Hippo signaling promotes JNK-dependent cell migration

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Edited by Norbert Perrimon, Harvard Medical School, Howard Hughes Medical Institute, Boston, MA, and approved January 12, 2017 (received for review December 28, 2016)

Overwhelming studies show that dysregulation of the Hippo pathway is positively correlated with cell proliferation, growth, and tumorigenesis. Paradoxically, the detailed molecular roles of the Hippo pathway in cell invasion remain debatable. Using a *Drosophila* invasion model in wing epithelium, we show herein that activated Hippo signaling promotes cell invasion and epithelial-mesenchymal transition through JNK, as inhibition of JNK signaling dramatically blocked Hippo pathway activation-induced matrix metalloproteinase 1 expression and cell invasion. Furthermore, we identify *bantam*-Rox8 modules as essential components downstream of Yorkie in mediating JNK-dependent cell invasion. Finally, we confirm that YAP (Yes-associated protein) expression negatively regulates TIA1 (Rox8 ortholog) expression and cell invasion in human cancer cells. Together, these findings provide molecular insights into Hippo pathway-mediated cell invasion and also raise a noteworthy concern in therapeutic interventions of Hippo-related cancers, as simply inhibiting Yorkie or YAP activity might paradoxically accelerate cell invasion and metastasis.

Hippo | JNK | *Drosophila* | migration | Rox8

The Hippo pathway is a highly conserved tumor-suppressor pathway recently identified in *Drosophila melanogaster* via genetic screens for growth-regulating genes (1). In *Drosophila*, the core Hippo pathway acts through a serine-threonine kinase cascade, consisting of Hippo (Hpo) and Warts (Wts), to inactivate the transcriptional coactivator Yorkie (Yki) (2–7). Once the Hippo pathway is deactivated, Yki can translocate into the nucleus to interact with different DNA-binding transcription factors to initiate transcription of growth-regulating genes, including cyclin E (*cycE*), *dmyc*, bantam (*ban*), and *Drosophila* inhibitor of apoptosis protein 1 (*Diap1*) (1). However, despite the well-documented roles of the Hippo pathway in regulating various aspects of tumorigenesis, including cell growth, proliferation, and survival (1, 4–7), the role and underlying mechanism of Hippo signaling in tumor metastasis and cell invasion remains controversial. For one, overwhelming studies have shown hyperactivation of YAP (Yes-associated protein, Yki ortholog) in various human cancers (1, 7, 8), and YAP overexpression can promote cell invasion and the epithelial-mesenchymal transition (EMT) of cultured cells (9–13). Paradoxically, recent data suggest that YAP is silenced in a subset of highly aggressive human colorectal carcinomas (14), and acts as an inhibitor of cell invasion in some breast cancer cell lines (15). Furthermore, clinical data indicate that individuals affected by multiple myeloma with low *YAP1* expression had a significant shorter survival than those with high *YAP1* expression (16), suggesting that YAP also has a tumor-suppressor activity in some contexts.

To elucidate the Hippo pathway's contradictory roles in regulating cell migration and invasion in vivo, we use *Drosophila* as a model to investigate the underlying mechanism. Here we show that Hippo pathway activation induces JNK-dependent cell invasion and EMT through *ban* miRNA, and identify Rox8 as an essential downstream mediator for Hippo activation-induced cell invasion.

Results and Discussion

Hippo Pathway Activation Promotes Cell Invasion. In the *Drosophila* wing epithelia, knocking down the cell polarity gene scribbled (*scrib*) along the anterior/posterior boundary using a patched-Gal4 (*ptc*-Gal4) driver produces an invasive migration phenotype (17–19), which has been used to model cell invasion in vivo. First, to investigate whether Hippo signaling activation could modulate cell invasion, we overexpressed Hpo or Wts, or knocked down *yki*, by *ptc*-Gal4. Compared with controls (Fig. 1*A*), activated Hpo signaling triggered invasive migration toward the posterior part of discs, a significant number of GFP^+ cells detached and migrated away basally from the *ptc* expression domain (Fig. 1 *B′*–*D′* and Fig. S1), along with up-regulated matrix metalloproteinase 1 (MMP1) expression (Fig. 1 *B″*–*D″*), a protein required for basement membrane degradation and cancer malignant transformation (20). Furthermore, activated Hippo signaling resulted in down-regulation of E-cadherin and Laminin (Fig. 1 *E′*–*H′*), two common molecular characteristics of EMT. Taken together, these results suggest that Hippo activation in epithelia cells promotes cell invasion and EMT.

Hippo pathway activation regulates apoptosis through transcriptional regulation of DIAP1 (4, 5, 21). In accordance with this finding, we found that loss of *yki* induced strong apoptosis (Fig. S2*B*). Given that cell invasion is frequently accompanied with apoptosis (22), to test if Hippo pathway-induced cell

Significance

Cancer is the leading cause of death worldwide, although studies revealed that dysregulation of the Hippo pathway contributes to tumorigenesis, whereas its roles in tumor invasion and cell migration remain paradoxical and largely elusive. Using *Drosophila* as a model, we herein find cross-talk between the Hippo and JNK pathways in regulating cell migration and invasion. Mechanistically, we identify *bantam*-Rox8 modules as essential components downstream of Yorkie in mediating JNK-dependent cell invasion. Our finding is particularly important as it offers a wake-up call for therapeutic interventions of Hippo-related cancers, because simply increasing Hippo signal activity may paradoxically accelerate cell invasion and metastasis.

Author contributions: X.M., X.Z., J.C., and L.X. designed research; X.M., H.W., J.J., W.X., Y.S., and W.L. performed research; X.M., X.Z., J.C., and L.X. analyzed data; and X.M., X.Z., J.C., and L.X. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1621359114/-/DCSupplemental.

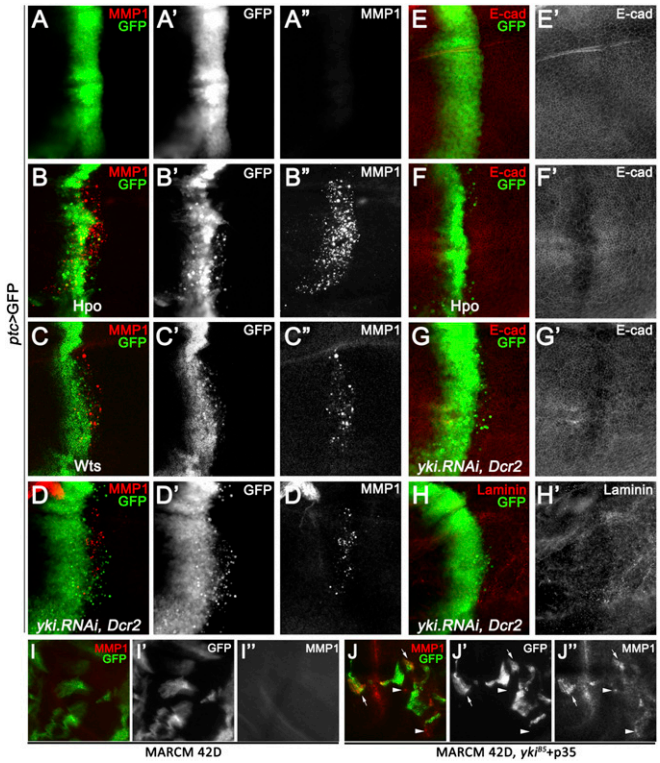

Fig. 1. Activation of Hpo signaling promotes cell invasion. (*A–H*) Fluorescence micrographs of wing discs are shown, anterior is to the *Left*, and cells are labeled with GFP expression. Compared with the control (*A*), ectopic expression of Hpo (*B*), or Wts (*C*), or loss of *yki* (*D*) induces cell invasion and MMP1 expression (red). (*E–G*) Compared with the control (*E*), cells expressing Hpo or knocking down *yki* show reduced E-cadherin expression (*F* and *G*). (*H*) *yki*-depleted cells show reduced Laminin B1 expression. (*I–J*) Compared with wild-type MARCM clones (*I*), *yki* mutant clones coexpressing p35 induces autonomous (arrow) and nonautonomous (arrowhead) MMP1 activation (*J*). [Magnification: (*A–H*) 20×; (*I–J*) 40×.] Genotypes: (*A* and *E*) *ptc*-Gal4 *UAS*-GFP/+; (*B* and *F*) *ptc*-Gal4 *UAS*-GFP/*UAS*-Hpo; *tub*-Gal80^{ts}/+; (*C*) *ptc*-Gal4 *UAS*-GFP/+; *UAS*-Wts/*tub*-Gal80^{ts}; (*D*, *G*, and *H*) *ptc*-Gal4 *UAS*-GFP/+; *UAS-yki.RNAi, UAS*-Dcr2/+; (*I*) *UAS*-GFP, *hs-Flp*; FRT42, *tub*-Gal80/FRT 42; *tub*-Gal4/+; and (*J*) *UAS*-GFP, *hs-Flp*; FRT42, *tub*-Gal80/FRT 42, *yki*^{B5}; *tub*-Gal4/+.

invasion is a result of apoptosis, we blocked caspase activity by coexpressing DIAP1 or a dominant-negative form of the caspase-9 homolog *Drosophila* Nedd-2-like caspase (DRONC), or by the deficiency *Df(3L)H99* that deletes three proapoptotic genes, reaper (*rpr*), head involution defective (*hid*), and *grim*. Interestingly, none of these alternations can significantly suppress loss of *yki*-induced cell invasion and MMP1 expression (Fig. S3 *A–D*), despite a complete inhibition of apoptosis (Fig. S3 *E–G*), confirming that the invasion behavior is not simply a secondary effect of apoptosis.

The induced MMP1 expression does not fully colocalized with GFP-labeled Hippo pathway-activating cells (Fig. 1 *B–D*), suggesting that the Hippo pathway triggers MMP1 activation both autonomously and nonautonomously. To confirm this theory, we blocked apoptosis by expressing p35 in *yki* mutant clones using the mosaic analysis with a repressible cell marker (MARCM) technique (23). We observed protrusions, like structure and distinct MMP1 activation, both cell autonomously (Fig. 1*J*, arrow) and nonautonomously (Fig. 1*J*, arrowhead). Taking these data together, we conclude that Hippo activation induces both autonomous and nonautonomous MMP1 activation and invasive behavior.

JNK Is Required for Hippo Activation-Induced Cell Invasion. MMP1 is a direct transcriptional target of JNK signaling (24), and JNK activation has been shown to play a critical role in modulating cell invasion (17, 18, 24–26). Thus, we hypothesized that the Hippo pathway might regulate cell invasion through activating JNK signaling. To test this idea, we first checked the expression of puckered (*puc*), a transcriptional target and readout of JNK pathway activation (27). Compared with the control (Fig. 2*A*), knocking down Yki by *ptc*-Gal4 resulted in a significant up-regulation of *puc*-LacZ (Fig. 2*B*). Next, we examined JNK activation directly using an antibody specific to the phosphorylated JNK (p-JNK). Elevated p-JNK staining was detected upon Yki depletion (Fig. 2 *C* and *D*). Furthermore, we found blocking JNK signaling by expressing a dominant-negative form of basket (Bsk^{DN}, *bsk* encodes the *Drosophila* JNK) completely impeded *yki* depletion, Wts or Hpo overexpression-induced cell invasion behavior, and MMP1 expression (Fig. 2 *E–J*). In addition, our genetic epistasis analysis showed that compromised JNK signaling by knocking down the JNK kinase hemipterous (*hep*) (27), JNKK kinase dTAK1 (28), or TNF receptor-associated factors 2 (dTRAF2) (29) all significantly suppressed Yki loss-induced invasion phenotype (Fig. S4 *A–E* and *G*), indicating Yki functions upstream of dTRAF2. As a negative control, inactivation of wallenda (*wnd*), a recently identified JNKK kinase that positively regulates cell invasion (18), failed to suppress the invasive behavior (Fig. S4 *F* and *G*). Intriguingly, blocking JNK signaling is not sufficient to suppress loss of *yki*-induced apoptosis (Fig. S2*C*). Together, these results suggest that JNK signaling is essential for activated Hippo signaling-induced cell invasion, but not apoptosis.

JNK Is Required for Hippo Activation-Induced Border Cell Migration. The epistasis data we present above compellingly suggest that Hippo modulates cell migration via JNK activation. Next, to

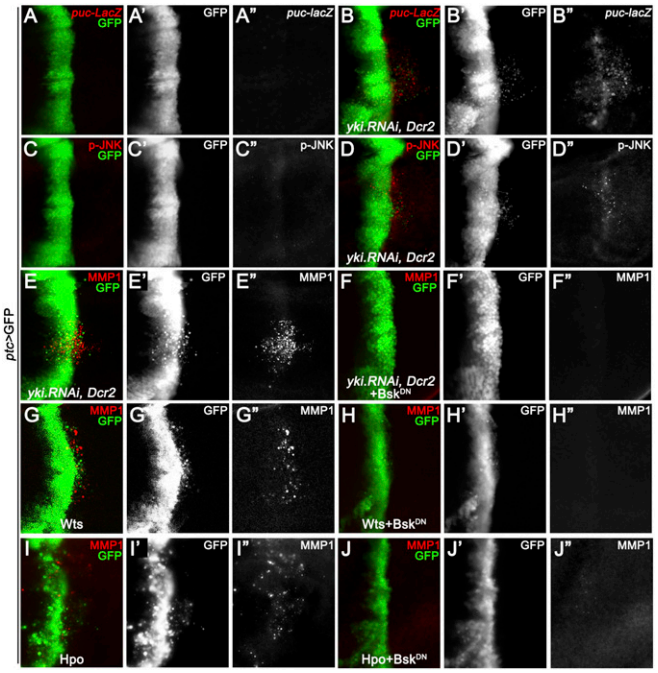

Fig. 2. Bsk is essential for Hpo activation-induced cell invasion. Fluorescence micrographs of wing discs are shown, anterior is to the *Left*, and cells are labeled with GFP expression. (*A–D*) Depletion of *yki* by *ptc*-Gal4 up-regulates *puc* transcription (*B*) and JNK phosphorylation (*D*). (*E–J*) Expression of Bsk^{DN} completely suppressed loss of *yki* (*E*), expression of Wts (*G*) or Hpo (*I*) induced cell invasion and MMP1 expression (*F*, *H*, and *J*). (Magnification: 20×.) Genotypes: (*A*) *ptc*-Gal4 *UAS*-GFP/+; *puc*^{E69}/+; (*B*) *ptc*-Gal4 *UAS*-GFP/+; *UAS-yki.RNAi, UAS*-Dcr2/*puc*^{E69}; (*C*) *ptc*-Gal4 *UAS*-GFP/+; (*D* and *E*) *ptc*-Gal4 *UAS*-GFP/+; *UAS-yki.RNAi, UAS*-Dcr2/+; (*F*) *ptc*-Gal4 *UAS*-GFP/+; *UAS-yki.RNAi, UAS*-Dcr2/*UAS*-Bsk^{DN}; (*G*) *ptc*-Gal4 *UAS*-GFP/+; *UAS*-Wts/*tub*-Gal80^{ts}; (*H*) *ptc*-Gal4 *UAS*-GFP/+; *UAS*-Wts/*UAS*-Bsk^{DN}, *tub*-Gal80^{ts}; (*I*) *ptc*-Gal4 *UAS*-GFP/*UAS*-Hpo; *tub*-Gal80^{ts}/+; and (*J*) *ptc*-Gal4 *UAS*-GFP/*UAS*-Hpo; *tub*-Gal80^{ts}/*UAS*-Bsk^{DN}.

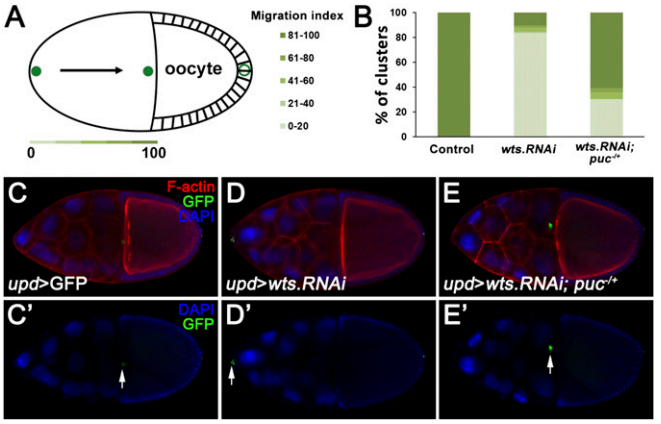

Fig. 3. Hippo pathway promotes JNK-dependent border cell migration in oogenesis. *upd*-Gal4 was used to overexpress or knockdown genes specifically in polar cells. (*A*) Stage-10 migration index for quantification of border cell migration. (*B*) Quantification of stage-10 migration index for the following genotypes: control ($n = 43$), *upd* > *wts.RNAi* ($n = 109$), and *upd* > *wts.RNAi* + *puc*$^{-/+}$ ($n = 109$). (*C*–*E*) Compared with controls, knockdown of *wts* induced border cell migration defect (*D*) was rescued by deleting one copy of *puc* (*E*). (Magnification: 20×.) Genotypes: (*C*) *upd*-Gal4, *UAS*-GFP/+; (*D*) *upd*-Gal4, *UAS*-GFP/+; *UAS-wts.RNAi*; and (*E*) *upd*-Gal4, *UAS*-GFP/+; *UAS-wts.RNAi*/*puc*^{E69}.

investigate the physiological role of Hippo-JNK cross-talk in regulating cell migration, we turn to oogenesis, a developmental process where both JNK and Hippo are required for correct border cell migration (30–32). During normal development, the border cell cluster arrives at the nurse cell–oocyte boundary by stage 10 (Fig. 3*A*) (33), so we selected stage-10 egg chambers to test their genetic interactions. Consistent with previous data (31), we found knocking down *wts* expression in polar cells by *upd*-Gal4 severely disrupted border cell migration (Fig. 3 *B*–*D*). Nevertheless, enhancing JNK signaling by simultaneously deleting one copy of *puc* (34) significantly rescued the border cell migration defect (Fig. 3 *B* and *E*), suggesting that JNK signaling also acts downstream of the Hippo pathway in regulating border cell migration. Interestingly, despite that Yki overexpression phenocopies *wts* knockdown-induced migratory defect (31), we found inhibition of Yki activity under *upd* promoter is not sufficient to accelerate border cell migration (Fig. S5 *A* and *B*), which is consistent with a previous study (31).

***ban* Is Essential for Loss of *yki*-Induced Cell Invasion.** To investigate the molecular mechanism by which JNK mediates Hippo activation-induced cell invasion, we dissected the role of Yki target genes individually, including *Diap1*, *dmyc*, and *ban* (21, 35–37). Overexpression of DIAP1 or Myc fails to suppress loss of *yki*-induced invasion (Fig. 4 *A*, *B*, and *E* and Fig. S3 *A* and *D*), whereas ectopic *ban* expression strongly impedes *ptc* > *yki.RNAi* and *ptc* > Hpo-induced invasive phenotype and MMP1 expression (Fig. 4 *C* and *E* and Fig. S6 *A* and *B*), and expression of *ban* alone has no obvious invasive phenotype (Fig. 4*D*). On the other hand, when *ban* activity was reduced along the anterior/posterior boundary, significant number of cells migrated toward the posterior part (Fig. 4*F*′ and Fig. S6*E*), coupled with increased MMP1 expression (Fig. 4*F*″) and JNK activation (Fig. 4 *H* and *I*), phenocopied loss of *yki* induced invasive behavior. More importantly, the cell invasion, MMP1 activation, and JNK activation phenotypes were all completely suppressed when JNK signaling was blocked (Fig. 4*G* and Fig. S6 *C*–*E*).

Next, to explore the detailed mechanism by which *ban* miRNA regulates JNK-mediated cell invasion, we checked the predicted *ban* binding targets by using an available algorithm called microRNA.org (38). Among all of the candidates, we called specific attention to one gene, *Rox8*, which harbors two potential *ban* binding targets in its 3′ UTR region (Fig. 5*A*). *Rox8* encodes a RNA-binding protein that controls important aspects of development, including alternative splicing and stress granule formation (39, 40). In addition, we have previously performed an unbiased genetic screen for factors modulating JNK signaling (41), and identified *Rox8* as a positive regulator of JNK signaling for Rox8 expression synergistically enhances Egr-induced JNK-dependent cell death (Fig. S7). Importantly, consistent with the computational prediction, we found knocking down *ban* significantly up-regulates Rox8 protein level (Fig. 5 *B* and *C*). Furthermore, depletion of *Rox8* dramatically suppressed loss of *ban*-induced cell invasion, MMP1 expression, and JNK activation (Fig. 5 *D*–*G*), as well as Hippo pathway activation-induced cell invasion and MMP1 expression (Fig. 5 *H* and *I*). These data indicate that *ban*-Rox8 signaling constitutes an essential module downstream of Yki in regulating JNK-mediated cell invasion.

YAP Negatively Regulates TIA1 and Suppress Cell Invasion. Having demonstrated that Hippo activation promotes cell invasion through inhibiting Rox8 in *Drosophila*, we next asked whether the Hippo pathway retains a conserved role in mammals. We examined various cancer cell lines of different origins, including lung (A549), colon (HT29), breast (MCF-7), and brain (U87), and generated stable cell lines with increased or decreased YAP expression using lentivirus (Fig. 6 and Fig. S8). We found YAP overexpression significantly decreases cell invasion in all cancer cell lines, as shown by a transwell assay (Fig. 6 and Fig. S8). Conversely, inhibition of YAP activity significantly increases invasion (Fig. 6 and Fig. S8). More importantly, we further showed that ectopic YAP significantly decreases, whereas YAP knockdown increases TIA1 (Rox8 ortholog) protein level (Fig. 6 and

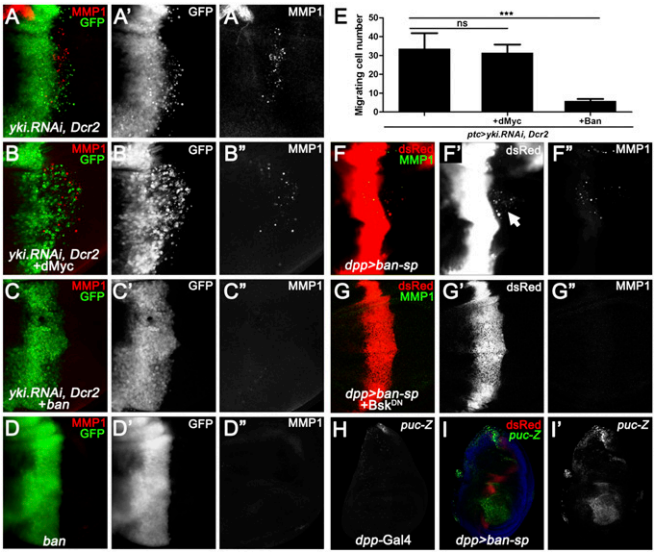

Fig. 4. *ban* is essential for loss of *yki*-induced cell invasion. Fluorescence micrographs of wing discs are shown, anterior in all panels is to the *Left*, and cells are labeled with GFP expression. (*A*–*D*) Overexpression of *ban* (*C*), but not dMyc (*B*), impedes loss of *yki*-induced cell invasion and MMP1 expression, whereas expression of *ban* alone gives no obvious invasion phenotype (*D*). (*E*) Quantification data of cell invasion phenotype in *A*–*C*. Data are presented as mean + SEM. *P* values were calculated using a one-way ANOVA. ***$P < 0.001$; n.s., no significant difference. (*F*–*H*) Expression of *ban sponge* driven by *dpp* promoter induces mild cell invasion, MMP1 expression (*F*), and intensive *puc* transcription (*I*). (*G*) Blocking JNK activity dramatically suppresses loss of *ban* induced cell invasion and MMP1 activation. [Magnification: (*A*–*D* and *F*–*I*) 20×.] Genotypes: (*A*) *ptc*-Gal4 *UAS*-GFP/+; *UAS-yki.RNAi, UAS*-Dcr2/+; (*B*) *ptc*-Gal4 *UAS*-GFP/+; *UAS-yki.RNAi, UAS*-Dcr2/*UAS*-dMyc; (*C*) *ptc*-Gal4 *UAS*-GFP/+; *UAS-yki.RNAi, UAS*-Dcr2/*ban*^{EP3622}; (*D*) *ptc*-Gal4 *UAS*-GFP/+; *ban*^{EP3622}/+; (*F*) *dpp*-Gal4 *UAS-ban-sponge*/+; (*G*) *dpp*-Gal4 *UAS-ban-sponge*/*UAS*-Bsk^{DN}; (*H*) *dpp*-Gal4/*puc*^{E69}; and (*I*) *dpp*-Gal4 *UAS-ban-sponge*/*puc*^{E69}.

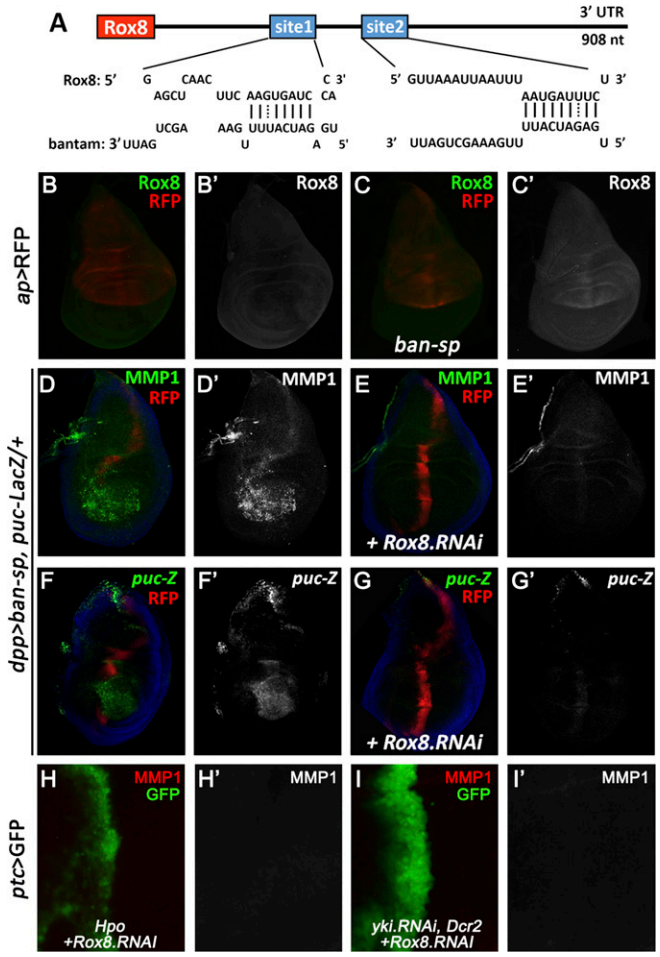

Fig. 5. *ban* down-regulates Rox8 to regulate cell invasion. (*A*) Schematic drawing of the 3′UTR regions of *Rox8* gene highlighting the *ban* seed sites. (*B* and *C*) Compared with controls (*B*), knocking down *ban* by *ap*-Gal4 significantly up-regulated Rox8 protein level (*C*). (*D*–*I*) Fluorescence micrographs of wing discs are shown, anterior in all panels is to the *Left*. Loss of *ban* induced MMP1 expression (*D′*) and JNK activation (*F′*) were both completely suppressed by knocking down *Rox8* activity (*E′* and *G′*). Reducing Rox8 activity impeded Hpo overexpression or loss of *yki*-induced cell invasion (*H* and *I*). (Magnification: 20×.) Genotypes: (*B*) *ap*-Gal4 *UAS*-RFP/+; (*C*) *ap*-Gal4 *UAS*-RFP/+; *UAS-ban-sponge*; (*D* and *F*) *dpp*-Gal4 *UAS-ban-sponge/puc*^{E69}; (*E* and *G*) *dpp*-Gal4 *UAS-ban-sponge/UAS-Rox8.RNAi*, *puc*^{E69}; (*H*) *ptc*-Gal4 *UAS*-GFP/*UAS*-Hpo; *tub-Gal80ts/UAS-Rox8.RNAi*; and (*I*) *ptc*-Gal4 *UAS*-GFP/+; *UAS-yki.RNAi, UAS*-Dcr2/*UAS-Rox8.RNAi*.

Fig. S8). Intriguingly, after analyzing tumor microarray data from the ONCOMINE database (https://www.oncomine.org/index.jsp), we also found a negative correlation between YAP1 and TIA1 levels in both normal lung cells and large cell lung carcinoma (Fig. S9). Together, these data suggest that apart from its tumor-promoting role, YAP can also function as an invasion suppressor.

Rox8 Induces JNK-Dependent Cell Invasion. In accordance with the physiological role of Rox8 in *yki* loss-induced cell invasion, we found overexpression of Rox8 is sufficient to induce JNK activation (Fig. 7 *A*–*D*), MMP1 expression (Fig. 7*E*), and dramatic basal side invasion of the wing epithelium (Fig. 7*F*), which can be strongly suppressed by blocking JNK signaling (Fig. 7 *G* and *H*). Consistent with the notion that invasive behavior is associated with disruption of epithelial integrity, Rox8-expressing cells exhibited increased actin accumulation (Fig. 7*J*), which was also suppressed by Bsk^{DN} expression (Fig. 7*K*). Conversely, we found Rox8-triggered apoptosis remained unaffected by Bsk^{DN} or expression of Timp (MMP inhibitor), even though the cell invasion behavior was completely impeded (Fig. S10), indicating that Rox8-induced cell invasion is also uncoupled from cell death. Taken together, these data suggest that JNK signaling is indispensable for Rox8 induced cell invasion, but not cell death.

Given RNA-binding proteins can directly bind to a specific sequence in mRNA to regulate its stabilization and translation to affect cancer progression (42), and taking into account that Rox8 is a positive regulator of JNK signaling, we cautiously examined the 3′UTR region of several well-known negative regulators of JNK signaling, including cell polarity complex components, disk large (*dlg*), lethal giant larvae (*lgl*), *scrib*, as well as C-terminal Src kinase (*Csk*). Interestingly, we identified five Rox8 putative binding sites (Fig. 7*L*) (CISBP-RNA Database) (43) in the 3′ UTR region of *dlg*, two in *scirb*, and one in *lgl* and *Csk*, indicating that Rox8 may also regulate mRNA level of those genes. To test this theory, we expressed Rox8 under the heat shock (*hs*) promoter in *Drosophila* and examined the mRNA level of these candidate genes. Remarkably, we found only the *dlg* mRNA level was significantly decreased after Rox8 expression, whereas *scrib*, *lgl*, and *Csk* mRNA levels remained unaffected (Fig. 7*M*). Because it has been shown previously that loss of *dlg* under the *ptc* promoter can also induce JNK-dependent cell invasion (44), we conclude that Rox8 expression decreases Dlg, which in turn activates JNK-mediated cell invasion.

Drosophila has been widely considered as an excellent organism to address cancer-related problems for the past decade (45, 46), several in vivo cell invasion and metastasis models were established to dissect genetic details of cancer progression (22, 47–49). Here, using *Drosophila* wing epithelium as a major model, we bring forward an interesting model that elevated

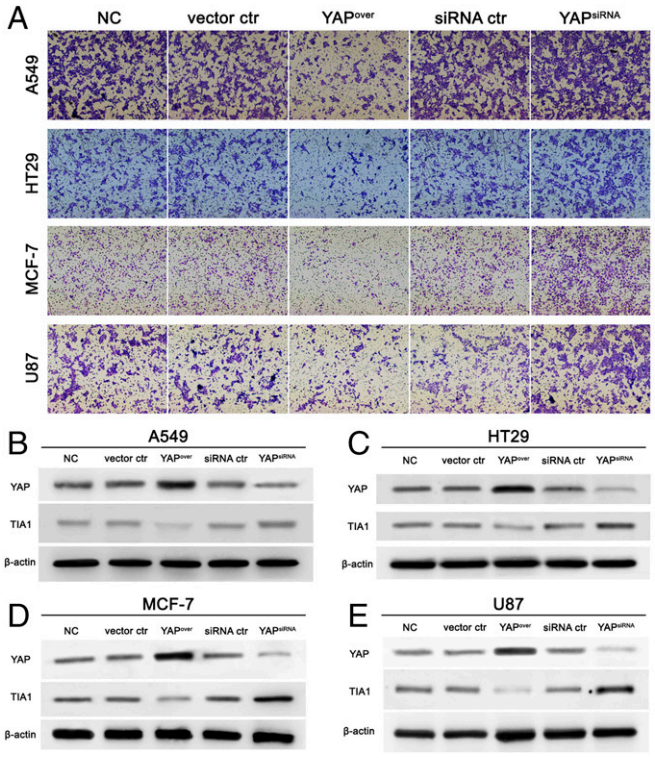

Fig. 6. YAP negatively regulates cell invasion and TIA1 expression. Stable YAP overexpression increased, whereas YAP depletion decreased cell migration (*A*) and TIA1 protein level (*B*–*E*) in cancer cell lines A549, HT29, MCF-7, and U87. NC, negative control; siRNA ctr: control siRNA lentivirus vector; vector ctr, empty lentivirus vector; YAP^{over}, YAP expression lentivirus vector; YAP^{siRNA}: YAP knockdown lentivirus vector. [Magnification: (*A*) 100×.]

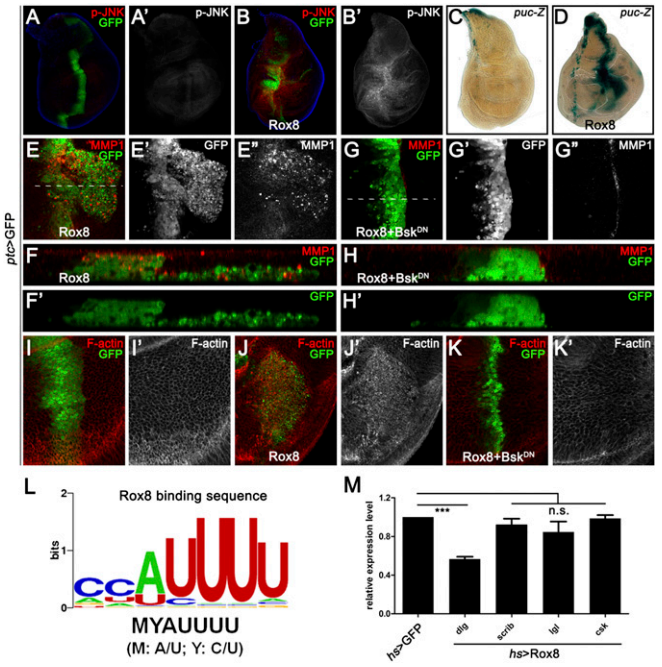

Fig. 7. Rox8 induces JNK-dependent cell invasion. (*A*–*D*) Expression of Rox8 activates JNK phosphorylation (*B*) and *puc* transcription (*D*). (*E*–*H*) Ectopic Rox8-induced MMP1 expression (*E′*) and basal cell invasion (*F*) and F-actin are dramatically suppressed by coexpression of Bsk^{DN} (*G*, and *H*). Compared with control (*I*), Rox8 expression-induced F-actin accumulation (*J*) is completely suppressed by JNK inhibition (*K*). (*L*) Rox8 binding sequence is shown. (*M*) Heat shock-induced Rox8 expression decreases *dlg* mRNA level, whereas *scrib*, *lgl*, and *Csk* mRNA levels remained unchanged, expression data were normalized to one using *hs* > GFP as the control. Results are shown as mean + SEM. *P* values were calculated using a one-way ANOVA. ***$P < 0.001$; n.s., no significant difference. [Magnification: (*A*–*D*) 10×; (*E*–*K*) 20×.] Genotypes: (*A* and *I*) *ptc*-Gal4 *UAS*-GFP/+; (*B*, *E*, *F*, *J*, and *M*–*O*) *ptc*-Gal4 *UAS*-GFP/+; *UAS*-Rox8/+; (*C*) *ptc*-Gal4 *UAS*-GFP/+; puc^{E69}/+; (*D*) *ptc*-Gal4 *UAS*-GFP/+; *UAS*-Rox8/puc^{E69}; and (*G*, *H*, and *K*) *ptc*-Gal4 *UAS*-GFP/+; *UAS*-Rox8/*UAS*-Bsk^{DN}.

Hippo signaling positively regulates cell migration/invasion through *ban*-Rox8 module-mediated JNK activation (Fig. S11). Moreover, we also demonstrated a conserved role of YAP in regulating cell invasion and TIA1 expression (Fig. 6). Consistent with our data, both TIA1 and YAP have nucleo-cytoplasmic shuttling ability (7, 50, 51). Interestingly, recent studies found that *hpo* and *wts* are both required for border cell migration during oogenesis (31, 32), highlighting the importance of cell migration promoting roles of the Hippo pathway. On the basis of that finding, our data further demonstrate that JNK acts downstream of Hippo pathway in inducing normal border cell migration (Fig. 3). Finally, given the fact that YAP is being considered as an important drug target (8, 52–54), our evidence presented herein offers a wake-up call for the therapeutic interventions of Hippo pathway-related cancers because inhibiting Yki (YAP) activity may paradoxically accelerate cell invasion.

Materials and Methods

***Drosophila* Strains and Husbandry.** All crosses were reared on standard *Drosophila* media at 25 °C first; 1 d after egg laying, the F1 generations were shifted to a 29 °C incubator unless indicated otherwise. For experiments involving Hpo and Wts overexpression, *tub*-Gal80ts was used, flies were first raised at 18 °C to restrict Gal4 activity for 5 d, then shifted to 29 °C for 2 d to inactivate Gal80ts. The following strains were used for this study: *ptc*-Gal4, *UAS*-GFP, *UAS*-Dcr2, *tub*-Gal80ts, nd $Rox8^{EP}$ were obtained from the Bloomington Stock Center; *UAS-yki RNAi* (#40497) was obtained from the Vienna *Drosophila* RNAi Center; *UAS*-Rox8 (GS17980) was a GS line obtained from the Kyoto *Drosophila* Genetic Resource Center; *UAS*-DIAP1, *UAS*-$DRONC^{DN}$, *Df(3L)H99*, *UAS-hep RNAi*, *UAS*-Bsk^{DN} (41), *UAS*-Egr, *UAS-dTAK1 RNAi, UAS-dTRAF2 RNAi* (17), *UAS-wnd RNAi* (18), *UAS*-Ban (55) were previously described; and *UAS*-dMyc (gift from Peter Gallant, University of Würzburg, Wuerzburg, Germany), *UAS-ban-sp* (bantam sponge, gift from Marco Milán, The Barcelona Institute of Science and Technology, Barcelona), *UAS*-Hpo and *UAS*-Wts (gifts from Shian Wu, Nankai University, Tianjin, China), *upd* > GFP (gift from Erika A. Bach, New York University School of Medicine).

Clonal Analysis. *yki* mutant clones were generated by crossing *hs-FLP*; *FRT 42D*, *tub-Gal80; tub*-Gal4, *UAS*-GFP with *FRT 42D*, yki^{B5}; UAS-p35/SM6-TM6B. Flp-out ectopic expression clones were generated by crossing *UAS*-transgenes with *y w hs-FLP;act* > y^{+} >Gal4 *UAS*-GFP. Clones were induced at the second instar: heat shock for 6 min at 37 °C 48–72 h after egg laying, and dissection was performed 36 h or 72 h after clone induction.

Immunostaining and X-Gal Staining. Third-instar larvae wing discs were dissected in cold PBS and fixed in freshly made 4% (wt/vol) paraformaldehyde and stained as described previously (18). A detailed description of antibodies used in this study is provided in the *SI Materials and Methods*.

Analysis of Border Cell Migration. Stage-10 egg chambers were selected and analyzed as previously described (31). As an index for migration, stage-10 egg chambers were categorized based on the location of the border cell cluster as depicted in Fig. 3*A*. Fig. 3*B* was generated with Excel (Microsoft).

Cell Invasion Assay. A total of 50,000 cells in suspension with trypsin treatment were added to the upper well of transwell chambers and incubated at 37 °C in 5% CO_2 for 48 h. The bottom chamber contained medium with 10% (vol/vol) FBS to serve as a chemoattractant. Cells that had invaded to the lower surface were fixed in 10% (vol/vol) formalin at room temperature for 30 min, stained with 0.05% Crystal violet, and counted by light microscopy. Mean invasion cells and SD were calculated. Invasion assays were performed in triplicate in three independent experiments. Mammalian cell culture, Western blot analysis, and quantitative real-time PCR are described in *SI Materials and Methods*.

Statistical Analysis. Quantification of the data was presented in bar graphs created with Graphpad Prism 5 (GraphPad Software). Data represent mean values + SD. *P* values were calculated using a one-way ANOVA with corrected Bonferroni multiple comparison tests to calculate statistical significance.

ACKNOWLEDGMENTS. We thank Duojia Pan, Shian Wu, Peter Gallant, Marco Milán, Henri-Marc Bourbon, the Bloomington Stock Center, Vienna *Drosophila* RNAi Center, Kyoto *Drosophila* Genetic Resource Center and the Core Facility of Drosophila Resource and Technology, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, for providing fly stocks and reagents; Dr. Hilal Kazan for helping finding the Rox8 binding motif; and Duojia Pan and Tian Xu. This work was supported by the National Natural Science Foundation of China (31371490, 31571516, 31601024, 81572501, and 81572626) and the Shanghai Committee of Science and Technology (09DZ2260100). Part of the work was conducted in Duojia Pan laboratory at the Department of Molecular Biology and Genetics, Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, and in the Tian Xu laboratory at Department of Genetics, Howard Hughes Medical Institute, Yale University School of Medicine.

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