the C terminus, were made in *Drosophila* tissue culture cells (the S2 cell line). We co-expressed each GST fusion protein with either wild-type Deltex or Dx^{mRZF} in S2 cells. The GST fusion form of the Deltex derivatives that bound to Glutathione-Sepharose 4B resin could be recovered and detected on a western blot using an anti-Deltex antibody. If Deltex formed homo-oligomers, non-GST fusion forms of Deltex should be co-purified with the fusion proteins and detected on the same western blot. We found that Deltex (non-GST fusion) bound to Dx^{full}+GST and was co-purified (Fig. 6, lane 11), but Dx^{mRZF} did not bind to the corresponding fusion protein (Fig. 6, lane 12). Neither wild-type Deltex nor Dx^{mRZF} bound to Dx^{mRZF}+GST under the same conditions (Fig. 6, lanes 13,14). These results show that Deltex self-associates and that the RING-H2 finger motif is required for this oligomerization.

We then tested the possibility that the oligomerization of Deltex that was mediated by the RING-H2 finger motif had a crucial role for the activity of Deltex. The GST protein forms a stable dimer complex, both in solution and in protein crystals (Lim et al., 1994; McTigue et al., 1995), and this dimerization can functionally substitute for the dimerization domain of a heterologous protein (Maru et al., 1996; Riley et al., 1996). The Deltex derivative-GST fusion proteins were expressed in vivo using the UAS/GAL4 system (Fig. 2B). A western blot analysis using the anti-GST antibody revealed that all the GST fusion proteins were expressed with the expected molecular weight (Fig. 2D). Each fusion protein was expressed under the control of the *ptc-GAL4* driver, and the transgenic flies were examined for formation of an ectopic wing margin-like structure and ectopic induction of SOPs (Fig. 7). Expression of Dx^{mRZF}+GST resulted in the formation of an ectopic wing margin-like structure and the ectopic induction of SOPs (Fig. 7D,J,P), while Dx^{mRZF} (the non- GST form) had no significant effect on either (Fig. 3E,K,Q). Therefore, the GST-mediated dimerization was sufficient to restore the function of Dx^{mRZF}. Furthermore, we found that overexpression of Dx^{mRZF}+GST under the control of a heat-shock promoter could rescue the mutant phenotype of the loss-of-function *deltex* in the wing veins (Fig. 8C). Dx^{mRZF}+GST was as competent as wild-type Deltex in rescuing the wing vein thickening phenotype (compare Fig. 8E with 8F). Occasionally, a wing vein was found to be missing in wings overexpressing Dx^{mRZF}+GST or wild-type Deltex (data not shown). By contrast, a control GST fusion form of wild-type Deltex did not show a substantial effect in the adult wing or on the formation of SOPs (Fig. 7A,G,M). These results suggest that the self-association of Deltex that is mediated by the RING-H2 finger motif in the wild-type protein is essential for the signaling activity of Deltex.

DISCUSSION

We have dissected the functions of three Deltex domains. These domains have been described previously, but their functions are not understood. Our results showed that the proline-rich and the RING-H2 finger motifs are required for distinct Deltex functions and are indispensable for Deltex activity. Although the identity of the proteins binding to these Deltex motifs and the nature of the protein-protein interactions

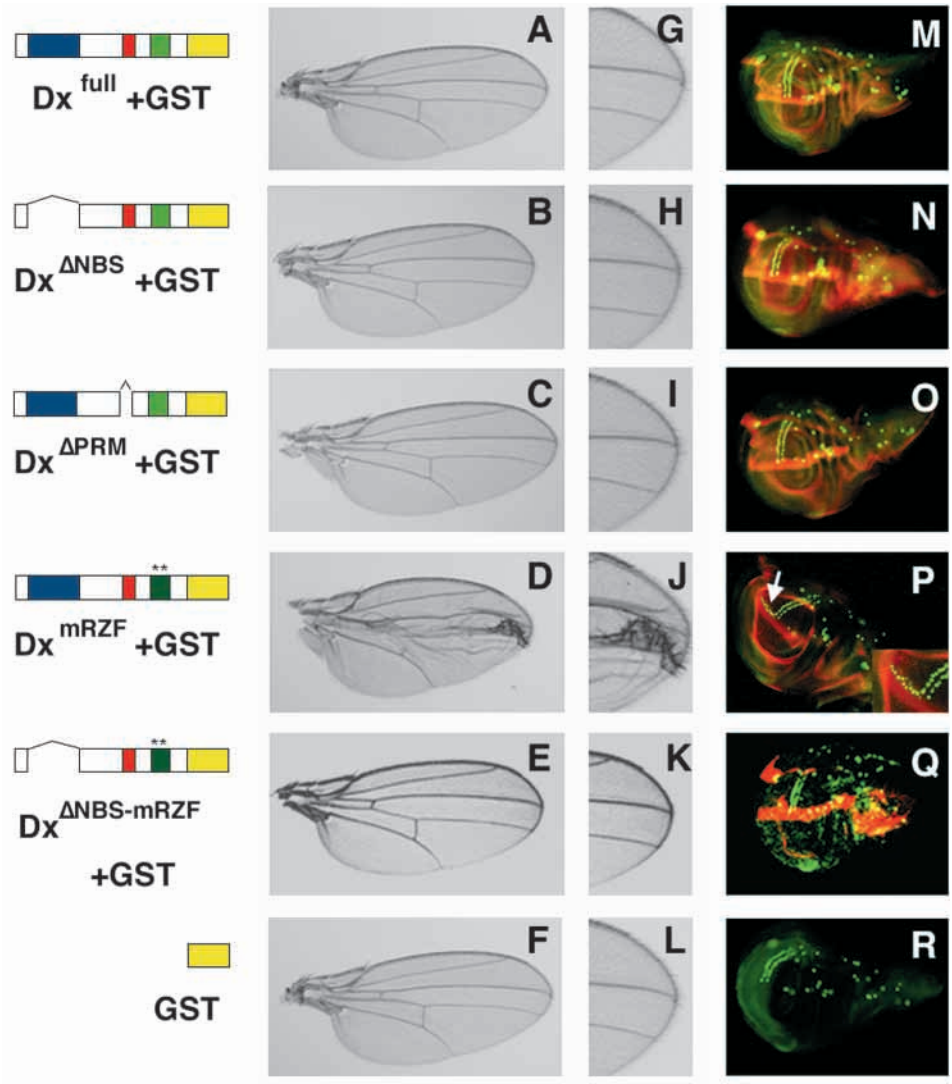

Fig. 7. GST-mediated dimerization substituted for the function of the Deltex RING-H2 finger motif. Transgenic flies carrying UAS constructs expressing Deltex derivatives fused to GST were crossed to *ptc-GAL4* or *ptc-GAL4/CyO;A101/TM6B*. (A-L) Adult wings and high-magnification photographs. (M-R) The SOP cells in the third-instar wing discs are shown in green, and Deltex derivatives are shown in red. An enhancer trap line, A101, was used to visualize SOP cells. (A,G,M) Overexpression of Dx^{full}+GST did not produce a noticeable effect. (B,H,N) Overexpression of $Dx^{\Delta NBS}$+GST resulted in wild-type wings and discs. (C,I,O) Overexpression of $Dx^{\Delta PRM}$+GST gave wild-type wings and discs. (D,J,P) Overexpression of Dx^{mRZF}+GST resulted in the induction of a secondary wing margin-like structure and ectopic SOP formation (indicated by an arrow), which resembled the effect of Dx^{full} overexpression (see Fig. 3B,H,N). Note that Dx^{mRZF} (non-GST form) did not show a substantial effect under the same conditions (see Fig. 3E,K,Q). (E,K,Q) Overexpression of $Dx^{\Delta NBS\text{-}mRZF}$+GST resulted in wild-type wings and discs. (F,L,R) Overexpression of GST alone did not show a substantial effect.

are still elusive, our results suggest that unidentified factor(s) are an integral component of Deltex function and the regulation of Notch signaling.

A dominant-negative form of Deltex

A proline-rich motif in the middle region of Deltex has been reported previously (Busseau et al., 1994; Matsuno et al., 1998). This motif shows homology to a consensus amino acid sequence of a binding site for SH3-domain proteins (Cohen et al., 1995; Di Fiore et al., 1997; Pawson and Scott, 1997; Kay et al., 2000). Indeed, we have previously demonstrated that human Grb-2, an SH3-domain protein, binds to Deltex (Lowenstein et al., 1992; Matsuno et al., 1998). In this paper, we show that Deltex lacking the proline-rich motif ($Dx^{\Delta PRM}$) behaves as a dominant-negative form. Based on these observations, we speculate that an as-yet-unidentified SH3-domain protein interacts with the proline-rich motif of Deltex and is an integral part of Deltex activity.

Nonetheless, the mechanism of the dominant-negative action of this mutant Deltex remains to be elucidated. Because proline-rich motifs are also found in the human, chicken and mouse Deltex homologs, the underlying mechanisms of this dominant-negative behavior may be evolutionarily conserved (Pampeno and Meruelo, 1996; Matsuno et al., 1998; Frolova and Beebe, 2000; Kishi et al., 2001). Previously, we showed that the expression of Deltex domain I fragment (amino acids 1-303), which lacks approximately two-thirds of the C-terminal region of the molecule, rescued a loss-of-function deltex phenotype and did not show dominant-negative function (Matsuno et al., 1995). Therefore, in addition to the absence of the proline-rich motif, the presence of some other part(s) of the Deltex domain II-III is required for the $Dx^{\Delta PRM}$ mutant to act as a dominant-negative form of the Deltex protein (see Fig. 2A).

While $Dx^{\Delta PRM}$ behaved as a dominant-negative protein during wing margin development, overexpression of $Dx^{\Delta PRM}$ under the control of a heat-shock promoter during early embryogenesis did not result in a neurogenic phenotype, which is an indication that Notch signaling was not disrupted (data not shown). Therefore, the dominant-negative action of $Dx^{\Delta PRM}$ may depend on the developmental context of cells, although the cellular component(s) responsible for this context-dependence remains to be identified. In this regard, it is noteworthy that none of the existing *deltex* alleles show the neurogenic phenotype (Xu and Artavanis-Tsakonas, 1990).

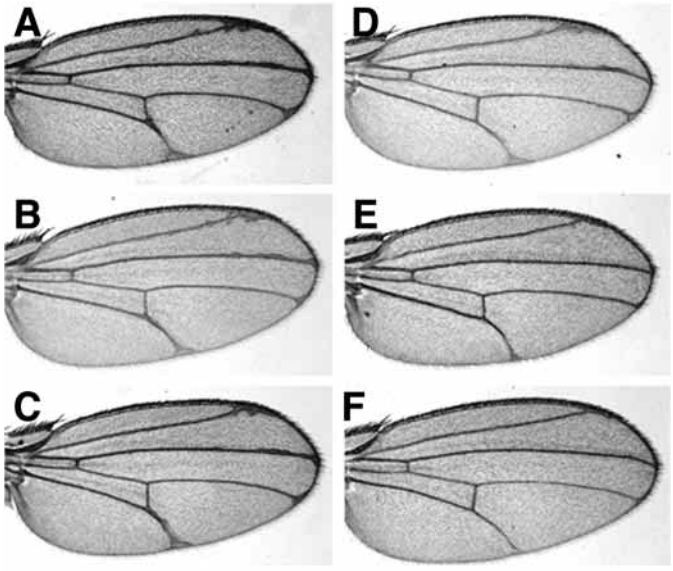

Fig. 8. Loss-of-function *deltex* phenotype was rescued by the overexpression of Dx^{mRZF}+GST. Adult wings from flies of the following genotypes. (C,F) *deltex*^{24} */ Y;; hs-GAL4/Dx*^{mRZF}*+GST*. Wing phenotype of *deltex* was rescued (27% showed complete rescue). (A-C) Wings without heat-shock treatment and (D-F) wings from flies heat-shocked at the early pupal stage. (A,D) *deltex*^{24} */ Y;; hs-GAL4*. Small deltas of extra vein material are visible where the veins reach the wing margin. (B,E) *deltex*^{24}*/Y;; hs-GAL4/UAS-Dx*^{ful}. Wing phenotype of *deltex* was rescued (18% showed complete rescue).

Oligomerization mediated by the RING-H2 finger motif is required for Deltex activity

In this paper, we have shown that Deltex forms homo-oligomers, and this oligomerization is integral for Deltex function. GST-mediated dimerization substituted for the function of the Deltex RING-H2 finger motif. The activity of Dx^{mRZF}+GST did not seem to be neomorphic, because the loss-of-function *deltex* phenotype was rescued by the expression of Dx^{mRZF}+GST. Furthermore, we often observed the partial loss of wing veins, which resembles the phenotypes of gain-of-function *Notch* mutants, or is also seen under the circumstances of constitutive activation of the Notch signal (Rebay et al., 1993; Struhl et al., 1993). However, the fusion of wild-type Deltex to GST (Dx^{full}+GST) did not show substantial activity in our system. Therefore, the GST-mediated dimerization may abolish the activity of wild-type Deltex; it is also possible that Dx^{full}+GST is not functional because the fusion to GST leads to some nonspecific disruption of the protein structure. However, it has been reported that the Ste5 oligomerization mediated by its RING-H2 finger motif serves as both a positive and negative regulatory step (Inouye et al., 1997). An oligomerization of Ste5 that regulates it negatively is relieved by the Ste5-Ste4 interaction, and this interaction then permits Ste5 to form an oligomer mediated by its RING-H2 finger motif, in that order. Therefore, sequential oligomerization taking place in the proper order may be also important for Deltex function. However, in the case of Deltex, the factor that might relieve it from its inhibitory oligomeric state remains to be identified. It is not likely that Notch functions as such a relieving factor, because a GST-fusion with a double mutant Deltex, $Dx^{\Delta NBS\text{-}mRZF}$ +GST, lost the activity of Dx^{mRZF} +GST (Fig. 7D,J,P,E,K,Q), suggesting that the binding of Deltex to the Notch CDC10/Ankyrin repeats was apparently still required in Dx^{mRZF} +GST, despite the fact that this protein presumably bypassed the inhibitory oligomerization state and was competent to signal. This also suggests that the binding of Deltex to Notch is not a prerequisite for the self-association of Deltex, as the Notch-binding domain of Deltex is still indispensable for the activity of the artificially dimerized Deltex GST (Fig. 7D,J,P,E,K,Q).

Implications from the dominant-negative form of Deltex

Previously, we have shown that the loss-of-function *deltex* phenotype could be rescued by the expression of an activated form of Notch (Matsuno et al., 1995). This observation suggested that Deltex might act upstream of the activated form of Notch, although the nature of the *deltex* alleles used in that study had not been characterized very well. The present study shows that the dominant-negative form of Deltex acts upstream of an activated form of Notch and downstream of wild-type Notch. Although we need to be cautious in using a dominant-negative form of a protein to speculate about an epistatic relationship, the above two results are consistent. Therefore, we speculate that this dominant-negative form of Deltex may inhibit the activation or maturation of the Notch receptor. For example, possible target steps include the ligand-dependent cleavage of Notch, the processing of Notch to its mature form or the ligand susceptibility of Notch. Alternatively, it is possible that the dominant-negative Deltex specifically decreases the stability of full-length Notch.

Differences in the inductive properties of Dx^{full} and N^{act}

We have shown that overexpression of Dx^{full} induces an ectopic wing margin-like structure, which is similar to the consequence of the ectopic expression of N^{act} (Dias-Benjumea and Cohen, 1995; de Celis and Bray, 1997). However, these two proteins appear to have distinct inductive properties in the wing pouch. As shown in Fig. 3N, Dx^{full} induces SOPs only in the ventral compartment of the wing pouch, while N^{act} induces SOPs in both the dorsal and ventral compartments (Fig. 5B). Furthermore, Dx^{full} induces SOPs in cells other than and distant from those expressing Dx^{full}. From these results, we speculate that induction of Serrate may be a part of these events. N^{act} has been shown to induce Serrate within the wing pouch, and Serrate effectively activates Notch only in the ventral compartments (Panin et al., 1997). The activation of Notch results in the Wg induction that in turn induces SOPs in the neighboring cells (Rulifson and Blair, 1995). Furthermore, high-level expression of Serrate autonomously inhibits the induction of the genes within the wing pouch that are dependent upon Notch signaling (Jonsson and Knust, 1996; Klein et al., 1997; Micchelli et al., 1997). Thus, the induction of Serrate would explain, at least in part, the result that Dx^{full} induced SOPs only in the ventral compartment, and ectopic SOPs were formed slightly remove from the cells expressing Dx^{full}.

Putative factor(s) binding to the proline-rich motif of Deltex

The dominant-negative behavior of $Dx^{\Delta PRM}$ suggests that

putative factor(s) that interact with the proline-rich motif might be essential for Deltex function. Suppressor of deltex [Su(dx)] is a good candidate. *Su(dx)* genetically suppresses *deltex* and *Notch* mutant phenotypes and encodes an E3-ubiquitin ligase (Fostier et al., 1998; Cornell et al., 1999). *Su(dx*) has WW domains that bind to proline-rich motifs in general (Cornell et al., 1999). In a mammalian system, a mammalian homolog of Su(dx), Itch, binds to the intracellular domain of Notch and ubiquitinates it (Qiu et al., 2000). Therefore, Deltex may function to suppress Su(dx), a negative regulator of Notch signaling, through an interaction that may be mediated by the proline-rich motif of Deltex and the WW domain of Su(dx).

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