Drosophila Deltex mediates Suppressor of Hairless-independent and late-endosomal activation of Notch signaling

Kazuya Hori[1], Maggy Fostier[2], Mikiko Ito[3], Takashi J. Fuwa[1], Masahiro J. Go[4], Hideyuki Okano[5], Martin Baron[2] and Kenji Matsuno[1,6,7,*]

[1]Department of Biological Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan
[2]School of Biological Sciences, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, UK
[3]Department of Nutrition, School of Medicine, University of Tokushima, 3-18-15 Kuramoto, Tokushima 770-8503, Japan
[4]Department of Neuroscience and Immunology, Kumamoto University, Graduate School of Medical Sciences, Honjo 2-2-1, Kumamoto 860-0811, Japan
[5]Department of Physiology, Keio University, School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[6]Genome and Drug Research Center, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan
[7]PRESTO, JST, 4-1-8 Honcho, Kawaguchi, Saitama, Japan
*Author for correspondence (e-mail: matsuno@rs.noda.tus.ac.jp)

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Summary

Notch (N) signaling is an evolutionarily conserved mechanism that regulates many cell-fate decisions. *deltex* (*dx*) encodes an E3-ubiquitin ligase that binds to the intracellular domain of N and positively regulates N signaling. However, the precise mechanism of Dx action is unknown. Here, we found that Dx was required and sufficient to activate the expression of gene targets of the canonical Su(H)-dependent N signaling pathway. Although Dx required N and a cis-acting element that overlaps with the Su(H)-binding site, Dx activated a target enhancer of N signaling, the dorsoventral compartment boundary enhancer of *vestigial* (vgBE), in a manner that was independent of the Delta (Dl)/Serrate (Ser) ligands- or Su(H). Dx caused N to be moved from the apical cell surface into the late-endosome, where it accumulated stably and co-localized with Dx. Consistent with this, the *dx* gene was required for the presence of N in the endocytic vesicles. Finally, blocking the N transportation from the plasma membrane to the late-endosome by a dominant-negative form of Rab5 inhibited the Dx-mediated activation of N signaling, suggesting that the accumulation of N in the late-endosome was required for the Dx-mediated Su(H)-independent N signaling.

Key words: Notch signaling, Deltex, Endocytic trafficking, Suppressor of Hairless, *Drosophila*

Introduction

Cell differentiation during development is often regulated by local cell-cell interactions, many of which involve signaling by the N family of receptors (reviewed by Greenwald, 1998). The N signaling pathway is an evolutionarily conserved mechanism that regulates many cell-fate decisions, cell death, cell division and pattern formation (reviewed by Greenwald, 1998; Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000; Klein, 2001). In *Drosophila*, the *N* gene encodes a 300 kD single-pass transmembrane receptor (Wharton et al., 1985). The binding of its ligand, Dl or Ser, leads to an extracellular cleavage of N (Brou et al., 2000), which is followed by another proteolytic cleavage within the transmembrane domain that releases the intracellular domain, N^{ICD} (Struhl and Greenwald, 2001). N^{ICD} then translocates to the nucleus and acts as a coactivator for the sequence-specific DNA-binding protein, Suppressor of Hairless [Su(H)] (Schroeter et al., 1998; Struhl and Adachi, 1998; Klein et al., 2000). This complex interacts with Mastermind and histone acetyl transferase, and activates the transcription of various target genes, including *vestigial* (*vg*), and multiple basic Helix-Loop-Helix and Bearded family genes in the *Enhancer of split* complex (Delidakis and Artavanis-Tsakonas, 1992; Kao et al., 1998; Wu et al., 2000; Lai et al., 2000).

Genetic and molecular studies in *Drosophila* have led to the identification of several additional components of the N signaling pathway. The *dx* gene encodes a cytoplasmic protein that binds to the intracellular domain of N and regulates N signaling in a positive manner (Xu and Artavanis-Tsakonas, 1990; Busseau et al., 1994; Diederich et al., 1994; Matsuno et al., 1995). In the Dx protein, domains involved in distinctive protein-protein interactions have been identified (Matsuno et al., 1997; Aravind, 2001; Matsuno et al., 2002). In addition, Dx has a RING-H2 finger motif often found in E3-ubiquitin ligase and human Dx homologs, the DTX proteins, were recently shown to have self-ubiquitination activity (Takeyama et al., 2003). However, the precise function of Dx in N signaling is still elusive.

Here, we investigated the function of *dx* during wing-margin development. *dx*, like *N* (reviewed by Brook et al., 1996; Cohen, 1996; Irvine and Vogt, 1997), was indispensable for this process. In addition, overexpressing Dx led to the expression of N downstream genes in the wing pouch of the third-instar wing disc. While the activation of vgBE by the

constitutively active N^{ICD} was eliminated in the *Su(H)* null mutant background, its activation by Dx was not. This is strong evidence that Dx-dependent N signaling occurs in a Su(H)-independent manner, as proposed previously (Ordentlich et al., 1998; Ramain et al., 2001; Hu et al., 2003). However, some of the nucleotide sequences in the Su(H)-binding site of vgBE were also required for this activation by Dx, leading us to speculate that this region might also bind to an as-yet-unidentified factor that mediates Dx-dependent signaling. Dx and N acted synergistically to increase N signaling. Furthermore, while Su(H) was dispensable for Dx-dependent N signaling, Dx required N to activate the expression of downstream N target genes. Our results also showed that the ectopic activation of N signaling associated with Dx overexpression occurred independent of the Dl/Ser ligands. We found that Dx promoted the relocation of N from the apical membrane to the late-endosome, where N was stabilized and co-localized with Dx. Finally, we demonstrated that blocking N trafficking to the late-endosome prevented the Dx-mediated activation of N signaling. Together, these results suggest that Dx-dependent activation of N, which is independent of Su(H), takes place in the late-endosomal compartments, unlike the N activation in Su(H)-dependent canonical N signaling, which is thought to occur at the plasma membrane (reviewed by Ray et al., 1999; Mumm and Kopan, 2000). This is a rare and perhaps unique example of two distinct signaling pathways downstream of a single receptor being activated in different membrane-bound compartments.

Materials and methods

Genetic strains

We used the following mutant alleles: dx^{24} (hypomorphic), $Su(H)^{\Delta 47}$ (null) (Morel and Schweisguth, 2000) and Dl^{REV10} and Ser^{RX106} double mutant (Ligoxygakis et al., 1998). We used the following enhancer trap lines: *vgBE-lacZ* (Kim et al., 1996), *vgBE Su(H)m-lacZ* (Kim et al., 1996), *wg-lacZ* (Kassis et al., 1992), *Dl-lacZ* (K. Matsuno, unpublished) and *N-lacZ* (de Celis et al., 1997). The UAS lines used were: *UAS-dx* (Matsuno et al., 2002), $UAS\text{-}dx^{\Delta Pro}$ (Matsuno et al., 2002), $UAS\text{-}N^{ICD}$ (Go et al., 1998), $UAS\text{-}N^{IR}$ (M. J. Go, unpublished), $UAS\text{-}N^{FL}$ (obtained from S. Artavanis-Tsakonas, unpublished), *UAS-Clc-GFP* (Chang et al., 2002), *UAS-Rab7-GFP* (Entchev et al., 2000) and $UAS\text{-}Rab5^{S43N}$ (Entchev et al., 2000) constructs. The UAS constructs were driven by *ptc-GAL4* (Johnson et al., 1995), *dpp-GAL4* (Klein and Arias, 1998), or *Act5C<FRT yellow$^+$ FRT>GAL4* (*AyGAL4*) (Ito et al., 1997), as indicated in the figure legends. The *AyGAL4* system was used to produce Flip-out GAL4 clones by a mechanism that combines the FLP/FRT and UAS/GAL4 systems. All crosses were cultured at 25°C unless otherwise stated.

Generation of mosaics

Mitotic clones were generated by Flp-mediated mitotic recombination (Xu and Rubin, 1993). Recombination was induced in the second-instar larvae by a 30-minute heat shock at 37°C. To generate the mutant clones of $Su(H)^{\Delta 47}$ in wing discs overexpressing Dx or N^{ICD} under the control of *dpp-GAL4*, $Su(H)^{\Delta 47}$ FRT^{40A}*/CyO,GFP; dpp-GAL4 vgBE-lacZ/TM6B* virgin females were crossed with either *UAS-dx/Y; Ubi-GFP* FRT^{40A}*/+; hsp70-flp/+* or *hsFLP/Y; Ubi-GFP* FRT^{40A}*/+;* $UAS\text{-}N^{ICD}$*/+* males, respectively. To generate the double mutant clones of Dl^{REV10} and Ser^{RX106} in wing discs overexpressing Dx under the control of *ptc-GAL4, hsFLP/+; ptc-GAL4/+; Ubi-GFP* FRT^{82B}*/TM6B* virgin females were crossed with *UAS-dx/Y; vgBE-lacZ/+;* Dl^{REV10}*,* Ser^{RX106} FRT^{82B}*/TM6B* males.

Generation of cells overexpressing Dx or N^{ICD}

Cells overexpressing Dx or N^{ICD} were generated using a technique that combines the FLP/FRT and UAS/GAL4 systems (Ito et al., 1997). To analyze the expression pattern of vgBE or *Dl* in the clones overexpressing Dx, *UAS-dx;; hsp70-flp* virgin females were crossed with either *AyGAL4 UAS-GFP/CyO; vgBE-lacZ/TM6B* or *AyGAL4 UAS-GFP/CyO; Dl-lacZ/TM6B* males, respectively. To analyze the expression pattern of *wg* in the clones overexpressing Dx, *UAS-dx/Y; wg-lacZ/+; hsp70-flp/+* males were crossed with *AyGAL4 UAS-GFP/CyO* virgin females. To analyze the expression pattern of *N* in the clones overexpressing Dx or N^{ICD}, *N-lacZ/FM6; AyGAL4 UAS-GFP/CyO* virgin females were crossed with either *UAS-dx;; hsp70-flp* or *hsp70-FLP1.22;;* $UAS\text{-}N^{ICD}$ males, respectively. Clones were induced 24-48 or 60-72 hours after egg laying by a 30-minute heat shock at 37°C, detected by the expression of GFP, and analyzed in third-instar larvae. To express N^+-GV3 in the eye imaginal discs of *GMR-dx* flies, *GMR-dx/CyO* virgin females were crossed with *y w;* $hs\text{-}N^+\text{-}GV3$ males (Struhl and Greenwald, 2001).

Immunohistochemistry and in situ hybridization

The wing imaginal discs dissected from third-instar larvae were stained as described previously (Matsuno et al., 2002). The following antibodies were used: rat anti-Dx (1:25) (Busseau et al., 1994); mouse anti-Wg (1:5) (van den Heuvel et al., 1989); mouse anti-Cut (1:100) (Jacobsen et al., 1998); mouse (Promega) and rabbit (Cappel) anti-β-GAL (1:1000); mouse anti-N^{ICD} (1:500) (Fehon et al., 1991); mouse anti-GAL4 (1:100) (Santa Cruz Biotechnology); and anti-Hook (1:500) (Kramer and Phistry, 1996). FITC- (Jackson Laboratories), Alexa 488- (Molecular Probes), rhodamine- (Chemicon) and Cy5- (Rockland) conjugated secondary antibodies were used at a dilution of 1:200. In situ hybridization with GAL4 or *wg* digoxigenin-labeled RNA probe was performed as described previously (Gonzalez-Crespo and Levine, 1993).

Detection of endocytic vesicles

Dissected third-instar larval disc complexes were incubated in 0.1 mg/ml fluorescein Dextran (3000 MW, anionic, lysine fixable; Molecular Probes) in M3 medium at 25°C for 10 minutes (pulse), then washed five times in ice-cold M3 medium. After a variable chase period (0-60 minutes), they were fixed as described previously (Matsuno et al., 2002). Dextran is taken up by endocytosis and marks progressively later endocytic compartments as the chase time is increased (Entchev et al., 2000). To visualize endocytic vesicles in the *Drosophila* cell line S2 overexpressing Dx, UAS-dx-YFP was driven by pWA-GAL4.

Results

dx is indispensable and sufficient for inducing wing-margin genes that are dependent on N signaling

Most of the existing *dx* alleles, such as dx^{24}, show recessive distal wing blade notching (Fig. 1B). This phenotype is similar to that of heterozygous *N* mutant alleles, suggesting the involvement of *dx* in N signaling during wing-margin development (Lindsley and Zimm, 1992). In wing-margin development, N signaling plays a pivotal role in the dorsoventral compartment boundary (DV boundary), which acquires an organizer-like function in the third-instar wing disc (de Celis et al., 1996; Neumann and Cohen, 1996). In the DV boundary, N is required for the localized expression of several genes that are essential for wing morphogenesis, including *wingless* (*wg*), *cut* and *vg* (Neumann and Cohen, 1996). To determine if the wing-blade notching phenotype of *dx* is a consequence of reduced N signaling in the DV boundary, we

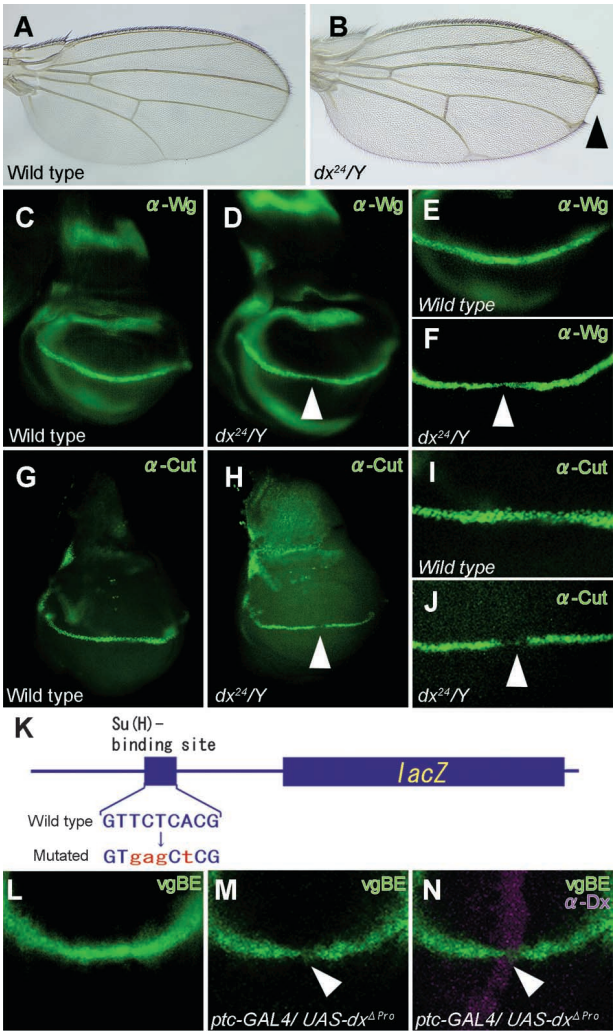


Fig. 1. *dx* is indispensable for the normal activation of N signaling at the DV boundary. (A) Wild-type adult wing. (B) Adult wing of dx^{24}/Y, a hypomorphic loss-of-function mutant of *dx*, showing recessive distal wing-blade notching (arrowhead). (C,E) Wg expression in the wild-type third-instar wing disc, detected in a narrow stripe along the DV boundary by an anti-Wg antibody (green). E is a higher magnification of C. (D,F) Wg expression (green) in the dx^{24}/Y third-instar wing disc, showing a reduction in the Wg protein at the intersection of the AP and DV boundaries that corresponds to the distal tip of the wing (black and white arrowhead in B,D,F). F is a higher magnification of D. (G,I) Cut expression in the wild-type third-instar wing disc, detected in a narrow stripe along the DV boundary by an anti-Cut antibody (green). I is a higher magnification of G. (H,J) Cut expression (green) in the dx^{24}/Y third-instar wing disc, showing an interruption at the intersection of the AP and DV boundaries that corresponds to the distal tip of the wing (black and white arrowheads in B,H,J). J is a higher magnification of H. (K) Schematic diagram of the *vgBE-lacZ* transgene constructs. Wild-type recognition sequences for Su(H) and its mutant derivative are shown in the upper and lower lines, respectively. Mutated nucleotides are shown in red. (L-N) Activation of vgBE at the DV boundary of the third-instar wing disc detected by *vgBE-lacZ* (green). (L) A wing disc of *vgBE-lacZ*. (M,N) A wing disc of vgBE-lacZ overexpressing $Dx^{\Delta Pro}$ (purple) under the control of *ptc-GAL4* at 18°C, showing suppression of vgBE activity in the region overexpressing $Dx^{\Delta Pro}$ (white arrowhead). M and N show a single green channel and a merged image, respectively.

examined the expression of Wg and Cut proteins in the wing disc of third-instar larvae. In the wild-type disc, Wg and Cut were detected in a narrow stripe along the DV boundary (Fig. 1C,E,G,I). Wg and Cut expression in the wing discs of third-instar dx^{24} larvae was reduced or interrupted (Fig. 1D,F,H,J). The *vg* gene, a target of N signaling, is an essential regulator of cell growth and differentiation in the *Drosophila* wing disc (Kim et al., 1996). Transcription of the *vg* gene within the wing disc is driven by two enhancers, the boundary and quadrant enhancers (Kim et al., 1996; Certel et al., 2000). The *vg* boundary enhancer (vgBE) is activated through Su(H)-dependent N signaling at the DV boundary of the third-instar wing disc (Kim et al., 1996). Activation of vgBE can be analyzed by detecting β-Galactosidase (β-GAL) driven by vgBE in a *vgBE-lacZ* transgenic line (Fig. 1K) (Kim et al., 1996). As reported previously and shown in Fig. 1L, vgBE is activated at the DV boundary in the wild-type mid-third-instar wing disc (Kim et al., 1996). Activation of vgBE in the dx^{24} disc was slightly but reproducibly reduced at the same stage (data not shown). Because the reduction of vgBE in dx^{24} was subtle, we expressed a dominant-negative form of Dx, $Dx^{\Delta Pro}$, to demonstrate a requirement of Dx for vgBE activation (Matsuno et al., 2002). Partial suppression of vgBE by $Dx^{\Delta Pro}$ was observed (Fig. 1M,N). These observations suggested that *dx* is required, at least in part, for the activity of N signaling during wing-margin formation.

Next, to study whether *dx* overexpression is sufficient to activate the N signaling pathway, we performed misexpression analyses by generating mosaic clones overexpressing Dx using a technique combining the FLP/FRT and UAS/GAL4 systems (Ito et al., 1997). Since endogenous Dx is expressed ubiquitously throughout the third-instar imaginal discs, the misexpression of Dx with GAL4 results in overexpression of the protein (Matsuno et al., 2002). First, we examined the effect of Dx overexpression on vgBE. vgBE was ectopically activated only in the cells overexpressing Dx (Fig. 2A-C), indicating that Dx function is cell autonomous. We also found that Dx overexpression was sufficient to ectopically activate *wg* gene expression, as detected by *wg-lacZ*, in a cell autonomous manner (Fig. 2D-F). Moreover, as shown in Fig. 2G-I, the expression of *Dl*, another known target gene of N signaling in this tissue, was also activated ectopically, as judged by *Dl-lacZ* (Panin et al., 1997). We noted that Dx overexpression weakly induced *Dl* expression cell non-autonomously and cell autonomously. This cell non-autonomous induction of *Dl* was probably due to the increased ectopic *wg* expression, which is known to activate Dl expression (Micchelli et al., 1997). In addition to its ligand, we found that the ectopic activation of N signaling upregulated the promoter activity of the *N* gene. As shown in Fig. 2K, promoter activity of the *N* gene was detected using an *N-lacZ* line. Misexpression of Dx (Fig. 2M-O) or N^{ICD} (Fig. 2P-R), activated the *N* promoter activity (Lieber et al., 1993; Struhl et al., 1993). Therefore, at least in some cellular contexts, the activation of N signaling induces the expression of both N and its ligand. Taken together, these results suggest that the overexpression of Dx ectopically activates the target genes of the N signal.

Dx activates N signaling in a Su(H)-independent manner

The activation of vgBE at the DV boundary is dependent on

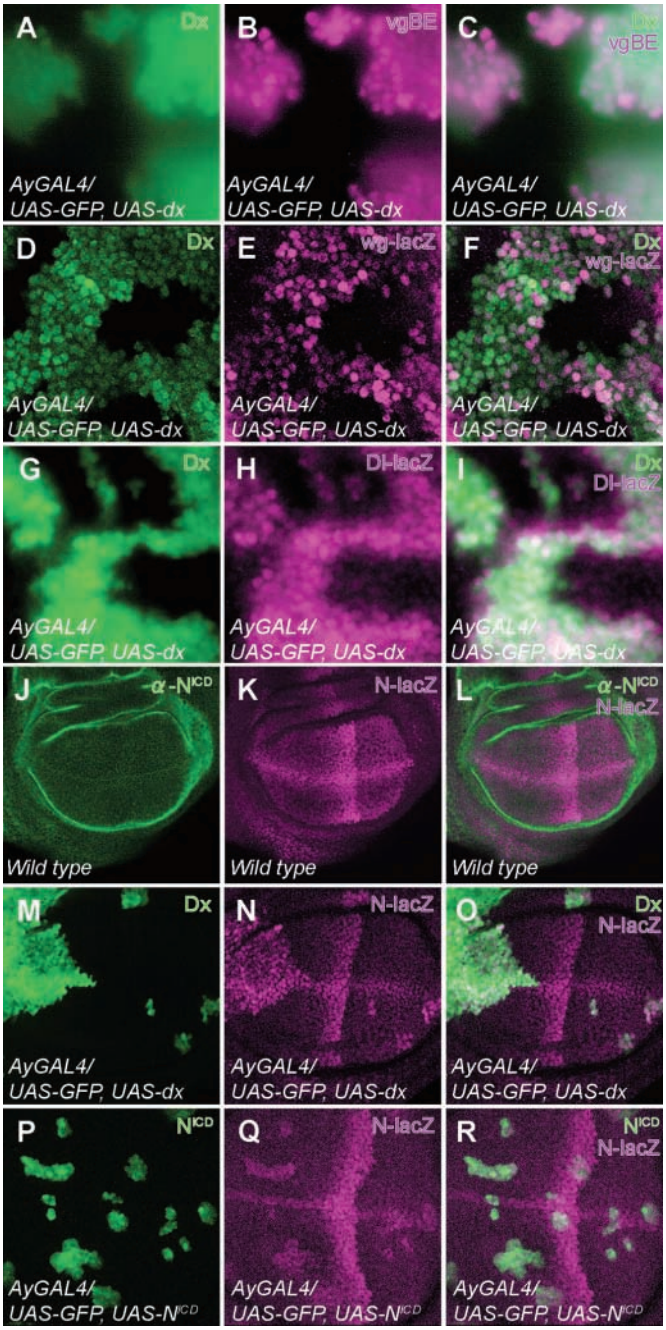


Fig. 2. Overexpression of Dx ectopically activates the target genes of N signaling. (A-C) Within the Dx-overexpressing clones (green), vgBE (purple) was activated in a cell autonomous manner. C is a merged image of A and B. (D-F) Within cells overexpressing Dx (green), the *wg* promoter was ectopically activated. The promoter activity of *wg* was detected by *wg-lacZ* (purple). F is a merged image of D and E. (G-I) Within cells overexpressing Dx (green), the *Dl* promoter was ectopically activated. The promoter activity of *Dl* was detected by *Dl-lacZ* (purple). I is a merged image of G and H. Weak cell non-autonomous induction of *Dl-lacZ* was also observed. (J,L) Expression of N in wild-type wing discs. N was detected by an anti-N^{ICD} antibody (green). (K,L) Activation of the *N* promoter was visualized by *N-lacZ* (purple). (M-O) Within the Dx-overexpressing clones (green), *N-lacZ* (purple) was activated in a cell autonomous manner. O is a merged image of M and N. (P-R) Within the N^{ICD}-overexpressing clones (green), *N-lacZ* (purple) was activated cell autonomously. R is a merged image of P and Q. All images were obtained from third-instar wing discs with respective genetic manipulation.

Su(H), the canonical effector protein of N signaling (Kim et al., 1996). However, several recent reports support the idea that Dx is involved in a Su(H)-independent signaling event (Ordentlich et al., 1998; Ramain et al., 2001). A Su(H)-binding site in vgBE is essential for its N-dependent activation at the DV boundary (Kim et al., 1996). To determine whether the induction of vgBE by Dx is also dependent on this Su(H)-binding site, we examined the effect of Dx on a mutant vgBE with point mutations at this site [*vgBE Su(H)m*] (Fig. 1K). These mutations completely abolish Su(H) binding, and vgBE Su(H)m is not activated by N signaling (Kim et al., 1996). Overexpression of Dx under the control of either *patched-GAL4* (*ptc-GAL4*) or *decapentaplegic-GAL4* (*dpp-GAL4*) (Johnson et al., 1995; Klein and Arias, 1998) directed the expression of Dx in a stripe along the anterior side of the anteroposterior compartment boundary (AP boundary) in third-instar wing discs (Fig. 3). Overexpression of Dx driven by *ptc-GAL4* and *dpp-GAL4* gave essentially the same results in the experiments below (data not shown). In agreement with our results above, overexpression of Dx resulted in the ectopic activation of vgBE along the AP boundary (compare Fig. 3A and B). On the other hand, as described previously, in the wing disc of third-instar larvae isolated from *vgBE Su(H)m-lacZ* transgenic lines, no induction of β-GAL along the DV boundary was detected (Fig. 3D). Notably, overexpression of Dx did not activate vgBE Su(H)m under the same conditions (Fig. 3E,F), indicating that the Su(H)-binding site of vgBE was also essential for the Dx-mediated signaling.

Thus, the ectopic activation of vgBE by Dx overexpression was dependent on the Su(H)-binding site. However, this does not necessarily mean that Su(H) is required for Dx to activate vgBE. We addressed this issue by generating *Su(H)* null mutant clones and analyzing the Dx activity in them. We found that in homozygous clones of a null allele, $Su(H)^{\Delta 47}$, Dx overexpression still activated vgBE (Fig. 3K-N). In contrast, as shown in Fig. 3G-J and reported previously (Lieber et al., 1993; Struhl et al., 1993), N^{ICD} failed to activate vgBE in these clones. We made similar observations using another strong loss-of-function allele of *Su(H)*, $Su(H)^{SF8}$ (data not shown). These results indicate that Dx activates vgBE in a Su(H)-independent manner. The different behavior of Dx and N^{ICD} in $Su(H)^{\Delta 47}$ homozygous clones was not attributable to their differential thresholds for inducing vgBE, because N^{ICD} was more potent than Dx in activating this enhancer (data not shown). Taken together, these results suggest that the downstream N activity that is induced by Dx expression depends on a mechanism distinct from that induced by N^{ICD}. This distinct activity may involve an unknown factor that interacts with vgBE at the same binding site as Su(H) or an overlapping one.

The Su(H)-independent function of Dx raised the possibility that Dx may not be directly involved in N signaling. Therefore, we examined whether the activity of Dx is dependent on N. Overexpression of Dx under the control of *ptc-GAL4* resulted in the ectopic activation of Wg, mostly, but not exclusively, in the ventral compartment (Fig. 3O). However, this preferential

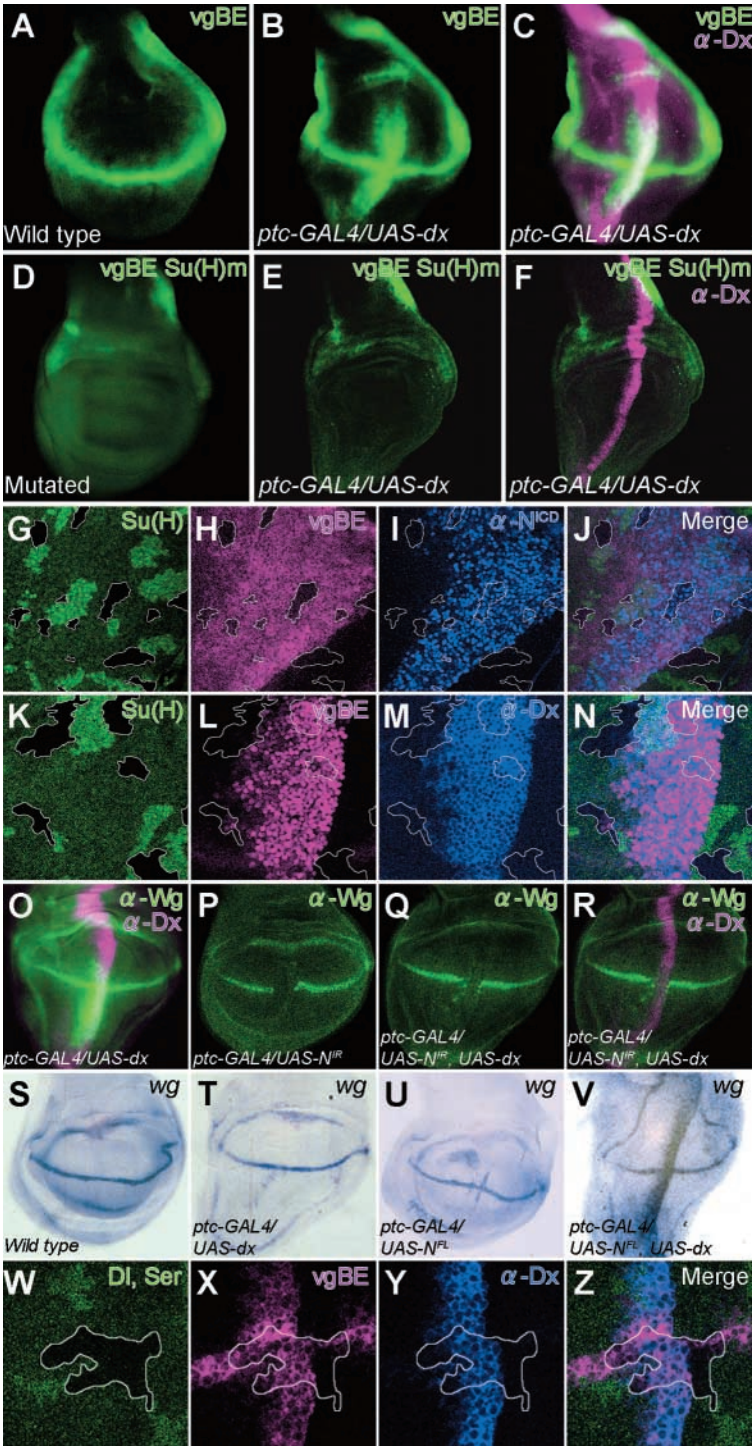


Fig. 3. Dx activates N signaling in a Su(H)-independent manner. (A) A mid-third-instar wing disc of *vgBE-lacZ*. β-GAL protein is shown in green. (B,C) Overexpression of Dx (purple) along the AP boundary in a mid-third-instar wing disc ectopically activated the vgBE (green) in both dorsal and ventral compartments of the wing pouch. B and C show a single green channel and a merged image, respectively. (D) In the wing disc dissected from a *vgBE Su(H)m-lacZ* transgenic line, β-GAL (green) was not detected along the DV boundary. (E,F) Overexpression of Dx (purple) along the AP boundary did not activate *vgBE Su(H)m-lacZ* (green). E and F show a single green channel and a merged image, respectively. (G-J) Within $Su(H)^{\Delta 47}/Su(H)^{\Delta 47}$ mutant clones (marked by an absence of green fluorescence) generated in the late-third-instar wing disc, an overexpression of N^{ICD} (blue) failed to activate the vgBE along the AP boundary (purple). J is a merged image of G, H and I. (K-N) Within $Su(H)^{\Delta 47}/Su(H)^{\Delta 47}$ mutant clones (marked by an absence of green fluorescence) generated in the late-third-instar wing disc, an overexpression of Dx (blue) still activated the vgBE (purple). N is a merged image of K, L and M. (O) Overexpression of Dx (purple) along the AP boundary induced ectopic expression of Wg mostly, but not exclusively, in the ventral region of the wing pouch (green). (P) Misexpression of double-strand RNA of *N* along the AP boundary resulted in the suppression of Wg expression (green) in the late-third-instar wing disc. (Q,R) Overexpression of Dx (purple) with double-strand RNA of *N* failed to induce the ectopic expression of Wg (green) in the late-third-instar wing disc. Q and R show a single green channel and a merged image, respectively. (S-V) Expression of the *wg* gene (blue) detected by in situ hybridization in the late-third-instar disc. (S) *wg* expression in the wild-type wing disc. (T) Overexpression of Dx along the AP boundary induced a weak ectopic *wg* expression mostly in the ventral region of the wing pouch. (U) Overexpressed N^{FL} induced a weak ectopic *wg* expression. (V) Co-expression of Dx and N^{FL} resulted in a synergistic enhancement of N signaling revealed by strong *wg* expression along the AP boundary. (W-Z) Within Dl^{REV10} and Ser^{RX106} double mutant clones (marked by an absence of green fluorescence) generated in the late-third-instar wing disc, an overexpression of Dx (blue) still activated the vgBE (purple). Z is a merged image of W, X and Y. *UAS-dx* (B,C,E,F,O-R,T-V,W-Z) and *UAS-*N^{IR} (P-R) were driven by *ptc-GAL4*. *UAS-dx* (K-N) and *UAS-*N^{ICD} (G-J) were driven by *dpp-GAL4*.

activation of Wg was not strictly determined, because in cells overexpressing Dx the *wg* promoter was activated equally well in the dorsal and ventral compartments (data not shown). This Dx activity was then examined when N was knocked down by RNA interference. An inverted repeat RNA corresponding to N (N^{IR}) was co-expressed with Dx, under the control of *ptc-GAL4*. Under this condition, N protein was barely detected in the region expressing N^{IR} (data not shown). Co-expression of N^{IR} inhibited the ectopic activation of *wg* associated with the overexpression of Dx, as well as endogenous *wg* expression (Fig. 3Q,R). The ectopic activation of vgBE by Dx was also abolished by co-expression with N^{IR} (data not shown). These results indicate that Dx function requires N and suggest that Dx functions upstream of N. In agreement with this, we found that co-expression of Dx and N showed a synergistic effect on the ectopic activation of wg (Fig. 3T-V). Similar synergistic activation was observed using vgBE (data not shown). In contrast, we found that the ectopic activation of vgBE by the overexpression of Dx occurred independently of the Dl/Ser ligands. In clonal cells simultaneously homozygous for both Dl and Ser, Dx overexpression still activated vgBE equally well as in wild-type cells (Fig. 3W-Z).

Dx promotes the relocalization of N from the apical plasma membrane to the intracellular vesicles

Based on the finding that Dx functions upstream of N, we decided to examine the possibility that Dx might affect the cellular N protein directly. In epidermal cells, the N protein

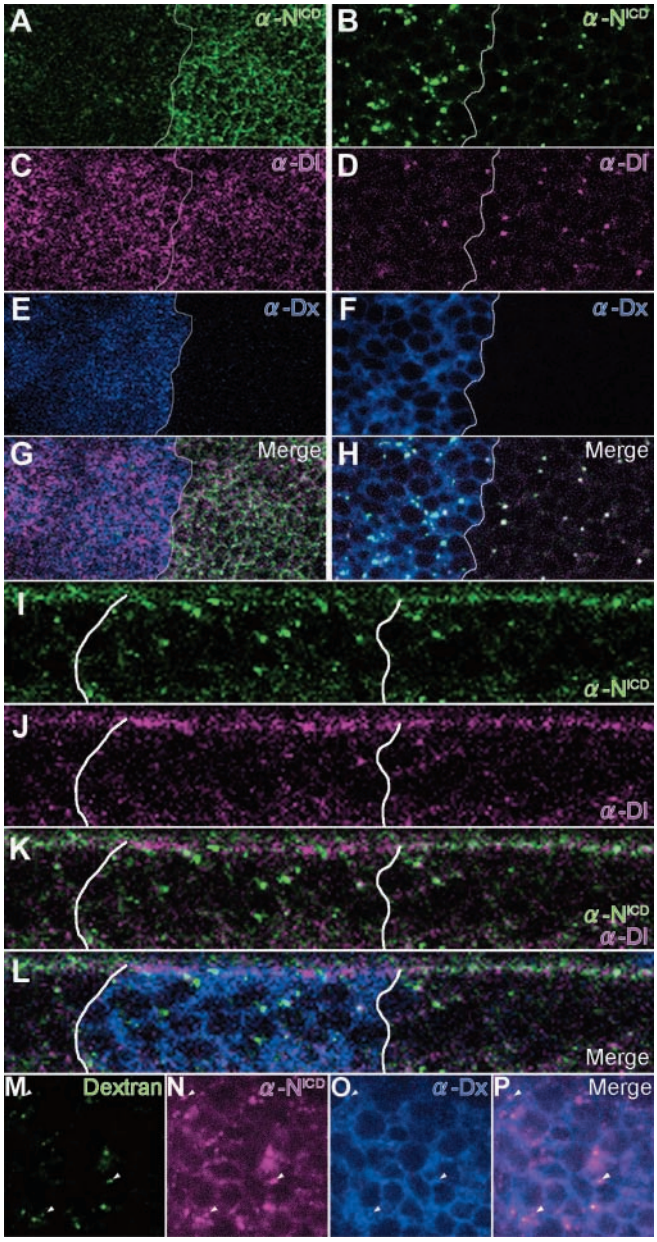


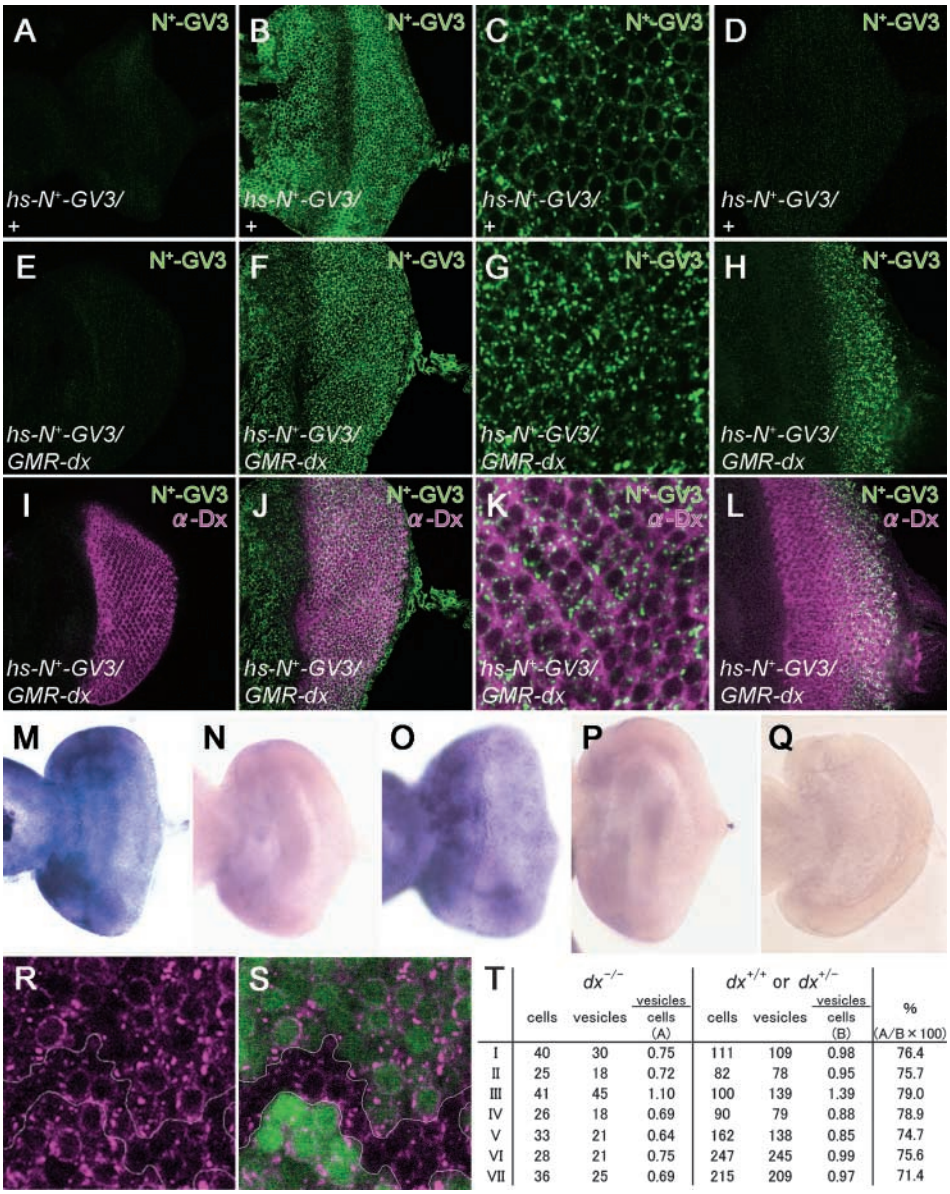
Fig. 4. Dx modulates the intracellular distribution of N. (A-L) Confocal microscopic images of third-instar wing discs overexpressing Dx. The boundaries of the region overexpressing Dx are shown by white lines. (A,C,E,G) Overexpression of Dx (blue in E and G) resulted in the depletion of N (green in A and G), but not Dl (purple in C and G), from the apical cell surface. G is a merged image of A, C and E. (B,D,F,H) In the basal plane, overexpression of Dx (blue in F and H) increased N (green in B and H) in intracellular vesicles. In the cells overexpressing Dx, Dx, but not Dl, was frequently co-localized with N in these vesicles (the left part of B-H). In contrast, N co-localized with Dl in the wild-type cells (the right part of B-H). Similar results were obtained when N was stained either with antibodies against the intracellular domain or against the extracellular domain of N. H is a merged image of B, D and F. (I-L) An optical cross-section of a wing disc overexpressing Dx, stained as in A-H. N (green) and Dl (purple) staining are shown in I and J, respectively. K is a merged image of I and J. L shows Dx (blue) staining merged with K. (M-P) A third-instar wing disc overexpressing Dx (blue) was incubated with fluorescein Dextran (green) to label the endocytic compartments. Fluorescein Dextran (green) and endogenous N (purple) were co-stained. Some of the vesicles containing Fluorescein Dextran and N, although not all, were also marked with Dx (shown by white arrowheads). P is a merged image of M, N and O. *UAS-dx* was driven by *ptc-GAL4*.

was localized to the apical lateral adhesion junction with only a small proportion in the basal intracellular vesicles (Fig. 4A-L). This distribution is consistent with previous reports (Fehon et al., 1991). However, when Dx was overexpressed, N was considerably depleted from the cell surface (Fig. 4A). In the basal region of Dx-expressing cells, vesicular staining of N became more prominent (Fig. 4B). In the optical vertical section, the relocalization of N protein from the apical surface to basal intracellular vesicles was observed in the region overexpressing Dx (Fig. 4I). The numbers of N-containing vesicles in the wild-type and Dx-overexpressing cells were counted to quantify this result. Given the number in each wild-type cell as 100, each Dx overexpressing cell had 269±18 vesicles. In contrast, the number of Dl-containing vesicles was not altered significantly in the Dx-overexpressing cells (114±38). Next, we analyzed the nature of these vesicles. Fluorescent Dextran added extracellularly was internalized in the vesicles containing N, indicating that these vesicles were of endocytic origin (Fig. 4M,N). Dx was often but not always associated with these vesicles (Fig. 4O). Markers for the endoplasmic reticulum (ER) and Golgi apparatus did not overlap with these vesicles (data not shown). It has been reported that mammalian homologs of Dx are localized to the nucleus in cultured cells (Yamamoto et al., 2001; Hu et al., 2003). In contrast, we did not observe the nuclear localization of *Drosophila* Dx under any of the conditions tested in vivo or in cultured cells (data not shown). We also noted that, in the basal region of wild-type cells, the Dl protein was mostly co-localized with N in intracellular vesicles (Fig. 4B,D). In contrast, in Dx-overexpressing cells, the Dl protein was not detected in the vesicles containing N, but accumulated slightly at the apical surface (Fig. 4B-D). These results suggest that Dx selectively affects the relocalization of N to the basal intracellular vesicles.

Dx stabilizes N in intracellular vesicles

Our results raised the possibility that Dx promotes the activation of Su(H)-independent N signaling by relocating N to endocytic vesicles. However, such action is apparently contradictory to the observation that Dx activates N signaling, because receptors transported to the endocytic pathway are often marked for degradation as a way to downregulate signaling (reviewed by Gruenberg, 2001). Therefore, we next examined whether N internalization could be linked to altered protein stability. Because ectopic Dx induced endogenous *N* expression (Fig. 2M-O), we were unable to investigate the effect of Dx on N stability by detecting endogenous protein. Thus, we used N^+-GV3, a heat-shock promoter-inducible chimeric protein of N that contains GAL4-VP16 (GV) inserted after the transmembrane domain in an otherwise wild-type N protein (Struhl and Adachi, 1998). It was previously confirmed that N^+-GV3 functions like wild-type N in vivo. The N^+-GV3 protein could be distinguished from endogenous N by anti-GAL4 antibody staining. To determine the effect of Dx on N^+-

GV3, we expressed Dx in the cells posterior to the morphogenetic furrow in the eye imaginal discs, under the control of the GMR promoter (Hay et al., 1994). We expressed N^+-GV3 under control of a heat-shock promoter in wild-type and *GMR-dx* eye imaginal discs, then examined the distribution of N^+-GV3. Before heat-shock treatment, no N^+-GV3 expression was detected in the eye imaginal discs (Fig. 5A,E,I). Thirty minutes after heat shock, we detected N^+-GV3 throughout the wild-type eye imaginal disc, except for the morphogenetic furrow (Fig. 5B,C). N^+-GV3 was primarily localized to the plasma membrane and small vesicles. N^+-GV3 in the wild-type eye imaginal disc disappeared 12 hours after heat shock, probably due to the degradation of N (Fig. 5D). In *GMR-dx* eye discs, N^+-GV3 was also detected throughout the eye imaginal disc 30 minutes after heat shock (Fig. 5F,J). In agreement with our results in the wing disc, in the region expressing Dx, N^+-GV3 was not localized to the plasma membrane but to vesicles (Fig. 5G,K); furthermore, it was still detected in the vesicles 12 hours after heat shock (Fig. 5H,L), unlike in the wild-type eye discs (Fig. 5D). Note that cells in the adjacent region posterior to the morphogenetic furrow did not contain detectable N^+-GV3 12 hours after heat shock (Fig. 5H,L), because these cells did not yet express Dx during the heat-shock treatment. The transcriptional efficiency of *N^+-GV3* mRNA did not contribute to this difference, because, as seen in the eye imaginal disc 30 minutes after heat shock, the amount of N^+-GV3 protein was comparable in wild-type and Dx-overexpressing eye imaginal discs (Fig. 5B,F). Furthermore, in situ hybridization using a probe for GAL4 revealed that the amount and stability of *N^+-GV3* mRNA did not differ substantially in these two discs (Fig. 5M-Q). Consistent with the results obtained with N^+-GV3, the ectopic



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	$dx^{-/-}$			$dx^{+/+}$ or $dx^{+/-}$			
	cells	vesicles	vesicles/cells (A)	cells	vesicles	vesicles/cells (B)	% (A/B × 100)
I	40	30	0.75	111	109	0.98	76.4
II	25	18	0.72	82	78	0.95	75.7
III	41	45	1.10	100	139	1.39	79.0
IV	26	18	0.69	90	79	0.88	78.9
V	33	21	0.64	162	138	0.85	74.7
VI	28	21	0.75	247	245	0.99	75.6
VII	36	25	0.69	215	209	0.97	71.4

Fig. 5. Overexpressed Dx increases the number of endocytic vesicles containing N and stabilizes the N in these vesicles. (A-L) The expression of N^+-GV3 protein (green) in eye discs was detected by anti-GAL4. Dx was driven by the GMR promoter (purple in I-L). (A,E,I) Wild-type (A) and *GMR-dx* (E,I) eye discs before heat shock. No expression of N^+-GV3 (green) was detected. I is a merged image. (B,C) In wild-type eye discs, N^+-GV3 expression (green) was observed throughout the eye disc, except for the morphogenetic furrow, 30 minutes after heat shock. The N^+-GV3 expression was primarily localized to the plasma membrane and within a small number of intracellular vesicles. C is a higher magnification of B. (D) In the wild-type eye disc, no N^+-GV3 expression (green) was observed 12 hours after heat shock. (F,G,J,K) In *GMR-dx* eye discs, N^+-GV3 expression (green) was observed throughout the eye disc, except for the morphogenetic furrow, 30 minutes after heat shock. In the region expressing Dx (purple), N^+-GV3 expression was primarily localized to the intracellular vesicles. G and K are a higher magnification of F and J, respectively. J and K are merged images. (H,L) In the region expressing Dx (purple), N^+-GV3 (green) was still detected. L is a merged image. (M-Q) In situ hybridization of wild-type (M,N,Q) and *GMR-dx* (O,P) eye discs using antisense (M-P) and sense (Q) GAL4 probes. *N^+-GV3* mRNA was detected using the antisense GAL4 probe. (M,O) In wild-type and *GMR-dx* eye discs, *N^+-GV3* mRNA was observed throughout the eye disc 30 minutes after heat shock. (N,P) In wild-type and *GMR-dx* eye discs, no N^+-GV3 mRNA was detected 12 hours after heat shock. (Q) In situ hybridization using a sense-strand probe for GAL4. (R,S) Within the dx^-/dx^- clones (patches without green fluorescence), the number of vesicles containing N^+-GV3 (purple) was reduced, compared with wild-type and $dx^-/+$ cells. (T) The number of vesicles containing N^+-GV3 per cell was compared in seven independent measurements. The relative number of vesicles in the dx^-/dx^- cells are shown as percentages.

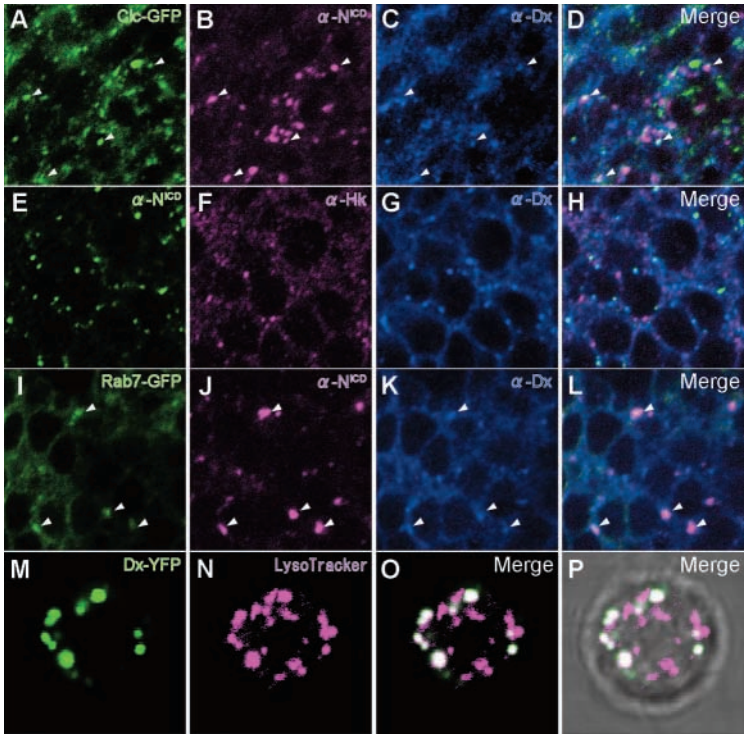

Fig. 6. Dx co-localizes with N in the late-endosomes. (A-L) Confocal microscopic images of third-instar wing discs overexpressing Dx. (A-D) Some of the Dx (blue in C and D) immunoreactivity co-localized with Clc-GFP (green in A and D) and N (purple in B and D). D is a merged image of A, B and C. (E-H) *Drosophila* Hk exists exclusively in the early-endosomes. Neither N (green in E and H) nor Dx (blue in G and H) co-localized with Hk (purple in F and H). H is a merged image of E, F and G. (I-L) *Drosophila* Rab7-GFP accumulates in the late-endosomal compartment. Dx (blue in K and L) and endogenous N (purple in J and L) co-localize in the late-endosome (green in I and L). L is a merged image of I, J and K. (M-P) A fusion protein of Dx and YFP (green in M, O and P) was expressed in the *Drosophila* S2 cell line. The lysosomes were visualized by LysoTracker (purple in N, O and P). O is a merged image of M and N. P is a merged picture of the optical microscopic image of a cell with O. (A-L) *UAS-dx* was driven by *ptc-GAL4*. (M-P) UAS-YFP-dx was driven by pWA-GAL4.

expression of Dx led to the accumulation of the endogenous N protein (data not shown). From these observations, it is likely that Dx directly or indirectly prolongs the half-life of N in these vesicles, consequently leading to the accumulation of N in them, at least under conditions involving the overexpression of these proteins.

We next examined whether Dx is required for the presence of N in these vesicles. We generated dx^{-}/dx^{-} clones in imaginal discs and overexpressed N^{+}-GV3 under the control of the heat-shock promoter (Fig. 5R,S). The numbers of N-containing vesicles per cell were compared (Fig. 5T). The average number of vesicles in each dx^{-}/dx^{-} cell was 76.1±2.58% of that in wild-type and $dx^{-/+}$ cells. A paired *t*-test showed that this reduction was statistically significant ($P<0.01$). Therefore, *dx* is partly required for the internalization and/or stabilization of N in these vesicles. This result also suggested that *dx* is not essential for the formation of N-containing vesicles, because a significant number of vesicles were formed in the dx^{-}/dx^{-} clones (Fig. 5R,S).

Transportation of N to the late-endosome is required for Dx-mediated activation of the N signal

We next attempted to define the nature of the endocytic vesicles associated with Dx-mediated signaling. Clathrin-dependent endocytosis involves the formation of vesicles with a clathrin coat, which is visualized by clathrin-GFP (Chang et al., 2002). Most of the vesicles labeled with Clathrin-GFP did not stain for N, although a small population of clathrin-positive vesicles did contain it (Fig. 6A-D). The N protein did not co-localize with Hook (Hk), a marker for early-endosomes (Fig. 6E-H) (Kramer and Phistry, 1996). However, N and Dx co-localized with Rab7-GFP, a marker for late-endosomes (approximately 80% of the Rab7-GFP-positive vesicles were also N-positive) (Fig. 6I-L) (Entchev et al., 2000). Dx-YFP also co-localized with vesicles stained with LysoTracker, a marker for acidic intracellular vesicles, which are mostly late-endosomes and lysosomes in the *Drosophila* S2 cell line (Fig. 6M-O). These results suggest that Dx probably promotes the accumulation of N in the late-endosomal compartment, where Dx co-localizes with N.

Next, we wished to find out whether the accumulation of N in the late-endosome is an essential process for Dx-mediated signaling. Rab5 is a small GTPase that is necessary for endosome fusion to form early-endosomes (reviewed by Clague and Urbe, 2001). Expression of a dominant-negative form of Rab5 ($Rab5^{S43N}$) blocks the early steps of endocytosis, including internalization and early-endosome fusion (Entchev et al., 2000). In wild-type discs, the ectopic expression of $Rab5^{S43N}$ under the control of *dpp-GAL4* did not show a detectable effect on vgBE activation (Fig. 7A-C). When $Rab5^{S43N}$ was co-expressed with Dx, the ectopic activation of vgBE associated with Dx overexpression was inhibited (compare Fig. 3B,C,L,M with Fig. 7D-F). Under this condition, N accumulated in unusually large Hk-positive vesicles located in the apical region of the epithelial cells, which were not observed in the wild-type discs (Fig. 7G-J). In the basal region of these cells, vesicles containing N had disappeared (Fig. 7K-N), in contrast to the basal late-endosome accumulation of N in cells overexpressing Dx alone (Fig. 4B,H and Fig. 6I-L). Thus, $Rab5^{S43N}$ appeared to interfere with the transportation of N from the plasma membrane to the late-endosome. This result suggests that the relocation of N from the apical plasma membrane to the late-endosome is essential for Su(H)-independent N signaling that is mediated by Dx.

Discussion

Dx activates a Su(H)-independent N signal

Recent studies suggest that Dx might not participate in the canonical N pathway (Ordentlich et al., 1998; Ramain et al., 2001; Yamamoto et al., 2001; Hu et al., 2003). In *Drosophila*, it was suggested that Dx has a Su(H)-independent function in the development of bristles on the notum and the eye (Ramain et al., 2001). Here, we showed that a null mutation of *Su(H)* prevented N^{ICD} from activating vgBE, but the same mutation did not interfere with the Dx-dependent activation of the same

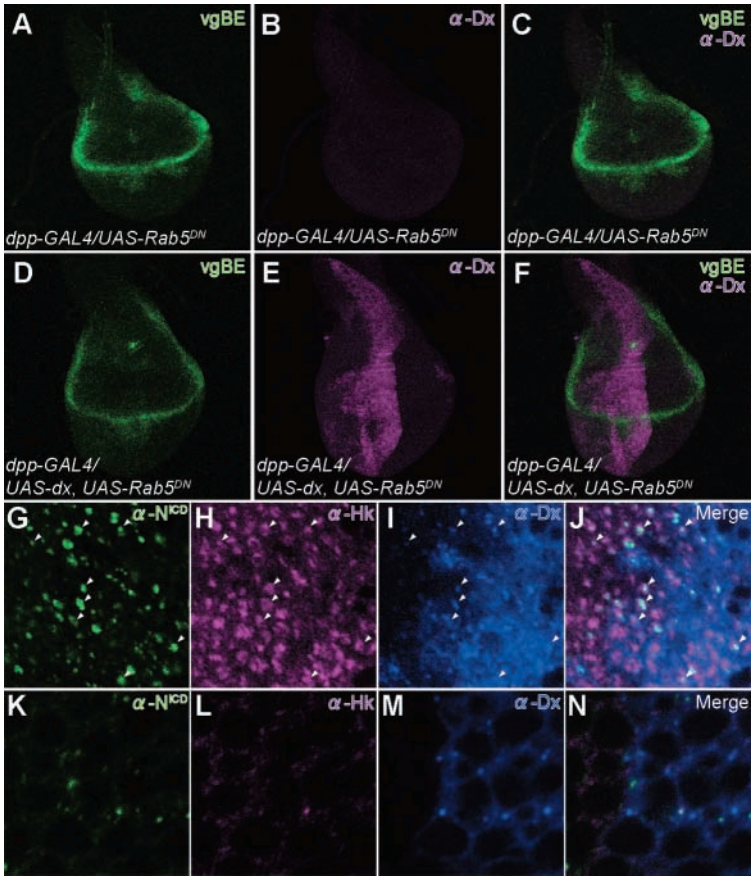

Fig. 7. Blockage of N delivery to the late-endosome inhibits the activation of Dx-mediated N signaling. (A-C) Overexpression of $Rab5^{S43N}$, a dominant-negative form of Rab5, along the AP boundary of the third instar wing disc did not show a detectable effect on endogenous vgBE activation (green in A and C). Endogenous expression of Dx was also detected (purple in B and C). (D-F) Co-expression of Dx (purple in E and F) and $Rab5^{S43N}$ along the AP boundary resulted in inhibition of the ectopic activation of vgBE (green in D and F) associated with Dx overexpression. (G-J) In the apical region of the epithelial cells overexpressing Dx (blue in I and J) with $Rab5^{S43N}$, N (green in G and J) accumulated in unusually large Hk-positive vesicles (purple in H and J), where Dx was co-localized with N (shown by white arrowheads). (K-N) In the basal region of the cells overexpressing Dx (blue in M and N) with $Rab5^{S43N}$, the accumulation of N (green in K and N) in the late-endosomes was not detected (compare to Fig. 4B). *UAS-dx* and *UAS-Rab5*^{S43N} were driven by *dpp-GAL4* (A-F) or *ptc-GAL4* (G-N).

vgBE construct. This finding indicated that the Dx-induced signaling occurred by a mechanism that is independent of Su(H), although our results do not exclude the possibility that Dx also contributes to Su(H)-dependent N signaling. On the other hand, we also found that vgBE Su(H)m, which has mutations in the Su(H)-binding site, was not activated by either N^{ICD} or Dx. Thus, we speculate that Dx signaling is mediated by another factor that recognizes a DNA sequence that overlaps with the Su(H)-binding site. Investigation of another protein that binds to the DNA sequence around the Su(H)-binding site of vgBE may allow us to identify a novel effector protein involved in Dx-mediated N signaling. Based on the mutant phenotypes of *dx* and *Su(H)*, the Dx-mediated Su(H)-independent pathway is probably only critical in a small subset of N functions in *Drosophila*, although a null mutation allele of *dx* has not been reported.

Dx regulates the membrane trafficking of N

Here, we demonstrated that the overexpression of Dx depleted N from the apical cell surface and increased the number of endocytic vesicles containing N. Dx extended the half-life of N, although it was not clear whether this was due to the prolonged half-life of the vesicles or to stabilization of the N protein itself inside them. N accumulated in the late-endosomal compartment, which was identified by the Rab7-GFP marker. Several models could explain this accumulation of N. First, Dx may promote the initiation of endocytic vesicle formation. However, we think this is unlikely, because we did not observe an increase in N-containing vesicles at the early stage of hs-N^{+}-GV3 turnover (data not shown). Second, Dx may interfere with membrane-trafficking, consequently preventing N from becoming degraded, or sustaining the half-life of vesicles containing N. There is accumulating evidence that the degradation of many transmembrane receptors, which leads to the downregulation of signaling, occurs in the lysosome. Thus, we speculate that Dx interferes with the delivery of N to the lysosome. In *dx* mutant cells, we observed a reduced number of N-containing vesicles, which is consistent with our idea that in wild-type cells, Dx also prevents N from relocating to the lysosomes, where it would be degraded. In *Drosophila*, it is known that Scabrous and Gp150, which localize to the late-endosome, negatively regulate N signaling; however, whether there is any functional relationship between Dx and these proteins remains to be studied (Li et al., 2003). In addition, Dx may play a role in receptor recycling, another process known to involve protein sorting to multivesicular bodies (MVBs), given that N at the apical plasma membrane was significantly depleted by Dx overexpression. However, the precise functions of Dx in these poorly understood processes remain to be addressed.

Two distinct N signaling pathways may be activated in different membrane compartments

It is known that receptor-mediated signaling can be upregulated by the inhibition of receptor degradation by preventing its endosome-to-lysosome delivery (Entchev et al., 2000). Although Dx overexpression resulted in the accumulation of N in the late-endosome, our results suggest that this triggered a signaling event that was distinct from canonical N signaling, rather than merely upregulating signaling by increasing the availability of N. Indeed, we found that the consequence of overexpressing full-length N was very different from that of overexpressing Dx [figure 5F in Matsuno et al. (Matsuno et al., 2002)]. In this respect, it is notable that two contradictory views have been reported regarding the intracellular compartments where Presenilin cleaves N in mammalian cells, although this issue has not been addressed in *Drosophila*. One view is that the cleavage of N occurs at the plasma membrane (Ray et al., 1999; Brown et al., 2000), while another group showed that Presenilin has a low optimal pH, raising the possibility that it is active in the acidic endocytic

compartments, such as late-endosomes (Pasternak et al., 2003; Gupta-Rossi et al., 2004). This discrepancy can be resolved by a hypothesis that two distinct N signaling pathways are executed in different membrane-bound compartments. Namely, the Su(H)-dependent canonical pathway and the Dx-mediated signaling pathway occur at the plasma membrane and the late-endosome, respectively. However, the biochemical mechanism of N activation in the late-endosomal compartment is virtually unknown. We also found that the ectopic activation of N signaling associated with Dx overexpression does not depend on the Dl/Ser ligands, which has been suggested before (Ramain et al., 2001). However, it was recently reported that F3/Contactin, a novel ligand for mammalian N, specifically activates Dx-mediated N signaling (Hu et al., 2003). Therefore, *Drosophila* Dx may need an F3/Contactin ortholog to activate vgBE. It is possible that the Su(H)-dependent and -independent N pathways are selectively activated by specific sets of N ligands, such as Dl/Ser and F3/Contactin.

In *Drosophila*, the *dx* wing-margin phenotype is completely suppressed by mutations of *Suppressor of deltex* [*Su(dx)*], which encodes a HECT domain E3 ubiquitin ligase, and this product binds to the intracellular domain of N (Fostier et al., 1998; Cornell et al., 1999). Indeed, itch, a mouse homolog of Su(dx), binds to the intracellular domain of mouse notch-1 through its WW domains and promotes the ubiquitination of N (Qiu et al., 2000). Recently, it was shown that the mono-ubiquitination of transmembrane proteins facilitates their incorporation into endocytic vesicles and lysosomal delivery (reviewed by Katzmann et al., 2002). Given that Dx is also an E3 ubiquitin ligase and affects membrane trafficking, a balance between Dx and Su(dx) activity may be important for controlling the rate of lysosomal delivery. Studies in progress should increase our understanding of the trafficking of N protein, which is probably a pivotal element in both the positive and negative regulation of N signaling.

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