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The WW Domain Protein Kibra Acts Upstream of Hippo in *Drosophila*

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SUMMARY

The conserved Hippo kinase pathway plays a pivotal role in organ size control and tumor suppression by restricting proliferation and promoting apoptosis. Whereas the function of the core kinase cascade, consisting of the serine/threonine kinases Hippo and Warts, in phosphorylating and thereby inactivating the transcriptional coactivator Yorkie is well established, much less is known about the upstream events that regulate Hippo signaling activity. The FERM domain proteins Expanded and Merlin appear to represent two different signaling branches that feed into the Hippo pathway. Signaling by the atypical cadherin Fat may act via Expanded, but how Merlin is regulated has remained elusive. Here, we show that the WW domain protein Kibra is a Hippo signaling component upstream of Hippo and Merlin. Kibra acts synergistically with Expanded, and it physically interacts with Merlin. Thus, Kibra predominantly acts in the Merlin branch upstream of the core kinase cascade to regulate Hippo signaling.

INTRODUCTION

Understanding organ size control is a major task of developmental biology and affects our conception of tumorigenesis. The conserved Hippo tumor suppressor pathway has recently emerged as a crucial mechanism to restrict tissue growth by promoting cell cycle exit and apoptosis (Harvey and Tapon, 2007; Pan, 2007; Saucedo and Edgar, 2007). The core of the Hippo pathway is composed of two serine/threonine kinases, the Ste20-like kinase Hippo (Hpo) and the NDR family kinase Warts (Wts). Hpo phosphorylates and activates Wts (Harvey et al., 2003; Pantalacci et al., 2003; Tapon et al., 2002), which in turn phosphorylates and thereby inactivates the transcriptional coactivator Yorkie (Yki) (Huang et al., 2005; Oh and Irvine, 2008). Yki binds the transcription factor Scalloped (Sd) (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008) to regulate the expression of *Cyclin E* (*CycE*), *Diap1*, and *bantam*, thereby promoting cell proliferation and inhibiting apoptosis (Bandura and Edgar, 2008; Edgar, 2006; Hariharan and Bilder, 2006; Pan, 2007; Reddy and Irvine, 2008; Saucedo and Edgar, 2007).

Whereas the function of the core Hpo kinase cascade is well established, much less is known about the upstream events ensuring context-dependent regulation of Hippo signaling. Two membrane-associated FERM domain proteins, Merlin (Mer) and Expanded (Ex), have been suggested to function in parallel to activate the Hippo pathway (Hamaratoglu et al., 2006; Zeng and Hong, 2008). Mutations in *Mer* or *ex* result in milder tissue overgrowth as compared to mutations in Hippo signaling core components, but the *Mer ex* double mutant phenotype closely resembles the *hpo* loss-of-function phenotype (Hamaratoglu et al., 2006). The atypical cadherin Fat (Ft) and its ligand Dachsous (Ds) appear to signal through Ex to activate the Hippo pathway (Bennett and Harvey, 2006; Cho et al., 2006; Tyler and Baker, 2007; Willecke et al., 2006). Badouel and colleagues recently showed a physical interaction between Ex and Yki that may directly inhibit Yki activity (Badouel et al., 2009), and Oh and colleagues also identified Ex, Wts, and Hpo as Yki binding partners that mediate a phosphorylation-independent repression of Yki activity (Oh et al., 2009). By contrast, the molecular details of Mer function have remained elusive. Since FERM domain proteins serve as linkers between the cytoskeleton and transmembrane proteins and have been implicated in the signal transduction from the plasma membrane to cytosolic signaling complexes (Chishti et al., 1998), uncovering the signaling inputs of Mer may help to explain how cell-cell contact activates Hippo signaling to inhibit growth and restrict organ size (Zhao et al., 2007).

Here, we show that the WW domain protein Kibra is a Hippo signaling component. Kibra was originally identified as a binding partner of human Dendrin (Kremerskothen et al., 2003). It contains two amino-terminal WW domains and a C2-like domain. In mammals Kibra is predominantly expressed in kidney and brain, and it has mainly been associated with memory performance (Papassotiropoulos et al., 2006; Schaper et al., 2008). Apart from a carboxy-terminal extension, the *Drosophila* homolog of Kibra displays overall homology (51% similarity, 32% identity) to its human counterpart, and they share their domain structure. Our genetic analysis revealed that Kibra acts upstream of Hpo and Mer to control organ size in *Drosophila*. *Kibra* genetically synergizes with *ex*, and the Kibra protein physically interacts with Mer. We propose a model whereby Kibra functions in the Mer branch upstream of the Hippo core kinase cascade to regulate Hpo activity.

RESULTS

Kibra Regulates Growth and Restricts Organ Size by Controlling Cell Number

Overexpression of *Drosophila Kibra* in the developing eye decreased the size of the adult organ (Figure 1B) (Tseng and

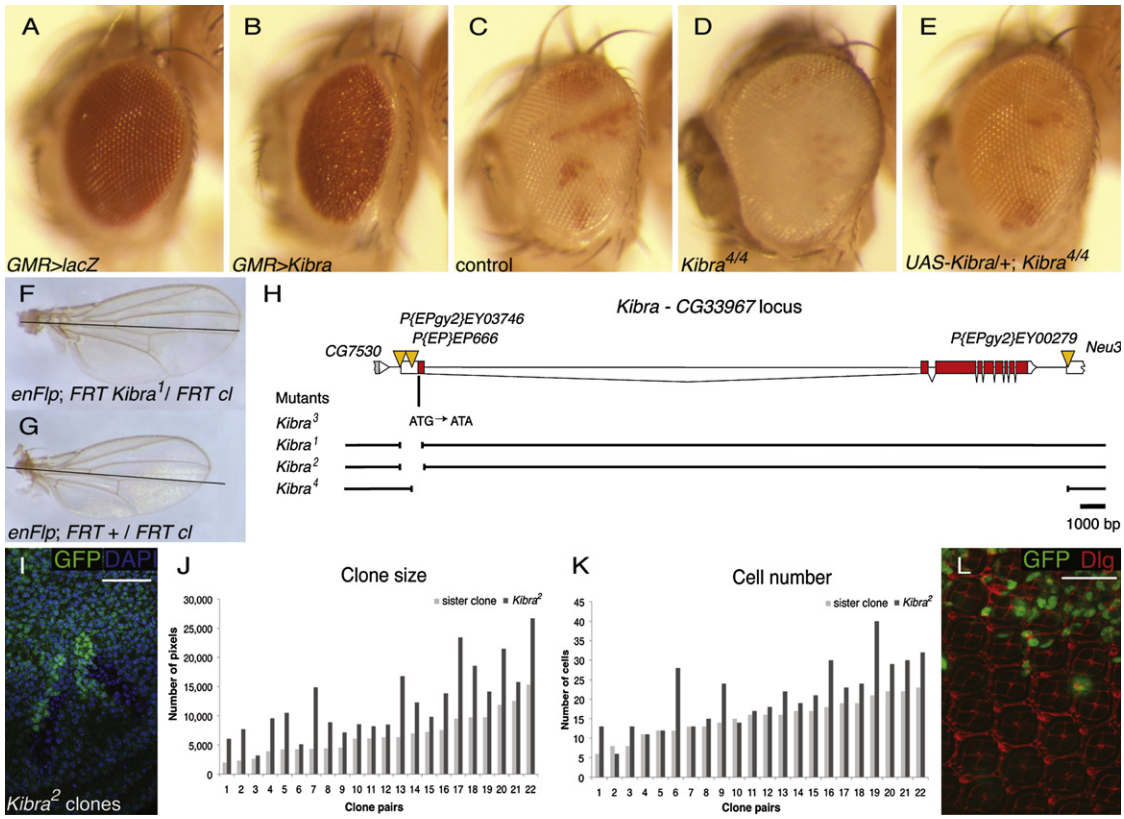

Figure 1. Kibra Regulates Tissue Size by Controlling Cell Number

(A and B) Eyes overexpressing the indicated *UAS* transgenes under the control of *GMR-Gal4*.

(C and D) Eye-specific reduction of *Kibra* function by eyFlp/FRT-mediated mitotic recombination (D) as compared to the control (C).

(E) Overgrowth of the *Kibra* mutant eye is rescued by the presence of a *UAS-Kibra* transgene.

(F and G) Compartment-specific reduction of *Kibra* function in the wing by enFlp/FRT-mediated mitotic recombination (F) as compared to the control (G).

(H) The *Kibra* locus (drawn to scale) spans 26 kb and contains nine exons (filled boxes). The initiating ATG is indicated. $Kibra^1$, $Kibra^2$, and $Kibra^4$ are deletions and the EMS allele $Kibra^3$ carries a point mutation in the Start codon.

(I) A part of a wing imaginal disc containing two clones of *Kibra* mutant cells (lacking GFP expression) and their twin clones is shown. The scale bar represents 50 μm.

(J and K) Statistical analyses of twin-spot clones (n = 22). *Kibra* mutant clones cover significantly larger areas (12,524 ± 6,083 pixels) than their sister clones (7,057 ± 3,750 pixels; p = 0.0000001) (J). *Kibra* mutant clones contain more cells (21 ± 9) than their sister clones (16 ± 7; p = 0.001) (K). Note that clones homozygous for a precise jump out allele are not enlarged (7,304 ± 3,015 pixels) when compared to their sister clones (7592 ± 2724 pixels; p = 0.590), and both contain the same number of cells (17 ± 7 and 19 ± 8; p = 0.329). Analyses were done with Student's t test (two-tailed).

(L) A part of a mid-pupal retina containing $Kibra^4$ mutant tissue (marked by the absence of GFP) and neighboring wild-type tissue (bright green) is shown. The tissue was stained with α-Discs large antibodies to visualize cell outlines (red). The scale bar represents 25 μm.

Hariharan, 2002). We generated four different loss-of-function alleles of *Kibra* to define its function in growth control (Figure 1H). Deletion of the first exon (harboring the translational start site) by imprecise excision of a P element resulted in the alleles $Kibra^1$ and $Kibra^2$. $Kibra^3$, a mutation in the initiating ATG, was generated by means of an EMS reversion mutagenesis of the EP-mediated *Kibra* overexpression phenotype. Finally, the entire *Kibra* locus was removed by the hybrid element insertion (HEI) technique (Parks et al., 2004) ($Kibra^4$). All alleles were lethal when homozygous and failed to complement each other but were complemented by the precise P element excision used as a control throughout this study. All mutants displayed the same growth phenotypes, and homozygous mutant animals died as first-instar larvae. We conclude that all *Kibra* alleles are genetically null.

Kibra mutant heads were enlarged in comparison to controls (Figures 1C and 1D). Similarly, wings containing posterior compartments largely mutant for *Kibra* were larger than control wings (Figures 1F and 1G). The presence of a *UAS-Kibra* overexpression construct, without any Gal4 driver, rescued the lethality of *Kibra* homozygous mutant flies as well as the size defects of *Kibra* mutant organs (data not shown and Figure 1E), proving that the growth alterations are caused by the loss of *Kibra* function. Thus, *Kibra* is a general regulator of growth that is required to restrict organ size.

To determine the cause of the *Kibra* mutant overgrowth phenotypes, we performed a clonal analysis in wing imaginal discs. Clones of *Kibra* mutant cells were larger than their corresponding wild-type sister clones (Figures 1I and 1J). The number of cells per clone was increased in *Kibra* mutant clones

compared to wild-type clones but not to the same extent as the clone size (Figure 1K). However, FACS analysis revealed that cell size was unchanged in *Kibra* mutant cells (data not shown), suggesting a change in cellular architecture in cells devoid of *Kibra* function. We conclude that *Kibra* mutant clones in the wing imaginal disc were enlarged because *Kibra* mutant cells exhibit a proliferative advantage over wild-type cells.

We also analyzed tangential sections of mosaic compound eyes consisting of *Kibra* mutant cells surrounded by heterozygous cells. The mutant ommatidia were normally structured and the different cell types properly differentiated, but the interommatidial regions were enlarged compared to the control (data not shown). The increased distance between mutant ommatidia was due to more cells, because clones of *Kibra* mutant cells in the pupal retina displayed an increase in the number of interommatidial cells (Figure 1L). Supernumerary interommatidial cells are a hallmark of inactivation of the Hippo pathway (Kango-Singh et al., 2002; Tapon et al., 2002). Whereas a complete loss of Hippo signaling causes a pronounced excess of interommatidial cells, a mild extra interommatidial cell phenotype is observed in mutants that reduce but do not abrogate Hippo signaling, such as *ex* or *Mer* (Hamaratoglu et al., 2006).

Overexpression of *Kibra* Causes Apoptosis

A reduction in Hippo signaling activity results in extra interommatidial cells because the developmental apoptosis in pupal retinae is largely eliminated (Harvey and Tapon, 2007). Conversely, overexpression of *hpo* or *ex* induces apoptosis in third instar eye discs (Pantalacci et al., 2003; Pellock et al., 2007). Overexpression of *Kibra* in clones in the wing imaginal disc reduced clone size (see Figures S1A–S1D available online). *Kibra*-overexpressing clones contained fewer cells than control clones (Figure S1E). To investigate whether overexpression of *Kibra* induces apoptosis, we generated *Kibra* overexpression clones in the third instar eye disc by using the Gene-Switch system (Rogulja and Irvine, 2005). Indeed, the *Kibra*-overexpressing clones located anterior to the morphogenetic furrow (MF) showed an increase in programmed cell death as judged by staining for cleaved Caspase-3 and TUNEL staining (Figures S1F and S1G; data not shown), suggesting that overexpression of *Kibra* induces inappropriate apoptosis of proliferating cells. Consistently, co-overexpression of *Diap1*, a direct Yorkie transcriptional target (Wu et al., 2008), partially rescued the small eye phenotype associated with *Kibra* overexpression (Figure S1M). Co-overexpression of *CycE*, another target of the Hippo pathway (Udan et al., 2003), also resulted in a partial rescue of the small eye (Figure S1N). The size of *Kibra*-overexpressing eyes was further restored by concomitant overexpression of *Diap1* and *CycE* (Figure S1O). These results suggest that the effects elicited by *Kibra* overexpression are at least partly due to a reduction in the expression of the Hippo pathway target genes *Diap1* and *CycE*.

Kibra Acts Genetically Upstream of *yki*, *hpo*, and *Mer* and Synergizes with *ex*

The striking similarities of the *Kibra*, *ex*, and *Mer* phenotypes prompted us to test genetically whether Kibra restricts tissue size via Hippo signaling. We started our interaction studies at the level of the transcriptional coactivator *yki*, which induces target genes promoting cell proliferation and cell survival and is inactivated by Hippo signaling. Three lines of evidence suggest that Kibra acts via inactivation of Yki. First, the coexpression of *Kibra* and *yki* during eye development suppressed the eye size reduction caused by *Kibra* and resulted in the same overgrowth phenotype as observed in eyes overexpressing *yki* alone (Figures 2A–2C). Second, the growth advantage of *Kibra* mutant cells was completely abolished by the concomitant loss of *yki* function (Figures 2D–2F). Third, a pupal lethal hypomorphic combination of *Kibra* alleles was rescued to viability by removal of a single copy of *yki* (Figures 2G and 2H).

To determine whether (and at which level) Kibra acts in the Hippo pathway to inactivate Yki, we performed a series of epistasis tests. We found that the loss-of-function phenotypes of *hpo*, *sav*, and *wts* were epistatic to the *Kibra* overexpression phenotype (Figures 2J–2L; Figures S2A–S2D), indicating that Kibra acts upstream of Hpo.

Next, we tested for interaction with the upstream components Ex and Mer. Overexpression of *ex* in a *Kibra* mutant background resulted in an intermediate phenotype (Figures S2E–S2G). Vice versa, overexpression of *Kibra* also yielded an additive effect in an *ex* mutant head (Figures S2H–S2J). Conversely, *Kibra* overexpression failed to reduce organ size in a *Mer* mutant head (Figures 2M and 2N), indicating that Kibra requires Mer to exert its function. We used the eyFlp/FRT recombination system (without cell lethal) to generate mosaic animals with heads largely homozygous for *ex* and *Mer* mutations, as well as *ex Kibra* and *Mer Kibra* double mutations, respectively. Both *ex* and *Mer* mosaic heads showed only mild overgrowth (Figures 2O and 2Q). Strikingly, pupae with mosaic heads doubly mutant for *ex* and *Kibra* did not eclose, and normal head structures were displaced by overgrown tissue (Figure 2P). In contrast, flies with *Mer Kibra* mosaic heads were viable. However, *Mer Kibra* double mutant clones showed stronger overgrowth than *Mer* clones (Figures 2Q and 2R). Reducing *ex* function during eye development by the expression of a hairpin RNAi construct (Dietzl et al., 2007) did not alter the wild-type eye size but resulted in a severe enhancement of the *Kibra* loss-of-function phenotype (Figures 2U and 2V), and the resulting eyes resembled those of *hpo* mutants. Reducing *Mer* function caused subtle overgrowth but enhanced the *Kibra* mutant phenotype much less (Figures 2W and 2X).

Whereas single mutants for *ex* and *Mer* cause a mild overgrowth phenotype, *ex Mer* double mutants display strong synergistic effects, suggesting that the two FERM domain proteins act in separate branches to activate Hippo signaling (Hamaratoglu et al., 2006; Pellock et al., 2007). Our findings suggest that Kibra acts primarily upstream of Mer. However, since *Mer Kibra* double mutant clones show stronger overgrowth than *Mer* mutant clones and a reduction of *Mer* function enhances the *Kibra* loss-of-function phenotype, Kibra also contributes to Mer-independent regulation of Yki activity.

Kibra Regulates a Minimal Hippo-Responsive Element

To confirm that Kibra acts via Hippo signaling, we also tested whether *Kibra* mutant clones upregulated the expression of a *Diap1* enhancer element (*diap1-GFP4.3*) that had been published to be a minimal Hippo responsive element (HRE; Zhang et al., 2008). A pronounced upregulation of *diap1-GFP4.3* was evident in clones of *hpo* mutant cells posterior and, to a weaker

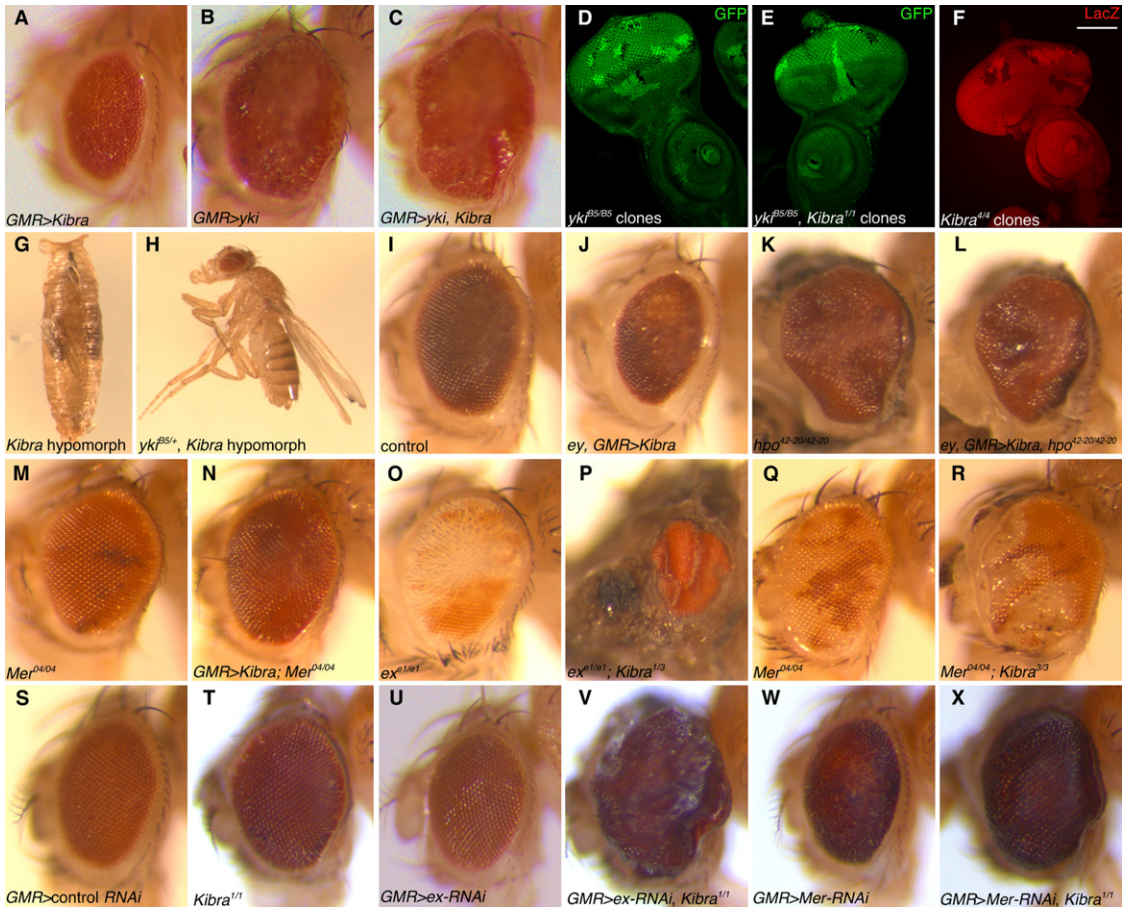

Figure 2. *Mer*, *hpo*, and *yki* are Epistatic to *Kibra*, and *ex* Synergizes with *Kibra*

(A–C) Eyes overexpressing the indicated *UAS* transgenes under the control of *GMR-Gal4*.

(D–F) Eye imaginal discs containing *yki* mutant clones (D), *yki Kibra* double mutant clones (E), and *Kibra* mutant clones ([F]; all black) and their wild-type sister clones (bright green or red, respectively). Quantification of clone sizes for *yki* (2,448 ± 1,075 pixels whereas the corresponding twin spots yield 7,563 ± 3,830 pixels; p = 0.0007) and *yki Kibra* (2,612 ± 1,688 pixels whereas the corresponding twin spots yield 10,195 ± 5,704 pixels; p = 0.0009) mutant clones revealed no significant difference (p = 0.825). Analyses were done with Student's t test (two-tailed). The scale bar represents 100 μm.

(G and H) The hypomorphic combination of *Kibra* alleles *Kibra*^{1}/ *P{PZ}l(3)neo42[02404]* results in pupal lethality (G) and is rescued to viability by removal of one copy of *yki* (H).

(I–L) Eyes mutant for *hpo* and overexpressing *Kibra* (L) are compared to control eyes (I), to eyes overexpressing *Kibra* (J), and to *hpo* mutant eyes (K).

(M and N) Eyes mutant for *Mer* and overexpressing *Kibra* (N) are compared to *Mer* mutant eyes (M).

(O–R) Eyes partially mutant (generated by eyFlp mediated mitotic recombination; without cell lethal) for *ex* (O), *ex Kibra* (P), *Mer* (Q), or *Mer Kibra* (R). The head shown in (P) is from a pharate adult.

(S–X) Eyes mutant for *Kibra* and overexpressing *UAS-ex-RNAi* or *UAS-Mer-RNAi* under the control of *GMR-Gal4* (V and X) are compared to control eyes (S), to *Kibra* mutant eyes (T), to eyes overexpressing *UAS-ex-RNAi* (U), and to eyes overexpressing *UAS-Mer-RNAi* (W).

extent, anterior to the MF in eye imaginal discs (Figure 3A). Cells lacking *Kibra* function also upregulated *diap1-GFP4.3* expression, although to a lesser degree and with restriction to the differentiating tissue posterior to the MF (Figure 3B). Clones of *ex* mutant cells, in resemblance to *hpo* clones, upregulated *diap1-GFP4.3* strongly behind and somewhat weaker before the MF (Figure 3C), whereas *Mer* mutant cells, like *Kibra* mutant cells, upregulated *diap1-GFP4.3* expression weakly and solely posterior to the MF (Figure 3D). Thus, the loss of *Kibra* results in an upregulation of a Hippo signaling reporter gene. The similar response of *diap1-GFP4.3* to loss of *Kibra* or *Mer* suggests that Kibra and Mer act in the same way on Hippo signaling to regulate the HRE.

ex Kibra double mutant clones showed strong upregulation of *diap1-GFP4.3* on both sides of the MF (Figure 3E). Note that both *ex* mutant clones as well as *ex Kibra* double mutant clones were homozygous for the enhancer trap insertion *ex*^{e1} *lacZ.* Clones in Figures 3C and 3E are therefore marked by the absence of cytoplasmic LacZ plus upregulation of nuclear LacZ, since Ex is induced upon Yki activity (Hamaratoglu et al., 2006). Although *Kibra* mutant cells in the eye disc did not regulate *ex*^{e1} *lacZ* (data not shown), upregulation of nuclear LacZ and, accordingly, Yki activity was much higher in the case of *ex Kibra* double mutant clones (Figure 3E) as compared to *ex* mutant clones (Figure 3C). Additionally, clones of *ex Kibra* double mutant cells were very large and invariantly adopted a rounded shape

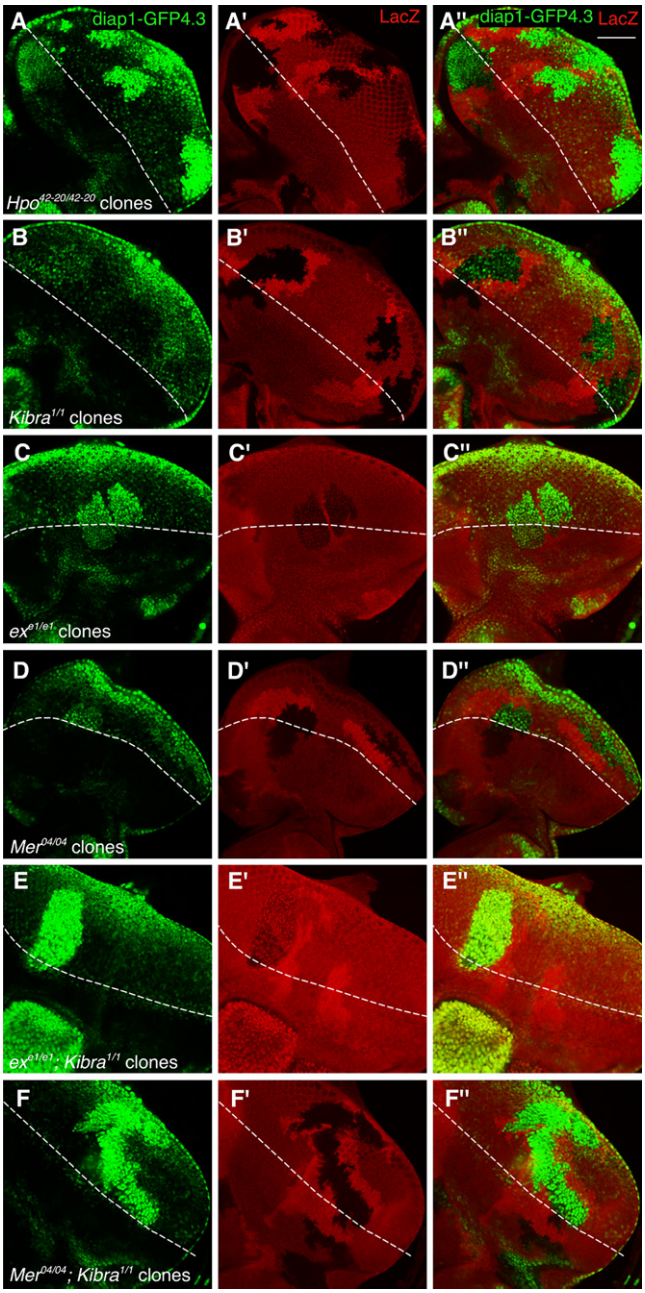

Figure 3. *Kibra*, *hpo*, *ex* and *Mer* Regulate a Minimal Hippo-Responsive Element

(A–F) Eye imaginal discs bearing *hpo* mutant clones (A–A″), *Kibra* mutant clones (B–B″), *ex* mutant clones (C–C″), *Mer* mutant clones (D–D″), *ex Kibra* double mutant clones (E–E″), or *Mer Kibra* double mutant clones (F–F″). The clones are marked by the absence of LacZ (red, [A′, B′, D′, and F′]) or by the absence of cytoplasmic LacZ (red, [C′ and E′]), respectively. Expression of GFP driven by the *Diap1* enhancer element *diap1-GFP4.3* is shown in green. Dotted lines indicate the MF. The scale bar represents 50 μm.

(Figure 3E), reminiscent of *yki*-overexpressing clones (Huang et al., 2005). These phenotypes suggest a high level of Yki activity in *ex Kibra* mutant cells. In contrast, *Mer Kibra* double mutant cells showed strong upregulation of *diap1-GFP4.3* posterior to the MF but none in the proliferative region anterior to the MF (Figure 3F). Thus, both *ex* and *Mer* act synergistically with *Kibra* to activate *diap1-GFP4.3* posterior to the MF. The additional synergism of *Kibra* and *ex*, but not *Mer*, anterior to the MF most likely explains the strong overgrowth phenotype of *ex Kibra* double mutant tissue as compared to the overgrowth of *Mer Kibra* tissue (Figures 2P, 2R, 2V, and 2X). We conclude that *Kibra* acts together with *Mer*, in parallel to *ex*, to regulate *diap1-GFP4.3* activity in both proliferating and postmitotic cells. In addition, *Kibra* contributes to Mer-independent regulation of Yki activity in differentiating cells.

One particularly interesting observation was that *Mer*, *Kibra*, and *Mer Kibra* mutant clones, but not *hpo* or *ex* mutant clones, seemed to exert a weak non-autonomous effect on *diap1-GFP4.3* activity of individual wild-type cells along clone borders (Figures S3A–S3D and S3F). In the case of *ex Kibra* double mutant clones, we even observed an upregulation of *diap1-GFP4.3* activity in an entire row of wild-type cells adjacent to the clone border (Figure S3E). It will be important to learn more about this non-autonomous regulation of a Hippo signaling reporter gene in order to gain mechanistic insight into *Kibra*-mediated regulation of Hippo target genes.

Kibra Binds Mer in *Drosophila* S2 Cells

Consistent with our genetic findings, Kibra has been identified in a yeast two-hybrid candidate screen as physical interactor of Mer (Formstecher et al., 2005). Coimmunoprecipitation experiments in *Drosophila* S2 cells revealed an interaction of the two proteins in both directions (Figures 4A and 4B). Interestingly, the binding was weak with full-length Kibra but stronger with a truncated version of Kibra lacking both N-terminal WW domains (ΔWW Kibra) (Figures 4A and 4C). Overexpressed Mer was localized at the cortex as well as in cytoplasmic punctae, whereas both overexpressed full-length Kibra and ΔWW Kibra were found exclusively in cytoplasmic punctae. When Mer was overexpressed together with full-length Kibra, we did not observe any colocalization (Figure 4D). In contrast, Mer colocalized weakly at the cortex and in a more pronounced way in cytoplasmic punctae with ΔWW Kibra (Figure 4D).

We also checked whether Kibra and Ex/Mer would affect each other's localization in vivo. Loss of *Kibra* did not affect the cortical localization of Mer or Ex (Figures S4A–S4E). Vice versa, the localization of Kibra at the apical cortex of epithelial cells, as seen in *Kibra*-overexpressing cells (Figures S4F–S4H) or in *hpo* mutant cells (Figures S4I–S4K) (Genevet et al., 2010 [this issue of *Developmental Cell*]), was not changed in *Mer ex* double mutant cells (Figures S4L–S4N). Further studies will be required to analyze the dynamics of the subcellular localization of the Hippo signaling upstream components.

DISCUSSION

Our study provides genetic and biochemical evidence that the WW domain protein Kibra is a Hippo signaling component. Several lines of evidence indicate that Kibra acts predominantly in the Mer branch (Figure 4E). First, the mild overgrowth phenotype caused by loss of *Kibra* function is akin to the *Mer* phenotype. Second, genetic epistasis experiments place *Kibra* upstream of *Mer*. Third, the effects of *Kibra* and *Mer* loss-of-function on a reporter for Hippo signaling activity are very similar.

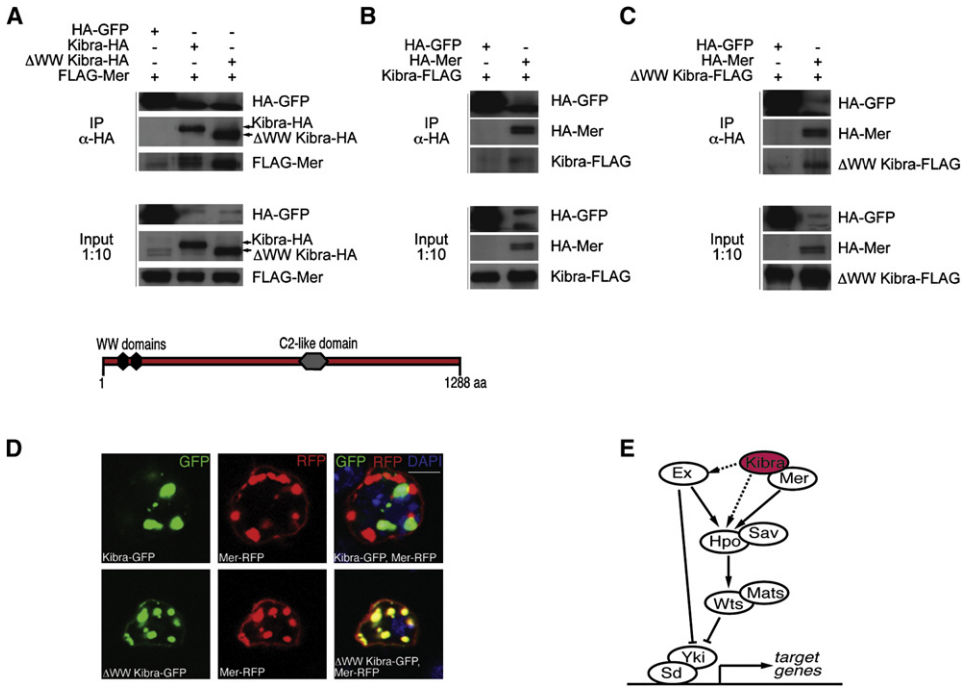

Figure 4. Kibra Is a Binding Partner of Mer in *Drosophila* S2 Cells

(A) Mer coimmunoprecipitates with both full-length Kibra and ΔWW Kibra in S2 cells. Kibra-HA (or ΔWW Kibra-HA) and Mer-FLAG were cotransfected in S2 cells. HA immunoprecipitates were blotted for Mer-FLAG. HA-GFP was used as a negative control. A schematic representation of the Kibra domain structure is shown below the blot.

(B and C) Both full-length Kibra (B) and ΔWW Kibra (C) coimmunoprecipitate with Mer in S2 cells. Kibra-FLAG (B) (or ΔWW Kibra-FLAG [C]) and Mer-HA were cotransfected in S2 cells. HA immunoprecipitates were blotted for Kibra-FLAG (or ΔWW Kibra-FLAG). HA-GFP was used as a negative control.

(D) S2 cells were cotransfected with Kibra-GFP and Mer-RFP (upper panel) or with ΔWW Kibra-GFP and Mer-RFP (lower panel). The scale bar represents 5 μm.

(E) Model of the Hippo signaling pathway.

Forth, *Kibra* and *Mer* synergise with *ex* in a similar fashion. Fifth, Kibra physically interacts with Mer. However, since our genetic analysis of *Kibra* also revealed a synergism with *Mer*, *Kibra* also acts on Yki activity in a *Mer*-independent manner.

FERM domain proteins, such as Mer, have been suggested to connect membrane proteins with the underlying cortical cytoskeleton in order to integrate signals from the membrane and initiate intracellular signaling cascades (McClatchey and Fehon, 2009). Thus, it is conceivable that Mer, together with as yet unknown proteins, assembles downstream cytoplasmic components of the Hippo pathway at the membrane and that controlled assembly and stabilization of such multiprotein complexes regulates the activity of the Hippo kinase cascade. In such a scenario, adaptor proteins providing multiple protein-protein interaction domains are of special interest.

The WW domain protein Kibra binds Mer and could enable signaling events at the membrane/cytoskeleton interface that activate the Hpo kinase cascade. Since a truncated Kibra protein lacking the WW domains interacts more fiercely with Mer, it is likely that the physical association of Kibra and Mer is modulated by binding of other factors to the WW domains of Kibra.

Interestingly, the effects caused by the concomitant loss of *ex* and *Kibra* functions are more severe than those elicited by mutated Hippo signaling core components. In addition to massively overgrowing, clones of *ex Kibra* double mutant cells round up, a behavior that we never observed in clones of *hpo* mutant cells. Furthermore, the *diap1-GFP4.3* reporter indicates higher Yki activity in proliferating *ex Kibra* mutant eye imaginal disc cells as compared to *hpo* mutant cells. It thus appears that Yki activity is unleashed in cells lacking both *ex* and *Kibra* functions. Since Ex has been shown to directly bind Yki (Badouel et al., 2009; Oh et al., 2009), it is tempting to speculate that Kibra participates in a distinct (Mer-independent) mechanism to prevent nuclear Yki localization.

EXPERIMENTAL PROCEDURES

Mutants and Transgenes

The generation of the *Kibra* loss-of-function alleles is described in the Supplemental Experimental Procedures. The lethality of *Kibra* mutants and the *Kibra* overgrowth phenotype were rescued by the presence of *UAS-Kibra 9* (2nd chromosome). *Kibra* overexpression was achieved by either *UAS-Kibra 9* or by the weaker *UAS-Kibra 18* (3rd chromosome), respectively. One copy of *UAS-Kibra 9* was used as a *Kibra*$^{+}$ rescue construct for *ex Kibra* double mutant clones. All other mutations and overexpression constructs are indicated in the Supplemental Experimental Procedures.

Clonal Analysis

Negatively marked mutant clones were generated using the hsFLP/FRT recombination system. *Kibra*-overexpressing clones were generated either by the *Actin-flp-out-Gal4* technique (Neufeld et al., 1998) or by the GeneSwitch system (Rogulja and Irvine, 2005). For the quantification of clones in imaginal discs, cell number and clone area were determined using Adobe Photoshop 7.0. Student's t tests were used to test for significance. Details

on fly lines and heat-shock protocols can be found in the Supplemental Experimental Procedures.

Immunostaining

Larval and pupal discs as well as S2 cells were fixed in 4% PFA, permeabilized with PBT, and blocked in 2% NDS. Antibodies used in this study were mouse α-β-Galactosidase (1:400; Promega), rabbit α-cleaved Caspase-3 (1:300; Cell Signaling), mouse α-Discs large (1:100; Developmental Studies Hybridoma Bank), rabbit α-Expanded (1:200; gift of A. Laughon), guinea pig α-Merlin (1:5,000; gift of R. Fehon), and rabbit α-Kibra (1:200; generated and kindly provided by N. Tapon; Genevet et al., 2010). Pictures were taken using a Leica SPE confocal laser scanning microscope.

Cell Culture, Transfection, Cloning, Immunoprecipitation, and Western Blot

S2 cells were cultured and transfected according to standard protocols. For expression of tagged proteins in S2 cells, the full-length cDNA of *Kibra* and a fragment encoding a truncated Kibra protein lacking both N-terminal WW domains (ΔWW Kibra, aa 166–end) were cloned into Gateway vectors pMtWH, pMtWF, and pMtWG. A full-length cDNA of *Mer* was cloned into pMtHW, pMtFW, and pAWR.

Coimmunoprecipitation experiments and western blots were performed according to standard protocols. Antibodies were mouse α-HA (1:3,000; Jackson ImmunoResearch), mouse α-FLAG (1:1,000; Sigma), and mouse α-HRP (1:10,000; Jackson ImmunoResearch).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, Supplemental Experimental Procedures, and Supplemental References and is available with this article online at doi:10.1016/j.devcel.2009.12.013.

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REFERENCES

Badouel, C., Gardano, L., Amin, N., Garg, A., Rosenfeld, R., Le Bihan, T., and McNeill, H. (2009). The FERM-domain protein Expanded regulates Hippo pathway activity via direct interactions with the transcriptional activator Yorkie. Dev. Cell *16*, 411–420.

Bandura, J.L., and Edgar, B.A. (2008). Yorkie and Scalloped: partners in growth activation. Dev. Cell *14*, 315–316.

Bennett, F.C., and Harvey, K.F. (2006). Fat cadherin modulates organ size in *Drosophila* via the Salvador/Warts/Hippo signaling pathway. Curr. Biol. *16*, 2101–2110.

Chishti, A.H., Kim, A.C., Marfatia, S.M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S.C., Low, P.S., Rouleau, G.A., Mohandas, N., et al. (1998). The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. Trends Biochem. Sci. *23*, 281–282.

Cho, E., Feng, Y., Rauskolb, C., Maitra, S., Fehon, R., and Irvine, K.D. (2006). Delineation of a Fat tumor suppressor pathway. Nat. Genet. *38*, 1142–1150.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature *448*, 151–156.

Edgar, B.A. (2006). From cell structure to transcription: Hippo forges a new path. Cell *124*, 267–273.

Formstecher, E., Aresta, S., Collura, V., Hamburger, A., Meil, A., Trehin, A., Reverdy, C., Betin, V., Maire, S., Brun, C., et al. (2005). Protein interaction mapping: a Drosophila case study. Genome Res. *15*, 376–384.

Genevet, A., Wehr, M.C., Brain, R., Thompson, B.J., and Tapon, N. (2010). Kibra is a regulator of the Salvador/Warts/Hippo signalling network. Dev. Cell *18*, this issue, 300–308.

Goulev, Y., Fauny, J.D., Gonzalez-Marti, B., Flagiello, D., Silber, J., and Zider, A. (2008). SCALLOPED interacts with YORKIE, the nuclear effector of the hippo tumor-suppressor pathway in Drosophila. Curr. Biol. *18*, 435–441.

Hamaratoglu, F., Willecke, M., Kango-Singh, M., Nolo, R., Hyun, E., Tao, C., Jafar-Nejad, H., and Halder, G. (2006). The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. Nat. Cell Biol. *8*, 27–36.

Hariharan, I.K., and Bilder, D. (2006). Regulation of imaginal disc growth by tumor-suppressor genes in Drosophila. Annu. Rev. Genet. *40*, 335–361.

Harvey, K., and Tapon, N. (2007). The Salvador-Warts-Hippo pathway—an emerging tumour-suppressor network. Nat. Rev. Cancer *7*, 182–191.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. (2003). The *Drosophila* Mst ortholog, *hippo*, restricts growth and cell proliferation and promotes apoptosis. Cell *114*, 457–467.

Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. (2005). The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the *Drosophila* Homolog of YAP. Cell *122*, 421–434.

Kango-Singh, M., Nolo, R., Tao, C., Verstreken, P., Hiesinger, P.R., Bellen, H.J., and Halder, G. (2002). Shar-pei mediates cell proliferation arrest during imaginal disc growth in Drosophila. Development *129*, 5719–5730.

Kremerskothen, J., Plaas, C., Büther, K., Finger, I., Veltel, S., Matanis, T., Liedtke, T., and Barnekow, A. (2003). Characterization of KIBRA, a novel WW domain-containing protein. Biochem. Biophys. Res. Commun. *300*, 862–867.

McClatchey, A.I., and Fehon, R.G. (2009). Merlin and the ERM proteins—regulators of receptor distribution and signaling at the cell cortex. Trends Cell Biol. *19*, 198–206.

Neufeld, T.P., de la Cruz, A.F., Johnston, L.A., and Edgar, B.A. (1998). Coordination of growth and cell division in the *Drosophila* wing. Cell *93*, 1183–1193.

Oh, H., and Irvine, K.D. (2008). In vivo regulation of Yorkie phosphorylation and localization. Development *135*, 1081–1088.

Oh, H., Reddy, B.V., and Irvine, K.D. (2009). Phosphorylation-independent repression of Yorkie in Fat-Hippo signaling. Dev. Biol. *335*, 188–197.

Pan, D. (2007). Hippo signaling in organ size control. Genes Dev. *21*, 886–897.

Pantalacci, S., Tapon, N., and Léopold, P. (2003). The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nat. Cell Biol. *5*, 921–927.

Papassotiropoulos, A., Stephan, D.A., Huentelman, M.J., Hoerndli, F.J., Craig, D.W., Pearson, J.V., Huynh, K.D., Brunner, F., Corneveaux, J., Osborne, D., et al. (2006). Common Kibra alleles are associated with human memory performance. Science *314*, 475–478.

Parks, A.L., Cook, K.R., Belvin, M., Dompe, N.A., Fawcett, R., Huppert, K., Tan, L.R., Winter, C.G., Bogart, K.P., Deal, J.E., et al. (2004). Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. Nat. Genet. *36*, 288–292.

Pellock, B.J., Buff, E., White, K., and Hariharan, I.K. (2007). The Drosophila tumor suppressors Expanded and Merlin differentially regulate cell cycle exit, apoptosis, and Wingless signaling. Dev. Biol. *304*, 102–115.

Reddy, B.V., and Irvine, K.D. (2008). The Fat and Warts signaling pathways: new insights into their regulation, mechanism and conservation. Development *135*, 2827–2838.

Rogulja, D., and Irvine, K.D. (2005). Regulation of cell proliferation by a morphogen gradient. Cell *123*, 449–461.

Saucedo, L.J., and Edgar, B.A. (2007). Filling out the Hippo pathway. Nat. Rev. Mol. Cell Biol. *8*, 613–621.

Schaper, K., Kolsch, H., Popp, J., Wagner, M., and Jessen, F. (2008). KIBRA gene variants are associated with episodic memory in healthy elderly. Neurobiol. Aging *29*, 1123–1125.

Tapon, N., Harvey, K.F., Bell, D.W., Wahrer, D.C., Schiripo, T.A., Haber, D.A., and Hariharan, I.K. (2002). *salvador* Promotes both cell cycle exit and apoptosis in *Drosophila* and is mutated in human cancer cell lines. Cell *110*, 467–478.

Tseng, A.S., and Hariharan, I.K. (2002). An overexpression screen in Drosophila for genes that restrict growth or cell-cycle progression in the developing eye. Genetics *162*, 229–243.

Tyler, D.M., and Baker, N.E. (2007). *expanded* and *fat* regulate growth and differentiation in the Drosophila eye through multiple signaling pathways. Dev. Biol. *305*, 187–201.

Udan, R.S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. (2003). Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat. Cell Biol. *5*, 914–920.

Willecke, M., Hamaratoglu, F., Kango-Singh, M., Udan, R., Chen, C.L., Tao, C., Zhang, X., and Halder, G. (2006). The Fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. Curr. Biol. *16*, 2090–2100.

Wu, S., Liu, Y., Zheng, Y., Dong, J., and Pan, D. (2008). The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. Dev. Cell *14*, 388–398.

Zeng, Q., and Hong, W. (2008). The emerging role of the hippo pathway in cell contact inhibition, organ size control, and cancer development in mammals. Cancer Cell *13*, 188–192.

Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B., and Jiang, J. (2008). The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. Dev. Cell *14*, 377–387.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., et al. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev. *21*, 2747–2761.