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

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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 β heavyweight-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 rhodomere, 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 rhodomere (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 β heavyweight-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 Crbf 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 γ-secretase complex (Herranz et al., 2006). During wing development, N upregulates crb transcription...
at the dorsoventral (D/V) boundary, and the ability of crb to inhibit the activity of the γ-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 γ-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 DI, 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× 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 (Cell Signaling Technology), rabbit phospho-histone H3 (Ser10) 1:250 (Cappell), rat ELAV 1:50, mouse NEDC 1:50, mouse NICD 1:50, mouse DI 1:50, mouse Ht 1:10 (Developmental Studies Hybridoma Bank), guinea pig Di 1:3000 (Hupperet 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× NA 0.7 multi-immersion, 40× NA 0.75-1.25 and 63× 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: yw; FRT82B, crb

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/FRTGMRhdcI 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

To dissect and manipulate the expression of crb in the eye disc, we used the eyFlp; FRT82B, Minute, ArmlacZ (Stowers and Schwarz, 1999) and eyFlp; FRT82B, 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).

Dextan 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 μl M3 media for 20 minutes before washing in 1× PBS, followed by fixation in 4% formaldehyde for 20 minutes at room temperature. For the dextan labelling experiment with the metalloliprotease inhibitor 1,10 o-phenanthroline (OP) (Sigma), 5 mM OP was added to the sample with the dextan.

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 μm² areas in each eye disc. Data were compared using a 2-tailed Student’s t-test.

Scanning electron microscopy (EM)

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’- CACCATGTGTATTCGTCGCCCTCAATGGGACCCGGCTCTCGCT-3’; SP reverse 5’-AATTAGTGCAATCGTGCACCGCCACTGAGGG-3’; TM forward 5’-GCGGTGCGCCGATGGCTATGGGAATTTACCCGGTGTG- GTG-3’; TM reverse 5’-GGCCCATCACCAGGAAGGT-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, pUAST-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).
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*11A22 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*11A22 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*11A22 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*11A22 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* system (Brand and Perrimon, 1993) to express *crb* transgenes under the control of the early eye driver *eyGAL4*, in otherwise *crb*11A22 mutant eye discs. We were able to fully rescue the *crb*11A22 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 (this study; see also Fig. S1 in the supplementary material) alone failed to rescue the *crb*11A22 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* or *lin* 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 used a previously published RNAi line against *patj* (Nak 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* alleles (Hong et al., 2001) and found that the *sdt* 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...
crb11A22 clones in eye imaginal discs (Fig. 2A,B). Surface area quantification revealed that the total area of crb11A22 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 crb11A22 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 crb11A22 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 crb11A22 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 crb11A22 mosaic third instar eye discs, we did not detect any significant differences in the number of apoptotic events in crb11A22 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 crb11A22 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 crb11A22 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 crb11A22 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 γ-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 haploinsufficiency 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-haploinsufficient 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 γ-secretase complex and flies heterozygous for the aph1 allele have smaller eyes than WT flies (Fig. 3C’,C’,D). Consistent with a link between γ-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 haploinsufficiency 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.
Crb is required for head size control

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*054-39* heterozygous eye. (A”) N*054-39* and *crb*1422 heterozygous eye. (B) WT eye. (B’) D*mdf10* heterozygous eye. (B”) D*mdf10* and *crb*1422 heterozygous eye. (C) WT eye. (C’) aph1*1D35* heterozygous eye. (C”) aph1*1D35* and *crb*1422 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 μm in A-C”; 20 μm 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, mβgalZ (Nellesen et al., 1999), which is expressed in the differentiating R4 photoreceptor. In *crb*1422 mutant clones, we detected an increase in the mβgalZ staining in the mutant R4 cells compared with neighbouring WT cells (Fig. 4A-A”). In addition, we made use of a dominant-negative form of mastermind, *mam*DN (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*DN in *crb*1422 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 Dl endocytosis

It has previously been proposed that at the photoreceptor stalk membrane, the association of Crb with β*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*1422 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 (Klueg and Muskavitch, 1999; Parks et al., 2000). We therefore examined N and Dl endocytosis using antibodies specific for the NICD or NECD epitopes to stain WT and *crb*1422 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*1422 mutant eye discs and observed a statistically significant increase in the mean number of DI-positive endosomes in *crb*1422 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*1422 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 the mean number of Dl-positive endosomes in *crb*1422 mutant discs compared with WT discs (37% increase; Fig. 5D; see also Fig. S11E-F’ in the supplementary material).

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).
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, tsg101 mutant clones result in ectopic expression of Hnt in the follicular epithelium at stage 5 (Vaccari et al., 2008) (Fig. 6B). By contrast, crb11A22 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 receiving cell for further processing by the γ-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 crb11A22 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 endocytosis seen in crb11A22 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...
evidence for the requirement of the S2 cleavage to promote endocytosis of N with Dl in cis. Moreover, Dl 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

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**Fig. 5.** crb loss of function leads to increased N and DI endocytosis. (A) Electron micrograph of the stalk membrane region from a crb11A22 mutant photoreceptor. Arrowheads indicate coated pit-like structures. (B) The mean number of NECD- and Hrs-positive vesicles in WT and crb11A22 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 crb11A22 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 crb11A22 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) crb11A22 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 crb11A22 mutant discs. WT, n=5; crb, n=7; ***, t-test: P<0.001. (H) Proportion of dextran vesicles positive for NICD in WT and crb11A22 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) crb11A22 mutant disc. (K) Number of Hrs-positive vesicles in WT and crb11A22 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.
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 endocytosis through the connection of Crb to βH-spectrin (Medina et al., 2002; Pellikka et al., 2002; Richard et al., 2009). Interestingly, crb function in the developing pupal photoreceptor has been linked to stalk membrane endocytosis supported by the finding that overexpression of Crb in either the gastrulating fly ectoderm or fly pupal photoreceptor leads to an increase in the length of the apical and stalk membranes, respectively (Pellikka et al., 2002; 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 βH-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 in 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, 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 endocytosis through the connection of Crb to βH-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 in 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/Dl endocytosis. Moreover, our data indicate that Crb function in regulating N activity does not depend on the ability for Crb to interact with βH-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 NIDC in the developing eye discs leads to overgrowth of the eyes (Dominguez and de Celis, 1998). Indeed, flies heterozygous for N, Dl 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 endocytosis 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 γ-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 γ-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 γ-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 determine what is the exact mechanism 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 γ-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

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