nature cell biology

Regulation of Notch signalling by non-visual β-arrestin

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Signalling activity of the Notch receptor, which plays a fundamental role in metazoan cell fate determination, is controlled at multiple levels. We uncovered a Notch signal-controlling mechanism that depends on the ability of the non-visual β-arrestin, Kurtz (Krz), to influence the degradation and, consequently, the function of the Notch receptor. We identified Krz as a binding partner of a known Notch-pathway modulator, Deltex (Dx), and demonstrated the existence of a trimeric Notch–Dx–Krz protein complex. This complex mediates the degradation of the Notch receptor through a ubiquitination-dependent pathway. Our results establish a novel mode of regulation of Notch signalling and define a new function for non-visual β-arrestins.

The Notch receptor is the central element of an evolutionarily conserved signalling mechanism that has a fundamental role in metazoan development. Notch signalling guides the establishment of distinct developmental cell lineages, as well as differentiation, proliferation and apoptotic events[1]. Malfunction of Notch leads to mutant phenotypes that affect a broad spectrum of cell types, whereas Notch-signalling abnormalities have been associated with diverse pathologies, including tumorigenesis, in humans[2].

Normal Notch function is sensitive to the gene dosage of the Notch receptor and other Notch-pathway elements. For example, both *Drosophila melanogaster* and humans display haploinsufficiency for the Notch-pathway ligands, whereas the gene that codes for the single Notch receptor in flies is not only haploinsufficient but is also associated with a mutant phenotype if the animal carries three, as opposed to the normal two, copies of the gene[3]. This exquisite sensitivity to dosage seems to be controlled by a complex and poorly understood repertoire of mechanisms that evolved to modulate the levels of the receptor and its ligands on the cell surface, as well as to provide links between Notch signalling and other cellular processes.

In an effort to identify novel components that are involved in Notch signalling and its regulation, we carried out biochemical screens to define the molecular partners of established Notch-pathway elements. We identified Kurtz (Krz), the single *Drosophila* homologue of mammalian non-visual β-arrestins[4], as an interacting partner of Deltex (Dx), a known modulator of Notch activity[5,6]. The function of Dx is not well understood, but several lines of evidence indicate that it may have an E3 ubiquitin-ligase activity[7–9].

The mammalian non-visual arrestins, β-arrestin-1 and β-arrestin-2, have a major role in desensitization and endocytosis of G-protein-coupled receptors (GPCRs)[10,11]. In addition, β-arrestins have recently been implicated in the regulation of other types of receptors[12–17], as well as being shown to function as signalling adaptors that connect GPCRs with other signalling pathways[18,19].

Here, we provide evidence that directly implicates Krz in the regulation of Notch signalling. We demonstrate that loss of *krz* function results in an accumulation of the Notch receptor and consequently, depending on the cellular context, an upregulation of markers of Notch-signalling activity. Using molecular and genetic analyses, we found that Krz, Dx and Notch form a protein complex that mediates the degradation of the Notch receptor. We, therefore, define a novel Notch-signal attenuation mechanism and a new role for non-visual β-arrestin as a regulator of Notch signals.

RESULTS

Identification of the non-visual arrestin, Krz, as a Dx interactor

In an effort to identify elements that are integrated into the molecular circuitry affecting Notch signalling, we carried out two independent protein-interaction screens: one based on the yeast two-hybrid system[20], and the other based on the identification of cellular protein complexes using the tandem affinity purification (TAP)-liquid chromatography (LC)-mass spectrometry (MS)/MS approach[21] (see Methods). Both methods identified Krz as an interacting partner of Dx.

A yeast two-hybrid screen of 6×10^6 cDNAs from a *Drosophila* 0–24 h embryonic library was carried out using full-length Dx as bait. Eight positive clones (His^+, see Methods) were isolated and found to encode overlapping *krz* cDNAs. Sequence analysis revealed that the amino-terminal half of Krz (amino acids 10–251) is necessary and sufficient for binding Dx (Fig. 1a). The corresponding domain of mammalian non-visual β-arrestins, which consists of the amino-terminal half of the protein, was shown to interact with activated GPCRs[11].

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Received 6 September; accepted 24 October 2005; published online: 13 November 2005; DOI: 10.1038/ncb1327

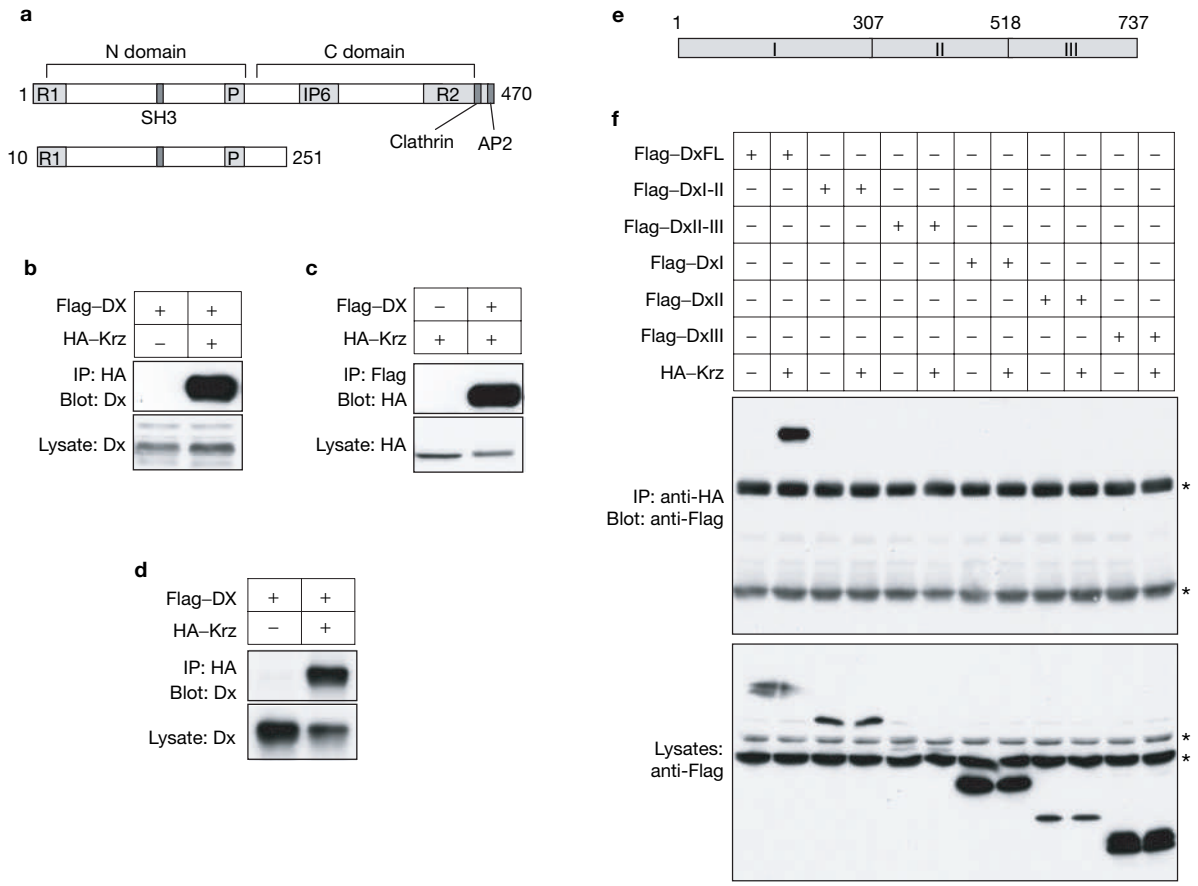

Figure 1 The Krz and Dx proteins interact. (**a**) Schematic representation of the Krz protein and its conserved domains, based on its homology with the mammalian non-visual β-arrestins (see refs 4, 11). N indicates an amino-terminal domain that is homologous to the region that is known to interact with activated G-protein-coupled receptors (GPCRs) in mammalian β-arrestins. R1 and R2 are regulatory domains, SH3 indicates c-Src homology domain 3 binding site (PXXP), P is a phosphate sensor domain and IP6 is the inositol phospholipid recognition domain. Predicted clathrin and AP2 interaction sites are also indicated. A region of Krz (amino acids 10–251) that was sufficient for binding to Dx, based on yeast two-hybrid analysis, is shown below the full-length protein. (**b**–**d**) Co-immunoprecipitation (IP) of Flag–Dx and HA–Krz from S2 cells (**b**, **c**) and imaginal discs (**d**). Flag–Dx and HA–Krz proteins were co-expressed and immunoprecipitated with either anti-HA agarose (**b**, **d**) or anti-Flag agarose (**c**). (**e**) A diagram of the Dx protein showing three major domains[8]. (**f**) Co-immunoprecipitation of full-length HA–Krz and Flag-tagged deletion fragments of Dx corresponding to amino-acid positions indicated in **e**. Only full-length Dx (Flag–DxFL) interacted with HA–Krz. Asterisks indicate immunoglobulins (upper panel) or non-specific bands (lower panel).

Four Krz peptides (VGEQPSIEVSK, VFELCPLLANNK, HEDTNLASSTLITNPAQR and ESLGIMVHYK) were also identified, using LC-MS/MS, among proteins in the 50,000–55,000 relative molecular mass range that co-purified with full-length amino-terminally TAP-tagged Dx (NTAP–Dx) that was isolated from stably transfected Kc167 cells (see Methods). These peptides correspond to endogenous Krz protein (with a predicted relative molecular mass of 51,200) that is expressed at normal levels. Krz was also identified as a Dx-interacting partner in an independent experiment involving another cell line (S2) that was stably transfected with the *NTAP–Dx* transgene.

Co-immunoprecipitation experiments confirmed the interaction between Krz and Dx. Using extracts from S2 cells expressing HA-tagged Krz and Flag-tagged Dx proteins, we demonstrated that both Krz and Dx could be immunoprecipitated with either anti-HA or anti-Flag antibodies (Fig. 1b, c). Furthermore, Flag–Dx protein was immunoprecipitated with HA–Krz from larval imaginal discs when both proteins were co-expressed (Fig. 1d). Deletion analysis demonstrated that full-length Dx protein is required for binding to Krz (Fig. 1e, f). Taken together, these experiments indicate that the Krz and Dx proteins directly interact.

To ensure that the tagged constructs encode functional proteins, we used the *GAL4–UAS* system to examine whether the *UAS–HA–Krz* and *UAS–Flag–Dx* transgenes were capable of rescuing loss-of-function *krz* and *dx* mutations, respectively. We found that the *UAS–Flag–Dx* transgene, which was expressed using the *C96–GAL4* driver, rescued the wing phenotype that was associated with a null mutation in *dx* (see Supplementary Information, Fig. S1a, b). A ubiquitous expression of *UAS–HA–Krz* with an *arm–GAL4* driver allowed the development of homozygous krz^1 mutants to the crawling third instar larval stage to the same extent as did the *UAS–ARRT5* transgene, which expresses an untagged Krz protein[4]. Therefore, both *UAS–Flag–Dx* and *UAS–HA–Krz* constructs were functional *in vivo*.

krz and *dx* interact genetically

To address the functional implications of the association between the Krz and Dx proteins, we investigated whether mutations in *krz* and *dx* display genetic interactions. We used two independent loss-of-function *dx* alleles, *dx* and dx^{SM} (ref. 22), and two independent *krz* loss-of-function alleles, krz^1 and krz^2 (ref. 4). A transheterozygous combination of *dx* and *krz* alleles (*dx*/+; *krz*/+ females) resulted in wings that were

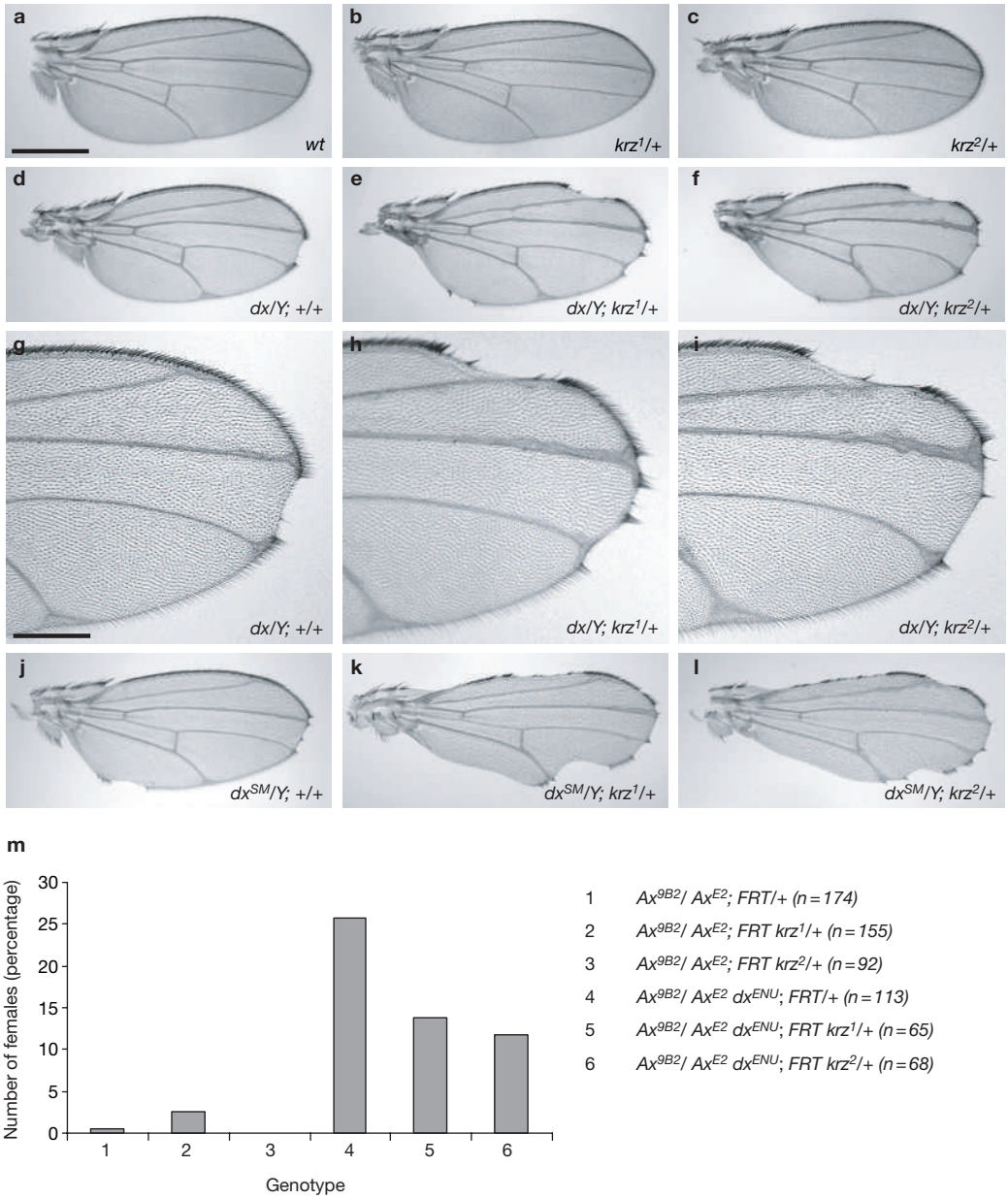

Figure 2 Genetic interactions between *dx* and *krz* mutations. (**a**–**l**) Representative wings from males with indicated genotypes. Wings from krz^1 (**b**) and krz^2 (**c**) heterozygotes are indistinguishable from wild-type (wt) control wings (**a**). *dx* (**d**, **g**) and dx^{SM} (**j**) show wing phenotypes that consist of notched wing margins and extra vein material, especially at the distal ends of wing veins. Both *dx* alleles show an enhancement of wing notching and vein thickening in hemizygous combinations with either krz^1 (**e**, **h**, **k**) or krz^2 (**f**, **i**, **l**) heterozygotes. Images in **g**–**i** are high-magnification images of the distal ends of wings shown in **d**–**f**, respectively. (**m**) Mutations in *krz* suppress the ability of *dx* to rescue the semilethality that is associated with a transheterozygous combination of two *Notch* alleles. Survival of females carrying two *Abruptex* alleles, Ax^{9B2} and Ax^{E2}, was tested in the presence of a control *FRT82B* chromosome (column 1) or in the presence of the same chromosome carrying krz^1 or krz^2 mutations (columns 2 and 3). Mutations in *krz* did not rescue Ax^{9B2}/Ax^{E2} semilethality. However, introduction of one copy of the dx^{ENU} allele rescued *Ax* semilethality to 26% (column 4), whereas further addition of one copy of either krz^1 or krz^2 reduced this rescue to 14% and 12%, respectively (columns 5 and 6). Scale bars, 500 μm (**a**–**f** and **j**–**l**), 200 μm (**g**–**i**).

indistinguishable from the wild type (data not shown). However, reducing the dose of *krz* in a genetic background that further reduces or eliminates *dx* (in *dx/Y; krz/+* males) elicited enhanced wing notching and vein thickening, compared with *dx* hemizygotes in a *krz* wild-type background[5] (Fig. 2d–l). Similar results were obtained using two other *dx* alleles, dx^{ENU} (ref. 22) and, importantly, a recently identified *dx* null allele, dx^{152} (provided by K. Matsuno, data not shown). Given that the genetic interaction between *dx* and *krz* was observed in the absence of all *dx* functions, it is clear that a complete absence of *dx* creates a sensitized genetic background that makes development of the wing margin sensitive to a decrease in the dosage of *krz*.

We extended these observations by examining the ability of *krz* alleles to influence the lethality that is associated with heteroallelic combinations of a class of gain-of-function *Notch* alleles, the *Abruptex* (*Ax*) mutations. Two *Ax* alleles, Ax^{9B2} and Ax^{E2}, display negative complementation such that the heteroallelic combination is semilethal[22]. This genetic background has proven to be a sensitive assay for identifying modulators of Notch activity[22]. For example, lowering the dosage of *dx* results in an

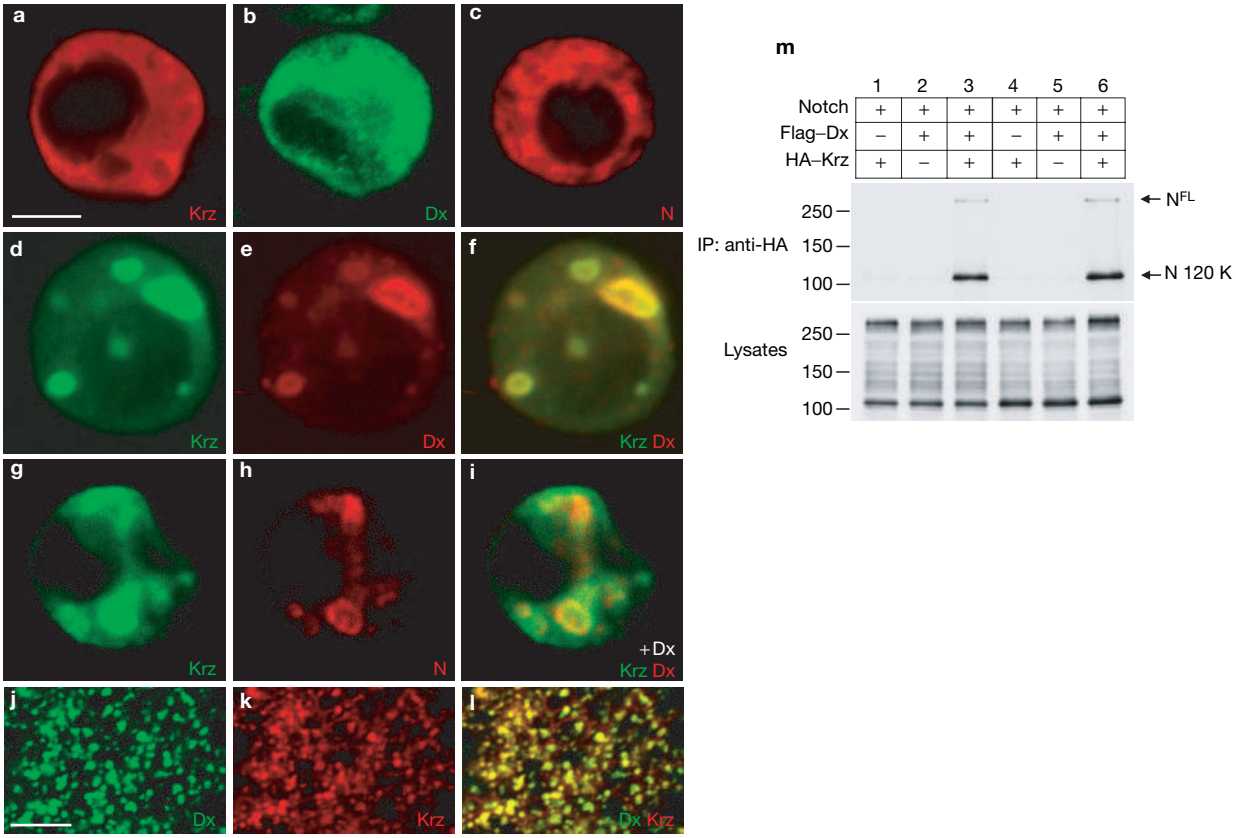

Figure 3 Krz, Dx and Notch colocalize and form a trimeric complex. (**a**–**i**) Confocal images of *Drosophila* S2 cells expressing HA–Krz alone (**a**), Flag–Dx alone (**b**), Notch (N) alone (**c**), HA–Krz and Flag–Dx together (**d**–**f**), and HA–Krz and Notch in the presence of Flag–Dx (**g**–**i**). Images in **f** and **i** are merges of those in **d** and **e**, and **g** and **h**, respectively. Co-expression of Krz and Dx leads to the redistribution of Krz and Dx into intracellular vesicles, where they colocalize (**d**–**f**). Notch is also present in those vesicles when co-expressed with HA–Krz and Flag–Dx (**g**–**i**). (**j**–**l**) Co-localization of Flag–Dx and HA–Krz in wing imaginal discs. *UAS–Flag–Dx* and *UAS–HA–Krz* were expressed under the control of the *C96–GAL4* driver. Image **l** is a merge of those in **j** and **k**. (**m**) Krz, Dx and Notch form a trimeric complex. S2N cells were transfected with HA–Krz alone, Flag–Dx alone, or HA–Krz and Flag–Dx together, and were incubated for 45 min with an equal number of either S2 cells (lanes 1–3) or S2Dl cells expressing full-length Delta (lanes 4–6). Cell extracts were immunoprecipitated (IP) with anti-HA antibody and immunoblotted with anti-Notch antibody. Notch was immunoprecipitated with HA–Krz, but only in the presence of Dx (lanes 3 and 6), and the formation of this trimeric complex was not affected by the presence of the ligand (Delta). Note that the Notch fragment with a relative molecular mass of 120,000 (N 120 K) was more efficiently bound than the full-length Notch receptor (N^{FL}). The bottom panel shows that Notch was present at equal levels in all lysates. Scale bars, 5 μm (**a**–**i**), 10 μm (**j**–**l**).

efficient suppression of the lethality that is associated with the Ax^{9B2}/Ax^{E2} heteroallelic combination (compare columns 1 and 4, Fig. 2m). Neither krz^1 nor krz^2 on their own were able to provide any significant rescue (columns 2 and 3, Fig. 2m). However, eliminating one copy of *krz* suppressed *dx*-mediated rescue of *Ax* lethality by approximately 50% (columns 5 and 6, Fig. 2m). Therefore, a compromised *Notch* genetic background reveals the ability of *krz* to interact with *dx* even in a transheterozygous combination (in Ax^{9B2}+/Ax^{E2} dx^{ENU}; *krz*/+ females).

Krz, Dx and Notch form a protein complex

To extend the analysis of the interactions between Krz, Dx and Notch, we tested the relative subcellular localization of these proteins when they were co-expressed in cultured cells. Immunocytochemical analysis revealed that the expression of either HA–Krz or Flag–Dx alone resulted in a diffuse distribution throughout the cytoplasm (Fig. 3a, b). By contrast, co-expression of both proteins led to a redistribution of Krz and Dx into intracellular vesicles, where they co-localized (Fig. 3d–f). Colocalization of co-expressed Krz and Dx was also observed *in vivo* in the wing imaginal discs (Fig. 3j–l). The nature of these vesicles remains to be determined, but several known intracellular trafficking markers — which label early and late endosome compartments, the Golgi apparatus and the endoplasmic reticulum — did not seem to co-localize with the Krz and Dx proteins (see Supplementary Information, Fig. S2).

As the Notch receptor is known to physically interact with Dx[6,8], we further investigated the relative distribution of Notch in the presence of Krz and Dx. Expression of Notch alone in S2 cells revealed a typical distribution of the protein on the cell surface and in the cytoplasm (Fig. 3c)[23]. Co-transfection of Notch with HA–Krz did not change the localization pattern of either protein (data not shown). However, when Notch was co-transfected with Krz and Dx, the Notch protein could be detected in the same cytoplasmic vesicles that contain Krz and Dx (Fig. 3g–i), raising the possibility that all three proteins form a complex.

The existence of such a trimeric complex was confirmed biochemically by immunoprecipitation studies involving protein extracts from S2 cells that had been stably transfected with full-length Notch (S2N cells) expressing HA-tagged Krz and/or Flag-tagged Dx. An anti-HA antibody immunoprecipitated HA–Krz, together with Notch, from these extracts only in the presence of Flag–Dx (Fig. 3m, lanes 1–3). We conclude that Notch, Dx and Krz form a protein complex and, in this complex, Krz interacts with Notch via Dx. The formation of the Notch–Dx–Krz complex was

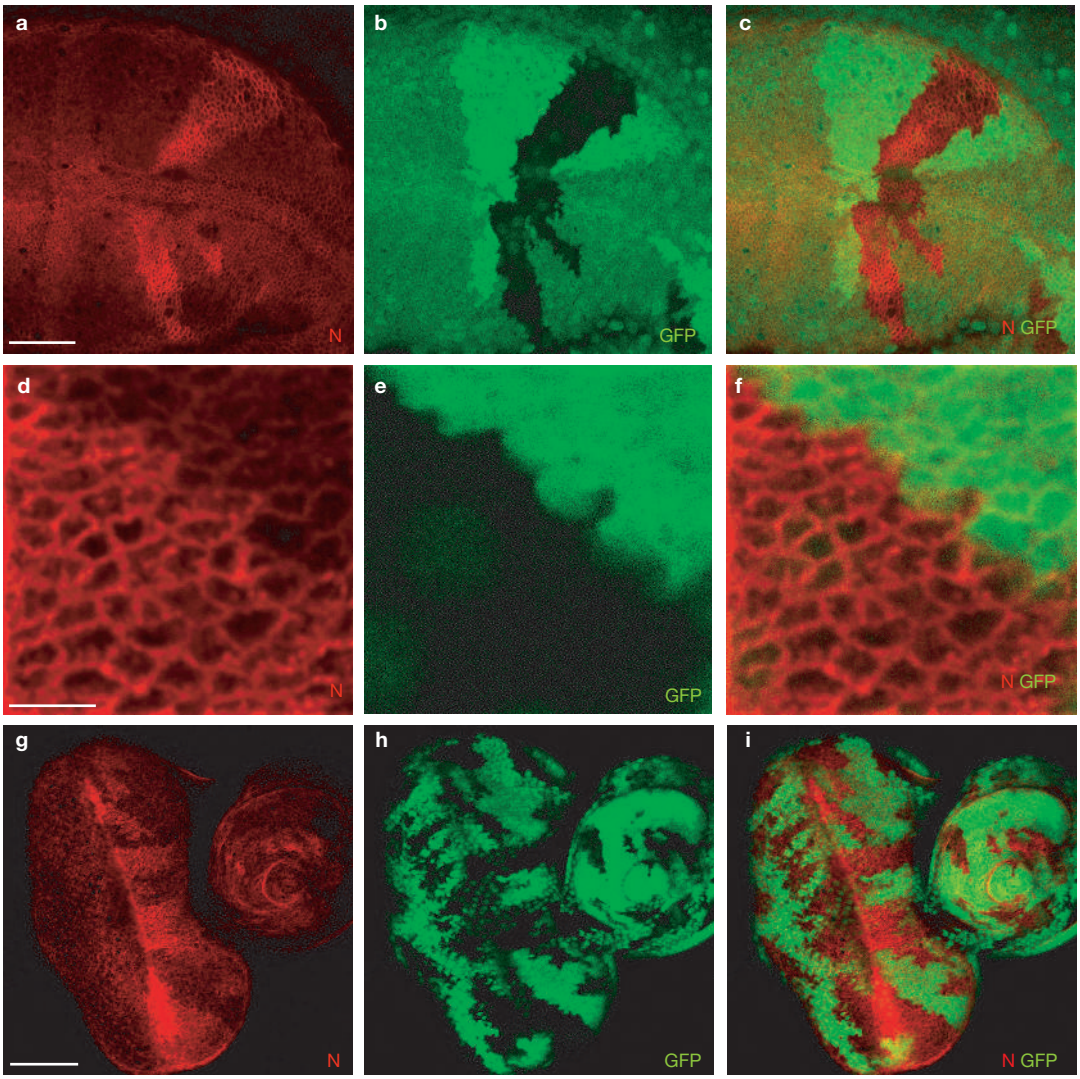

Figure 4 Loss of *krz* upregulates Notch protein levels. (**a**–**i**) Levels of Notch (N) protein in third instar wing discs (**a**–**f**) and eye-antennal discs (**g**–**i**) that contain *krz* mutant clones marked by the absence of green fluorescent protein (GFP). Images in **c**, **f** and **i** are merges of those in **a** and **b**, **d** and **e**, and **g** and **h**, respectively. High magnification of *krz* clones in **a**, **b** and **c** are shown in **d**, **e** and **f**, respectively. Note the increased levels of Notch in cells lacking *krz*. Scale bars, 30 μm (**a**–**c**), 5 μm (**d**–**f**), 50 μm (**g**–**i**).

unaltered in the presence of the Notch ligand, Delta (Fig. 3m, lanes 4–6). Our analysis does not exclude the possibility that additional proteins may be part of this complex, nor does it address the issue of stoichiometry.

krz loss of function affects Notch protein levels and signalling activity

To probe the functional significance of an interaction between Krz, Dx and Notch *in vivo*, we examined the effects of *krz* loss of function on the endogenous Notch receptor. To this end, we generated *krz* loss-of-function clones in two different tissues — the wing and eye-antennal imaginal discs — using the krz^1-null mutant[4] and the FLP/FRT system[24]. We found that the levels of the Notch protein, normally expressed throughout these discs, were substantially elevated in *krz* mutant cells compared with the surrounding wild-type cells. This increased level of Notch was observed in both the wing and eye-antennal discs (Fig. 4). We note, however, that in the eye discs, this elevated level of Notch was more prominent in *krz* clones that were located anterior to the morphogenetic furrow (Fig. 4g–i). In contrast with the upregulation of Notch, the levels of Dx were unaltered in *krz* mutant clones (see Supplementary Information, Fig. S3m–o).

We sought to determine whether the accumulation of the Notch receptor, as observed in *krz* mutant cells, affects signalling by examining the expression of two markers of Notch activity, Wingless (Wg) and Cut[25–27]. The increased levels of Notch that were seen in *krz* clones in the wing disc were accompanied by an upregulation of Wg in a subset of *krz* mutant cells that were located in the ventral middle region of the disc that was in close proximity to the dorsal/ventral boundary, a region that is known to depend on Notch signals for its development (Fig. 5a, b). Out of 11 *krz* mutant clones located in that particular area of the wing disc, 7 showed ectopic Wg expression. Ectopic expression of another marker of Notch activity, Cut, was also observed in a subset of cells in *krz* mutant clones in the eye imaginal disc (Fig. 5c–e). However, we have not detected ectopic expression of E(spl) proteins, that are recognized by mAb323 antibodies, in *krz* clones in the wing or eye discs (data not shown). These observations indicate that loss of *krz* function results in elevated levels of Notch that are sometimes accompanied by upregulation of downstream

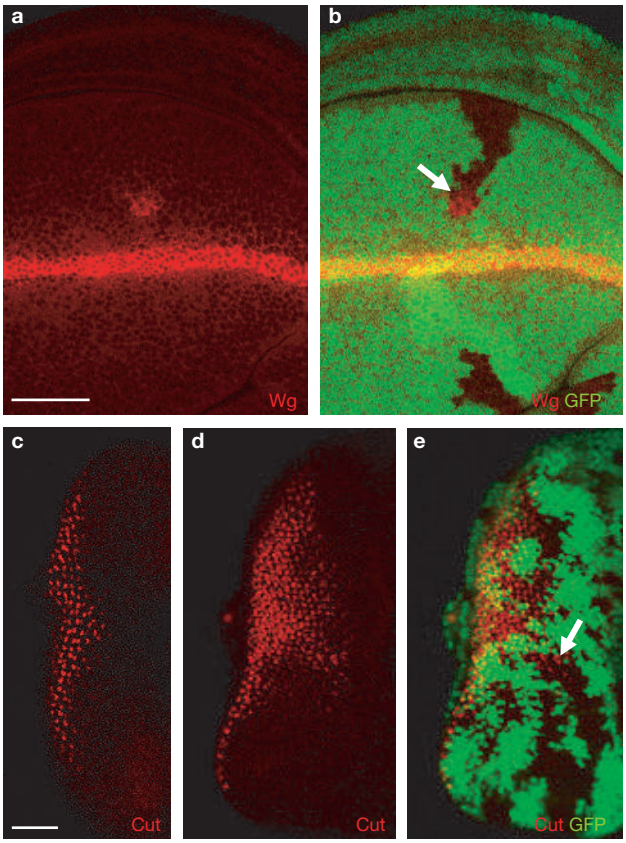

Figure 5 Upregulation of markers of Notch activity in *krz* mutant clones. Ectopic expression (arrows) of Wg in the wing disc (**a**, **b**) and Cut in the eye disc (**d**, **e**) was observed in a subset of cells in *krz* mutant clones, as marked by the absence of green fluorescent protein (GFP) (**b**, **e**). Image **c** shows the wild-type pattern of Cut expression in the eye imaginal disc. Scale bars, 30 μm.

effectors in a context-dependent manner. Consistent with a context-specific upregulation of Notch signalling in *krz* mutant clones, we occasionally observed, with a frequency of less than 1%, ectopic bristles and vein material (indicating Notch gain-of-function effects) in the adult wings of individuals carrying *krz* clones (data not shown).

Krz and Dx modulate Notch protein stability *in vivo*

We sought to corroborate the conclusions drawn from the *krz* loss-of-function studies by a gain-of-function analysis, monitoring the effects of ectopic expression of Krz and Dx on Notch.

Expression of HA–Krz alone in cells of the wing margin, under the control of the *C96–GAL4* driver, did not visibly affect wing development (Fig. 6a). Expressing only Flag–Dx with the same driver resulted in mild irregularities of bristle pattern along the wing margin (Fig. 6e). However, expression of both HA–Krz and Flag–Dx with *C96–GAL4* led to notching around the entire wing margin (Fig. 6i), which is reminiscent of Notch loss-of-function phenotypes. This synergistic effect was also observed in the eye, when HA–Krz and Flag–Dx were co-expressed using the *GMR–GAL4* driver (see Supplementary Information, Fig. S1c–e). Therefore, in two different tissues, Krz and Dx act synergistically to elicit phenotypes that are consistent with a downregulation of Notch activity.

In parallel with the gain-of-function phenotypic analysis in the wing and the eye, we examined the levels of endogenous Notch protein in wing imaginal discs in a background of HA–Krz and Flag–Dx expression that was driven by *C96–GAL4*. Consistent with an absence of wing phenotypes (Fig. 6a), expression of HA–Krz alone did not detectably affect the levels or localization of the Notch protein (Fig. 6b–d). When Flag–Dx was expressed alone, Flag–Dx and Notch co-localized in intracellular vesicles, with no apparent reduction in Notch protein levels (Fig. 6f–h). The localization of Dx and Notch in intracellular vesicles has also been reported by Hori *et al.*[28]. Coexpression of HA–Krz, together with Flag–Dx, resulted in a significant depletion of Notch from Dx-positive vesicles and an overall reduction in Notch protein levels (Fig. 6j–l). Therefore, the synergistic effects of coexpression of Krz and Dx, which mimic Notch loss-of-function phenotypes (Fig. 6i), seem to be caused by the decrease in Notch protein levels.

The synergistic effect of Krz and Dx on Notch could also be observed in cultured cells (Fig. 7a–f). Expression of HA–Krz or Flag–Dx alone did not affect Notch protein levels in S2 cells that were stably transfected with full-length Notch (S2N cells; Fig. 7a–c, and data not shown). When HA–Krz and Flag–Dx were co-expressed in S2N cells, the levels of Notch protein were markedly reduced (Fig. 7d–f). We noted that such reduction of Notch levels was observed only when Notch was present in limiting amounts, such as in imaginal discs or in S2N cells (Fig. 6k, l; Fig. 7e, f), but not in cells in which Notch was expressed at higher levels, when it was co-transfected along with Krz and Dx (Fig. 3h–i). The level of Notch on the cell surface was also reduced in S2N cells that were cotransfected with Krz and Dx (see Supplementary Information, Fig. S4a–f).

Using a cell-culture-based ligand-dependent Notch-activity reporter assay (see Methods), we found that Delta-dependent Notch signalling was reduced by 45% when the Notch protein was simultaneously expressed with Flag–Dx and HA–Krz, compared with cells expressing Notch alone (Fig. 8a). This experiment demonstrated that, together, Krz and Dx can downregulate ligand-dependent Notch activity. However, the effects of Krz and Dx on Notch are also, at least in part, ligand-independent, as in S2N cells, which are known to lack detectable Delta protein, Notch levels are reduced in the presence of Krz and Dx (Fig. 7e, f; and see Supplementary Information, Fig. S4a–f).

We conclude that Dx, together with Krz, triggers the degradation of the Notch protein, a process that is mediated by the formation of the Notch–Dx–Krz complex. The reduction in Notch protein levels results in the downregulation of Notch signalling.

Krz and Dx synergistically affect Notch ubiquitination

In an attempt to gain insight into the biochemical mechanisms that underlie the reduction of Notch protein levels, we examined whether the degradation of Notch that was observed in the presence of Krz and Dx is mediated by ubiquitination. Indeed, non-visual β-arrestins can facilitate the ubiquitination and downregulation of GPCRs and the IGF-1R[29,30].

Full-length Notch was transfected into S2 cells, along with Flag–Dx, Myc–Krz and HA-tagged ubiquitin (HA–Ub). Transfected cells were incubated in the presence of the proteasome inhibitor MG132, and Notch was immunoprecipitated with a Notch-specific antibody, C17.9C6. In the absence of Myc–Krz or Flag–Dx, Notch was weakly ubiquitinated, as seen by a smear that was recognized by the anti-HA antibody that detects HA–Ub moieties (Fig. 8b, upper panel, lane 2). Co-expression of Notch with Myc–Krz did not change this ubiquitination pattern (lane 4). Co-expression with Flag–Dx gave a slight but reproducible enhancement of Notch ubiquitination (lane 3). However, when Notch was co-expressed with both Myc–Krz and Flag–Dx, we observed significantly higher ubiquitination of Notch (lane 5).

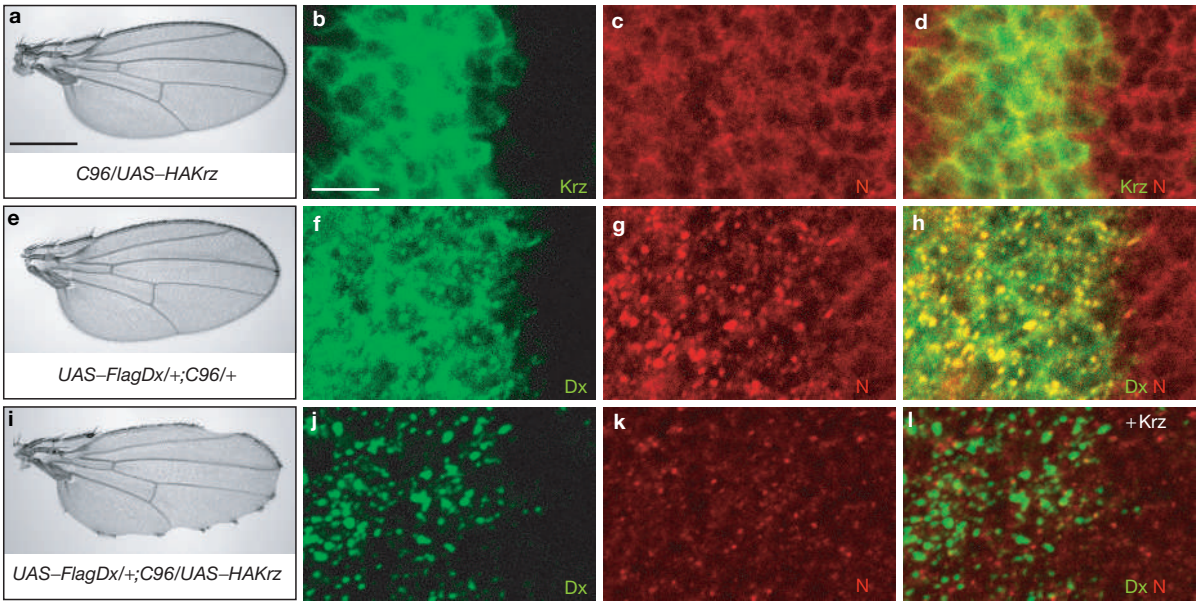

Figure 6 Coexpression of Krz and Dx results in the reduction of Notch protein levels *in vivo*. *UAS–HA–Krz* and *UAS–Flag–Dx* transgenes were expressed under the control of the *C96–GAL4* driver, which is expressed in cells corresponding to the dorsal–ventral boundary of the wing imaginal disc. Endogenous Notch (N) levels were monitored with the C17.9C6 antibody. (**a**–**d**) Expression of HA–Krz alone resulted in a wing phenotype that was indistinguishable from the wild type (**a**), and did not affect the levels or localization of the Notch protein (**b**–**d**). (**e**–**h**) Expression of Flag–Dx alone led to mild irregularities of bristle pattern along the anterior wing margin (**e**) and Flag–Dx protein was co-localized with Notch in intracellular vesicles (**f**–**h**). (**i**–**l**) Co-expression of both HA–Krz and Flag–Dx resulted in wing notching that resembled defects associated with reduced Notch signalling (**i**), and led to a marked depletion of Notch from Dx-positive vesicles (**j**–**l**). Images in **d**, **h** and **l** are the merges of **b** and **c**, **f** and **g**, and **j** and **k**, respectively. Quantification of the fluorescence intensity corresponding to the Notch signal in the *C96–GAL4* expression domain in **c**, **g** and **k** gave the following values (arbitrary units): **c**, 34.36; **g**, 31.65; and **k**, 24.98. Scale bars, 500 μm (**a**, **e**, **i**), 10 μm (**b**–**d**, **f**–**h**, **j**–**l**).

We have also observed that addition of the proteasome inhibitor, MG132, resulted in stabilization of Notch in S2N cells, including those transfected with HA–Krz and Flag–Dx (Fig. 7g–i). Therefore, an outcome of the formation of Notch–Dx–Krz complexes is the enhancement of ubiquitination of the Notch receptor, which results in its proteasomal degradation.

DISCUSSION

Notch signalling is known to affect a broad spectrum of cell-fate decisions throughout development. Genetic studies revealed an uncommon sensitivity of normal development to the dosage of Notch signals and, therefore, controlling the protein quantity of the Notch receptor and its ligands on the surface of cellular neighbours is an essential aspect of Notch regulation. The present study reveals the existence of a hitherto unknown Notch-signal controlling mechanism that relies on modulating Notch-receptor levels through the activity of the *krz* gene that encodes the single non-visual β-arrestin in *Drosophila*. Consequently, our analysis unveils a new role of β-arrestins as regulators of Notch signalling.

Mammalian non-visual β-arrestins were originally thought to function exclusively in the desensitization and clathrin-mediated internalization of GPCRs (reviewed in refs 10, 11). The range of β-arrestin activity has been recently extended by uncovering their involvement in the regulation of other receptor systems[12–19]. The data we present here further extend the spectrum of β-arrestin functions, given the demonstration that the *Drosophila* non-visual β-arrestin, Krz, can modulate the protein levels of the Notch receptor and, consequently, Notch signalling. Our analysis indicates that the interaction between Krz and Notch is mediated by Dx.

The biochemical nature of Dx and its full spectrum of activities are not yet fully understood. *dx* was first implicated in Notch signalling as a modifier of Notch phenotypes[22]. Indirect evidence implied that Dx may have a role in the transcriptional regulation of Notch targets[31,32]. Additional studies postulated that *dx* may define a node in the Notch-signalling pathway that is independent of Suppressor of Hairless (CBF1 in mammals), the classical effector of Notch signals[28,33,34]. In mammals, Deltex seems to be an antagonist of Notch signals[35–37]. However, overexpression of Dx in *Drosophila* can mimic the phenotypes that are associated with *Notch* gain-of-function mutations, and loss of *dx* function results in wing-margin phenotypes that are reminiscent of loss of Notch function, indicating a positive rather than a negative role in Notch signalling[8,38]. Although our data do not exclude the possibility that Dx may have a positive role in Notch signalling in certain cellular contexts, the evidence presented here unambiguously demonstrates that Dx, in combination with Krz, functions as a negative regulator of Notch. The results of the present analysis, together with the previously published genetic studies, indicate that Dx may behave both as an agonist and as an antagonist of Notch signalling, depending on the specific cellular context[8,28,35,37].

Notwithstanding the lack of direct evidence regarding the biochemical nature of Dx[8,28,32–34,38], the fact that Dx contains a RING-H2 and two WWE domains indicates that Dx may function as an E3 ubiquitin ligase[7]. In fact, E3 ubiquitin-ligase activity has been shown to exist for mammalian homologues of *Drosophila* Dx[9]. Mammalian β-arrestins have also been implicated in receptor ubiquitination events. A stable association between β-arrestins and Class B GPCRs was shown to promote receptor ubiquitination and degradation by recruiting E3 ubiquitin ligases, such as Mdm2, to the receptor[29,30,39].

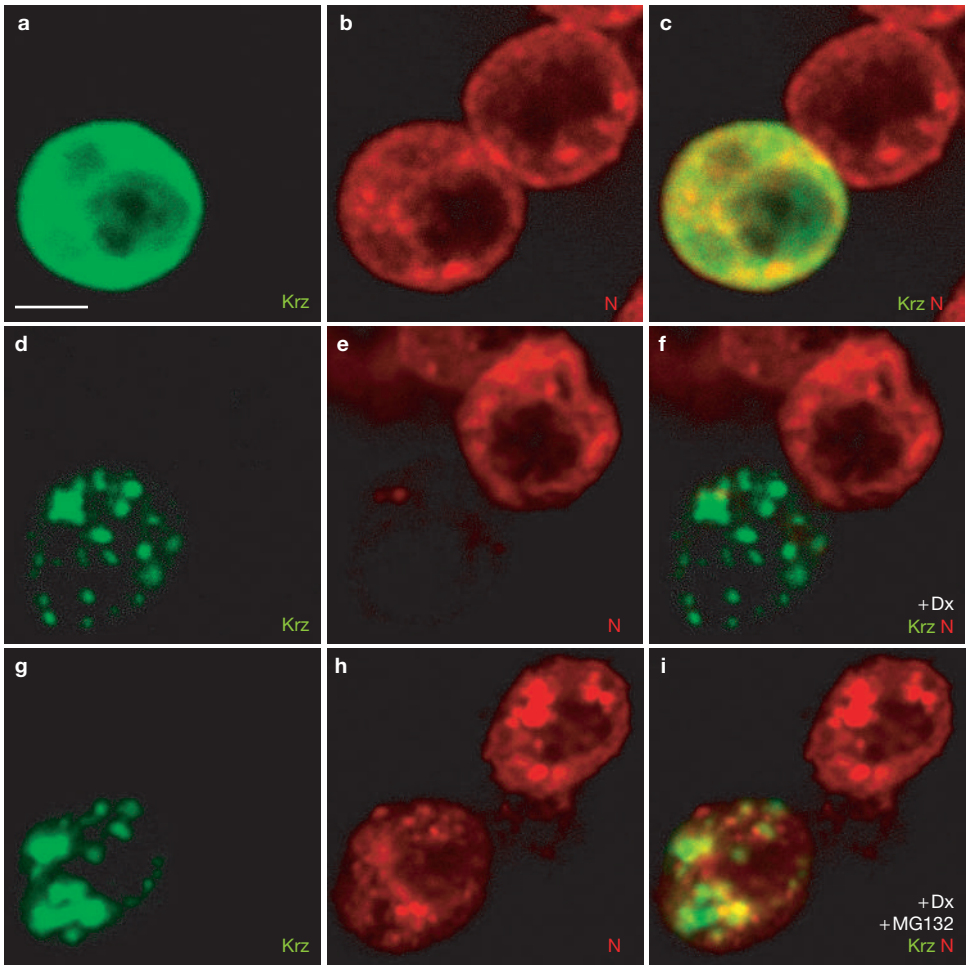

Figure 7 Krz and Dx synergistically affect the stability of Notch. (**a**–**i**) S2N cells carrying a stably integrated full-length Notch (N) construct were transfected with pMT–HA–Krz alone or in combination with pMT–Flag–Dx and stained with anti-HA and anti-Notch antibodies. (**a**–**c**) Expression of HA–Krz alone did not affect the level or distribution of Notch in S2N cells, and the HA–Krz protein showed diffuse cytoplasmic localization. (**d**–**f**) Co-expression of HA–Krz and Flag–Dx significantly reduced the amount of Notch in S2N cells (compare the level of Notch in the Krz-positive cell with an adjacent cell that was not transfected). In the presence of Flag–Dx, the HA–Krz protein localized in vesicles. Relative Notch levels in transfected cells were assessed by scoring for the presence or absence of the protein by immunofluorescence. Medium to high Notch levels were observed in 100% of S2N cells that were singly transfected with either HA–Krz or Flag–Dx ($n = 32$ and $n = 28$ cells, respectively). By contrast, Notch was reduced to undetectable levels in 51% of S2N cells that were transfected with both HA–Krz and Flag–Dx ($n = 39$). (**g**–**i**) Addition of the proteasome inhibitor MG132 prevented depletion of Notch from S2N cells that had been co-transfected with HA–Krz and Flag–Dx. Scale bar, 5 μm.

We reproducibly observed a small increase of Notch ubiquitination in the presence of Dx, which was further enhanced following addition of Krz (Fig. 8b). Previous studies implicated Notch in both poly- and monoubiquitination events[40–42]. We did not detect an increase in Notch monoubiquitination following addition of Dx, Krz or both (see Supplementary Information, Fig. 4g), so we attribute an increase in ubiquitination, as seen in Fig. 8b, to polyubiquitination of Notch. Our study, therefore, associated the formation of the Notch–Dx–Krz complex with polyubiquitination of the Notch receptor and a subsequent reduction of Notch levels, apparently via proteasomal degradation. The underlying mechanism is unknown at this point, but it is possible that the incorporation of Krz into the Notch–Dx–Krz complex may promote polyubiquitination of Notch by facilitating the ubiquitin-ligase activity of Dx, by recruiting additional E3 ligases or perhaps by inducing an altered conformation of the Notch receptor.

It is worth mentioning that additional E3 ligases, such as Suppressor of deltex (Su(dx)) and Nedd4, have been associated with Notch signalling[43–45]. However, we did not observe co-localization of Su(dx) with vesicles containing Krz and Dx following co-transfection of these three proteins in S2 cells (data not shown). Our data support a connection between the formation of the Notch–Dx–Krz complex and the proteasomal rather than the lysosomal degradative pathway. However, an involvement of the Krz–Dx vesicles in the intracellular trafficking of the Notch receptor cannot be excluded, despite the fact that our marker analysis has not revealed the identity of these vesicles (see Supplementary Information, Fig. S2).

It has been documented that non-visual β-arrestins are involved in trafficking of GPCRs and other types of receptors[12–17]. Given that *krz* seems to be the only β-arrestin in the *Drosophila* genome, the question is raised as to whether other, non-seven-transmembrane-receptor systems are affected in *krz* mutant cells. We asked whether, similar to the Notch receptor, the levels of Frizzled or the epidermal growth factor receptor (EGFR) are affected in loss-of-function *krz* clones in the wing or the eye imaginal discs, and did not find any change in their levels or localization (see Supplementary Information, Fig. S3a–l). However, these observations do not exclude the possibility that *krz* is still involved in the regulation of these and other receptors, as is the case in mammals. If, in mammalian systems, Notch is regulated by a similar mechanism, then

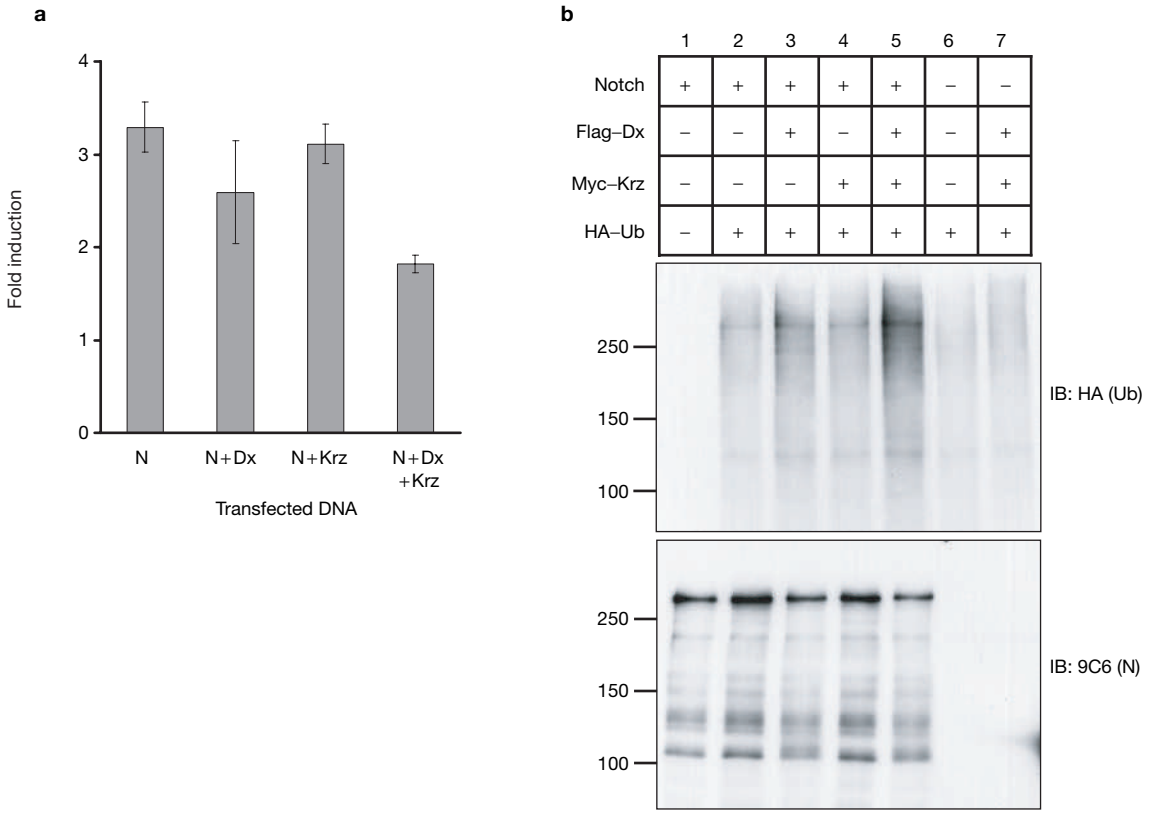

Figure 8 Krz and Dx downregulate Notch activity and enhance Notch ubiquitination. (**a**) Ligand-dependent luciferase assay using the 2 × m3 reporter was performed as described in Methods. Activation of Notch (N) by S2DI cells resulted in a 3.3-fold increase in luciferase signal over controls, which were incubated with S2 cells (DI protein is undetectable in S2 cells). Co-expression of Notch with Flag–Dx reduced this activation to approximately 2.5-fold (a 25% reduction), whereas co-expression of Notch with HA–Krz alone had no effect. When both Flag–Dx and HA–Krz were present, activation of 2 × m3 reporter by Notch was reduced by 45%, at $P < 0.035$. Error bars indicate standard deviation values. (**b**) Krz and Dx synergistically enhance Notch ubiquitination. S2 cells were transfected with pMT–NcDNA, pMT–Flag–Dx, pMT–Myc–Krz and HS–HA–Ub constructs in the indicated combinations, and protein expression was induced as described in Methods. Cells were lysed and Notch protein was immunoprecipitated with C17.9C6 monoclonal antibody. Immunoprecipitated proteins were analysed by western blotting (IB) with anti-HA (upper panel) and anti-Notch (lower panel) antibodies. No ubiquitination was observed without transfected HA–Ub (lane 1), and low-level background ubiquitination was observed in the absence of transfected Notch (lanes 6 and 7). Coexpression of Notch and HA–Ub resulted in some ubiquitination (lane 2). However, when Notch was co-expressed with Flag–Dx and HA–Ub, overall ubiquitination increased (lane 3) but remained unchanged in combination with Myc–Krz alone (lane 4). When all four proteins were co-expressed, Notch ubiquitination was significantly enhanced (lane 5). The results of the assay were quantified by individually dividing the ubiquitination signal from lanes 2–5 (upper panel) by the Notch signal in the corresponding lanes in the lower panel. The relative values for Notch ubiquitination levels, normalized by the lane 2 value, were the following: lane 2, 1.0; lane 3, 5.2; lane 4, 2.4; and lane 5, 12.8.

loss-of-function mutations in β-arrestins may result in the upregulation of Notch signals in certain tissues. This would be particularly significant in tissues in which Notch activation has a role in tumorigenesis[36,46].

Together, our loss-of-function and the complementary gain-of-function analyses indicate that Krz in involved in the regulation of Notch signalling. We propose that one of the biological functions of Krz is to modulate the level of the Notch receptor in the cell and thereby to optimize the amount of Notch that can participate in signalling. Such regulation of Notch by Krz is likely to be, at least in part, constitutive and may not require its interaction with a ligand.

It seems unlikely that the action of the *Drosophila* β-arrestin Krz is confined to the Notch signalling pathway, but further studies will be necessary to establish the spectrum of Krz function. An association of other signalling receptors with Krz would not only link them to non-visual β-arrestin function, but it would also provide a potential mode of cross-talk with Notch. These links may be important for defining the cellular framework within which controlling mechanisms have evolved to act on evolutionarily conserved signalling pathways such as Notch. □

METHODS

Yeast two-hybrid and tandem affinity purification. The full-length *Drosophila* Deltex cDNA (accession number U09789) was amplified by polymerase chain reaction (PCR) and cloned into the pB27 vector (Hybrigenics, Paris, France) using Sfi1 restriction sites. Deltex cDNA was fused in-frame with the sequence encoding the LexA DNA-binding domain of pB27. This construct was used as bait to screen oligo(dT)-primed *D. melanogaster* 0–24 h embryo cDNA libraries cloned in pGAD prey vectors containing GAL4 activation domains. A yeast two-hybrid screen was carried out using a mating approach with L40ΔGAL4 and Y187 yeast strains[20]. His^+ colonies were selected on media lacking tryptophan, leucine and histidine, and all positive pGAD plasmids from His^+ colonies were isolated and sequenced to identify interactors.

For TAP, full-length Deltex cDNA was amplified by PCR and cloned into the pMK33–NTAP vector, so that it carried the TAP tag at the amino terminus[21]. pMK33–NTAP–Dx was transfected into *Drosophila* Kc167 cells, and a stable line was selected in the presence of Hygromycin B (Sigma, St Louis, MO). Protein extracts were prepared from 1 L of cell culture after an overnight induction of NTAP–Dx protein expression with 0.07 mM $CuSO_4$. Cells were lysed in a lysis buffer containing 50 mM Tris, pH 7.5, 125 mM NaCl, 5% glycerol, 0.2% NP-40, 1.5 mM $MgCl_2$, 1 mM DTT, 25 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA and complete protease inhibitor (Roche, Indianapolis, IN). Supernatants were cleared by

ultracentrifugation and subjected to TAP, following established protocols[21]. Final protein fractions were separated on 4–12% Novex gels (Invitrogen, Carlsbad, CA) and stained with colloidal Coomassie (Sigma). LC-MS/MS analysis and peptide identification were performed as described previously[47].

Cell transfections, immunoprecipitations and immunoblotting. *Drosophila* S2 and Kc167 cells were maintained at 25°C in standard Schneider M3 medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum. Full-length Dx and Krz open reading frames were amplified by PCR with oligonucleotides containing sequences encoding appropriate affinity tags and cloned into the pMT/V5-His–TOPO vector (Invitrogen) to generate amino-terminally tagged Flag–Dx, HA–Krz and Myc–Krz. pMT–NcDNA containing full-length Notch was described previously[48]. All transfections were performed using Effectene transfection reagent (Qiagen, Valencia, CA). For immunoprecipitations, cells were grown on 10-cm plates. Equal amounts of plasmid were transfected, and the total amount of DNA was kept constant (6 μg) by adding empty vector. Proteins were induced with 0.35 mM $CuSO_4$ overnight, cells were lysed in the lysis buffer (see above), lysates were cleared by centrifugation at 14,000 *g* for 20 min and incubated with anti-Flag or anti-HA affinity beads (Sigma) for 3 h. After three washes with lysis buffer, protein complexes were eluted with SDS sample buffer, separated on SDS protein gels, transferred onto Immun-Blot PVDF membranes (Bio-Rad, Hercules, CA) and probed with monoclonal anti-Flag or anti-HA antibodies (Sigma). Similar procedures were used for immunoprecipitations from imaginal discs, in which Flag–Dx and HA–Krz proteins were expressed under the control of the *T113–GAL4* driver. For detection of Deltex, we also used monoclonal rat anti-Dx antibody[5].

Immunostaining of imaginal discs and cultured cells. Imaginal discs were dissected from third instar larvae in phosphate-buffered saline (PBS) and kept on ice until fixation in a 1:1 mixture of 3% paraformaldehyde in PBS and heptane. After 1 min, the paraformaldehyde and heptane mixture was replaced by 3% paraformaldehyde and 5% DMSO and incubated for 20 min. Discs were washed twice in Tri-PBS (PBS + 0.2% Triton-X-100). Discs were then washed four times in Tri-PBS with 1% bovine serum albumin (BSA) for 10 min each, followed by incubation for 30 min in Tri-PBS with 0.1% BSA and 8% normal goat serum. Primary antibodies, mouse anti-Notch (C17.9C6 and C458.2H) at 1:300 (ref. 23), rat anti-Deltex at 1:25 (ref. 5), mouse anti-Wg (4D4) and mouse anti-Cut (2B10) at 1:100 (Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-HA at 1:100 (Sigma), rabbit anti-Flag at 1:300 (Sigma), mouse anti-Frizzled at 1:5 (Developmental Studies Hybridoma Bank) and rat anti-EGFR at 1:200 (a gift from P. Rorth) were diluted in Tri-PBS, added to the discs and incubated for 12 h at room temperature. After four washes in Tri-PBS with 1% BSA, the discs were incubated with fluorescently labelled secondary antibodies (goat anti-mouse, goat anti-rat and goat anti-rabbit antibodies conjugated to FITC or Cy3, at a 1:100 to 1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for 90 min at room temperature, followed by washing four times in Tri-PBS. Discs were mounted in FluoroGuard Antifade Reagent (Bio-Rad), and images were obtained with a Zeiss LSM510 confocal microscope.

Drosophila S2 cells were washed twice with cold PBS, followed by fixation in 4% methanol-free formaldehyde for 20 min. Subsequent washes, antibody incubations and visualization were performed as described above. For proteasome inhibitor treatment, transfected cells were incubated with 50 μM MG132 for 4 h.

***Drosophila* genetics.** All stocks were maintained on standard cornmeal/yeast/molasses/agar medium at 25°C. The krz^1/*TM3*, krz^2/*TM3* and *UAS–ARRT5* lines were obtained from R.L. Davis[4]. We used the following *dx* alleles: *dx*, dx^{ENU}, dx^{SM} (ref. 22) and dx^{152} (a gift from K. Matsuno). Two alleles of *Abruptex*, Ax^{9B2} and Ax^{E2} were used to test the ability of *krz* to modify the *deltex*-mediated suppression of the negative complementation between *Abruptex* alleles[22]. Stocks used for generation of FLP-induced mosaics were constructed from *y w; FRT 82B*, *y w hsFLP*; *FRT82B Ubi–GFP/TM6B*, and *y w ey–FLP*; *FRT82B Ubi–GFP/TM6B,* which were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). Mosaic analysis using the FLP/FRT system was performed as described previously[24]. Recombination was induced in second instar larvae by a 30 min heat shock at 37°C.

For generation of the *P[UAS–HA–krz]* and *P[UAS–Flag–dx]* strains, a full-length *krz* cDNA with a HA tag at the amino-terminus and a full-length *dx* cDNA with a Flag tag at the amino-terminus were cloned in the pUAST vector. These constructs were introduced into w^{1118} embryos by germline transformation according to the standard procedures. Multiple independent insertions were obtained for each construct. The UAS constructs were expressed under the control of *C96–GAL4*, *GMR–GAL4*, *arm–GAL4* and *T113–GAL4* drivers. The rescuing ability of *UAS–HA–Krz* was tested with the *arm–GAL4* driver in homozygous krz^1 mutant animals. The majority of homozygous krz^1 mutants die as embryos, and less than 25% reach the third instar larval stage[4].

Ligand-dependent luciferase assay. A 1,665 base pair (bp) fragment of the *E(spl)m3* promoter was PCR-amplified from Canton S genomic DNA with oligonucleotides containing engineered *Kpn*I and *Sac*I sites and directionally cloned into the pGL2-Basic vector (Promega, Madison, WI) to make m3-Luc. This fragment contains 1,519 bp of upstream regulatory sequences of the *E(spl)m3* gene and 146 bp of its 5′ untranslated region[49] and corresponds to nucleotides 132,284 to 133,948 of GenBank entry AE003754.3 (GI:23172333). A *Sma*I/*Apa*I fragment from m3-Luc was made blunt and ligated into *Sma*I-cut m3-Luc to generate 2 × m3-Luc. This final reporter construct contains a tandem duplication of 1.4 kb of *E(spl)m3* upstream regulatory sequences.

Ligand-dependent Notch luciferase assays were performed in duplicate. Kc167 cells were transfected with 2 × m3-Luc, pMT–NcDNA, pMT–Flag–Dx and pMT–HA–Krz constructs in equal proportions, at a cell density of 10^6 cells ml^{-1}, and immediately induced overnight with 0.07 mM $CuSO_4$. In parallel, separate cultures of S2 and S2Dl cells were induced overnight with the same concentration of $CuSO_4$. S2 and S2Dl cell concentrations were adjusted to 10^6 cells ml^{-1}, and 0.5 ml of the adjusted cultures were mixed with 0.5 ml of transfected Kc167 cells (from the same transfection sample) and rocked at room temperature. Samples of 100 μl were taken at 0 and 4 h, and luciferase signals were developed using a Luc-Screen assay (Applied Biosystems, Foster City, CA) and measured using a Microlumat LB 96P fluorimeter (EG&G Berthold, Oak Ridge, TN). To quantify the fold induction after ligand activation, values at 0 h were subtracted from values at 4 h to correct for basal expression, then the corrected values at 4 h for S2Dl cells were divided by the corrected values at 4 h for S2 cells.

Ubiquitination assay. Ubiquitination assays were based on a protocol provided by R. Fehon. A total of 1 μg of pMT–NcDNA was co-transfected into S2 cells growing on 10-cm plates in various combinations with 2 μg each of pMT–Flag–Dx, pMT–Myc–Krz and HS–HA–Ub (a gift from R. Fehon[50]). After 24 h of transfection, cells were induced for 12 h with 0.35 mM $CuSO_4$. Plates were heat-shocked for 30 min at 37°C, recovered for 2 h at 25°C, after which MG132 was added to a final concentration of 50 μM and cells were incubated for 4 h at 25°C. Cells were lysed in lysis buffer as described above, except that the NP-40 final concentration was increased to 0.5% and the buffer contained 1 mM EGTA instead of EDTA, 1 mM N-ethyl-maleimide and 10 μM MG132. Notch was immunoprecipitated as described above, with 5 μl of C17.9C6 ascites and 25 μl of Protein G-agarose (Sigma) per sample. Immunoprecipitated proteins were analysed by western blotting with C17.9C6 and anti-HA antibodies.

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

The authors wish to thank their colleagues D. Finley, M. Baron, R. Lake, M. Kankel and L. Grimm for helpful discussions about the manuscript. We are also grateful to M. Gonzalez-Gaitan and H. Chang for their comments and reagents. *krz* alleles were kindly provided by R. Davis and the dx^{152} allele was a gift from K. Matsuno. The anti-EGFR antibody was a gift from P. Rorth. R. Fehon provided a ubiquitination assay protocol and an HS–HA–Ub construct. pMT–Flag–Ub^{WT} and pMT–Flag–Ub^{MONO} were a gift from S. Bray. The GFP–Sec61 construct was a gift from G. Voeltz. Several fly lines were provided by the Bloomington Drosophila Stock Center, and some of the antibodies used in this work were obtained from the Developmental Studies Hybridoma Bank, Iowa. This work was supported by grants NS26084, GM62931 and CA098402 to S.A.-T. A.V. was supported by a postdoctoral fellowship from the Massachusetts General Hospital Fund for Medical Discovery.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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ERRATUM

Owing to a technical error, the pages of this manuscript were originally mis-numbered by a 100 pages. This has now been corrected online. The corrected online manuscript is numbered 100 pages higher than the mis-numbered version.

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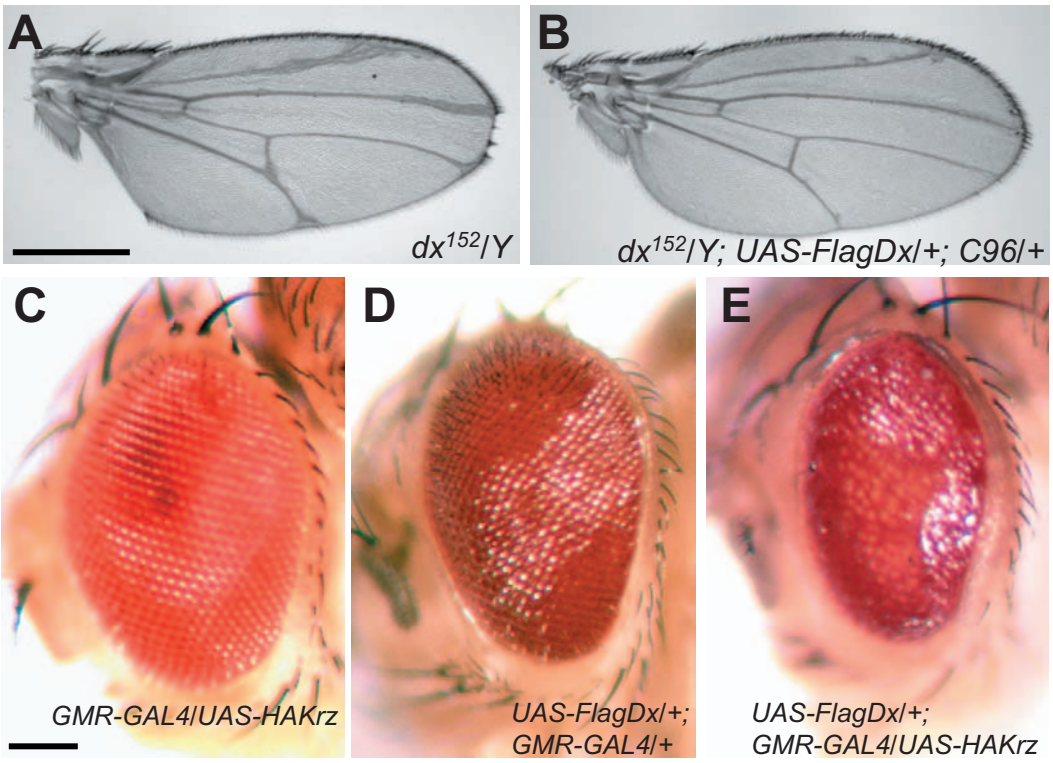

Figure S1 Rescue of the dx^{152} mutant wing phenotype and synergistic effects of Krz and Dx co-expression**.** (A) A null allele of *dx* (dx^{152}) showed characteristic wing tip notching and vein thickening. (B) The dx^{152} null phenotype was rescued when the *UAS-Flag-Dx* transgene was expressed in the wing margin under the control of the *C96-GAL4* driver. (C-E) *UAS-HA-Krz* and *UAS-Flag-Dx* transgenes were expressed in the eye under the control of the *GMR-GAL4* driver. (C) Expression of HA-Krz alone resulted in no abnormalities in eye development. (D) Expression of Flag-Dx led to some disorganization of ommatidia and a slight reduction in eye size. (E) Co-expression of HA-Krz and Flag-Dx resulted in a severe rough and glazed eye phenotype and the eye was reduced in size.
Scale bar, 500 μm (A, B), 100 μm (C-E).

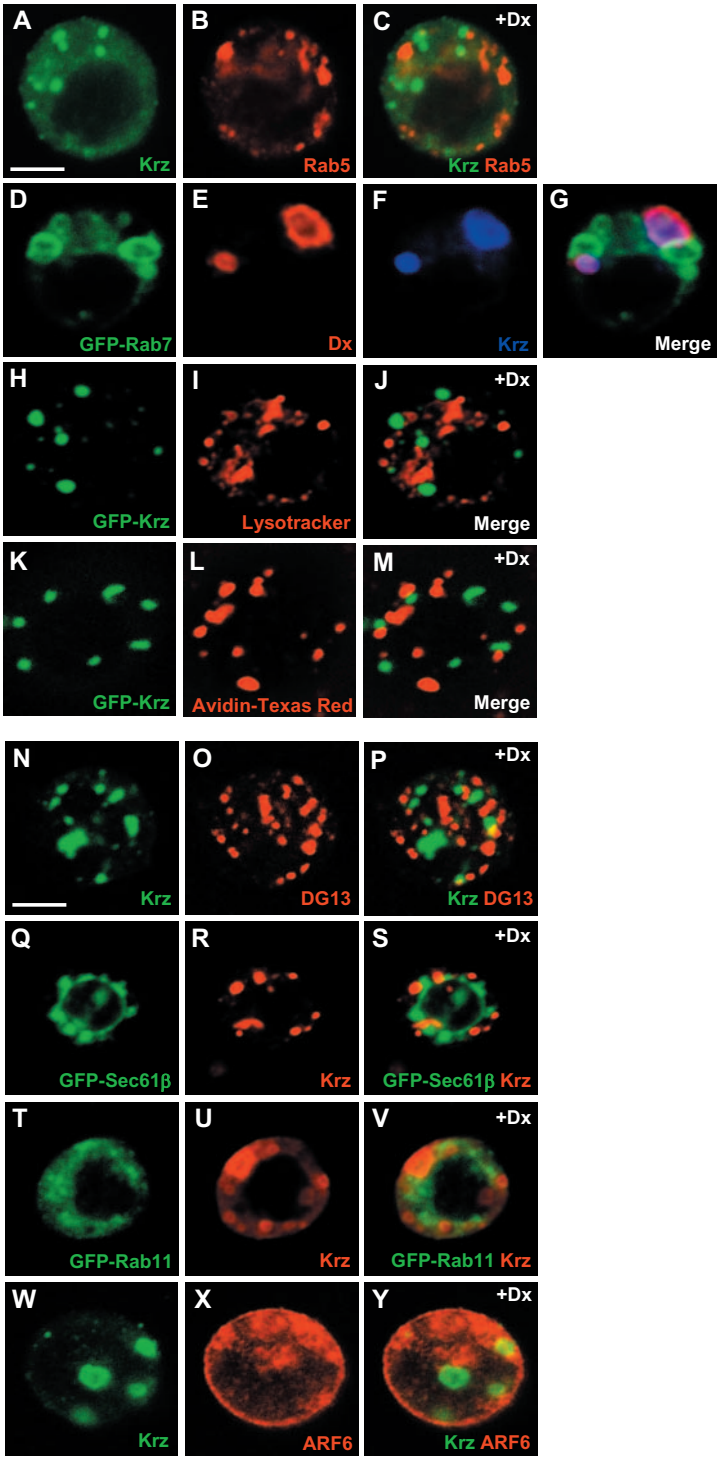

Figure S2 Vesicles containing Dx and Krz proteins do not colocalize with several known intracellular trafficking markers. (A-Y) S2 cells were transfected with the indicated combinations of Krz- and Dx-expressing plasmids and various markers of intracellular trafficking. (A-C) HA-Krz and Dx were co-expressed with Flag-Rab5 (early endocytic marker) and detected with anti-HA and anti-Flag antibodies. (D-G) HA-Krz, Flag-Dx and GFP-Rab7 (late endocytic marker, a gift from M. Gonzalez-Gaitan) were co-expressed and visualized by GFP fluorescence and by immunostaining with anti-HA and anti-Flag antibodies. (H-M) Cells were transfected with GFP-Krz and Flag-Dx and incubated for 10 min with Lysotracker Red (Molecular Probes) or for 40 min with Avidin-Texas Red conjugate (Molecular Probes), after which live cells were observed by confocal microscopy. (N-P) Cells were transfected with HA-Krz and Flag-Dx and stained with anti-HA and DG13 antibodies (Golgi marker). (Q-S) Cells were transfected with HA-Krz, Flag-Dx and GFP-Sec61((endoplasmic reticulum marker, a gift from G. Voeltz). Proteins were visualized by GFP fluorescence and by immunostaining with anti-HA antibodies. (T-V) Cells were co-transfected with GFP-Rab11 (recycling endosome marker), HA-Krz and Flag-Dx, which were visualized by GFP fluorescence and immunostaining with anti-HA antibodies. (W-Y) HA-Krz, ARF6-Myc and Flag-Dx were co-transfected and detected with anti-Myc and anti-HA antibodies. Scale bars, 5 μm.

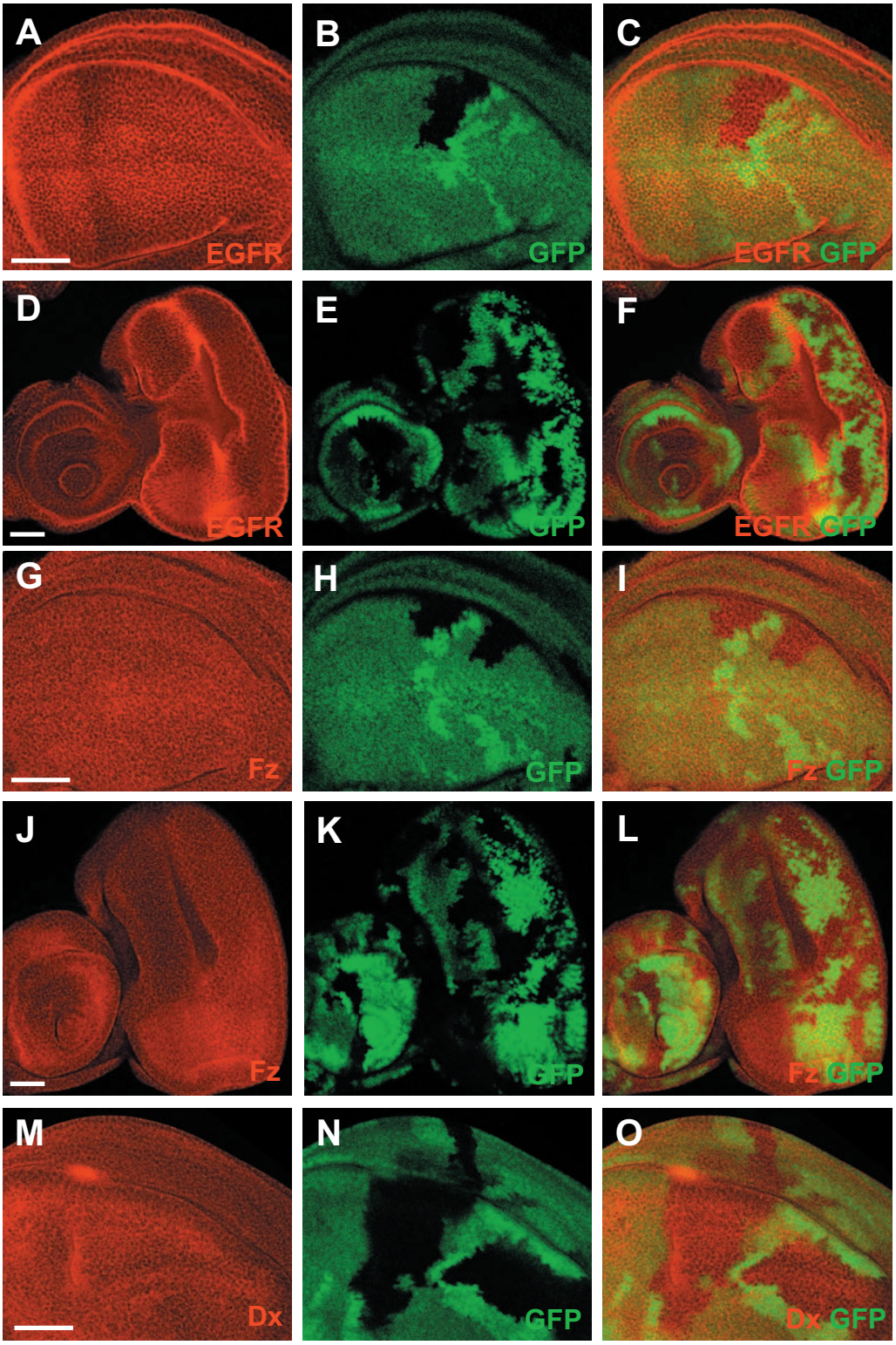

Figure S3 Loss of *krz* function has no effect on EGFR, Frizzled and Dx expression. Levels of EGFR in third instar wing discs (A-C) and eye discs (D-F), Frizzled in wing discs (G-I) and eye discs (J-L), and Dx in wing discs (M-O) remain unaltered in *krz* mutant clones marked by absence of GFP. Images in (C), (F), (I), (L) and (O) are merges of (A and B), (D and E), (G and H), (J and K), (M and N), respectively. Scale bars, 30 µm.

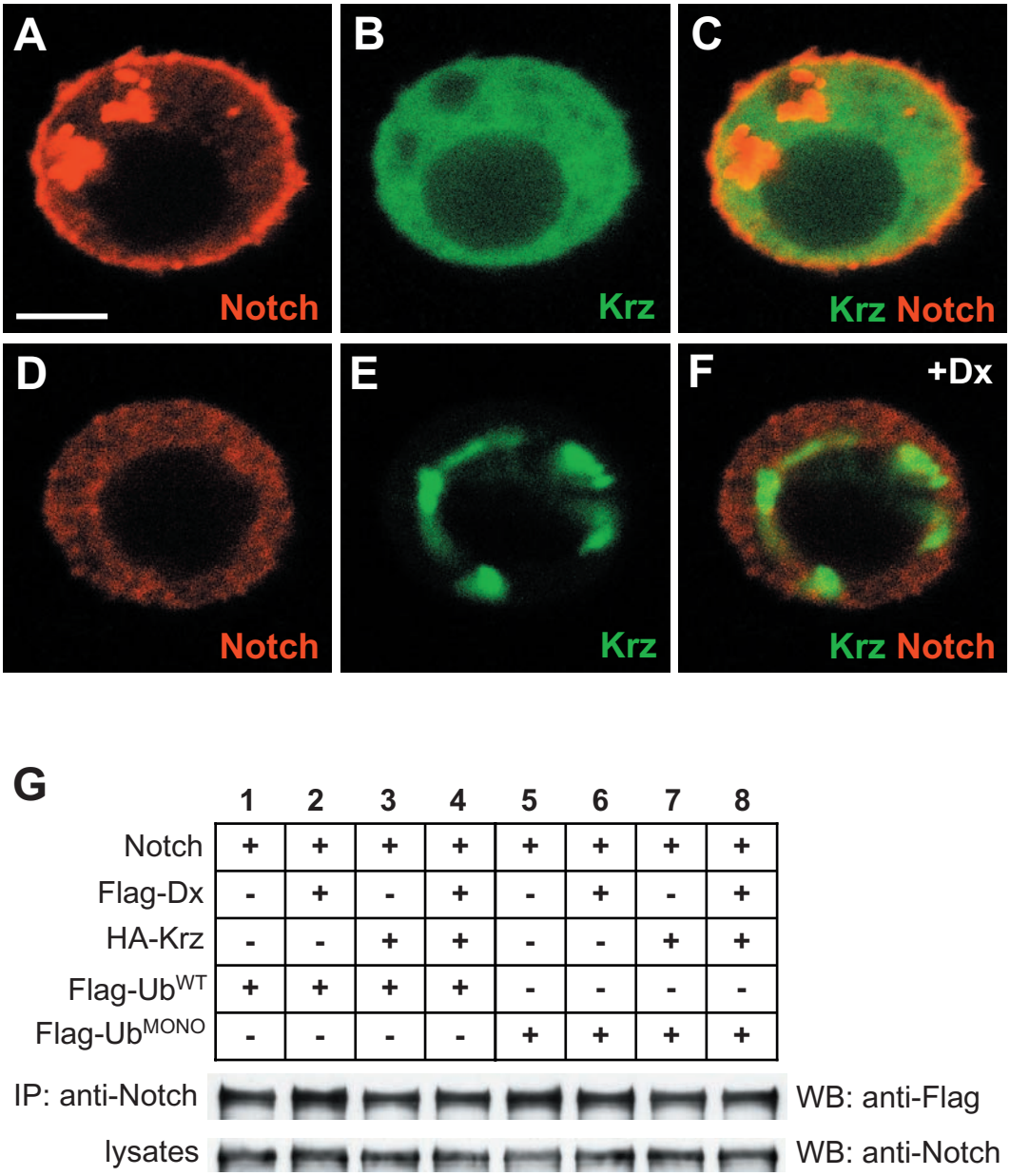

Figure S4 Effects of co-expression of Krz and Dx on Notch surface levels and monoubiquitination. S2N cells were transfected with GFP-Krz (A-C) or GFP-Krz and Flag-Dx (D-F), fixed and stained without permeabilization using a monoclonal antibody against Notch extracellular domain (C458.2H). Signal was detected using GFP fluorescence (for Krz, green) and Cy3-conjugated goat anti-mouse antibodies (for Notch, red). In S2N cells co-expressing Krz and Dx, the levels of Notch on the cell surface were significantly reduced (D-F). (G) S2 cells were transfected with pMT-NcDNA, pMT-Flag-Dx, pMT-HA-Krz and pMT-Flag-Ub^{WT} or pMT-Flag-Ub^{MONO} constructs in indicated combinations, and protein expression was induced overnight with 0.35 mM $CuSO_4$. Flag-Ub^{MONO} protein is a Flag-tagged mutated version of ubiquitin that can only participate in monoubiquitination events. Cells were lysed and Notch protein was immunoprecipitated with C17.9C6 monoclonal antibody. Immunoprecipitated proteins were analyzed by Western blotting with anti-Flag antibodies (upper panel). Lower panel shows the level of full-length Notch protein in the lysates. The addition of either Krz or Dx alone or in combination did not change the monoubiquitination level of full-length Notch. Scale bar, 5 μm (A-F).