Impaired Hippo signaling promotes Rho1–JNK-dependent growth

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The Hippo and c-Jun N-terminal kinase (JNK) pathway both regulate growth and contribute to tumorigenesis when dysregulated. Whereas the Hippo pathway acts via the transcription coactivator Yki/YAP to regulate target gene expression, JNK signaling, triggered by various modulators including Rho GTPases, activates the transcription factors Jun and Fos. Here, we show that impaired Hippo signaling induces JNK activation through Rho1. Blocking Rho1–JNK signaling suppresses Yki-induced overgrowth in the wing disk, whereas ectopic Rho1 expression promotes tissue growth when apoptosis is prohibited. Furthermore, Yki directly regulates Rho1 transcription via the transcription factor Sd. Thus, our results have identified a novel molecular link between the Hippo and JNK pathways and implicated the essential role of the JNK pathway in Hippo signaling-related tumorigenesis.

Hippo | JNK | growth | Rho1 | *Drosophila*

Tumor progression often involves deregulated signaling pathways that lead to unchecked proliferation and evasion of apoptosis, which are considered two important hallmarks of cancer development (1). Recently, the Hippo pathway was shown to play an evolutionarily conserved role in regulating cell growth, proliferation, and survival in tumorigenesis (2, 3). In *Drosophila*, the core Hippo pathway acts through a kinase cascade consisting of Hippo (Hpo) and Warts (Wts) to inactivate the transcriptional coactivator Yorkie (Yki; YAP/TAZ in mammals) (4, 5). Yki/YAP interacts with different DNA-binding transcription factors to activate transcription of growth-regulating genes, including *cyclin* E (*cycE*), *E2f1*, and *Drosophila inhibitor of apoptosis protein 1* (*Diap1*) (6).

The c-Jun N-terminal kinase (JNK) pathway plays a crucial role in regulating a wide range of cellular activities including proliferation, differentiation, migration, and cell death during tumor progression (7). JNK signaling is also required for tumor growth triggered by loss of cell polarity and oncogenic Ras cooperation in *Drosophila* (8, 9), and for progenitor cell proliferation and Ras-induced tumorigenesis in mammals (10, 11).

Although both Hpo and JNK signaling have been implicated in cell proliferation and tissue growth, it has remained elusive how the two pathways are coordinated in vivo. Recently some contradictory findings were reported in *Drosophila*. On one hand, JNK signaling induces Yki activation during compensatory cell proliferation and neoplastic tumor growth (12–14); on the other hand, JNK suppressed Yki elevation in *scrib* mutant cells during growth regulation (15, 16). However, it remains unknown whether JNK signaling is regulated by the Hpo–Yki pathway and contributes to its growth function.

Here we show that impaired Hpo signaling activates the Rho1–JNK pathway in *Drosophila*. Compromised Rho1–JNK signaling suppresses Yki-induced cell proliferation and tissue overgrowth in the developing wing, whereas activated Rho1–JNK signaling promotes proliferation and growth when cell death is blocked. Sd is necessary and sufficient for Yki-induced JNK activation. We have further identified *rho1* as a direct transcriptional target of the Yki/Sd complex. Our data in *Drosophila* suggest a possible interaction in growth control between Hpo–Yki and Rho–JNK signaling in mammals.

Results

Impaired Hpo Signaling Up-Regulates JNK Activity. To investigate whether Hpo signaling could modulate JNK activity, we checked the expression of *puc*, a transcriptional target and a readout of JNK activity in vivo (17). Compared with the control (Fig. 1*A*), inactivation of Hpo signaling by *sd*–Gal4-driven Yki expression resulted in up-regulated *puc*–LacZ expression in the overgrown wing pouch (Fig. 1*C*). As a positive control, *puc*–LacZ expression was strongly elevated by expressing the JNK kinase *hemipterous* (*hep*) (Fig. 1*B*). To probe whether JNK is activated by Yki in an autonomous and/or a spatial manner, we examined *puc* transcription in Flp-out clones expressing a hyperactive form of Yki (Yki^{S168A}). JNK activation was observed throughout the wing disk autonomously (Fig. S1), although occasionally cells adjacent to Yki overexpression clones were also found to have increased *puc* activity (Fig. S1, white arrow), which is likely caused by the supercompetitive activity of Yki clones (18).

Next, to further verify the result, we checked the expression of matrix metalloprotease1 (MMP1), another direct transcriptional target of the JNK pathway (19). MMP1 was normally detected in trachea and a small region of notum in wild-type wing discs (Fig. S2*A*) (20). We generated loss-of-function and gain-of-function clones in wing discs and found that loss of the *wts* gene and forced expression of Yki or Yki^{S168A} all resulted in strong MMP1 activation in the clones (Fig. 1 *D*–*G*). In addition, both MMP1

Significance

Both Hippo and c-Jun N-terminal kinase (JNK) signaling have well-established roles in promoting tissue growth, while it remains elusive whether and how the two pathways interact or coordinate in growth control. We report in the present study that the Hpo–Yki pathway modulates Rho1–JNK signaling-mediated growth in *Drosophila* wing development. We show that the impaired Hpo pathway activates JNK through transcriptional up-regulation of Rho1. These findings expands our understanding about the cross-talk between two important signaling pathways, namely Hippo–Yki and Rho1–JNK, in regulating growth.

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The authors declare no conflict of interest.

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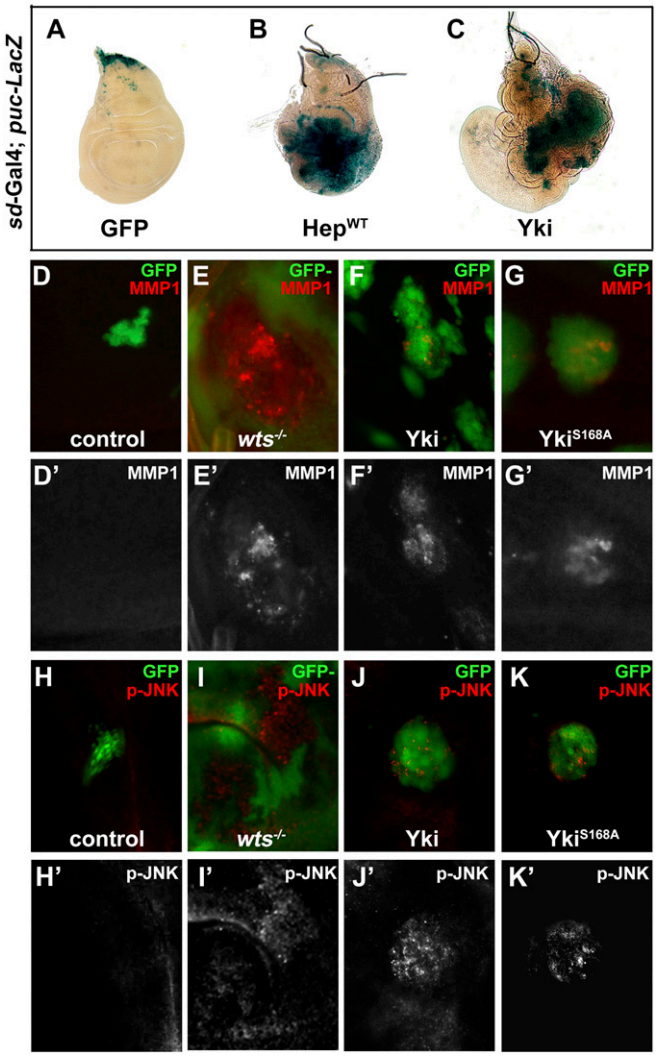

Fig. 1. Yki activates JNK signaling in *Drosophila*. Third instar wing discs are shown. Ectopic Yki activates JNK target *puc* expression. *puc*-LacZ expression is shown in *sd* > GFP control (*A*), *sd*>Hep^{WT} (*B*), and *sd* > Yki (*C*) wing discs. Compared with control clones (*D* and *H*), impaired Hippo signaling activates MMP1 expression and JNK phosphorylation in *wts* mutant clones (*E* and *I*), or clones expressing Yki (*F* and *J*) or Yki^{S168A} (*G* and *K*). Clones were labeled by the presence (*D*, *F*, *G*, *H*, *J*, and *K*) or absence (*E* and *I*) of GFP expression. See also Figs. S1 and S2.

and *puc–LacZ* expression were up-regulated by ectopic Yki or Yki^{S168A} driven by *ptc*–Gal4 (Figs. S2 *B* and *C* and S3*F*). Finally, we examined JNK activation directly using an antibody specific to the phosphorylated JNK (p-JNK). Elevated p-JNK staining was detected in *wts* mutant clones as well as cells expressing Yki or Yki^{S168A} (Fig. 1 *H–K* and Fig. S2*E*). Together, these data indicate that impaired Hpo signaling results in JNK activation in vivo.

JNK Is Required for Yki-Induced Overgrowth. The Hpo pathway plays a key role in organ size control through the regulation of cell number (21). Consistently, immunostaining with an antibody against phospho-histone 3 (PH3), a specific marker for mitotic cells, indicated an increase of PH3$^+$ cells in *ptc* > Yki^{S168A} wing discs (Fig. 2 *C* and *E*). Blocking JNK signaling by expressing a dominant negative form of *basket* (Bsk^{DN}, *bsk* encodes the *Drosophila* JNK) dramatically suppressed such increase of PH3$^+$ cells and the expansion of the GFP$^+$ region (Fig. 2 *D* and *E*). Consistently, reducing JNK activity also strongly impeded loss of *wts*-induced overgrowth phenotype (Fig. S2 *F* and *G*). In addition, clone overgrowth in wing discs resulting from expression of

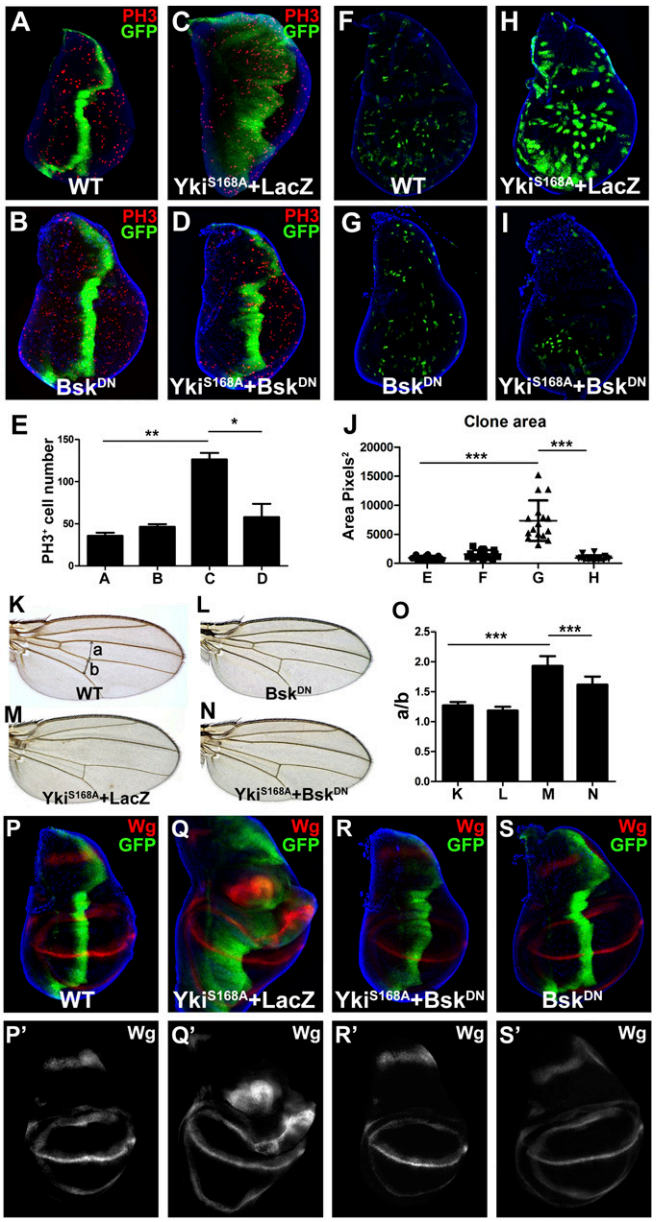

Fig. 2. JNK is required for Yki-induced overgrowth and Wg expression. (*A–J*) Yki induces JNK-dependent overgrowth in wing discs. Third instar wing discs of *ptc* > GFP (*A*), *ptc*> Bsk^{DN} (*B*), *ptc* > Yki^{S168A} + LacZ (*C*), or *ptc* > Yki^{S168A} + Bsk^{DN} (*D*) were stained for PH3 and DNA (DAPI). (*E*) Average PH3$^+$ cells within *ptc* expression domain in *A–D* are shown. $^{**}P < 0.01$, $^{*}P < 0.05$ (mean + SD, $n \geq 5$). (*F–I*) Third instar wing discs bearing flip-out clones expressing GFP without (*F*) or with Bsk^{DN} (*G*), Yki^{S168A} + LacZ (*H*), or Yki^{S168A} + Bsk^{DN} (*I*). (*J*) Statistical analyses of clone sizes in *F–I*. $^{***}P < 0.001$ (mean ± SD, $n = 15$). (*K–N*) Blocking JNK activity suppressed Yki-induced expansion of the intervein region between L3 and L4 in adult wings. Light micrographs of adult wings from *ptc* > GFP (*K*), *ptc*> Bsk^{DN} (*L*), *ptc* > Yki^{S168A}, *tub*-Gal80^{ts} + LacZ (*M*), or *ptc* > Yki^{S168A}, *tub*-Gal80^{ts} + Bsk^{DN} (*N*) flies. (*O*) Average ratio of a/b in *K–N*. a, distance between L3 and L4; b, length of posterior cross-vein between L4 and L5. Positions of a and b are shown in *K*. $^{***}P < 0.001$ (mean + SD, $n = 10$). (*P–S*) JNK is required for Yki-induced ectopic Wg expression. Third instar wing discs of *ptc* > GFP (*P*), *ptc* > Yki^{S168A} + LacZ (*Q*), *ptc* > Yki^{S168A} + Bsk^{DN} (*R*), or *ptc*> Bsk^{DN} (*S*) were stained with DNA (DAPI) and Wg. See also Figs. S3–S5.

Yki^{S168A} was also suppressed by Bsk^{DN} (Fig. 2 *F–J*). Furthermore, expression of Yki^{S168A} along the anterior-posterior (A-P) compartment boundary driven by *ptc*–Gal4 resulted in an expansion of the intervein region between L3 and L4 in adult wings, which was also suppressed by expression of Bsk^{DN} (Fig. 2 *K–O*). However, expression of Bsk^{DN} by itself had no effect on growth (Fig. 2 *B*, *G*, and *L*) and did not affect Yki^{S168A}-triggered cell death, as revealed by immunostaining for active caspase 3 (Casp3) (Fig. S3 *A–C*). The small amount of Casp3 activation observed in *ptc* > Yki^{S168A} discs (Fig. S3*B*) is consistent with a previous report (18). Together, these results suggest that JNK signaling is required for Yki-induced cell proliferation and organ growth.

Next, to investigate the molecular mechanism by which JNK mediates Yki-induced overgrowth, we analyzed the expression of Yki target genes, including *wingless* (*wg*), *Expanded* (*ex*), *Diap1*, *dmyc*, *bantam* (*ban*), and *CycE*, that are required for growth control (18, 22–24). Blocking JNK signaling fully suppressed Yki-induced up-regulation of Wg (Fig. 2 *P–S*), but not that of *diap1*–LacZ, *ex*–LacZ, *dmyc*–LacZ, and *ban*–LacZ (Fig. S4 *A–F* and *I–N*). Furthermore, consistent with Wg's role in regulating Yki-triggered growth, we found Yki-induced proliferation and growth phenotype were significantly blocked by knocking down *wg* (Fig. S4 *G* and *H*). We noted that Yki failed to induce CycE expression in the developing wing (Fig. S4 *O* and *P*), in accordance with a previous study that the Hippo pathway regulates CycE in a tissue-specific manner (4). As a positive control, CycE was detected when ectopically expressed in wing discs (Fig. S4*Q*). As expected, elevated expression of *puc* and *MMP1* induced by Yki^{S168A} were both suppressed by Bsk^{DN} (Fig. S3 *H* and *I*). Together, these data suggest that *wg* is not a direct transcriptional target of Yki, but rather, regulated by the Hpo–Yki pathway indirectly through JNK signaling. Consistent with this explanation, Wg was reported to act downstream of JNK signaling to promote compensatory cell proliferation (25).

Moreover, to find out whether Yki induces JNK-mediated growth in a tissue-specific manner, we checked genetic interactions between Hpo–Yki and JNK signaling in the developing eye. First, we found expression of Yki or a *wts* RNAi under *GMR* promoter induces overgrowth in the adult eye, which cannot be suppressed by coexpression of Bsk^{DN} (Fig. S5 *A–E*). Second, the overgrowth phenotype of eye disk clones expressing Yki^{S168A} cannot be suppressed by blocking JNK signaling (Fig. S5 *F* and *G*). Thus, the role of JNK in regulating loss of Hpo signal-induced growth is highly context dependent.

Yki Regulates JNK Signaling Through Rho1. To investigate the molecular mechanism underlying Yki-induced JNK activation, we

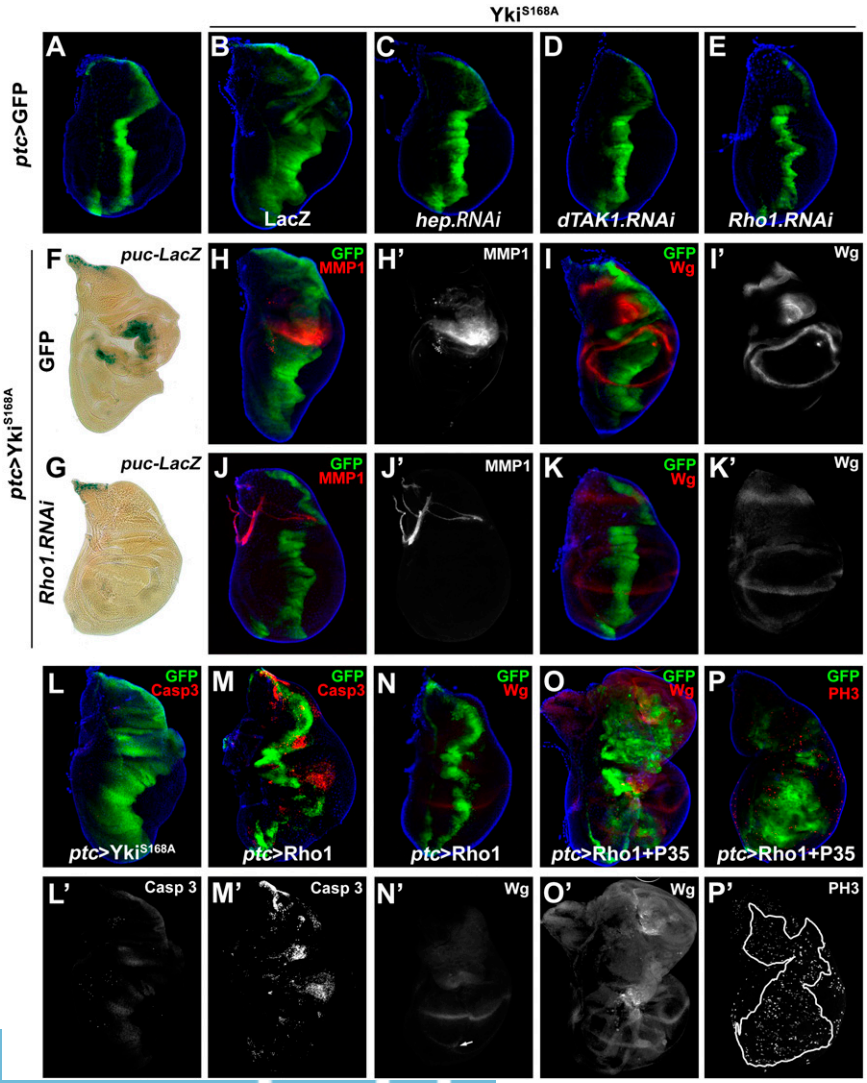

Fig. 3. Rho1 is required for Yki-induced overgrowth and JNK activation. (*A–E*) Third instar wing discs of control (*A*) or expressing Yki^{S168A} + LacZ (*B*), Yki^{S168A} + *hep* RNAi (*C*), Yki^{S168A} + *dTAK1* RNAi (*D*), and Yki^{S168A} + *Rho1* RNAi (*E*) are shown. (*F–K*) Third instar wing discs stained with X-Gal (*F* and *G*), MMP1 (*H* and *J*), or Wg (*I* and *K*). Yki-induced expansion of GFP^{+} region (*H* and *I*), JNK target gene expression (*F* and *H*), and Wg expression (*I*) were all suppressed by knocking down *Rho1* (*G*, *J*, and *K*). (*L* and *M*) Third instar wing disk stained with cleaved caspase 3. Strong apoptosis was induced by *ptc* > Rho1 (*M*), but not *ptc* > Yki^{S168A} (*L*). (*N–P*) Blocking Rho1-triggered apoptosis by P35-stimulated Wg production (*O*) and cell proliferation (*P*). See also Figs. S6 and S7.

examined genetic epistasis between Yki and upstream components of JNK signaling. Compromised JNK signaling by knocking down *hep* or JNKK kinase *dTAK1* significantly suppressed *ptc* > Yki^{S168A}-induced disk overgrowth (Fig. 3 *A–D*), indicating Yki functions upstream of dTAK1 and Hep. The Rho GTPase family members were known to regulate JNK signaling upstream of dTAK1 (26, 27), and in particular, Rho1 has been shown to induce JNK-mediated apoptosis and compensatory proliferation (28, 29). Consistently, Rho1 expression driven by *ptc*–Gal4 resulted in elevated *puc* expression (Fig. S6 *D* and *E*). Knocking down *Rho1* expression by two independent RNAi significantly blocked Yki-triggered overgrowth (Fig. 3*E* and Fig. S6*C*), as well as Yki-induced up-regulation of *puc–LacZ*, MMP1, and Wg (Fig. 3 *F–K*). Taken together, these results suggest that Rho1 mediates Yki-induced JNK activation and overgrowth.

To examine whether increased Rho1 activity is able to drive cell proliferation and tissue growth, we expressed Rho1 in the wing discs under *ptc* promoter. In agreement with a previous study (28), Rho1 expression induced strong cell death (Fig. 3*M*) and mild Wg expression (Fig. 3*N*) without expansion of the GFP^{+} region (Fig. 3 *M* and *N*). Intriguingly, when cell death was blocked by P35, Rho1 was able to phenocopy Yki^{S168A}-induced overgrowth (Fig. 3 *L* and *O*) and proliferation (Fig. 3*P*), coupled with Wg induction (Fig. 3*O*). Interestingly, we also observed increased Yki nuclear accumulation in Rho1 + p35 expression cells (Fig. S7*D*), an indication of Yki activation (4), suggesting a positive feedback loop exits between Yki and Rho1 in growth control. In agreement with this view, we found reducing JNK or Yki activity both significantly impeded Rho1-induced growth (Fig. S7 *A–C*). Hence, the above data indicate Yki promotes tissue growth by concerted action of downstream signaling pathways of both Rho1–JNK–mediated Wg expression, cell proliferation, and apoptosis and dIAP1-mediated inhibition of apoptosis.

Sd Is Essential for Yki-Induced JNK Activation. Sd is the best-characterized DNA-binding partner of Yki, and loss of *sd* fully suppresses Yki-induced tissue overgrowth and elevated target gene transcription (24). Consistently, we found Yki^{S168A}-induced overgrowth and MMP1 expression were dramatically suppressed by knocking down *sd* (Fig. 4 *A* and *B*), whereas ectopic expression of a constitutively active form of Sd (Sd^{GA}) (30) was sufficient to induce MMP1 expression (Fig. S8*A*) and *puc* transcription (Fig. S8*B*). Apart from Sd, Mad was recently reported as another transcription factor that cooperates with Yki to regulate growth (31). Consistently, we found loss of *mad* partially suppressed Yki-induced growth (Fig. 4*C′* and Fig. S9); however, the induction of MMP1 remained unaffected (Fig. 4*C′′*). Collectively, these data indicate that Yki acts through Sd to promote JNK-mediated growth.

Yki Directly Induces Rho1 Expression. To further explore the molecular mechanism by which the Hippo–Yki pathway modulates Rho1–JNK signaling, we examined whether Yki is able to induce *Rho1* transcription. To this end, we first checked Rho1 expression by quantitative real-time PCR (qRT-PCR) and Western blot. Expression of Yki^{S168A} driven by *ptc*–Gal4 resulted in a significant elevation of *Rho1* mRNA (Fig. 5*G*) and protein (Fig. 5*H*) in wing discs. This result was confirmed by in situ hybridization, in which *Rho1* transcription was significantly up-regulated in wing discs (Fig. 5 *A* and *B*) and salivary glands (Fig. 5 *D* and *E*). Knocking down *sd* fully suppressed Yki-induced *Rho1* transcription (Fig. 5 *C* and *F*), indicating Yki acts through Sd to induce *Rho1* transcription.

To investigate whether the Yki–Sd complex activates *Rho1* transcription directly, we examined the *Rho1 cis*-regulatory region and identified a putative Sd binding motif (CATTCCA) (24, 30) in the first intron (Fig. 5*I*). To verify this motif as a true Sd binding site, we generated two firefly luciferase reporters containing either the first half (E1) or the second half (E2) of the intron, with the putative Sd binding motif located in the middle of E2 (Fig. 5*I*), and performed transient dual luciferase assay in S2 cells. Consistent with this prediction, we found E2, but not E1, was able to significantly induce luciferase expression upon Yki transfection (Fig. 5*J*). Moreover, deletion of the putative Sd binding motif (E3, Fig. 5*I*) abrogated the increase in luciferase expression (Fig. 5*J*). To validate the in vitro results, we generated LacZ reporter driven by E2 or E3. Whereas E2–LacZ was significantly induced in the wing disk upon Yki expression (Fig. 5 *K* and *L*), E3–LacZ was barely responsive to Yki (Fig. 5 *M* and *N*). Collectively, these data suggest that the CATTCCA motif is a true recognition site required for the Yki–Sd complex to activate *Rho1* transcription.

Finally, to determine whether the Yki–Sd complex could directly bind to the putative Sd binding motif, we performed chromatin immunoprecipitation (ChIP) experiments in S2 cells

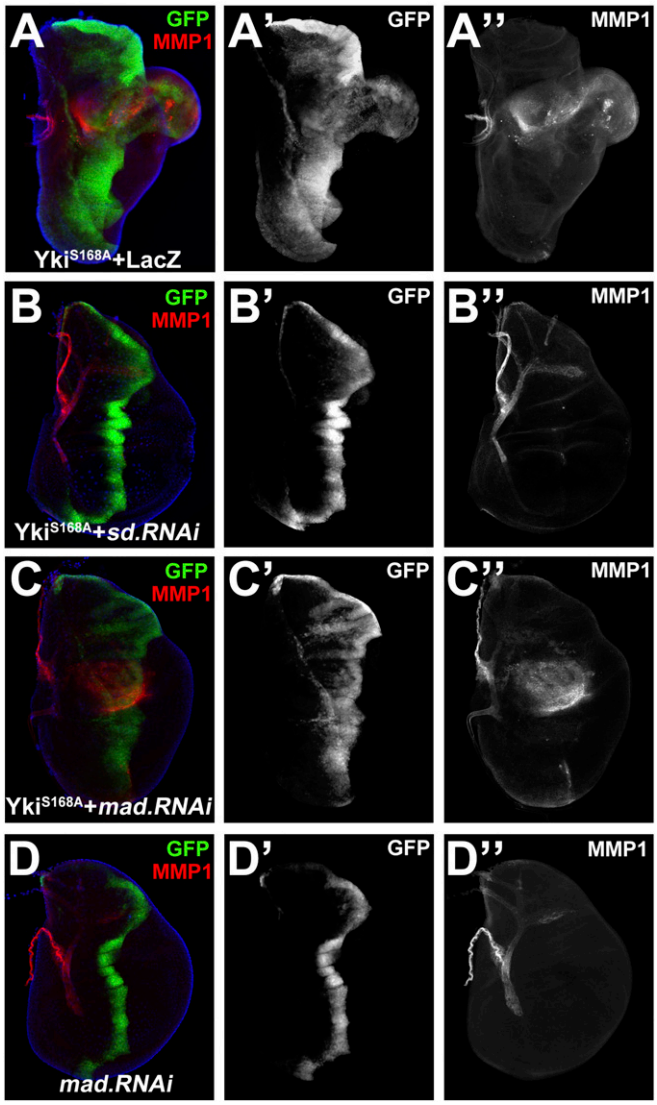

Fig. 4. Yki induces Sd-dependent JNK activation. Knocking down *sd* suppressed Yki-induced JNK activation. Third instar wing discs were stained for MMP1. Yki^{S168A}-induced MMP1 up-regulation (*A*) is suppressed by expression of *sd* RNAi (*B*), but not that of *mad* RNAi (*C* and *D*). Genotype: *ptc* > Yki^{S168A} + LacZ (*A*), *ptc* > Yki^{S168A} + *sd* RNAi (*B*), *ptc* > Yki^{S168A} + *mad* RNAi (*C*), and *ptc*> *mad* RNAi (*D*). See also Figs. S8 and S9.

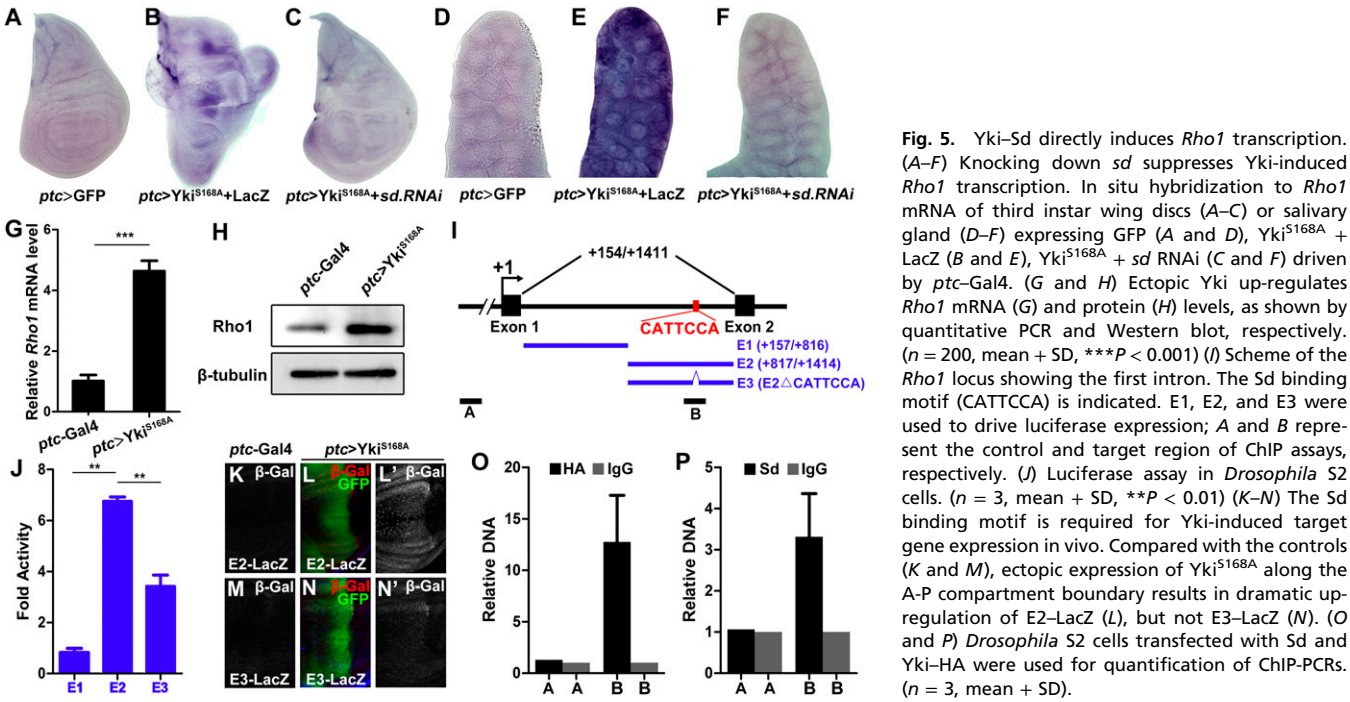

Fig. 5. Yki–Sd directly induces *Rho1* transcription. (*A–F*) Knocking down *sd* suppresses Yki-induced *Rho1* transcription. In situ hybridization to *Rho1* mRNA of third instar wing discs (*A–C*) or salivary gland (*D–F*) expressing GFP (*A* and *D*), Yki^{S168A} + LacZ (*B* and *E*), Yki^{S168A} + *sd* RNAi (*C* and *F*) driven by *ptc*–Gal4. (*G* and *H*) Ectopic Yki up-regulates *Rho1* mRNA (*G*) and protein (*H*) levels, as shown by quantitative PCR and Western blot, respectively. ($n = 200$, mean + SD, ***$P < 0.001$) (*I*) Scheme of the *Rho1* locus showing the first intron. The Sd binding motif (CATTCCA) is indicated. E1, E2, and E3 were used to drive luciferase expression; *A* and *B* represent the control and target region of ChIP assays, respectively. (*J*) Luciferase assay in *Drosophila* S2 cells. ($n = 3$, mean + SD, **$P < 0.01$) (*K–N*) The Sd binding motif is required for Yki-induced target gene expression in vivo. Compared with the controls (*K* and *M*), ectopic expression of Yki^{S168A} along the A-P compartment boundary results in dramatic up-regulation of E2–LacZ (*L*), but not E3–LacZ (*N*). (*O* and *P*) *Drosophila* S2 cells transfected with Sd and Yki–HA were used for quantification of ChIP-PCRs. ($n = 3$, mean + SD).

cotransfected with HA-tagged Yki (HA–Yki) and Sd, followed by qPCR to generate DNA fragments that match either region A located upstream of the *Rho1* transcription start site or region B that harbors the Sd binding motif (Fig. 5*I*). We found that DNA amplified from chromatin precipitated with antibodies against HA (Fig. 5*O*) or Sd (Fig. 5*P*) were appreciably enriched in region B, but not in region A. Whereas neither region was enriched with control IgG antiserum (Fig. 5 *O* and *P*). Together, the above results demonstrate that Yki–Sd directly regulates *Rho1* transcription by targeting to the Sd binding motif located in the first intron of *Rho1*.

Discussion

Recent studies have revealed a complex interaction network between Hippo and other key signaling pathways, including TGF-β/SMAD and Wnt/β-catenin pathways (32), whereas its communication with JNK signaling remains elusive (12, 14, 15). Here we provide genetic evidences that impaired Hippo signaling promotes overgrowth through Rho1–JNK signaling in *Drosophila*. First, loss of Hippo signaling induces JNK activation and its target gene expression. Second, Yki-induced overgrowth is suppressed by blocking Rho1–JNK signaling. Third, ectopic Rho1 expression phenocopies Yki-triggered overgrowth and proliferation when cell death is compromised.

Yki/YAP's ability in promoting tissue growth depends on transcription factors, including Sd/TEADs and SMADs (33). Consistent with this notion, we found Sd, but not Mad, is essential for Yki-induced JNK activation, whereas ectopic Sd expression is sufficient to activate JNK signaling by itself. We further implicated the Rho1 GTPase as the critical factor that bridges the interaction between Hippo and JNK signaling. Rho1 not only mediates Yki-induced JNK activation and overgrowth, but also serves as a direct transcriptional target of Yki/Sd complex. Intriguingly, we also found that Rho1 activation promotes nuclear translocation of Yki in wing discs, and reducing Yki activity significantly impeded Rho1 induced growth (Fig. S7), implying the existence of a potential positive feedback loop to amplify Yki-induced overgrowth and to help maintain signaling in a steady state. Consistent with our observation, recent studies reported that GPCRs could activate YAP/TAZ through RhoA in mammals (34, 35), whereas elevated JNK signaling in *Drosophila* could stimulate Yki nuclear translocation during regeneration and tissue growth (12–14). Thus, our results provide the other side of the story about a novel cross-talk between Hippo and JNK signaling.

Intriguingly, we found that ectopic Yki expression driven by *ptc*–Gal4 induced MMP1 activation (Fig. S2 *B′–C′*), *puc*–LacZ expression (Fig. 3*F*), *rho1* transcription (Fig. 5*B*), and Yki target gene transcription (Fig. S4) predominantly in the proximal region of wing disk, but not that of the dorsal/ventral boundary. This is consistent with a recently published paper showing that tension in the center region of *Drosophila* wing disk is lower than that in the periphery, which correlates with lower Yki activity (36). It is also worth noting that despite the requirement of JNK signaling in Yki-induced wing overgrowth, JNK was not activated strictly in an autonomous manner upon Yki overexpression (Fig. S1). This could be caused by supercompetitive activity of Yki expression clones (18), or, alternatively, through a propagation of JNK signal into neighboring cells (37), which would be very interesting to study further.

Apart from its role in growth control, the Hippo pathway also regulates tumor invasion and metastasis (2). Similarly, JNK signaling plays a major role in modulating metastasis in both flies and mammals (38, 39). Rho1 was also reported to cooperate with oncogenic Ras to induce large invasive tumors (40). Hence, it is likely that Rho1 also acts as the molecular link between Yki and JNK signaling in modulating metastasis as well.

Materials and Methods

***Drosophila* Genetics and Stocks.** All stocks were reared on standard *Drosophila* media at 25 °C unless otherwise indicated. For experiments involving *tub*-Gal80^{ts}, flies were raised at 18 °C to restrict Gal4 activity for 5 d, then shifted to 29 °C for 2 d to inactivate Gal80^{ts}. The following stocks were used for this study: *sd*-Gal4, *ptc*-Gal4, *UAS*-GFP, *UAS*-p35, *UAS*-Rho1 (no. 7334), *UAS*-Yki^{S168A}, *UAS-Rho1* RNAi (nos. 27727 and 9910), *UAS*-LacZ (no. 3956), *dmyc*-LacZ (no. 11981), *ban*-LacZ (no. 10154), and *tub*-Gal80^{ts} were obtained from the Bloomington Stock Center; *UAS-sd* RNAi (no. 101497), UAS-*wg* RNAi (no. 13352), and UAS-*yki* RNAi (no. 40497) were obtained from the Vienna *Drosophila* RNAi Center; *UAS*-Sd^{GA} (30), *puc-LacZ*, *UAS*-Bsk^{DN}, *UAS-hep* RNAi,

UAS-dTAK1 RNAi (41), *ex-LacZ*, *diap1-LacZ*, *UAS*-Yki (24), *UAS-mad* RNAi (42), and *wts*^{X1} (43) were previously described. Clonal analysis is described in *SI Materials and Methods*.

Immunostaining and in Situ Hybridization. Wing discs of third instar larvae were fixed in 4% (wt/vol) paraformaldehyde and stained as described previously (24), using mouse anti-MMP1 (1:200; Developmental Studies Hybridoma Bank, DSHB), mouse anti–β-Gal (1:1,000; DSHB), mouse anti-Wg (1:300; DSHB), rabbit antiphospho-JNK (1:200; Calbiochem), rabbit anti-PH3 [1:100; cell signaling technology (CST)], rabbit antiactive caspase 3 (1: 400; CST), Rat anti-CycE (1:200; gift from Helena Richardson, Peter MacCallum Cancer Centre, Melbourne), rabbit anti-Yki (1:1,000; gift from Duojia Pan, Johns Hopkins University School of Medicine, Baltimore). Secondary antibodies were anti-rabbit Alexa (1:1,000; CST) and anti-mouse Cy3 (1:1,000; Jackson Immuno Research). In situ hybridization was performed on wing discs and salivary glands as previously described (44). Antisense probes for full-length *Rho1* were generated with digoxigenin-labeled deoxyribonucleotide triphosphates (Roche) using a pCS2–Rho1 construst and synthesized by T7 polymerase.

X-Gal Staining. Eye and wing discs were dissected from third instar larvae in PBT (1× PBS pH 7.0, 0.1% Triton X-100) and stained for β-galactosidase activity as previously described (45).

Cell Culture, Transfection, Western Blot Analysis, Luciferase Reporter Assay, qRT-PCR, and ChIP Assay. S2 cells were cultured in Insectagro DS2 (Corning) supplemented with 10% (vol/vol) FBS (HyClone), 50 units/mL of penicillin and 50 μg/mL of streptomycin. Transfection was performed using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. qRT-PCR, Western blot analysis, luciferase reporter assay, and ChIP assay are described in *SI Materials and Methods*.

Statistical Analysis. Quantification of the data is presented in bar graphs created with GraphPad Prism 5. Data represent mean values ± SD. We used one-way ANOVA with corrected Bonferroni multiple comparison test to calculate statistical significance in all our experiments.

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