

# Crumbs is required to achieve proper organ size control during *Drosophila* head development

Emily C. N. Richardson<sup>1,2</sup> and Franck Pichaud<sup>1,2,\*</sup>

## SUMMARY

Crumbs (Crb) is a conserved apical polarity determinant required for zonula adherens specification and remodelling during *Drosophila* development. Interestingly, *crb* function in maintaining apicobasal polarity appears largely dispensable in primary epithelia such as the imaginal discs. Here, we show that *crb* function is not required for maintaining epithelial integrity during the morphogenesis of the *Drosophila* head and eye. However, although *crb* mutant heads are properly developed, they are also significantly larger than their wild-type counterparts. We demonstrate that in the eye, this is caused by an increase in cell proliferation that can be attributed to an increase in ligand-dependent Notch (N) signalling. Moreover, we show that in *crb* mutant cells, ectopic N activity correlates with an increase in N and Delta endocytosis. These data indicate a role for Crb in modulating endocytosis at the apical epithelial plasma membrane, which we demonstrate is independent of Crb function in apicobasal polarity. Overall, our work reveals a novel function for Crb in limiting ligand-dependent transactivation of the N receptor at the epithelial cell membrane.

**KEY WORDS:** Crumbs, Endocytosis, Notch, Cell proliferation, *Drosophila*

## INTRODUCTION

Crumbs (Crb) is a conserved, single pass, transmembrane (TM) protein that is essential for the establishment of epithelial cell polarity in the gastrulating *Drosophila* embryo (Knust et al., 1987; Tepass et al., 1990). It is localized to the apical domain of epithelial cells, where it is involved in specifying the zonula adherens (za), thereby setting up the apicobasal axis of the cell. Crb forms a complex with Stardust (Sdt/Pals1) (Bachmann et al., 2001; Roh et al., 2002; Tepass and Knust, 1993), which binds to the intracellular domain of Crb and recruits Pals-associated tight junction protein (Patj) (Bhat et al., 1999; Pielage et al., 2003) and Lin7 (veli – FlyBase) (Bachmann et al., 2008). The intracellular domain of Crb is also able to bind to a variety of conserved proteins including Par6, aPKC (Hurd et al., 2003; Nam and Choi, 2003; Sotillos et al., 2004) and the Ferm (band 4.1, ezrin, radixin, moesin) protein Yurt (Laprise et al., 2006). Crb is also apically associated with  $\beta_{\text{Heavy}}$ -spectrin and Moesin through its intracellular domain (Medina et al., 2002; Pellikka et al., 2002). Importantly, in the gastrulating fly embryo, the intracellular domain of Crb is sufficient to rescue the loss of apicobasal polarity observed in the absence of *crb* function (Klebes and Knust, 2000; Tepass et al., 1990; Wodarz et al., 1993; Wodarz et al., 1995).

In the *Drosophila* photoreceptor, *crb* is required for the proper morphology of the apical light-gathering organelle called the rhabdomere, as well as for photoreceptor elongation along the proximodistal axis of the retina. In addition, *crb* is required for the formation of the photoreceptor stalk membrane, a subapical membrane domain supporting the rhabdomere (Izaddoost et al.,

2002; Pellikka et al., 2002). The activity of *crb* in promoting stalk membrane morphogenesis is antagonized by the conserved basolateral protein Yurt (Laprise et al., 2006). Importantly, *crb* overexpression is sufficient to expand the stalk membrane, as well as the apical domain of the developing fly ectoderm (Pellikka et al., 2002; Wodarz et al., 1995). However, the molecular basis for *crb* function in regulating apical membrane and stalk length is not well understood. The ability of Crb to recruit  $\beta_{\text{Heavy}}$ -spectrin, and Moesin (Medina et al., 2002; Pellikka et al., 2002) raises the possibility that Crb might promote stalk membrane morphogenesis through the recruitment of F-actin at the cell cortex. This, in turn, has been proposed to limit endocytosis at the photoreceptor stalk membrane (Pellikka et al., 2002; Pichaud and Desplan, 2002). Consistent with a potential role for *crb* in regulating endocytosis at the apical membrane, work in zebrafish has recently revealed that the *crb* orthologue Crb2a regulates the length of the photoreceptor inner segment (IS), a membrane domain analogous to the *Drosophila* photoreceptor stalk (Omori and Malicki, 2006; Pellikka et al., 2002). Similarly, in mice, mutations in *Crb1* lead to shortening of the photoreceptor IS and outer segments (Mehalow et al., 2003). Finally, in *Drosophila*, *crb* function is also required to prevent light-induced photoreceptor apoptosis. Although the nature of this role for *crb* is not well understood, it depends on the extracellular domain of Crb rather than on its short intracellular domain (Johnson et al., 2002). Such a role for Crb is particularly relevant as mutations in the human homologue of *crb*, *CRB1*, are associated with retinitis pigmentosa Leber's congenital amaurosis (den Hollander et al., 2001; den Hollander et al., 1999; Lotery et al., 2001).

Although *crb* is required for apicobasal polarity specification and remodelling in epithelial cells such as the fly embryonic ectoderm and renal tubules (Tepass et al., 1990; Wodarz et al., 1993; Campbell et al., 2009), it is largely dispensable for maintaining polarity in epithelia such as the eye imaginal disc. In the wing disc however, *crb* has recently been shown to regulate Notch (N) signalling, by suppressing the activity of the  $\gamma$ -secretase complex (Herranz et al., 2006). During wing development, N upregulates *crb* transcription

<sup>1</sup>MRC Laboratory for Molecular Cell Biology and Cell Biology Unit, and <sup>2</sup>Research Department of Cell and Developmental Biology, University College London, Gower Street, WC1E 6BT, London, UK.

\*Author for correspondence (f.pichaud@ucl.ac.uk)

at the dorsoventral (D/V) boundary, and the ability of *crb* to inhibit the activity of the  $\gamma$ -secretase complex has been proposed to help refine this domain of N activity (Herranz et al., 2006). In addition, in the developing *Drosophila* gut, the expression of *crb* at the D/V boundary is dependent on the activation of N target genes (Fusse and Hoch, 2002). These observations are compatible with Crb being part of negative-feedback loop during N signalling. However, this might be specific to *Drosophila* as it has recently been shown that all three human *crb* orthologues are unable to interact with or regulate the activity of the  $\gamma$ -secretase complex in mammalian cells (Pardossi-Piquard et al., 2007).

Here, we show that the *crb* locus is not required for maintaining apicobasal polarity during head and eye morphogenesis. However, we find that *crb* mutant heads and eyes are significantly larger than their wild-type (WT) counterparts. We demonstrate that the loss of *crb* function in the developing eye imaginal disc leads to an overproliferation phenotype, mediated by an increase in N signalling activity. Further, our data suggest a role for *crb* in limiting endocytosis of the N receptor and its ligand D1, a process that we attribute to a function for *crb* in regulating endocytosis at the apical epithelial cell membrane.

## MATERIALS AND METHODS

### Antibodies and immunostaining

Eye discs from L3 larvae and pupal retinas were dissected in  $1 \times$  PBS and fixed in 4% formaldehyde for 20 minutes at room temperature before staining. Pupae were staged at 20°C as previously described (Walther and Pichaud, 2006). The following antibodies were used in this study: rabbit cleaved Caspase-3 1:500 (Cell Signaling Technology), rabbit phosphohistone H3 (Ser10) 1:250 (Millipore), rabbit anti- $\beta$ -galactosidase 1:2000 (Cappel), rat ELAV 1:50, mouse NECD 1:50, mouse NICD 1:50, mouse D1 1:50, mouse Hnt 1:10 (Developmental Studies Hybridoma Bank), guinea pig D1 1:3000 (Huppert et al., 1997), guinea pig Hrs 1:1000 (Lloyd et al., 2002), rat Crb 1:500 (Richard et al., 2006). Alexa goat (1:400) and Jackson donkey secondary antibodies (1:100) were used. Discs were mounted in VectaShield (Vector Labs) and imaged at room temperature using a Leica SP5 confocal microscope. The following lenses were used: 20 $\times$  NA 0.7 multi-immersion, 40 $\times$  NA 0.75–1.25 and 63 $\times$  NA 0.6–1.4 oil immersion (Leica). ImageJ and Photoshop CS (Adobe) were used to edit the images and Illustrator CS was used to assemble the figures.

### Fly strains and genetics

The following fly strains were used: *y,w;*; *FRT82B, crb<sup>11A22</sup>* (Johnson et al., 2002), *w;*; *FRT82B, crb<sup>36</sup>* and *w;*; *FRT82B, crb<sup>4</sup>* (Pichaud and Desplan, 2001), *crbRNAi<sup>39178</sup>* (Vienna *Drosophila* RNAi Center), *UAS-crExtTMGFP* (Pellikka et al., 2002), *UAS-crExt* (Wodarz et al., 1995), *lin-7<sup>54</sup>* and *lin-7<sup>66</sup>* (Bachmann et al., 2004), *UAS-dpatjRNAi* (Nam and Choi, 2006), *sdt<sup>XP96</sup>* (Hong et al., 2001), *N<sup>264-39</sup>* and *N<sup>55e11</sup>* (Bloomington Stock Center), *Dl<sup>revF10</sup>* (Fanto and Mlodzik, 1999), *aph1<sup>D35</sup>* (Hu and Fortini, 2003), *E(spl) m $\beta$ lacZ* (Nellesen et al., 1999), *UAS-mam<sup>DN</sup>* (Giraldez et al., 2002), *Hrs<sup>D28</sup>* (Bloomington Stock Center) and *w;*; *FRT80B, tsg101<sup>2</sup>* (Moberg et al., 2005). To generate *crb<sup>11A22</sup>* whole mutant eyes, we used the *w;*; *EGUF; FRT82B, GMRhid, Cl/TM2* (Stowers and Schwarz, 1999) and *eyFLP;*; *FRT82B, Minute, ArmlacZ* (Newsome et al., 2000) fly strains. Mosaic analyses in the eye disc were performed using the *eyFLP-FRT* (Newsome et al., 2000) and Mosaic Analysis with a Repressible Cell Marker (MARCM) system (Lee and Luo, 2001). The *crbRNAi* was expressed in the wing using *enGAL4* (Bloomington Stock Center).

### Scanning electron microscopy (SEM)

Whole flies were fixed in 2% paraformaldehyde, 2% glutaraldehyde and 0.1 M cacodylate for 2 hours and then serially dehydrated in ethanol, as previously described (Sullivan, 2000). The flies were then critical-point dried and mounted on aluminium stubs before gold coating. Imaging was carried out on a JEOL Variable Pressure scanning electron microscope (SEM).

### *crbTMGFP* transgene

Cloning was carried out using the Gateway cloning system (Invitrogen). The Crb signal peptide (SP) (a.a. 69–91) and TM domain (a.a. 86–112) were amplified using PCR with the following primers: SP forward 5'-CACCATGGTTTATTCGTCGCCTCAATGGATACCGCTC-3'; SP reverse 5'-AATGATGGCAATCGTCGGCACCCGACTGAGGC-3'; TM forward 5'-GCGGTGCCGACGATTGCCATCATTGTAATACCCGTAGTGGTG-GTG-3'; TM reverse 5'-GGCCATCACCAGGAAGGT-3'. The SP and TM PCR products were used as a template and run on PCR together, using the SP forward primer and the TM reverse primer. The PCR product was then cloned into *pENTR-TOPO* (Invitrogen) before being recombined into pTWG (*Drosophila* Genomics Resource Center). The corresponding C-terminal GFP fusion, *pUAS-CrbTMGFP*, was used to generate transgenic flies. Transgenic flies were generated by the Bestgene *Drosophila* embryo injection service. Expression of the transgene was verified by western blot (see Fig. S1 in the supplementary material).

### Dextran uptake assay

Eye discs were dissected from L3 larvae in M3 media + 10% FCS. They were then incubated in 10 mg/ml 3000 molecular weight dextran TexasRed (Invitrogen) in 200  $\mu$ l M3 media for 20 minutes before washing in  $1 \times$  PBS, followed by fixation in 4% formaldehyde for 20 minutes at room temperature. For the dextran labelling experiment with the metalloprotease inhibitor 1,10 o-phenanthroline (OP) (Sigma), 5 mM OP was added to the sample with the dextran.

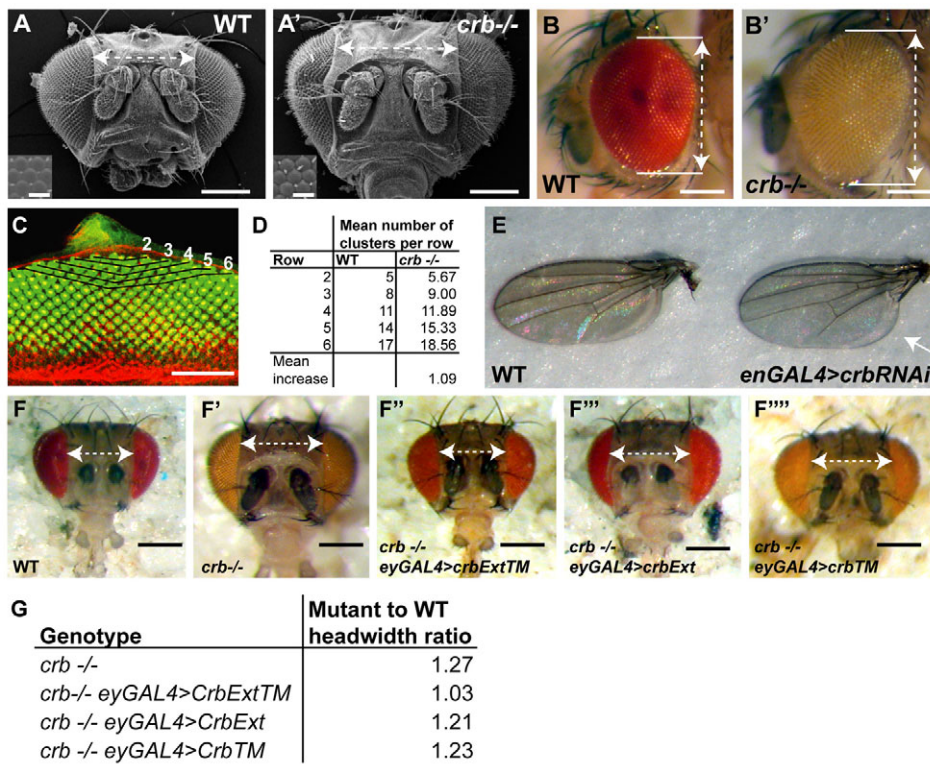
### Quantification and statistical analysis

Quantification was carried out using ImageJ. Vesicle counts were carried out in the anterior compartment of the eye disc by counting the number of vesicles in a sample of 15  $\mu$ m<sup>2</sup> areas in each eye disc. Data were compared using a 2-tailed Student's *t*-test.

## RESULTS

### *crb* function is required for proper head, eye and wing size control

*crb* function is crucial for establishing or remodelling apicobasal polarity in epithelial cells. However, *crb* function in developing epithelia such as the eye imaginal disc remains unclear. To assay *crb* function in the eye disc, we used the *EGUF/FRTGMRhidCl* system to generate whole mutant eyes (Stowers and Schwarz, 1999). Surprisingly, this approach allowed us to recover viable adult animals with whole mutant heads for the null allele *crb<sup>11A22</sup>*. These flies did not display any notable defects in either overall head capsule structure or gross eye morphology (Fig. 1A,A'; see also Fig. S2 in the supplementary material), indicating that *crb* function is largely dispensable during the extensive pupal tissue remodelling involved in generating the adult head. Yet, sectioning mutant adult eyes confirmed that *crb* is required for proper photoreceptor morphogenesis, as demonstrated by the disorganization of their apical domain and abnormally short stalk membranes (Izaddoost et al., 2002; Pellikka et al., 2002) (see Fig. S3 in the supplementary material). Interestingly, we observed that the *crb* mutant heads were significantly bigger than wild-type (WT) heads (Fig. 1A,A') and that this was not owing to any overall systemic increase in size caused by the *EGUF/FRTGMRhidCl* system (see Fig. S4A,B in the supplementary material). This was also the case for two other *crb* alleles, *crb<sup>4</sup>* and *crb<sup>36</sup>*, which were generated from an ethyl methane sulfonate mutagenesis screen (Pichaud and Desplan, 2001). In addition, we made use of a *crb* RNAi line to abolish *crb* expression in the eye. The specificity of this RNAi line was tested in mosaic eye discs stained with an antibody against Crb (see Fig. S5A,A' in the supplementary material). When expressed with the *eyGAL4* promoter, this RNAi line also led to an increase in head and eye size (see Fig. S5B,B' in the supplementary material).



The *crb* phenotype is characterized by a size increase that is proportional across the head, in that the ratio between major landmarks of the head remained the same in *crb* and WT heads (see Table S1 in the supplementary material). Comparison of the width of the head capsule, as measured by the distance between the two compound eyes, indicated that *crb*<sup>11A22</sup> heads were approximately 1.3 times bigger than those of the WT. This was also the case for eye size (Fig. 1B,B'). Quantification of the eye surface area indicated that *crb*<sup>11A22</sup> eyes were 1.3 times bigger than those of the WT ( $n=10$ ). Consistent with a global increase in head and eye size, we also found that the number of photoreceptor clusters present in the differentiating eye disc was greater in *crb*<sup>11A22</sup> mutant discs than in the WT discs (Fig. 1C,D). When this analysis was extended to counting all the ommatidia specified in the pupal eye, we found that *crb*<sup>11A22</sup> mutant retinas had, on average, approximately 170 more ommatidia than WT retinas ( $n=6$ ,  $t$ -test:  $P<0.001$ ). Collectively, these data indicate that the *crb* locus is required for normal head and eye size control during development. To assess whether this was specific to eye and head development, we made use of the *enGAL4* driver to express *crb* RNAi in the posterior compartment of the wing, and found that this led to an overgrowth of this compartment (Fig. 1E). These data demonstrate that *crb* function in regulating organ size is not limited to the eye-antennal imaginal disc but extends to other imaginal discs such as the wing.

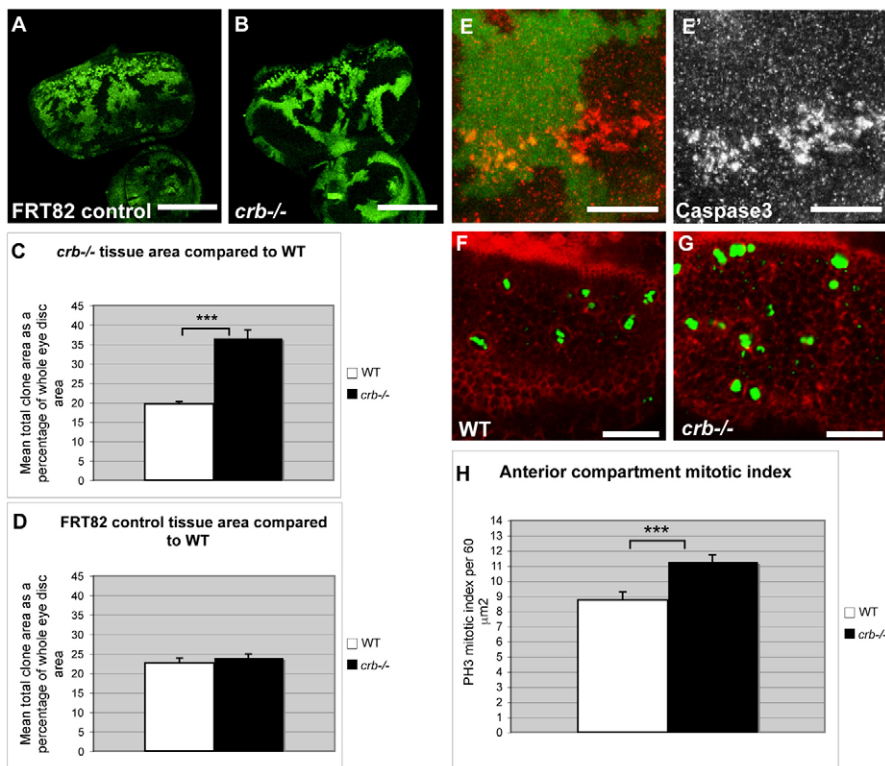
We next sought to dissect *crb* function during head development, using the *GAL4/UAS* system (Brand and Perrimon, 1993) to express various *crb* transgenes under the control of the early eye driver *eyGAL4*, in otherwise *crb*<sup>11A22</sup> mutant eye discs. We were able to fully rescue the *crb*<sup>11A22</sup> head overgrowth phenotype with a *crb* transgene encoding the extracellular domain and TM domain, but lacking the intracellular domain, which is essential for *crb* function in specifying apicobasal polarity (Klebes and Knust, 2000; Tepass et al., 1990; Wodarz et al., 1993; Wodarz et al., 1995) (Fig. 1F'',G). Expression of the extracellular (Wodarz et al., 1995) or TM domain

**Fig. 1. *crb* function is required for proper head, eye and wing size development.** (A,A') Wild-type (WT) *Drosophila* WT head (A). *crb*<sup>11A22</sup> whole mutant head (A'). Dashed arrows indicate the width of head capsule. Inset shows close up of facet lenses. (B,B') WT eye (B). *crb*<sup>11A22</sup> whole mutant eye (B'). Dashed arrow and solid lines indicate eye height. (C) WT eye imaginal disc stained with ELAV (green) and Phalloidin (red). Numbers indicate row number. (D) Mean number of ommatidia per row as indicated in C in WT and *crb*<sup>11A22</sup> discs. WT,  $n=11$ ; *crb*<sup>11A22</sup>,  $n=9$ . (E) Left, WT wing; right, *enGAL4* driving *crbRNAi*<sup>9178</sup> in the posterior compartment of the wing (arrow). (F-F''') WT head (F). (F'-F''') *crb*<sup>11A22</sup> whole mutant heads expressing *crb* transgenes under the control of the *eyGAL4* promoter. (F') *crb*<sup>-/-</sup>. (F'') *UAS-crExtTMGFP*. (F''') *UAS-crExt*. (F''') *UAS-crExtTMGFP*. (G) Mean ratio of the head width of the genotypes indicated compared with that of the WT. Scale bars: 250  $\mu$ m in A,A',B,B',F-F'''; 20  $\mu$ m in insets; 50  $\mu$ m in C.

(this study; see also Fig. S1 in the supplementary material) alone failed to rescue the *crb*<sup>11A22</sup> head overgrowth phenotype (Fig. 1F''',F''',G). Expressing full-length Crb using *eyGAL4* leads to a very strong gain-of-function phenotype characterised by a complete loss of the head, thus preventing us to use this transgene in rescue experiments (data not shown). Nevertheless, these data demonstrate that, in the developing eye imaginal disc, both the extracellular and TM domains are required to fully rescue the *crb* head and eye overgrowth phenotype. The lack of requirement for the intracellular of Crb to rescue the head and eye overgrowth, suggests that during this process, Crb function is independent of its intracellular binding partners, Sdt, Patj and Lin7. To investigate this further, we examined the contribution of these genes during eye development. Mutations in *lin7* are homozygous viable, and *lin7*<sup>54</sup> or *lin7*<sup>66</sup> mutant heads do not show any size differences compared with those of WT (see Fig. S6A,B in the supplementary material). In the case of Patj, there are no mutant alleles currently available, so we made use of a previously published RNAi line against *patj* (Nam and Choi, 2006). When expressed using *eyGAL4*, the corresponding adult heads did not show any size differences compared with those of WT (see Fig. S6A,C in the supplementary material). The *sdt* locus is on the X chromosome, thus preventing us from generating whole mutant heads. We therefore tested this gene by generating mosaic eye discs for the *sdt*<sup>XP96</sup> allele (Hong et al., 2001) and found that the *sdt*<sup>XP96</sup> clones did not have a growth advantage compared with the WT clones (see Fig. S7 in the supplementary material).

### *crb* regulates cell proliferation during eye development

The overgrowth phenotype we describe here could reflect either excessive proliferation within the presumptive eye and head capsule fields, decreased apoptosis, increased cell growth or some combination of these. To help distinguish between these possibilities, we first made use of the FLP-FRT system to analyze



**Fig. 2. *crb* regulates cell proliferation during *Drosophila* eye development.**

(A) Control clones generated using a WT FRT82. (B) *crb*<sup>11A22</sup> clones. (C) Quantification of the total size of the *crb*<sup>11A22</sup> mutant tissue and WT homozygous tissue. *n*=10 discs per genotype. \*\*\*, *t*-test: *P*<0.001. (D) Quantification of the total size of the FRT82 control tissue and WT tissue. *n*=8 discs per genotype. *t*-test: *P*>0.05. (E) *crb*<sup>11A22</sup> clones in the eye imaginal disc marked by lack of GFP staining (green) and stained for cleaved Caspase 3 (red). (E') Cleaved Caspase 3. (F,G) Anterior compartments of the eye imaginal disc stained for phospho-Histone H3 (green) and with Phalloidin (red). WT (F) and *crb*<sup>11A22</sup> (G) whole mutant eye discs. (H) Quantification of the mitotic index in WT and *crb*<sup>11A22</sup> mutant discs. *n*=8 discs per genotype. \*\*\*, *t*-test: *P*<0.001. (C,D,H) Error bars represent the s.e. Scale bars: 50 μm in A,B; 10 μm in E-G.

*crb*<sup>11A22</sup> clones in eye imaginal discs (Fig. 2A,B). Surface area quantification revealed that the total area of *crb*<sup>11A22</sup> mutant tissue was significantly larger than that of WT homozygous tissue (Fig. 2C,D). To assess cell size, we counted the number of facet lenses in a set surface area in WT and *crb*<sup>11A22</sup> mutant adult eyes. Each facet lens corresponds to one ommatidium and defects in cell size lead to variations in the size of the corresponding facet lens (Bridges, 1925). We found that the number of facets per unit of surface area in *crb*<sup>11A22</sup> and WT eyes was identical (Fig. 1A,B; see also Fig. S8A-C in the supplementary material), suggesting that loss of *crb* function in the developing eye does not affect cell size. Consistent with this conclusion, we did not detect any differences in apical cell perimeter between *crb*<sup>11A22</sup> mutant and WT cells in third instar eye discs (Fig. S8D,D').

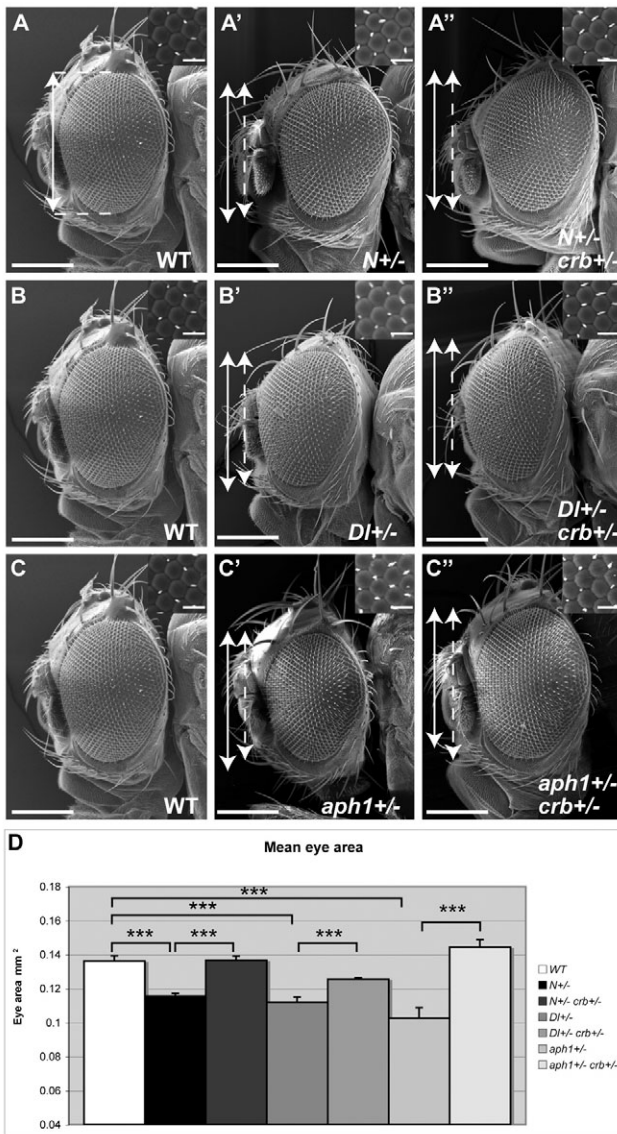
In order to test a role for *crb* in regulating apoptosis, we used an antibody directed against activated Caspase 3. When staining *crb*<sup>11A22</sup> mosaic third instar eye discs, we did not detect any significant differences in the number of apoptotic events in *crb*<sup>11A22</sup> mutant and WT clones (Fig. 2E,E'). In addition, we analysed the patterning and final ommatidial cell number in pupal retinas, which is dependent on developmentally controlled apoptosis, and found no differences between *crb*<sup>11A22</sup> mutant and WT retinas (see Fig. S8E,F in the supplementary material). These data indicate that *crb* function is not required to regulate the rate of apoptosis in the eye imaginal disc and pupal retina.

We next assessed the cell proliferation rates in *crb*<sup>11A22</sup> mutant and WT eye discs using a phospho-histone H3 (PH3) antibody to detect mitotic cells. The anterior-most region of the eye imaginal disc, located ahead of the morphogenetic furrow, is where the bulk of cell proliferation occurs during eye development. We found that in the *crb*<sup>11A22</sup> mutant, the number of mitotic cells in this region of the eye disc was greater than in the WT (Fig. 2F,G). Quantification of the number of mitotic events indicated that this increase was statistically

significant (Fig. 2H). Altogether, our findings demonstrate that increased cell proliferation is the cause of the overgrowth phenotype we observe in the eye in the absence of *crb* function.

### ***crb* modifies N function in controlling *Drosophila* eye size**

N signalling is a key regulator of eye growth in *Drosophila*, and previous work has shown that *crb* acts to limit N signalling through suppressing the activity of the  $\gamma$ -secretase complex (Herranz et al., 2006). Indeed, flies heterozygous for *N*, *Dl* and *Ser* have smaller eyes than WT flies (Fig. 3A-B',D; see also Fig. S9A,A' in the supplementary material). However, the *N*, *Dl* and *Ser* haplo-insufficiency does not affect the size of the head capsule (see Table S2 in the supplementary material). These observations raise the possibility that Crb might regulate N signalling during eye development. Accordingly, removing a copy of the *crb* locus was able to suppress both the notched wing (Herranz et al., 2006) (see Fig. S10 in the supplementary material) and small eye phenotypes (Fig. 3A",D) observed for two independent alleles of *N*. This suppression was also observed for *Dl*- and *Ser*-heterozygous flies (Fig. 3B",D; see also Fig. S9A" in the supplementary material). In every case, this was achieved without altering the size of the facet lenses. *anterior pharynx defective 1* (*aph1*) encodes a component of the  $\gamma$ -secretase complex and flies heterozygous for the *aph1*<sup>D35</sup> allele have smaller eyes than WT flies (Fig. 3C,C',D). Consistent with a link between  $\gamma$ -secretase activity and Crb, this small eye phenotype can also be suppressed by removing a copy of the *crb* locus (Fig. 3C',C",D). Collectively, these data suggest that during eye development, *crb* functions as a negative regulator of the N signalling pathway. However, because the *N*, *Dl* and *Ser* haplo-insufficiency does not affect the width of the head capsule, we conclude that in this particular epithelia, *crb* function is not necessarily related to that of *N*.



**Fig. 3. *crb* modifies N function in controlling *Drosophila* eye size.** (A–C'') White solid arrow represents the WT eye height for comparison and white dashed arrow represents the actual eye height. Inset shows a close up of facet lenses. (A) WT eye. (A') *N<sup>264-39</sup>* heterozygous eye. (A'') *N<sup>264-39</sup>* and *crb<sup>11A22</sup>* heterozygous eye. (B) WT eye. (B') *DI<sup>revF10</sup>* heterozygous eye. (B'') *DI<sup>revF10</sup>* and *crb<sup>11A22</sup>* heterozygous eye. (C) WT eye. (C') *aph1<sup>D35</sup>* heterozygous eye. (C'') *aph1<sup>D35</sup>* and *crb<sup>11A22</sup>* heterozygous eye. (D) Mean eye size represented by the eye area.  $n=6$  eyes per genotype. \*\*\*,  $t$ -test:  $P<0.001$ . Error bars represent the s.e. Scale bars: 200  $\mu\text{m}$  in A–C''; 20  $\mu\text{m}$  in insets.

### The *crb* eye overgrowth phenotype is dependent on N signalling

To further study the link between *crb* and N activity in the developing eye imaginal disc, we used the reporter of N transcriptional activation, *m $\beta$ lacZ* (Nellesen et al., 1999), which is expressed in the differentiating R4 photoreceptor. In *crb<sup>11A22</sup>* mutant clones, we detected an increase in the *m $\beta$ lacZ* staining in the mutant R4 cells compared with neighbouring WT cells (Fig. 4A–A'',B). In addition, we made use of a dominant-negative form of *mastermind*, *mam<sup>DN</sup>* (Giraldez et al., 2002), which acts as an inhibitor of N signalling. Mam

normally acts in conjunction with the N intracellular domain (NICD) to promote transcriptional activation of the downstream targets of the N signalling pathway. Expression of *mam<sup>DN</sup>* in *crb<sup>11A22</sup>* mutant clones suppressed the over-proliferation seen in *crb* mutant clones (Fig. 4C–G), suggesting that, in the developing eye disc, N activity is epistatic to *crb* during cell proliferation control. These data suggest that *crb* limits N signalling during eye development.

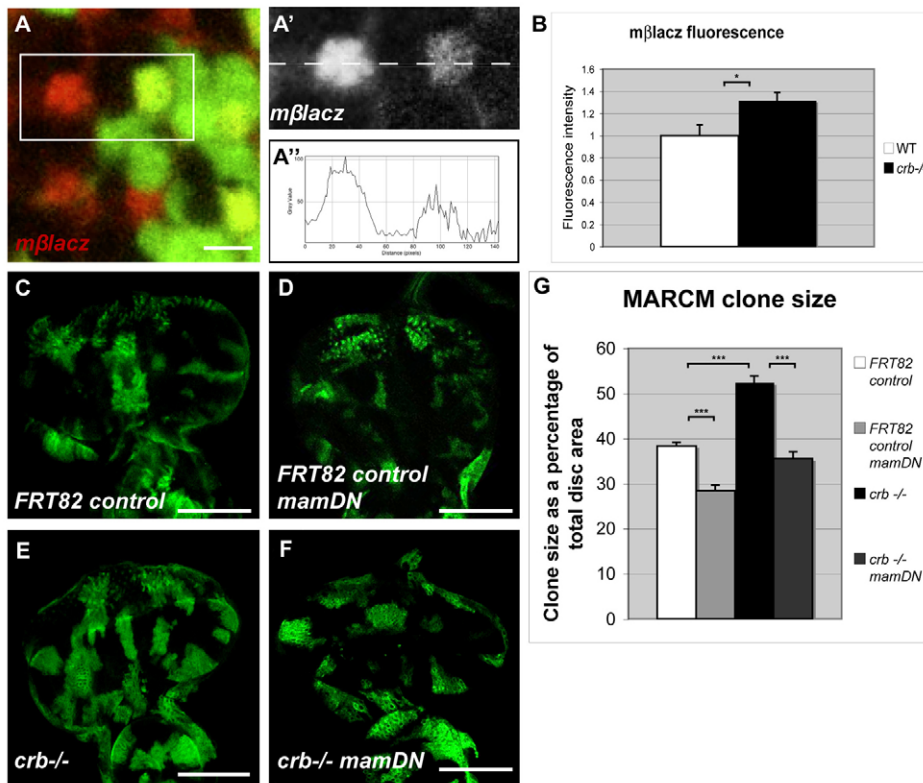
### Loss of *crb* function leads to increased N and DI endocytosis

It has previously been proposed that at the photoreceptor stalk membrane, the association of Crb with  $\beta_{\text{Heavy}}$ -spectrin might limit the rate of endocytosis at that particular membrane domain (Pellikka et al., 2002; Pichaud and Desplan, 2002). Accordingly, we detected a high frequency of clathrin-coated-like pits at the stalk membrane of *crb<sup>11A22</sup>* mutant adult photoreceptors (Fig. 5A), which are rarely detected in WT photoreceptors. Importantly, endocytosis is a crucial regulator of N signalling. In particular, endocytosis/recycling of the N ligands is required for its activation (Lai et al., 2005; Le Borgne et al., 2005; Le Borgne and Schweisguth, 2003a; Le Borgne and Schweisguth, 2003b; Wang and Struhl, 2004). In addition, the transendocytosis of the N extracellular domain (NECD) bound to its ligand allows the S2 cleavage of N, a crucial step for promoting N signalling (Klug and Muskavitch, 1999; Parks et al., 2000). We therefore examined N and DI endocytosis using antibodies specific for the NICD or NECD epitopes to stain WT and *crb<sup>11A22</sup>* mutant eye discs. In the absence of *crb* function, we found a statistically significant increase in the mean number of NECD- or NICD-positive vesicles labelled by the endosomal marker Hrs, representing a 40% and 30% increase, respectively (Fig. 5B,C; see also Fig. S11A–D'' in the supplementary material). These data suggest that the loss of *crb* function leads to an increase in the endocytosis of either S2 cleaved or full-length N. We next quantified the rate of DI endocytosis in WT and *crb<sup>11A22</sup>* mutant eye discs and observed a statistically significant increase in the mean number of DI-positive endosomes in *crb<sup>11A22</sup>* mutant discs compared with WT discs (37% increase; Fig. 5D; see also Fig. S11E–F'' in the supplementary material).

We next carried out a dextran uptake assay on eye discs (Entchev et al., 2000). Analysis of the proportion of dextran vesicles, that were positive for NECD or NICD showed that, in *crb<sup>11A22</sup>* mutant eye discs, there was a statistically significant increase of 19% and 27%, respectively, compared with WT eye discs (Fig. 5G,H). This set of data further indicates that the loss of *crb* function leads to an increase in NECD and NICD endocytosis. Interestingly, we did not detect any differences in the mean number of dextran-positive vesicles in *crb<sup>11A22</sup>* and WT eye discs (WT=8.88 vesicles/15  $\mu\text{m}^2 \pm 1.37$  s.d.,  $n=5$  discs; *crb*=8.92 vesicles/15  $\mu\text{m}^2 \pm 1.93$  s.d.,  $n=6$  discs), suggesting that the loss of *crb* function does not cause an overall increase in endocytosis. This is further supported by the quantification of the number of Hrs-positive endosomes in the anterior compartment of the eye, which show no significant difference between *crb<sup>11A22</sup>* and WT discs (Fig. 5I–K). Additionally, removing a copy of the *Hrs* locus in a *crb<sup>11A22</sup>* mutant background does not modify the *crb* overgrowth phenotype (data not shown). In conclusion, these data indicate that during eye development, *crb* plays a role in limiting the endocytosis of N and DI.

### Loss of *crb* function causes ligand-dependent N activation

The ectopic activation of N observed in the absence of *crb* function could result from either ligand-dependent or ligand-independent N signalling (for a review, see Furthauer and Gonzalez-Gaitan, 2009).



**Fig. 4. The *crb* overgrowth phenotype is dependent on N signalling.**

(A) *crb*<sup>11A22</sup> clones in the eye imaginal disc marked by lack of GFP staining (green) and expressing *mβlacZ* (red). (A') Close up of boxed area in A. (A'') Fluorescence intensity along the dashed line in A'. (B) Normalized *mβlacZ* fluorescence intensity per cell in WT and *crb* mutant clones. *n*=15 cells per genotype. \*, *t*-test: *P*<0.05. (C) FRT82 control clones. (D) FRT82 control clones expressing *mam*<sup>DN</sup>. (E) *crb*<sup>11A22</sup> clones. (F) *crb*<sup>11A22</sup> clones expressing *mam*<sup>DN</sup>. Clones are positively labelled with GFP (green). (G) Clone size area. *n*=10 discs per genotype. \*\*\*, *t*-test: *P*<0.001. (B,G) Error bars represent the s.e. Scale bars: 5 μm in A; 50 μm in C-F.

To help distinguish between these two possibilities, we used the developing egg chambers of the *Drosophila* ovary, a system in which N activation in the follicular epithelium depends on the presentation of DI by the germline cells at stage 6-7 (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). We monitored N activation in the follicular epithelium by the expression of the transcription factor Hindsight (Hnt; Pebbled – FlyBase): in WT ovaries at stage 5, when DI is not yet expressed in the germline cells, no Hnt expression is detected in the follicular epithelium (Sun and Deng, 2007); however, if ligand-independent activation of N is ectopically induced at this stage, then the follicular epithelium exhibits ectopic expression of Hnt (Vaccari et al., 2008). Indeed, *tsg*<sup>101</sup> mutant clones result in ectopic expression of Hnt in the follicular epithelium at stage 5 (Vaccari et al., 2008) (Fig. 6B). By contrast, *crb*<sup>11A22</sup> mutant clones did not reveal any ectopic Hnt expression in the follicular epithelium at stage 5 (Fig. 6A). These data indicate that removing *crb* in N-expressing cells is not sufficient to promote ectopic signalling.

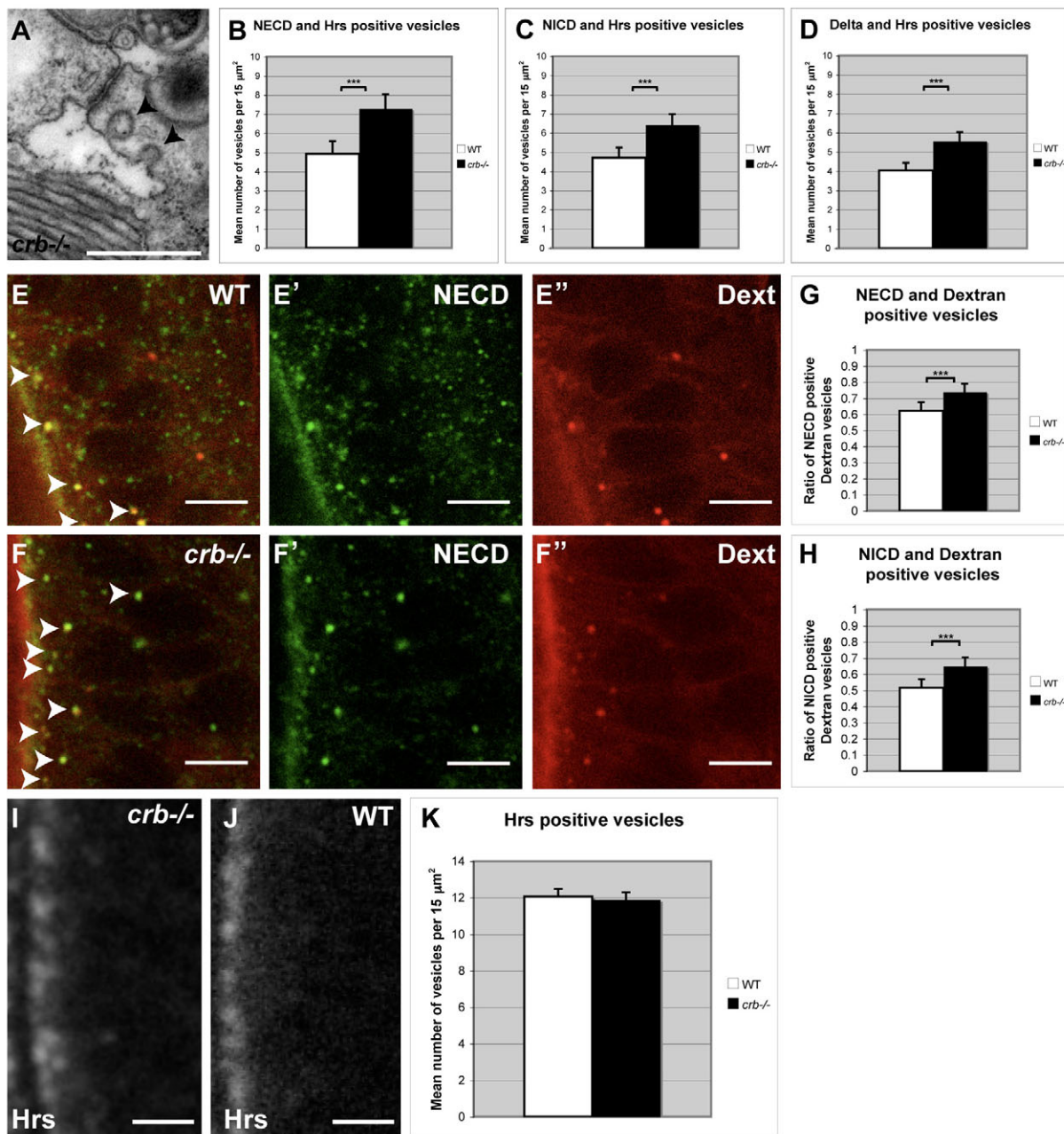
### The increase in N endocytosis is dependent upon the S2 cleavage of N

Typically, activation of the N pathway requires the S2 cleavage of the N receptor and the subsequent endocytosis of NECD bound to DI into the signal-sending cell. This is accompanied by the endocytosis of NICD into the signal receiving cell for further processing by the  $\gamma$ -secretase complex. Our work in the follicular epithelium suggests that the N activation we report here requires the transactivation of the N receptor by DI. Compatible with this model, we found that the mean number of vesicles containing both NICD and DI was very similar between *crb*<sup>11A22</sup> mutant and WT eyes discs (Fig. 6C; see also Fig. S12A-B'' in the supplementary material), whereas the number of NECD- and DI-positive vesicles was increased in mutant eye discs (Fig. 6D; see

also Fig. S12C-D'' in the supplementary material). From these data, we conclude that the increase in NECD- and DI-positive vesicles in *crb* mutant eye discs probably reflects an increase in the DI-dependent S2 cleavage of N. If this is the case, then inhibiting this cleavage should suppress the increase in NECD and DI endocytosis measured in the absence of *crb* function. In order to block the S2 cleavage, we made use of the metalloprotease inhibitor 1,10 o-phenanthroline (OP), a potent inhibitor of the Adam protease family (Mumm et al., 2000). Treatment with OP, concomitant with the onset of dextran uptake, reproducibly suppressed the increase in NECD and DI co-endocytosis seen in *crb*<sup>11A22</sup> whole mutant eye discs when compared with WT (Fig. 6E; see also Fig. S12E-F'' in the supplementary material). This also revealed that OP treatment led to a similar suppression of NECD/DI endocytosis in WT discs. These findings indicate that the increase NECD and DI endocytosis we observe in the absence of *crb* function is dependent upon the S2 cleavage of N, an event that requires ligand-dependent transactivation of the N receptor.

### DISCUSSION

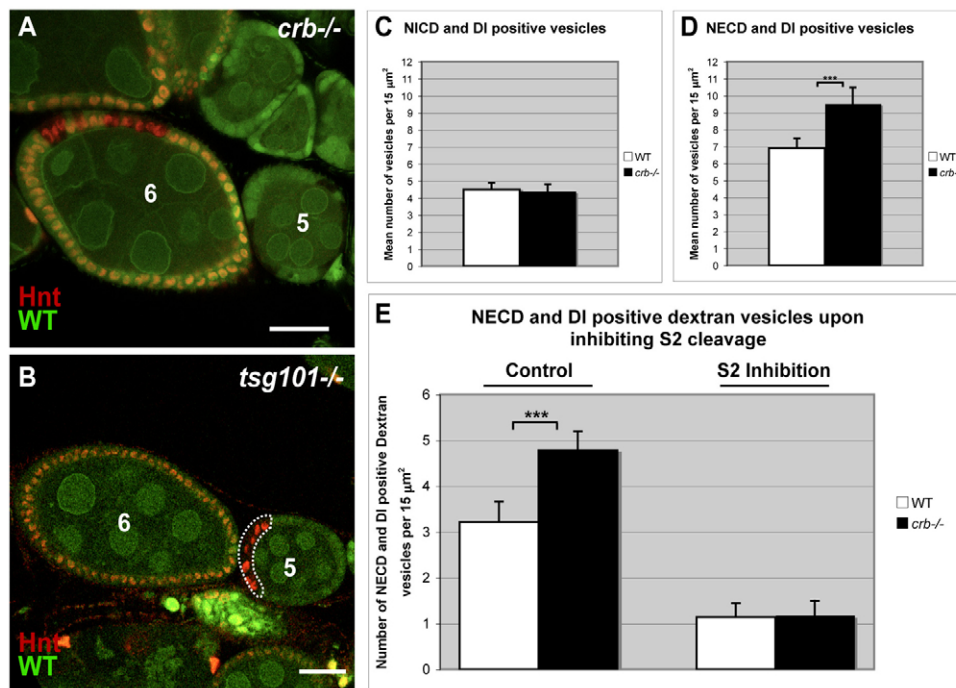
The work presented here demonstrates a novel function for *crb* in the proper control of head and eye size during *Drosophila* development. This function is not restricted to the fly head and eye, but also extends to other tissues such as the wing. In the case of the eye, our data indicate that this is linked to a function for Crb in limiting ligand-dependent transactivation of N. In support of this model, we observed a significant increase in endosomes positive for NICD, NECD and DI in *crb* mutant eye discs, that correlates with excessive cell proliferation. We also found that the eye overgrowth phenotype associated with the loss of *crb* function is correlated with an increase in NECD/DI co-endocytosis. Our data further indicate that this increase is dependent on the S2 cleavage of N. There is currently no



**Fig. 5. *crb* loss of function leads to increased N and DI endocytosis.** (A) Electron micrograph of the stalk membrane region from a *crb*<sup>11A22</sup> mutant photoreceptor. Arrowheads indicate coated pit-like structures. (B) The mean number of NECD- and Hrs-positive vesicles in WT and *crb*<sup>11A22</sup> mutant discs. WT, *n*=7; *crb*, *n*=6; \*\*\*, *t*-test: *P*<0.001. (C) The mean number of NICD- and Hrs-positive vesicles in WT and *crb*<sup>11A22</sup> mutant discs. WT, *n*=5; *crb*, *n*=6; \*\*\*, *t*-test: *P*<0.001. (D) The mean number of DI- and Hrs-positive vesicles in WT and *crb*<sup>11A22</sup> eye discs. WT, *n*=5; *crb*, *n*=6; \*\*\*, *t*-test: *P*<0.001. (E–F'') Eye discs after 20 minute dextran uptake. Anterior compartment, apical left. (E) WT disc. Merge of NECD (green) and dextran (red). Arrowheads indicate NECD- and dextran-positive vesicles. (E') NECD. (E'') Dextran. (F) *crb*<sup>11A22</sup> mutant disc. Merge of NECD (green) and dextran (red). Arrowheads indicate NECD- and dextran-positive vesicles. (F') NECD. (F'') Dextran. (G) Proportion of dextran vesicles positive for NECD in WT and *crb*<sup>11A22</sup> mutant discs. WT, *n*=5; *crb*, *n*=7; \*\*\*, *t*-test: *P*<0.001. (H) Proportion of dextran vesicles positive for NICD in WT and *crb*<sup>11A22</sup> mutant discs. WT, *n*=5; *crb*, *n*=4; \*\*\*, *t*-test: *P*<0.001. (I, J) Anterior compartment of the eye disc stained for Hrs (green), apical left. (I) WT disc. (J) *crb*<sup>11A22</sup> mutant disc. (K) Number of Hrs-positive vesicles in WT and *crb*<sup>11A22</sup> mutant discs. WT, *n*=8; *crb*, *n*=8; *t*-test: *P*>0.05. *n*, number of discs. Vesicles were counted in multiple sample areas per disc. Error bars represent the s.e. Scale bars: 0.5 μm in A; 5 μm in E–F''; 2 μm in I, J.

evidence for the requirement of the S2 cleavage to promote endocytosis of N with DI in cis. Moreover, DI in cis is thought to inhibit N activation (de Celis and Bray, 1997; Micchelli et al., 1997). We therefore conclude that during eye development, Crb limits ligand-dependent transactivation of the N receptor.

Mutations in the *crb* gene are associated with a failure to properly polarise the ectoderm along the apicobasal axis in the gastrulating embryo. In human, three Crb orthologues, CRB1, 2 and 3, have been described to date. Interestingly, CRB1 can be differentially spliced to produce the CRB1b isoform, which contains only the extracellular



**Fig. 6. *crb* limits DI-dependent N transactivation and S2 cleavage.** (A,B) *crb*<sup>11A22</sup> mutant clones marked by lack of GFP (green) as outlined by the white dotted line in the follicular epithelium (FE) of the egg chamber. At stage 5, no Hnt (red) expression in either the *crb*<sup>11A22</sup> clones or WT tissue is detected. *tsg101* clones (B) marked by lack of GFP (green) lead to ectopic Hnt expression (red) at stage 5. (C) Number of DI- and NICD-positive vesicles in WT and *crb*<sup>11A22</sup> eye discs. WT, *n*=6; *crb*, *n*=8; *t*-test: *P*>0.05. (D) Number of DI- and NECD-positive vesicles in WT and *crb*<sup>11A22</sup> eye discs. WT, *n*=5; *crb*, *n*=4; \*\*\*, *t*-test: *P*<0.001. (E) The effect of S2 cleavage inhibition upon the number of dextran vesicles positive for NECD and DI in WT and *crb*<sup>11A22</sup> mutant discs. Left-hand bars, control with no S2 cleavage inhibitor; right-hand bars, 5 mM OP. WT, *n*=6; *crb*, *n*=6; \*\*\*, *t*-test: *P*<0.001. *n*, number of discs. Vesicles were counted in multiple sample areas per disc. Error bars represent the s.e. Scale bars: 25 μm.

domain, whereas CRB3 lacks the conserved extracellular domain and comprises just the TM and intracellular domains. This suggests independent function for the extracellular and intracellular domains. The function of *crb* in establishing apicobasal polarity can be rescued in the *Drosophila* embryo using its intracellular domain anchored to the plasma membrane via the TM domain (Klebes and Knust, 2000; Tepass et al., 1990; Wodarz et al., 1993; Wodarz et al., 1995). Interestingly, *crb* function in the developing pupal photoreceptor has been linked to stalk membrane endocytosis through the connection of Crb to β<sub>Heavy</sub>-spectrin (Medina et al., 2002; Pellikka et al., 2002; Richard et al., 2009). This idea is supported by the finding that overexpression of Crb in either the gastrulating fly ectoderm or fly pupal photoreceptor leads to an increase the length of the apical and stalk membranes, respectively (Pellikka et al., 2002; Wodarz et al., 1995). Interestingly, when examining *crb* mutant adult photoreceptors, we were able to detect clathrin-coated-like pits in the region of the stalk membrane, which are not normally readily detectable in the WT. Importantly, overexpression of the extracellular domain of Crb linked only to its TM domain is sufficient to cause a striking increase in the length of the stalk membrane in the developing fly photoreceptor (Pellikka et al., 2002). Consistent with this finding is our observation that this transgene can rescue the overgrowth phenotype in *crb* mutant heads, a phenotype that is correlated with an increased in N/DI endocytosis. Moreover, our data indicate that Crb function in regulating N activity does not depend on the ability for Crb to interact with β<sub>Heavy</sub>-spectrin, Moesin, Yurt, Sdt, Patj, or Lin7. This suggests that the extracellular domain of Crb might bind to a component of the

extracellular matrix or with itself. It is therefore probable that in the WT, both the extracellular and intracellular domain could synergise to limit endocytosis at the apical membrane.

During *Drosophila* eye development, N activation at the D/V boundary is thought to promote cell proliferation within the eye primordium via activation of the JAK-STAT pathway (Chao et al., 2004). Consistent with this model, overexpression of the NICD in the developing eye discs leads to overgrowth of the eyes (Dominguez and de Celis, 1998). Indeed, flies heterozygous for *N*, *DI* and *Ser* have smaller eyes than WT flies. However, the width of the corresponding head capsules remains unchanged, arguing that N activity is not required during the head capsule growth. These data suggest that *crb* function in this epithelium might not be related to N activity, but is instead linked to that of another growth signalling pathway. Our data in the eye strongly argue that loss of *crb* function causes ectopic activation of the N signalling pathway. Moreover, the overgrowth of *crb* mutant eye tissue can be suppressed by inhibiting the N pathway. Finally, our analysis of *crb* mutant clones during oogenesis, together with the inhibition of the S2 cleavage, indicates that *crb* limits ligand-dependent transactivation of N.

Trafficking, and in particular endocytosis, plays a major role in modulating the N signalling pathway. A steady level of N at the cell surface is achieved by a balance between its activation on the way to the plasma membrane and its endocytosis and degradation (Acar et al., 2008; Okajima et al., 2005; Sakata et al., 2004; Wilkin et al., 2004). Ligand activation also requires the endocytosis and recycling of the ligand back to the cell surface in the signal-sending cell (Lai et al., 2005; Le Borgne et al., 2005; Le Borgne and Schweisguth, 2003a;



Le Borgne and Schweisguth, 2003b; Wang and Struhl, 2004). Upon ligand binding, the transendocytosis of NECD bound to its ligand into the signal-sending cell allows the S2 cleavage of N (Klueg and Muskavitch, 1999; Parks et al., 2000), and recent work indicates that proteolytic cleavage of N by the  $\gamma$ -secretase complex occurs in the endocytic compartment (Gupta-Rossi et al., 2004; Lu and Bilder, 2005; Moberg et al., 2005; Vaccari and Bilder, 2005; Vaccari et al., 2008; Yan et al., 2009). In normal conditions, the S2 cleavage of N is required for its subsequent S3/S4 cleavage by the  $\gamma$ -secretase complex (De Strooper et al., 1999). The increase in the number of NECD/DI endosomes we observed in the absence of *crb* function cannot be explained by Crb's link to the  $\gamma$ -secretase complex (Herranz et al., 2006) and indicates that in the developing eye disc, *crb* is also required to limit ligand-dependent transactivation of N.

How does Crb act to regulate ligand-dependent N signalling activity? Structural analysis of Crb, N and DI shows that these proteins all contain extracellular domains that contain multiple EGF-like repeats (Knust et al., 1987). This raises the possibility that the extracellular domain of Crb could interact with N and/or DI via their EGF-like repeats, thus preventing N-DI binding. Such specificity towards the N pathway is supported by our observation that there is no significant increase in Hrs-positive endosomes in the absence of *crb* function. Interestingly, other EGF repeat-containing proteins have been shown to inhibit N signalling; Dlk, for example, interacts and inhibits Notch1 in mammalian cells (Baladron et al., 2005). Alternatively, Crb might limit the rate of endocytosis of N or DI. One outcome of this could be that Crb limits DI activation by regulating its endocytosis and subsequent recycling back to the plasma membrane in order for it to become competent for signalling (Wang and Struhl, 2004). Another possibility is that Crb might limit the endocytosis of NECD and DI into the signal-sending cell, which is proposed to be required for the S2 cleavage of N (Chitnis, 2006; Le Borgne and Schweisguth, 2003a; Le Borgne and Schweisguth, 2003b). An increase in either of these endocytic events due to Crb loss-of-function could result in ectopic N activity. It is difficult to differentiate signal-sending versus signal-receiving cells in the context of the early developing eye epithelium, which prevents us from determining whether *crb* function is required in one of these cell types or in both. In addition, it will be interesting to determine how exactly the extracellular domain of Crb modulates endocytosis at the apical membrane, and whether, as suggested by the present study, this might target specific signalling pathways such as the N pathway. Finally, given that *crb* itself is a transcriptional target of the N pathway, its ability to limit the activity of the  $\gamma$ -secretase complex (Herranz et al., 2006), together with its function in limiting ligand-dependent transactivation of N (this study), is likely to provide a very robust negative-feedback loop mechanism to regulate N activity during organogenesis.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Author contributions

F.P. and E.R. planned the project and designed experiments. E.R. conducted the experiments and carried out the data analysis. F.P. supervised the project. The manuscript was written by F.P. and E.R.

#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.041913/-/DC1>

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**Table S1. The ratio of major landmarks of the head is the same in *crb* mutant heads compared with WT**

	Ratio of eye height to antenna length	Ratio of eye height to head width
<i>crb</i> <sup>-/-</sup> female	1.99	1.03
WT female	1.99	1.01

*n*=10 per genotype.

**Table S2. The *N*, *DI* and *Ser* haplo-insufficiency does not reduce head capsule size**

Genotypes	Mutant to WT head width ratio	<i>n</i>
<i>N</i> <sup>+/-</sup>	0.99	7
<i>N</i> <sup>+/-</sup> <i>crb</i> <sup>+/-</sup>	0.99	8
<i>DI</i> <sup>+/-</sup>	0.98	7
<i>Ser</i> <sup>+/-</sup>	0.98	5