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Kibra and Merlin activate the Hippo pathway spatially distinct from and independent of Expanded

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SUMMARY

The Hippo pathway is emerging as a key evolutionarily conserved signaling mechanism that controls organ size. Three membrane-associated proteins, Kibra, Merlin, and Expanded regulate pathway activity, but the precise molecular mechanism by which they function is still poorly understood. Here we provide evidence that Merlin and Kibra activate Hippo signaling in parallel to Expanded at a spatially distinct cellular domain, the medial apical cortex. Merlin and Kibra together recruit the adapter protein Salvador, which in turn recruits the core kinase Hippo. In addition, we show that Crumbs has a dual effect on Hippo signaling. Crumbs promotes the ability of Expanded to activate the pathway, but also sequesters Kibra to downregulate Hippo signaling. Together, our findings elucidate the mechanism of Hippo pathway activation by Merlin and Kibra, identify a subcellular domain for Hippo pathway regulation, and demonstrate differential activity of upstream regulators in different subcellular domains.

eTOC Blurb

Merlin, Kibra, and Expanded are believed to act in a complex at intercellular junctions to control Hippo pathway activity. Su et al. show that instead Merlin and Kibra function at the *Drosophila* apical medial cortex separately from Crumbs and Expanded, thereby identifying an additional subcellular domain for Hippo pathway regulation.

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AUTHOR CONTRIBUTIONS

T.S. and R.G.F. conceived the project. T.S. performed most of experiments with the exception of generating the *Mer-YFP*, *ex-YFP*, *hpo-YFP* and *yki-YFP* transgenes, which were generated by M.Z.L. J.X. contributed to the design and implementation of the *kib:GFP* CRISPR allele. T.S. and R.G.F. analyzed the data and interpreted the results. T.S. and R.G.F. wrote the manuscript. R.G.F. supervised all aspects of the project.

Keywords

Hippo pathway; growth control; Kibra; Merlin; Expanded; Crumbs; Salvador; Hippo; Warts; Yorkie; apical medial domain

INTRODUCTION

How multicellular tissues reach and maintain appropriate size is a fundamental and fascinating question in developmental biology. Pioneering work in *Drosophila* has identified the Hippo pathway as having a key role in regulating organ growth (Huang, et al., 2005; Harvey, et al., 2003; Wu, et al., 2003; Tapon, et al., 2002; Xu, et al., 1995). The Hippo pathway is composed of upstream activators consisting of Merlin (Mer), Expanded (Ex), Kibra (Kib) and Salvador (Sav), a core kinase cassette consisting of Tao1, Hippo (Hpo) and Warts (Wts), and a downstream transcriptional co-activator, Yorkie (Yki) (Boggiano and Fehon, 2012). Parallel studies in mammals have demonstrated that the components and overall functions of the Hippo pathway are evolutionarily conserved (Yu, et al., 2015). Genetic perturbation of Hippo pathway members can lead to tumorigenesis in mice (Dong, et al., 2007) and the Neurofibromatosis 2 (NF2) tumor syndrome in humans (Rouleau, et al., 1993). Additionally, a variety of human cancers exhibit Hippo pathway deregulation (Harvey, et al., 2013).

A striking aspect of Hippo pathway function and regulation is its close relationship with the cell cortex and intercellular junctions (Enderle and McNeill, 2013; Boggiano and Fehon, 2012). In polarized epithelia such as the Drosophila imaginal discs, the cortex and associated plasma membrane are organized into apical, junctional and basolateral domains (Tepass, 2012) (Figure 1A). Hippo pathway components including Crumbs (Crb), Ex, Mer, Echinoid (Ed), Ajuba (Jub), Sav, Hpo and Wts have been shown to localize junctionally (Enderle and McNeill, 2013; Boggiano and Fehon, 2012), and recent studies indicate that the junctional domain is a primary site of pathway activation (Chung, et al., 2016; Sun, et al., 2015). Mer, Ex and Kib also are known to form a complex, colocalize at the junctions, and function upstream of the core kinases, suggesting that these three proteins function together to regulate pathway activity (Enderle and McNeill, 2013; Yu, et al., 2010). However, Mer and Ex localize at the cortex independent of each other and in some contexts appear to function in parallel genetically (Reddy and Irvine, 2011; Milton, et al., 2010; Hamaratoglu, et al., 2006; McCartney, et al., 2000). Furthermore, both Ex and Mer can recruit Wts to the plasma membrane (Sun, et al., 2015; Yin, et al., 2013). Thus, despite considerable progress in understanding how Hippo pathway activity is regulated, we do not yet understand how these proteins are organized and function at the cell cortex, particularly in the context of living tissues.

Here we use endogenously expressed, fluorescently-tagged proteins in intact tissues to explore the functional relationships between upstream Hippo pathway components and the core kinases. We show that Mer and Kib function at the medial apical cell cortex where they can recruit Sav, Hpo and Wts to promote Hippo pathway activation, all independently of Ex. Furthermore, Mer promotes accumulation not only of Wts in this domain, but also Sav and

Hpo. Strikingly, although Crb synergizes with Ex at junctions to promote HSW pathway activation, Crb antagonizes Kib's ability to restrict growth by recruiting it to the junctions. Together, these findings reveal a previously unrecognized second cortical domain for pathway activation, elucidate the cellular basis underlying observed genetic redundancies between Mer and Ex, and provide a mechanistic better understanding for how Mer, the product of the NF2 tumor suppressor, regulates Hippo signaling.

RESULTS

Mer, Kib and Sav colocalize at a non-junctional site

Previous studies have shown that the upstream Hippo pathway regulators Mer, Kib and Ex primarily localize to the junctional domain in imaginal epithelia cells (Sun, et al., 2015; Enderle and McNeill, 2013; Genevet, et al., 2010). However, close examination of Mer antibody staining shows significant non-junctional staining at the medial apical cell cortex (McCartney, et al., 2000; McCartney and Fehon, 1996). To assess the subcellular localization of endogenously expressed Mer and other Hippo pathway components in living tissues, we generated endogenously expressed, fluorescently tagged forms of Mer, Kib and Ex either using CRISPR/Cas-9 genome editing (*kib:GFP*) or transgenes made by recombineering tags into genomic sequences contained within bacterial artificial chromosome (BAC) clones (*Mer-YFP* and *ex-YFP*). All tagged, endogenously expressed proteins used in this study are fully functional, as judged by the ability to rescue null mutations in the corresponding genes (YFP-tagged transgenes) or homozygous viability (*kib:GFP*; data not shown).

In living wing imaginal discs, Ex-YFP appeared exclusively localized to the junctions, while both Mer-YFP and Kib:GFP localized both junctionally and to non-junctional punctae localized within the medial apical cortex (Figures 1B–1D). Using antibody staining on fixed tissues, we then compared the localizations of Kib and Mer and found that they normally colocalize both junctionally and medially (Figure 1E—1E″).

We next examined the localization of Sav, a scaffolding protein critical for recruitment and activation of the Hpo kinase to the signaling complex (Wu, et al., 2003). A previous study has reported that Sav localizes junctionally, where it interacts with the transmembrane protocadherin Ed (Yue, et al., 2012). We have not yet tagged Sav for imaging in live tissues, but published antisera recognize endogenous Sav (Yue, et al., 2012). When we co-stained Mer and Sav in the wing imaginal disc, we found that these proteins also co-localized both junctionally and medially at the apical ends of imaginal epithelial cells (Figure 1F—1F″).

To further define the specific apical domain at which these components localize, we co-stained for Merlin and three apically-localized markers - Cad99C, a marker for apical microvilli (Glowinski, et al. 2014), Squash-mCherry, which localizes to the medial and junctional apical cortex, and Rab5-CFP, an early endosomal component. Serial optical sectioning of co-stained tissues revealed that the plane of maximal Merlin staining intensity coincides well with that of Cad99C (Figure S1A) and Squash (Figure S1C), but is apical to the maximal plane of Rab5-CFP (Figure S1B). These results indicate that Merlin, and by association Kib and Sav, localize to the apical cortex, immediately adjacent to the apical

membrane. Thus, our analysis reveals that three important Hippo pathway regulators, Kib, Mer and Sav, localize to the medial apical cortex, a previously unrecognized subcellular localization for pathway components (Figure 1G).

Kib and Mer work together to promote accumulation of Sav

The co-localization of Mer, Kib and Sav suggests the possibility that these proteins function together within the medial apical domain. Because Sav is recruited to the junctions through interactions with the cytoplasmic tail of Ed (Yue, et al., 2012), we first asked if a separate mechanism involving Kib and Mer recruits Sav medially. Consistent with this idea, depletion of both Mer and Kib by RNAi resulted in a strong reduction in medial Sav, while junctional Sav was largely unaffected (Figures 2A—2A″). In contrast, loss of either Mer or Kib alone has no detectable effect on Sav antibody staining (Figure S3A–3A‴ and S3B–S3B‴). These results suggest that medial Sav is recruited by Kib and Mer, and supports the idea that the medial apical domain might serve as a site of Hippo pathway activation.

We next asked if ectopic expression of Kib, which promotes pathway activation, also affected the localization and level of Mer and Sav. Ectopic expression of *UAS-kib* at moderate levels in the posterior compartment under the *hedgehog-Gal4* (*hhGal4*) driver results in modest pathway activation and an undergrowth phenotype in the adult wing (Baumgartner, et al., 2010; Yu, et al., 2010; data not shown). In the wing imaginal disc, ectopically expressed Kib accumulated apically in punctae both medially and junctionally (Figures 2B and 2B″–2B‴), similar to endogenous Kib (Figures 1C and 1E′–1E″). In *UAS-kib* expressing cells, staining for endogenous Mer and Sav showed increased apical accumulation in a punctate pattern (Figures 2B—2B′ and 2C–2C′). Mer strongly colocalized with Kib (Figures 2B″–2B‴) and Sav (Figure 2C″–2C‴) apically, and Mer abundance was reduced basally (Figures S3C–S3C‴). Thus expression of Kib alone was sufficient to recruit both Mer and Sav to the apical domain. In contrast, ectopically expressed Mer or Sav does not promote accumulation of the other two proteins (data not shown).

The results presented so far suggest that Kib has a unique function in organizing pathway members at the apical cell cortex. To investigate the functional relationships between Kib, Mer and Sav, we examined the effect of removing Mer or Sav either by RNAi or in mutant clones in the presence of ectopically expressed Kib. We found that depleting Sav by RNAi does not reduce Kib-induced Mer accumulation, suggesting that Kib recruits Mer independently of Sav (Figures 2D, S3D–S3D″ and S3F–S3F″). In contrast, in Mer depleted cells, Sav accumulation was significantly reduced but still modestly higher than in wild-type cells, suggesting that Mer promotes the ability of Kib to recruit Sav (Figures 2E, S3E–S3E″ and S3G–S3G″). This result is consistent with a previous study showing that loss of Mer suppresses the effect of ectopic Kib expression on growth in the eye (Baumgartner, et al., 2010) and the wing (data not shown).

Mer and Kib promote apical accumulation and activation of Hpo in a Sav-dependent manner

The data presented suggest the possibility of a second site of Hippo pathway activation in the medial apical domain that is regulated by Kib, Mer and Sav. If so, then we predict that

the downstream kinases, in particular Hpo and Wts, should also be recruited medially. To investigate the potential involvement of Hpo, we generated a YFP-tagged, *hpo* transgene expressed under its endogenous promoter and polyclonal anti-Hpo antisera. Consistent with a previous report using antibody staining (Sun, et al., 2015), Hpo-YFP showed a mostly cytoplasmic localization with weak accumulation across the apical cortex (Figure S4A). In live wing imaginal cells that ectopically expressed Kib, Hpo-YFP accumulated apically (Figure 3A—3A″) in a punctate pattern similar to that we observed for Mer and Sav (Figures 2C—2C‴). In fixed tissues stained for Hpo, Kib and E-cad, we found that Kib and Hpo strongly co-localized at both medially and junctionally (Figure 3B—3B‴). In addition, staining with anti-phospho Mst1/2 showed that the accumulated Hpo was active (Figure 3C—3C‴).

To examine how Hpo-YFP is recruited into Kib punctae, we used RNAi to deplete other pathway components in live wing epithelial cells and examined the effects on Kib-promoted Hpo-YFP accumulation. The efficiency of the RNAi lines was confirmed by testing their ability to deplete endogenously expressed proteins (Figures S4B, S4C and S2B). Consistent with Sav's known role to bind Hpo directly (Wu, et al., 2003), depletion of Sav by RNAi abolished Kib-induced Hpo-YFP accumulation (Figures 3D and S5A–S5A″). In addition, we found that depletion of Mer also significantly reduced Hpo recruitment by Kib, assayed by either Hpo-YFP in live tissues (Figures 3D and S5B–S5B″) or anti-Hpo staining (Figures S5D–S5D‴). This finding is consistent with a previous study showing that Mer, Sav and Hpo form a complex in S2 cells (Reddy and Irvine, 2011; Yu, et al., 2010). To rule out the possibility that the residual Hpo accumulation is due to incomplete removal of Mer by RNAi, Mer^4 null mutant clones were generated in the background of *nub-Gal4*-driven ectopic expression of *UAS-kib* in the wing pouch region. Consistent with the RNAi experiments, apical Hpo-YFP was reduced but still modestly accumulated in Mer^4 mutant cells that ectopically expressed Kib (Figure S5E–S5E′). Thus Mer promotes, but is not required for Sav's ability to recruit Hpo in concert with Kib. This observation is consistent with previous genetic studies showing that while loss of *Mer* suppresses the effects ectopic Kib expression with respect to growth in the eye, the *Mer; kib* double mutant phenotype is stronger than either single mutant alone (Baumgartner, et al., 2010; Yu, et al., 2010).

To ensure that the ability of Kib and Mer to assemble pathway components is not dependent on ectopic expression, we used RNAi to deplete these proteins and examined endogenously expressed Hpo-YFP in the wing epithelium, which as indicated previously is diffusely enriched in the apical domain and does not display pronounced medial or junctional accumulation (Figure S4A). As expected, removal of Sav severely reduced Hpo-YFP apically (Figure 3E, 3G—3G′). Additionally, simultaneous depletion of both Kib and Mer also significantly reduced Hpo-YFP levels in the apical domain (Figures 3E, 3F—3F″). Taken together, these gain and loss of function results indicate that Kib and Mer work together to promote apical accumulation and activation of Hpo in a Sav-dependent manner.

Mer, Kib and Sav function independently of Ex

Previous studies have suggested that Mer, Kib and Ex function as a complex (Enderle and McNeill, 2013; Genevet, et al., 2010; Yu, et al., 2010), and Ex has been proposed to be a key

upstream regulator of Hippo pathway activation (Sun, et al., 2015). Therefore, we next asked if ectopic Kib expression had a similar effect on Ex as we observed for Mer. In marked contrast to Mer and Sav, we did not observe recruitment of Ex to the medial apical domain by ectopic Kib expression. Indeed, neither the abundance nor localization of endogenous Ex was noticeably altered (Figures 4A—4A‴; Figure S6A–6A″). This suggests that Kib assembles Mer and Sav independently of Ex, though we did note that Kib at the junctions colocalized with Ex (Figure 4A‴).

To confirm that Kib can assemble other upstream components medially independently of Ex, and to explore the relationship between Ex and Kib at the junctions, we ectopically expressed Kib in *ex* null clones using mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999). Kib apical localization, either medially or junctionally, was unchanged in the absence of Ex (Figures 4B—4B‴). Furthermore, Kib-induced apical accumulation of Mer and Sav was unaffected by loss of Ex (Figures 4C—4C‴). We next asked if Ex is required for accumulation and activation of Hpo in cells that ectopically express Kib. Ex depletion by RNAi did not significantly affect either apical Hpo level as assayed by Hpo-YFP or anti-Hpo (Figures 3D, 4D—4D‴ and S5C–S5C″) or anti-phospho Mst1/2 staining (Figure S6B–S6B″). Taken together, these results indicate that Kib promotes assembly and activation of Hippo pathway components medially, independent of and spatially distinct from Ex.

To further examine functional interactions between *kib*, *Mer,* and *ex*, we tested the effects of ectopic *kib* expression on growth in normal or *ex* deficient tissues. We found that *UAS-kib* expression in the wing suppressed the overgrowth phenotype due to *ex* depletion to near wild-type (Figures 4E–4F and S6C–S6E). This result is consistent with our molecular observation that Kib can promote accumulation and activation of Hpo in wing epithelial cells in the absence of Ex.

Kib recruits Wts in a Mer-dependent manner

If Kib organizes Mer, Sav and Hpo medially into sites of Hippo signaling, then we expect that Wts, which acts downstream of Hpo, should also be recruited there. In imaginal epithelial cells, most endogenous Wts is tethered by Jub at the adherens junction, spatially segregated from other pathway components (Sun, et al., 2015; Rauskolb, et al., 2014). To maximize our ability to detect whether Wts is recruited medially by Kib, we examined a GFP-tagged version of Wts expressed under its endogenous promoter (Rauskolb, et al., 2014) in the background of *UAS-kib* driven by *hhGal4*. Using live imaging of the wing imaginal disc, we observed that Wts accumulated medially in Kib expressing cells (Figures 5A—5A′).

Previous studies have shown that both Ex and Mer are capable of recruiting Wts to the cell cortex (Sun, et al., 2015; Yin, et al., 2013). To determine the role of these proteins in the medial apical domain, we depleted each protein using *UAS-RNAi* transgenes in the background of *UAS-kib* and *hhGal4*. While depletion of Mer prevented Wts accumulation medially, depletion of Ex had no observable effect (Figures 5B—5B′ and 5C–5C′). Neither manipulation affected junctional Wts.

Crumbs sequesters Kibra junctionally to repress its function in growth control

To this point, we have focused on Kibra function in the medial apical domain. However, in living tissues we observed a significant fraction of Kib localized to the junctions, raising the questions 1) how does Kib localize there, and 2) does it function there? Previous biochemical studies have shown that Kib interacts with both Mer and Ex (Genevet, et al., 2010; Yu, et al., 2010), so we asked whether loss of either gene affects localization of endogenously expressed Kib:GFP in living tissues. Although we found that Kib is up-regulated in both *Mer* and *ex* mutant clones, Kib localization did not change noticeably in either mutant background (Figures S7A–S7B). Additionally, simultaneous depletion of both Mer and Ex had no effect on Kib:GFP localization (data not shown), suggesting that another mechanism is responsible for Kib's junctional localization.

The transmembrane protein Crb is known to function upstream in Hippo signaling, at least in part by regulating stability and localization of Ex (Zhang, et al., 2015; Ribeiro, et al., 2014; Chen, et al., 2010; Ling, et al., 2010; Robinson, et al., 2010) (Figures S7C and S7E–S7E′). Crb is also strongly junctionally localized, so we wondered if Crb might promote Kib junctional localization. Depletion of Crb by RNAi severely disrupted the junctional localization of Kib:GFP in wing epithelium, resulting in accumulation of Kib punctae primarily at the AMR (Figures 6A—6A′, 6B–6B″). Basal Kib levels were unaffected (Figure S7D), suggesting Kib was retained at the apical cortex. In contrast, Crb depletion results in basal mislocalization of Ex (Chen, et al., 2010; Ling, et al., 2010) (Figures S7D–S7D′).

The Crb intracellular domain has two short motifs, the juxtamembrane FERM-binding motif (FBM) and the C-terminal PDZ-binding motif (PBM). Ex binds the FBM and is mislocalized basally in mitotic clones homozygous for a *crb* allele in which the FBM is deleted (Ling, et al., 2010). Using MARCM, we ectopically expressed Kib in either *crb* null or $crb^{\Delta FBM}$ mutant cells. Ectopically expressed Kib was strongly medially localized in *crb* null clones (Figures 6C—6C‴ and 6D–6D‴), similar to our observations for endogenous Kib:GFP. In contrast, Kib localization was unchanged compared to controls in $crb^{\Delta FBM}$ cells (Figures 6C—6C‴ and 6E–6E‴). Thus junctional localization of Kib is dependent upon Crb, but does not require the FERM binding domain of Crb. We could not ask if the PDZ-binding motif is needed for proper Kib localization because Crb lacking this domain did not localize junctionally (Figure S7F–S7F′ and S7G–S7G′).

If Kib functions at the medial apical domain, then we predict that loss of Crb, which causes strong medial localization of Kib, should promote Kib-dependent pathway activity. To test this, we compared the effect of ectopic Kib expression alone to its effect when Crb was simultaneously depleted by RNAi. Loss of Crb alone results in a modest overgrowth phenotype in the wing, while ectopic expression of either Kib or Ex causes a mild undergrowth phenotype (Figures 7A–7B). These observations suggest that all three proteins function synergistically with respect to growth. Consistent with the idea that Crb promotes Ex function, Crb depletion strongly suppressed the undergrowth phenotype caused by ectopic Ex expression (Figures 7A–7B). In marked contrast, Crb depletion enhanced the undergrowth phenotype caused by ectopic Kib expression (Figures 7A–7B), indicating that *crb* genetically antagonizes *kib* function. To ask if these effects on growth are due to Hippo

pathway activation, we generated a YFP-tagged version of Yki expressed under its endogenous promoter (Yki-YFP) and used it as a biosensor for pathway activity. Depletion of Wts resulted in strong nuclear re-localization of Yki-YFP (Figure S7H–S7H″), demonstrating that Yki-YFP is a functional biosensor for pathway activity. Although ectopic Kib expression alone did not have a detectable effect on Yki subcellular localization, depletion of Crb by RNAi caused Yki-YFP to become more nuclear. In contrast, simultaneous depletion of Crb by RNAi and ectopic Kib expression resulted in decreased Yki nuclear accumulation (Figure 7C). Taken together, our observations are consistent with the idea that Kib promotes Hippo signaling medially, and that Crb represses Kib activity by sequestering it at the junctions. We speculate that in loss of Crb alone, increased Kib activity is counterbalanced by decreased Ex function, with the net result being an overall slightly overgrown phenotype.

DISCUSSION

To address the question of how the upstream Hippo pathway components Kib, Mer and Ex regulate pathway activity, we examined their functional interactions in the imaginal epithelium using endogenously expressed, tagged proteins in both fixed and living tissues. Our results identify a cellular domain for Hippo pathway activation (Figure 7D), the non-junctional, medial region of the apical cell cortex. We also show that Kib, together with Mer and Sav, function at this site to organize the core kinases Hpo and Wts to promote pathway activity. Hippo signaling at the medial apical domain occurs independently of Ex, which localizes at the junctional cortex together with Crb. Additionally, we have discovered that Kib's ability to promote pathway activity is repressed by Crb. Together these studies reveal the existence of parallel, spatially distinct signaling mechanisms that together regulate Hippo pathway output and tissue growth. The discovery of these distinct signaling domains is consistent with and explains previous genetic studies showing that Mer and Kib function in parallel to Crb and Ex (McCartney et al., 2000; Yu et al., 2010; Ling et al., 2010).

A striking aspect of our findings is they reveal a novel role for Crb in regulating Hippo pathway activity. Previous studies have shown two opposing Crb functions with respect to Ex and tissue growth: 1) Crb promotes Ex function by recruiting Ex to the junctional domain where it can assemble and activate Hippo pathway components, and 2) Crb represses Ex function by promoting its ubiquitination and degradation (Zhang, et al., 2015; Ribeiro, et al., 2014; Chen, et al., 2010; Ling, et al., 2010; Robinson, et al., 2010). Surprisingly, our results indicate that Crb also promotes growth by repressing Kib function, possibly by sequestering it away from the medial apical domain. These results highlight Crb as a key regulator of pathway output that may serve as a nexus for crosstalk between Kib- and Ex-mediated parallel upstream inputs, and raise the intriguing question of how these different functions of Crb might be regulated. They also raise the question of how Kib activity is regulated by Crb at the junctional domain. Previous studies have demonstrated molecular interactions between Crb and Ex (Ling et al. 2010), and between Ex, Kib and Mer (Yu et al. 2010; Genevet et al. 2010; Baumgartner et al. 2010), suggesting that Crb might regulate Kib through Ex. However, our observations that depletion of Ex suppresses the *UAS-kib* phenotype while depletion of Crb enhances *UAS-kib* argues strongly that the functional interaction between Crb and Kib is not mediated by Ex or other downstream components.

Our findings also highlight the question of why there are two mechanisms, one mediated by Ex at the junctions and a second organized medially by Kib and Mer, for controlling Hippo pathway output. In some cases these apparently parallel inputs might reflect tissue specific differences (Reddy and Irvine, 2011; Milton, et al., 2010). However, in the wing imaginal epithelium, a tissue in which Hippo signaling has been extensively characterized, both mechanisms clearly operate in parallel in the same cells. At the moment, we understand very little about the external cues that regulate key upstream pathway components such as Kib and Ex, although recent studies on Hippo signaling have revealed the importance of tension in regulating pathway output. In Drosophila, previous studies have demonstrated tension-sensing mechanisms localized to the junctional domain that regulate Hippo pathway activity (Leckband and de Rooij, 2014; Rauskolb, et al., 2014). Junctional mechanisms are ideally positioned to detect forces exerted across fields of cells in tissues. However, recent studies have shown that intracellular contractile forces, such as those that mediate apical constriction during tissue morphogenesis, are generated by networks of actomyosin contractile bundles that localize within the medial apical cortex (Levayer, et al., 2011; Martin, et al., 2009). One intriguing possibility raised by our discovery that Kib and Mer function in the same apical domain is that their output is sensitive to intracellularly generated cortical tension. The ability to sense tension in individual cells during tissue morphogenesis – such as the formation of epithelial folds – could be critical to coordinate cell shape changes and cell division during development.

STAR Methods Text

CONTACT FOR REAGENT AND RESOURCE SHARING

Please contact R.G.F. (rfehon@uchicago.edu) for reagents and resources generated in this study.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila melanogaster was cultured using standard techniques at 25°C (unless otherwise noted). Both male and female animals were used, except that females were used for studies in which wing size was measured.

METHOD DETAILS

Drosophila Genetics—For expression of UAS lines, the following drivers were used: *hh-Gal4, ap-Gal4, and nub-Gal4.* The UAS lines used in this study are listed in Key Resources table.

To generate endogenously expressed, fluorescent protein forms of Mer, Ex and Hpo, YFP tags were inserted into genomic BAC clones encompassing the entire *Mer, ex and Hpo* genes using recombineering (details provided upon request). Transgenes of these tagged genomic constructs were then generated at the VK37 (22A3, chromosome 2L) and VK1 (59D3, chromosome 2R) landing sites using phiC31 integration (Ludwig, et al., 2011). Each transgene rescues the lethality of corresponding null alleles, indicating that the tags do not interfere with normal function (data not shown). C-terminally GFP-tagged Kibra was generated using CRISPR/Cas9 genome editing. A plasmid including a 993 bp left homology

arm fused in-frame to GFP and followed by a 1001 bp right homology arm inserted into pBlueScript was used as the donor template. This plasmid was co-injected with a pU6-Bbs1-chiRNA based plasmid expressing the guide RNA (5′-TACCAGCACCAGTTGTAACTCC-3′) into vas-Cas9 embryos. The resulting allele, *kib:GFP,* is homozygous viable indicating it is fully functional.

To generate mutant clones or MARCM clones, the following genotypes were used:

Mer clones:

y w hsflp Ubi-RFP FRT19A/Mer^{4} FRT19A; kib:GFP/+

y w hsflp Ubi-RFP FRT19A/Mer^{4} FRT19A; nub-Gal4, Hpo-YFP/+; UAS-kib/+

ex clones:

ex^{e1} FRT40/Ubi-RFP FRT40; hsflp, Sb/kib:GFP

y w hsflp tub-Gal4 UAS-GFP/+/Y; ex^{e1} FRT40/tubGal80 FRT40; UAS-kib/+

sav clones:

y w hsflp/+/Y; nub-Gal4/UAS-kib; FRT 82B sav^{3}/FRT 82B Ubi-RFP

crb clones:

y w hsflp UAS-GFP/+/Y; UAS-kib/+; FRT 82B/FRT 82B tub-Gal80

y w hsflp UAS-GFP/+/Y; UAS-kib/+; FRT 82B crb^{1}/FRT 82B tub-Gal80

y w hsflp UAS-GFP/+/Y; UAS-kib/+; FRT 82B crb $^{\Delta FBM}$/FRT 82B tub-Gal80

Immunostaining and Imaging—Wing imaginal discs from late third instar larvae were fixed and stained as previously described (McCartney and Fehon, 1996). Primary antibodies, listed in Key Resources table, were diluted as follows: anti-Kib (1:2000), anti-Sav (1:2000), anti-Ex (1:5000) anti-Mer (1:10000), anti-E-cad (1:1000), anti-Crb (1:1000), anti-pMst1/2 (1:2000) and anti-Cad99c (1:1000). For anti-Crb staining, dissected imaginal discs were treated with methanol for 5 min following fixation. Secondary antibodies (diluted 1:1000) were from Jackson ImmunoResearch Laboratories. Immunostaining samples were imaged using either a Zeiss LSM 510 or LSM 880 confocal microscope and the images were analyzed with *Image J.*

Wing growth assessment—For assessment of wing growth, adult female wings were mounted in methyl salicylate and photographed with the same settings on a Zeiss Axioplan microscope using a Canon camera (EOS rebel T2i). Subsequent measurements of wing size and statistical analyses were processed using *Image J* and Graphpad Prism, respectively.

Live Imaging—For live imaging, freshly dissected wing discs from third instar larvae were mounted in a glass bottom petri dish (MatTeK Corporation, Part No.: P35G-1.5-14-C) and cultured in Schneider’s Drosophila Medium supplemented with 10% Fetal Bovine Serum. The mounted samples were immediately imaged on a Zeiss LSM 880 confocal microscope.

QUANTIFICATION AND STATISTICAL ANALYSIS

Image J was used to quantify mean fluorescence intensity in selected regions of the posterior and anterior compartment in Figures 2D–2E and 3D–3E, and *Graphpad Prism* was then used to perform statistical analysis. A nonparametric Mann Whitney test was used in Figures 2D–2E. A one-way ANOVA test was used in Figures 3D–3E, 4F and 7B. Statistical significance of results between compared groups was indicated as follows: **** ($p \leq 0.0001$), *** ($p \leq 0.001$), ns ($p > 0.05$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Kibra, Merlin and Salvador promote Hippo pathway activity from a non-junctional site.
- Kibra, Merlin and Salvador recruit Hippo and Warts independently of Expanded.
- Crumbs sequesters Kibra junctionally to repress its function in growth control.

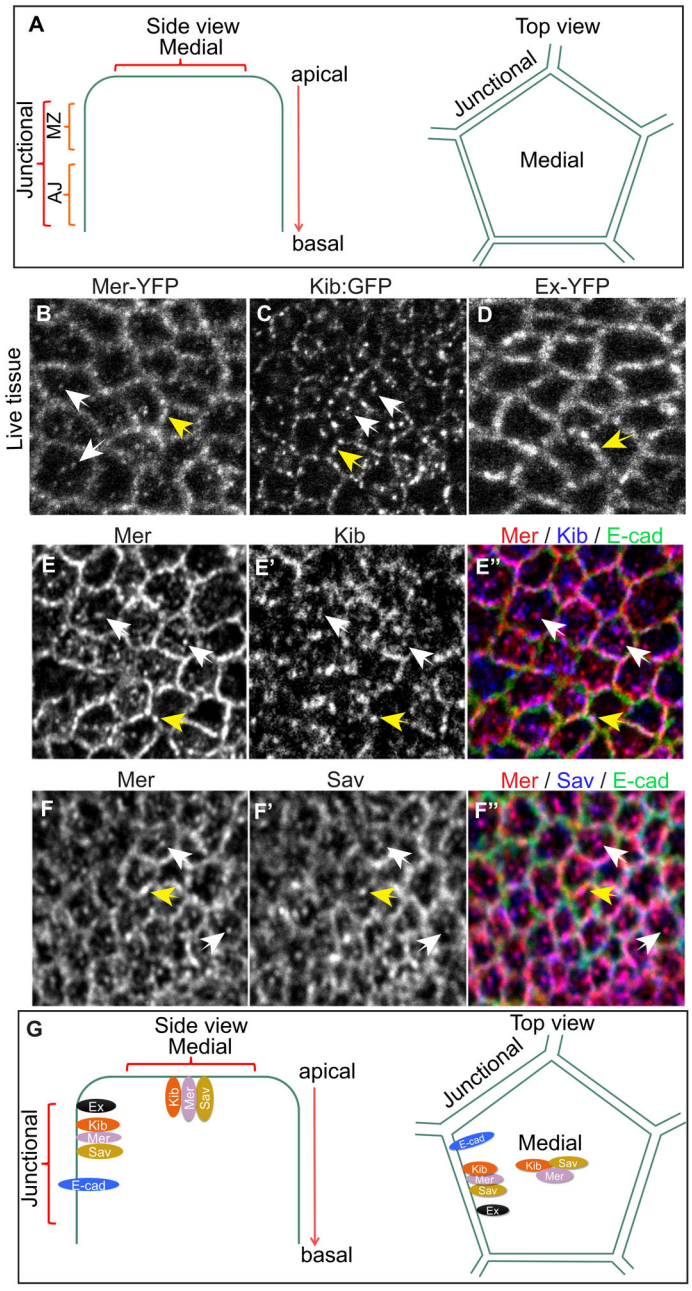

Figure 1. Mer and Kib accumulate at both the junctional and medial domains of wing imaginal epithelial cells, while Ex only localizes to junctions

(A) Cartoons showing side (cross section) and top (tangential section) views of a typical imaginal disc cell. The junctional domain is composed of the adherens junction (AJ) and marginal zone (MZ).

(B–D) Tangential sections through the apical domain of wing epithelial cells expressing Mer-YFP, Kib:GFP and Ex-YFP, respectively. Junctional accumulation is indicated by yellow arrows and medial accumulation by white arrows.

(E–E″) Co-staining of Mer and Kib in the wing epithelium. Mer and Kib show extensive co-localization junctionally (yellow arrows) and medially (white arrows).

(F–F″) Co-staining of Mer and Sav shows co-localization at the junctions and medially. (G) Cartoons summarizing the observed localizations of proteins examined in this figure. All panels are taken from the blade region of third-instar wing imaginal discs. See also Figure S1 and S2

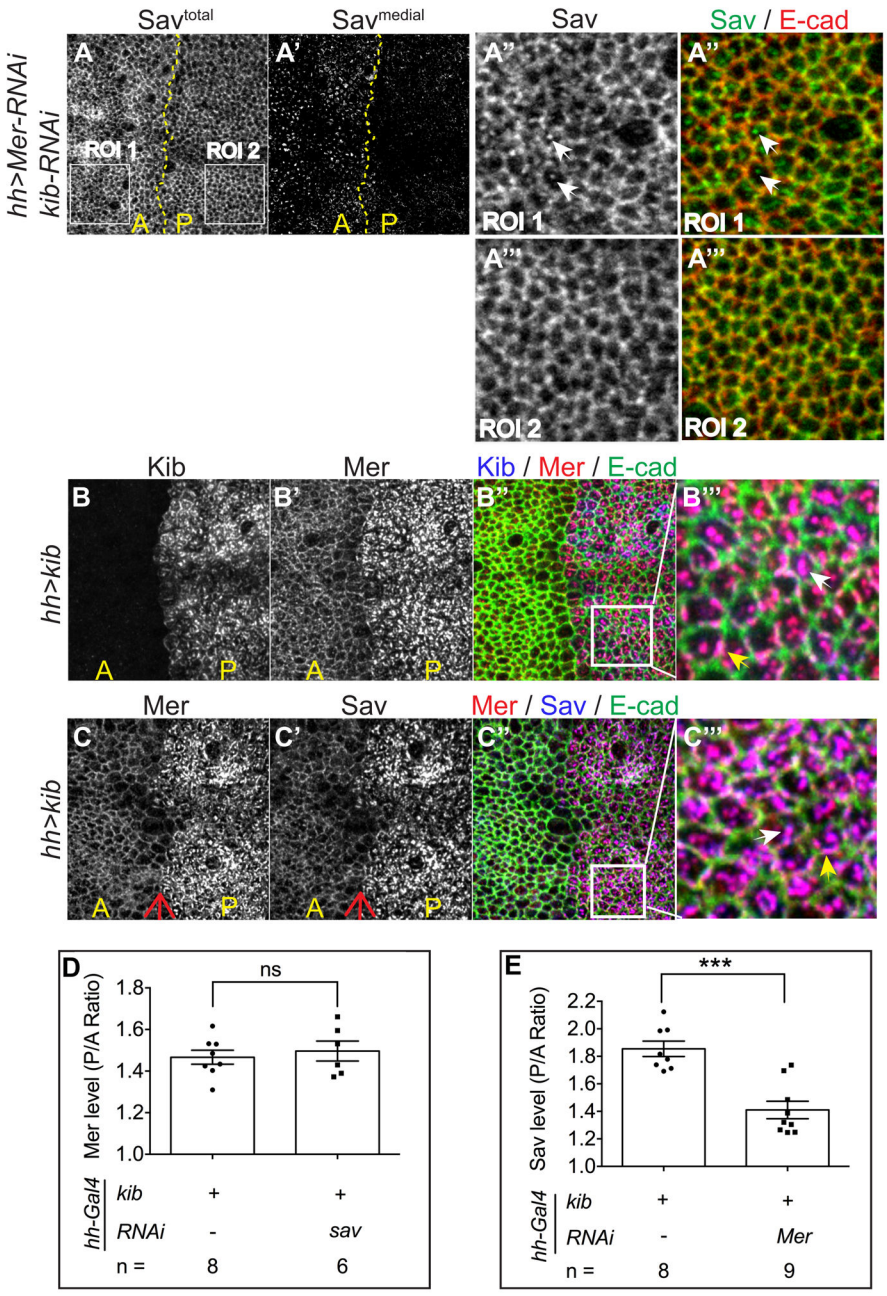

Figure 2. Kib and Mer work together to promote accumulation of Sav at the medial apical domain

(A–A‴) Sav localization in the wing epithelium expressing both *UAS-Mer-RNAi* and *UAS-kib-RNAi* in the posterior compartment under *hh-Gal4* (dashed line indicates anterior-posterior compartment boundary). Anti-E-cad staining (not shown) was used to mark the junctions and generate a binary mask to remove junctional Sav from the image (compare Sav^{total} in A to Sav^{medial} in A′). Note that while total Sav staining is not strongly affected by reduction of Mer and Kib, Sav^{medial} is dramatically reduced. High magnification insets of anterior (*wild-type*; ROI 1) in A″ and posterior (*Mer-RNAi, kib-RNAi*; ROI 2) in A‴ cells show loss of medial Sav in Kib/Mer depleted cells.

(B–C) Kib expression enhances Mer and Sav abundance apically. Co-staining of wing disc epithelial cells expressing *UAS-kib* in the posterior compartment under *hh-Gal4* with anti-Kib, anti-Mer and anti-Ecad (B-B‴), and with anti-Mer, anti-Sav and anti-Ecad (C–C‴). The composite images indicate co-localization of Kib and Mer (B′–B‴) or Mer and Sav (C′–C‴) at the junctions (yellow arrows) and medially (white arrows).

(D) Increased Mer staining promoted by expression of *UAS-kib* under *hh-Gal4* is not affected by Sav depletion by RNAi. Scatter plots show that the level of Mer staining between posterior (*hh-Gal4*) and anterior (*wild-type*) cells (P/A Ratio) is unchanged in discs expressing *UAS-kib* alone or *UAS-kib* and *UAS-sav-RNAi*. Representative images are shown in Figures 2C—2C′ and S1B–S1B′, respectively.

(E) Depletion of Mer reduces but does not eliminate Kib-induced recruitment of apical Sav. Representative images are shown in Figures 2C—2C′ and S1C–S1C′.

The number of samples for each group is indicated. Data are represented as mean ± SEM. Asterisks represent statistical significance of difference between selected groups (see STAR Methods). ns: not significant.

See also Figure S3.

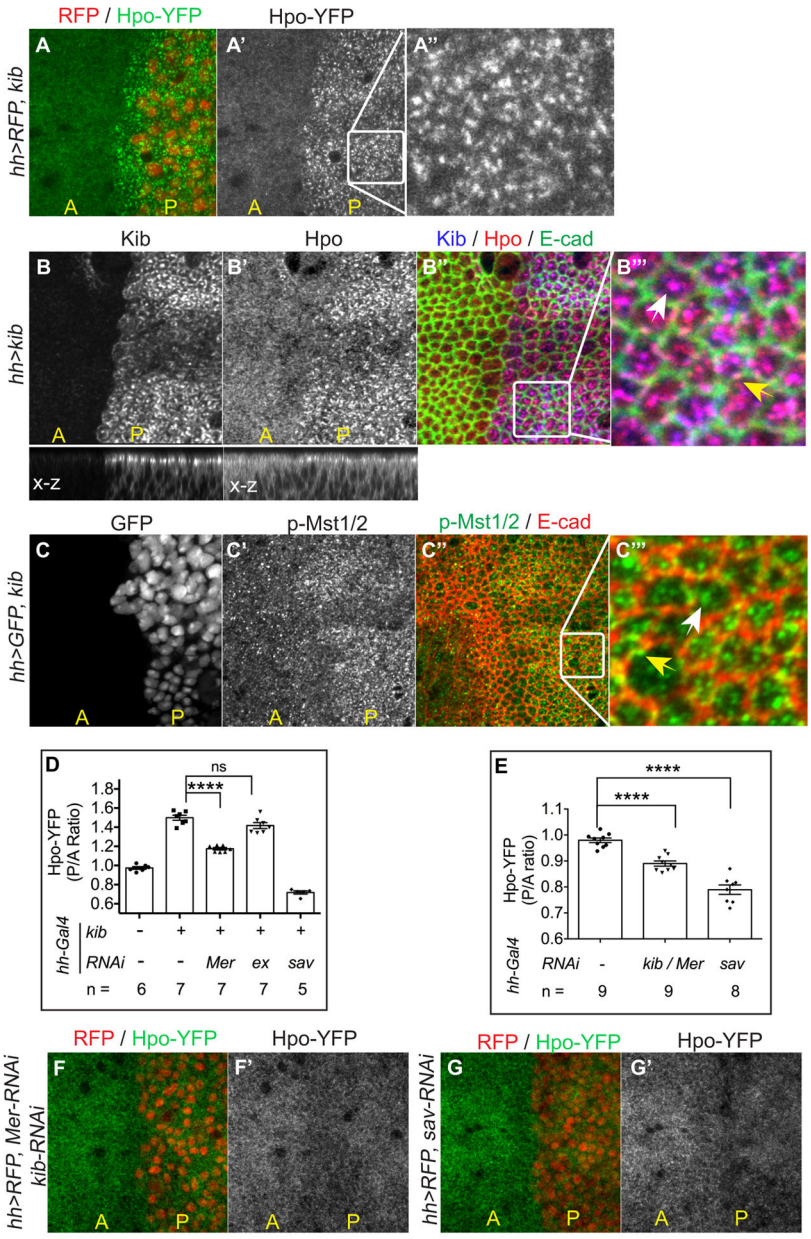

Figure 3. Kib, Mer and Sav together promote apical accumulation and activation of Hpo

(A–A″) Kib, expressed in the posterior compartment of a living wing disc, induces accumulation of Hpo-YFP apically. RFP expression, driven by *hh-Gal4*, marks the posterior compartment.

(B–B‴) Co-staining of anti-Hpo, anti-Kib and anti-E-cad in the wing epithelium expressing *UAS-kib* under *hh-Gal4*. There is strong co-localization between Hpo and Kib. Cross-section (X-Z) views of Kib and Hpo staining show apical accumulation (apical is up). Yellow and white arrows in the inset indicate junctional and medial localization, respectively.

(C–C‴) Hpo recruited by ectopically expressed Kib is active. Anti-phospho-Mst1/2 and anti-Ecad staining of the wing epithelium expressing *UAS-kib* under *hh-Gal4* shows strong accumulation of phospho-Hpo medially.

(D) Scatter plots (mean ± SEM) showing P/A ratio of apical Hpo-YFP in discs expressing RFP (control), or *UAS-kib* and RNAi transgenes for *Mer*, e*x* or *sav* as indicated. Although depletion of either Mer or Sav reduced Kib-induced accumulation of Hpo-YFP, depletion of Ex had no effect. Representative images of these genotypes are presented in Figures S5.

(E–G) Depletion of Kib and Mer reduces the level of apically localized Hpo-YFP in living tissues. Scatter plots show the P/A ratio of apical Hpo-YFP abundance in discs of the indicated genotypes (E). Data are represented as mean ± SEM. Representative images are shown in F–G′. Hpo-YFP abundance was reduced in the posterior compartment, marked by the expression of RFP, either by simultaneous depletion of Kib and Mer (F–F′) or depletion of Sav alone (G–G′).

See also Figure S4 and S5.

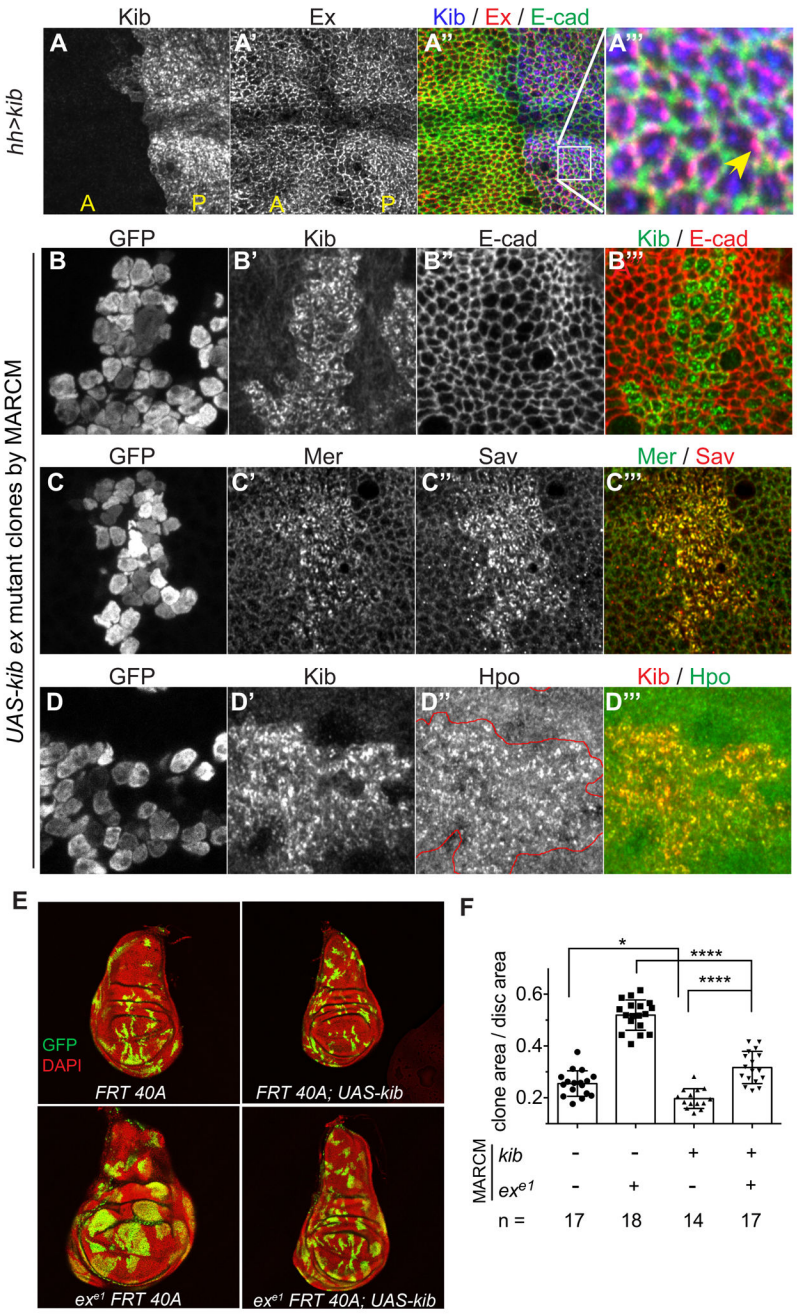

Figure 4. Kib induces Hippo signaling independently of Ex

(A–A‴) Kib expression does not obviously affect Ex abundance or localization. Co-staining of anti-Kib, anti-Ex and anti-Ecad in the wing epithelium expressing *UAS-kib* under *hh-Gal4*. High magnification inset shows that Kib and Ex co-localize at the junctions (yellow arrows). Note that there is no observable Ex staining medially.

(B–D) The organization of punctate structures containing Kib (B–B‴), Mer (C–C‴), Sav (C–C‴) and Hpo (D–D‴) is not affected by loss of Ex. *ex* mutant clones that expressed *UAS-GFP* and *UAS-kib* were induced in the wing using the MARCM technique. Compare

these images to those in Figures 2B (Kib and Mer), 2C (Sav) and 3B (Hpo) in which *UAS-kib* is expressed in a wild-type background.

(E–F) Kib and Ex have additive effects with respect to growth of the wing imaginal disc. MARCM clones (marked by the presence of GFP) of the indicated genotypes were induced using hsFlp expression (E). Ectopic Kib expression suppressed the overgrowth phenotype of *ex* null clones. Scatter plots show clone area (marked by GFP) relative to the entire wing imaginal disc (marked by DAPI) for the indicated clone genotypes (F). The number of samples for each group is indicated. Data are represented as mean ± SEM

See also Figure S6.

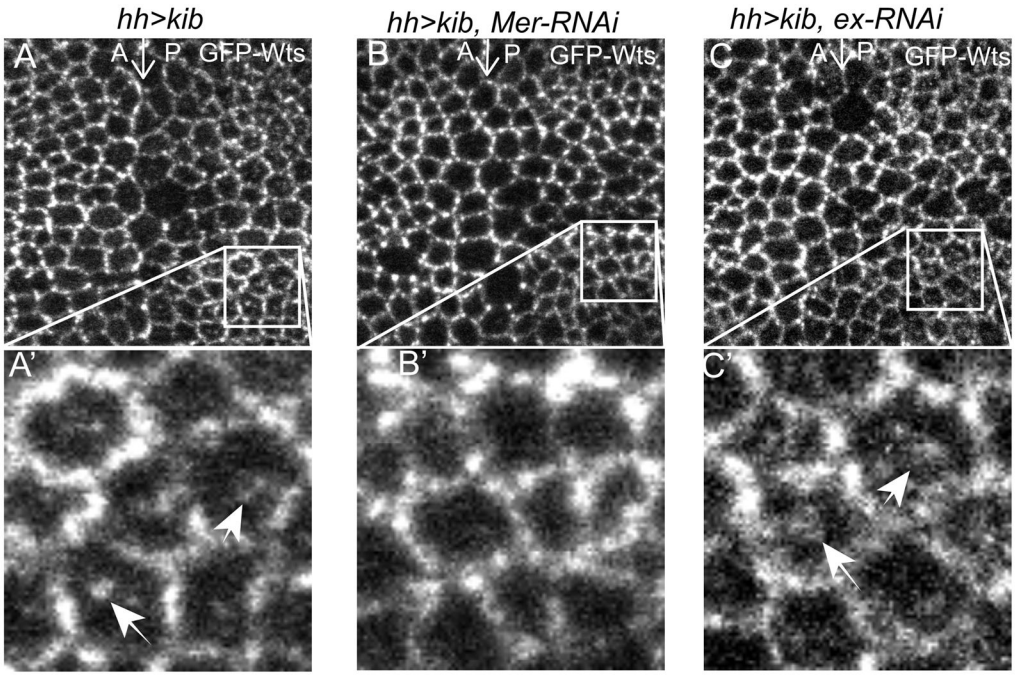

Figure 5. Kib recruits Wts to the medial domain in a Mer-dependent manner

(A–C) *GFP-wts* in wing discs expressing *UAS-kib* alone, or together with *UAS-Mer-RNAi* or *ex-RNAi* as indicated under the *hh-Gal4* driver. GFP-Wts accumulated medially (white arrows) with ectopic Kib expression (A–A′). While medial GFP-Wts accumulation was suppressed by depletion of Mer (B–B′), medial GFP-Wts accumulation was unaffected by Ex depletion (C–C′).

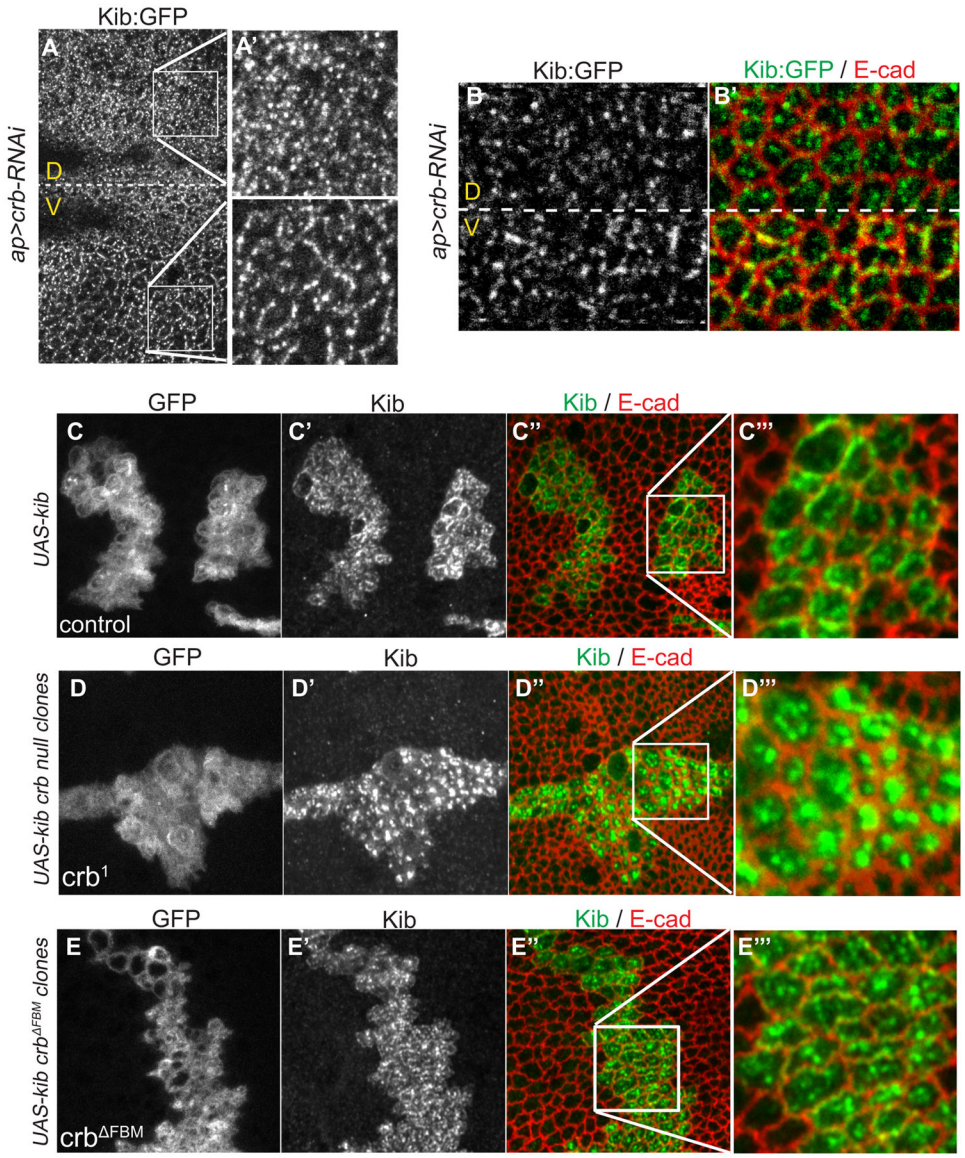

Figure 6. Crb tethers Kib junctionally

(A–B′) Crb is required to localize Kib at the junctions. In live wing discs, endogenously expressed Kib:GFP is predominantly junctional with some medial staining in wild type cells (ventral compartment, V). However, when Crb is depleted in dorsal (D) cells using *ap>crb-RNAi*, the junctional appearance of Kib:GFP is lost (A–A′). Co-staining with anti-Ecad shows that when Crb is depleted in the dorsal compartment, Kib:GFP becomes primarily medial (B–B′). The dashed white line marks the approximate D/V boundary.

(C–E‴) The FERM binding domain in Crb is not required for Kib junctional localization. Wing epithelial cells containing GFP-marked MARCM clones ectopically expressing Kib alone (C–C‴) (control), in a crb^{1} null clone (D–D‴), or a $crb^{\Delta FBM}$ clone (E–E‴).

See also Figure S7.

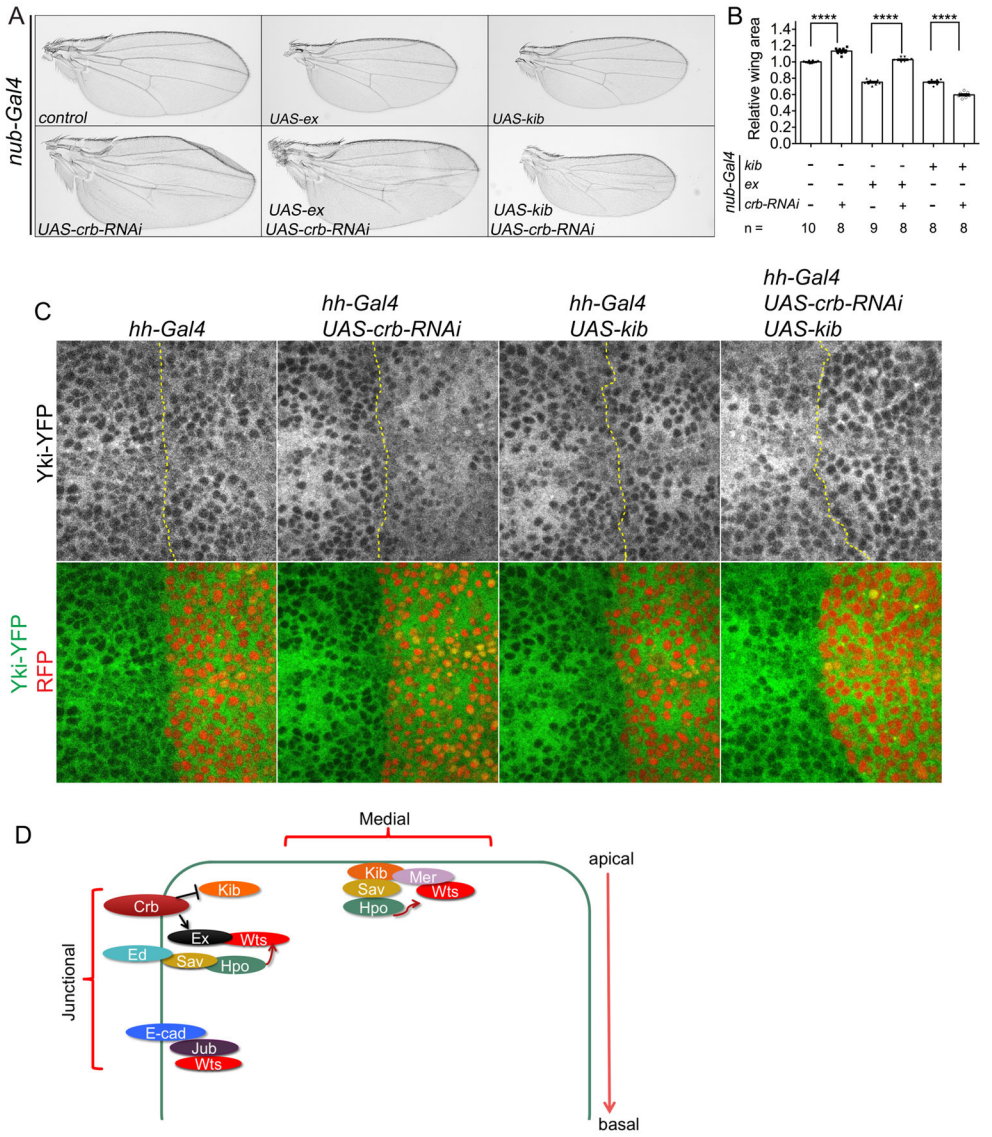

Figure 7. Crb represses Kib's function in growth control

(A–B) Crb promotes Ex function but antagonizes Kib. Adult wings from flies with the indicated *UAS* transgenes under the control of *nub-Gal4* driver (A). While depleting Crb by RNAi suppresses the undergrowth caused by ectopic Ex expression, it enhances the undergrowth phenotype of ectopic Kib expression. (B) Scatter plots quantifying wing area for the genotypes shown in A. Data are represented as mean ± SEM.

(C) A biosensor for Hippo pathway activity, endogenously expressed Yki-YFP, shows that Crb regulates Kib activity in growth control via Hippo pathway activity. As expected from its overgrowth phenotype, depletion of Crb results in greater nuclear localization of Yki-YFP, as evidenced by less obvious dark nuclear 'holes' in the posterior compartment where *hh-Gal4* is expressed. Kib expression alone has little or no detectable effect on Yorkie subcellular (nuclear) localization. In contrast, expressing Kib and simultaneously depleting of Crb results in decreased Yki-YFP nuclear accumulation, indicating increased upstream pathway activation.

(D) A working model for upstream activation of Hippo signaling. We propose that the Hippo signaling occurs at two different cortical sites. The first is organized by Ex at the marginal zone (MZ), as proposed previously (Sun et al., 2015). We demonstrate a second site at the medial apical domain where Kib, together with Mer and Sav, promote pathway activity independently of Ex. Crb represses Kib function by sequestering it to the junctions. The indicated protein associations and functional relationships are based on published literature and the results of this study.

See also Figure S7.