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RESEARCH REPORT

Ebi modulates wing growth by ubiquitin-dependent downregulation of Crumbs in *Drosophila*

Minh Binh Nguyen, Linh Thuong Vuong* and Kwang-Wook Choi‡

ABSTRACT

Notch signaling at the dorsoventral (DV) boundary is essential for patterning and growth of wings in *Drosophila*. The WD40 domain protein Ebi has been implicated in the regulation of Notch signaling at the DV boundary. Here we show that Ebi regulates wing growth by antagonizing the function of the transmembrane protein Crumbs (Crb). Ebi physically binds to the extracellular domain of Crb (Crb^{ext}), and this interaction is specifically mediated by WD40 repeats 7-8 of Ebi and a laminin G domain of Crb^{ext}. Wing notching resulting from reduced levels of Ebi is suppressed by decreasing the Crb function. Consistent with this antagonistic genetic relationship, Ebi knockdown in the DV boundary elevates the Crb protein level. Furthermore, we show that Ebi is required for downregulation of Crb by ubiquitylation. Taken together, we propose that the interplay of Crb expression in the DV boundary and ubiquitin-dependent Crb downregulation by Ebi provides a mechanism for the maintenance of Notch signaling during wing development.

KEY WORDS: Drosophila, Notch, Crumbs, Ebi, Ubiquitin

INTRODUCTION

Notch signaling is a conserved mechanism that regulates diverse developmental events, including tissue growth, cell fate specification and cell polarity (Artavanis-Tsakonas et al., 1999; Lai, 2004). Thus, how Notch signaling is regulated is an important developmental issue. Notch signaling is activated by the interaction between the extracellular domains (ECDs) of the Notch receptor and its transmembrane protein ligands, Delta and Serrate (de Celis et al., 1996). Ligand binding to Notch results in the cleavage of the intracellular domain (ICD) by γ-secretase (Mumm et al., 2000). The ICD fragment of Notch enters the nucleus, leading to transcriptional activation of specific target genes (Lieber et al., 1993; Lai, 2004).

Crumbs (Crb) is a transmembrane protein with EGF-like repeats in the extracellular domain, a structural feature shared with Notch (Tepass et al., 1990). Crb has multiple functions, including roles in apicobasal cell polarity (Tepass et al., 1990; Wodarz et al., 1995; Tanentzapf et al., 2000), morphogenesis (Izaddoost et al., 2002; Pellikka et al., 2002), Hippo signaling (Parsons et al., 2010; Laprise, 2011; Ribeiro et al., 2014) and mitosis (Yeom et al., 2014). Interestingly, recent studies have provided evidence that Notch activity is affected by Crb. For example, loss of Crb in the eye imaginal disc results in head overgrowth by increased cell proliferation due to ectopic Notch activity. This activation of Notch signaling is correlated with an increased endocytosis of Notch and its ligand Delta. Hence, independent of its function in cell polarity, Crb acts as an inhibitory factor to Notch activation by limiting endocytosis (Richardson and Pichaud, 2010). Crb is also involved in the inhibition of Notch signaling in vertebrates like zebrafish, in which Notch activity is necessary for the apical mitosis of neuroepithelial cells during embryogenesis (Ohata et al., 2011). The inhibition of Notch activity by Crb is due to a direct interaction between the extracellular domains of Crb and Notch. Further, Mosaic eyes (Moe, a homolog of *Drosophila* Yurt) antagonizes the Crb function in a positive feedback loop to maintain the apical basal gradient of Notch activity in neuroepithelial cells, thus restricting their mitosis to the apical area (Ohata et al., 2011).

These interactions between Crb and Notch described above suggest that Crb plays distinct roles in the regulation of Notch signaling in different developmental contexts. In *Drosophila*, activation of Notch signaling at the DV boundary of wing discs is pivotal for wing growth (de Celis et al., 1996). It has been shown that while *crb* gene expression is induced by Notch signaling, Crb protein antagonizes Notch signaling by interfering with γ-secretase (Herranz et al., 2006), a protease necessary for Notch signaling (Schweisguth, 2004). Thus, the level of Crb at the DV boundary needs to be downregulated to maintain Notch signaling in wing disc. However, the mechanism underlying the regulation of Crb remains to be understood.

Genetic studies have shown that the *ebi* gene is required for wing growth by activating or de-repressing transcription of Notch target genes in the DV boundary (Marygold et al., 2011). Although both Ebi and Crb are involved in the modulation of Notch signaling at the DV boundary of wing disc, the relationship between these two proteins has not been studied. Ebi protein contains a LisH domain and an F box-like motif in the N-terminal region and eight WD40 repeats in the C-terminal region (Marygold et al., 2011). Interestingly, Ebi is known to function in the degradation of specific target proteins by interacting with an E3 ubiquitin ligase Seven-in-absentia (Sina), the founding member of the SIAH family proteins (Matsuzawa and Reed, 2001; Tsuda et al., 2006). This raises a possibility that Ebi might be involved in downregulation of Crb by directly interacting with a specific region of Crb. Crb protein consists of a large extracellular domain (Crb^{ext}) and a short intracellular domain (Crb^{intra}) (Tepass et al., 1990). While the Crb^{intra} domain and its interaction partners have been extensively studied (Bachmann et al., 2001; Hong et al., 2001; Izaddoost et al., 2002; Nam and Choi, 2003; Tepass, 2012), few binding partners of the Crb^{ext} domain have been identified thus far (Hafezi et al., 2012; Roper, 2012; Zou et al., 2012; Letizia et al., 2013; Pocha and Knust, 2013).

In this work, we identified Ebi as an interacting partner of the Crb^{ext} domain. Genetic evidence indicates that Ebi is antagonistic to Crb function in wing development. We show that Ebi is required for

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea.
*Present address: Department of Developmental and Regenerative Biology and Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA.

‡Author for correspondence (kchoi100@kaist.ac.kr)

K.-W.C., 0000-0003-2884-4996

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ubiquitin-dependent downregulation of Crb. This study provides a novel mechanism for the regulation of Notch signaling through the antagonistic interaction between Ebi and Crb.

RESULTS AND DISCUSSION

Crumbs extracellular domain binds to the WD40 repeats of Ebi

To gain insights into the function of Crb^{ext}, we searched for binding partners of a conserved domain of Crb^{ext} using a yeast two-hybrid screen. The bait was the fourth laminin G domain (Crb^{lam4}). This domain is most closely related to the third laminin G domain of human CRB1 in which many mutations associated with retinal diseases have been identified (den Hollander et al., 2004). One of the Crb^{lam4}-interacting clones contained a 230 bp cDNA insert that encodes WD40 repeats 7 and 8 located in the C-terminal region of Ebi (Fig. 1A). To further confirm the binding between Ebi and Crb, we examined their interaction by co-immunoprecipitation (co-IP). In the assay using S2 cells transfected with Crb^{lam4TM}-V5 and Flag-Ebi^{full}, these two proteins were coimmunoprecipitated (Fig. 1B), indicating that the extracellular domain of Crb can form a protein complex with Ebi.

To identify the specific region of Ebi involved in Crb^{lam4} binding, we performed pull-down experiments using GST-fusion proteins. The N-terminal region of Ebi has a LisH domain, while the C-terminal part consists of eight WD40 repeats. Consistent with the two-hybrid interaction, pull-down assays confirmed the specific binding between Crb^{lam4} and the WD40 7-8 repeats of Ebi (hereafter $Ebi^{WD7/8}$). Additional binding assays with truncated mutant Ebi proteins showed that Crb^{lam4} did not bind the WD40 1-6 repeats or the N-terminal region of Ebi (Fig. 1C). Furthermore, other domains of Crb such as the laminin G domains 1-3, the EGF repeat domain or the intracellular domain also did not bind to the $Ebi^{WD7/8}$ (Fig. 1D). These data indicate that the Crb^{lam4} domain specifically interacts with the $Ebi^{WD7/8}$ repeats.

Ebi is antagonistic to Crb in wing development

To check for physiological relevance of the binding between Crb and Ebi, we tested whether these two genes show any genetic interaction in wing development. Consistent with a previous study (Marygold et al., 2011), RNAi knockdown of Ebi in the DV boundary region driven by *C96-Gal4* (*C96>ebi* RNAi) caused notching along the wing margin resulting in a 33±3% (n=10) reduction of the wing size (Fig. 2B,G) compared with the control (Fig. 2A). Three independent *ebi* RNAi lines showed similar wing

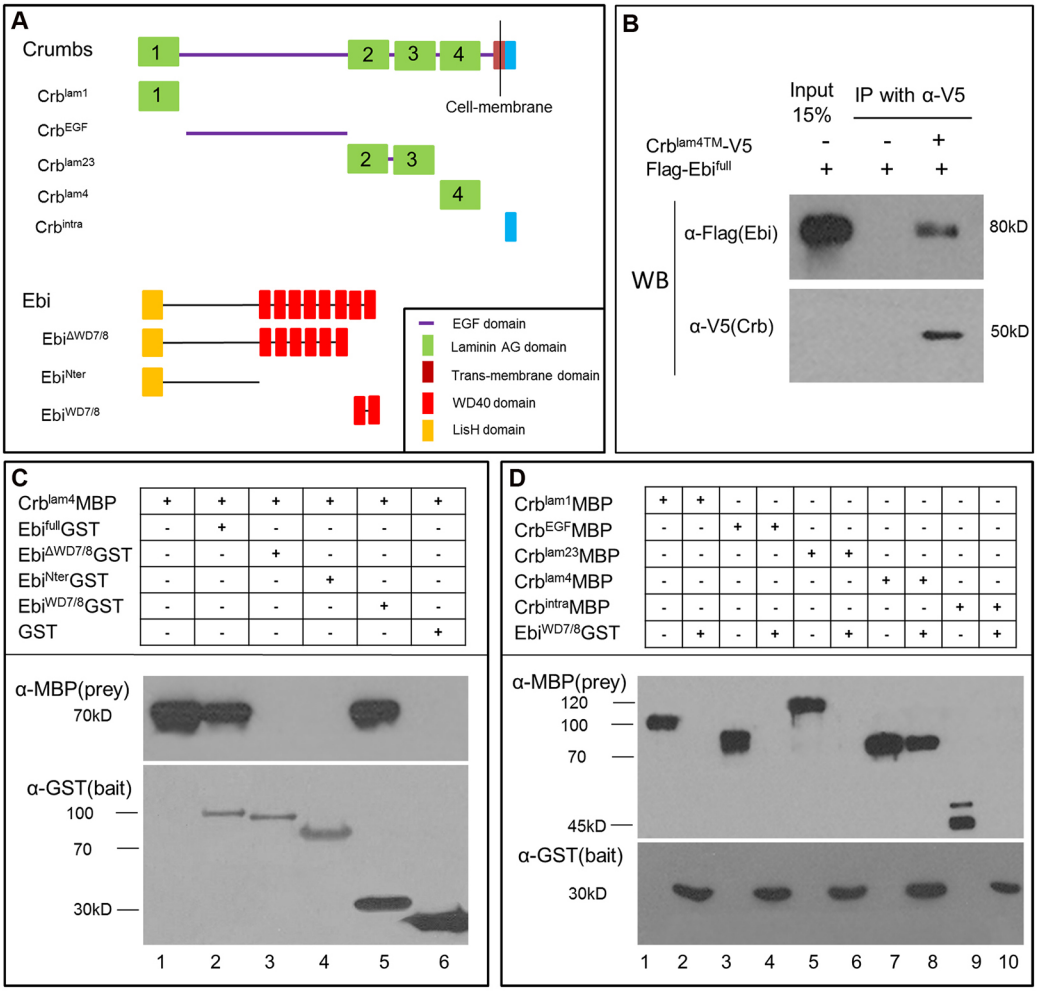

Fig. 1. Ebi physically interacts with Crb. (A) Schematic domain structures of wild-type Crb and Ebi proteins and their mutant constructs. (B) Co-immunoprecipitation of Crb extracellular domain and Ebi. S2 cells were transfected with Flag-Ebi^{full} or co-transfected with Flag-Ebi^{full} and Crb^{lam4TM}-V5, as indicated. Anti-V5 and anti-Flag antibodies were used for IP and detection, respectively. (C) Direct binding of Crb and WD40 7/8 repeats of Ebi ($Ebi^{WD7/8}$). The Crb^{lam4} extracellular domain (lane 1, input 10%) was pulled down by Ebi full length (Ebi^{full}) and $Ebi^{WD7/8}$ (lanes 2 and 5) but not by $Ebi^{\Delta WD7/8}$, Ebi N-terminal region (Ebi^{Nter}) or GST (lanes 3, 4, 6). (D) Direct binding of $Ebi^{WD7/8}$ and Crb^{lam4}. The $Ebi^{WD7/8}$ domain pulled down the Crb^{lam4} (lane 8) but not the other domains of Crb (lanes 2, 4, 6 and 10). 10% inputs are shown in lanes 1, 3, 5, 7 and 9.

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defects (Fig. S1B-D). Because Notch signaling in the wing disc induces expression of its target gene *cut* along the DV boundary (de Celis et al., 1996; Micchelli et al., 1997), we examined the effects of *ebi* knockdown on the level of Cut protein. As expected from the notching phenotype in the adult wing, *ebi* RNAi by *C96-Gal4* resulted in reduced Cut expression along the DV boundary (50±2% reduction, n=5) (Fig. S1F). This *ebi* RNAi wing phenotype was significantly suppressed in 70% (n=10) of examined wings by reducing the *crb* gene dosage in the crb^{11A22}/+ heterozygote. The size of *ebi* RNAi wings was increased by 85±3% (n=10) in the crb^{11A22}/+ background (Fig. 2C,G) compared with the control (Fig. 2A). Similarly, reduced Cut expression by *ebi* RNAi was significantly restored by crb^{11A22}/+ (Fig. S1G). These results suggest that *ebi* and *crb* function antagonistically.

Since Ebi binds to the extracellular domain of Crb, we checked whether there is any specific genetic interaction between Ebi and Crb^{ext}. *ebi* RNAi in the wing pouch by *nub-Gal4* led to a 47±3% (n=10) reduction of the wing size at 25°C (Fig. S2B). Similarly, overexpression of Crb^{ext} ($Crb^{extTMGFP}$) by *nub-Gal4* resulted in 50 ±3% (n=10) reduction of the wing size (Fig. S2C). Both *nub>*$crb^{extTMGFP}$ and *nub>ebi RNAi* wings also showed loss of crossveins in the wing (Fig. S2B,C). *ebi* RNAi and Crb^{ext} overexpression caused a 80±3% reduction of wing size at 29°C (Fig. 2D,D′,H). Under these conditions, overexpression of wild-type Ebi (Ebi^{full}) resulted in partial suppression of the reduced wing phenotype caused by $Crb^{extTMGFP}$ (Fig. 2E,E′,H), although overexpression of Ebi alone as a control did not affect wing development (Fig. S2D). These results indicate that Ebi can antagonize the effects of Crb^{ext} overexpression.

To test whether the $WD40^{7/8}$ repeats that bind to Crb are important for the function of Ebi, we generated *UAS-*$ebi^{\Delta WD7/8}$ to express a mutated Ebi protein deleted in the $WD40^{7/8}$ repeats. In contrast to the wild-type Ebi (Ebi^{full}) (Fig. 2E,E′), $Ebi^{\Delta WD7/8}$ failed to suppress the *nub>*$crb^{extTMGFP}$ phenotype (Fig. 2F,F′,H). Hence, the $WD40^{7/8}$ domains of Ebi are not only important for binding Crb^{lam4} but also required for Ebi to antagonize Crb.

Reduced levels of Ebi elevate Crb at the dorsoventral wing boundary

To understand the mechanism for the antagonistic genetic interaction between Ebi and Crb, we checked whether Ebi regulates the level of Crb protein. As shown previously (Herranz

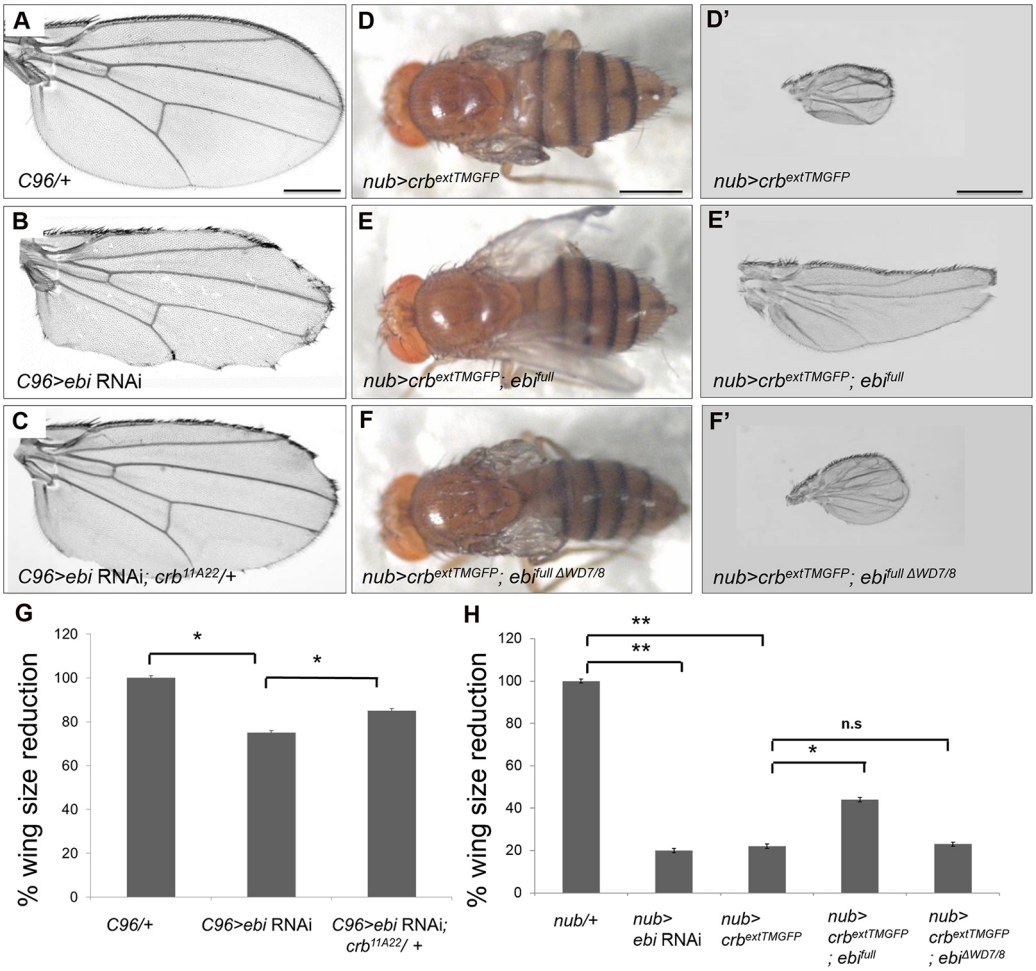

Fig. 2. Genetic interaction between *crb* and *ebi*. (A-C) Effects of *ebi* RNAi and Crb overexpression by *C96-Gal4*. (A) *C9*/+ control shows normal wing. (B) *C96>ebi* RNAi causes large notching in the wing margin. (C) *crb*/+ heterozygote in *ebi* RNAi knockdown background (*C96>ebi* RNAi*;* crb^{11A22}/+) suppresses the wing notching phenotype of *ebi* RNAi. (D,D′) Overexpression of Crb^{ext} (*nub>*$crb^{extTMGFP}$) causes severe reduction of wing size. (E,E′) Overexpression of Ebi^{full} in $Crb^{extTMGFP}$ overexpression background (*nub>*$crb^{extTMGFP}$*;* ebi^{full}) partially suppresses the small wing phenotype. (F,F′) Mutated Ebi lacking WD40 7/8 repeats ($Ebi^{full\Delta WD7/8}$) fails to rescue the small wing phenotype. Experiments were performed at 29°C. Scale bars: 100 µm (A-F′). (G,H) Quantification of percentage wing size reduction shown in A-C and D-F′, respectively. **P<0.001, *P<0.05; n.s, not significant (t-test; n=10).

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et al., 2006), the *crb-lacZ* reporter is strongly expressed in the DV boundary region of all wing discs examined (Fig. 3D-D′′). In contrast to *crb-lacZ* expression, Crb protein is expressed uniformly in most wing discs with no significant increase in the DV boundary region (Fig. 3A,A′), although increased Crb expression in the DV boundary was found in about 10±2% (n=50) of wing discs examined. Thus, we reasoned that excessive Crb protein induced in the DV boundary might be unstable and subject to degradation. If Ebi is involved in the downregulation of Crb, reduction of Ebi may increase the Crb protein level in the DV boundary region. Indeed, Ebi knockdown by *C96>ebi* RNAi resulted in upregulation of Crb in the DV boundary region compared with the surrounding areas (42±2% upregulation in ∼50% of wing discs examined, n>50) (Fig. 3B). We also tested whether Ebi knockdown in the anterior compartment by *Ci-Gal4* is sufficient to increase the Crb level in the targeted region. Consistent with the result from *C96>ebi* RNAi, Ebi knockdown by *Ci-Gal4* led to upregulation of Crb in the anterior region but not in the posterior control region in about 50% of wing discs examined (n>30) (Fig. 3C-C′′).

To determine whether Ebi regulates *crb* expression at the transcriptional level, we examined the effects of Ebi knockdown on the expression of a *crb-lacZ* reporter (Herranz et al., 2006). *Crb-lacZ* in the wild-type background was strongly induced along the DV boundary compared with surrounding regions of the wing pouch (Fig. 3D′). In contrast to the increase of Crb protein level by *ebi* RNAi, *Ci>ebi* RNAi did not significantly alter the level of *crb-lacZ* expression in the anterior wing compartment (Fig. 3D-D″). Thus, Ebi seems to downregulate the level of Crb protein post-translationally rather than by transcriptional repression.

In addition, we tried to examine the effects of an *ebi* null mutation on Crb expression, but mutant clones could not survive or were too small to determine the effect, as also previously reported (Marygold et al., 2011). As an alternative, we generated *ebi* RNAi clones by flp-out recombination (Theodosiou and Xu, 1998) to compare the Crb expression level in *ebi* RNAi clones and the adjacent wild-type cells. An early induction of flippase resulted in large areas of *ebi* RNAi clones marked with GFP. Similar to the results from *Ci>ebi* RNAi, the level of Crb was increased in the DV boundary region within *ebi* RNAi clones compared with the wild-type control area

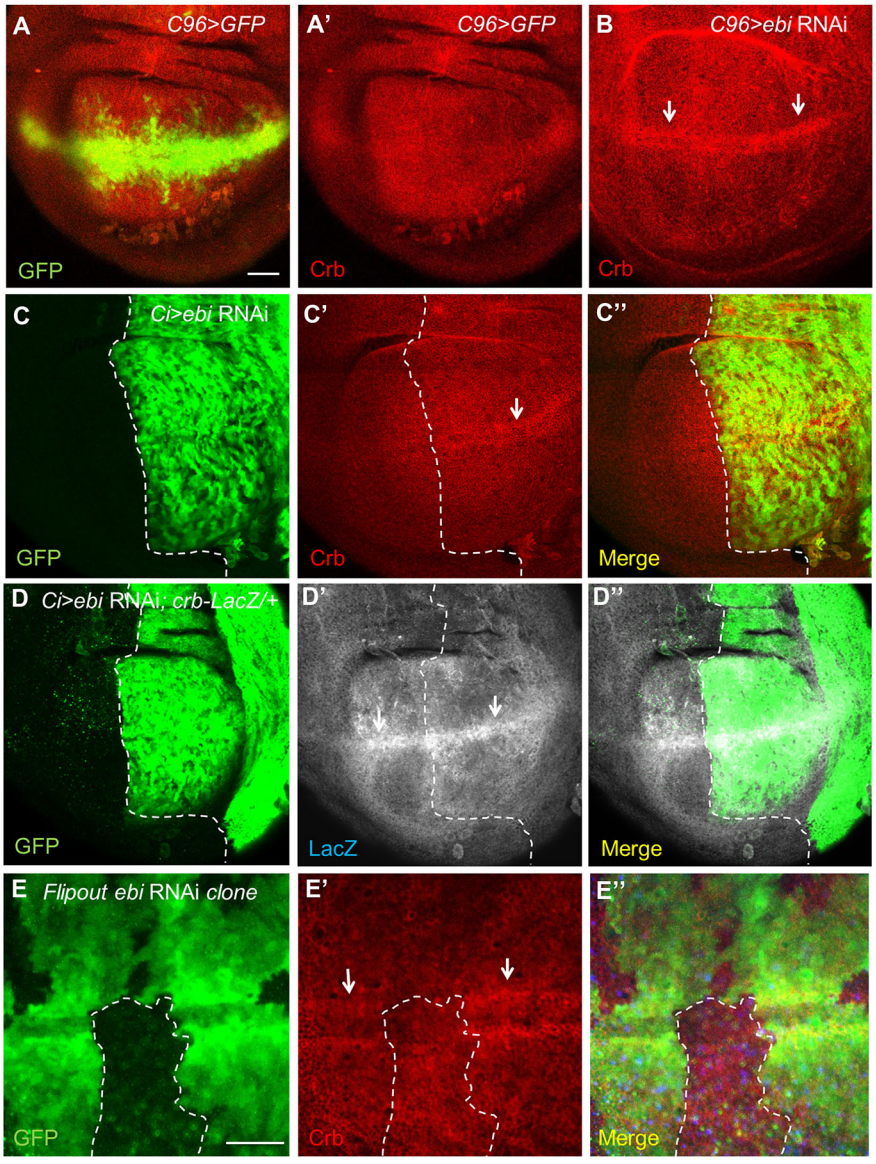

Fig. 3. Effect of Ebi knockdown on Crb. (A,B) Effect of *ebi* RNAi on Crb level. (A) *C96>GFP* expression pattern in wing disc. (A′) *C96>GFP* control wing disc shows a low level of ubiquitous Crb staining by anti-Crb^{intra} antibody and no obvious enhancement of Crb in the DV boundary region (GFP^+ region in A). (B) *C96>ebi* RNAi causes upregulation of Crb along the DV boundary (arrows). (C-C″) Effects of Ebi knockdown by *Ci-Gal4 (Ci>GFP)* on Crb protein level. In the wing disc of *Ci>ebi* RNAi*; crb-lacZ*, GFP is expressed in the anterior compartment (C). Ebi knockdown in the anterior compartment (GFP^+ region) results in an enhancement of the Crb level along the DV boundary in the anterior compartment (arrows in C′). (D-D″) Effects of Ebi knockdown by *Ci-Gal4* on *crb-lacZ* level. The level of *crb-lacZ* reporter expression in the DV boundary region (arrows) was not affected by *ebi* RNAi in the anterior wing compartment (D′). (E-E″) Flipout *ebi* RNAi clones (GFP^+) show enhanced Crb levels in the DV boundary (arrows in E′) compared with the GFP-negative control region. Scale bars: 25 µm (A-E″).

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(Fig. 3E-E″). Taken together, these results show that Ebi is required for downregulation of Crb protein in the DV boundary.

Ebi downregulates Crb by ubiquitylation

To confirm the role of Ebi in Crb downregulation, we examined the level of Crb protein from fly extracts by western blot analysis. Crb protein levels in crb^{11A22}/+ heterozygotes were reduced by 48±2% (n=3) compared with wild-type level (*Canton-S* or w^{1118}) (Fig. 4A,A′). In contrast, the level of Crb protein was about 60±2% (n=3) higher in ebi^{E4}/+ heterozygotes than the wild-type level (Fig. 4A,A′). These results suggest that Ebi is required for the downregulation of Crb protein level.

It has been shown that Ebi is involved in proteasome-dependent degradation (Matsuzawa and Reed, 2001; Tsuda et al., 2002). Thus, we postulated that Crb might be a target for ubiquitin-mediated degradation by Ebi. To test this idea, first we checked whether reducing proteasome activity could increase the Crb level. In control wing discs without *ebi* RNAi, anti-ubiquitin staining was almost uniformly distributed within the wing pouch area (Fig. 4B,B″). Interestingly, when a 26S proteasome subunit was knocked down by *Prosβ6* RNAi with *C96-Gal4*, the level of ubiquitin was consistently reduced in the DV boundary region (Fig. 4C). In contrast, the level of Crb was enhanced in the area of Prosβ6 knockdown (Fig. 4C′,C′′). These results suggest that Ebi

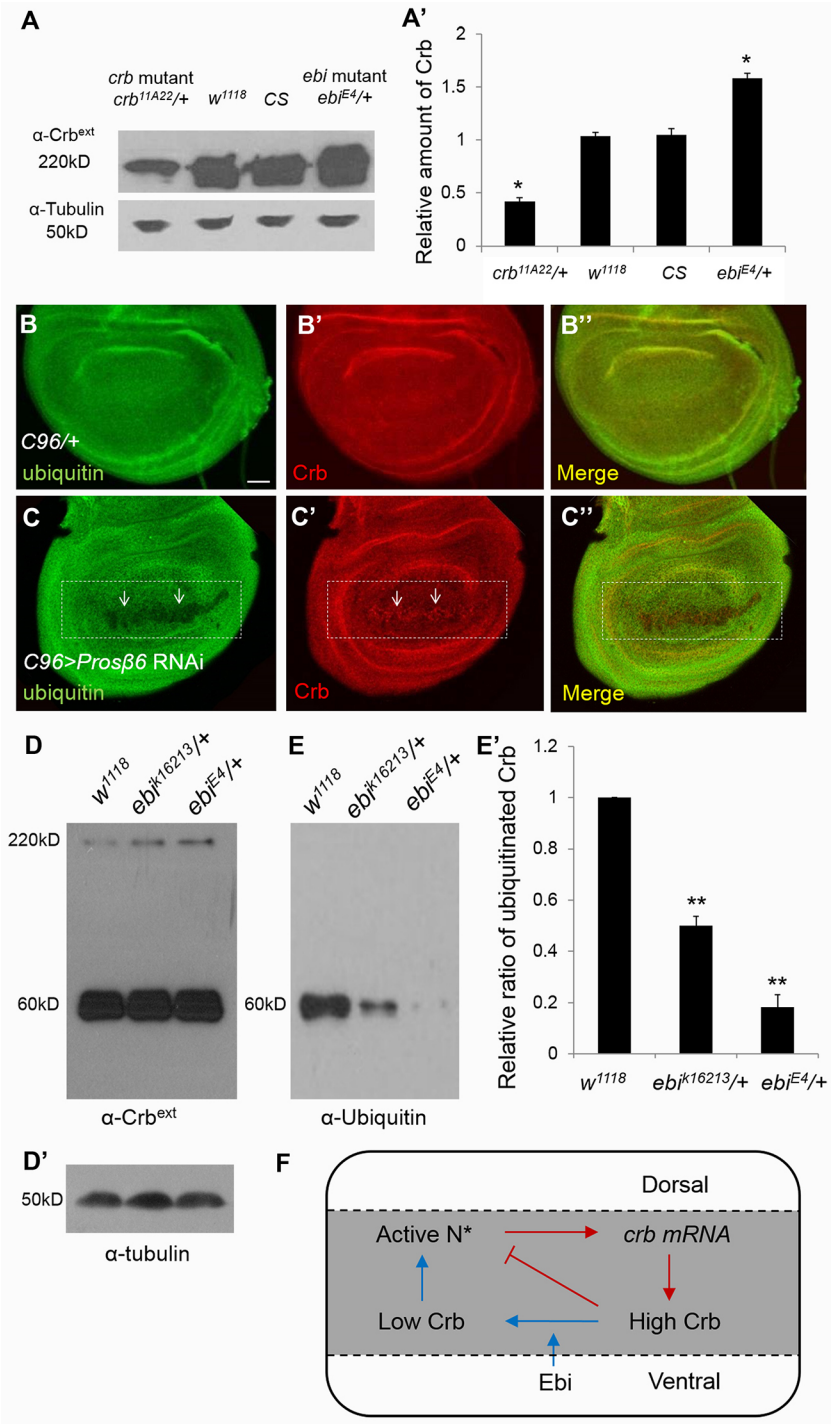

Fig. 4. Ebi is required for downregulation of Crb through ubiquitylation. (A) Effects of *ebi* mutation on the Crb protein level in whole adult flies. Crb protein level is decreased in crb^{11A22}/+ heterozygote compared with wild-type controls [w^{1118}; Canton-S (CS)]. Crb protein level was increased in ebi^{E4}/+ heterozygous mutant files compared with wild-type controls. (A′) Quantification of data in A. (B,C) Anti-ubiquitin staining in wing disc. Ubiquitin expression level was relatively even in the wing pouch of *C96*/+ control (B-B″). (C-C″) The level of ubiquitin was reduced in the DV boundary region by *Prosβ6* RNAi with *C96-Gal6* (*C96>Prosβ6* RNAi) (arrows in C). In contrast, Crb level was enhanced in the DV boundary region (arrows in C′). Scale bar: 50 µm (B,C). (D-E′) Effects of *ebi* mutations on Crb ubiquitylation. Whole adult fly extracts were immunoprecipated by anti-Crb^{ext} antibody. Western blot stained with anti-Crb antibody (D) shows Crb bands at 220 kDa and 60 kDa. Western blot stained with anti-ubiquitin antibody shows ubiquitin labeling on the 60 kDa Crb band. Both *ebi*/+ mutant heterozygotes have reduced ubiquitin labeling at the 60 kDa Crb band (E). The western blot with anti-tubulin staining shows input levels of tubulin prior to anti-Crb immunoprecipitation (D′).
(E′) Quantification of the data in (E). (F) A model for the role of Crb-Ebi interaction in wing development. Activated Notch signaling induces *crb* transcription in the DV boundary (gray region). Hyperactivation of Notch can be restricted by the inhibitory function of Crb. Ebi-dependent downregulation of Crb prevents excessive inhibition of Notch activity, thus providing a regulatory loop for stable Notch signaling. Red and blue arrows indicate negative and positive pathways for activated Notch signaling, respectively. Data are mean±s.d. of n=3; **P<0.001, *P<0.05 (t-test).

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might be involved in ubiquitin/proteasome-dependent degradation of Crb in the DV boundary region.

Next, we tested whether Crb is ubiquitylated to be targeted by the proteasome. Western blots of protein extracts from wild-type and *ebi*/+ heterozygous adult tissues showed similar patterns of many ubiquitin-labeled protein bands stained by an anti-Ubiquitin antibody (Fig. S3). For identification of ubiquitylated Crb, we carried out immunoprecipitation of Crb from protein extracts of wild-type and *ebi* heterozygous mutant adult flies. From immunoprecipitation with anti-Crb, the majority of Crb was detected as a 60 kDa band whereas the 220 kDa full length Crb was a minor form (Fig. 4D), suggesting that the 60 kDa band is a cleaved form of Crb. Interestingly, only the 60 kDa form of Crb was ubiquitylated whereas the full length Crb was not, as if ubiquitylated full-length Crb proteins were degraded. The amount of the full length Crb was increased in two different alleles of *ebi*/+ heterozygotes (Fig. 4D). In contrast, ebi^{E4}/+ heterozygous flies showed strong reduction of ubiquitylation in the 60 kDa Crb (Fig. 4E,E′), whereas the levels of α-tubulin were similar (Fig. 4D′). Similar results were found in another allele, ebi^{k16213}/+. Taken together with increased Crb levels in *ebi* mutants and Ebi-depleted wing discs, these data suggest that Ebi is required for ubiquitin-dependent downregulation of Crb.

Overlapping localization of Crb and Notch in the cell membrane and intracellular vesicles

It has been reported that the extracellular domains of Crb and Notch physically interact in mammalian cells (Ohata et al., 2011). We tested whether Crb puncta of wing disc cells overlap with Notch detected by anti-NICD (Notch intracellular domain) antibody. At the apical level of wing disc epithelium, Crb and Notch showed nearly identical patterns of membrane staining (Fig. S4A). Many NICD puncta were detected at more basal sections (Fig. S4B). Interestingly, approximately 40% of NICD puncta overlapped with Crb puncta based on ImageJ quantification. Ebi overexpression by *C96-Gal4* did not significantly alter the pattern of NICD and Crb staining (Fig. S4C,D). In contrast, Ebi knockdown by *C96>ebi* RNAi increased the Crb level in the apical region of the DV boundary (Fig. S4E′). Numbers of both Notch and Crb puncta were also increased by Ebi knockdown (Fig. S4F,F′,G). These results suggest that loss of Ebi leads to increased internalization of NICD and Crb and accumulation of Crb in the apical cell membrane for Notch inhibition.

In this study, we have shown that Ebi promotes wing development by downregulating the Crb protein level. Crb is known to antagonize Notch signaling by interfering with γ-secretase, which is necessary for Notch cleavage (Herranz et al., 2006). Our data indicate that the level of Crb protein is regulated post-translationally by Ebi. Thus, Notch signaling at the DV boundary in the wing disc seems to be regulated by two opposing activities, that is, the activation of *crb* transcription by Notch and the downregulation of Crb protein by Ebi. In a model proposed in Fig. 4F, Notch is activated in the DV boundary for active growth of wing discs, which will also induce upregulation of *crb* mRNA and protein. When Notch signaling reaches a level that is higher than optimal, accumulated Crb protein may antagonize Notch signaling (Herranz et al., 2006; Ohata et al., 2011). However, excessive Notch inhibition by Crb can be prevented by Ebi-dependent downregulation of Crb, thus maintaining the homeostasis of growth signaling. This feedback control of the Crb protein level might help keep Notch signaling at a proper level during wing development.

Ebi and mammalian Ebi orthologs can act as corepressors/coactivator exchange factors (Tsuda et al., 2002; Perissi et al., 2008). Thus, it has been proposed that Ebi might participate in the dissociation or degradation of the corepressor(s) via the proteasome and facilitates the recruitment of coactivators for transcriptional activation of Notch target genes in wing development (Marygold et al., 2011). However, knockdown of Ebi did not significantly alter the level of *crb-lacZ* (Fig. 3D′), a reporter gene induced by Notch signaling (Herranz et al., 2006). Instead, our data suggest that Ebi activates Notch signaling in wing development by proteasome-dependent downregulation of Crb. This function of Ebi is consistent with the role of Ebi in degradation of Tramtrack in *Drosophila* (Dong et al., 1999; Boulton et al., 2000) and the mammalian Ebi function in β-catenin downregulation by ubiquitylation (Matsuzawa and Reed, 2001).

While the intracellular domain of Crb has been extensively studied, little is known about protein partners that physically interact with Crb^{ext}. In zebrafish, the extracellular domains of the Crb family proteins can bind to the Notch extracellular domain to inhibit Notch activation (Ohata et al., 2011). In addition, Crb^{ext} is known to dimerize with itself for cell adhesion between photoreceptors in zebrafish (Zou et al., 2012; Pocha and Knust, 2013). In this study, we have shown that the lam4 domain of Crb^{ext} directly interacts with repeats 7-8 of the WD40 domain of Ebi. The mechanism underlying this physical interaction between Ebi and the extracellular domain of Crb is currently unknown. One possibility is that Crb protein may be internalized into the cytoplasm by endocytosis (Roeth et al., 2009). In this case, Crb^{ext} will be located inside the endosomal vesicles, hence cytosolic Ebi still cannot access Crb^{ext}. Secondly, Ebi might interact with the extracellular domain of newly synthesized Crb protein prior to its targeting to the cell membrane. Thirdly, Ebi protein may be secreted from the cell and bind to Crb^{ext}. It remains to be determined whether Ebi uses one of these methods or another unknown mechanism to interact with the lam4 domain of Crb. It would also be interesting to see whether the Ebi-dependent downregulation of Crb plays a conserved role for Notch signaling in other organisms.

MATERIALS AND METHODS

Fly genetics

All *Drosophila* strains were maintained at room temperature, unless stated otherwise. Ebi knockdown was induced by crossing *UAS-ebi* RNAi lines with *nub-Gal4* and *C96-Gal4*. Gal4 and RNAi lines were obtained from the Bloomington Stock Center, Vienna Drosophila Resource Center (Austria) and National Institute of Genetics (Japan). *UAS-*$Crb^{extTMGFP}$ fly strain was provided by Elizabeth Knust (Max Planck Institute, Dresden, Germany). ebi^{E4} and ebi^{k16213} mutants were provided by Leo Tsuda (National Center for Geriatrics and Gerontology, Japan). Ebi overexpression was induced by crossing *UAS-*ebi^{Exell} (ebi^{full}) with *C96-Gal4*. Flp-out *ebi* RNAi clones were made by crossing: *yw; hs-flp22; ebi* RNAi with *Ay≫Gal4,UAS-GFP* (Kyoto Stock Center #107-724). F1 progeny was heatshocked at 38°C for 60 min at first instar larval stage. Then, wing imaginal discs from 3rd instar larvae were analyzed by immunostaining.

Yeast two-hybrid screening

Matchmaker Gold yeast two-hybrid system (Clontech) was used to screen for Crb^{lam4} binding proteins. Firstly, the Crb^{lam4} coding sequence was cloned in pGBKT7 vector to make a fusion construct of Gal4 DNA-binding domain and Crb^{lam4} in the yeast strain Y2HGold. The culture of Y2HGold containing the fusion bait construct was mixed with the adult *Drosophila* Mate and Plate cDNA library (Clontech), which expresses *Drosophila* proteins fused with the Gal4 activation domain in the yeast strain *Y187*. After 24 h, mated diploid cells contain four reporter genes, *HIS3*, *ADE2*, *MEL1* and *AUR1-C* that can be activated in response to two-hybrid interaction. Approximately 10^7 transformants were screened, and positive clones obtained were selected by the ability to activate β-galactosidase and

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grow in restricted medium by expression of four reporter genes under the control of Gal4-responsive promoters. The strength of interactions was determined by the growth rate and color of clones on selective plates. Finally, inserts from positive clones were sequenced and identified by BLAST search in Flybase.

Immunostaining

Wing imaginal discs were fixed with 1% paraformaldehyde for 15 min and 100% methanol for 5 min. Fixed discs were immunostained, as described (Yeom et al., 2014). Briefly, after washing twice with PBS (pH 7.3), disc samples were blocked in 0.5% BSA. Samples were incubated with primary antibodies at the following concentrations: rabbit anti-Ebi (from Leo Tsuda, National Center for Geriatrics and Gerontology, Japan) at 1:200, rat anti-Crb^{intra} at 1:100, rabbit anti-Dlg (from Kyungok Cho, Korea Advanced Institute of Science and Technology, Daejeon, Korea) at 1:100, sheep anti-GFP at 1:100 (Bio-Rad, 4745-1051), mouse anti-Cut at 1:100 (DSHB, 2B10) and rabbit anti-ubiquitin at 1:50 (Abcam, ab8134). Then, secondary antibodies conjugated with Cy3 (1:600), Cy5 (1:400) or FITC (1:100) (Alexa Fluor, Molecular Probes) were used. Specificity for anti-Crb^{intra} antibody was tested in *crb* null mutant clones (Fig. S5). Fluorescent images were acquired using a Carl Zeiss LSM710 confocal microscope. The puncta colocalization was quantified by ImageJ-Fiji plugin colocalization analysis (http://fiji.sc/Cookbook). Manders' coefficients were used for percentage puncta colocalization measurements.

Generation of anti-Crb^{ext} antibody

A DNA fragment for the fourth laminin domain of Crb was amplified and cloned into the pMal vector. MBP-Crb^{lam4} fusion protein was expressed in *E.coli R2* cells by isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. Purified fusion protein was used to inject into rats to raise antibody (Abfrontier, Seoul, Korea). Anti-Crb^{ext} was validated by immunostaining in western blots (Fig. S6).

Cell culture, transfection, immunoprecipitation

S2 cells were grown in M3 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma) and 1% penicillin-streptomycin solution. For transfection, 3×10^6 to 4×10^6 cells were seeded in 4.5 ml medium per 50 ml flask and allowed to adhere. Then, transfection was performed using Cellfectin II reagent, according to the manufacturer's protocol (Invitrogen). A total of 1-2 µg DNA was used for each transfection. Cells were collected for analysis 36-48 h after transfection. For immunoprecipitation, cells were lysed in 0.1% CHAPS buffer, and the lysates (1 mg total protein) were precleared by incubating with Protein G-Sepharose beads (Amersham Bioscience) for 1 h at 4°C. The beads were removed after centrifugation and the clear lysates in the supernatant were kept. The beads were coupled with Flag-tag (Abcam) polyclonal antibodies at 4°C for 2 h and were incubated with the clear lysates overnight at 4°C. Samples were washed four times with IP buffer (20 mM HEPES pH 7.5, 100 mM KCl, 0.05% Triton X-100, 25 mM EDTA, 5 mM DTT, 5% glycerol and protease inhibitor cocktail). Immunoprecipitates were resuspended in 5× SDS sample buffer, heated for 5 min at 95°C, separated by SDS-PAGE and transferred to nitrocellulose membrane. Proteins were detected by a standard immunostaining protocol and enhanced chemiluminescence (ECL) (Gendepot, USA).

Protein constructs

Crb^{lam1} [amino acids (aa) 1-465], Crb^{EGF} (aa 466-981), Crb^{lam23} (aa 982-1575), Crb^{lam4} (aa 1576-1792) and Crb^{intra} (aa 2108-2146) were cloned into pMal (New England) to generate N-terminal MBP fusion proteins. Ebi^{Full} (aa 1-696), $Ebi^{\Delta WD7/8}$ (aa 1-613), Ebi^{Nter} (aa 1-343) and $Ebi^{WD7/8}$ (aa 614-696) were cloned into pGex4T1 (Pharmacia) to generate N-terminal GST fusion proteins. Crb^{lam4TM} (aa 1576-2106) was cloned into pAC5.1V5 (Invitrogen) and Ebi^{Full} was cloned into pAC5.1Flag (Invitrogen) for expressing protein in S2 cells.

GST pull down assay

For GST pull down, IPTG-inducible *E. coli R2* cells (*BL21* derivative) were transformed with plasmid constructs for fusion proteins MBP-Crb^{lam4}, GST Ebi^{Full}, GST $Ebi^{\Delta WD7/8}$, GST Ebi^{Nter} and GST $Ebi^{WD7/8}$. Bacterial cell lysates were prepared using a standard method. Equal amounts of blocked glutathione Sepharose 4B beads (Bioprogen) with GST, GST fusion protein or beads alone were incubated in pull down buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10% glycerol, 0.1% Triton X-100, 1 mM DTT and protease inhibitor cocktail), 1× sample buffer was added, beads were boiled, and proteins were resolved on SDS-PAGE. Proteins were electrophoretically transferred onto nitrocellulose membranes, blocked in 5% skimmed milk (Bio-Rad) for 1 h and incubated with primary goat anti-GST (Santa Cruz) or rabbit anti-MBP antibody (Santa Cruz). Goat anti-rabbit or anti-goat antibodies (Molecular Probes) were used as secondary antibodies. Pulled down proteins were visualized using an ECL kit (Thermo Fisher Scientific).

Primers

The following constructs and primers were used: crb^{lam1}, Fwd-ATGAGGATCCGCCAATGCGTCACTGTCGCAA and Rev-ATGAGTCGACTGGCAGCAGTCATATTGTGCTG; crb^{EGF}, Fwd-ATGAGAATTCTGCTTCCAGTCAGACTGCAAA and Rev-ATGAGTCGACCAGTAAATGTGGTGTTCGTAACAC; crb^{lam23}, Fwd-ATGAGTCGACAGGTAACTCATCTGTACAACTGC and Rev-ATGAGTCGACCAGTAAATGTGGTGTTCGTAACAC; crb^{lam4}, Fwd-ATGAGTCGACGTGTTACGAACACCACATTTACTG and Rev-ATGAGTCGACTCAATCGCCCTCGAATCCAGGCTG; crb^{intra}, Fwd-ATGAGAATTCATGGCCAGGAACAAGCGAGC and Rev-ATGAGTCGACCTAAATTAGTCGCTCTTCCGG; crb^{lam4TM}, Fwd-ATGAGTCGACGTGTTACGAACACCACATTTACTG and Rev-ATGAGAATTCGCTCGCTTGTTCCTGGCCAT; Ebi^{full}, Fwd-ATGAGAATTCATGAGTTTTTCCAGCGACGAGG and Rev-ATGACTCGAGTCAGAACTTTCGCAGGTCCAAC; $Ebi^{\Delta WD7/8}$, Fwd-ATGAGAATTCATGAGTTTTTCCAGCGACGAGG and Rev-ATGACTCGAGTCACCACAGTCTTACCGTGGAAT; Ebi^{Nter}, Fwd-ATGAGAATTCATGAGTTTTTCCAGCGACGAGG and Rev-ATGACTCGAGTATTTCGATGTTCTCGTCAATC; $Ebi^{WD7/8}$, Fwd-ATGAGAATTCGACGTGGAGAGGGGCAGCTG and Rev-ATGA-CTCGAGTCAGAACTTTCGCAGGTCCAAC.

crb and *ebi* cDNA (Flybase) were used as templates for PCR amplification.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.W.C., M.B.N.; Investigation: M.B.N., L.T.V.; Writing - Review & Editing: M.B.N., K.W.C.; Funding acquisition: K.W.C.; Supervision: K.W.C.

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Supplementary information

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