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RESEARCH ARTICLE

Drosophila Crumbs prevents ectopic Notch activation in developing wings by inhibiting ligand-independent endocytosis

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ABSTRACT

Many signalling components are apically restricted in epithelial cells, and receptor localisation and abundance is key for morphogenesis and tissue homeostasis. Hence, controlling apicobasal epithelial polarity is crucial for proper signalling. Notch is a ubiquitously expressed, apically localised receptor, which performs a plethora of functions; therefore, its activity has to be tightly regulated. Here, we show that *Drosophila* Crumbs, an evolutionarily conserved polarity determinant, prevents Notch endocytosis in developing wings through direct interaction between the two proteins. Notch endocytosis in the absence of Crumbs results in the activation of the ligand-independent, Deltex-dependent Notch signalling pathway, and does not require the ligands Delta and Serrate or γ-secretase activity. This function of Crumbs is not due to general defects in apicobasal polarity, as localisation of other apical proteins is unaffected. Our data reveal a mechanism to explain how Crumbs directly controls localisation and trafficking of the potent Notch receptor, and adds yet another aspect of Crumbs regulation in Notch pathway activity. Furthermore, our data highlight a close link between the apical determinant Crumbs, receptor trafficking and tissue homeostasis.

KEY WORDS: Crb–Notch interaction, Ligand-independent Notch pathway, Wing vein refinement, *Deltex*

INTRODUCTION

The Crumbs (Crb) protein complex is an evolutionarily conserved key regulator of epithelial apicobasal polarity (Rodriguez-Boulan and Macara, 2014; Tepass, 2012). Its central constituent is the eponymous transmembrane protein Crb, initially identified in *Drosophila*. In *Drosophila* embryos, loss of *crb* function results in embryonic lethality, caused by the breakdown of many epithelia (Grawe et al., 1996; Tepass, 1996; Tepass and Knust, 1990). Comparable phenotypes are observed in mouse embryos lacking *Crb2* or *Crb3*, two of the three mammalian *Crb* genes (Charrier et al., 2015; Szymaniak et al., 2015; Whiteman et al., 2014; Xiao et al., 2011).

Although Crb is expressed in all cells of *Drosophila* imaginal discs, the anlagen of the external organs of the adult fly, epithelial integrity is not affected by the loss of *crb* function in these tissues (Hafezi et al., 2012; Herranz et al., 2006; Ribeiro et al., 2014). However, *crb* has been associated with various other functions in imaginal discs, such as regulation of Notch receptor processing in the wing margin (Herranz et al., 2006), cell survival (Hafezi et al., 2012) and organ size control by the Notch or Hippo pathways (Chen et al., 2010; Ling et al., 2010; Ribeiro et al., 2014; Richardson and Pichaud, 2010). In the pupal retinal epithelium, morphogenesis of photoreceptor cells is impaired upon loss of *crb* (Izaddoost et al., 2002; Johnson et al., 2002; Pellikka et al., 2002). Finally, in adult photoreceptor cells *crb* prevents light-dependent retinal degeneration (Chartier et al., 2012; Johnson et al., 2002).

These examples illustrate that *Drosophila* Crb executes a plethora of functions, raising the question whether individual regions of the protein may exert specific functions. Crb is a type I transmembrane protein, the large extracellular region of which contains an array of epidermal growth factor (EGF)-like repeats, intermingled with repeats similar to the globular domain of laminin A. The short, highly conserved cytoplasmic tail recruits the other core members of the Crb complex, Stardust, Patj and Lin-7 (also known as Veli) (Bulgakova and Knust, 2009). Results from structure-function analyses assigned specific functions to individual motifs within the short cytoplasmic tail. While the C-terminal PSD-95–Discs large–ZO-1 (PDZ) domain-binding motif of the cytoplasmic tail is essential for the regulation of polarity in embryonic epithelia (Klebes and Knust, 2000; Klose et al., 2013), its protein 4.1–ezrin–radixin–moesin (FERM) domain-binding motif participates in controlling dorsal closure during embryogenesis (Flores-Benitez and Knust, 2016; Klose et al., 2013) and Hippo-mediated growth of wing imaginal discs (Chen et al., 2010; Ling et al., 2010; Ribeiro et al., 2014). Less is known about the function of the huge extracellular domain. It was reported to modify ligand-dependent Notch activity (Herranz et al., 2006; Richardson and Pichaud, 2010), and to mediate non-autonomous effects on cell survival (Hafezi et al., 2012). Other results suggested that the extracellular domain is engaged in homophilic interactions to stabilise Crb in the membrane, both in the *Drosophila* follicular epithelium (Fletcher et al., 2012) and in the zebrafish retina (Zou et al., 2012).

In order to identify novel functions of the extracellular domain of Crb, we applied a genetic approach, using the *Drosophila* wing as a read-out. The wing is an ideal system to study the genetic control of pattern formation and growth, and has been extensively used to unravel genetic interactions and epistatic relationships (reviewed in Blair, 2007; Ribeiro et al., 2014). We screened for mutations that dominantly modify the wing vein phenotype induced by overexpression of the membrane-bound extracellular domain of Crb. Mutations in the Notch receptor turned out to dominantly enhance this vein phenotype, and to suppress the *crb* loss-of-function vein phenotype. At the cellular level, loss of Crb induces endocytosis of Notch, followed by the activation of the ligand-independent Notch signalling pathway, thus adding yet another mechanism to fine-tune this potent and ubiquitously expressed receptor.

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RESULTS

Crb negatively regulates Notch activity via its extracellular domain

We used the adult wings of *Drosophila* to identify novel functions mediated by the extracellular domain of Crb. In wings of wild-type flies, the five longitudinal veins L1-L5 and the two cross-veins are arranged in a stereotypic pattern (Fig. 1A). L1-L5 are smooth and straight and meet the margin in a distinct point (Fig. 1A,A′). Overexpression of UAS-$crb^{extraTM}$-*GFP*, which encodes a Crb protein in which the intracellular domain is replaced by GFP (Pellikka et al., 2002), resulted in veins that are thicker and irregular (Herranz et al., 2006) and exhibited delta-shaped broadenings when reaching the margin (Fig. 1B,B′). These deltas were most pronounced in L5 (Fig. 1B′).

To understand how Crb contributes to vein formation we screened a large number of deficiencies and mutations for their ability to dominantly suppress or enhance the $crb^{extraTM}$-*GFP*-induced vein phenotype (unpublished results). We observed a strong enhancement of the venation defects in $crb^{extraTM}$-*GFP*-expressing flies that carry only one functional copy of *Notch*: nearly all veins were thicker and formed broad deltas (Fig. 1C,C′). This phenotype mirrors aspects of the *Notch* haplo-insufficiency phenotype (Fig. 1D,D′), suggesting that $crb^{extraTM}$-*GFP* overexpression interferes with the Notch signalling pathway. Knocking down Crb levels by expression of crb^{RNAi} induced the opposite phenotype; L4 and L5 were shorter, leaving a gap between the end of the veins and the margin (Fig. 1E,E′). This phenotype is a phenocopy of the *Notch* gain-of-function allele *Abruptex* (Ax^{M1}, also known as $N^{Ax\text{-}M1}$) (Fig. 1F,F′), suggesting that Notch and Crb act antagonistically on vein formation. Only the *Notch* loss-of-function thick-vein phenotype, but not the notched-wing phenotype, could be rescued by expressing crb^{RNAi} in wings of *Notch* heterozygous animals (Fig. 1G,G′). Similarly, the gain-of-function phenotype of Ax^{M1} could be rescued by $crb^{extraTM}$-*GFP* expression (Fig. 1H,H′). In both cases, the majority of the wings showed veins that looked completely wild-type (quantified in Fig. 1I). These results confirm previous observations (Herranz et al., 2006) and suggest that in developing wild-type wings *crb,* or more specifically the extracellular domain of Crb, negatively regulates Notch pathway activity, at least in some areas of the wing.

Two different periods of Notch activity during wing development have been determined by using a temperature-sensitive *Notch* allele. Whereas early temperature shifts (second or third larval instar) result

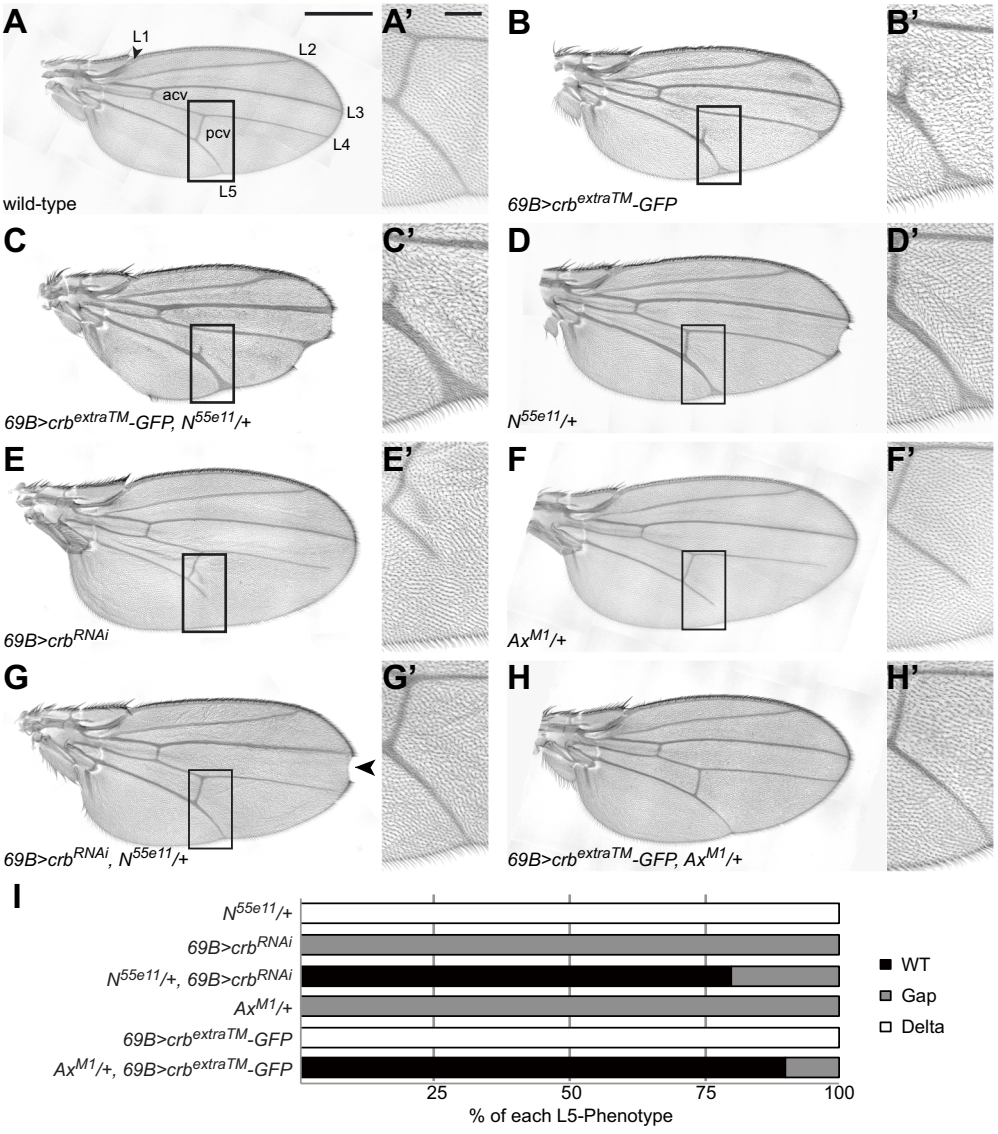

Fig. 1. *crb* negatively regulates *Notch* activity. (A-H) Wings of female flies with different genotypes. (A′-H′) Magnifications of boxed L5 region in A-H. (A) Wild-type wing showing the longitudinal veins (L1-L5), the anterior crossvein (acv) and the posterior crossvein (pcv). (B) Overexpression of $crb^{extraTM}$-*GFP* leads to vein thickening and formation of deltas. (C) Removing one copy of *Notch* (*N*) enhances the $crb^{extraTM}$-*GFP* phenotype. (D) *Notch* haploinsufficiency results in vein thickening and delta formation similar to $crb^{extraTM}$-*GFP* overexpression. (E) *crb* knockdown by RNAi induces shortening of L5 ('gap' phenotype), phenocopying the *Notch* gain-of-function allele Ax^{M1} (F). (G) Knockdown of *crb* supresses the *Notch* haploinsufficiency phenotype. (H) Overexpression of $crb^{extraTM}$-*GFP* rescues the *Notch* gain-of-function phenotype of Ax^{M1}. Note that *crb* knockdown does not rescue the wing notching phenotype of *N*/+ wings (arrowhead in G). (I) Quantification of the L5 phenotypes in the rescue experiments (G,H), *n*=10 for all genotypes. Scale bars: 500 µm in A, 100 µm in A′.

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in wing notches, shifting the temperature after pupariation induces thickening of the veins, but no notches at the wing margin (Huppert et al., 1997; Shellenbarger and Mohler, 1978; Sturtevant and Bier, 1995). The thick-vein phenotype is a consequence of a failure to properly partition vein and intervein cell fates during pupal stages (De Celis, 1998). Knocking down *crb* suppressed only the thick-vein *Notch* loss-of-function phenotypes, but not the notched-wing phenotype, suggesting that *crb* regulates *Notch* activity only at later stages. To substantiate this assumption, we used the temperature-dependent GAL80 to activate GAL4-mediated crb^{RNAi} during 24 h intervals, from first larval instar to late pupal development, to score the phenocritical period at which knockdown of *crb* activates the Notch pathway. This approach showed that *crb* is required to suppress Notch activity between 120 and 144 h after egg laying (Fig. S1). This period overlaps with that during which Notch activity is required to partition vein and intervein cell fates (Sturtevant and Bier, 1995).

Taken together, the data suggest that *crb* is a suppressor of Notch activity during vein/intervein fate refinement.

The extracellular domain of Crb is required for apical localisation of Notch

Given the genetic interactions between *Notch* and *crb*, we asked whether Crb has an effect on Notch protein at the cellular level. Therefore, we first analysed Notch expression and localisation throughout wing development in animals that clonally overexpressed $crb^{extraTM}$-*GFP*. In wild type, Notch is localised apically (Fehon et al., 1991; Sasaki et al., 2007). In wing discs of third instar larvae (Fig. 2A-B″) as well as in pupal wings at 28 h after puparium formation (APF) (Fig. 2C-D″) overexpression of $crb^{extraTM}$-*GFP*

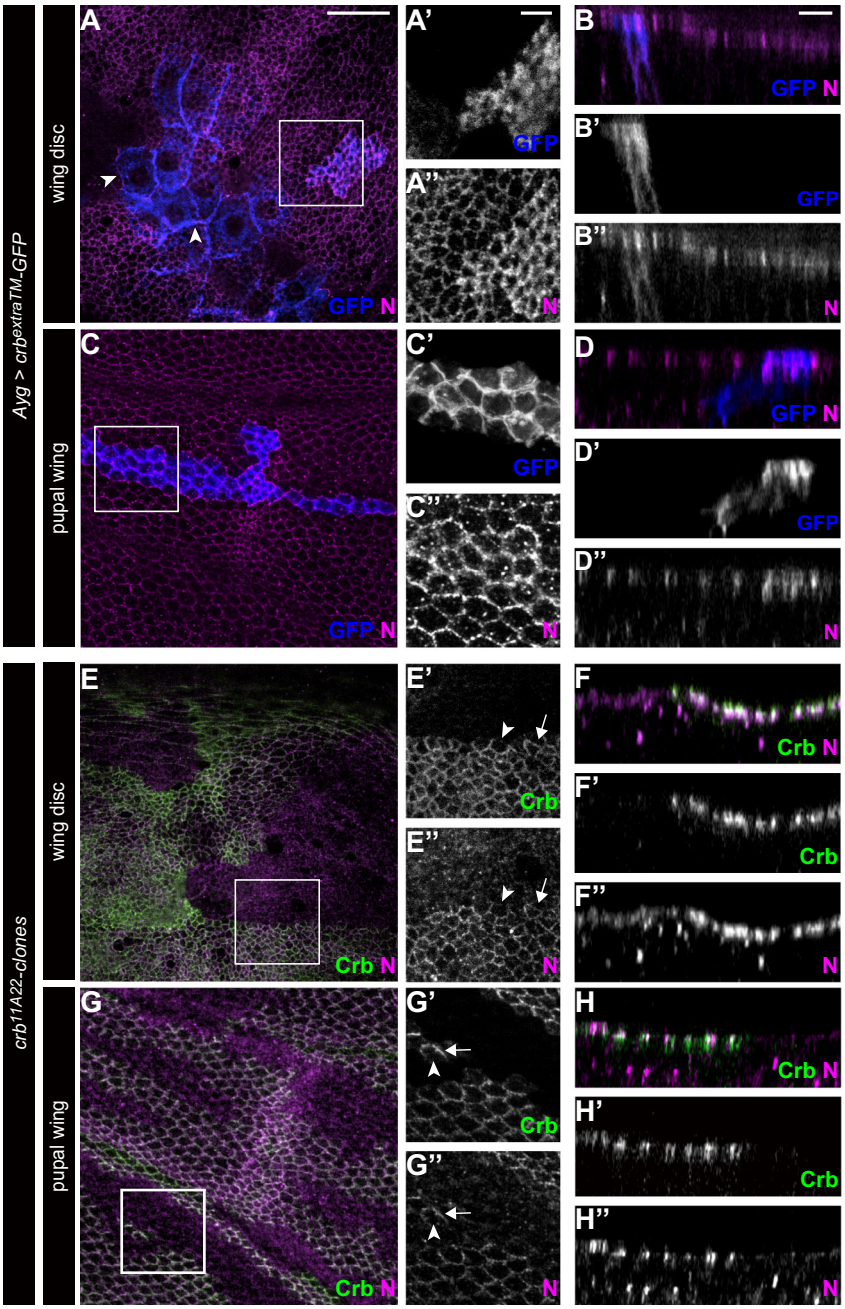

Fig. 2. Crb is required for apical membrane localization of Notch. (A-D″) $crb^{extraTM}$-*GFP* expression under the control of flip-out-GAL4 (*Ayg*). Areas expressing $crb^{extraTM}$-*GFP* are GFP-positive (blue). (A) Apical section of a third instar wing disc. (A′-A″) Magnifications of the boxed area in A. More Notch accumulates in cells that express $crb^{extraTM}$-*GFP*. Arrowheads in A mark $crb^{extraTM}$-*GFP* expression in cells of the peripodial membrane. (B-B″) Transverse view of a third instar wing disc. Notch expands laterally in cells expressing $crb^{extraTM}$-*GFP*. (C) Apical section of a pupal wing. (C′-C″) Magnifications of the boxed area in C. More Notch accumulates in cells that express $crb^{extraTM}$-*GFP*. (D-D″) Transverse view of a pupal wing. Notch expands laterally in cells expressing $crb^{extraTM}$-*GFP*. (E-H″) crb^{11A22} clones, marked by the absence of Crb staining (green). (E) Apical section of a third instar wing disc. (E′-E″) Magnifications of the boxed area in E. Notch is missing from the apical plasma membrane of *crb* mutant cells. (F-F″) Transverse view of a third instar wing disc. (G) Apical section of a pupal wing. (G′-G″) Magnifications of the boxed area in G. Notch is missing from the apical plasma membrane of *crb* mutant cells. (H-H″) Transverse view of a pupal wing. Notch localises in the subapical region in the presence of Crb. In both larval and pupal stages, Crb localises at the apical membrane at the border between two wild-type cells (arrows in E′,G′), but is missing from the membrane between wild-type and *crb* mutant cells (arrowheads in E′,G′). Notch distribution exactly follows the Crb pattern (arrows and arrowheads in E″,G″). Apical is up in B-B″,D-D″,F-F″,H-H″. Magnifications in A′-B″,C′-D″,E′-F″,G′-H″ are scalings of the region of interest using a bicubic algorithm. Scale bars: 20 µm in A, 5 µm in A′,B. All Notch stainings in this work were done using an antibody against the extracellular domain of the protein. Stainings with an antibody against the Notch intracellular domain (Nicd) gave the same results (example shown in Fig. S3).

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[GFP-positive cells (blue) in Fig. 2A,A′,C,C′] caused a two-fold increase of apical Notch (Fig. 2A″,C″; quantification in Fig. S2) and spreading of Notch towards the lateral side of the cells, particularly in larval discs (Fig. 2B,B″,D,D″). In clones lacking *crb*, Notch protein was reduced and no longer accumulated at the apical membrane as in wild type. This was more obvious in pupal clones (Fig. 2G-H″; quantification in Fig. S2), but also in larval clones, which is in contrast to what has been reported (Herranz et al., 2006). This effect of *crb* on Notch localisation was not limited to developing wings, but was also observed in eye and leg discs (Fig. S3).

To better understand how Crb affects Notch localisation, we took advantage of a recent observation showing that localisation of Crb at the apical plasma membrane of an epithelial cell requires Crb expression in its neighbouring cell (Hafezi et al., 2012; Letizia et al., 2013; Pellikka et al., 2002; Pocha and Wassmer, 2011). This means that the plasma membrane of a wild-type cell abutting a *crb* mutant cell was devoid of Crb (Fig. 2E′,G′, arrowheads), whereas Crb was localised apically on the plasma membranes between two wild-type cells (Fig. 2E′,G′, arrow). Similarly, Notch was absent from the plasma membranes between wild-type and *crb* mutant cells (Fig. 2E″,G″, arrowheads), whereas Notch co-localised with Crb in plasma membranes between two wild-type cells (Fig. 2E″,G″, arrows). In contrast, wild-type cells adjacent to *Notch* mutant cells had an even distribution of both Notch and Crb around the cell. Furthermore, loss of *Notch* did not affect Crb localisation (Fig. 3A-A″). This suggests that anisotropic distribution of Notch in wild-type cells at the *crb* and wild-type clone boundary is a consequence of the absence of Crb at this membrane.

To test whether loss of apical Notch in *crb* mutant cells is the result of a specific role of Crb in regulating Notch and not just due to the loss of apicobasal polarity, we analysed the localisation of other apical transmembrane proteins in *crb* mutant clones in pupal wings. The atypical cadherins Fat (Ft) and Dachsous (Ds) and the seven-pass membrane cadherin Flamingo [Fmi, also known as Starry night (Stan)] were still localised at the apical membrane in the absence of *crb* (Fig. 3B-C″ and data not shown).

Recent experiments in cell culture using recombinantly expressed proteins suggested that zebrafish Notch1a and zebrafish Crb (Crb1, Crb2a and Crb2b) interact via their extracellular domains (Ohata et al., 2011). Here, we applied the proximity ligation assay (PLA) (Söderberg et al., 2006) to analyse any direct association between Notch and Crb *in-situ*, using antibodies directed against the extracellular domains of Notch and Crb. A moderate amount of PLA signal was observed in wild-type tissue in pupal wings. This signal was strongly increased in cells overexpressing $crb^{extraTM}$-*GFP* (Fig. 4A-A″), and reduced in *crb* mutant cells (Fig. 4B-B″). From this we conclude that Crb directly controls Notch localisation.

Taken together, our results indicate that *crb* specifically facilitates accumulation of Notch through direct interaction of its extracellular domain with that of the Notch receptor.

In the absence of Crb, Notch is endocytosed from the apical plasma membrane independent of ligands

Since Crb regulates Notch localisation, we next asked whether Crb affects Notch transport to, or its stability at, the plasma membrane. In order to distinguish between these two possibilities we blocked endocytosis in pupal wings containing *crb* mutant clones. If Crb is important for stabilising Notch at the plasma membrane, blocking endocytosis would restore Notch at the membrane in *crb* mutant cells. If Crb is involved in trafficking of Notch to the plasma membrane we would expect no difference in Notch localisation. After 1 h incubation in dynasore, a well-established inhibitor of Dynamin (also known as Shibire) (Macia et al., 2006), Notch was localised at the apical plasma membrane not only in wild-type cells, but also in *crb* mutant cells (Fig. 5A-A″), as shown by co-localisation with the adherens junction-associated protein Canoe (Cno) (Fig. 5B-B‴), suggesting that Crb inhibits endocytosis of Notch. To confirm this result, we blocked endocytosis by using $shibire^{1}$ (shi^{1}), a temperature-sensitive allele of the gene encoding Dynamin. In shi^{1} mutant cells at the restrictive temperature, Notch is retained at the apical plasma membrane of *crb*-negative cells (Fig. S4). These results suggest that Crb stabilises Notch at the apical plasma membrane by preventing its endocytosis.

It has been proposed that in *Drosophila* eye discs the absence of Crb facilitates incorporation of Notch into early endocytic vesicles marked by Hrs (Richardson and Pichaud, 2010). Therefore, we asked whether increased Notch endocytosis in *crb* mutant cells observed in pupal wings depends on the Notch ligands Delta (Dl) and Serrate (Ser). If Notch endocytosis in the absence of Crb is ligand-dependent, we would not detect loss of Notch from the membrane in

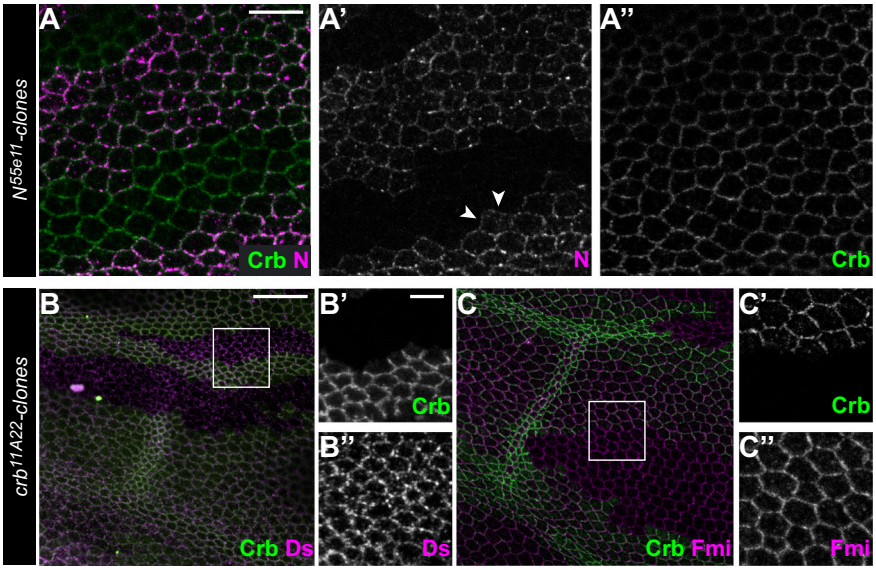

Fig. 3. Localisation of apical proteins in *Notch* and *crb* mutant clones. (A-A″) Apical section of a pupal wing with N^{55e11} clones, marked by the absence of Notch staining (magenta). Notch is localised along the whole circumference of the cell, including membranes between wild-type and *Notch* mutant cells (arrowheads in A′). Crb localisation is not affected in *Notch* mutant cells. (B-C″) Apical sections of pupal wings with crb^{11A22} clones, marked by the absence of Crb staining (green). Neither the localisation of Ds (B-B″) nor of Fmi (C-C″) are affected by the loss of Crb. Magnifications in B′,B″,C′,C″ are scalings of the region of interest using a bicubic algorithm. Scale bars: 10 µm in A, 20 µm in B, 5 µm in B′.

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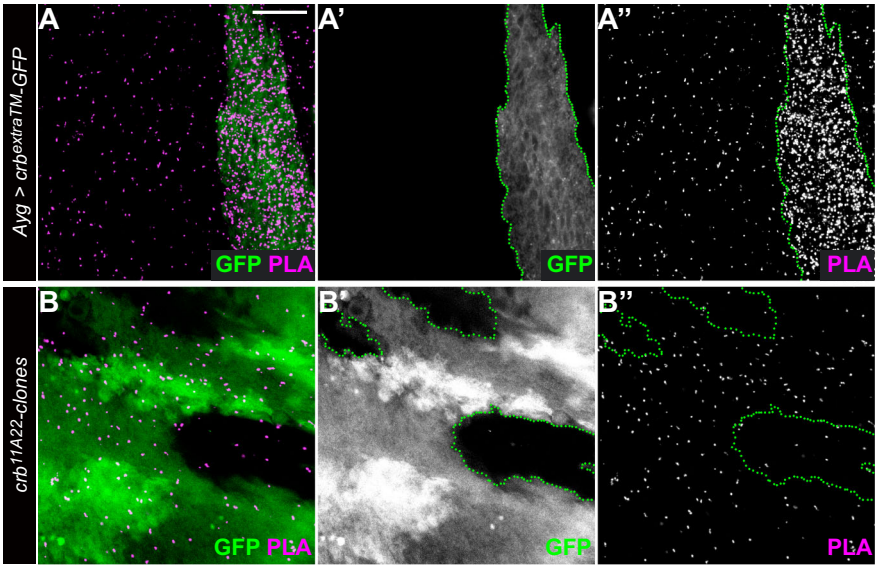

Fig. 4. Crb interacts with Notch *in-situ* via the extracellular domain. (A-A″) Apicolateral section (3 µm) of a pupal wing expressing $crb^{extraTM}$-*GFP* under the control of flip-out GAL4 (*Ayg*). The area expressing $crb^{extraTM}$-*GFP* is GFP-positive (green) and outlined by the green dotted line in A′,A″. PLA signal shows a moderate number of direct interactions between the extracellular domains of Crb and Notch in wild-type tissue and a strong increase in the number of interactions if $crb^{extraTM}$-*GFP* is expressed. (B-B″) Apicolateral section (3 µm) of a pupal wing with crb^{11A22} clones. *crb*-negative tissue is marked by loss of GFP and clones are outlined by the green dotted line in B′,B″. The PLA signal is almost gone in *crb* clones. Scale bar: 20 µm.

cells that lack *crb* as well as *Dl* and *Ser*. In cells that are wild-type for *crb*, Notch was localised at the membrane (Fig. 6A-B′). This localisation was independent of whether Dl and Ser were expressed [GFP-positive cells (blue) in Fig. 6A,B″] or not (Fig. 6A,B″, GFP-negative cells). In cells that were mutant for *crb*, Notch was lost from the plasma membrane (Fig. 6A,C,C′) independent of the presence (GFP-positive cells in Fig. 6A,C″) or absence (Fig. 6A,C″, GFP-negative cells) of Dl and Ser. This implies that loss of Notch from the membrane in the absence of *crb* is not dependent on these ligands.

Beside ligand-mediated endocytosis of processed Notch (Le Borgne et al., 2005), internalisation of the full-length receptor can also occur in a ligand-independent way, a process that requires ubiquitination by the ubiquitin ligase Deltex (Dx) (Andersson et al., 2011; Baron, 2012; Guruharsha et al., 2012; Hori et al., 2004, 2014, 2012; Kopan, 2012). We reasoned that if the removal of Notch from the plasma membrane in the absence of *crb* is mediated by Dx, concomitant removal of *crb* and *dx* should prevent Notch endocytosis. Pupal wings at 28 h APF mutant for *dx* showed increased Notch accumulation at the apical membrane (Fig. 6E,F′,F″, RFP-negative cells), similar to what has been reported for wing discs (Yamada et al., 2011). In contrast, loss of *dx* did not affect Crb (Fig. 6E,F,F″). Cells mutant for *crb*, but otherwise dx^{+} lost most of their apical Notch (Fig. 6E,G-G″, RFP-positive cells). Upon simultaneous loss of *dx* and *crb*, Notch was still lost from the apical membrane, but was retained in the apical region of the cell (Fig. 6E,G-G″, RFP-positive cells). Loss of Notch from the apical membrane in these doubly mutant cells was confirmed by staining pupal wings under detergent-free conditions (Fig. S5).

It has been proposed that one role of Crb is to reduce the ligand-dependent signalling activity of Notch in the margin of larval wing discs by repressing the activity of the γ-secretase complex (Herranz et al., 2006). This complex is required to proteolytically process the intracellular domain of Notch, thereby allowing it to enter the nucleus and activate Notch target genes (reviewed in Le Borgne et al., 2005). To determine whether upregulation of γ-secretase activity is responsible for the mis-localisation of Notch in pupal wing cells lacking Crb, we analysed Notch localisation in cells that concomitantly downregulate *crb* and *Presenilin* (*Psn*), the gene encoding the enzymatic component of the γ-secretase complex. These cells lost Notch from their apical membrane and exhibited the same intracellular distribution of Notch as cells mutant for just *crb*, suggesting that *Psn* is not required for Notch endocytosis in the absence of *crb* (Fig. 7A-B″).

Taken together, our results imply that in pupal wings, (1) absence of Crb induces Notch endocytosis; (2) endocytosis of Notch from the apical membrane in the absence of Crb is independent of Notch ligands, of the γ-secretase complex and of Dx, and (3) Dx or ubiquitination of Notch by Dx is needed for further transport of endocytosed Notch.

crb prevents activation of the ligand-independent Notch signalling pathway

Ligand-independent internalisation of the full-length Notch protein from the plasma membrane and trafficking through the degradation pathway has been suggested as a means to prevent excess Notch activation (Pratt et al., 2011). Conversely, it has been shown that the endocytosed Notch receptor can escape lysosomal degradation and becomes involved in signalling in a Dx-dependent way (Andersson et al., 2011; Baron, 2012; Guruharsha et al., 2012; Hori et al., 2004, 2014, 2012; Kopan, 2012). In fact, *dx/Y* wings show a weak *Notch* loss-of-function phenotype (Hori et al., 2004).

Given the weak Notch gain-of-function wing vein phenotype upon loss of *crb* (Fig. 1), we asked whether absence of *crb* results in enhanced activation of the Notch signalling pathway. Therefore, we monitored the expression of the Notch activity reporter *Su(H)-Gbe-GFP:nls* (de Navascues et al., 2012) in pupal wings, which contained areas expressing crb^{RNAi}. In wild-type wings at 26-28 h APF the reporter revealed Notch activity along the wing margin and adjacent to the developing veins (not shown). Wing areas in which *crb* is downregulated via RNAi expression (outlined in magenta in Fig. 8A″) exhibit ectopic activation of the Notch reporter in vein tissues (Fig. 8A-B″, quantified in Fig. 8C). In contrast, *crb*-expressing vein cells show little or no activity of the reporter. These data suggest that during development of wild-type wings Crb negatively regulates Notch signalling in the veins.

Since Notch trafficking is impaired in cells doubly mutant for *dx* and *crb* (see Fig. 6), we hypothesise that the gain-of-function Notch phenotype observed upon *crb* knockdown in the wing is dependent on *dx*. To test this hypothesis we expressed crb^{RNAi} in animals that carry dx^{1}, a hypomorphic allele of *dx* (Fig. 9A-C). Wings of flies mutant for dx^{1} showed a weak *Notch* loss-of-function phenotype with small delta-shaped broadening of L5 (Fig. 9A). The *Notch*

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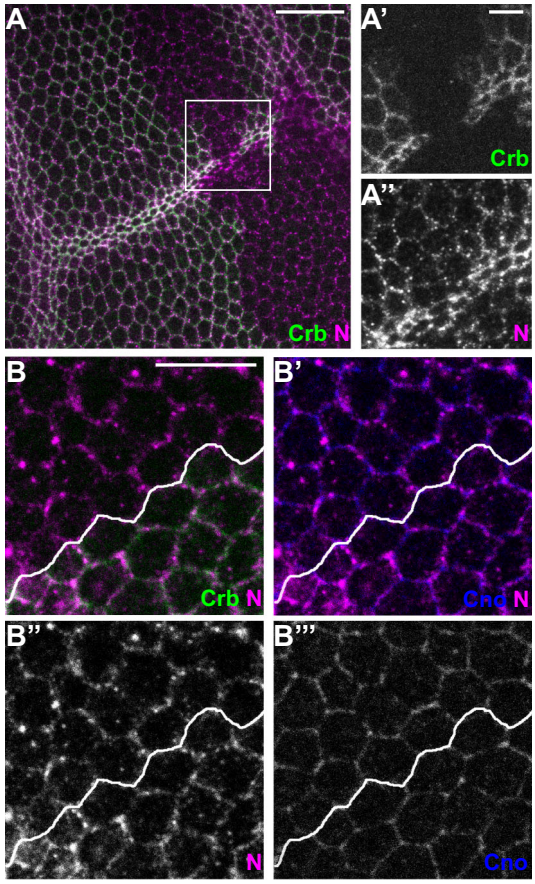

Fig. 5. Blocking endocytosis in *crb* cells rescues the membrane localization of Notch. (A-B‴) Apical sections of pupal wings with crb^{11A22} clones [marked by the absence of Crb staining (green)] after 60 min incubation in 60 µm dynasore. (A′,A″) Magnifications of the boxed area in A. The white line in B-B‴ marks the clone border between crb^{11A22} cells (above) and wild-type cells (below). Note that Notch co-localises with the adherens junction marker Cno (blue) in crb^{11A22} cells. Magnifications in A′,A″ are scalings of the region of interest using a bicubic algorithm. Scale bars: 20 µm in A, 5 µm in A′,B.

gain-of-function phenotype obtained upon downregulation of *crb* (Fig. 9B) was nearly completely reverted to wild-type when combined with dx^1 (Fig. 9C), in that L5 reached the wing margin in the vast majority of the animals (quantified in Fig. 9H). These results suggest that ectopic activation of Notch in *crb* mutant cells requires *dx* activity for further trafficking of Notch to a late endosomal compartment, where the receptor is activated.

Ligand-independent activation of Notch in a late endosomal compartment depends on components of the HOPS (homotypic fusion and protein sorting) and the AP-3 (Adaptor Protein 3) complexes. The activity of these complexes is required to maintain full-length Notch at the limiting membrane of multivesicular bodies (MVB), thereby preventing Notch internalisation and degradation in the lumen of the late endosome and/or lysosome (Wilkin et al., 2008). For example, lack of *carnation* (*car*) or *garnet* (*g*) suppresses ectopic Notch activation resulting from overexpression of Dx (Wilkin et al., 2008). *car* encodes the homolog of the yeast protein VPS33, a constituent of the HOPS complex (Mullins and Bonifacino, 2001). Car protein is localised on large Rab7-positive MVBs and is required for the fusion of the Rab7-positive late endosome with the lysosome (Sevrioukov et al., 1999; Sriram et al., 2003; reviewed in Balderhaar and Ungermann, 2013). *g* is the *Drosophila* homolog of the human AP-3 complex member AP-3δ (Ooi et al., 1997). The AP-3 complex is required for selective transport to the lysosome (Cowles et al., 1997; Dell'Angelica et al., 1997; Newell-Litwa et al., 2007). Reduction of *car* or *g* function in an otherwise wild-type background using hypomorphic alleles car^1 and g^1 had no effect on the formation of L5 (Fig. 9D,E). However, Notch activation induced by knockdown of *crb* was suppressed upon reduced function of *car* or *g* (compare Fig. 9B with F and G, quantification in Fig. 9H). Notch activation in the absence of *crb* was not the result of a general trafficking defect, since expression of dominant-negative versions of Rab4, Rab6 or Rab8 (Zhang et al., 2007) did not rescue the L5 phenotype of crb^{RNAi} expressing wings (Fig. S7).

Taken together, our results show that the extracellular portion of Crb is directly involved in Notch stabilisation. In the absence of Crb the bulk of Notch is cleared from the apical membrane by endocytosis. Internalised Notch results in ectopic activation of the Notch pathway in the veins in a ligand-independent, but *dx*-dependent manner.

DISCUSSION

Since the discovery of *Drosophila* Crb as a regulator of apicobasal polarity (Grawe et al., 1996; Tepass, 1996; Tepass and Knust, 1990), a variety of other functions have been assigned to this highly conserved protein, all of which take place in the apex of epithelial cells, where Crb is localised. Imaginal epithelia of the developing fly are ideally suited to dissect specific functions of Crb, since loss of *crb* in these epithelia does not induce breakdown of tissue integrity. We now provide evidence that direct interaction between Crb and Notch stabilises Notch by preventing its endocytosis and thereby fine-tunes Notch-mediated signalling in the developing wing.

Previous genetic interaction studies suggested a role of Crb in regulating the ligand-dependent Notch pathway. Herranz et al. (2006) concluded that in larval wing discs absence of Crb enhances intramembrane proteolytic processing of Notch by γ-secretase, called S3 cleavage, which depends on ligand-induced S2 cleavage (Mumm et al., 2000) and is followed by the translocation of the intracellular portion of Notch into the nucleus and activation of downstream genes. Along the same line, Richardson and Pichaud (2010) proposed, based on inhibition experiments, that absence of Crb in eye imaginal discs enhances Dl-mediated, metalloprotease-dependent S2 cleavage of Notch, and showed increased internalised Notch in uptake assays in the absence of Crb. Our results differ from those previously published in that: (1) in pupal wings, endocytosis of Notch in the absence of *crb* does not depend on the ligands Dl and Ser nor on Psn, suggesting that it is independent of the S2 and S3 cleavage of Notch; and (2) endocytosis of Notch in *crb* mutant pupal cells activates the *dx*-dependent ligand-independent Notch pathway. The obvious differences between our data and those published previously could be explained by the fact that we analysed a different process – wing vein refinement in pupal wings rather than wing margin specification or eye/head development. Our results underscore the importance of tightly controlling such a potent receptor at various levels in a context-dependent manner, and point to more than a single mechanism by which Crb regulates Notch. This behaviour is not unique to Crb and has been suggested for another transmembrane protein, Sanpodo (Spdo). In the daughter cells of asymmetrically dividing sensory organ precursor cells, *spdo* enhances Notch signalling in one daughter by interacting with γ-secretase, while it suppresses Notch signalling in the other, Numb-expressing daughter by causing Notch internalisation (Babaoglan et al., 2009; Upadhyay et al., 2013).

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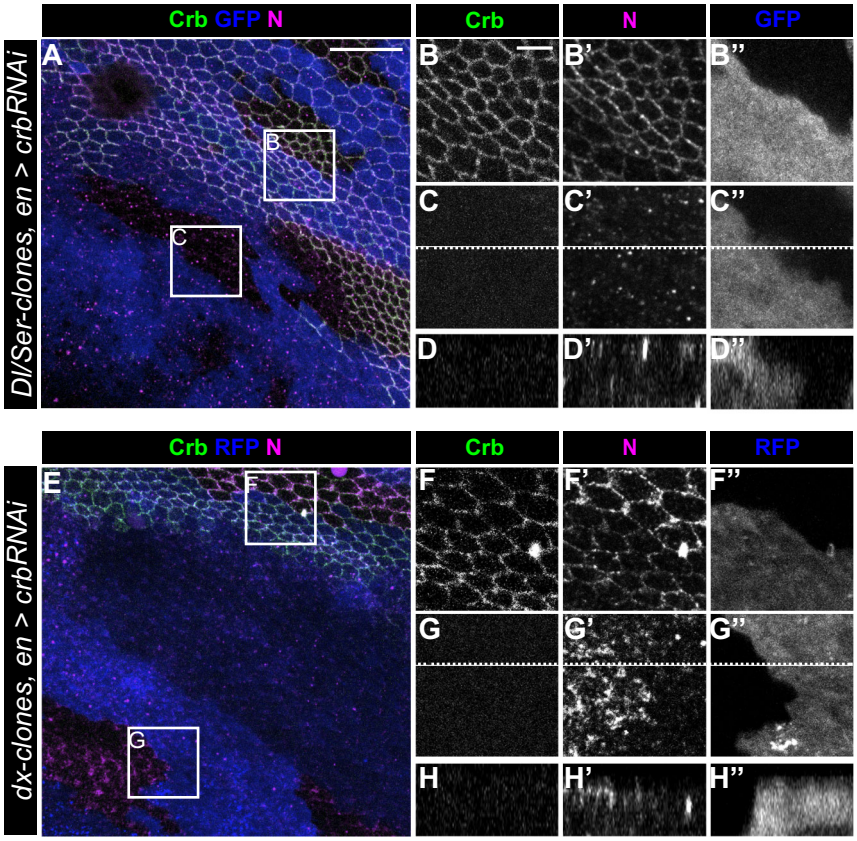

Fig. 6. Crb affects Notch localisation in a ligand-independent manner. (A-C″) Apical sections of a pupal wing carrying Dl^{revF10}, Ser^{RX82} clones [marked by the absence of GFP (blue)] and expressing $en{>}crb^{RNAi}$ [marked by the absence of Crb staining (green)]. (B-C″) Magnifications of the boxed areas in A. (B-B″) Localisation of neither Crb (B) nor Notch (B′) is affected by loss of Dl and Ser alone (GFP-negative cells in B‴). (C-C‴) In Crb-negative cells (C) membrane localisation of Notch (C′) is not rescued by the absence of Dl and Ser (GFP-negative cells in C″). (D-D″) Transverse view of the area marked by the dotted lines in C-C″. (E-G″) Apical sections of a pupal wing carrying dx^{ENU} clones [marked by the absence of RFP (blue)] and expressing $en{>}crb^{RNAi}$ [marked by the absence of Crb staining (green)]. (F-G″) Magnifications of the boxed areas in E. (F-F″) Crb localisation at the membrane (F) is not affected by loss of *dx* (RFP-negative cells in F″), whereas Notch accumulates at the membrane of *dx*-negative cells (F′,F″). (G-G″) Absence of Dx (RFP-negative cells in G″) in Crb-negative cells (G) does not restore membrane localisation of Notch but results in Notch accumulation in the apical cortex (G′). (H-H″) Transverse view of the area marked by the dotted lines in G-G″. All pictures except A and E are scalings of the region of interest using a bicubic algorithm. Scale bars: 20 µm in A, 5 µm in B.

A wealth of data document that a complex network of genes regulates Notch levels through endocytosis and trafficking. For example, mutations in the E3 ubiquitin ligase-encoding genes *Nedd4* and *Suppressor of deltex* [*Su(dx)*], in components of the endosomal sorting complex required for transport (ESCRT) complex and in *lethal giant discs* [*lgd*, also known as *l(2)gd1*], as well as overexpression of *dx* alter Notch trafficking (Hori et al., 2004, 2011; Jaekel and Klein, 2006; Matsuno et al., 2002; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). Our results add yet another level to this regulatory network by demonstrating that Crb ensures the stability of Notch at the apical plasma membrane, thereby preventing Notch endocytosis and activation. Crb exerts this function by close association of its extracellular domain with the extracellular domain of Notch, thus supporting previous conclusions obtained from studies using recombinant Notch and Crb proteins from zebrafish (Ohata et al., 2011). We would like to stress that this relationship between Crb and Notch should not be generalised, as increased accumulation of Crb3 in the zebrafish midgut mutant for *plasmolipin* increased Notch internalisation, but reduced

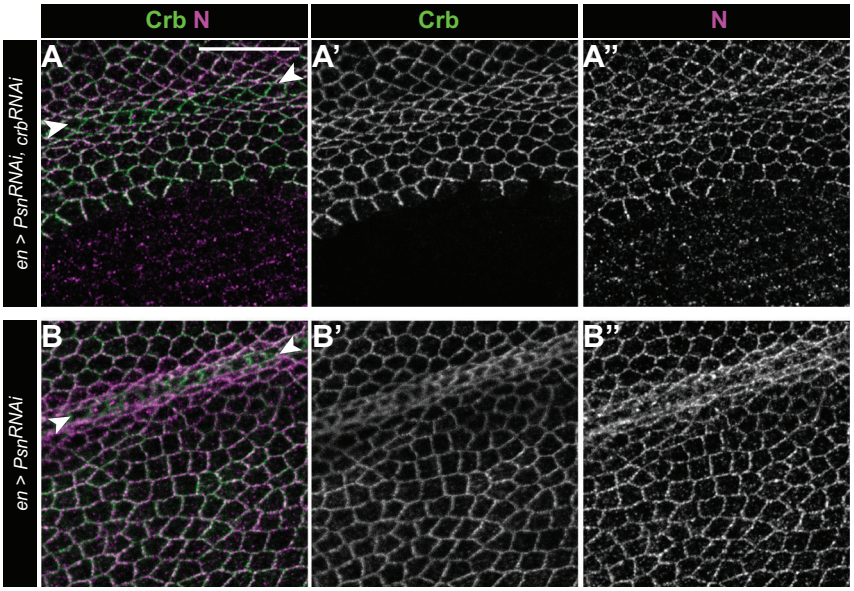

Fig. 7. Crb affects apical localisation of Notch independent of *Presenilin*. (A-A″) Apical section of pupal wings expressing $en{>}Psn^{RNAi}$, crb^{RNAi}. *crb* and *Psn* expression are knocked down in the posterior half of the wing, starting a few cell rows posterior to the L3 (marked by arrowheads). In cells with knocked down *crb* and *Psn* (A′) Notch is lost from the membrane (A″), similar to cells lacking Crb alone (compare with Fig. 3). (B-B″) Apical section of pupal wings expressing $en{>}Psn^{RNAi}$. Knockdown of *Psn* occurs in the posterior half of the wing, starting a few cell rows below the L3 (marked by arrowheads, compare with panel A). Knockdown of *Psn* alone has no effect on the apical localisation of either Crb (B′) or Notch (B″). Scale bar: 20 µm.

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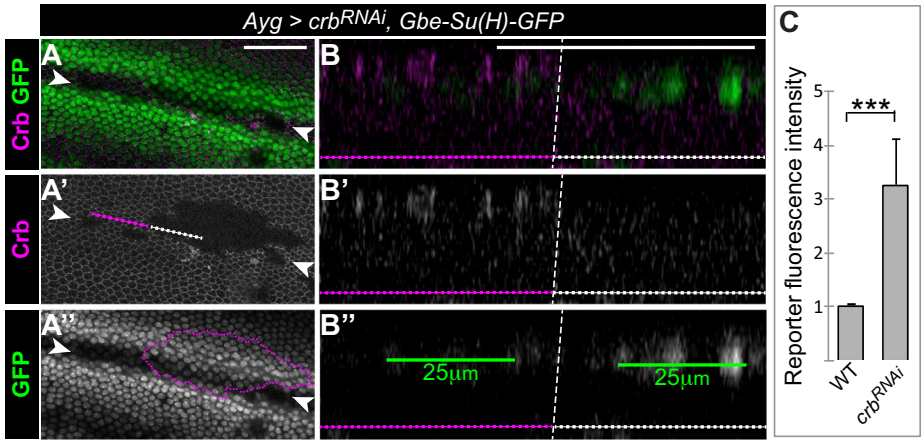

Fig. 8. Notch activity is ectopically upregulated in *crb*-negative vein cells. (A-A″) Section of a pupal wing expressing *crb*^{RNAi} under the control of flip-out GAL4 (*Ayg*) [*crb* knockdown areas are marked by the absence of Crb-staining (magenta)] and the Notch (N) reporter *Gbe-Su(H)-GFP* (green). Crb staining is shown at the apical level, GFP staining is shown at the level of the nuclei. The arrowheads bracket L3. *crb*-negative vein cells show ectopic activation of *Gbe-Su(H)-GFP* (outline of the *crb* knockdown area in magenta in A″). (B-B″) Transverse section of the area marked by the dotted line in A′, the magenta section of the line marks wild-type tissue, the white section marks *crb*-negative cells. (C) Quantification of Notch reporter activity in *crb*-negative vein areas (*n*=5). Reporter activity was determined as average fluorescence intensity along an apical 25 µm line. For each wing wild-type levels were set to 1. An example is given in B″. Error bars represent the standard deviation. Scale bars: 200 µm in A,B. Further examples are shown in Fig. S6. ***$P \leq 0.001$ by Student's *t*-test.

Notch signalling (Rodríguez-Fraticelli et al., 2015). Hence, Crb can be added to the growing list of non-canonical Notch ligands that modify and/or buffer its activity. Some of these contain tandem EGF-like repeats similar to Crb and are known to positively or negatively regulate Notch signalling, e.g. the mammalian proteins Delta and Notch-like epidermal growth factor-related receptor (DNER) and Delta-like 1/Delta-like 2 (Dlk1/Dlk2, also known as Protein delta homolog 1/2), or the *Drosophila* Notch-activating protein Weary (Wry) (reviewed in Kopan and Ilagan, 2009; Wang, 2011).

Concomitant with Notch endocytosis in the absence of Crb, the *dx*-dependent Notch pathway is activated. It has been debated whether this pathway can be activated under physiological conditions, or whether activation occurs only accidentally as a result of impaired Notch degradation (reviewed in (Palmer and Deng, 2015). Support for the presence of ligand-independent Notch activation was provided by demonstrating that follicle cells lacking both *trans*- and *cis*-acting ligands can activate Notch target genes (Palmer et al., 2014).

Although reduction of Crb results in widespread endocytosis of Notch, Notch pathway activation occurs only very locally, i.e. in veins, suggesting context-dependent regulation. Both Notch and Crb are upregulated in cells adjacent to the veins (data not shown) and it is conceivable that activation of the reporter gene in the absence of Crb requires a critical threshold level of Notch endocytosis, which is only achieved in veins. Alternatively, activation of internalised Notch might depend on cofactors that are only present in veins, but not in intervein regions and/or that Crb regulates additional processes differently in vein and intervein regions. Finally, the difference observed in Notch activation in the absence of Crb may point to differences in Notch degradation in different regions of the developing wing as suggested previously (Hori et al., 2011). The effect of loss of *crb* on the phenotype of adult wings is even more restricted, namely to the distal portion of L4 and L5, suggesting variable spatial or temporal sensitivity of different parts of the wing veins. A similar temporally controlled effect on wing vein development has also been described for *Su(dx)*, mutations in which show a much milder phenotype in the adult wing compared

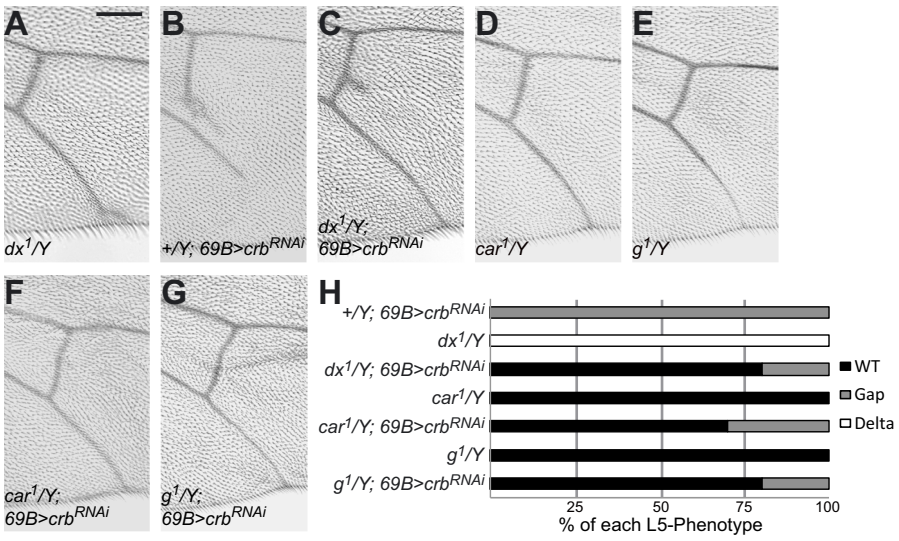

Fig. 9. Loss of Crb leads to activation of the ligand-independent Notch pathway. (A-G) L5 area of male wings. (A) L5 of wings hemizygous for the hypomorphic allele *dx*^{1} shows a weak *Notch* loss-of-function phenotype. The 'gap' phenotype of Crb-negative wings (B) is rescued upon simultaneous loss of Dx (C). L5 of wings hemizygous for the hypomorphic alleles *car*^{1} (D) or *g*^{1} (E) do not show a *Notch* gain- or loss-of-function phenotype. The 'gap' phenotype of Crb-negative wings (B) is rescued upon simultaneous loss of *car* (F) or *g* (G). (H) Quantification of the L5 phenotypes in the rescue experiments, *n*=10 for all genotypes. Scale bar: 100 µm.

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with the pupal wing (Mazaleyrat et al., 2003). In contrast to the restricted *Notch* gain-of-function phenotype achieved upon loss of *crb*, overexpression of the membrane-bound extracellular domain of Crb mimics the loss of Notch in all veins. This is accompanied by increased accumulation of Notch at the apical membrane throughout the wing, which might lead to cis-inactivation of the ligand-dependent pathway (Sprinzak et al., 2010). However, the *Notch*-typical notched-wing phenotype was not observed, underlining the conclusion that Crb modulates Notch signalling in a strictly context-dependent manner. Widespread endocytosis of Notch upon reduction of Crb does not affect the ligand-dependent Notch pathway, suggesting that residual Notch at the membrane is sufficient for proper ligand-dependent signalling. This conclusion gets support from the finding that concomitant knockdown of *crb* and components of the ligand-independent pathway (e.g. *dx*, *car* and *g*) does not lead to *Notch* loss-of-function phenotypes in the wing.

Taken together, our data provide a molecular basis to explain how the apical determinant Crb can regulate homeostasis of epithelial cells by stabilising Notch at the apical membrane, thereby fine-tuning Notch pathway activity. Interestingly, reduction of Crb3 expression correlates with an increase in the tumorigenic potential in mouse epithelial kidney cells, defining Crb3 as a tumour suppressor gene (Karp et al., 2008; reviewed in Laprise, 2011). Given the well-established role of Notch in tumour formation, it will be appealing to explore whether deregulation of Notch in some of these cancers is associated with the loss and/or reduction of Crumbs proteins.

MATERIALS AND METHODS

Fly strains and genetics

All flies and crosses were kept at 25°C unless stated otherwise. The following fly strains were used: *FRT82B, crb^{11A22}/TM6B* (Johnson et al., 2002), *Dl^{revF10}, Ser^{RX82}, FRT82B/TM6B* [Bloomington *Drosophila* Stock Center (BDSC), 6300], *shi^{1}* (BDSC, 7068), *dx^{ENU}, FRT19A/FM7* (Xu and Artavanis-Tsakonas, 1990), *dx^{1}* (BDSC, 34), *ec^{1}, N^{55e11}/FM7* (Lindsley and Zimm, 1992), *N^{55e11}, FRT19A/FM7C* (BDSC, 28813), *Ax^{M1}/FM7* (gift from J. de Celis CBMSO, Madrid, Spain and J. de Navascues European Cancer Stem Cell Research Institute, Cardiff, UK). *FRT82B, ubi GFP/TM6B* (BDSC, 5188), *FRT82B, arm-lacZ/TM6* (BDSC, 7369), *Ubi-mRFP-nls, hs-Flp22* (BDSC, 8862), *FRT19A* (BDSC, 31418), *car^{1}* (BDSC, 19), *g^{1}* (BDSC, 3958), *UAS-crb^{extraTM}-GFP* (Pellikka et al., 2002), *UAS-crb^{RNAi}* (VDRC #39177), *UAS-Psn^{RNAi}* (VDRC #43082), *Ay*-GAL4 (BDSC, 3953), *69B*-GAL4 (BDSC, 1774), *C765*-GAL4 (BDSC, 36523), *en^{hen}*-GAL4 (gift from C. Dahmann, Institute of Genetics, TU Dresden, Dresden, Germany), *tub*-GAL80^{ts20} (BDSC, 7019), *UAS-Rab4 DN37* (BDSC, 9768), *UAS-Rab6 DN03* (BDSC, 23250), *UAS-Rab8 DN09* (BDSC, 23271), *Gbe+Su(H)-nlsGFP* (de Navascues et al., 2012).

Mutant clones were generated using the FLP/FRT System (Xu and Rubin, 1993), larvae were heat-shocked 72 h after egg laying (AEL) for 90 min at 37°C. GAL4 flip-out clones (Ito et al., 1997) were generated by heat-shocking larva 72 h AEL for 10 min at 37°C.

Time-course experiment

Flies expressing *C765*-GAL4 (Brand and Perrimon, 1993) and *tub*-GAL80^{ts20} were crossed to flies carrying UAS-*crb^{RNAi}*. Eggs were collected for 4 h at 25°C and shifted to the restrictive temperature (29°C) for the intervals indicated.

Immunohistochemistry

Third instar larvae were dissected in Grace's insect medium and fixed in 4% PFA in Grace's for 30 min at room temperature (RT). After washing, discs were incubated for 1 h at RT in blocking solution [0.1 mg/ml BSA in PBT (0.1% w/v Triton X-100/PBS)].

White prepupae were collected and aged for 26-28 h at 29°C. Pupal cases were opened in 4% PFA in PBS and pupae were fixed overnight at 4°C. After washing, pupal wings were dissected in PBS and incubated for 1 h at RT in blocking solution. Wings and discs were incubated with antibody overnight at 4°C, washed in PBT and incubated with secondary antibody for 2 h at RT. Samples were mounted in Vectashield (Vector Laboratories) and imaged with a LSM 700 Laser Scanning Confocal Microscope (Carl Zeiss). Images were processed with Fiji (Schindelin et al., 2012), and Photoshop (Adobe) software. Unless otherwise stated all images shown represent apical projections of 1 µm thickness that include the subapical region and the adherens junctions.

The following primary antibody were used, diluted in blocking solution: rat anti-Crb, 1:2000 (Tepass et al., 1990); mouse anti-Necd, 1:800 (C458.2H, DSHB); mouse anti-Nicd, 1:5 [C17.9C6, DSHB (supernatant)]; rabbit anti-GFP 1:2000, (A11122, Invitrogen); rabbit anti-Cno, 1:1000 (Matsuo et al., 1999); mouse anti-Ds, 1:400 (gift from S. Eaton, MPI-CBG, Dresden, Germany); rat anti-Ft, 1:1000 (Gift from H. McNeill, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada); mouse anti-Fmi, 1:20 (Flamingo #74, DSHB), rabbit anti-Ed 1:5000 (Laplante and Nilson, 2006).

Proximity ligation assay (PLA)

Antibodies against the extracellular domains of Crb and Notch were directly conjugated to PLA oligonucleotides using the Duolink Probemaker Plus and Minus Kits (Sigma Aldrich). The assay was performed on pupal wings with the Duolink PLA Kit (Sigma Aldrich) following the manufacturer's instructions. In brief, pupal wings were dissected as described above and incubated with the conjugated primary antibodies overnight at 4°C, followed by ligation at 37°C for 30 min and amplification at 37°C for 100 min. Pupal wings were mounted in Duolink mounting media.

Dynasore treatment of pupal wings

Pupae were dissected in Grace's insect medium and incubated in 60 µM dynasore in Grace's at RT for 1 h. The dynasore was washed out and wings were fixed in 4% PFA in PBS for 1 h at RT.

Analysis of adult wings

Wings were fixed in 70% ethanol, rehydrated in PBS and mounted on microscopy slides in Hoyer's medium. The slides were left at 65°C for 24 h and imaged with an AxioImager.Z1 microscope (Carl Zeiss). Images were processed with Fiji and Photoshop software.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

L.N. conceived the studies, designed and performed the experiments, interpreted results, and wrote and edited the manuscript. E.K. conceptualized the goal, supported data interpretation, wrote and edited the manuscript, and acquired funding.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.141762.supplemental

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