The apical-basal cell polarity determinant Crumbs regulates Hippo signaling in Drosophila

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Defects in apical-basal cell polarity and abnormal expression of cell polarity determinants are often associated with cancer in vertebrates. In Drosophila, abnormal expression of apical-basal determinants can cause neoplastic phenotypes, including loss of cell polarity and overproliferation. However, the pathways through which apical-basal polarity determinants affect growth are poorly understood. Here, we investigated the mechanism by which the apical determinant Crumbs (Crb) affects growth in Drosophila imaginal discs. Overexpression of Crb causes severe overproliferation, and we found that loss of Crb similarly results in overgrowth of imaginal discs. Crb gain and loss of function cause defects in Hippo signaling, a key signaling pathway that controls tissue growth in Drosophila and mammals. Manipulation of Crb levels caused the up-regulation of Hippo target genes, genetically interacted with known Hippo pathway components, and required Yorkie, a transcriptional coactivator that acts downstream in the Hippo pathway for target gene induction and overgrowth. Interestingly, Crb regulates growth and cell polarity through different motifs in its intracellular domain. A juxtamembrane FERM domain-binding motif is responsible for growth regulation and induction of Hippo target gene expression, whereas Crb uses a PDZ-binding motif to form a complex with other polarity factors. The Hippo pathway component Expanded, an apically localized adaptor protein, is mislocalized in both Crb mutant cells and Crb overexpressing tissues, whereas the other Hippo pathway components, Fat and Merlin, are unaffected. Taken together, our data show that Crb regulates growth through Hippo signaling, and thus identify Crb as a previously undescribed upstream input into the Hippo pathway.

Proper establishment and maintenance of apical-basal cell polarity in epithelial tissues is essential for animal development. Epithelial cell polarity is established and maintained through the concerted actions of three conserved polarity modules, the Crumbs (Crb), atypical protein kinase C (aPKC), and Discs large (Dlg) polarity modules (1–4). The Crb complex, composed of Crb, PALSI-associated tight junction protein (Patj), and Stardust (Sdt), and the aPKC complex, composed of aPKC, Par6, and Bazooka, localize to the subsarcal and apical sites and are important for the establishment and maintenance of the apical domain (1–4). Disruption of either apical complex in Drosophila embryos, for example, causes loss of apical-basal cell polarity, loss of apical markers, and expansion of the basolateral domain. The Dlg module contains Dlg, Lethal giant larvae (Lgl), and Scribble (Scrib). These proteins localize to the basolateral membrane and are required for each other’s localization, and perturbation of the Dlg complex causes loss of basolateral markers and expansion of apical markers (1–4). Interestingly, altering the expression of genes involved in the regulation of apicobasal polarity is often associated with the development of cancer in vertebrates and can lead to the development of neoplastic tumors in Drosophila imaginal discs (2, 4, 5). Imaginal discs are epithelial precursors of adult fly tissues and a widely used model system for the study of tissue growth and patterning. Imaginal discs that are homozygous mutant for scrib, dlg, or lgl lose their apical-basal polarity and severely overgrow (1–4). Similarly, overexpression of the apical determinant Crb causes overgrowth of Drosophila imaginal discs in addition to causing defects in cell polarity and expansion of apical domain markers to the basolateral domain (6, 7). Each of these situations thus promotes an overabundance of the apical domain. Several mechanisms have been suggested to explain the observed phenotypes. For example, defects in apical-basal polarity may cause overgrowth as a result of deregulation of many signaling pathways as a consequence of mis trafficking of receptors, or the polarity complex proteins may specifically modulate one or more growth-controlling pathways (5, 8). However, although abnormal expression of apical-basal determinants can cause dramatic overgrowth phenotypes, the mechanisms through which they affect growth are poorly understood.

Here, we provide evidence that Crb acts through the Hippo pathway to regulate tissue size. Crb is a transmembrane domain protein with a large extracellular domain and a short intracellular domain (9). The extracellular domain contains 28 epidermal growth factor-like repeats and 3 laminin G-like repeats, and the intracellular domain contains two conserved motifs, a juxtamembrane motif (JM) and a PDZ-binding motif (PBM) (10). The Hippo pathway has emerged as a key signaling pathway that regulates growth of imaginal discs (11–14). Hippo signaling limits cell proliferation in imaginal discs, and flies that lack Hippo pathway activity have severely overgrown discs and corresponding adult structures. Several components of the pathway have been discovered, and a signal transduction pathway from the plasma membrane into the nucleus has emerged. Central to the Hippo pathway is a kinase cascade involving the Hippo (Hpo) and Warts (Wts) kinases and their regulatory proteins Salvador and Mags. Active Hpo phosphorylates and activates Wts, which then phosphorylates and inhibits the activity of the transcriptional coactivator Yorkie (Yki). Active Yki translocates to the nucleus, where it forms a complex with the transcription factor Scalloped to induce the expression of target genes that drive cell proliferation and cell survival, such as cyclin E, diap1, and the bantam miRNA. Thus, when active, Hpo and Wts suppress cell proliferation by restraining the activity of Yki.

Several components are known to act upstream of Hpo and Wts. The atypical cadherin Fat was identified as a receptor that regulates the Hippo pathway (11–14). It was proposed that Fat transduces signals from Dachsous (Ds), an atypical cadherin related to Fat, and Four-jointed, an extracellular kinase that phosphorylates Fat and Ds (12, 14, 15). Fat signal transduction involves an unconventional myosin, Dachs (D), the kinase Discs...
overgrown, and a 4.1, Ezrin, Radixin, Moesin (FERM) domain-containing adaptor protein Expanded (Ex), although through poorly understood processes (14). Ex localizes to the subapical region, where it cooperates with Merlin (Mer), another FERM domain-containing protein, and Kibra, a WW domain protein, in regulating the activity of the Hippo pathway (12, 14, 16–18).

Notably, although these upstream components are required for normal activity of the Hippo pathway, their mutant phenotypes are relatively weak when compared with mutations in more downstream components, indicating that other inputs into the Hippo pathway exist. Our data now link the transmembrane protein Crb to the activity of the Hippo pathway, and thereby identify a previously undescribed input into the Hippo pathway.

**Results**

**Crb Gain and Loss of Function Cause Overgrowth and Up-Regulation of Hippo Pathway Target Genes.** Overexpression of full-length Crb or a truncated version of Crb that lacks the extracellular domain (Crb<sup>intratrans</sup>) during wing development causes significant enlargement of the overexpression domain in wing discs and results in overgrown wings (Fig. 1 A–G) (6, 7). The enlargement of the overexpression domain was associated with extra cell proliferation, as evidenced by higher levels of BrdU incorporation, which marks cells in S-phase of the cell cycle (Fig. 1 D and E). Overexpression of Crb therefore stimulates cell proliferation in wing discs. These overgrowth phenotypes resemble hypomorphic hpo phenotypes in that they affect cell proliferation and the size of the wing but do not show strong effects on patterning. We thus wanted to test for possible effects of Crb on the activity of the Hippo pathway. We monitored the activity of the Hippo pathway by assaying the expression of ex, which is regulated by the Hippo pathway in a negative feedback loop, using a lacZ enhancer trap insertion (ex-lacZ) (19); the expression of diap1 using a diap1-GFP reporter transgene that is directly regulated by the Hippo pathway (20); and the expression of Wingless (Wg), which is regulated by Hippo signaling in the prospective hinge region of the wing disc (21). We found that overexpression of full-length Crb or Crb<sup>intratrans</sup> in wing discs along the anterior-posterior compartment boundary by dpp-Gal4 caused strong up-regulation of ex-lacZ throughout the Crb overexpression domain (Fig. 1 F and G). Similarly, overexpression of Crb or Crb<sup>intratrans</sup> induced the expression of diap1-GFP and Wg in wing discs (Figs. S1 and S2). These effects are similar to loss of Hippo signaling, and thus indicate that Crb overexpression affects the activity of the Hippo pathway.

The observation that Crb overexpression causes up-regulation of Hippo target genes raises the question as to whether loss of crb affects growth. We thus analyzed the phenotypes of homozygous crb mutant cells in imaginal discs. To generate tissues that were nearly entirely mutant for crb, we flanked chromosomes carrying the crb null allele crb<sup>14122</sup> against chromosomes carrying a Minute mutation in addition to the GFP marker. We found that crb mutant heads and wings are larger than controls and that crb mutant wings showed venation defects (Fig. 2 A–D), as was previously observed (22). To assay for defects in the regulation of cell proliferation, we tested for ectopic proliferation in eye discs. In WT animals, cells posterior to the second mitotic wave, a synchronized cell division of cells just posterior to the morphogenetic furrow, do not divide anymore. However, crb mutant cells in this region failed to arrest the cell cycle and, instead, continued to proliferate, as evidenced by ectopic BrdU incorporation (Fig. 2E, arrowhead). This result indicates that Crb is required for cell proliferation arrest posterior to the morphogenetic furrow. Crb is thus a growth regulator required to restrict cell proliferation and the size of imaginal disc-derived tissues.

Given the effects of Crb overexpression on Hippo signaling, we tested whether loss of crb also affects Hippo target gene expression. We found that the ex-lacZ reporter is up-regulated in crb mutant clones, which was especially evident in the hinge region of wing discs (Fig. 2F, arrowhead) and was weaker in the wing pouch. These results indicate that Crb is required for proper regulation of Hippo target genes.

Interestingly, both crb loss of function and Crb overexpression cause overgrowth and induction of Hippo target genes, although the Crb overexpression phenotypes are stronger than the loss of

![Fig. 2. Crb is required for proper tissue size, cell-cycle arrest, and Hippo target gene expression.](image)

**Fig. 1.** Crb overexpression causes overgrowth and up-regulation of Hippo pathway target genes. (A) WT wing. (B) Wing that overexpressed Crb during development under the control of C765>Gal4. (C) Overlay of the images in A (red) and B (blue) shows that the Crb-expressing wing is overgrown. Confoocal images of wing imaginal discs of third instar larvae expressing GFP under the control of the dpp-Gal4 driver (D and F) or larvae overexpressing Crb in addition to GFP(F and G). (D and E) Discs are stained for BrdU incorporation to mark cells in S-phase (red in D and E, gray in D′ and E′). (F and G) Discs are stained to reveal the expression of the Hippo pathway reporter ex-lacZ (red in F and G, gray in F′ and G′). For disc panels, anterior is to the left and ventral is up.
function phenotypes. The similarity of the loss and overexpression phenotypes indicates that WT levels of Crb are essential for normal functioning of the Hippo pathway.

Crb Genetically Interacts with Hippo Pathway Components and Requires Yki for Overgrowth and Hippo Target Gene Induction. To test more directly the hypothesis that Crb acts through the Hippo pathway, we first investigated whether Crb genetically interacts with known Hippo pathway components. The overgrowth phenotypes of crb mutant clones are similar but not as strong as those of hpo mutants (23). Rather, the crb mutant phenotypes resemble partial loss of Hippo signaling. Loss of hpo or wts causes massive increases in the number of interommatidial cells (23), whereas crb mutant retinae showed no significant increase (Fig. 3). Given that Crb is a transmembrane protein localized to the subapical region, where Fat, Ex, and Mer localize, Crb may also act upstream in the Hippo pathway. The fat, ex, and mer mutants also have relatively mild phenotypes, but mer;fat and mer;crb double mutants have synergistic phenotypes and produce many extra interommatidial cells (19, 24, 25). Similarly, we found that knockdown of mer from crb mutant retinae resulted in the production of extra interommatidial cells, which was not observed in the crb mutants or the mer knockdown retina (Fig. 3B). Thus, Crb and Mer cooperate to regulate cell number in the retina. Moreover, we observed strong genetic interaction between crb and the deregulation of Fat signaling by overexpression of D, which mimics loss of Fat activity (26). Knocking down crb in developing wings by nubbin-Gal4-driven UAS-crbrNAi caused mild overgrowth phenotypes (Fig. 3 C and D), whereas overexpression of D resulted in slightly larger wings compared with WT wings (Fig. 3E). However, the combined knockdown of crb with overexpression of D caused synergistic effects and resulted in significantly larger wings (Fig. 3F). We conclude that loss of crb genetically interacts with mutations in Hippo pathway components.

Second, we asked whether Crb functions as an input into the Hippo pathway and whether the Hippo pathway is necessary for its growth control function. To determine this, we tested whether Yki is required for the overgrowth phenotype induced by Crb overexpression. We found that heterozygosity for yki dominantly suppressed the overgrowth phenotype induced by overexpressing Crb in wing discs (Fig. 3 G and H). Additionally, knockdown of yki by RNAi suppressed the overgrowth phenotype caused by Crb overexpression and also reversed the induction of ex-lacZ (Fig. 3I–K). Yki is thus required for the overgrowth and Hippo pathway target gene induction in response to Crb overexpression.

Crb Regulates Hippo Signaling and Apical-Basal Polarity Through Different Domains. Crb is a transmembrane protein with a very short intracellular domain of 37 amino acids that contains two conserved protein-binding motifs (Fig. 4E): a C-terminal PBM that binds to Sdt and is required for the action of Crb in apical-basal polarity (10, 27–29) and a FERM domain-binding motif, the JM, that binds to the FERM domain of Yurt (30) and complexes with Moesin and β-Spectrin (31). In the pupal retina, overexpressed Crbintra mislocalizes throughout the cell and causes mislocalization of Patj and Armadillo (Arm), a marker for adherens junctions (29). The effects on Patj require the PBM but not the JM, whereas, conversely, the effects on Arm require the JM but not the PBM. Similarly, overexpression of Crbintra in the embryo recruits Sdt to the basolateral membrane, an effect that requires the PBM but not the JM (10). When we assayed the different motifs for their effects on Hippo signaling, we found that Crb requires the JM but not the PBM to affect the Hippo pathway, as seen by lack of diap1-GFP expression (Fig. 4 and Figs. S1 and S2). Thus, mutation of the JM completely abolished the effects of Crbintra on growth as well as the induction of Hippo reporters ex-lacZ (Fig. 4 A–D) and diap1-GFP (Fig. S1D) and Wg expression (Fig. S2D). In contrast, overexpression of CrbintraPBM still caused overgrowth phenotypes and induction of ex-lacZ, diap1-GFP, and Wg as strong as those caused by Crbintra overexpression (Fig. 4C and Figs. S1E and S2E). Removal of both domains abrogated the effects of Crbintra (Fig. 4D and Figs. S1F and S2F). We quantified the overgrowth phenotypes by measuring the relative size of the dpp-Gal4 expression domains by the ratio of the GFP-positive area to the overall size of the discs (Fig. 4F). We found that the overexpression of full-length Crb and Crbintra induced around 4-fold overgrowth of the expression domain. However, mutation of the JM eliminated the growth effects, whereas deletion of the PBM did not affect the growth-inducing activity of Crbintra. In summary, these data show that Crb regulates Hippo signaling and cell polarity through different mechanisms because of the differential requirement of the JM and PBM.

Crb Is Required for Localization of Ex to the Subapical Membrane. Crb is a transmembrane protein, and it may therefore act as a receptor of the Hippo pathway. Fat, Ex, and Mer as well as Crb localize to the subapical plasma membrane, raising the possibility that they affect each other’s localization. However, fat, ex, and mer were not required for correct localization of Crb: ex mutant cells as well as fat mutant cells had increased amounts of Crb at the cell boundaries (Fig. 5B). Thus, Crb and Ex localize to the same subapical membranes.

Fig. 3. Crb genetically interacts with Hippo pathway components and requires Yki for overgrowth and regulation of Hippo target genes. (A and B) Confocal images of pupal retinae with crbex-Z-driven mutant clones marked by the absence of GFP expression (green) stained with antibodies to detect Dlg to visualize cell outlines (red in A and B, gray in A’ and B’). The retina in A is otherwise WT, whereas the retina in B also has knockdown of Mer by a GMR-Gal4-driven UAS-merRNAi construct. The crb mutant clones are normal in A but show extra interommatidial cells in B (arrowheads). (C–H) Adult wings of the indicated genotypes. Wing-specific overexpression of crbex-Z or D using nub-Gal4 did not cause significant overgrowth (D and E), but coexpression caused synergistic overgrowth effects (F), (G and H) Overexpression of Crbintra caused overgrowth, which was suppressed by removing one copy of yki. Confocal images of wing imaginal discs that expressed Crbintra (I), ykiRNAi (J), and Crbintra and ykiRNAi (K) in the posterior compartment driven by hh-Gal4 and marked by coexpression of GFP. These discs are also stained for β-Gal to reveal the expression of the Hippo reporter ex-lacZ. Crbintra overexpression caused overgrowth and induction of ex-lacZ, whereas ykiRNAi expression caused reduced ex-lacZ expression and compartment size. Coexpression of ykiRNAi with Crbintra suppressed the induction of ex-lacZ and overgrowth. Arrowheads point to the compartment boundaries.
membrane, whereas mer mutant cells had similar amounts of Crb to that in WT cells (Fig. S3). Strikingly, however, Ex was largely absent from the plasma membrane of crb mutant cells (Fig. 5, E and F). Large crb mutant clones often did not show any cytoplasmic ex expression. The defects in Hippo pathway activity may thus be synergized with loss of crb in the pupal retina, indicating that Crb and Mer cooperate to modulate Hippo signaling. This is also supported by our finding that Ex but not Mer localization depends on Crb.

How does Crb regulate Hippo pathway activity? We found that crb mutant clones had reduced levels of Ex localized to the plasma membrane even though the transcription of ex was increased, as evidenced by the up-regulation of ex-lacZ. Thus, Crb affects Ex posttranscriptionally and is required for the localization of Ex to the apical membrane. Similarly, overexpression of Crb caused loss of Ex from the subapical membrane, although increasing ex-lacZ expression. The defects in Hippo pathway activity may thus be a consequence of the loss of Ex from the plasma membrane and disruption of subapical Hippo signaling complexes (16, 36, 37). In this scenario, Crb may act as a scaffold that is required to recruit Ex to the membrane, making it available for another regulator or receptor. Alternatively, Crb may act as a receptor and modulate the activity of Ex. Notably, Crb is nonautonomously required for Ex localization at the apical membrane. These results thus identify a cell-cell interaction-dependent mechanism that regulates the Hippo pathway.

Crb appears to regulate cell polarity and Hippo signaling through different mechanisms. A version of Crb that lacks the extracellular domain, Crbintra, is sufficient to mediate the pleio-

Discussion
Our data functionally link the growth regulatory activity of Crb with the Hippo pathway. Several lines of evidence indicate that Crb acts through the Hippo pathway to regulate tissue size. First, loss of crb and Crb overexpression cause overgrowth, excess proliferation, and the induction of Hippo pathway target genes. Second, loss of Crb genetically interacts with mutations in known Hippo pathway components. Third, the overgrowth phenotypes of crb and the induction of Hippo target genes depend on normal Yki levels, indicating that Yki acts downstream of Crb. Fourth, Crb is required for the localization of Ex to the subapical plasma membrane. Taken together, our data place Crb upstream of Ex to regulate the activity of Yki, and thereby organ growth. Similar conclusions were recently presented by Ling et al. (34) and Robinson et al. (35) while this manuscript was under review.

To date, the Hippo pathway comprises a signal transduction cascade that includes the Fat cadherin, a transmembrane protein that acts upstream of several components that form a kinase cascade, which culminates in the regulation of gene expression through the transcriptional coactivator Yki (11–14). However, the mutant phenotypes of fat and other components that act upstream in the pathway are generally weaker than those of mutants in genes that act more downstream, such as hpo and wts, indicating that other unknown inputs into the Hippo pathway may exist. Here, we identify Crb as a previously undescrbed upstream input into the Hippo pathway. Interestingly, knockdown of mer synergized with loss of crb in the pupal retina, indicating that Crb and Mer cooperate to modulate Hippo signaling. This is also supported by our finding that Ex but not Mer localization depends on Crb.

Fig. 4. JM of the Crb intracellular domain mediates the regulation of growth and the Hippo pathway. (A–D) Confocal images of third instar wing discs overexpressing different mutant versions of Crbintra driven by the dpp-Gal4 driver. The expression domain is marked by coexpression of GFP (green). Discs are stained for the expression of ex-lacZ (red in A–D, gray in A–D). Overexpression of the WT version of Crbintra and the Crbintra/JM mutant is able to drive growth and ex-lacZ induction, but mutation of the JM domain abolishes these effects. (E) Alignment of the intracellular domain sequence of Drosophila Crb (Dm) with that of the three human Crb homologs (Hs Crb1–3). Conserved residues are red, and the extent of the JM and PBM is indicated with blue bars. (F) Quantification of the overgrowth phenotypes shown in A–D. Labeling indicates overexpressed protein. FL, Crb full-length. For disc panels, anterior is to the left and ventral is up.
tropic functions of Crb in regulating cell polarity (29, 38) and Hippo signaling. Significantly, the effects on cell polarity and Hippo signaling require different motifs: the JM is required for the effects on Hippo signaling, whereas the PBM, which binds to Sdt, is required for the effects on apical complexes. The JM is a FERM domain-binding motif that directly interacts with the FERM domain protein Yurt during photoreceptor morphogenesis (30) and complexes with Moesin and β-Spectrin (31). Yurt and Moesin do not have Hippo-like growth defects, and thus may not mediate the function of Crb in growth. However, Crb can bind to Ex (34), and direct effects of Crb on Ex thus appear to mediate the effects of Crb on the Hippo pathway. The fact that the JM but not the PBM is required for the regulation of the Hippo pathway indicates that Crb has separate functions in polarity and growth control. Interestingly, mutant cells do not have obvious morphological cell polarity defects in imaginal discs, although membrane levels of Patj and aPKC are reduced in mutant cells (39, 40). However, Fat and Mer are not lost from the membrane of mutant cells, and mutant cells thus do not have a general loss of apically localized proteins. Altogether, our results indicate that Crb affects Hippo signaling through a specific mechanism rather than as a secondary consequence of defects in apical-basal polarity. These data suggest that Crb is required to recruit and/or assemble a complex that regulates Hippo signaling and contains Ex. Importantly, the observation that mutant clones, which have reduced apical membranes (29, 39, 41), show overproliferation argues that it is not just the size of the apical membrane that dictates growth behavior, and thus further supports a model in which Crb plays a direct role in the generation of a growth-controlling signal.

It has recently been reported that Crb affects the activity of Notch signaling (22, 42). In contrast to our results, however, the effects of Crb on Notch are mediated by its extracellular domain. Because Notch also regulates growth in imaginal discs, Crb may regulate growth through multiple mechanisms.

Does a Crb-like receptor act upstream of Hippo signaling in vertebrates? Most of the known components of the Hippo signaling pathway in Drosophila are highly conserved in vertebrates, where they also act together in a signaling pathway (11–14, 43). Several of the vertebrate Hippo pathway components act as tumor suppressor genes and have been implicated in regulating cell proliferation and apoptosis. Vertebrates have three Crb homologs, Crbl–3, all of which have at least some conservation of the intracellular domain and function in apical-basal polarity (44). Importantly, Crb3 has been implicated as a tumor suppressor in selected immortal baby mouse kidney epithelial cells that acquired tumorigenicity (45). There, Crb3 expression is lost during the selection of tumorigenic cell lines, and reexpression of Crb3 restores contact inhibition and cell polarity and suppresses cell migration and metastasis. It will be interesting to test whether Crb3 acts through the Hippo pathway for its function as a tumor suppressor. Our data linking Crb to Hippo signaling may thus have important implications for the study and treatment of cancer.

**Methods**

Mutant clones were induced using the FLP/FRT system. For generating crb, ex, mer, fat, or d mutant clones, the following alleles were flipped against corresponding ubi-GFP and marked FRT chromosomes: crb11A22 (null), ex1′ (null), mer1 (null), fat422 (null), and dcr1. To generate crb mutant heads and wings nearly entirely mutant for crb, we induced mitotic recombination by flipping crb1 into Minute chromosomes using ey-FLP and ubx-FLP, respectively. The UAS-Gal4 system in combination with temperature-sensitive Gal80 was used for overexpression with the following stocks: UAS-Crb63 (from E. Knust); UAS-Crb63, UAS-Crb63, UAS-Crb63, UAS-Crb63, and UAS-Crb63 (all from K. Choi); UAS-D (from K. Irvine); UAS-crb63 (nos. 39177 and 39178; Vienna Drosophila RNAi Center (VDRC)); UAS-megDUC (no. 142288; National Institute of Genetics Fly Stock Center); UAS-ykiB5 (no. 40497; VDRC); and C765-Gal, nub-Gal, dpp-Gal, hh-Gal4, and en-Gal4. The C765-Gal and nub-Gal crosses to UAS-Crb63 were kept at 18 °C. For hh-Gal4 and en-Gal4 crosses with different Crb constructs, crosses included Gal80 and were kept at 18 °C until they were shifted to 30 °C for 5 or 24 h, respectively, before dissection. Other crosses were kept at 25 °C. Other stocks were ex797 and ykiB5.

The quantification of the overgrowth phenotypes was done using ImageJ (National Institutes of Health), marking the GFP-positive region or the entire disc with the "analyze particle" function. The ratio of the GFP-positive area over the total disc area was calculated.

SEM of adult flies was done following the Hexamethyldisilazane method. Antibody stainings of imaginal discs and BrdU incorporations were done as described by Haramaroglu et al. (19). The following antibodies were used: guinea pig anti-Mer (R. Feihon, 1:4,000), rabbit-anti-Ex (A. Laughon, 1:2,000), and mouse-anti-Dlg (M. Simon, 1:2,000), rat-anti-Ecad (T. Uemura, 1:30), mouse-anti-Arm (Developmental Studies Hybridoma Bank, 1:30), mouse anti-BrdU (Becton Dickinson, 1:50), mouse anti-Dlg (Developmental Studies Hybridoma Bank, 1:300), mouse anti-β-Gal (Promega, 1:2,000), mouse anti-Crb (Developmental Studies Hybridoma Bank, 1:300).

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Supporting Information

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Fig. S1. Crb regulates the Hippo target diap1-GFP, for which it requires the JM. (A–F) Confocal images of third instar wing imaginal discs overexpressing full-length Crb (B) or different mutants of Crb\textsuperscript{intra} (C–F) along the anterior-posterior compartment boundary driven by the dpp-Gal4 driver. The anterior compartments are marked by staining for Cubitus interruptus (Ci) (green). Discs are stained for the expression of the Hippo pathway reporter diap1-GFP (red in A–F, gray in A′–F). Labeling indicates overexpressed Crb protein. For all disc panels, anterior is to the left and ventral is up.

Fig. S2. Crb regulates the Hippo target Wg, for which it requires the JM. (A–F) Confocal images of third instar wing imaginal discs overexpressing full-length Crb (B) or different mutants of Crb\textsuperscript{intra} (C–F) in the posterior compartment driven by the en-Gal4 driver. The anterior compartments are marked by staining for Cubitus interruptus (Ci) (green). Discs are stained for the expression of Wg, which is regulated by the Hippo pathway in the hinge region but not in the presumptive margin of wing discs (red in A–F, gray in A′–F). Labeling indicates overexpressed Crb protein. For all disc panels, anterior is to the left and ventral is up.
Fig. S3. Crb is localized normally in fat, ex, and mer mutant clones. The fat^{424} (A), ex^{e7} (B), and mer^{6} (C) mutant clones in wing imaginal discs are marked by the absence of GFP expression (green). Crb (red in A–C, gray in A′–C′) is localized at the plasma membrane in mutant cells similar to WT cells. Crb levels are slightly elevated in all three mutants. Arrowheads point to clone borders.

Fig. S4. Crb is not required for Mer, Fat, and D localization. (A) crb^{11A22} mutant clones in wing discs that are marked by the absence of GFP expression (green). Mer (red in A, gray in A′) localization is not significantly affected in mutant clones. (B) Fat localization is not significantly affected in crb^{11A22} mutant clones. (C) Ex was lost from the membranes of crb^{11A22} mutant clones that were produced in a d^{GC13} homozygous mutant wing disc. Mutant clones are marked by the absence of GFP expression (green), and Ex staining is in red (C) and gray (C′). (D) crb^{11A22} mutant clones in wing discs that are marked by the absence of GFP expression (green). D (red in D, gray in D′) localization is not significantly affected in mutant cells. D localization was analyzed by anti-V5 staining for a V5-tagged D that was overexpressed in the wing disc by the nub-Gal4 driver. Arrowheads point to clone borders in A–C and to mutant cells in D.