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Self-refinement of Notch activity through the transmembrane protein Crumbs: modulation of γ-Secretase activity

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Cell interactions mediated by Notch family receptors have been implicated in the specification of tissue boundaries. Tightly localized activation of Notch is crucial for the formation of sharp boundaries. In the *Drosophila* wing imaginal disc, the Notch receptor is expressed in all cells. However, Notch activity is limited to a narrow stripe of cells along the dorsal–ventral compartment boundary, where it induces the expression of target genes. How a widely expressed protein becomes tightly regulated at the dorsal–ventral boundary in the *Drosophila* wing is not completely understood. Here, we show that the transmembrane protein Crumbs is involved in a feedback mechanism used by Notch to refine its own activation domain at the *Drosophila* wing margin. Crumbs reduces the activity of the γ-Secretase complex, which mediates the proteolytic intracellular processing of Notch. These results indicate a novel molecular mechanism of the regulation of Notch signal, and also that defects in Crumbs might be involved in similar abnormal γ-Secretase complex activity observed in Alzheimer's disease.

Keywords: *Drosophila* wing; Crumbs; Notch; presenilin

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INTRODUCTION

Notch signalling is involved in a variety of cell-fate decisions during development. Ligand binding to the Notch receptor results in a γ-Secretase-mediated proteolytic intracellular processing of Notch. The Notch intracellular domain fragment (N^{icd}) translocates to the nucleus, where it interacts with the Suppressor of Hairless (Su(H)) DNA-binding protein (reviewed by Lai, 2004). In cooperation with other transcriptional activators, including Mastermind, transcription of Notch target genes can be induced.

In the wing imaginal disc, Notch is activated at the boundary between dorsal and ventral compartments. Several mechanisms have been implicated in limiting Notch activity to cells immediately adjacent to the dorsal–ventral (DV) boundary. Early in development, restricted expression of the glycosyltransferase Fringe in dorsal cells modifies the receptor protein Notch in the dorsal compartment (Bruckner *et al*, 2000; Moloney, 2000). Fringe activity makes dorsal cells more sensitive to Delta, a ligand expressed by ventral cells, and less sensitive to Serrate, the ligand expressed by dorsal cells (Fig 1A; Diaz-Benjumea & Cohen, 1995; de Celis *et al*, 1996; Doherty *et al*, 1996; Fleming *et al*, 1997; Panin *et al*, 1997). Consequently, signalling by each ligand is limited to nearby cells on the opposite side of the boundary, with the result that high levels of Notch activity are limited to a narrow band of cells along the DV boundary. Later in development, another set of cell interactions takes over to maintain Notch activity along the DV boundary (Fig 1A; de Celis & Bray, 1997; Micchelli *et al*, 1997). Notch activation induces Wingless expression in boundary cells. Wingless expression limits Notch activity to cells immediately adjacent to the DV boundary.

The transmembrane protein Crumbs is a central regulator of epithelial apical–basal polarity in *Drosophila*, and loss-of-function mutations in the human homologue of Crumbs, CRB1 (RP12), cause recessive retinal dystrophies, including retinitis pigmentosa (den Hollander, 1999). Here, we present evidence of a new function of Crumbs in *Drosophila*. Crumbs mediates a novel negative feedback loop of Notch to restrict its own activation domain to a thin stripe corresponding to the DV boundary. We show that Crumbs attenuates Notch signalling by repressing the activity of the γ-Secretase complex. This complex also mediates the intracellular cleavage of the amyloid precursor protein (APP), thus leading to accumulation of the Aβ peptide in plaques in Alzheimer's disease (AD). We postulate that Crumbs may also be a potential modulator of AD pathogenesis.

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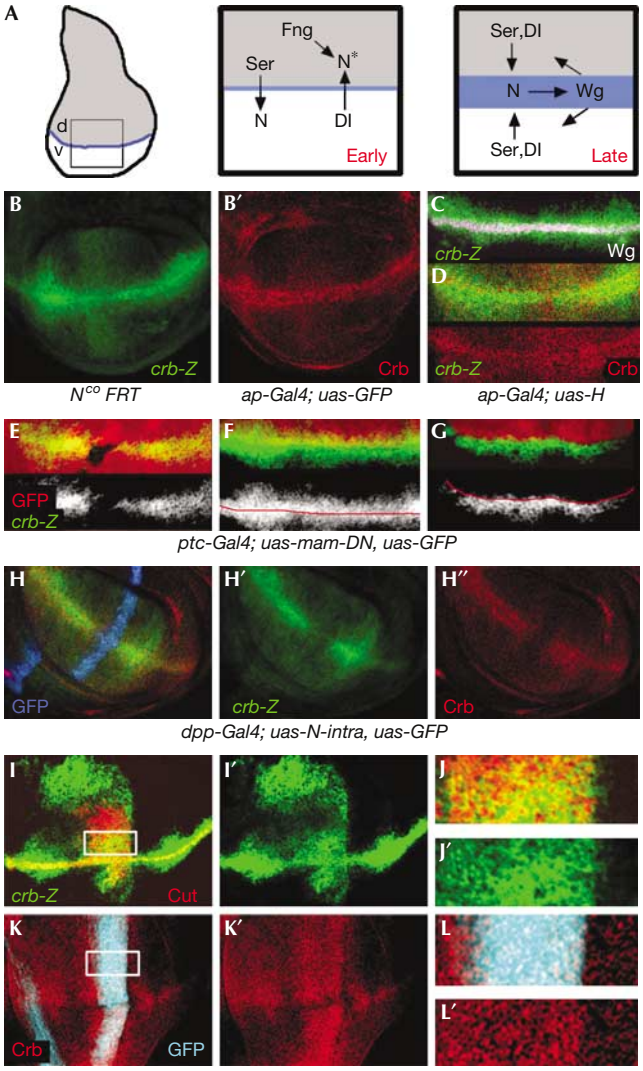

Fig 1 | *crb* expression in the wing imaginal disc depends on Notch activity. (**A**) Establishment and maintenance of the dorsal–ventral (DV) organizing centre. Early in development, Serrate (Ser) signals to ventral cells and Delta to dorsal cells to activate Notch along the DV boundary. Fng modifies Notch (N*) in dorsal cells, thus making it sensitive to Dl and not to Serrate. Later in development, a positive feedback loop between Wg- and Ser/Dl-expressing cells maintains the signalling centre along the DV boundary. (**B–D**) Crumbs protein (red) and *crb-lacZ* expression (green) in third instar wing discs. *crb-lacZ* expression was visualized by an antibody against β-galactosidase (β-Gal). Wg protein expression is shown in white in (**C**). (**E**) Clones of cells lacking *Notch* activity marked by the absence of green fluorescent protein (GFP; red). *crb-lacZ* expression (green) is reduced in the mutant clones. (**F,G**) Wing discs that expressed GFP (**F**, in red) or Hairless and GFP (**G**) under the control of ap^{Gal4}. *crb-lacZ* expression (green) is reduced in the ap^{Gal4} expression domain. (**H–H″**) Wing disc that expressed GFP (blue) and a dominant-negative form of Mastermind (mam-DN) under the control of ptc^{Gal4}. *crb-lacZ* expression (green) and Crb protein (red) are reduced in the ptc^{Gal4} expression domain. (**I,I′**) Wing disc that expressed the intracellular domain of Notch under the control of dpp^{Gal4}. *crb-lacZ* expression is shown in green and Cut protein expression in red. (**J,J′**) High magnification of the squared region shown in (**I**). (**K,K′**) Wing disc that expressed the intracellular domain of Notch and GFP (blue) under the control of dpp^{Gal4}. Crumbs protein expression is shown in red. (**L,L′**) High magnification of the squared region of the disc shown in (**K**).

RESULTS AND DISCUSSION

We were interested in the function of Crumbs (Crb) on the basis of its expression pattern in the developing wing imaginal disc. A *piggyBac-lacZ* insertion (see Methods for details) that lies 240 bp upstream of the *crumbs* transcription start site is expressed at higher levels along the DV boundary (Fig 1B–D). Low level of expression is detected in all wing cells. Expression of Crb protein coincides with that of *crb-lacZ* expression (Fig 1B,D), which depends on Notch activity. Clones of cells mutant for the Notch receptor reduce *crb* expression (Fig 1E). Blocking Notch activation by driving the expression of *Hairless* (a repressor of the Notch signalling pathway at a transcriptional level) or a dominant-negative form of the nuclear Notch effector Mastermind (Giraldez *et al*, 2002) reduces expression of *crb* in a cell-autonomous manner (Fig 1F–H). Expression of an activated form of the Notch receptor (N^{icd}) in a perpendicular stripe to the DV boundary induces ectopic *crb* expression in the wing pouch in a cell-autonomous manner (Fig 1I–L). In wing discs mutant for the *Notch* gain-of-function allele $Abruptex^{M1}$, the expression domain of *crb* is expanded and ectopically expressed in the wing pouch (Fig 3K,M).

We noticed that the expression domain of *crb* is broader than that of Wingless (Wg) or Cut, two known target genes of Notch at the DV boundary (Fig 1C; data not shown). This observation suggests that *cut* and *wg* may be high-threshold targets of Notch, and *crumbs* may require lower levels of Notch activation to be induced. Indeed, *crb* responds better to ectopic activation of the Notch signalling pathway than Wg and Cut. In $Abruptex^{M1}$ (Fig 3J–M) and *dpp-gal4; uas-*N^{icd} wing discs (Fig 1I), the regions of ectopic expression of *crb* are larger than those of Cut and Wg. We monitored Cut, Wg and Crb expression in a thermosensitive Notch background (N^{ts2}) reared for 24 h at permissive (18 °C) or restrictive (25 and 29 °C) temperatures. At 18 °C, all target genes are expressed along the DV boundary (supplementary Fig 1 online). At 25 °C, Cut expression is almost completely lost, and Wg and Crb expression levels are slightly reduced. At 29 °C, expression of Cut and Wg is not observed along the DV boundary, and expression levels of Crb are reduced. When reared at 29 °C for longer periods, Crb expression is lost.

The *crb* gene was genetically identified as an essential component for organizing apical–basal polarity and adherens junctions (AJs) in embryonic epithelia (Tepass *et al*, 1990). Crb is a transmembrane protein with a long extracellular domain with 28 EGF repeats and a short cytoplasmic tail. This intracellular domain is required and is sufficient to exert Crb function in setting apical–basal polarity. To assess the role of Crb in wing development, clones of *crb* mutant cells were generated and analysed in wing discs and adult wings. Mutant clones for *crb* (crb^1 and crb^{11A22}) are able to cover large areas of the wing, indicating that loss of *crb* does not compromise cell viability. Apical–basal polarity and AJs are not affected in these clones, as previously reported in the eye imaginal disc (Izaddoost *et al*, 2002; Pellikka *et al*, 2002).

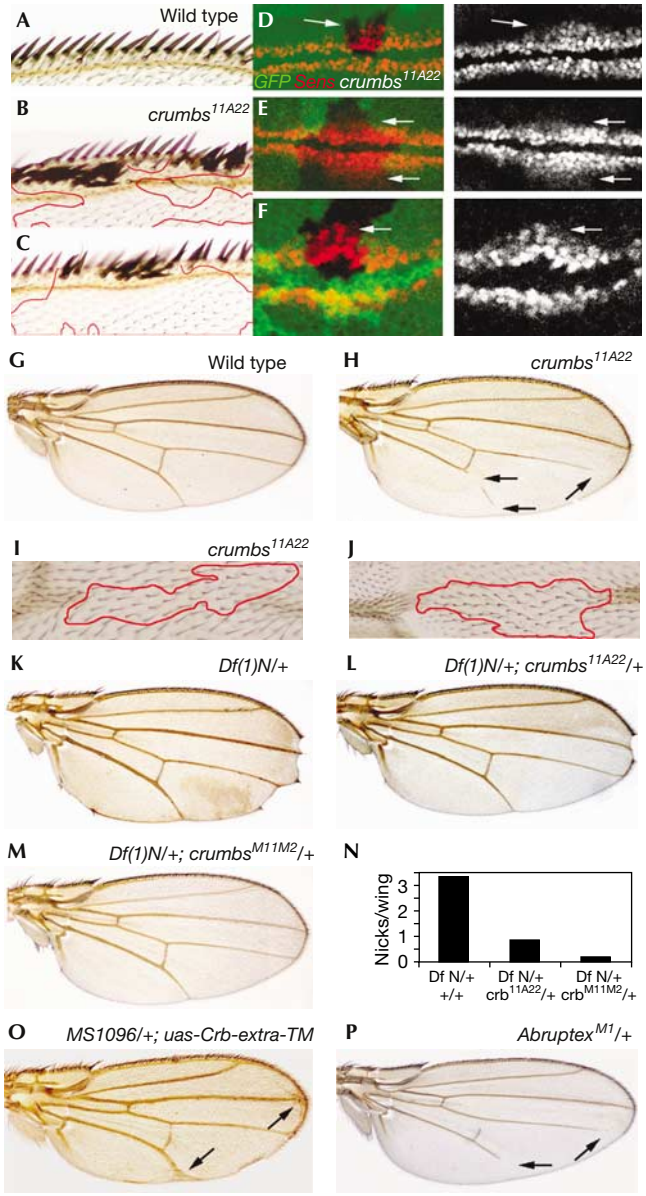

Fig 2 | *crb* mutants cause Notch gain-of-function phenotypes. (**A**) Cuticle preparation of a wild-type anterior wing margin (AWM) in the dorsal compartment. Note the single, well-organized row of mechanosensory bristles and the thin chemosensory bristles in every fourth position. (**B**,**C**) Two examples of AWM in the dorsal compartment carrying clones mutant for crb^{11A22} and marked with *forked*. Note the extra mechanosensory and chemosensory bristles. The contour of the clones is highlighted in red. (**D**–**F**) Wing discs with clones of cells lacking *crb* activity and marked by the absence of the green fluorescent protein (GFP; green) marker. Expression of Senseless protein (red) visualizes the domain in which the sensory organs of the wing margin will develop. Note the ectopic expression of Senseless in mutant cells (arrows). (**G**–**J**) Cuticle preparations of a wild-type wing (**G**) and wings with *crb* mutant clones running along the veins (**H**–**J**). Note the absence of vein differentiation (arrows in (**H**), and clones highlighted in red in (**I**,**J**)). (**K**–**M**) Cuticle preparations of *Df(1)Notch-8/+* (**K**), *Df(1)Notch-8/+; $crumbs^{11A22}$/+* wing (**L**) and *Df(1)Notch-8/+; $crumbs^{M11M2}$/+* wings (**M**). (**N**) Histogram showing the number of nicks per wing in the genotypes corresponding to (**K**–**M**). Nicks per wing(*Df(1)Notch-8/+*) = 3.3 ± 1; nicks per wing(*Df(1)Notch-8/+; $crumbs^{11A22}$/+*) = 0.7 ± 0.5; nicks per wing(*Df(1)Notch-8/+; $crumbs^{M11M2}$/+*) = 0.2 ± 0.6. n(*Df(1)Notch-8/+*) = 10, n(*Df(1)Notch-8/+; $crumbs^{11A22}$/+*) = 18, n(*Df(1)Notch-8/+; $crumbs^{M11M2}$/+*) = 11. (**O**) Cuticle preparation of a wing expressing a truncated form of Crumbs lacking the intracellular domain (Crb-extra-TM) in the MS1096-Gal4 domain. Note that veins are thicker and deltas are in the distal part of the veins (arrows). (**P**) Cuticle preparation of an Ax^{M1}/+ wing. Note the loss of the distal part of the veins (arrows).

Localization of Armadillo and E-Cadherin proteins, two components of the AJs, is not affected (supplementary Fig 2 online). Thus, the maintenance of apical–basal polarity and AJs in the wing epithelia does not seem to depend on Crb activity. When abutting the DV boundary, *crb* mutant clones produce a broadening of the wing margin in a cell-autonomous manner (Fig 2A–C), mimicking a gain-of-function activity of Notch. The adult wing is decorated by five longitudinal veins (Fig 2G). Increased levels of Notch activity induce loss of vein tissue (Fig 2P). *crb* mutant clones lose vein differentiation in a cell-autonomous manner (Fig 2H–J). The haploinsufficient phenotype of *Notch* (loss of wing margin and vein thickening; Fig 2K) is rescued in a heterozygous *crb* mutant background (Fig 2L–N). These results indicate that Crb attenuates Notch signalling in the developing wing.

The wing margin is decorated by sensory bristles (Fig 2A). The domain in which the precursors of sensory bristles form can be visualized in developing wing discs by the expression of the transcription factor Senseless (Nolo *et al*, 2000). Clones mutant for *crumbs* form more rows of Senseless-expressing cells (Fig 2D–F). Wg is expressed along the DV boundary by the activity of Notch. High levels of Wg signalling in nearby cells specify the domain of Senseless expression. The broadening of the Senseless domain suggests that Wg might be ectopically expressed owing to ectopic activation of the Notch signalling pathway. We then analysed Wg protein and *wg-lacZ* expression in clones of *crb* mutant cells. *wg* was ectopically expressed in these cells (Fig 3A–C), although at lower levels than the endogenous *wg* expression domain. The largely autonomous effect of *crb* mutant clones on bristle specification may be due to the relatively low levels of ectopic Wg. Cut, a target of Notch that requires high levels of Notch activity (supplementary Fig 1 online), was not affected in these clones (data not shown). $Abruptex^{M1}$ is a gain-of-function allele of Notch, in which the expression domain of the Notch target genes Wg and Cut along the DV boundary is broader than in wild-type discs (Fig 3J,L). In *crb* heterozygous wing discs, this phenotype is enhanced (Fig 3K,M). These results indicate ectopic activation of the Notch signalling pathway in the absence of Crb activity.

We wondered at which level Crb affects the Notch signalling cascade. The absence of Crb activity induces hyperactivation of the Notch signalling pathway. We then tried to rescue the wing phenotype by coexpressing dominant-negative forms of the nuclear Notch effector Mastermind (Giraldez *et al*, 2002), the Notch receptor

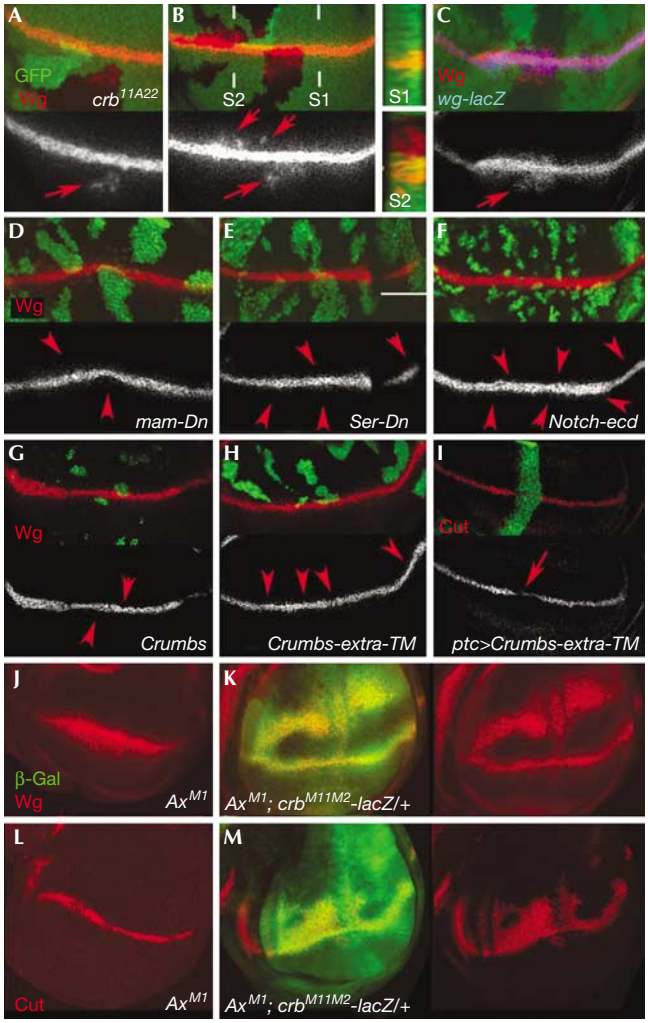

Fig 3 | Epistatic analysis of Crb activity. (**A–C**) Wing discs with clones of cells lacking *crb* activity and marked by the absence of the green fluorescent protein (GFP; green) marker. Wg protein distribution (**A–C**, red) and *wg-lacZ* (**C**, blue) expression were monitored. Note ectopic expression of Wg and *wg-lacZ* in the cells of the clones close to the dorsal–ventral (DV) boundary (red arrows). (**D–F**) Wing discs with crb^{11A22} mutant clones positively labelled by the GFP (green) marker. Activity of the Notch signalling pathway was blocked in these clones using a dominant-negative form of Mastermind (Mam-DN; **D**), Serrate ligand (Ser-DN; **E**) and Notch receptor (Necd; **F**). Note the absence of ectopic expression of Wg in the clones (red arrowheads). (**G,H**) Wing discs with crb^{11A22} mutant clones positively labelled by the GFP marker and expressing the full-length form of Crumbs (**G**) or a truncated form of Crumbs lacking the intracellular domain (Crb-extra-TM; **H**). (**I**) Overexpression of Crb-extra-TM (green) in the *ptc-Gal4* domain leads to reduced expression of Cut (red) at the DV boundary (red arrow). (**J–M**) Wingless (**J,K**) and Cut (**L,M**) protein expression (red) in Ax^{M1} (**J,L**) and Ax^{M1}; $crumbs^{M11.M2}$-*lacZ*/+ third instar wing imaginal discs (**K,M**). $crumbs^{M11.M2}$-*lacZ* expression is shown in green.

(N^{ecd}; Parks *et al*, 2000) and the Notch ligands (Ser^{DN} or Dl^{DN}; see Methods for details). Mastermind blocks Notch signalling at the transcriptional level. The dominant-negative form of Notch blocks signal reception and its ligands titrate out Notch receptor (Sun & Artavanis-Tsakonas, 1997; Parks *et al*, 2000; Bardot *et al*, 2005), thus blocking Notch signalling at the receptor level. Interestingly, in all cases, ectopic expression of Wingless was rescued, suggesting that Crb acts upstream or at the level of the Notch receptor (Fig 3D–F). The subcellular localization of Crb partially overlaps that of Notch at the Zonula Adherens (supplementary Fig 2 online). In *crb* mutant clones, Notch receptor subcellular localization is not affected.

Ligand binding to the Notch receptor results in a γ-Secretase-dependent proteolytic intracellular processing of Notch that gives rise to the Notch intracellular domain fragment (N^{icd}), which translocates to the nucleus, where it interacts with the Suppressor of Hairless (Su(H)) DNA-binding protein (Lai, 2004). We then monitored γ-Secretase activity in the absence of Crb activity. For this purpose, we used three different reporters of γ-Secretase activity. The first two reporters express, either specifically in the eye under the control of the eye-specific GMR promoter or ubiquitously under the control of heat-shock promoter, a truncated form of β-APP, a transmembrane protein also cleaved by γ-Secretase (Struhl & Adachi, 2000) in which the intracellular domain is substituted by the Gal4 protein (Struhl & Adachi, 2000). The γ-Secretase reporter output constructs consist of a Gal4-responsive transcriptional cassette driving either the expression of the *Drosophila* cell death activator GRIM (Guo *et al*, 2003) or green fluorescent protein (GFP; Struhl & Adachi, 2000). Cleavage of APP releases from the membrane a fragment consisting of the intracellular domain of APP and Gal4, which translocates to the nucleus and activates GRIM or GFP expression, thus leading to reduced or green fluorescent adult eyes (Fig 4B,D). Flies mutant for *crumbs* enhance γ-Secretase cleavage of APP in the adult eye, indicating that Crumbs attenuates γ-Secretase activity (Fig 4C–F). In the wing imaginal discs, γ-Secretase cleavage of APP is reduced in the domain of *crb* expression (Fig 4H,I). Flies heterozygous for *crb* enhance the activity of γ-Secretase (compare Fig 4G,H). Similar results were obtained when γ-Secretase cleavage of Notch was measured (Fig 4J–L). In this case, a reporter ubiquitously expressing a truncated form of the Notch receptor lacking the extracellular domain (N-ECN) was used. The intracellular domain was substituted by the Gal4 protein (Struhl & Adachi, 2000).

Crumbs associates with the Stardust and DPATJ proteins through its short cytoplasmic tail to establish apical–basal cell polarity in the embryo (Bhat *et al*, 1999; Bachmann *et al*, 2001). Expression of this cytoplasmic tail in a mutant background for *crb* is sufficient to partially rescue the failure in apical–basal cell polarity, indicating that the large extracellular domain of Crb is dispensable for this process. Three different observations indicate that the extracellular domain of Crb is required to attenuate Notch signalling and that the intracellular domain is dispensable. Mutant clones for a null allele of *stardust* (sdt^{XP96}) are able to cover large areas of the wing without any overt phenotype when abutting the DV boundary or when running along the longitudinal veins (data not shown). The *crb* mutant wing phenotype can be rescued when simultaneously expressing either full-length Crb (Fig 3G) or a truncated form of Crb lacking the whole intracellular tail (Crb-Extra-TM; Fig 3H). Overexpression of Crb-Extra-TM leads to a mild downregulation of the Notch signalling pathway (Figs 2O,3I). In the adult wing, veins are thicker, resembling a *Notch*

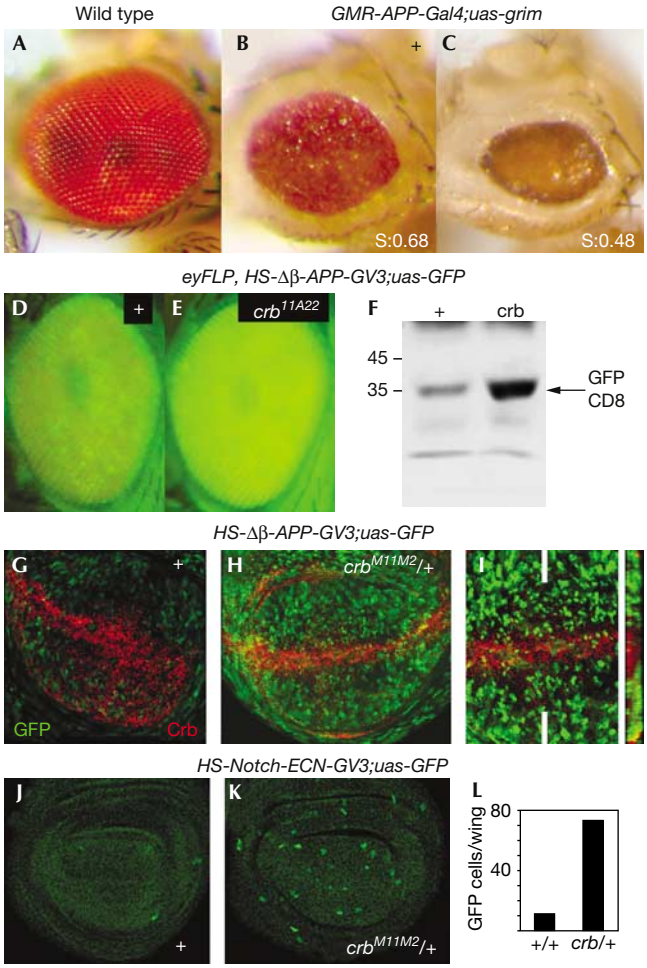

Fig 4 | Crb reduces γ-Secretase activity. (**A–C**) Adult fly eyes of the following genotypes: (**A**) wild type; (**B**) *GMR–APP–GAL4, UAS–GRIM/+* and (**C**) *GMR–APP–GAL4, UAS–GRIM/crb$^{M11.M2}$*. The final size of the adult flies was measured and compared with wild-type eyes. The ratio (mutant eye size/wild-type eye size) is shown as S in (**B**,**C**). S(*GMR–APP–GAL4, UAS–GRIM/+*) = 0.68 ± 0.1, $n = 60$; S(*GMR–APP–GAL4, UAS–GRIM/crb$^{M11.M2}$*) = 0.48 ± 0.06, $n = 57$. (**D**,**E**) Adult fly eyes of the following genotypes: (**D**) eyFLP,*HS–*Δβ*–APP–GAL4-VP16, UAS–GFP* and (**E**) eyFLP,*HS–*Δβ*–APP–GAL4-VP16, UAS–GFP; FRT82/FRT82 crb^{11A22}*. (**F**) Western blot showing the green fluorescent protein (GFP) amounts of eyFLP,*HS–*Δβ*–APP–GAL4-VP16, UAS–GFP* (control) and eyFLP,*HS–*Δβ*–APP–GAL4-VP16, UAS–GFP; FRT82/FRT82 crb^{11A22}* adult heads. (**G–I**) *HS–*Δβ*–APP–GAL4-VP16, UAS–GFP* (**G**) and *HS–*Δβ*–APP–GAL4-VP16, UAS–GFP; crb^{M11M2}/+* wing discs (**H**,**I**). Crb protein expression (red) is shown in (**G**). *crb-lacZ* expression (red) was visualized in (**H**,**I**) by an antibody against β-galactosidase. The right panel in (**I**) shows an XZ confocal section of the wing disc at the level of the white lines. (**J**,**K**) *HS–Notch-ECN–GAL4-VP16, UAS–GFP* (**J**) and *HS–Notch-ECN–GAL4-VP16, UAS–GFP; crb^{M11M2}/+* wing discs (**K**). (**L**) Histogram showing the number of GFP-positive cells per wing in the genotypes corresponding to (**J**,**K**). The number of discs per genotype was 6.

loss-of-function phenotype (de Celis & Garcia Bellido, 1994). In the wing imaginal disc, Crb-Extra-TM overexpression reduces the expression levels of Cut at the DV boundary, a target of Notch that requires high levels of Notch activity. Wg expression is not affected (data not shown).

Signalling centres along compartment boundaries are required to organize the growth and pattern of the surrounding tissue. However, too much of a signal has deleterious effects. The Notch signalling centre organizes the growth and pattern of the developing wing primordium, partially through the secreted protein Wingless. Wingless activity contributes to limit Notch activity to cells immediately adjacent to the DV boundary. Here, we present evidence that Notch also contributes to the refinement of its activation domain through its target gene *crumbs*. Crumbs attenuates Notch signalling by repressing the activity of the γ-Secretase complex. Many loss-of-function mutations in the human homologue of Crumbs, CRB1, cause recessive retinal dystrophies, including retinitis pigmentosa (den Hollander, 1999). Given the fact that the γ-Secretase complex also mediates the intracellular cleavage of the transmembrane protein APP, leading to accumulation of the Aβ peptide in plaques in AD, we postulate that Crumbs may also be involved in modulating AD pathogenesis. Our analysis indicates a role for the extracellular part of the Crb protein in this process. It is interesting to note that many mutations that give rise to retinal dystrophies are missense mutations that affect different EGF or LG domains of CRB1 (den Hollander, 1999). Thus, molecular interactions mediated by the extracellular domain of Crb may be crucial in both types of disease.

METHODS

***Drosophila* strains.** *crb$^{M11.M2}$* (*crb-lacZ* in the text) is a *piggyBac-lacZ* insertion 240 bp upstream of the *crumbs* transcription start site. Homozygous *crb$^{M11.M2}$* embryos show a strong *crumbs* loss-of-function phenotype (data not shown). The *piggyBac-lacZ* reporter plasmid was constructed as described in supplementary Methods. *EP-mam^{DN}* is described by Giraldez *et al* (2002). *UAS-Ser^{DN}* and *UAS-Dl^{DN}* were constructed as described in supplementary Methods. Other stocks are described in FlyBase. Genotypes of larvae used for genetic mosaic analyses are described in supplementary Methods.

Antibodies. Rat anti-Crb was a gift from Choi (Izaddoost *et al*, 2002). Guinea-pig anti-Senseless was a gift from H. Bellen (Nolo *et al*, 2000). Other antibodies are commercially available.

Monitoring γ-Secretase activity in *Drosophila*. Three different reporter constructs were used. The first reporter construct consists of the full-length APP and the yeast transcription factor GAL4 appended to the APP carboxyl terminus. It is expressed in the adult eye under the control of the GMR promoter (Guo *et al*, 2003). The second and third reporter constructs consist of truncated forms of APP (Δβ-APP) or Notch (Notch-ECN) lacking the extracellular domain and the transmembrane domain joined to a Gal4–VP16 fusion protein (Struhl & Adachi, 1998). They are expressed under the control of the *hsp70* promoter. Induction of expression is carried out as described by Struhl & Adachi (2000). The γ-Secretase reporter output constructs consist of Gal4-responsive transcriptional cassettes driving the expression of the *Drosophila* cell death activator GRIM or the GFP protein.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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