

Cooperative Regulation of Growth by Yorkie and Mad through *bantam*

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SUMMARY

The Dpp and Fat-Hippo signaling pathways both regulate growth in *Drosophila*. Dpp is a BMP family ligand and acts via a Smad family DNA-binding transcription factor, Mad. Fat-Hippo signaling acts via a non-DNA-binding transcriptional coactivator protein, Yorkie. Here, we show that these pathways are directly interlinked. They act synergistically to promote growth, in part via regulation of the micro-RNA gene *bantam*, and their ability to promote growth is mutually dependent. Yorkie and Mad physically bind each other, and we identify a 410 bp minimal enhancer of *bantam* that responds to Yorkie:Mad *in vivo* and in cultured cells, and show that both Yorkie and Mad associate with this enhancer *in vivo*. Our results indicate that in promoting the growth of *Drosophila* tissues, Fat-Hippo and Dpp signaling contribute distinct subunits of a shared transcriptional activation complex, Yorkie:Mad.

INTRODUCTION

Metazoan development relies on the reiterative deployment of multiple, conserved intercellular signaling pathways. The developing *Drosophila* wing has served as a model for identifying and characterizing pathways that regulate growth and patterning. For example, the Decapentaplegic (Dpp), Wingless (Wg), Notch, Hedgehog, Epidermal Growth Factor Receptor, and Fat-Hippo pathways all play important roles during wing development, and key components of each pathway were first identified through studies in the wing. The action of these pathways must be coordinated to ensure proper development. Here, we describe an unexpected intertwining of the Fat-Hippo and Dpp signaling pathways that regulates the growth of *Drosophila* tissues.

The Dpp pathway is named for its ligand, Dpp, a member of the BMP family. Dpp signals through type I (Thickveins, Tkv) and type II (Punt) receptors to promote phosphorylation of an R-Smad transcription factor, Mad (reviewed in Affolter and Basler, 2007). Mad can then act in conjunction with a co-Smad, Medea (Med), to activate the transcription of downstream genes. Alternatively, Mad and Med can activate downstream genes through a derepression mechanism, acting in concert

with Schnurri to repress the expression of the transcriptional repressor Brinker (Brk).

The Fat-Hippo pathway plays a conserved role in growth control and oncogenesis (reviewed in Reddy and Irvine, 2008). Fat-Hippo signaling controls growth by regulating transcription, and the critical mediator of this is a transcriptional coactivator protein, known as Yorkie (Yki) in *Drosophila* and Yap in vertebrates (reviewed in Oh and Irvine, 2010). Multiple upstream branches of Fat-Hippo signaling have been identified, but they all act via a kinase, Warts, which inhibits Yki activity by phosphorylating it. Several downstream genes that contribute to organ growth have been identified as targets of Yki in *Drosophila*, including the micro-RNA (miRNA) gene *bantam* (*ban*), Cyclins B and E, E2f1, and the inhibitor of apoptosis Diap1 (encoded by *thread*). Yki does not bind DNA itself but instead acts in conjunction with DNA-binding partner proteins. Several DNA-binding partners for Yap were identified in mammals before Yap was linked to Hippo signaling (reviewed in Bertini et al., 2009; Oh and Irvine, 2010). One of these, Sd/Tea, was subsequently identified as a key Yki/Yap partner, contributing to the influence of Fat-Hippo signaling on growth in both *Drosophila* and vertebrates (Goulev et al., 2008; Ota and Sasaki, 2008; Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008). However, because *sd* is only expressed and required in a fraction of the cells where *yki* is required, the identification of Sd as a Yki partner raised the question of how Yki controls growth in areas where *sd* appears to play no role. More recently, Hth was identified as a second Yki partner in *Drosophila*, promoting growth in the anterior eye in conjunction with Yki, in part by regulating *ban* (Peng et al., 2009). However, Hth also exhibits spatially restricted expression and genetic requirements, and so its identification still did not provide a general solution to the question of how Yki acts to promote growth in diverse places.

The role of Dpp signaling in controlling growth and patterning has been extensively studied (reviewed in Affolter and Basler, 2007). As its name suggests, Dpp is broadly required for growth in all of the imaginal discs. In the wing, Dpp is secreted by a stripe of cells along the anterior-posterior (A-P) compartment boundary and then spreads from its site of synthesis, forming a morphogen gradient. The Dpp gradient plays an important role in wing patterning, and genes expressed in distinct domains in response to different thresholds of Dpp pathway activity have been identified. Much of the downstream patterning and growth control by Dpp in the wing is thought to be regulated by establishment of an inverse gradient of Brk expression, although some genes are also directly activated by Mad-Med transcription complexes.

The observations that both the Dpp and the Fat-Hippo pathways are required for growth regulation in *Drosophila* raise the

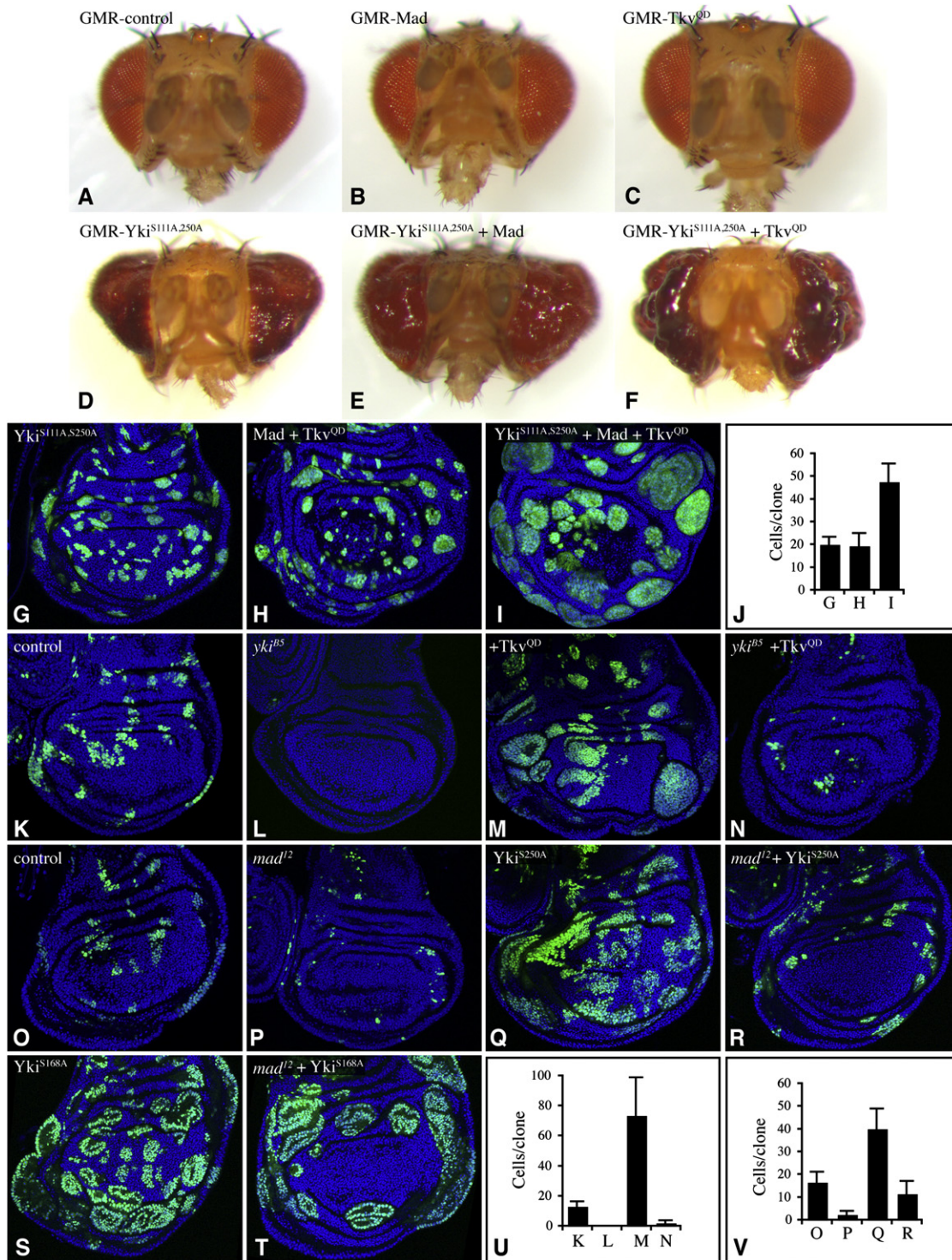


Figure 1. Yki and Tkv/Mad Act Cooperatively to Promote Growth

(A-F) Heads of adult females from *GMR-Gal4* and (A) control (B) *UAS-Flag:Mad*, (C) *UAS-Tkv^{Q235D}*, (D) *UAS-Yki^{S111A,250A}:V5*, (E) *UAS-Yki^{S111A,250A}:V5 UAS-Flag:Mad*, and (F) *UAS-Yki^{S111A,250A}:V5 UAS-Tkv^{Q235D}*.

(G-I) Wing discs with clones (*AyGal4*) marked by *UAS-GFP* (green) and expressing (G) *UAS-Yki^{S111A,250A}:V5*, (H) *UAS-Flag:Mad UAS-Tkv^{Q235D}*, (I) *UAS-Yki^{S111A,S250A}:V5 UAS-Flag:Mad UAS-Tkv^{Q235D}*, grown for 48 hr, nuclei are labeled with Hoechst (blue).

(J) Average cells/clone in genotypes shown in (G)-(I), as indicated.

(K-N) Wing discs with MARCM clones expressing *UAS-GFP*, grown for 72 hr. (K) control, (L) mutant for *yki^{B5}*, (M) expressing *UAS-Tkv^{Q235D}*, (N) expressing *UAS-Tkv^{Q235D}* and mutant for *yki^{B5}*.

question of how they are related. One link between them was identified through studies that established that Dpp signaling helps to establish gradients of expression of regulators of Fat-Hippo signaling, including the Fat ligand Dachshous (Ds) and the Fat/Ds kinase Four-jointed (Fj) (Rogulja et al., 2008). In Fat signaling, the gradient, rather than just the absolute levels, of Fat regulators is critical for pathway regulation, presumably because their ability to polarize cells impinges on Warts regulation (Rogulja et al., 2008; Willecke et al., 2008). Thus, the role of the Fj and Ds gradients in wing growth helped to explain how cell proliferation could be evenly distributed in the developing wing, despite being promoted by a gradient of Dpp pathway activity (Rogulja and Irvine, 2005). However, studies of Dpp signaling have established that the Dpp pathway also possesses an autonomous ability to promote wing growth (Martín et al., 2004; Martín-Castellanos and Edgar, 2002; Rogulja and Irvine, 2005; Schwank et al., 2008). In this manuscript we show that this autonomous growth promotion involves a second intertwining with Fat-Hippo signaling. Remarkably, we find that the transcription factors at the end of each pathway, Yki and Mad, can interact directly to form a transcription factor complex that regulates the expression of downstream target genes crucial for growth, including *ban*.

RESULTS

Coregulation of Growth by Yki and Mad

In a test of the functional relationship between Fat-Hippo and Dpp signaling, we explored the consequences of simultaneous elevation of the activity of both pathways. For Fat-Hippo this was achieved by expressing isoforms of Yki activated by mutations in one or more Warts phosphorylation sites (Dong et al., 2007; Oh and Irvine, 2008, 2009). For Dpp this was achieved either by expressing an activated form of the Tkv receptor, Tkv^{QD} (Nellen et al., 1996), or by overexpressing Mad. Transgenes encoding these proteins were expressed in vivo under UAS-Gal4 control. When *GMR-Gal4* was used to drive expression in the eye disc, activated forms of Yki (Yki^{S168A}:V5, or Yki^{S111A,S250A}:V5) (Oh and Irvine, 2009) induce enlarged and irregular eyes (Figure 1D; see Figure S1A available online), whereas Tkv^{QD} or Mad did not significantly increase eye size (Figures 1B and 1C). However, when activated Yki was combined with Mad or Tkv^{QD}, the eye overgrowth phenotype was enhanced (Figures 1E and 1F; Figures S1B and S1C). In a second assay, clones of cells expressing transgenes in the developing wing disc were examined. A mildly activated form of Yki, Yki^{S111A,S250A}:V5, induces only mild growth enhancement (Figure 1G), and Mad and Tkv^{QD} on their own modestly enhance the growth of clones in lateral regions (Figure 1H). However, when they were all coexpressed, a stronger overgrowth phenotype was observed (Figures 1I and 1J). Thus, Fat-Hippo and Dpp signaling can act cooperatively to promote growth in both eyes and wings.

To further investigate this cooperativity, we created clones of cells in which one pathway was activated, while the other was inactivated. Mutation of *yki* suppressed the ability of Tkv^{QD} to stimulate growth in the wing disc (Figures 1M, 1N, and 1U), which indicates that autonomous growth promotion by Dpp signaling requires Yki. In complementary experiments the overgrowth that would normally be induced by expressing activated-Yki (Figures 1Q and 1S) was partially suppressed by loss-of-function mutations in *mad* (Figures 1R, 1T, and 1V; Figure S1). With high-level Yki activation (Yki^{S168A}:V5), suppression of growth by mutation of *mad* was evident in the distal wing, but not in the proximal wing (Figures 1S and 1T). With low-level activation of Yki (Yki^{S250A}:V5), suppression of growth was evident throughout the wing disc (Figures 1Q, 1R, and 1V). These observations imply that Yki and Mad act in parallel to promote growth. In principle this could occur either because they each independently regulate distinct downstream genes required for growth, or because they cooperate to regulate one or more common downstream genes. As described below, we identified a downstream gene essential for normal growth, *ban*, which is directly regulated by the concerted action of Yki and Mad.

ban Is a Common Downstream Target of Yki and Mad

ban is a miRNA gene that affects growth in developing tissues: loss of *ban* reduces growth, whereas overexpression of *ban* enhances growth (Brennecke et al., 2003; Hipfner et al., 2002). Expression of *ban* can be detected using a *GFP-ban* sensor, which inversely reports *ban* levels by virtue of a *ban* recognition sequence in the 3'UTR of a *GFP*-expressing transgene (Brennecke et al., 2003). *ban* is a downstream target of Yki (Nolo et al., 2006; Thompson and Cohen, 2006), and consistent with published reports, *GFP-ban* sensor expression was decreased in clones expressing activated Yki, and increased in clones in which *yki* levels were decreased by RNAi (Figures 2A and 2B). Most clones of cells expressing Tkv^{QD} in wing discs did not noticeably influence *ban* sensor expression, although a slight decrease was observed in a fraction of clones in lateral regions of the wing disc, where Tkv^{QD} clones overgrow (Figure 2C), consistent with an earlier report (Martín et al., 2004). However, when coexpressed, Tkv and Yki acted synergistically to decrease *GFP-ban* sensor expression. Thus, whereas expression of wild-type Yki:V5 alone did not significantly influence *ban* sensor expression (Figure 2D), and Tkv^{QD} clones only rarely influenced *ban* sensor expression, coexpression of Tkv^{QD} and wild-type Yki:V5 consistently decreased *GFP-ban* sensor expression (Figure 2E). A similar synergy was observed when wild-type Mad and wild-type Yki were coexpressed: neither transcription factor significantly decreased *GFP-ban* sensor expression on its own (Figures 2D and 2F), but when coexpressed, they decreased *GFP-ban* sensor expression (Figure 2G). Moreover, *mad* RNAi, like *yki* RNAi, increased *GFP-ban* sensor expression (Figure 2H). These observations indicate that Fat-Hippo and Dpp

(O–T) Wing discs with MARCM clones grown for 72 hr, expressing UAS-*GFP*. (O) control, (P) mutant for *mad*¹², (Q) expressing UAS-Yki^{S250A}:V5, (R) expressing UAS-Yki^{S250A}:V5 and mutant for *mad*¹², (S) expressing UAS-Yki^{S168A}:V5, (T) expressing UAS-Yki^{S168A}:V5 and mutant for *mad*¹².

(U) Average cells/clone in genotypes shown in (K)–(N), as indicated.

(V) Average cells/clone in genotypes shown in (O)–(R), as indicated. Error bars show standard deviation.

See also Figure S1.

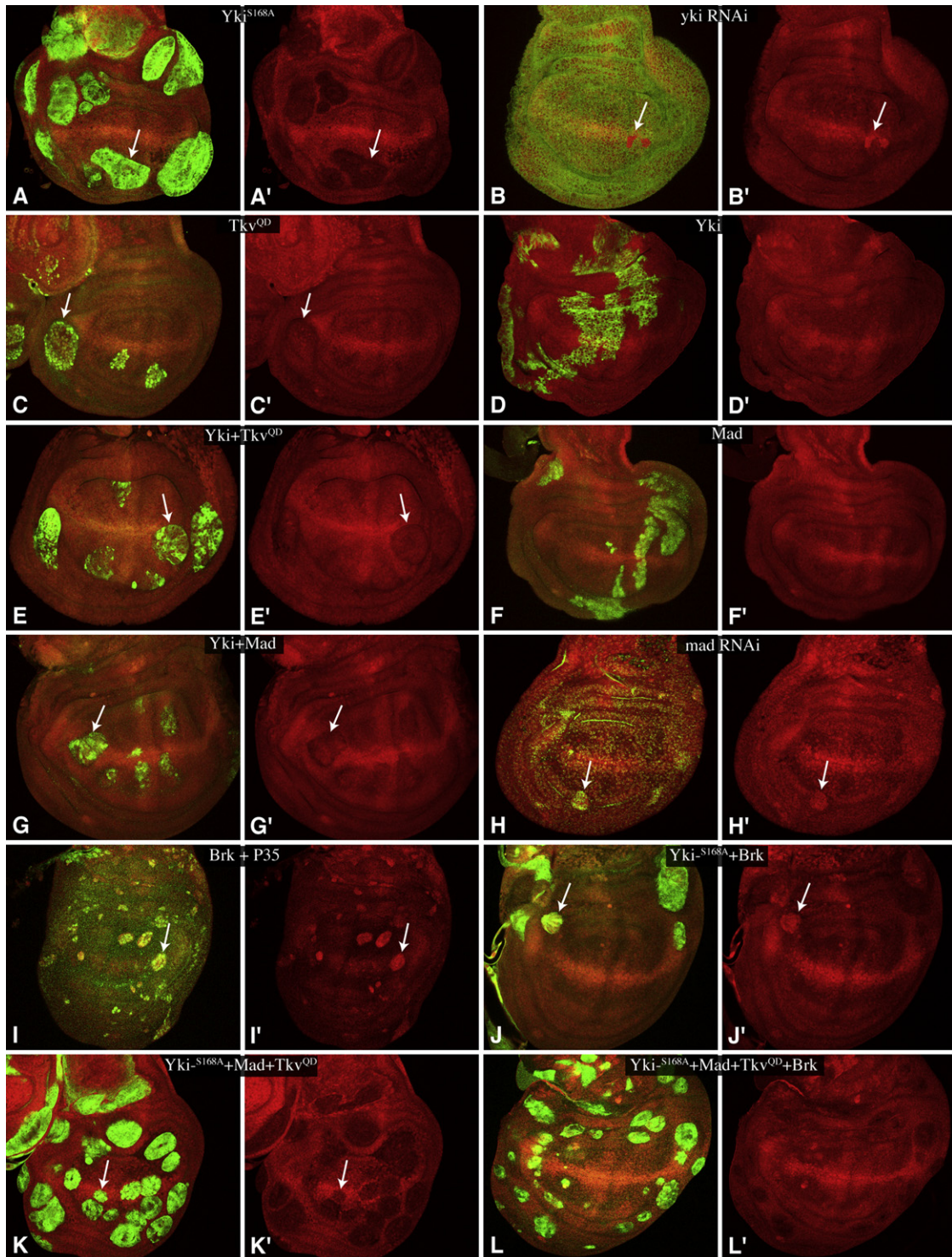


Figure 2. Regulation of *ban* by Yki, Mad/Tkv, and Brk

Wing discs expressing *GFP-ban* sensor (red) with (A) Flip-out clones expressing *UAS-Yki^{S168A}:V5* (marked by anti-V5, green), (B) *UAS-RNAi-yki* (anti-Yki, green), (C) *UAS-Tkv^{Q235D} UAS-lacZ:NL* (β -gal), green), (D) *UAS-Yki:V5* (anti-V5, green), (E) *UAS-Yki:V5 UAS-Tkv^{Q235D}* (anti-V5, green), (F) *UAS-Flag:Mad* (anti-Flag, green), (G) *UAS-Yki:V5 UAS-Flag:Mad* (anti-V5, green), (H) *UAS-RNAi-mad UAS-Dcr2* (anti-Dcr2, green), (I) *UAS-Brk UAS-p35* (anti-Brk, green), (J) *UAS-Yki^{S168A}:V5 UAS-Brk* (anti-V5, green), (K) *UAS-Yki^{S168A}:V5 UAS-Flag:Mad UAS-Tkv^{Q235D}* (anti-V5, green), and (L) *UAS-Yki^{S168A}:V5 UAS-Flag:Mad UAS-Tkv^{Q235D} UAS-Brk* (anti-V5, green). Panels marked prime show the *GFP-ban* sensor alone. In (A)–(K) arrows highlight clones with visible increases or decreases in *GFP-ban* sensor expression. Late third instar larvae were dissected either 48 hr (B, H, I, K, and L) or 72 hr (A, C–G, and J) after clone induction.

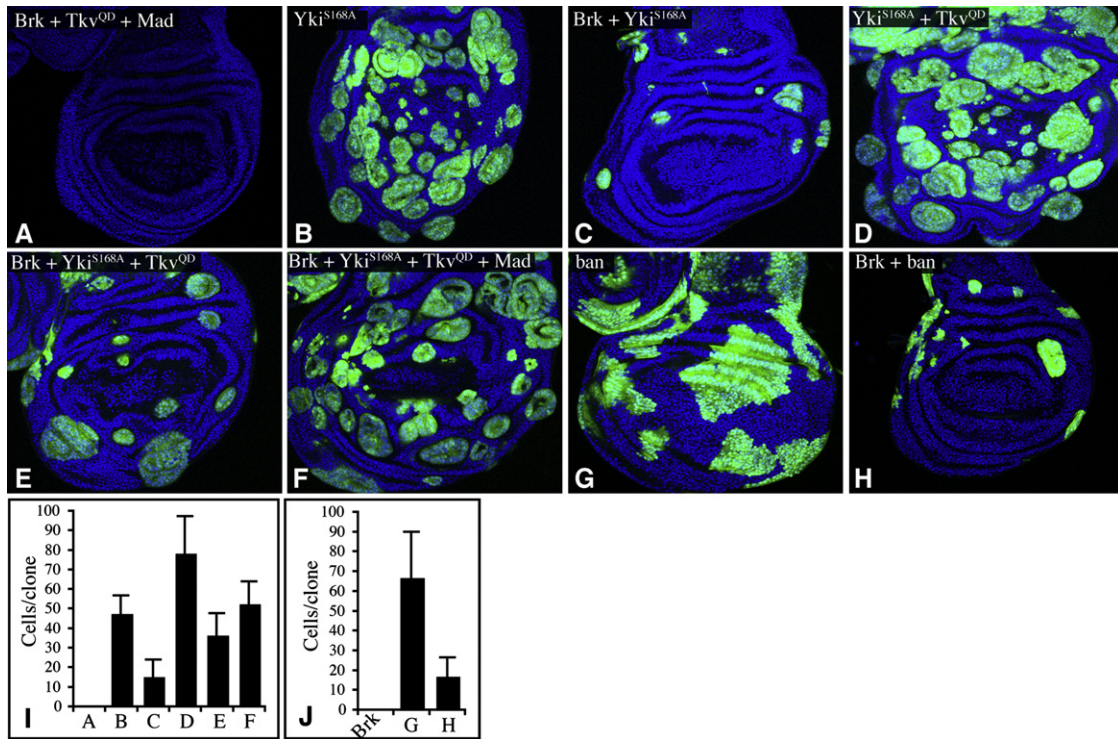


Figure 3. Brk Represses *ban* and Yki-Mad Dependent Growth

(A–H) Wing discs (nuclei labeled by Hoechst, blue) with Flip-out clones (marked by UAS-GFP, green) expressing (A) UAS-*brk* UAS-Tkv^{Q235D} UAS-Flag:Mad, (B) UAS-*yki*^{S168A}:V5, (C) UAS-*brk* UAS-*yki*^{S168A}:V5, (D) UAS-*yki*^{S168A}:V5 UAS-Tkv^{Q235D}, (E) UAS-*brk* UAS-*yki*^{S168A}:V5 UAS-Tkv^{Q235D}, (F) UAS-*brk* UAS-*yki*^{S168A}:V5 UAS-Tkv^{Q235D} UAS-Flag:Mad, (G) GS-*ban*, and (H) GS-*ban* UAS-*brk*. Third instar larvae were dissected 48 hr (A–F) or 72 hr (G and H) after heat shock. (I and J) Average cells/clone in genotypes shown in (A)–(H), as indicated.

See also Figure S2. Error bars show standard deviation.

signaling coregulate *ban* through the key downstream transcription factors in each pathway, Yki and Mad.

It has been reported that *yki* mutant clones can be partially rescued by overexpression of *ban*, consistent with the inference that *ban* is a key downstream target of Yki (Nolo et al., 2006; Thompson and Cohen, 2006). To investigate whether *ban* could also rescue *mad* mutant clones, we used the MARCM technique to make clones of cells mutant for *mad* and overexpressing *ban*. Both *mad*¹⁰ and *mad*¹² mutant clones were partially rescued by *ban* overexpression (Figure S1).

Brk Represses *ban* Expression and Yki-Mad-Dependent Growth

The results described above indicate that Yki and Mad act synergistically to promote growth, at least in part by regulating *ban*. However, prior studies of growth regulation by Dpp indicated that Mad regulates growth principally through repression of expression of the transcriptional repressor Brk, which is a negative regulator of growth (Martín et al., 2004; Muller et al., 2003; Schwank et al., 2008). The disparate observations that Mad regulates growth through Brk, and that Mad cooperates with Yki to regulate growth by promoting *ban* expression, could be reconciled if Brk also regulates *ban*. Indeed, evidence for regulation of GFP-*ban* sensor expression by Brk has been reported (Martín et al., 2004). We performed a series of experiments to confirm and extend characterization of *ban* regulation by Brk.

Clones of cells overexpressing Brk grow poorly and are normally not recovered in wing imaginal discs. However, when the apoptosis inhibitor P35 was coexpressed with Brk, clones could be recovered, and these clones upregulated GFP-*ban* sensor expression (Figure 2I). The growth and survival of Brk-expressing clones could also be partially rescued by coexpression of activated Yki (Figures 2J and 3C). Remarkably, even though activated Yki upregulated *ban* expression (indicated by repression of GFP-*ban* sensor expression) (Figure 2A), clones of cells coexpressing Brk and activated Yki often repressed *ban* expression (Figure 2J). Thus, Brk is able to repress *ban* even in the presence of Yki. Coexpression of Mad and TkV^{QD} with Yki counteracted this repressive effect of Brk (Figure 2L).

We also examined relationships among Brk, Yki, and *ban* in growth control. Coexpression of Mad and TkV^{QD} was not sufficient to reverse the failure of Brk-expressing clones to survive (Figure 3A). However, the rescue of Brk-expressing clones by coexpression of activated Yki was enhanced by expression of TkV^{QD}, resulting in some clone overgrowth (Figure 3E), and when TkV^{QD} and Mad were coexpressed together with Yki^{S168A}, strong growth of Brk-expressing clones was observed (Figures 3F and 3I). Thus, Yki and Mad can act together to oppose growth repression by Brk. To investigate the opposing effects of Brk and Yki-Mad on growth in another context, we also examined eyes of animals expressing different combinations of transgenes under *GMR-Gal4* control. Expression of Brk

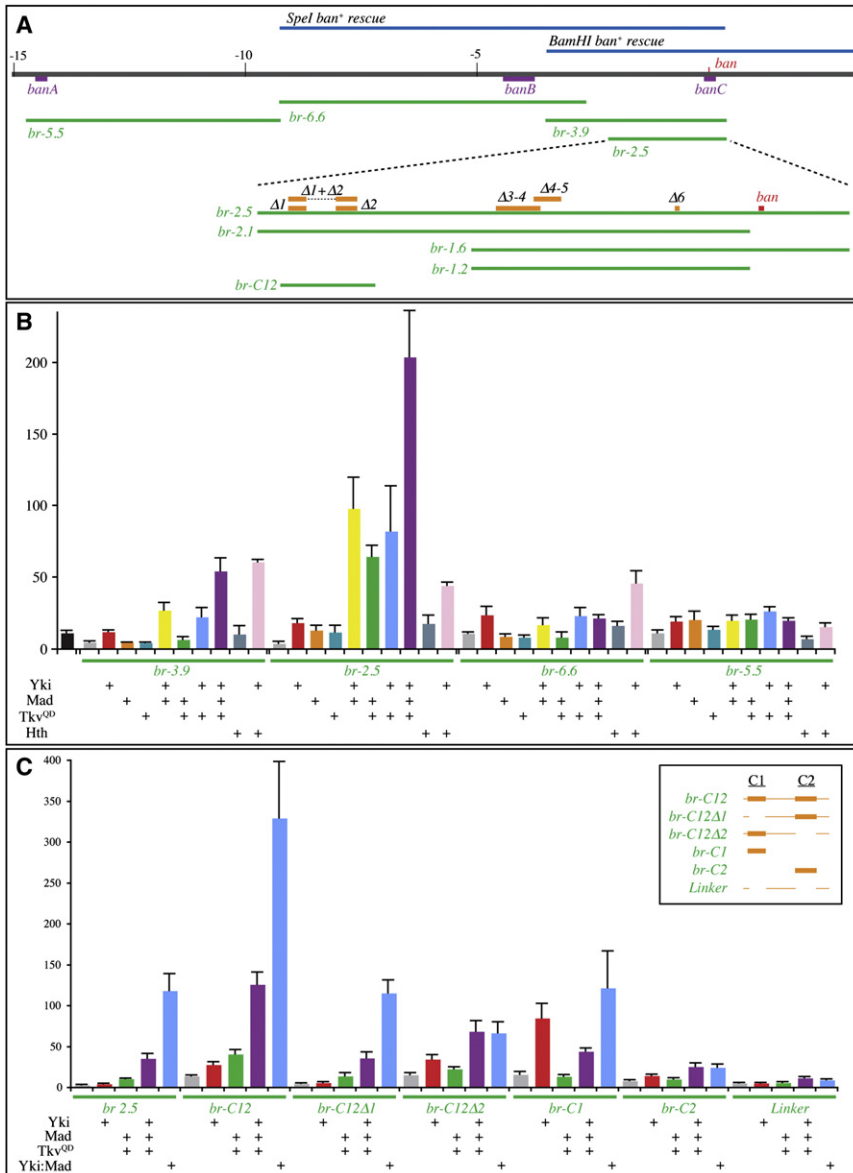


Figure 4. Identification of *ban* Enhancers

(A) Features of the *ban* locus. Blue lines indicate genomic rescue constructs (Brennecke et al., 2003), purple lines regions tested for Hth/Yki binding (Peng et al., 2009), green lines *ban* reporter constructs, orange lines deletions of sequences conserved among *Drosophila* species, and red line *ban* miRNA.

(B and C) Histograms depicting average ratios of firefly luciferase (experimental)/Renilla luciferase (control) from triplicate experiments; error bars indicate standard deviation.

(B) Expression from the indicated *ban* luciferase reporters in lysates of S2 cells transfected to express Yki, Mad, Tkv^{OD}, and/or Hth, as indicated. Black bar represents a firefly luciferase reporter with a minimal Hsp70 promoter.

(C) Expression from the indicated *ban* luciferase reporters in lysates of S2 cells transfected to express Yki, Mad, Tkv^{OD}, and/or Yki:Mad, as indicated. Inset indicates the DNA present in *br-C12* and subfragments, where the thick lines are the C1 and C2 regions identified in Figure S4. See also Figures S3 and S4.

Yki and Mad Coregulate a *ban* Enhancer

To determine whether the synergistic activation of *ban* expression by Yki and Mad reflects direct coregulation, we sought to identify and characterize *ban* regulatory sequences. Two overlapping *ban* genomic rescue constructs (*SpeI-ban+* and *BamHI-ban+*) together define a 3.9 kb region, which should thus contain all sequences essential for *ban* function (Brennecke et al., 2003), although we note that a Yki and Hth binding site was recently identified 14.5 kb upstream of the *ban* miRNA (Figure 4A) (Peng et al., 2009). A reporter construct (*br-3.9*), including the entire 3.9 kb present in the overlap of *ban* rescue constructs, was created by cloning this

results in smaller eyes, and this small eye phenotype was suppressed by coexpression of Flag:Mad and Tkv^{OD} (Figure S2). The small eye phenotype of Brk overexpression was also reversed by expression of activated-Yki, and coexpression of Yki and Tkv^{OD} together completely overcame the effect of Brk, resulting in overgrown eyes (Figure S2). Because Brk was under a heterologous promoter when expressed in clones or under *GMR-Gal4* control, the growth-promoting activity of Tkv and Mad in this context cannot be ascribed to their repression of Brk but instead must reflect parallel influences of these genes on growth, which our results suggest are at least in part due to regulation of *ban*, in concert with Yki. Consistent with the importance of *ban* in growth regulation downstream of Brk, forced expression of *ban* could also partially rescue the growth of Brk-expressing clones (Figures 3H and 3J), and Brk-expressing eyes (Figure S2).

DNA upstream of *luciferase*. In addition we created a smaller *ban* reporter construct (*br-2.5*) from sequences within *br-3.9* and two reporter constructs containing sequences farther upstream (*br-5.5* and *br-6.6*, Figure 4A). Their transcriptional activity was assayed by measuring Luciferase in transfected *Drosophila* S2 cells in the presence or absence of cotransfected transcription factors. Each of these *ban* reporters was stimulated to some degree by Yki (Figure 4B). The greatest stimulation was observed using the *br-2.5* reporter, which suggests that the 1.4 kb region that differs between *br-3.9* and *br-2.5* might contain elements that repress transcription. To investigate the influence of Dpp signaling, combinations of Yki, Tkv^{OD} and Mad were cotransfected together with the *ban* reporters (Figure 4B). Tkv^{OD} or Mad, or both in combination, enhanced transcription from the *br-2.5* reporter. Interestingly, they did not significantly enhance transcription from the *br-3.9* or *br-6.6* reporters, and the

br-5.5 reporter was only modestly affected. Notably, when Yki was cotransfected together with Mad and/or Tkv^{OD}, a synergistic enhancement of transcription was detected from the *br-3.9* and *br-2.5* reporters, but not the *br-6.6* or *br-5.5* reporters. These observations support the conclusion from *in vivo* studies that the Fat-Hippo and the Dpp pathways can cooperate to promote *ban* expression and define a 2.5 kb region that is sufficient to mediate this cooperative effect. For comparison we also characterized the responsiveness of these enhancers to cotransfection with Hth; the *br-2.5*, *br-3.9*, and *br-6.6* reporters were all modestly stimulated by cotransfection of Yki and Hth (Figure 4B).

To further narrow down *cis*-regulatory elements responsible for Yki-Mad mediated activation, we next made three smaller reporter constructs (*br-2.1*, *br-1.6*, *br-1.2*) by deleting portions of the *br-2.5* reporter (Figure 4A). The *br-1.6* and *br-1.2* reporters failed to respond to Yki and Mad (Figure S3A; data not shown), implicating the 900 bp region between the 5' ends of *br-1.6* and *br-2.5* as essential for Yki-Mad mediated transcriptional activation. However, because the *br-2.1* reporter was less active than *br-2.5*, we suspected that there might be multiple Yki-Mad responsive elements. To identify potential regulatory elements, we compared the sequence of the *br-2.5* region among several *Drosophila* species (*D. erecta*, *yakuba*, *simulans*, *sechellia*, *melanogaster*, *persimilis*, and *pseudoobscura*), and identified seven conserved stretches of nucleotides, comprising the stem-loop region of the *ban* miRNA and six upstream regions (Figure 4A; Figure S4; data not shown). To evaluate requirements for these conserved sequences, reporter constructs in which one or two of them were deleted within the context of *br-2.5* were examined. Among the individual conserved motif deletions, $\Delta 1$ and $\Delta 2$ significantly lowered transcriptional activation, whereas $\Delta 3$, $\Delta 4$, $\Delta 5$, or $\Delta 6$ had no effect (Figure S3A; data not shown). Among three double-deletion constructs ($\Delta 1$ - $\Delta 2$, $\Delta 3$ -4, $\Delta 4$ -5), $\Delta 1$ - $\Delta 2$ almost completely eliminated the responsiveness of *br-2.5* to Yki and Mad, whereas $\Delta 3$ -4 and $\Delta 4$ -5 had no effect (Figure S3A). To investigate whether this region is not only necessary, but also sufficient, for Yki-Mad regulation, a 410 bp region including conserved sequences 1 and 2 (*br-C12*, Figure 4; Figure S4) was assayed. Because multimerization of minimal transcription elements is sometimes needed for effective responses, we also made multimers of this enhancer. Both *br-C12*, and an 8x-*br-C12* multimer, responded robustly to Yki and Mad (Figure 4; Figure S3). Thus, the 410 bp *br-C12* contains sequence elements sufficient for Yki-Mad-activated transcription. Because activation of *br-C12* was even higher than that of *br-2.5*, *br-2.5* might also include sequences that direct repression.

To delineate responsive elements with the C12 region, we characterized smaller subfragments, but these failed to fully recapitulate the Yki-Mad responsiveness of *br-C12* (Figure 4C). For example *br-C12* expression was induced 9-fold by coexpression of Yki, Mad, and Tkv^{OD}, whereas *br-C1* or *br-C2* expression was induced only 3-fold, and expression regulated by the less-conserved "Linker" sequences was induced only 2-fold. *br-C1* was also strongly induced by Yki alone, which suggests that this region contains an element that responds to Yki independently of Mad. Addition of the Linker region to C1 (*br-C12 Δ 2*) slightly increased (to 4-fold) responsiveness to Yki, Mad, and Tkv^{OD}. Combining the Linker region and C2

(*br-C12 Δ 1*) recreated a more robust (7-fold) responsiveness, although the absolute levels of expression were substantially lower than for *br-C12* (Figure 4C). These observations indicate that multiple sequence elements are required for effective core-sponsiveness to Yki and Mad.

Two other DNA-binding proteins have previously been identified as partners for Yki in *Drosophila*: Sd and Hth (Goulev et al., 2008; Peng et al., 2009; Wu et al., 2008; Zhang et al., 2008). Previously mapped Hth-binding sites at the *ban* locus lie outside *br-C12* (Peng et al., 2009). There are no consensus Sd-binding site sequences within the *br-C12* sequence, and when Sd expression was reduced in S2 cells by RNAi, no effect on activation of *br-C12* was observed (Figure S3D), even though the same RNAi treatment reduced expression of the *3xsd-luciferase* and *Diap1-3.5-luciferase* reporters (Figures S3E and S3F).

Activity of Yki-Mad Responsive *ban* Enhancers In Vivo

To examine the activity and regulation of *ban* enhancers *in vivo*, we first created transgenic flies that expressed a *lacZ* reporter under *br-2.5* control. *br2.5-lacZ* expression was barely detectable with one copy of the reporter, although as a homozygote *br2.5-lacZ* expression could be detected, especially in the proximal, medial wing (Figure 5A). Importantly, expression of activated forms of Yki or Tkv substantially increased *br2.5-lacZ* expression. Activated Yki was most effective in the medial, proximal wing disc (Figures S5A and S5C), whereas Tkv^{OD} could increase *br2.5-lacZ* expression in lateral regions (Figure S5B), but to a variable degree. Coexpression of Yki and Tkv^{OD} was more effective at inducing *br2.5-lacZ*, both medially and laterally (Figure 5C). The preferential expression of *br2.5-lacZ* in medial regions, and its induction there by Yki, is consistent with an influence of Dpp signaling because this is where endogenous pathway activity is highest.

To confirm the importance of the C12 region *in vivo*, we created and characterized two additional transgenes, one comprising *br-C12* driving *lacZ* expression, and the other comprising the *br-2.5-lacZ* reporter with C1 and C2 deleted. Transgenic flies containing insertions of these reporters at the same cytological location as *br2.5-lacZ* were examined. By contrast to *br2.5-lacZ*, *br2.5 Δ 1- Δ 2-lacZ* was not detectably expressed even as a homozygote, nor was its expression induced by activation of Yki or Tkv (Figures 5D and 5E; Figure S5D). Similar to *br-2.5-lacZ*, *br-C12-lacZ* was expressed most strongly in the medial, proximal wing, but its expression was at a higher level because it was readily detected with only one copy of the transgene (Figure 5B). The observation that *br-C12-lacZ* expression was strongest in the medial area of the wing disc, and lowest at the lateral edges, is consistent with regulation by Dpp signaling (Figure 5B). Moreover, *br-C12-lacZ* expression was elevated by activation of Yki or Tkv (Figures 5G and 5I) and reduced by RNAi of *yki* or *Mad* (Figures 5H and 5J), confirming its regulation by Yki and Mad *in vivo*.

In S2 reporter assays, Brk only reduced Yki-Mad mediated activation of *br-C12* by half (Figure S3B). However, in wing discs, *br2.5-lacZ* and *br-C12-lacZ* reporter expression were partially complementary to Brk expression (Figures 5A and 5B), and their expression was repressed by Brk overexpression and increased by *brk* RNAi (Figure 5F; Figure S5; data not shown), indicating that they respond to Brk.

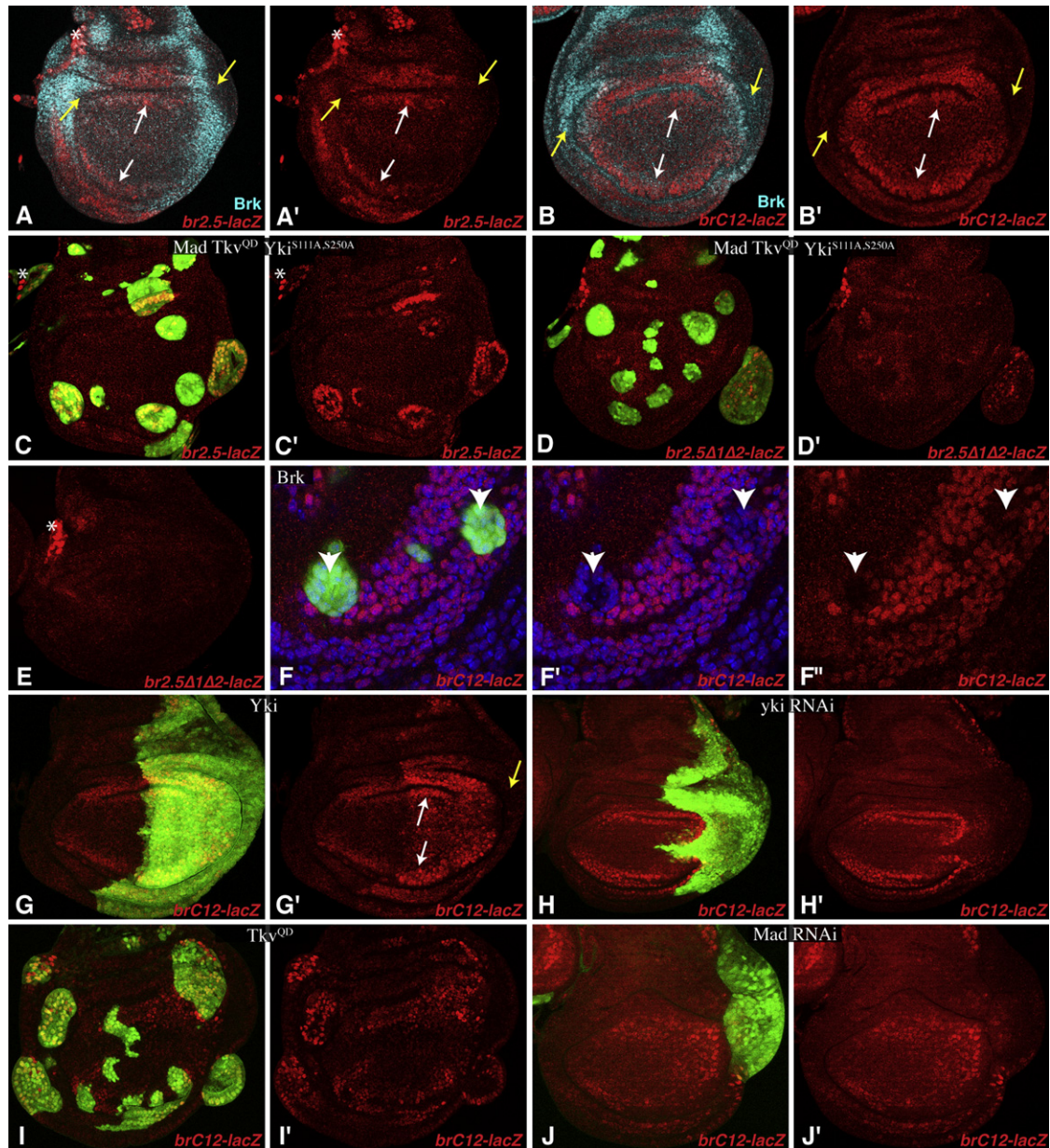


Figure 5. In Vivo Analysis of *ban* Enhancers

Wing discs from flies expressing *ban-lacZ* reporters. Panels marked prime show individual channel for β -gal (red); expression of activated or RNAi transgenes is marked by coexpression of GFP (green). Nonspecific staining in the trachea (asterisk) is sometimes visible. Yellow arrows point to lateral regions with low expression; white arrows point to medial regions with higher expression. Brk expression is shown in cyan (A and B), and nuclei (F) in blue.

(A) With two copies of *br-2.5-lacZ*, expression is visible in the proximal wing and is complementary to Brk.

(B) With one copy of *br-C12-lacZ*, expression is visible throughout the medial wing and is complementary to Brk.

(C and D) Expression of one copy of *br2.5-lacZ* (C) and *br2.5-Δ1Δ2-lacZ* (D) in wing discs with Flip-out clones (marked by UAS-GFP, green) expressing UAS-Flag:Mad UAS- Tkv^{Q235D} UAS-Yki^{S111, 250A}:V5.

(E) Even with two copies of *br-2.5Δ1Δ2-lacZ*, no expression is detected.

(F) Flip-out clones expressing UAS-*brk* UAS-*p35* repress *br-C12* expression (arrowheads); nuclei (marked by Hoechst, blue) are shown here to indicate that lack of staining is not an artifact of loss or movement of nuclei.

(G–J) Expression of *br-C12-lacZ* in cells with transgenes activating or depleting Yki or Mad are marked by GFP (green). (G) *en-Gal4* UAS-GFP UAS-Yki:V5. (H) *en-Gal4* UAS-GFP UAS-*yki* RNAi. (I) *Ay-Gal4* UAS-GFP UAS-Tkv^{Q235D}. (J) *en-Gal4* UAS-GFP UAS-Mad RNAi.

See also Figure S5.

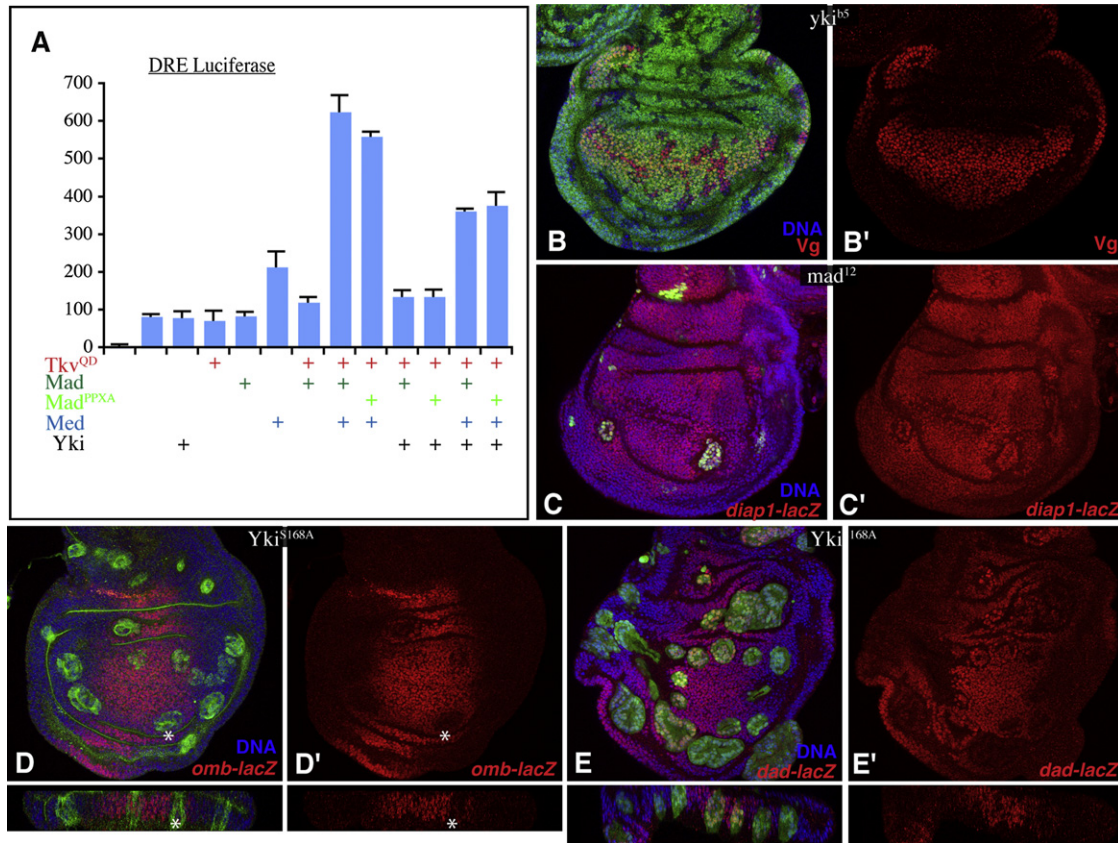


Figure 6. Independently Regulated Targets of Yki and Mad

(A) Histogram of luciferase assay results (average firefly/Renilla ratio from triplicate experiments, error bars indicate standard deviation) using *Ubx DRE* reporter in S2 cells transfected to express Yki, Tkv^{Q235D}, Mad, Mad^{PPXA}, and/or Med as indicated. (B–E) Horizontal (square panels) and vertical (thin rectangles) sections of wing discs, with nuclei labeled by Hoechst (blue). Panels marked by prime symbols show stains without the clone marker. Black bar (first bar) represents a firefly luciferase reporter with a minimal Hsp70 promoter.

(B) Vg (anti-Vg, red) expression in *yki^{BS}* clones (marked by lack of β-gal, green).

(C) *diap1-lacZ* expression (marked by β-gal, red) in *mad¹²* MARCM clones (marked by presence of GFP, green).

(D and E) Flip-out clones marked by UAS-GFP (green) expressing UAS-*yki^{S168A}:V5* and stained for (D) *omb-lacZ* or (E) *dad-lacZ* (β-gal, red); asterisk marks decreased expression. Third instar larvae were dissected 48 hr after heat shock.

See also Figure S6.

Independently Regulated Downstream Targets of Yki and Mad

To investigate how generally Yki and Mad require each other to effect transcriptional activation, we examined their influence on previously identified downstream genes. A Dpp-responsive element (DRE) within an enhancer of the *Ubx* gene has been characterized previously (Kirkpatrick et al., 2001). In luciferase reporter assays in S2 cells, Yki did not stimulate DRE-mediated transcription, nor did it enhance the effects of Mad and Tkv^{QD} on the DRE (Figure 6A). Regulation of the DRE and *br-2.5* also differed in other respects. For the DRE, exogenous expression of the co-SMAD Med stimulated activation (Figure 6A), and depletion of Med reduced activation (Figure S6B). Conversely, addition or depletion of Med had little effect on *br-2.5* (Figure 7A; Figure S6A).

We also characterized downstream targets of Dpp signaling in wing discs (reviewed in Affolter and Basler, 2007). Four genes that are positively regulated by Dpp signaling (*vestigial [vg]*, *optomotor blind [omb]*, *daughters against dpp [dad]*, *spalt-related*

[*salr*]) and one gene that is negatively regulated (*brinker [brk]*) were examined. By contrast to the upregulation of *ban*, expression of activated-Yki did not lead to detectable increases in the expression of *omb*, *salr*, or *dad*, nor decreases in the expression of *brk* (Figure 6; Figure S6). In fact, expression of *omb* appeared to be slightly decreased by expression of activated Yki. Vg was upregulated, but only in medial, proximal clones (Figure S6D). Because *yki* mutant clones did not decrease endogenous Vg expression (Figure 6B), the ectopic induction of Vg by Yki likely reflects activation of Vg-dependent feed-forward signaling (Zecca and Struhl, 2010), or an ability of overexpressed Yki to effect a Vg-like activity that autoregulates Vg in conjunction with Sd (Halder et al., 1998; Simmonds et al., 1998), rather than a normal role for Yki as a partner for Mad in activating Vg. In sum, whereas Yki functions as a Mad coregulator for some genes (e.g., *ban*), Mad regulation of other genes is Yki independent.

We also investigated the potential requirement for Mad in regulating previously identified direct targets of Yki. A 3.5 kb

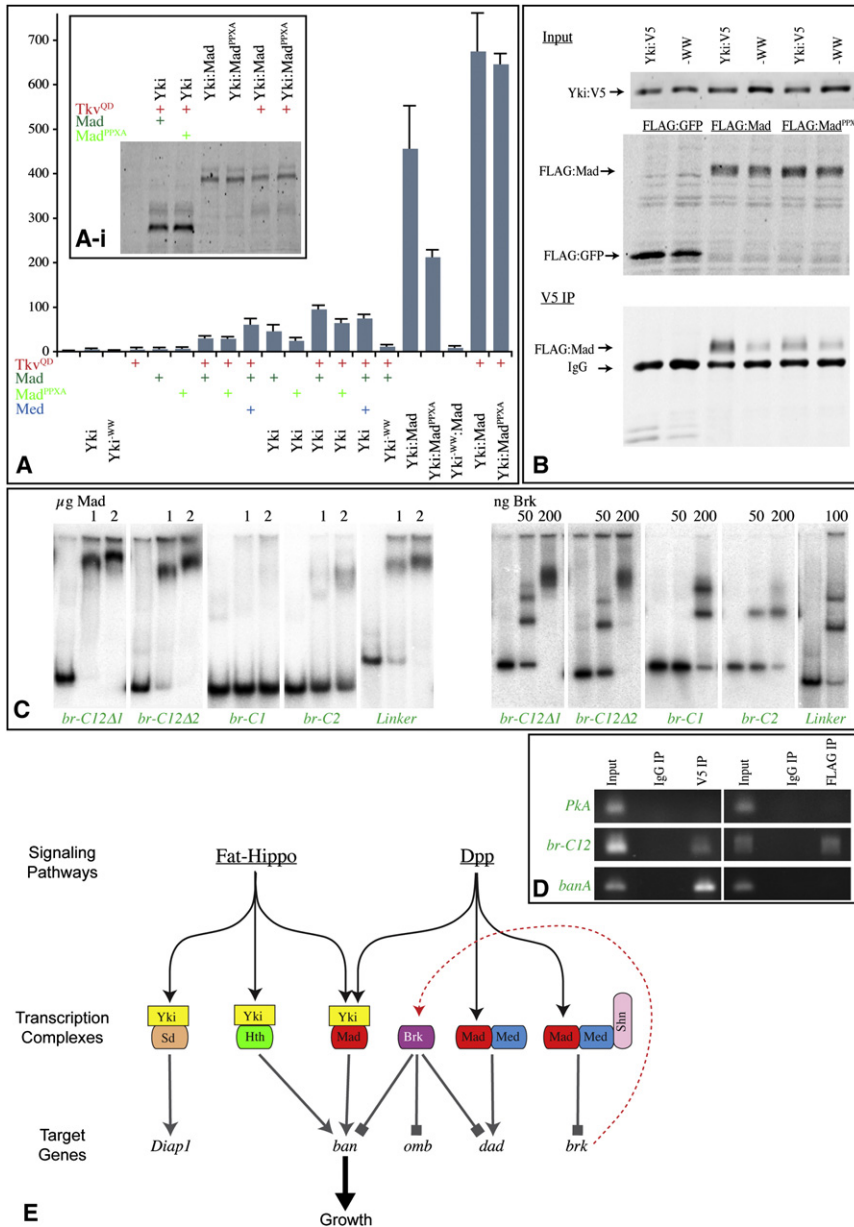


Figure 7. Interaction between Yki and Mad and Association with *ban* Enhancers

(A) Histogram of luciferase assay results (average firefly/Renilla ratio from triplicate experiments, error bars indicate standard deviation) using *br-2.5* in S2 cells transfected to express Yki, Yki^{-WW}, TkV^{Q235D}, Mad, Mad^{PPXA}, Med, Yki:Mad, Yki:Mad^{PPXA}, and/or Yki^{-WW}:Mad, as indicated. Inset (A-i) shows western blot (anti-FLAG) on lysates used for luciferase assays; Flag:Mad, Flag:Mad^{PPXA}, Yki:Flag:Mad, and Yki:Flag:Mad^{PPXA} were detected using anti-Flag antibodies.

(B) Coimmunoprecipitation of Yki:V5 and FLAG:Mad expressed in S2 cells. Upper panels (Input) show western blots (anti-V5, anti-FLAG) on lysates; lower panel (IP) shows western blot (anti-FLAG) on material precipitated using anti-V5 beads.

(C) EMSA of *ban* enhancer fragments, using the indicated amounts of Mad or Brk proteins.

(D) ChIP analysis of *ban* enhancers. Chromatin from eye discs of late third instar larvae expressing *UAS-Yki^{S168A}:V5* and *UAS-Flag:Mad* under *GMR-Gal4* control was immunoprecipitated using anti-V5, anti-Flag, or mouse IgG (control) and amplified by PCR using primers for *PkA* (negative control), *banA* (positive control for Yki), and *ban C12* region.

(E) Summary model. Fat-Hippo signaling regulates the activity of Yki, and Dpp signaling regulates the activity of Mad (black arrows). Three different DNA-binding partners for Yki have been identified: Sd, Hth, and Mad. Several downstream targets of Yki have been identified, but only *Diap1* and *ban* have been shown to be direct. Yki activates (gray arrows) *Diap1* expression with Sd, and *ban* expression with Hth or Mad. Mad and Med activate some targets directly (e.g., *dad*), and others indirectly through repression (gray block lines) of *brk*. Production of Brk protein (dashed red line) generates a repressor of Dpp pathway targets, some of which are also regulated by Mad complexes (e.g., *dad*, *ban*), others of which are regulated only by Brk (e.g., *omb*). Both pathways control growth in part through regulation of *ban*. See also Figure S7.

enhancer from the *Diap1* (*thread*) gene is directly regulated by Sd and Yki (Zhang et al., 2008). However, a *Diap1-3.5-GFP* reporter was not regulated by TkV^{QD}, either alone or in combination with Yki^{S250A}, even though TkV^{QD} and Yki^{S250A} exert synergistic effects on growth (Figure S6). To confirm the lack of responsiveness of this *Diap1-3.5* kb enhancer to Dpp signaling, we also assayed a *Diap1-3.5-luciferase* construct in S2 cells. *Diap1-3.5-luciferase*, as well as another direct target of Yki and Sd (*3x-sd-luciferase*) (Wu et al., 2008), was activated by Yki in a Sd-dependent fashion (Figure S3). However, they were not stimulated by Mad or TkV^{QD} (Figures S3E and S3F). Moreover, an enhancer trap insertion in *Diap1* (*thread-lacZ*) was unaffected by *mad* mutant clones in vivo (Figure 6C). Thus, *Diap1* is a Yki:Sd target, but not a Yki:Mad target. Altogether, our observations

indicate that Yki and Mad coregulate one or more target genes important for growth control, including *ban*, but they also each independently regulate distinct sets of downstream target genes in combination with other factors.

Yki and Mad Interact Directly to Regulate a *ban* Enhancer

The coregulation of *ban* enhancers by Yki and Mad could in principle be achieved by independent, parallel action. However, we considered the possibility that it might be mediated by direct association between Mad and Yki, acting as subunits of a shared transcriptional activation complex. Yki contains two WW domains, which can associate with PPXY motifs in other proteins to mediate direct binding (Macias et al., 2002). Mad has a PPXY

motif in the linker region between its two Mad homology domains. To assay for direct interaction between Mad and Yki, S2 cells were cotransfected with FLAG:Mad and Yki:V5. Coimmunoprecipitation experiments confirmed Yki-Mad binding (Figure 7B). Mutations in either the WW domains of Yki or in the PPXY motif of Mad reduced, but did not abolish, Yki-Mad binding, which suggests that WW domain-PPXY interactions contribute to, but do not completely explain, the physical association between these proteins. Our detection of Yki-Mad binding is consistent with a recent study (Alarcon et al., 2009).

Because WW domain-PPXY interactions contribute to Yki-Mad binding, we investigated whether they contribute to Yki-Mad-mediated transcriptional activation of *ban*. When the PPXY motif of Mad was mutated (Mad^{PPXA}), the ability of Mad to enhance Yki-dependent activation of *br-2.5* was reduced but not abolished (Figure 7A), consistent with the effect of this mutation on Yki-Mad binding. Mad^{PPXA} was similar to wild-type Mad on the Mad-Med responsive DRE (Figure 6A). Mutation of the WW domains of Yki (Yki^{WW}) abolished its ability to stimulate *ban* reporter expression, either alone or in combination with Tkv and Mad (Figure 7A), consistent with observations that the WW domains of Yki are essential for its ability to promote transcriptional activation (Oh et al., 2009; Zhang et al., 2009). To further investigate the significance of Yki-Mad binding, we created transgenes that expressed Yki:Mad fusion proteins. A wild-type Yki:Mad fusion was a potent activator of *br-2.5* and *br-C12* expression, significantly more active than the Yki, Tkv^{OD}, and Mad combination, which indicates that direct interaction between Yki and Mad promotes *ban* transcription (Figures 4C and 7A). A Yki:Mad^{PPXA} fusion protein was also an activator of the *br-2.5* reporter, but Yki^{WW}:Mad was not (Figure 7A), which implies that the requirement for the WW domains is not solely related to Mad recruitment.

Yki, Mad, and Brk Bind the *ban* C12 Enhancer

The observations that Yki and Mad directly bind to each other, and act synergistically to regulate *ban* through the C12 enhancer, suggest that they can act together in a transcriptional activation complex that directly regulates *ban* via this enhancer. This hypothesis predicts that Mad and Yki should be coassociated with *ban* enhancer DNA in vivo. To test this we performed chromatin immunoprecipitation (ChIP) experiments. Sheared genomic DNA was immunoprecipitated, using anti-V5 and anti-FLAG antibodies, from eye imaginal discs expressing Yki^{S168A}:V5 and Flag:Mad under *GMR-Gal4* control. (The *br-C12-lacZ* reporter also responds to Yki and Tkv in eye discs [Figures S5G and S5H].) The 410 bp C12 region was amplified by PCR from DNA immunoprecipitated using either anti-V5 or anti-FLAG (Figure 7D). As negative controls, DNA was precipitated with nonspecific mouse IgG, or primers for an unrelated locus (protein kinase A) were employed; in neither case was a PCR band amplified from the precipitated material (Figure 7D). Thus, Yki and Mad colocalize to the C12 region of *ban* in vivo. We also confirmed the association of Yki with a previously identified Yki-Hth site (*banA*) (Peng et al., 2009). Mad was not detected at *banA* (Figure 7D), which suggests that distinct Yki-Mad and Yki-Hth complexes regulate *ban* through separate enhancers. Although antibodies suitable for ChIP of endogenous Mad were not available, we confirmed the association of endogenous

Yki with the *br-C12* enhancer in wild-type wing discs by ChIP using anti-Yki sera (Figure S7A).

To confirm that Mad can directly associate with *br-C12*, we performed electrophoretic mobility shift assays (EMSA), using a Mad polypeptide including the DNA binding domain. In this assay, Mad also bound to *br-C12*, and not to *banA* (Figure S7B). When subfragments of *br-C12* were assayed, the strongest binding was detected to the Linker regions, and Mad also bound detectably to C2, but not C1 (Figure 7C). Thus, *br-C12* contains multiple Mad-binding sites, despite the absence of sequences matching previously identified binding sites within the conserved C1 and C2 sequences. Notably, the regions that bind Mad in vitro (C2 and the Linker) together direct robust Yki:Mad responsiveness in S2 cells (Figure 4C). Brk could also bind directly to *br-C12* in vitro (Figure 7C; Figure S7B), suggesting that its repression of *ban* is direct. Brk and Mad binding sites are related, and in some instances they compete for binding to these overlapping sequences (Affolter and Basler, 2007). However, Brk did not detectably compete for Mad binding within *br-C12* (Figure S7C), which suggests that Brk represses *ban* expression through distinct sites within this enhancer.

DISCUSSION

Although substantial progress has been made toward identifying the regulatory pathways that control organ growth during development, elucidating how these pathways interact with each other to achieve proper growth control remains a significant challenge. Here, we have described an unanticipated intertwining of the Dpp and Fat-Hippo pathways that is required for growth control during imaginal development in *Drosophila*. Our observations argue that this involves a shared transcription factor complex, Yki-Mad, which includes subunits that are the key downstream transcriptional effectors for their respective pathways. The link between Yki and Mad is supported by several observations. The ability of each transcription factor to promote growth in vivo is partially dependent upon the other. They act synergistically to promote growth and to regulate the expression of *ban*, a key regulator of growth in *Drosophila*. They bind directly to each other, and a mutation that decreases binding decreases their transcriptional activity, whereas directly fusing them together dramatically enhances it. And they regulate *ban* through the same minimal enhancer, to which they both bind in vivo, and to which Mad can bind in vitro.

An interaction between Yap and an inhibitory Smad, Smad7, was first reported several years ago (Ferrigno et al., 2002), and more recently interactions between Yap and Smad1, between Yki and Mad, and between Taz (a Yap homolog) and Smad2/3-4 have been reported (Alarcon et al., 2009; Varelas et al., 2008). However, the relevance of these interactions to either Fat-Hippo or Dpp signaling was not established. An obstacle to divining the general relevance of interactions between Smads and Yap family members has been their promiscuity—over a dozen partners for Yap have been identified previously (Bertini et al., 2009), and over 50 partners have been identified for Smads (Feng and Derynck, 2005; Ross and Hill, 2008). Importantly then, our observations establish Mad and Yki as critical partners for growth regulation in *Drosophila*, which is a crucial biological function of their respective pathways in imaginal discs.

Transcriptional Regulation by Dpp Signaling

Smads are DNA-binding proteins, but on their own they generally bind with relatively low specificity (Feng and Derynck, 2005; Ross and Hill, 2008). This has led to a paradigm in which Smads bind regulatory sequences in conjunction with one or more cofactors. Dozens of cofactors have been identified in vertebrates, but far fewer in *Drosophila*. Instead, it has been concluded that most Dpp signaling in *Drosophila* is effected through transcriptional repression of Brk (Affolter and Basler, 2007). Repression of Brk is effected by a complex that includes Mad, Med, and the DNA-binding transcriptional repressor Schnurri, which together bind to “silencer elements.” However, genes that are directly activated by Dpp signaling have also been identified (Affolter and Basler, 2007). The best known of these is *dad*, which is activated by Dpp signaling via an activating element that binds a Mad-Medea complex without Schnurri (Weiss et al., 2010). The identification of Yki as a transcriptional coactivator for Mad raises the question of whether it is also a coactivator for other Dpp target genes. The lack of effect of Yki on *dad* expression, and on other Dpp pathway targets, argues that Yki is not a universal Mad coactivator but rather acts as a coactivator for a subset of Mad target genes.

Although Smad-mediated transcription generally appears to involve complexes that include both R-Smads and co-Smads, some instances of Smad complexes composed only of R-Smads have been described in vertebrates (Ross and Hill, 2008). Our studies suggest that a Yki:Mad complex need not include Med. For example in cultured cells, addition or depletion of Med did not significantly affect expression of *br-2.5* but did affect the *Ubx-DRE*.

The Role of Brk in Growth Control

Our delineation of a role for a Yki:Mad complex in growth control has to be squared with prior observations, which led to the conclusion that Dpp signaling regulates growth via repression of Brk. We propose that normal growth regulation by Dpp signaling is effected by two, parallel mechanisms—direct activation of downstream targets by Yki:Mad complexes, and derepression of downstream targets by repression of Brk (Figure 7E). This model fits studies of *ban* expression because both Brk and Yki:Mad regulate *ban* and bind the C12 enhancer. Importantly, because Yki:Mad can influence growth and *ban* expression even when Brk is expressed under the control of a heterologous promoter, its effects cannot be ascribed simply to transcriptional repression of Brk: Yki:Mad and Brk must compete to regulate *ban*. This parallels studies of *salM* and *dad* regulation by Dpp signaling, for which Mad activates transcription both directly, and indirectly, via Brk (Affolter and Basler, 2007). In the parallel regulation model, the regulatory links could be partially redundant. Thus, the lack of requirement for Mad for growth in the absence of Brk could be explained by derepression of Yki:Mad target genes like *ban*.

The sharing of components (e.g., Mad) between the direct (Yki-Mad) and indirect (Brk) growth regulatory pathways complicates assessment of their respective contributions, and indeed we would predict that their relative contributions could vary in time and space (e.g., depending on the level of Yki activation). However, if Yki-Mad activation is independent of Med, then *med* mutant clones might be expected to have weaker effects on

growth than *mad* mutant clones because Med would only participate in the Brk pathway. Notably, just such a difference has been reported (Wisotzkey et al., 1998). Moreover, Brk is evolutionarily derived because, thus far, Brk homologs have only been found in insects. Thus, whereas in *Drosophila* the Brk derepression pathway plays a major role in growth regulation, in other arthropods, direct activation by Yki-Mad might be more important.

Growth Regulation by Yki and Its DNA-Binding Partners

One of the puzzles of growth regulation by Yki in *Drosophila* has been the restricted distribution of its DNA-binding partners, Sd and Hth. Mad, by contrast, is expressed ubiquitously and is required for growth in all imaginal discs. Thus, it is well positioned to be a general partner for Yki in growth regulation.

Because Smads often form complexes with other transcription factors, a Yki-Mad complex might form in conjunction with other proteins, and indeed our observation that the linker region of *br-C12* exhibits the strongest binding to Mad and was necessary but not sufficient for strong Yki-Mad-mediated activation suggests that this might be the case. RNAi of *sd* did not significantly decrease the expression of *ban* reporters in S2 cells, which implies that Sd is not an obligate partner for Yki-Mad. RNAi of Hth also did not influence *ban* reporters (unpublished data), although this is subject to the caveat that we lacked Hth-responsive reporters with which to compare. However, as coregulators with Yki in overexpression experiments, Hth exhibited different profiles from Tk^v^{QD} and Mad in terms of which *ban* enhancers were most sensitive. Thus, we favor the hypothesis that distinct Yki-Hth and Yki-Mad complexes independently regulate *ban*, acting through distinct enhancers. This could also explain the observations that mutation of *mad* did not block the overgrowth of Yki^{S168A}-expressing clones in the proximal wing, where Hth expression is high, and did not block growth of *expanded* mutant clones in the eye imaginal disc, which upregulate Hth (Tyler and Baker, 2007); in these cases Hth might act in place of Mad to promote *ban* expression with Yki. Finally, we emphasize that whereas *ban* is clearly a key downstream target of Yki-Mad, there are also likely to be other targets important for growth control. Regardless of their number, our studies reveal that *Drosophila* tissues directly monitor the status of both the Dpp and Fat-Hippo pathways through a requirement for a shared transcription factor complex.

EXPERIMENTAL PROCEDURES

Histology and Imaging

Imaginal discs were fixed and stained as described previously (Cho and Irvine, 2004), using as primary antibodies rabbit anti-Yki (1:400), mouse anti-V5 (1:400; Invitrogen), mouse anti-Flag (1:2000; Sigma), rabbit anti-Vg (1:200; gift of S. Carroll), rabbit anti-Dcr2 (1:200; Abcam), goat anti- β -gal (1:500; BioGenesis), and guinea pig anti-Brk (1:2000; gift of G. Morata). Fluorescent stains were captured on a Leica TCS SP5 confocal microscope.

Clone sizes were measured by counting cell numbers (using nuclear staining) because in some instances clones induce folding, which makes area measurements inaccurate, and cell size is not significantly affected by activation of Yki or Tk^v. At least 30 clones, from multiple discs, were counted for the histograms in Figure 1, and at least 20 clones were counted for the histograms in Figure 7.

S2 Cell Assays and Coimmunoprecipitation

S2 cells were cultured with Schneider's *Drosophila* medium (Invitrogen) and 10% FBS (Sigma). For coimmunoprecipitation, transient transfections were

performed with equal amounts of DNA (1 μ g per construct) using Cellfectin (Invitrogen) in 6-well plates according to the manufacturer's protocol. Coimmunoprecipitation assays were performed as described previously (Chen et al., 2003). For western blotting, mouse anti-V5 (1:5,000; Invitrogen), IRDye 680 conjugated anti-mouse (goat, 1:10,000; Odyssey), and IRDye 800 conjugated anti-Flag (1:100,000, rabbit) were used. Blots were scanned and analyzed using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Luciferase reporter assays were performed using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. S2 cells were transfected in triplicate with Luciferase-reporter constructs (25 ng) and copia-*renilla* luciferase (8 ng) reporters in 48-well plates together with 25 ng of each plasmid in a pAc5.1 vector (Invitrogen) and incubated for 48 hr after transfection. For RNAi experiments, templates for *in vitro* transcription were amplified by PCR, which have T7 promoter at each end, then used for *in vitro* transcription using the MEGAscript Kit (Ambion) according to the manufacturer's instructions. RNAi was performed as described previously (Clemens et al., 2000) but modified in order to combine RNAi and transfection. Briefly, 24 hr after dsRNA was added to the cell, cells were washed once with serum-free medium and then transfection was performed, and the cells were incubated for 48 hr for luciferase assays.

ChIP

Late third instar larvae, wild-type, or overexpressing YkiS168A:V5 and Flag:Mad under the control of GMR-Gal4 were dissected, and wing or eye imaginal discs were collected in PBS on ice. Chromatin preparation and immunoprecipitation were performed as described in the protocol of the EZ ChIP Chromatin Immunoprecipitation Kit (Millipore). About 50 discs were used for an IP reaction. Rabbit normal IgG (1 μ g; Millipore), rabbit anti-Yki (1:400; Oh and Irvine [2008]), Mouse normal IgG (1 μ g; Millipore), mouse anti-V5 (1 μ g; Invitrogen), and mouse anti-Flag (0.5 μ g; Sigma) were used for immunoprecipitation. For PCR pairs of primers from Pka (5'-agccgcactcgcgcttctac/5'-caatcagcagattctccgct), *bana* (5'-aatccaacgtgcagcggc/5'-agcgggtctcaagcacagc) (Peng et al., 2009) and C12 region of *bantam* (5'-gcgactgagcgtgggtttttg/5'-cgactctcaaccattctaaacta) were used.

EMSA

As in earlier studies, we used the DNA-binding domains of Brk and Mad rather than full-length proteins, and we observed that Brk has significantly higher affinity for DNA than Mad (Kim et al., 1997; Kirkpatrick et al., 2001; Pyrowolakis et al., 2004; Weiss et al., 2010). DNA-binding domains of Mad (MadPvull, first 303 aa: MHI + Linker) (Kim et al., 1997) and Brk (Brk:DBD, first 99 aa) were amplified by PCR and inserted into SmaI site of pGEX-3X vector and transformed into BL21(DE3)pLysS to be induced by 0.5 mM IPTG. Purification of GST-MadPvull and Brk:DBD was performed using the B-PER GST Fusion Protein Spin Purification Kit (Thermo Scientific) according to manufacturer's instruction. Radioactive-labeled probes were generated by PCR followed by T4 PNK treatment in the presence of [γ -³²P] ATP. Binding reactions were carried out in 20 μ l of 100 mM KCl, 10 mM Tris (pH 7.5), 1 mM DTT, 5 mM MgCl₂, 0.05% NP-40, 1 μ g poly(dIdC), 2.5% Glycerol, 1 mM EDTA, and 0.3% BSA containing 10,000 cpm probes for 20 min at room temperature and then separated in 5% polyacrylamide gels containing 1 \times TBE (Bio-Rad), followed by autoradiography using phosphorimager.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2010.12.002.

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