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ORIGINAL ARTICLE

A novel partner of Scalloped regulates Hippo signaling via antagonizing Scalloped-Yorkie activity

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The Hippo (Hpo) pathway controls tissue growth and organ size by regulating the activity of transcriptional co-activator Yorkie (Yki), which associates with transcription factor Scalloped (Sd) in the nucleus to promote downstream target gene expression. Here we identify a novel protein Sd-Binding-Protein (SdBP)/Tgi, which directly competes with Yki for binding to Sd through its TDU domains and inhibits the Sd-Yki transcriptional activity. We also find that SdBP retains Yki in the nucleus through the association with Yki WW domains via its PPXY motifs. Collectively, we identify SdBP as a novel component of the Hpo pathway, negatively regulating the transcriptional activity of Sd-Yki to restrict tissue growth.

***Keywords*:** Hippo; Sd

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Introduction

Among developmental processes, organ size control is one of the critical steps. Failure of appropriate organ size control results in diseases including tumorigenesis. In the past decade, the Hippo (Hpo) signaling pathway was identified as one of the important signaling pathways that regulate organ growth [1-4]. This pathway was first identified in *Drosophila* and is highly conserved in mammals. The serine/threonine Ste20-like kinase Hpo and the nuclear Dbf-2-related (NDR) family kinase Warts (Wts) associate with two scaffold proteins Salvador (Sav) and Mob as tumor suppressor (Mats) to induce a series of phosphorylation events, which is considered to be the core of the Hpo pathway [5-12]. This kinase cascade results in the inactivation and cytoplasmic retention of transcriptional co-activator Yorkie (Yki, YAP in mammals) [13, 14]. In the absence of Hpo signaling, Yki translocates into the nucleus and binds to the transcription factors such as Scalloped (Sd, TEAD/TEF family of proteins in mammals) [15-17] to promote proliferation and inhibit apoptosis by regulating the expression of Hpo pathway target genes including *diap1*, *cyclin E* and *bantam* [18, 19].

Sd is the only TEAD/TEF protein family member in *Drosophila* [20]. Unlike *Drosophila*, mammals contain four closely related TEAD/TEF family members, TEAD1-TEAD4. They are expressed widely during development and regulate the development of various tissues, including heart, skeletal muscles, neural crest, notochord and trophoectoderm [21-25]. The function of Sd-Yki complex has been intensively studied. In mammals, the structure and function of TEAD-YAP were well characterized [26-28]. Recent studies revealed that mammalian TEAD proteins are involved in the regulation of cell proliferation by cooperating with YAP under the control of Hpo signaling [29-32]. Interestingly, both genetic studies in *Drosophila* and pharmacological studies in transgenic mice revealed that loss of *sd* or inhibition of TEAD has minimal impact on normal tissue homeostasis and physiology, suggesting that Sd/TEAD is required for Yki/YAP-mediated tissue overgrowth but is largely dispensable for normal tissue homeostasis [15, 16, 33].

As YAP has been implicated in organ overgrowth and various human cancers [4, 29, 34], this interesting prop-

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erty of TEAD may provide us a good opportunity to develop pharmacologically viable strategy against the YAP oncoprotein. However, so far, hardly any component(s) that directly inhibit Sd-Yki/TEAD-YAP complex activity has been identified. In this study, we report the discovery of a novel Sd binding protein, Sd-Binding-Protein (SdBP). We find that it directly interacts with Sd via its TDU domains and with Yki through its PPXY motifs. We also reveal that it serves as an inhibitor of Sd-Yki transcriptional complex. Moreover, we provide evidence that SdBP and Yki compete with each other for Sd binding to regulate the transcriptional activity of Sd-Yki complex, thereby keeping the homeostasis of tissue growth.

Results

Identification of SdBP as an Sd-binding partner

To gain understanding of the regulatory mechanism of Sd-Yki complex, we performed yeast two-hybrid (Y2H) analysis using Sd C-terminal fragment (208-440 aa) as a bait to screen the *Drosophila* embryo cDNA library. We found that a protein (named as Sd-Binding-Protein, SdBP, encoded by CG10741) associated with Sd in Y2H assay (Supplementary information, Figure S1A).

To verify the interaction between SdBP and Sd *in vitro*, we performed co-immunoprecipitation experiment using full-length Sd and SdBP in Schneider 2 (S2) cells. Strong binding between Sd and SdBP *in vitro* was observed (Figure 1A). Subsequent GST pull-down assay confirmed a direct interaction between Sd and SdBP (Supplementary information, Figure S1B). In addition, we found that SdBP is a nuclear protein and co-localizes with Sd in the nucleus when expressed by Gal4 driver *MS1096* (Supplementary information, Figure S1C-S1E'''). Taken together, these results identify SdBP as a novel binding partner of Sd.

There are two annotated transcripts of SdBP in FlyBase (http://flybase.org/reports/FBgn0036373.html). Expression of either isoforms of SdBP, SdBP-RA and SdBP-RB, under the control of *MS1096* resulted in growth inhibition (Supplementary information, Figure S1F-S1F'''). No distinguishable difference between SdBP-RA and SdBP-RB in function and subcellular localization was observed (Supplementary information, Figure S1F-S1G'); we chose SdBP-RB for further study.

SdBP overexpression antagonizes Sd-Yki-induced tissue overgrowth

To probe the function of SdBP, we first examined the expression pattern of SdBP in different tissues including eye discs and wing discs by immunostaining and *in situ* assay. An antibody of SdBP was generated and validated using immunostaining by expressing SdBP RNAi transgene under the control of *hh-Gal4*, which drives gene expression in the posterior compartment (P-compartment) of *Drosophila* imaginal discs (Supplementary information, Figure S2A-S2A"). We observed that SdBP is ubiquitously expressed in eye discs and wing discs without a specific pattern (Supplementary information, Figure S2B-S2C' and S2D-S2E'). It is known that Sd is a transcription factor, and forms a transcriptional complex with Yki to control tissue growth in the Hpo pathway [15-17]. As SdBP binds to and co-localizes with Sd *in vitro* and *in vivo* (Figure 1A and Supplementary information, Figure S1A-S1E'''), we performed analyses to investigate whether SdBP plays a role in regulating Sd-Yki transcriptional activity. SdBP was subjected to a dual luciferase assay that can reflect Sd-Yki transcriptional activity. In S2 cells, coexpression of Yki and Sd activated the luciferase (*Luc*) reporter gene, *3xSd2-Luc* [15] (Figure 1B). Coexpressing SdBP sharply decreased the activity of Sd-Yki complex (Figure 1B), suggesting that SdBP influenced Sd-Yki activity. In addition, this inhibition is specific as only mild change was observed when we tested SdBP using Wnt and Hh reporters (Supplementary information, Figure S2F-S2G).

To probe the function of SdBP on Sd-Yki activity *in vivo*, we overexpressed SdBP under the control of *GMR-Gal4* in *Drosophila* compound eyes. Overexpression of *UAS-SdBP* posterior to morphogenetic furrow using the *GMR-Gal4* driver resulted in mild growth defects in eyes, compared with controls (compare Figure 1D with 1C). Yki overexpression-induced overgrowth phenotype was completely suppressed by coexpression of SdBP (compare Figure 1F with 1E). Furthermore, the *GMR-Yki*-induced increase in BrdU (a DNA replication maker) incorporation was diminished by coexpression of SdBP (Figure 1C'-1F'). These results suggest that SdBP suppresses growth through antagonizing Sd-Yki activity.

Gain of function of SdBP inhibits tissue growth through regulating the Hpo pathway

To verify whether SdBP overexpression induced growth defects via regulating the Hpo pathway, we expressed SdBP in flip-out clones to examine the changes in the expression of the Hpo pathway target genes. We observed a downregulation of DIAP1 and Ex proteins in SdBP-overexpressed regions (Figure 2A-2B'). Similar changes in *diap1-lacZ* (an enhancer trap for *diap1* that reflects its transcriptional level) and *ex-lacZ* signals (an enhancer trap for *expanded* in response to Hpo signaling [35]) were observed (Supplementary information, Figure S3A-S3B"). The microRNA *bantam* is a Hpo pathway target that promotes cell growth [18, 19]. We found that

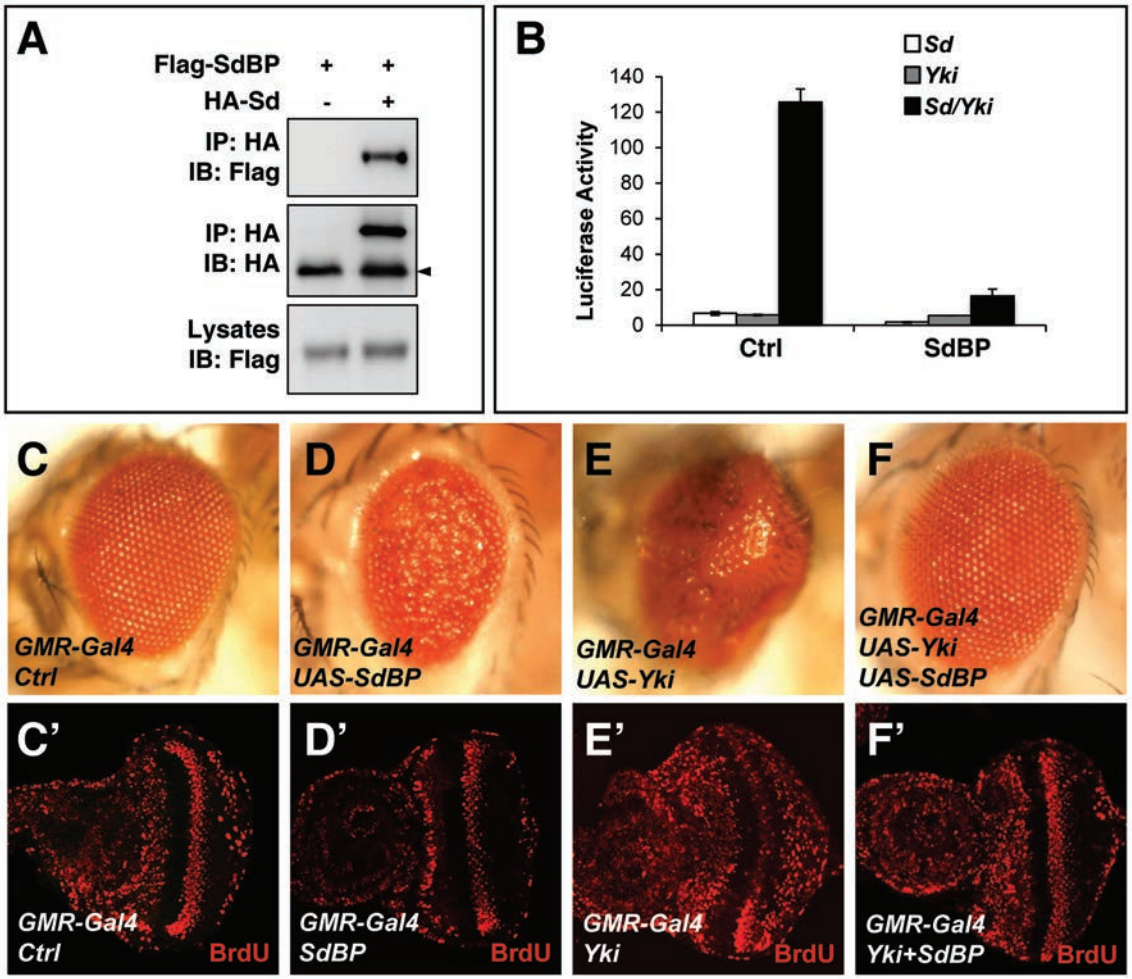


Figure 1 SdBP associated with Sd and antagonized Sd-Yki-induced overgrowth. **(A)** Co-immunoprecipation of HA-Sd and 3× Flag-SdBP in S2 cells. The indicated constructs were transfected into S2 cells, followed by immunoprecipitation and western blot analysis with the indicated antibodies. The arrowhead indicates IgG chain. **(B)** Luciferase activity of a luciferase reporter plasmid with 3× Sd-binding sites and the control vector in S2 cells cotransfected with the indicated constructs. Data were presented as mean ± s.d. (*n* = 3). **(C-F)** Gain of function of SdBP induced growth defects and also reduced *GMR-Yki*-induced overgrowth. Adult eyes expressing *GMR-Gal4* **(C)**, *GMR-Gal4/UAS-SdBP* **(D)**, *GMR-Gal4/UAS-Yki* **(E)**, and *GMR-Gal4/UAS-(SdBP+Yki)* **(F)**. **(C'-F')** BrdU staining of wild-type eye discs **(C')**, and eye discs expressing *UAS-SdBP* **(D')**, *UAS-Yki* **(E')** or *UAS-(Yki+SdBP)* **(F')** under the control of *GMR-Gal4*.

the expression of *bantam-GFP* reporter, which inversely correlates with the expression of *bantam*, was increased by overexpressing SdBP (Supplementary information, Figure S3C-S3C"). Briefly, SdBP inhibits tissue growth by restricting the expression of the Hpo pathway target genes.

To further dissect the function of SdBP in the Hpo pathway, we used mosaic analysis with a repressible cell marker (MARCM) system to express SdBP in *hpo* or *sav* mutant clones. We found that eye discs carrying hpo^{BF33} or sav^{SH13} mutant clones exhibited overgrowth and elevated DIAP1 levels, resulting in enlarged and folded adult eyes (Figure 2C-2C"' and 2E-2E"'). Over-expressing SdBP in these mutant clones antagonized the overgrowth of mutant clones and the increase of DIAP1 in clone regions, resulting in nearly normal adult eyes (compare Figure 2D-2D"' and 2F-2F"' with 2C-2C"' and 2E-2E"'). These pieces of evidence suggest that SdBP represses overgrowth phenotypes induced by loss of function of upstream Hpo signaling members.

TDU domains and PPXY motifs mediate the interactions of SdBP with Sd and Yki, respectively

We have demonstrated that SdBP induces growth suppression through antagonizing the activity of Sd-Yki complex. We next sought to investigate the mechanisms

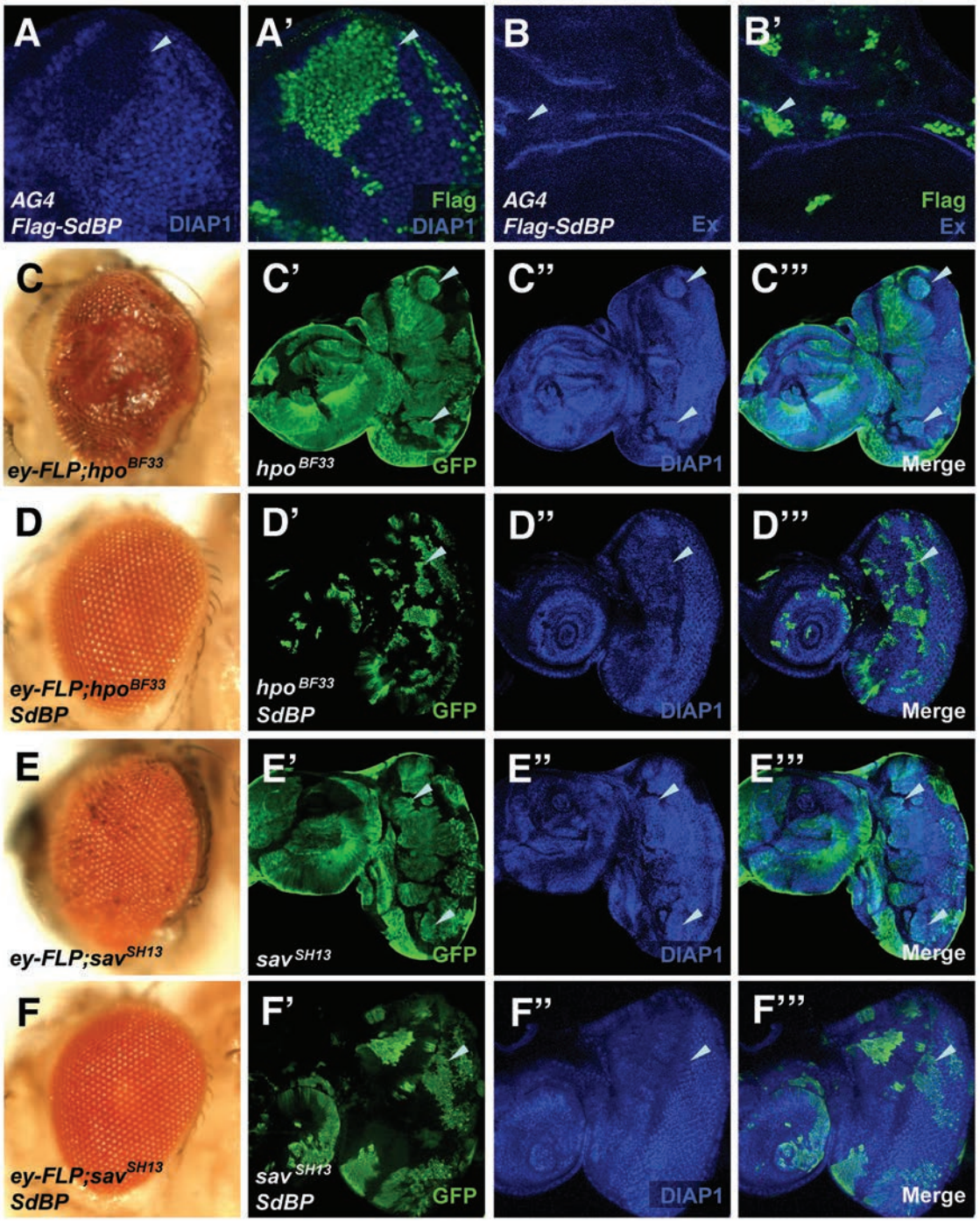


Figure 2 SdBP inhibited growth through downregulating the expression of the Hpo pathway target genes. **(A-B')** Overexpression of SdBP downregulated the expression of the Hpo pathway target genes under the control of *act > CD2 > Gal4 (AG4)*. SdBP overexpression region was marked by Flag (green). DIAP1 **(A-A')** and Ex **(B-B')** were stained (blue). Arrowheads indicated the clone regions. **(C-F''')** Eye discs bearing *hpo* mutants **(C-C''')**, *hpo* mutants overexpressing SdBP **(D-D''')**, *sav* mutants **(E-E''')**, and *sav* mutants overexpressing SdBP **(F-F''')**. GFP marked clones, and discs stained with DIAP1 (blue). Arrowheads indicated the clone regions.

by which SdBP regulates Sd-Yki activity. According to its primary sequence (Figure 3A), we generated several SdBP variants that contained mutations in its two TDU domains. TDU domain is highly conserved and has been

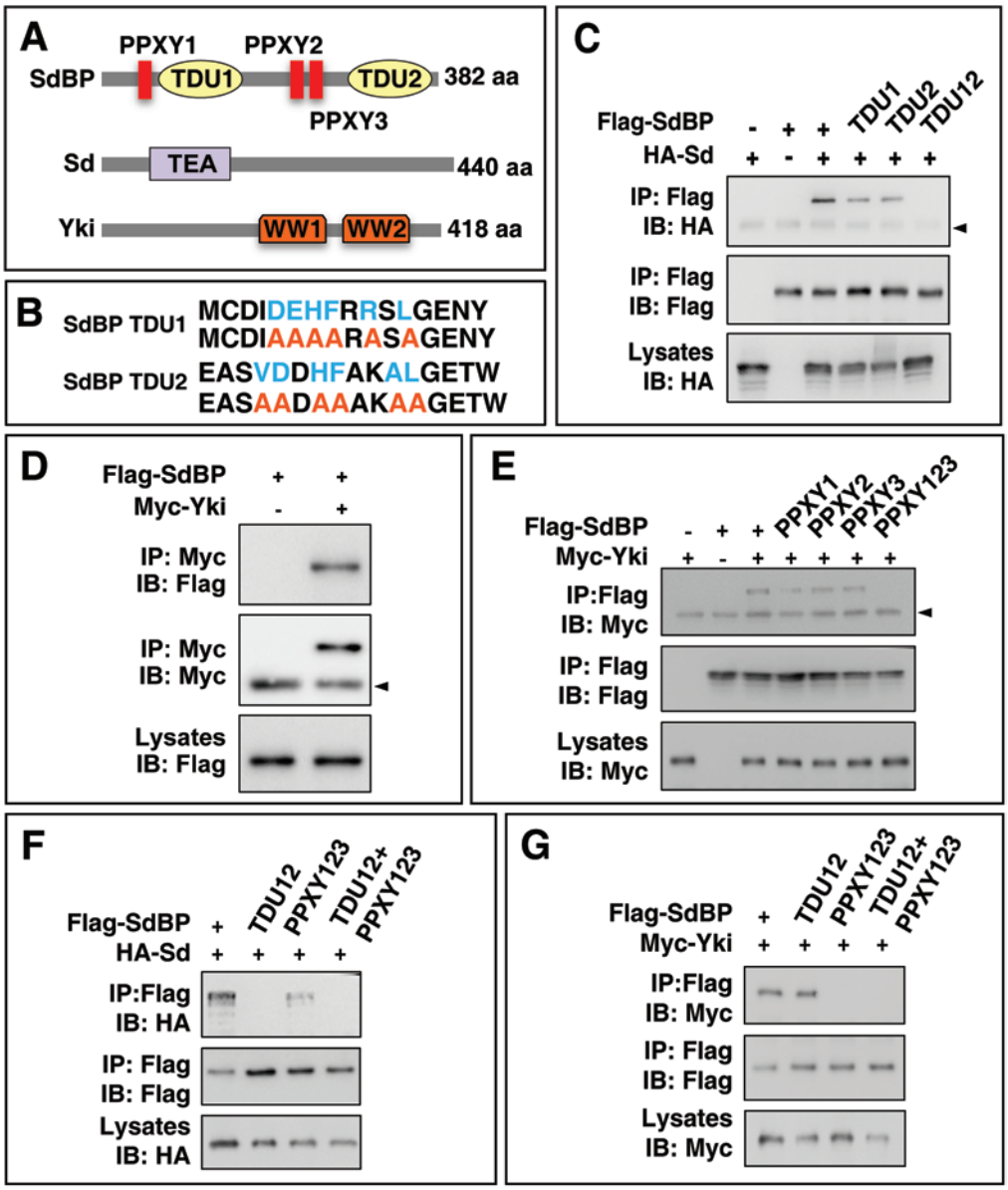


Figure 3 TDU domains and PPXY motifs mediated the interaction between SdBP and Sd/Yki. **(A)** Domain organization of SdBP, Sd and Yki. SdBP contains two TDU domains and three PPXY motifs, and Yki has two WW domains at its C terminus. **(B)** TDU domain mutations generated in SdBP. Wild-type amino acids were shown in blue, and corresponding mutations were shown in red. **(C)** Co-immunoprecipation of Sd and the indicated SdBP TDU variants. The arrowhead indicates IgG chain. **(D)** Myc-Yki and 3× Flag-SdBP interacted with each other in S2 cells. The arrowhead indicates IgG chain. **(E)** Co-immunoprecipitation between Yki and the indicated SdBP PPXY variants. The arrowhead indicates IgG chain. **(F, G)** Co-immunoprecipitation assay showed that SdBP interacted with Sd and Yki variants. SdBP TDU variants only disrupted binding between SdBP and Sd, but not Yki **(F)**, and *vice versa* **(G)**. However, SdBP-(TDU12+PPXY123) bound with neither Sd nor Yki. The indicated constructs in **C**-**G** were co-transfected into S2 cells, followed by western blot assay directly or after immunoprecipitation.

shown to interact with TEAD/TEF family members [36]; therefore, we speculated that these two TDU domains might mediate the binding between SdBP and Sd. To verify this hypothesis, we performed co-immunoprecipitation and GST pull-down experiments using SdBP variants that carry mutations either in both TDU domains (TDU12) or in one of the TDU domains (TDU1 and TDU2) (Figure 3A-3C and Supplementary information, Figure S4A). The association between SdBP and Sd was completely abolished when both TDU domains were mutated, while

the binding was still retained when single TDU domain was mutated (Figure 3C and Supplementary information, Figure S4A). These results suggest that both the TDU domains of SdBP are involved in the association of SdBP with Sd.

It was also reported that the Hpo pathway components utilize the WW domain and PPXY motif for protein-protein interactions in a common way [37, 38]. Noticing that SdBP contains three PPXY motifs (Figure 3A), we speculated that a binding between SdBP and Yki through PPXY motifs and WW domains might exist. To verify this hypothesis, we performed co-immunoprecipitation and Y2H assays. An interaction between SdBP and Yki was detected (Figure 3D and Supplementary information, Figure S4B). To confirm whether the binding is mediated by PPXY motifs and WW domains, SdBP variants carrying mutations either in all PPXY motifs (PPXY123) or in one of the PPXY motifs (PPXY1, PPXY2 and PPXY3) were used in co-immunoprecipitation (Figure 3E) and GST pull-down assays (Supplementary information, Figure S4C). We found that all three PPXY motifs are involved in mediating the binding between SdBP and Yki as only the triple mutation completely abolished the binding between SdBP and Yki (Figure 3E and Supplementary information, Figure S4C). In addition, deletion of both C-terminal WW domains of Yki abolished SdBP-Yki interaction (Supplementary information, Figure S4D). In brief, SdBP binds to Yki WW domains through its PPXY motifs.

To further explore the binding specificity between SdBP and Sd-Yki complex, we applied co-immunoprecipitation and GST pull-down assays, and found that TDU12 did not affect SdBP-Yki association, and PPXY123 did not affect SdBP-Sd interaction (Figure 3F and 3G and Supplementary information, Figure S4E), implying that SdBP-Sd association and SdBP-Yki association are independent of each other. Moreover, the SdBP variant carrying mutations in both TDU domains and all PPXY motifs (TDU12+PPXY123) did not bind to either Sd or Yki (Figure 3F and 3G and Supplementary information, Figure S4E). In addition, no direct interactions between SdBP and other known Hpo pathway components, such as Mer, Wts or Mats, were detected (Supplementary information, Figure S4F), suggesting that the function of SdBP *in vivo* in growth control is through its association with Sd-Yki complex.

TDU domains and PPXY motifs are critical for the function of SdBP in growth inhibition

We next focused on exploring whether the function of SdBP in growth inhibition was dependent on TDU domains and PPXY motifs. Using a dual luciferase assay, we found that both SdBP variants, TDU12 and PPXY123, weakened the strength of the function of SdBP on Sd-Yki activity, whereas TDU12+PPXY123 no longer suppressed Sd-Yki activity (Figure 4A), implying that both TDU domains and PPXY motifs played important roles in regulating Sd-Yki activity *in vitro*. To further confirm these findings, we generated transgenic flies carrying different combination of mutations of these domains. Similar to what have been observed in luciferase assay (Figure 4A), when coexpressing SdBP variants with Yki under the control of *GMR* driver, only TDU12+PPXY123 was totally incapable of inhibiting the overgrowth phenotype induced by *GMR-Yki* (compare Figure 4G with 4B-4F). TDU12+PPXY123 might also have a dominant-negative function as its expression drove the eyes even larger and more folded than the *GMR-Yki* control (compare Figure 4G with 4C). When expressing the SdBP variants under the control of *MS1096,* TDU12 showed slightly reduced wings compared with control while PPXY123 led to smaller wings (Figure 4H-4K and 4M). Similar to what have been observed in eyes, TDU12+PPXY123 had a dominant-negative effect and resulted in even bigger wings (compare Figure 4L with 4H, and 4M). Meanwhile, we also tested the changes of downstream markers of the Hpo pathway when expressing these variants under the control of *hhGal4* driver. Consistent with results presented above, TDU12 and PPXY123 decreased the protein level of DIAP1, while TDU12+PPXY123 led to no discernible change in DIAP1 level (compare Figure 4P-4P' and 4Q-4Q' with 4N-4O' and 4R-4R'). Of note, expression of a TDU domain deletion form of SdBP (SdBP-TDUdel, in which both TDU domains were deleted) by *hhGal4* also decreased the protein level of DIAP1 (Supplementary information, Figures S5), suggesting that the function of SdBP did not solely depend on TDU domains. Taken together, these findings indicate that both TDU domains and all PPXY motifs are involved in SdBP-induced growth inhibition.

SdBP forms a complex with Sd and Yki in the nucleus

We have demonstrated that SdBP binds to Sd and Yki independently and suppresses their activity, however, the detailed mechanism by which SdBP suppresses Sd-Yki activity is unclear. To figure out the underlying mechanism, we performed a two-step immunoprecipitation experiment. We found that SdBP, Sd and Yki associate with each other and form a ternary complex (Figure 5A). To confirm this, we examined the localization of these proteins in *Drosophila* wing discs by overexpression. To clearly visualize the localization of these proteins, we generated GFP-fused SdBP (SdBP-GFP) for

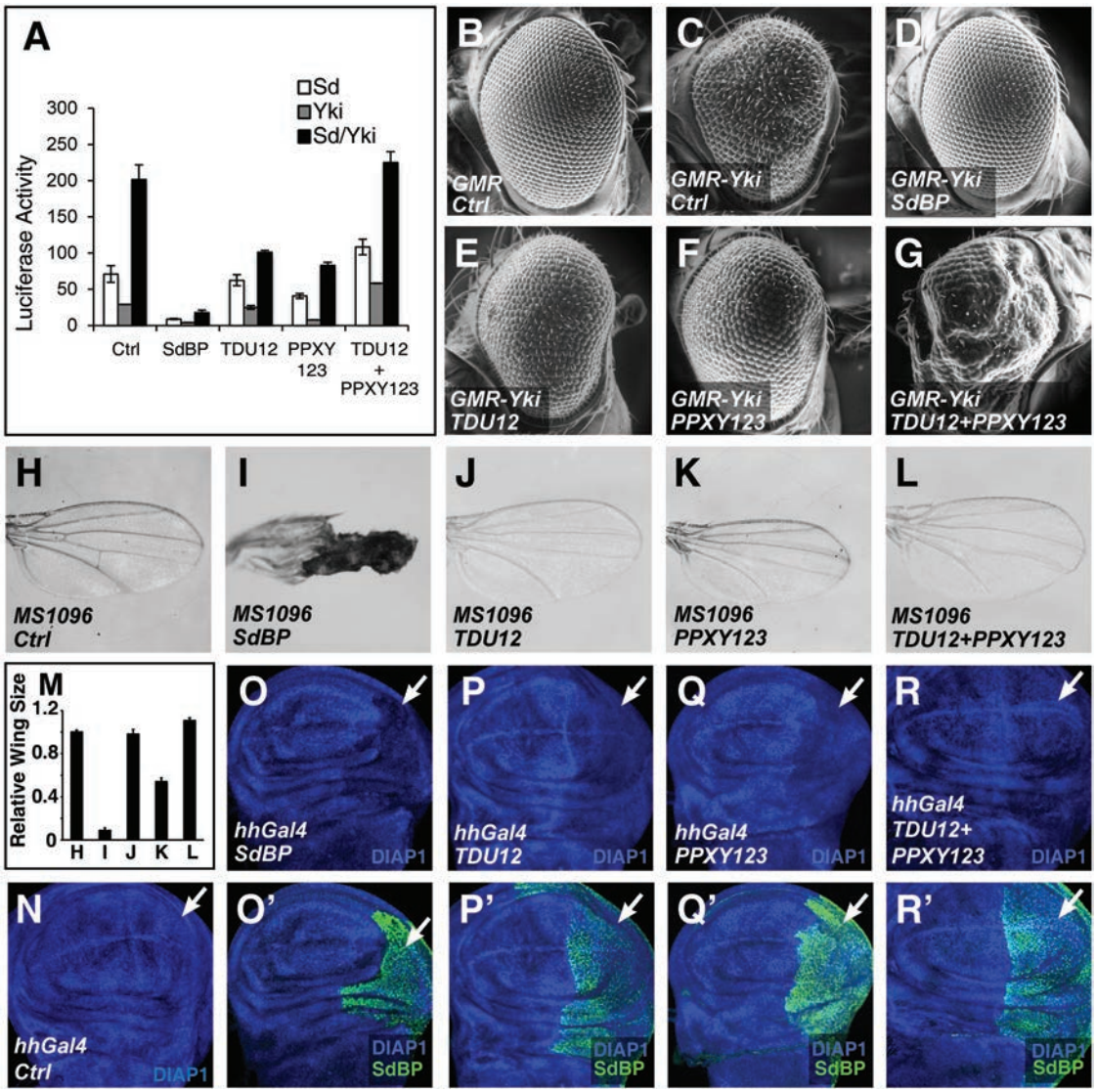


Figure 4 TDU domains and PPXY motifs are critical for SdBP function in growth inhibition. **(A)** Luciferase assay showed that SdBP variants inhibited Sd-Yki activity to different degrees. The indicated plasmids were co-transfected with 3× Sd luciferase reporter, and luciferase activity was measured by standard dual-luciferase protocols. Data were presented as mean ± s.d. (n = 3). **(B-G)** Scanning electron microscope images of *Drosophila* compound eyes from the following genotypes: *GMR-Gal4* **(B)**, *GMR-Gal4/UAS-Yki* **(C)**, *GMR-Gal4/UAS-(Yki+SdBP)* **(D)**, *GMR-Gal4/UAS-(Yki+TDU12)* **(E)**, *GMR-Gal4/UAS-(Yki+PPXY123)* **(F)**, and *GMR-Gal4/UAS-(Yki+(TDU12+PPXY123))* **(G)**. **(H-L)** Adult wings of wild-type **(H)**, or wings expressing *UAS-SdBP* **(I)**, *UAS-TDU12* **(J)**, *UAS-PPXY123* **(K)**, and *UAS-(TDU12+PPXY123)* **(L)** under the control of *MS1096*. **(M)** Quantification of relative wing sizes (fold change) of samples indicated in **H**-**L**. **(N-R')** Control wing discs **(N)** and wing discs expressing *UAS-SdBP* **(O-O')**, *UAS-TDU12* **(P-P')**, *UAS-PPXY123* **(Q-Q')** and *UAS-(TDU12+PPXY123)* **(R-R')** by *hhGal4*. SdBP (green) and DIAP1 (blue) were stained. Arrows indicated the P-compartment.

in vivo observation (Supplementary information, Figure S6A-S6A") and confirmed that there are no discernible functional differences between wild-type SdBP and SdBP-GFP (Supplementary information, Figure S6B-S6B"'). Interestingly, coexpression of SdBP-GFP and Yki resulted in an even distribution of Yki in cells unlike in the control where Yki mainly locates in the cytoplasm (compare Figure 5D and 5D" with 5B-5B'). This even distribution of Yki in cells was also observed when Sd and Yki were coexpressed (Figure 5C-5C'). When SdBP, Sd and Yki were coexpressed, the majority of Yki anchored in the nucleus (Figure 5E-5E"'), implying that Yki is retained in the nucleus upon SdBP coexpression. Moreover, expressing SdBP inhibited Sd-

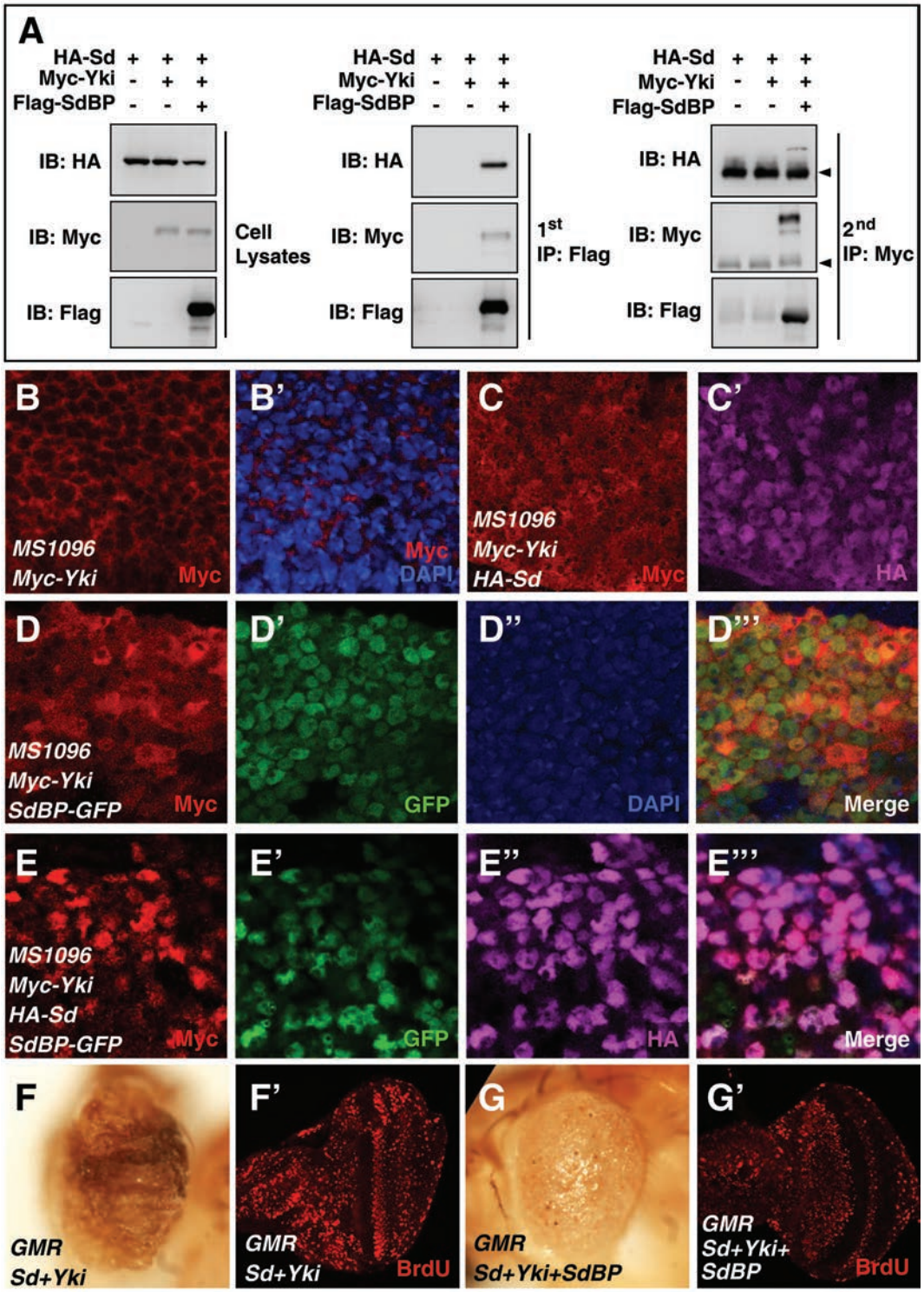


Figure 5 SdBP formed a complex with Sd-Yki. **(A)** S2 cells expressing the indicated proteins were collected for the two-step immunoprecipitation and analyzed by western blot. Cell lysates were first immunoprecipitated with M2-Flag beads, and then eluted for a second immunoprecipitation with Myc antibody. The arrowheads indicate IgG chain. **(B-E''')** Confocal images of third-instar wing discs expressing Myc-Yki **(B-B')**, or Myc-Yki with HA-Sd **(C-C')** or Myc-Yki with SdBP-GFP **(D-D''')** or Myc-Yki with HA-Sd and SdBP-GFP **(E-E''')**. Discs were stained with α-Myc (red), α-HA (magenta) antibodies and DAPI (blue). **(F-G')** Adult eyes expressing *GMR-Gal4/UAS-(Sd+Yki)* **(F)** and *GMR-Gal4/UAS-(Sd+Yki+SdBP)* **(G)**. BrdU staining of eye discs expressing *UAS-(Sd+Yki)* **(F')** and *UAS-(Sd+Yki+SdBP)* **(G')** under the control of *GMR-Gal4*.

Yki overexpression-induced overgrowth of adult eyes as well as BrdU increase (Figure 5F-5G'), further indicating that SdBP suppresses the activity of Sd-Yki complex. In summary, SdBP retains Yki in the nucleus and simultaneously forms a complex with Sd, while Sd-Yki activity is inhibited by the presence of SdBP.

SdBP and Yki compete with each other for Sd to control tissue growth

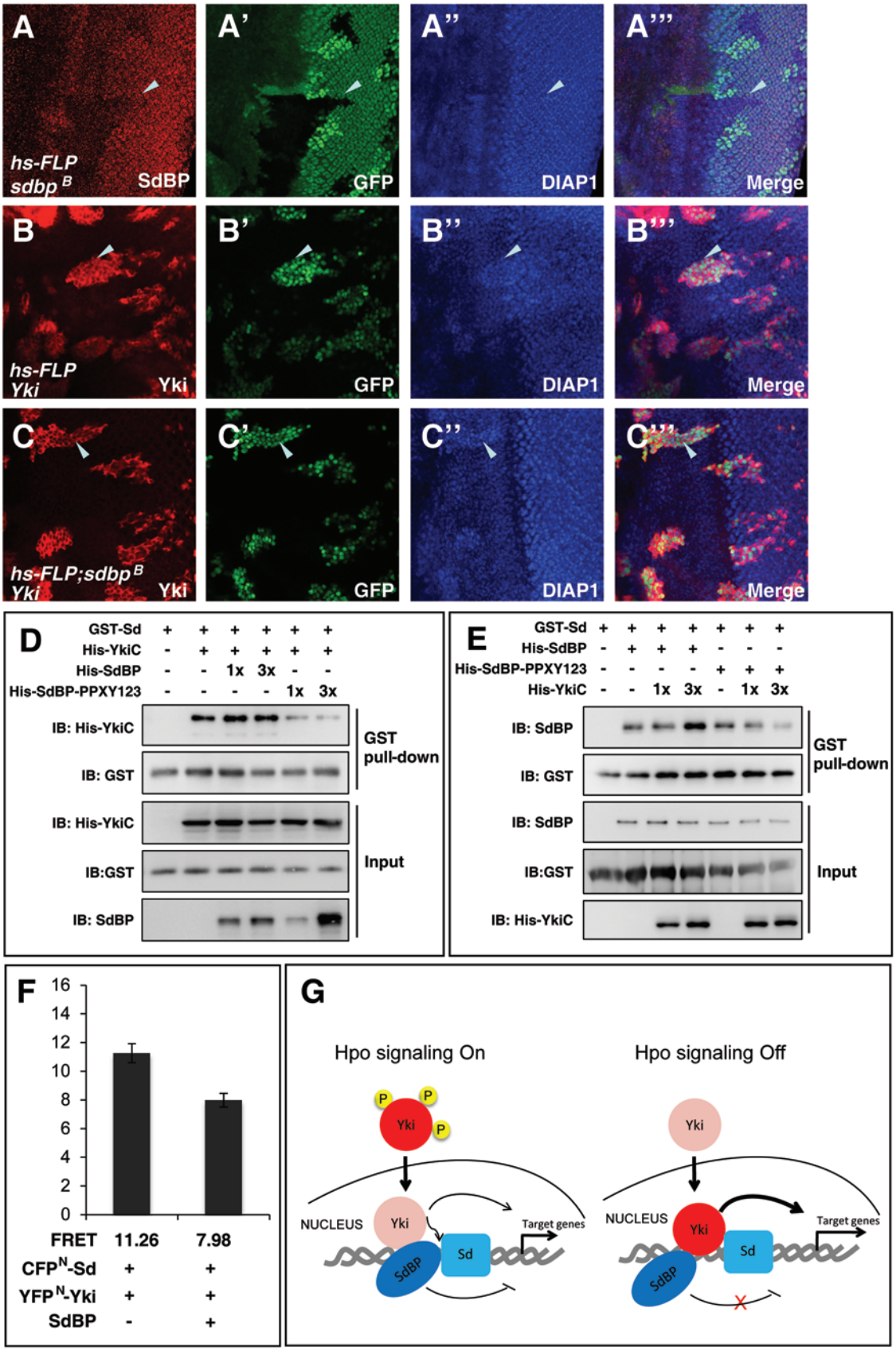
We showed that overexpressed SdBP forms a complex with Sd-Yki in the nucleus and inhibited their activity. To further explore the role of SdBP in the Hpo pathway, we generated a null mutation by "ends-out" gene targeting in which most of the *SdBP* coding sequence was replaced by *white* gene [39]. Three independent null alleles, $sdbp^{A}$, $sdbp^{B}$ and $sdbp^{36}$, were isolated. Homozygotes of these alleles exhibit lethal phenotypes before the third instar larval stage. We used $sdbp^{B}$ allele to carry out further analyses (Supplementary information, Figure S7A-S7A"). Interestingly, no discernible changes of clonal growth and the protein levels of DIAP1 and Ex were observed in the $sdbp^{B}$ mutant clones in eye and wing imaginal discs (Figure 6A-6A"' and Supplementary information, Figure S7B-S7B"). Similar to the phenotypes induced by the loss of *sd* in eye discs [15], loss of *sdbp* did not affect the expression of the Hpo pathway downstream markers. In addition, loss of *sdbp* did not affect the protein levels and subcellular localization of Sd or Yki (Supplementary information, Figure S7C-S7D"). It has been shown previously that Sd is absolutely required for tissue overgrowth induced by excessive Yki activity and loss of Sd suppresses the overgrowth phenotype as well as ectopic Hpo target gene expression caused by Yki overexpression [15]. Nevertheless, compared with Yki overexpression control (Figure 6B-6B"'), loss of *sdbp* exhibited neither decrease nor increase in clone size or ectopic *diap1* expression induced by Yki overexpression in $sdbp^{B}$ MARCM clones (Figure 6C-6C"'). Altogether, although neither *sd* nor *sdbp* is essential for normal eye development, the difference between them is that loss of *sdbp* does not influence the phenotypes induced by excessive Yki activity in eye imaginal discs.

In order to explain how the transcriptional activity of nuclear Yki is inhibited by SdBP coexpression, we performed dosage-dependent GST pull-down and co-immunoprecipitation assays. We found that, upon SdBP binding, wild-type SdBP did not affect the stability of Sd-Yki complex while its variant carrying PPXA mutation in all PPXY motifs (SdBP-PPXY123) competed with Yki for Sd (Figure 6D and Supplementary information, Figure S7E). Conversely, we found that, Yki also competed with SdBP-PPXY123 (Figure 6E and Supplementary information, Figure S7F). These findings suggest that SdBP and Yki compete with each other for Sd binding in a concentration-dependent manner. However, only SdBP PPXY variant shows the dual competition (Figure 6D-6E). Instead, wild-type SdBP forms a complex with Sd-Yki (Figure 5A). To further investigate this intriguing finding, we employed the fluorescence resonance energy transfer (FRET) assay to detect the influence exerted by SdBP on the protein-protein interaction between Sd and Yki. In FRET assay, the transfer of energy between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) was measured. Fusion proteins with CFP fused to the N-terminus of Sd (CFP^{N}-Sd) and YFP fused to the N-terminus of Yki (YFP^{N}-Yki) were generated to examine whether the interaction between Sd and Yki could be affected by coexpressing SdBP in S2 cells. We observed that the FRET efficiency was decreased between CFP^{N}-Sd and YFP^{N}-Yki when SdBP was cotransfected (Figure 6F), suggesting that SdBP competes with Yki for Sd while simultaneously interacts with Yki via its PPXY motifs, resulting in the nuclear retention and dysfunction of Yki protein.

Discussion

The evolutionarily conserved Hpo signaling pathway has emerged as a pivotal pathway that plays a critical role in controlling tissue growth and organ size. The Sd-Yki

Figure 6 SdBP and Yki competed with each other for Sd to control growth. **(A-C"')** Confocal images of third-instar wing discs bearing *hs-FLP* clones of $sdbp^{B}$ mutant **(A-A"')**, GFP-labeled MARCM clones with Yki overexpression **(B-B"')**, and GFP-labeled MARCM clones of $sdbp^{B}$ with Yki overexpression **(C-C"')**. Cells in clone regions were labeled by SdBP (red), Yki (red), DIAP1 (blue) and GFP (green). **(D)** GST pull-down assay showed that wild-type SdBP did not affect the stability of Sd-Yki complex while SdBP-PPXY123 competed with Yki for Sd. His-YkiC plus dosage increased wild-type SdBP or SdBP-PPXY123 was pulled down by GST-Sd, followed by western blot using the indicated antibodies. **(E)** GST pull-down assay showed that Yki also competed with SdBP-PPXY123 for Sd. Wild-type SdBP or SdBP-PPXY123 plus dosage increased His-YkiC were pulled down by GST-Sd, followed by western blot using the indicated antibodies. **(F)** S2 cells were cotransfected with CFP^{N}-Sd and YFP^{N}-Yki. The FRET efficiency was decreased in the presence of SdBP. Data are presented as mean ± s.d. ($n \geq 12$). **(G)** A model of how Hpo signaling regulates the competition between Yki and SdBP for Sd binding. See discussion for details.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His-SdBP-PPXY123 - - - - 1x 3x
IB: His-YkiC
IB: GST
GST pull-down
IB: His-YkiC
IB:GST
IB: SdBP
Input
E
GST-Sd + + + + + + +
His-SdBP - + + + - - -
His-SdBP-PPXY123 - - - - + + +
His-YkiC - - 1x 3x - 1x 3x
IB: SdBP
IB: GST
GST pull-down
IB: SdBP
IB:GST
IB: His-YkiC
Input
F
16
14
12
10
8
6
4
2
0
FRET 11.26 7.98
CFP N-Sd + +
YFP N-Yki + +
SdBP - +
G
Hpo signaling On
Hpo signaling Off
P
P
P
Yki
NUCLEUS
Yki
Target genes
Sd
SdBP
Yki
NUCLEUS
Yki
Target genes
Sd
SdBP
X

transcription complex serves as an essential downstream transcriptional effector and regulates Hpo target gene expression. In this study, using genetic and biochemical analyses, we identified SdBP as a novel negative regulator of Sd-Yki complex and found that it suppresses cell growth. Our data show that SdBP simultaneously binds to Sd through TDU domains and Yki via PPXY motifs (Figure 6). This association disrupts the direct interaction between Yki and Sd (Figure 6F), but results in a transcriptional inactivated ternary complex to precisely regulate the direction and intensity of Hpo signaling. We reason that SdBP competes with Yki for the binding to Sd, as a result, within the ternary Sd-SdBP-Yki complex, Yki binds to PPXY motifs of SdBP, leading to an inhibition of Yki activity. Indeed, ectopic SdBP expression clearly suppresses the activity of Yki despite its nuclear location (Figure 5).

We further uncover a mutual competition of SdBP and Yki in binding to Sd. SdBP and Yki compete for Sd with a balance in normal physiological condition, resulting in neither growth defects nor growth advances. The Yin-Yang harmonization might promote the relative equilibrium of Hpo signaling in growth control. In the normal physiological condition, SdBP forms a repressor complex with Sd through unknown mechanisms in the nucleus, while the majority of Yki proteins are restricted in the cytoplasm by the upstream Hpo signaling (Figure 6G). Of note, Yki should be more competitive compared with SdBP, suggested by the structure information of the YAP-TEAD complex and the Vgll1-TEAD complex [40]. Similar to the YAP/Vgll1 and TEAD complexes, Yki and SdBP may form interfaces 1 and 2 in the TEAD-interacting regions and may compete for binding to the same surface on TEAD, yet, Yki may contain an extra interface 3 compared with SdBP. Therefore, a low concentration of nuclear Yki protein may be sufficient to antagonize SdBP for normal tissue growth. However, a small amount of nuclear Yki is essential for tissue homoeostasis under normal conditions as suggested by previous finding that *yki* mutant clones grew poorly [41]. This might be due to the fact that in the absence of Yki, the fine balance between Yki and SdBP is disturbed, thus SdBP readily binds to Sd for efficient repression due to its nuclear localization. On the other hand, in the absence of SdBP, the existing small amount of nuclear Yki proteins can hardly induce the excessive expression of the Hpo pathway target genes, while the majority of Yki proteins are restricted in the cytoplasm by Hpo signaling; therefore, no obvious growth advances were observed in *sdbp* mutant clones (Figure 6A-6A''' and Supplementary information, Figure S7B). In the absence of Hpo signaling or in the case of Yki overexpression, abundant Yki proteins translocate into the nucleus and dominate for Sd regardless of whether SdBP existed (Figure 6G), resulting in elevated expression of Hpo pathway target genes (Figures 2C-2C''', 2E-2E''' and 6B-6C''').

It has been shown previously that Sd acts in conjunction with Vestigial (Vg) and other transcription factors to promote wing development by directly regulating the expression of wing patterning genes [42, 43]. In addition to its expression in wing discs as Vg, Sd is also expressed in other tissues and plays a broader role than Vg during development. In conjunction with Yki, Sd may be required for the basal expression of genes that can be suppressed by Hpo signaling in other tissues. Importantly, Sd is required for tissue overgrowth by regulating the expression of genes involved in cell proliferation, cell growth, and apoptosis when Yki is hyper-activated [15-17].

In mammals, four genes encode TDU domain-containing proteins. Vestigial like 1, 2 and 3 have only one TDU domain; vestigial like 4 (Vgll4/Vgl4) has two TDU domains. It has been reported that Vgll4 is the mammalian homolog of SdBP and binds to TEAD [44]. Interestingly, we identified that Vgll4 was frequently downregulated in human lung cancer specimens as a novel tumor suppressor through the direct competition with YAP for binding to TEAD4 (Zhang WJ *et al*., in submission). In contrast to SdBP, Vgll4 has 2 TDU domains but no PPXY motifs. Although Vgll4 cannot form a complex with YAP-TEADs in a way similar to SdBP, the competition of SdBP for Yki activity may be functionally and evolutionally conserved from *Drosophila* to mammals. This is consistent with the independent study by Koontz *et al*. showing that SdBP (named as Tondu-domain containing Growth Inhibitor (Tgi) in their study) and Vgll4 are conserved to work as negative regulators of Yki/YAP through a direct competition for Sd/TEAD2 binding [45].

Introduction of SdBP to Sd-Yki complex may bring us new ideas for the regulation of downstream Hpo pathway. As YAP is a known proto-oncogene in many cancers, the identification of SdBP/Vgll4 may provide us a promising therapeutic strategy for inhibiting YAP tumorigenic function in the future.

Materials and Methods

Y2H screens

Sd C-terminus (208-440 aa) fragment was used as baits to screen a *Drosophila* embryo cDNA library using the Matchmaker Gold Yeast Two-Hybrid system according to manufacturer's instructions (Clontech). SdBP-Sd interacting clones were selected for their ability to activate four independent reporter genes (*AUR1-C*, *ADE2*, *HIS3* and *MEL1*). The interaction between SdBP and Sd was further confirmed by co-immunoprecipitation. The interaction

between SdBP and Yki was confirmed by Y2H assays. pGBKT7-Sd-C/pACT2-Yki and pGBKT7-Yki/pACT2-Sd were used as a positive control mating. pGBKT7-Sd-C/pACT2 and pGBKT7-Yki/pACT2 were used as a negative control mating.

Cloning of expression constructs

SdBP cDNA or its variants were amplified by PCR and introduced into the pUAST-Flag or pUAST-3× Flag vector by using the restriction enzymes *Bgl*II and *Xho*I. pUAST-Myc-Yki and pUAST-HA-Sd constructs were previously described [15]. All PCR-amplified products were sequence-verified.

Drosophila genetics

All crosses and staging were done at 25 °C unless specifically indicated. *UAS-SdBP* or its variants flies were generated by cloning *SdBP* or its variants after PCR amplification into the *pUAST*, and injected into *yw* flies. For flip-out ectopic expression clones, UAS-transgenes with *Flag-SdBP* were crossed to *AG4* flies. All lines gave qualitatively identical phenotypes. *SdBP RNAi* stock (no. 34394) was provided by Bloomington *Drosophila* Stock Center (BDSC). *HA-Sd;Myc-Yki* recombinants were generated from the described stocks. Ectopic expression was induced in eyes using *GMR-Gal4*, in wings using *MS1096*, and in posterior cells using *hhGal4*. Mutant clones were generated using the MARCM system [46]. The genotypes for generated clones are as follows: (Figure 2C-2C''') *ey-FLP/+; FRT42D hpo^{BF33}/FRT42D tub-Gal80; +/tub-Gal4 UAS-GFP*. (Figure 2D-2D''') *ey-FLP/+; FRT42D hpo^{BF33}/FRT42D tub-Gal80; UAS-SdBP/tub-Gal4 UAS-GFP*. (Figure 2E-2E''') *ey-FLP; UAS-GFP tub-Gal4/+; FRT82B sav^{SH13}/FRT82B tub-Gal80*. (Figure 2F-2F''') *ey-FLP; UAS-GFP tub-Gal4/UAS-SdBP; FRT82B sav^{SH13}/FRT82B tub-Gal80*. (Figure 6B-6B''') *hs-FLP tub-Gal4 UAS-GFP; UAS-Yki/+; FRT80B/FRT80B tub-Gal80*. (Figure 6C-6C''') *hs-FLP tub-Gal4 UAS-GFP; UAS-Yki/+; FRT80B sdbp^{B}/FRT80B tub-Gal80*. (Figure 6A-6A''', Supplementary information, Figure S7A'', S7B-S7B'', S7C-S7C'', S7D-S7D'') *hs-FLP; ;FRT80B sdbp^{B}/FRT80B GFP*. For generated SdBP mutant, SdBP knockout construct was generated according to the ends-out gene targeting strategy [39]. The targeting construct is expected to replace most of the coding sequence of *sdbp* with *white* gene in pW25 vector. Two pairs of oligos: 1) 5′-ATTTGCGGCCGCCAGTTGCAGTTGCACTCAGGTTCGT-3′ and 5′-ATTTGCGGCCGCGCTGTGGCTTTGCGGTTGATTTGGG-3′ and 2) 5′-TTGGCGCGCCCCAATTCCCAACAGAACTCAACACA-3′ and 5′-TTGGCGCGCCCAGCATTATTGTTGCTCGACGTGTT-3′ were used to amplify DNA fragments of about 4.8 kb from *Drosophila* genomic DNA. These two fragments were cloned into the *Not*I and *Asc*I sites of pW25, respectively. Flies carrying the targeting construct on the second chromosome were crossed to *y[1] w[*]; P{70FLP}23 P{70I-SceI}4A/TM6* (BDSC), and the following steps were performed as described previously [39].

Cell culture, transfection, co-immunoprecipitation, western blot, real-time PCR and luciferase reporter assays

Drosophila S2 cells were maintained in Schneider's medium supplemented with 10% fetal bovine serum at 25 °C. Cells were transiently transfected using Lipofectamine (Invitrogen) according to the manufacturer's instructions. After 48 h of expression, cells were lysed in 1% Trition X-100 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100 and 1 mM EGTA) supplemented with Protease Inhibitor Cocktail (Roche). For co-immunoprecipitation, cells were lysed in NP-40 buffer (50 mM Tris-Cl, pH 8.0, 0.1 M NaCl, 1% NP-40, 10% Glycerol, 1.5 mM EDTA, 10 mM Sodium fluoride, 1 mM Sodium vanadate, Protease Inhibitor Cocktail) for 30 min at 4 °C. Lysates were incubated with the indicated antibodies for 2 h or overnight at 4 °C. Samples were combined with 25 μl Protein A/G PLUS agarose (Santa cruz) for 1 h at 4 °C. Beads were washed three times with NP-40 buffer, followed by western blot assay. For two-Step IP, Flag M2 affinity gel (Sigma) was used in the first-step IP, beads from the first IP were eluted with 3× Flag peptide (Sigma) and then mixed with Myc antibody in the second-step IP. Western blot was performed according to standard protocols. Protein gels were run and blotted using the Mini-PROTEAN Tetra Electrophoresis system (Biorad). Purified and associated proteins were detected by western blot analysis using chemiluminescence (Western Lightning Plus-ECL, PerkinElmer). Western blots were probed with antibodies against mouse α-HA (Sigma), mouse α-Flag (Sigma), mouse α-Myc (Sigma), mouse α-His (Sigma), and mouse α-HRP (Santa cruz) using dilutions of 1:5 000 for all antibodies. Rabbit α-SdBP antibody was generated using SdBP full-length protein, and used with dilution of 1:1 000 in western blot. Real-time PCR was performed using standard protocols. Luciferase reporter gene assay was performed using protocols as described previously [15].

GST pull-down assay

Glutathione agarose beads (GE) were used to purify the indicated proteins. These GST fusion beads were incubated with either S2 cell lysates or purified proteins in GST pull-down lysis buffer (20 mM Tris-Cl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% NP-40 and 1% PMSF) at 4 °C for 1 h, followed by washing three times with GST pull-down lysis buffer and western blot analysis.

Immunostaining

Wing and eye imaginal discs from third-instar larvae were dissected, fixed for 25 min in 4% formaldehyde and washed three times in PBS supplemented with 0.1% Triton X-100 (PBS-T). Discs were incubated in the primary antibody diluted in PBS-T for 2 h at room temperature, followed by three washes with PBS-T and incubation with a secondary antibody in PBS-T for 2 h at room temperature. After three further washes, discs were mounted in PBS/glycerol medium with DAPI. Dilutions for the antibodies against mouse α-Myc, mouse α-HA, mouse α-Flag, mouse α-CD2 (Invitrogen) and rabbit α-SdBP were 1:200. Antibodies: rabbit α-Sd (1:50, produced by immunizing rabbits with the peptide of Sd amino acids 208-440) and rabbit α-Yki (1:50, produced by immunizing rabbits with the peptide of Yki amino acids 180-418) were used. Fluorescent stains were captured on a Leica LAS SP5 confocal microscope.

FRET assay

CFP^{N}-Sd and YFP^{N}-Yki were transfected into S2 cells together with an ubi-Gal4 expression vector. Cells were washed with PBS, fixed with 4% formaldehyde for 20 min, and mounted on slides in 80% glycerol. Fluorescence signals were acquired with the 63× objective of a Leica LAS SP5 confocal microscope. Each data set was based on 12-15 individual cells. In each cell, three to four regions of interest in photobleached area were selected for analysis. The intensity change of CFP was analyzed using the Leica soft-

ware. The efficiency of FRET was calculated according to a previous paper [47].

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)