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Report

SCALLOPED Interacts with YORKIE, the Nuclear Effector of the Hippo Tumor-Suppressor Pathway in *Drosophila*

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Summary

In *Drosophila*, SCALLOPED (SD) belongs to a family of evolutionarily conserved proteins characterized by the presence of a TEA/ATTS DNA-binding domain [1, 2]. SD physically interacts with the product of the *vestigial* (*vg*) gene, where the dimer functions as a master gene controlling wing formation [3, 4]. The VG-SD dimer activates the transcription of several specific wing genes, including *sd* and *vg* themselves [5, 6]. The dimer drives cell-cycle progression by inducing expression of the *dE2F1* transcription factor [7], which regulates genes involved in DNA replication and cell-cycle progression. Recently, YORKIE (YKI) was identified as a transcriptional coactivator that is the downstream effector of the Hippo signaling pathway, which controls cell proliferation and apoptosis in *Drosophila* [8]. We identified SD as a partner for YKI. We show that interaction between YKI and SD increases SD transcriptional activity both ex vivo in *Drosophila* S2 cells and in vivo in *Drosophila* wing discs and promotes YKI nuclear localization. We also show that YKI overexpression induces *vg* and *dE2F1* expression and that proliferation induced by YKI or by a dominant-negative form of FAT in wing disc is significantly reduced in a *sd* hypomorphic mutant context. Contrary to YKI, SD is not required in all imaginal tissues. This indicates that YKI-SD interaction acts in a tissue-specific fashion and that other YKI partners must exist.

Results and Discussion

YORKIE (YKI) encodes a transcriptional coactivator that is the downstream effector of the Salvador-Warts-Hippo signaling pathway [8]. This pathway consists of the two serine-threonine kinases, HIPPO [9, 10] and WARTS (WTS, also known as Large Tumor Suppressor) [11], the adaptator molecules SALVADOR (SAV) [11, 12] and Mob as Tumor Suppressor (MATS) [13], the FERM domain proteins EXPANDED (EX) and MERLIN (MER) [14], and the protocadherin FAT (FT) [15]. In this pathway, the HPO kinase phosphorylates WTS, which in turn phosphorylates and inactivates YKI by excluding it from the nucleus [8, 16]. MER and EX colocalize at the cell cortex and act upstream of HPO to regulate the activity of YKI [14]. FAT acts upstream of the Hippo signaling cascade by recruiting EX to the apical plasma membrane and modulating the abundance of WTS [17–21]. Inactivation of *ft*, *hpo*, *wts*, and *sav*, inactivation of both *ex* and *mer*, and overexpression of *yki* all lead to increased proliferation and reduced apoptosis [8, 10, 11, 13, 14].

This pathway acts through YKI to regulate the expression of *cyclin E* (*cycE*) and the *Drosophila inhibitor of apoptosis 1* (*diap-1*), which are involved in cell-cycle progression and cell-death inhibition, respectively, and the microRNA *bantam*, which can promote growth and inhibit apoptosis [8, 22, 23].

YKI Interacts with SD and TEF Proteins

The YKI peptide shares significant homology with the mammalian Yes Associated Protein 65 (YAP) (31% identity with human YAP). A particular region near the amino terminus of YKI (amino acids 57–114) displays the highest homology with YAP (56.2% identity). This region in YAP corresponds to a domain that binds all mammalian transcription-enhancer factors (TEFs), which are the homologs of the *Drosophila* SD protein [24].

Conservation between mammalian YAP and YKI of the YAP domain that interacts with TEF suggests that YKI might interact with SD. To investigate this possibility, we performed glutathione S-transferase (GST)-pull-down experiments. As shown in Figure 1B, ^{35}S-labeled YKI specifically binds to GST-SD beads.

Our results are in good accordance with a large two-hybrid screen in which SD was identified as a binding partner for YKI [25]. To refine the SD domain that binds YKI and the region of YKI involved in the interaction with SD, we examined the binding of deleted SD or YKI peptides. Results that we obtained (Figures 1B and 1C) indicate a requirement of the N terminus of YKI and the C terminus domain of SD. These results implicate YKI as an auxiliary protein that specifically interacts with SD and demonstrate that this ability to interact is conserved between TEA factors (TEF-1 and SD) and YAP factors (YAP and YKI) (Figure 1B and Figure S1 available online).

SD Promotes YKI Nuclear Localization

To determine whether SD, which possesses a putative nuclear localization signal (NLS) within the TEA domain [26], can modify YKI localization, we performed transfection experiments with plasmids expressing SD fused to FLAG sequence and YKI fused to hemagglutinin (HA) sequence. We observed that in *Drosophila* S2 cells, YKI is mainly cytoplasmic (Figure 1D). We transfected S2 cells with YKI and SD and observed that YKI is exclusively nuclear (Figure 1E). The nuclear translocation of YKI when SD is expressed argues in favor of the idea that SD and YKI interact ex vivo.

The Interaction between YKI and SD Enhances SD Transcriptional Activity

In order to assess whether interaction between YKI and SD is able to modulate transcriptional activity conferred by SD, we tested, in cotransfection experiments, the transcriptional ability of a modified SD protein in which the TEA DNA-binding domain has been replaced by that of the yeast GAL4 protein (amino acids 1–147), under the control of the *hsp70* promoter (*hsp*-GAL4$_{db}$-SD). We used a GAL4-responsive reporter gene

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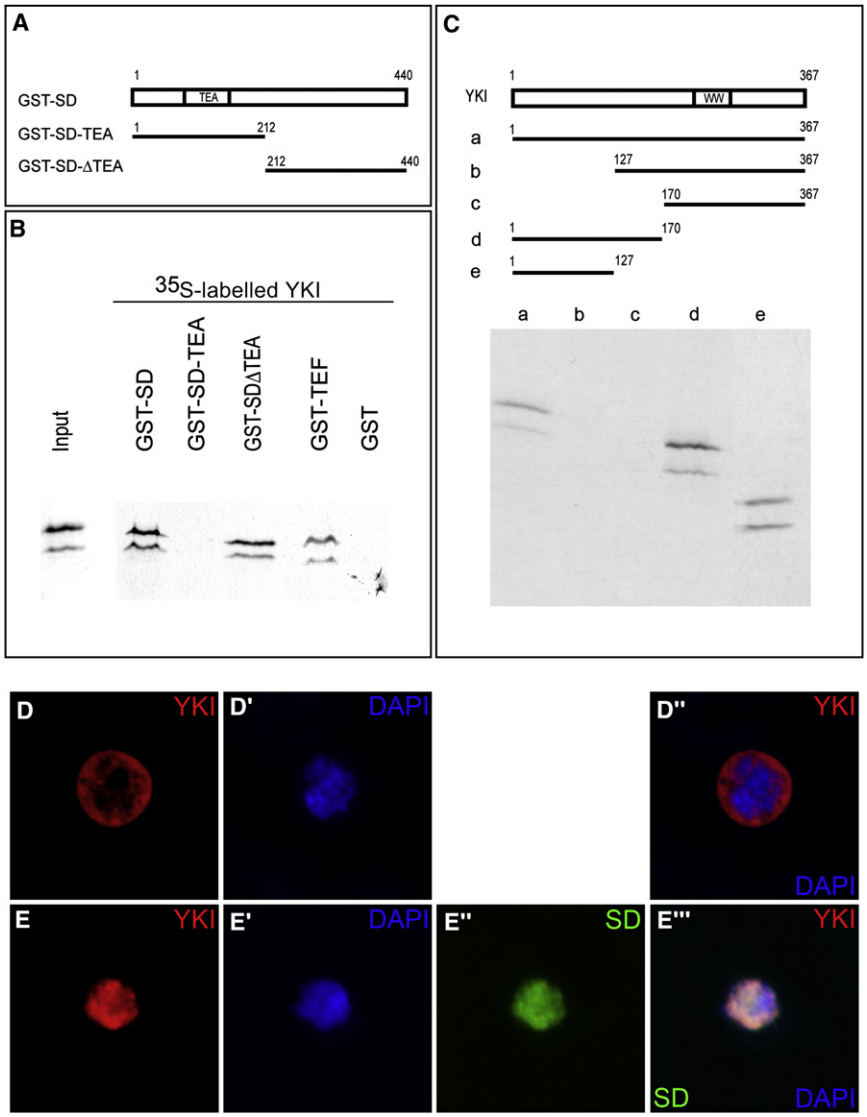


Figure 1. SD Physically Interacts with YKI in GST-Pull-Down Assays

(A) A schematic diagram indicating the structural features of the SD protein fused to GST peptide and the two truncated SD peptides (GST-SD-TEA and GST-SD-ΔTEA).

(B) Full-length YKI peptide was transcribed and translated in vitro with [^{35}S] methionine. The labeled peptide was incubated with GST-TEF-1, GST-SD, or the two GST-SD truncated peptide beads as indicated in figure. GST beads were used as a control. The materials retained by the beads were analyzed by autoradiography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Results indicate that YKI interacts specifically with the C terminus domain of SD and with human TEF-1. No interaction was observed with GST beads.

(C) A schematic diagram of the YKI peptide and the four truncated YKI peptides is indicated at the top of the figure. The indicated YKI peptides were transcribed and translated in vitro with [^{35}S] methionine. The labeled peptides were incubated with GST-SD beads. The materials retained by GST-SD beads were analyzed by autoradiography after SDS-PAGE. Results indicate that the N terminus domain of YKI peptide is required for interaction with SD.

(D–E‴) SD promotes YKI nuclear localization. *Drosophila* S2 cells were transfected with the following mixtures of plasmid: 100 ng of YKI-HA plasmid (D) and 100 ng of YKI-HA plasmid and 100 ng of SD-FLAG plasmid (E). Cells were stained with anti-HA (in red) (D, D″, E, and E‴) for the visualization of YKI and with anti-Flag (in green) (E″ and E‴) for the visualization of SD. Cells were counterstained with DAPI (in blue) (D′, D″, E′, and E‴) for the visualization of nuclei. Results indicate that YKI is mainly cytoplasmic (D–D″), but cotransfection of SD localizes YKI to the nucleus (E–E‴).

in which the *luciferase* gene is under the control of UAS sequences (pUAS-*luc*). Cotransfection of a mixture of these plasmids with increasing amounts of *yki*-expressing plasmid induced *luciferase* activity in a dose-dependent manner (up to 120-fold) (Figure 2A). This result indicates that YKI interacts in a cellular context with SD to stimulate the transcriptional activity conferred by SD. Next, we wanted to test whether YKI could stimulate SD transcriptional activity on the SD-VG-dependent promoter. VG-SD can activate *vg* expression by binding to a regulatory sequence named *vg*QE located in the fourth intron of the *vg* gene [5, 27]. We observed that transfection of SD results in a 2.5-fold increase in *vg*QE expression (Figure 2B). Transfection of SD and YKI results in a significant increase in luciferase *vg*QE expression, indicating that YKI affects the transcriptional activity of SD. Transfection of equal amounts of VG- and SD-expressing plasmids does not significantly increase *vg*QE expression compared to the experiments where only SD-expressing plasmid was transfected. However, a further increase in *vg*QE activity was observed when SD, VG, and YKI plasmids were cotransfected, suggesting that YKI acts cooperatively with VG to stimulate the transcriptional activity of SD. These observations are consistent with our results, which indicate that VG and YKI do not compete for binding to SD (Figure S2).

It has been previously shown that, in S2 cells, the transcriptional activity of YKI is suppressed by coexpression of upstream components of the Hippo pathway [8, 19]. We analyzed the transcriptional activity of SD-VG complex when HPO was overexpressed. Coexpression of GAL4$_{db}$-SD and VG results in a powerful induction of luciferase activity (Figure 2C). This induction was suppressed when we overexpressed HPO. Although we cannot exclude that HPO used a mechanism other than YKI inhibition, the most likely expanation for our results is that the Hippo pathway, and thus YKI function, is required for SD-VG activity.

It has been proposed that, in the SD-VG complex, VG provides the activating component [3], and that the binding of VG to SD switches the target selectivity of SD [5]. If we assume that the SD-VG-YKI complex is able to form into a cell, one attractive explanation for the role of interaction between YKI and SD is that YKI provides the main activating component and VG undergoes a conformational change in SD ensuring accurate DNA target selection.

To explore the possibility that YKI function is evolutionarily conserved, we tested the effects of YKI on TEF transcriptional

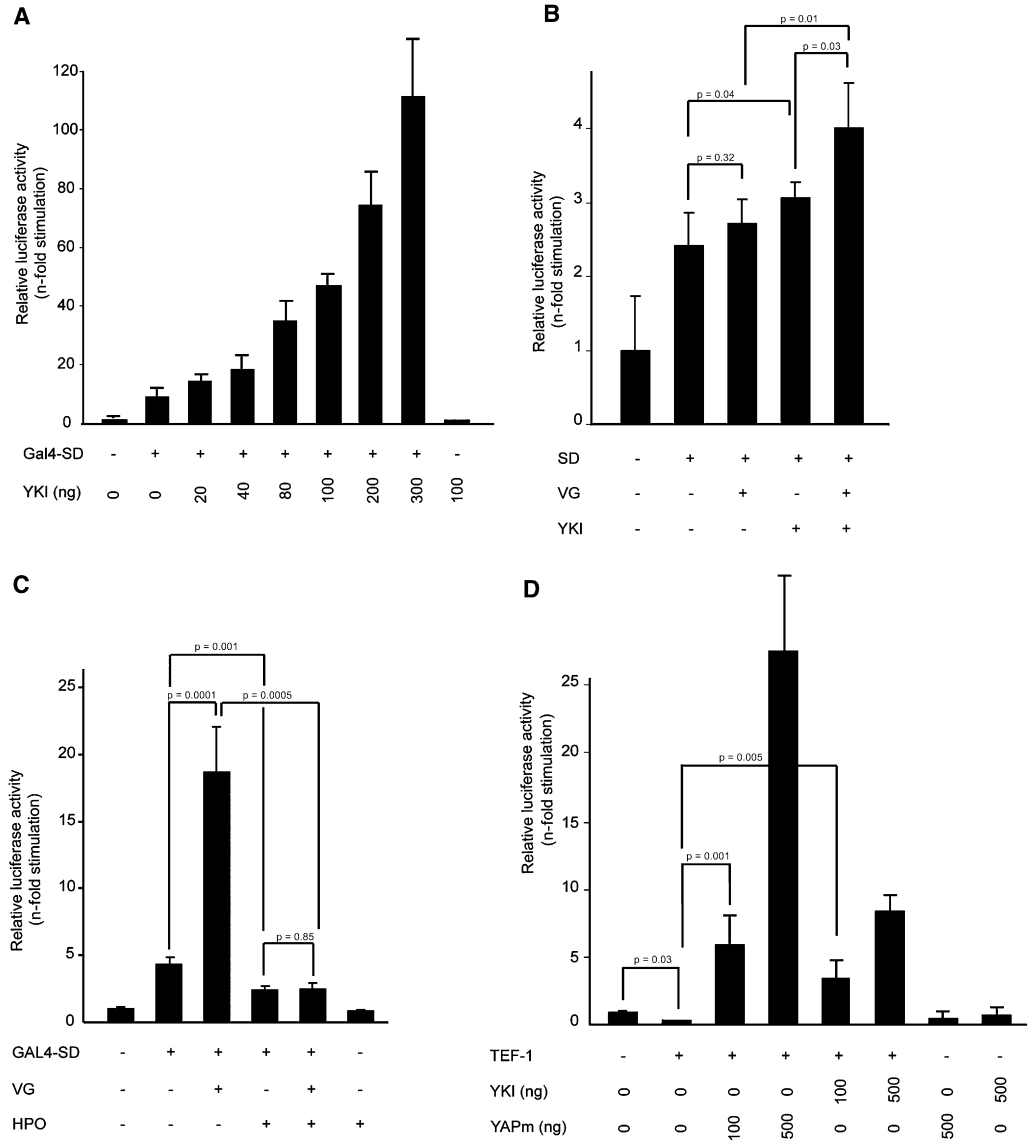


Figure 2. The Interaction between SD and YKI Enhances SD Transcriptional Activity

(A) *Drosophila* S2 cells were transfected with 100 ng of pUAS-*luc* (GAL4-responsive luciferase reporter) plasmid along with 100 ng of *hsp*-GAL4$_{db}$-SD plasmid expressing a modified SD protein in which the TEA domain was replaced by the GAL4 DNA-binding domain and the indicated amount of pCaSpeR-*hsp70-yki* plasmid expressing YKI. The data are expressed as the n-fold change relative to the negative control transfected with pUAS-*luc* plasmid.

(B) *Drosophila* S2 cells were transfected with 100 ng of *vg*QE-*luc* reporter plasmid with the indicated combination of plasmids encoding SD, VG, or YKI. The p values were calculated by Student's t test from six independent experiments. The data are expressed as the n-fold change relative to the negative control transfected with *vg*QE-*luc* plasmid.

(C) *Drosophila* S2 cells were transfected with 100 ng of pUAS-*luc* reporter plasmid with the indicated combination of plasmids encoding GAL4$_{db}$-SD, VG, or HPO. The p values were calculated as in (B).

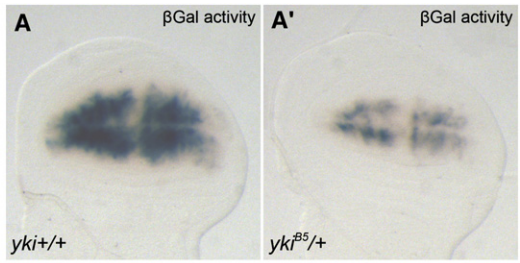
(D) Human HeLa cells were transfected with 100 ng of −2570/−2518-TK164-luc plasmid (TEF-1-responsive luciferase reporter), 100 ng of pXJ40-TEF-1 plasmid expressing TEF-1, and the indicated amount of pCMV-YAP plasmid expressing murine YAP or pXJ40-YKI plasmid expressing YKI. The p values were calculated as in (B). The data are expressed as the n-fold change relative to the negative control transfected with TK164-*luc* plasmid.

In all experiments, histograms show the mean of at least three independent experiments, and transfection efficiency normalization was performed by co-transfection of a β-galactosidase expression vector and assaying of β-gal activity in cell extracts. Error bars indicate standard deviation.

activity in a HeLa human cell line. We used a TEF1 responsive reporter plasmid (−2570/−2518-TK164-luc) [28]. We observed that YKI, similarly to YAP, stimulate TEF1 transcriptional activity (Figure 2D) [8]. These results reveal an evolutionarily conserved regulatory mechanism. Interestingly, it has recently been shown that YAP is the effector of the mammalian Hippo pathway. It is amplified in mouse tumor models and has several oncogenic properties [15, 26, 27]. Furthermore, YAP can functionally substitute for YKI in *Drosophila* [8]. The relevance of the YAP-TEF interaction in this pathway, however, remains to be determined.

YKI Is Required for SD Transcriptional Activity in Wing Imaginal Discs

To determine whether YKI-SD interaction regulates SD transcriptional activity in vivo in *Drosophila*, we used a sensor



B

		I	II	III	IV
w^{1118}	n = 45	100%			
yki^{B5}/+	n = 52	100%			
sd^{ETX4}/Y	n = 53		100%		
sd^{ETX4}/Y; yki^{B5}/+	n = 62		64.5%	35.5%	
sd^{ETX4}/Y; +/vg^{79d5}	n = 32			100%	
sd^{ETX4}/Y; yki^{B5},+/+,vg^{79d5}	n = 41			14.6%	85.4%
sd^{ETX4}/Y; +/vg^{83b27}	n = 60		100%		
sd^{ETX4}/Y; yki^{B5},+/+,vg^{83b27}	n = 40		45%	55%	

Figure 3. YKI Is Required for SD-VG Transcriptional Activity in Wing Discs

(A and A′) YKI is required for SD-VG transcriptional activity in the wing disc. All third-instar wing discs are orientated with the posterior compartment to the right and the ventral one at the top. In these experiments, X-gal staining was performed rigorously. Staining times were precisely the same, and limiting conditions were used so that the difference in *lacZ* expression could be distinguished. The images presented are representative of those seen in 50 discs from four different experiments. (A) shows a wing disc from *hsp70-GAL4$_{db}$-sd*/+; UAS-*lacZ*/+ heat shocked and stained with X-gal 24 hr later. *hsp70-GAL4$_{db}$-sd* transgene encodes, under the control of the hsp70 promoter, a modified SD protein in which the TEA domain was replaced by the GAL4 DNA-binding domain. X-gal staining is observed in the wing pouch, where SD-VG is transcriptionally active. (A′) shows wing discs from *hsp70-GAL4$_{db}$-sd*/*yki^{B5}*; UAS-*lacZ*/+ heat shocked and stained with X-gal 24 hr later. Compared to the control (A), a significant decrease in SD-VG activity can be observed.

(B) Genetic interactions between *yki*, *sd*, and *vg*. In these experiments, the defects in wing were divided into four classes, with an arbitrary scale illustrated in the figure: wild-type (I), mildly notched (II), severely notched (III), and reduction in wing size to an almost complete absence of wings (IV). Flies of the indicated genotype were analyzed and assigned to the phenotypic classes. n is the number of flies scored for each phenotype. We used the *sd^{ETX4}* weak allele of *sd* and the amorphic *yki^{B5}* allele of *yki*. Abnormal wing phenotype in the *sd^{ETX4}* allele results in only partial loss of wing structure (class II in [B]). Flies heterozygous for the *yki^{B5}* allele (B) or *sd^{ETX4}* allele (data not shown) exhibited wild-type wing in 100% of individuals according to the recessive nature of these alleles. Flies hemizygous for *sd^{ETX4}* allele exhibit notched wings (class II) in 100% of individuals. Flies hemizygous for *sd^{ETX4}* and heterozygous for *yki^{B5}* exhibit a stronger wing phenotype (class III) in 35.5% of individuals, suggesting genetic interaction of *yki* with *sd*. Flies hemizygous for *sd^{ETX4}* and heterozygous for *vg^{79d5}* display a stronger wing phenotype (class III) in 100% of individuals. We observed a shift toward a more extreme phenotype (class IV) in flies hemizygous for *sd^{ETX4}* and double heterozygous for *vg^{79d5}* and *yki^{B5}*. The *vg^{83b27}* allele does not genetically interact with *sd^{etx4}*. We did observe, however, a higher percentage of wings of class III from flies hemizygous for *sd^{ETX4}* and double heterozygous for *vg^{83b27}* and *yki^{B5}* compared to flies hemizygous for *sd^{ETX4}* and heterozygous for *yki^{B5}* (chi2-square, p = 0.04).

transgene, "*hsp70-GAL4$_{db}$-sd*," expressing a modified SD protein, in which the TEA DNA-binding domain has been replaced by that of the yeast GAL4 DNA-binding domain, under the control of the hsp70 promoter. We have previously shown that GAL4::SD activity, monitored by the *UAS-lacZ*-responsive reporter transgene, is restricted to the wing pouch, where SD dimerizes with VG (Figure 3A) [6, 29]. A significant reduction in *lacZ* activity was observed in wing discs heterozygous for the amorphic *yki^{B5}* allele compared to control wing discs (Figures 3A and 3A′). The fact that SD activity is sensitive to *yki* dosage in wing imaginal discs supports the idea that YKI is required for SD transcriptional activity in vivo.

scalloped and *yorkie* Interact during Wing Morphogenesis

To better understand the functional relationship between YKI and SD during wing development, we assessed genetic interactions between these genes. We used the *sd^{ETX4}* weak allele of *sd* and the amorphic *yki^{B5}* allele of *yki*. Abnormal wing phenotype in the *sd^{ETX4}* allele results in only partial loss of wing structure (class II in Figure 3B). One dose of the *yki^{B5}* allele enhances the hemizygous *sd^{ETX4}* wing phenotype and results in a more severely notched phenotype (class III in Figure 3B) in 35% of individuals. Therefore, in a hypomorphic *sd* background, reduction of the *yki* dose leads to an enhancement of the *sd* phenotype, showing that YKI is required for SD activity during wing development. During development, SD physically interacts with VG to promote wing-tissue formation. This can be visualized by genetic interaction between weak alleles of *sd* and *vg* at the homozygous state [3]. To further probe the link between *yki* and *sd*/*vg* function, we investigated their genetic interactions. We used the *vg^{83b27}* and *vg^{79d5}* alleles. Flies hemizygous for *sd^{ETX4}* and heterozygous for *vg^{83b27}* exhibit a wing phenotype similar to that of *sd^{ETX4}* flies. We observed a higher percentage of flies with a severe wing phenotype (class III) in flies hemizygous for *sd^{ETX4}* and double heterozygous for *vg^{83b27}*and *yki^{B5}* compared to *sd^{ETX4}*; *yki^{B5}*/+ flies (Figure 3B), indicating that reduction of *vg* dose increases genetic interaction between *sd* and *yki*. Similar results were observed with the *vg^{79d5}* allele (Figure 3B). Taken together, these results indicate that reduction of both *vg* and *yki* dosage enhances the *sd* phenotype and support the hypothesis that YKI works together with SD-VG during wing development.

YKI Induces *vg* and *vg*QE Expression in Wing and Haltere Discs

It has been shown that binding sites for SD and SD-VG are necessary for regulation of *vg* expression [30, 31] and thus may regulate by a feedback mechanism *vg* expression. Results obtained in S2 cells indicate that YKI increases SD transcriptional activity. This prompted us to investigate whether *yki* overexpression in imaginal discs enhances VG-SD activity, thus regulating *vg* expression and *vg*QE activity. We drove *yki* overexpression by using a UAS-GAL4 system or by flip-out (FLP-FRT) recombination in clones. In both cases, we observed that overexpression of *yki* induces *vg* and *vgQE* expression in a cell-autonomous manner in wing discs (Figures 4A–4A″ and 4B′) and in haltere discs (Figure S3).

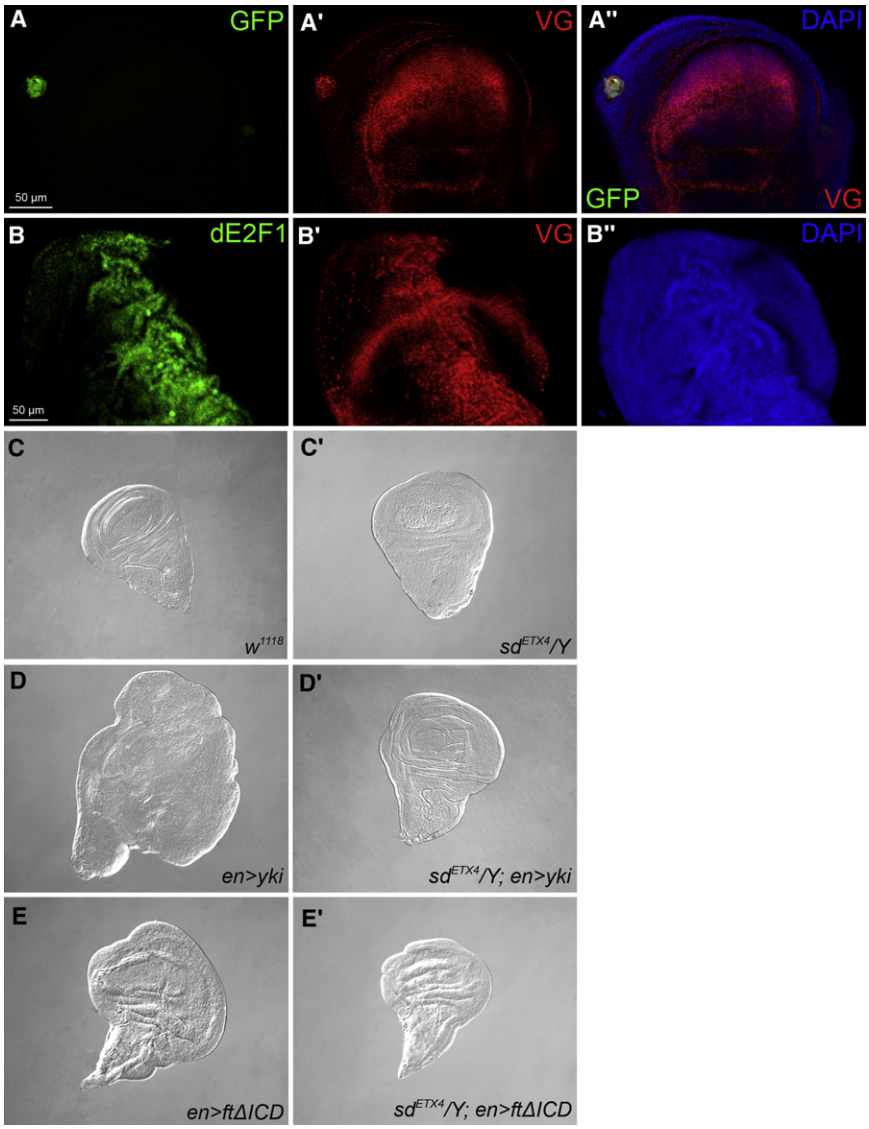


Figure 4. Overexpression of *yki* Induces *vg* and *dE2F1* Expression

(A–A″) Wing imaginal disc of *hsp70-flp/X*; *tub-FRT* > cd2 > *FRT-GAL4*, *UAS-GFP/UAS-yki*. Clones were induced at the second larval instar. Discs were stained with green fluorescent protein (GFP) so that the flip-out clone overexpressing *yki* could be highlighted (A) and with VG antibody (A′). In the merge (A″), nuclei are marked with DAPI.

(B–B″) Wing imaginal disc of *ptc-GAL4/dE2F1-lacZ*; *UAS-yki*. Wing discs were stained with β-gal antibody for the visualization of *d2EF1* expression (B), with VG antibody (B′), and with DAPI (B″). Overexpression of *yki* induces an increase of *d2EF1* (B) and *vg* (A′ and B′).

(C–E′) SD is required for cell proliferation induced by YKI and FTΔICD in wing imaginal disc. Wing disc from late third instar larvae of the following genotypes are shown: wild-type (C), sd^{ETX4}/Y (C′), *en-gal4*; UAS-*yki* (D), sd^{ETX4}/Y; *en-gal4*; UAS-*yki* (D′), *en-gal4*; UAS-*ftΔICD* (E), and sd^{ETX4}/Y; *en-gal4*; UAS-*ftΔICD* (E′). YKI or FTΔICD induces massive overgrowth of the posterior compartment (D and E). Overgrowth is severely reduced in wing discs hemizygous for sd^{ETX4} (D′ and E′).

YKI Induces Expression of *dE2F1*, a Target Gene of SD-VG

Several sets of data argue strongly in favor of an involvement of VG-SD in cell proliferation and cell-cycle progression in wing imaginal discs. vg^{null} cell clones do not proliferate in the wing pouch, whereas they can be recovered in the notum region, where *vg* is not expressed, indicating that *vg* is required for the proliferation of wing blade cells or for their survival [32]. On the other hand, *vg* ectopic expression induces wing outgrowths and *dE2F1* expression [7]. dE2F1 is the *Drosophila* homolog of the E2F transcription-factor family that plays a pivotal role during cell-cycle progression in controlling expression of different genes involved in G1-S transition (including *cycE*, a target gene of the Hippo pathway) and DNA replication [33]. We found that YKI overexpression with the *ptc*-GAL4 driver clearly upregulates expression of the *dE2F1-LacZ* reporter strain in a cell-autonomous manner (Figure 4B). This indicates that YKI and VG-SD both activate *dE2F1* expression and might work together in this process. We can hypothesize either that SD-VG is required for *dE2F1* induction by YKI or that YKI induces *dE2F1* independently from VG-SD and reinforces *dE2F1* expression through SD-VG induction.

SD Is Required for Cell Proliferation Induced by YKI in Wing Imaginal Discs

To determine more precisely the relationship between SD and YKI in cell proliferation, we tested the effect of YKI overexpression in a mutant context for *sd*. Strong hypomorph alleles of *sd* are characterized by an absence of wing pouch cells. In contrast, sd^{ETX4} corresponds to a weak allele of *sd* in which a large number of wing pouch cells are still present. We chose to use this allele to exclude the possibility that the observed effect resulted from an absence of cell proliferation in the wing pouch because of a lack of *sd* expression. Wing discs overexpressing *yki* with the *en*-GAL4 driver exhibit clear increases in size of the posterior region (Figure 4D and [23]), resulting in massive overgrowth. In contrast, the size of the posterior compartment was almost normal in wing discs from flies hemizygous for sd^{ETX4} and overexpressing *yki* with the *en*-GAL4 driver (Figure 4D′). This result shows that YKI needs SD to fully induce cell proliferation. Next, we asked whether SD-YKI interaction is acting downstream of the Hippo pathway. To test this, we made use of a dominant-negative form of FT that lacks the intracellular domain (FTΔICD) [34]. Indeed, FT is an upstream component of the Hippo pathway [17–21], and overexpression of FTΔICD induced overgrowth and the expression of Hippo target genes [19, 34]. We observed a significant reduction of induced overgrowth (Figures 4E and 4E′) in sd^{ETX4} wing discs. This result strongly suggests that SD-YKI interaction acts downstream of the Hippo pathway in wing discs.

In this paper, we addressed the role of SD-YKI interaction in the wing disc. The role of SD in other imaginal tissues is poorly understood. Strong alleles of *sd* are associated with lethality in early larval stage [1, 2, 35]. In the eye disc, clones homozygous

for a strong allele of *sd* die or poorly survived, whereas they are associated with truncated legs when generated in the leg disc, suggesting that SD should play a role in cell survival in these tissues [36]. In the wing disc, *sd* clones die in the wing pouch but can be easily recovered in regions that will give rise to the notum, whereas YKI is required in the entire wing disc [8, 32, 37]. This implies that other partners must interact with YKI to promote tissue growth in other structures. However, the induction of *vg* that we did observe in cells overexpressing *yki* and our results showing that proliferation induced by YKI in the wing disc is significantly reduced in *sd* mutant context suggest that SD-VG is required for YKI function in the wing disc.

Supplemental Data

Experimental Procedures and three figures are available at http://www.current-biology.com/cgi/content/full/18/6/435/DC1/.

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