Hippo signaling suppresses tumor cell metastasis via a Yki-Src42A positive feedback loop

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Metastasis is an important cause of death from malignant tumors. It is of great significance to explore the molecular mechanism of metastasis for the development of anti-cancer drugs. Here, we find that the Hippo pathway hampers tumor cell metastasis in vivo. Silence of hpo or its downstream wts promotes tumor cell migration in a Yki-dependent manner. Furthermore, we identify that inhibition of the Hippo pathway promotes tumor cell migration through transcriptional activating src42A, a Drosophila homolog of the SRC oncogene. Yki activates src42A transcription through direct binding its intron region. Intriguingly, Src42A further increases Yki transcriptional activity to form a positive feedback loop. Finally, we show that SRC is also a target of YAP and important for YAP to promote the migration of human hepatocellular carcinoma cells. Together, our findings uncover a conserved Yki/YAP-Src42A/SRC positive feedback loop promoting tumor cell migration and provide SRC as a potential therapeutic target for YAP-driven metastatic tumors.

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INTRODUCTION

The Hippo pathway controls organ size by coordinating cell proliferation and cell apoptosis through the transcriptional coactivator Yki in Drosophila and YAP/TAZ in mammals [1–5]. Deregulation of the Hippo pathway has been linked to numerous human disorders, including cancers [4–6]. The Hippo pathway consists of a core kinase cascade, wherein the kinase Hpo phosphorylates and activates the downstream kinase Wts which, in turn, phosphorylates the transcriptional coactivator Yki on multiple serine residues to prevent its nuclear accumulation [7, 8]. In the nucleus, Yki pairs with the DNA-binding transcriptional factor Sd to drive target gene expression [9–11]. Well-documented Yki/YAP/TAZ target genes include ex [12], cycE [13], myc [14, 15], ban [16], e2f1 [17], CTGF [11], and diap1 [13], which play pro-proliferative or anti-apoptotic roles. Target genes of Yki/YAP in other cellular processes, such as cell migration, are much less understood. Identifying novel targets downstream of Yki/YAP can not only deepen our understanding of the Hippo pathway, but also pave ways for novel treatment of Hippo-related cancers.

Activation of YAP/TAZ, as manifested by genomic locus amplification, gene fusion, increased expression, or enhanced nuclear translocation, is frequently observed in malignant tumors [5, 6, 18]. These observations strongly suggest that YAP/TAZ activation contributes to tumor progression and metastasis. Indeed, overexpression of YAP in nontransformed epithelial cells results in epithelial-to-mesenchymal transition (EMT), a critical process for cancer metastasis [19, 20]. In addition, ectopic expression of YAP can promote cell migration and invasion in cultured tumor cell line [21, 22]. The role of YAP/TAZ in promoting metastasis has been further implicated in several cancers [18]. Consistently, in Drosophila ovary, over-expression of hpo disrupts polarization of the actin cytoskeleton and thus attenuates border cell migration [23]. How Yki/YAP controls the key steps of metastasis, such as cell migration, remains poorly understood.

To explore the role of Hippo signaling in tumor cell migration, we employed an excellent Drosophila tumor model, in which RNAi of the neoplastic tumor suppressor gene scrib confers wing imaginal disc cells invasive characteristics [24]. We found that loss of hpo or wts enhanced scrib-RNAi-induced cell migration and this enhancement was mediated by Yki. Furthermore, we identified src42A, a Drosophila homolog of human oncogene SRC, as a direct target of Yki which is dispensable for Yki to promote tumor cell migration. Yki, through its DNA-binding partner Sd, directly binds to the second intron of src42A to drive its transcription. Intriguingly, Src42A enhances Yki transcriptional activity vice versa, forming a positive feedback loop. Finally, we showed that SRC is also a target of YAP in human hepatocellular carcinoma (HCC) cells and responsible for YAP-induced cell metastasis. Taken together, we unveiled Src42A/SRC is a critical transcriptional target for Yki/YAP-induced tumor cell migration, and provided SRC as a potential therapeutic target for Hippo-related cancers.
RESULTS
Inhibition of the Hippo pathway promotes tumor cell migration

In Drosophila wing disks, knockdown of scrib along the anterior/posterior (A/P) boundary using the ptc-gal4 driver induces an invasive cell migration phenotype, which has been widely used as model tumor cell migration [25]. To investigate whether the Hippo pathway is involved in regulating tumor cell migration, we genetically manipulated this pathway activity in this tumor cell migration model. Compared with the control disc (Fig. 1A, G), knockdown of hpo or wts enhanced the migration of scrib-RNAi cells (Fig. 1B, C, G). However, lacZ co-expression did not affect cell migration (Fig. S1A–C), removing the effect of Gal4 titration. This enhancement was mediated by Yki, as silencing src42A or wts-RNAi-induced enhancement of tumor cell migration (Fig. 1D, E, G). In contrast, knockdown of yki nearly blocked scrib-RNAi-induced cell migration (Fig. 1F, G). To confirm the RNAi efficiencies, we dissected the salivary gland for RT-qPCR assays. The immunostaining results showed that the ptc-gal4 was able to drive gene expression throughout the salivary gland (Fig. S1D, E). RT-qPCR data revealed that RNAi lines could silence corresponding genes (Fig. S1F). To show that the role of Yki in scrib-RNAi-induced cell migration is not confined to the ptc-expressing cells, we created random scrib RNAi clones using the FLP-out technique which were marked with green fluorescence (GFP) [26]. Knockdown of scrib induced moderate cell migration in these clones (Fig. 1H), which was enhanced by overexpressing yki (Fig. 1I). Taken together, these results indicate that inhibition of the Hippo pathway promotes scrib-RNAi-induced tumor cell migration.

**src42A is a transcriptional target of Yki**

To identify the related target gene(s) accounting for Yki-enhanced tumor cell migration, an RNA-seq analysis was carried out. We collected adult heads of control (GMR) and yki overexpression (GMR > yki) flies for RNA-seq analysis. Compared with the control, 1241 genes were upregulated in GMR > yki samples (Fig. S2A). By comparing our RNA-seq results with other published RNA-seq [27] and Yki ChIP-seq data [28], we found 123 overlapping genes (Fig. S2B). The list of overlapping genes encompasses several well-known Yki-Sd targets, such as ex [12], wg [29], rho1 [30], ds [31], and crb [32] (Fig. S2C), confirming the reliability of this analysis. We selected src42A (Fig. S2C), which is a Drosophila homolog of human oncogene SRC, for further investigation given its capability of promoting tumor metastasis [33, 34].

To validate our RNA-seq results, we used RT-qPCR analysis to examine the expression level of src42A as well as several other target genes. Consistent with the RNA-seq result, elevated src42A expression was detected by RT-qPCR in GMR > yki samples (Fig. S2D). In addition, analysis of published ChIP-seq data revealed robust enrichment of Yki on the genomic locus of src42A (Fig. S2F) as well as the known Yki target ex (Fig. S2E) [28]. Taken together, these results suggest that src42A is a potential transcriptional target of Yki.

To confirm that src42A is a bona fide target of Yki, we used a src42A-lacZ reporter, in which the lacZ sequence was inserted downstream of src42A promoter. Compared with the control disc (Fig. 2A), overexpression of yki increased src42A-lacZ level (Fig. 2B). Secondly, we generated mouse anti-Src42A antibody to verify the induction of endogenous Src421A by Yki. Overexpression of a
dominant negative form of src42A (src42A(ΔN)) elevated (Fig. S3A, B), while knockdown of src42A decreased staining signals (Fig. S3C), confirming the specificity of the antibody. Consistently, ectopic expression of yki also upregulated Src42A protein level (Fig. 2D), compared to the control disc (Fig. 2C).

**Yki activates src42A expression through Yki-Sd complex**

As a transcriptional coactivator, Yki acts with the DNA-binding partner Sd to drive gene expression [9, 10]. We next examined whether Sd is required for Yki to induce src42A expression. Compared with yki overexpression alone (Fig. 2B), simultaneous knockdown of sd repressed src42A-lacZ upregulation (Fig. 2E). A previous study showed that mutation of S94 of YAP abolishes its affinity to TEAD1 [35]. Intriguingly, this serine residue was evolutionarily conserved between Drosophila Yki and human YAP (Fig. 5A). Consistently, Yki-S74F with the conserved serine substituted by phenylalanine (F) indeed lost its binding activity to Sd (Fig. S4B, C). We next further assessed the Yki-S74F mutant by examining two well accepted transcriptional reporters of Yki-Sd complex. To ensure equal expression of Yki and Yki-S74F constructs were introduced into the same genomic locus using the phiC31 integrase system [36]. Compared with control disks (Fig. S4D, G), overexpression of yki markedly increased the expression levels of two reporters diap1-lacZ (Fig. S4E) and ex-lacZ (Fig. S4H), while Yki-S74F failed to do so (Fig. S4F, I), indicating that Yki-S74F is not able to interact with Sd to drive target gene expression. In keeping with the sd RNAi results (Fig. 2E), the Yki-S74F mutant was also not able to induce the expression of src42A-lacZ (Fig. 2F). Taken together, these results have clearly demonstrated that Yki activates src42A expression in an Sd-dependent manner.

**Src42A is required for Yki to induce tumor cell migration**

Having established src42A as a transcriptional target of Yki, we then assessed the functional relevance of Src42A in Yki-mediated tumor cell migration. Compared with hpo RNAi alone (Fig. 3A, E), simultaneous knockdown of src42A inhibited tumor cell migration (Fig. 3B, E). Similarly, silence of src42A also reduced wts-RNAi-induced tumor cell migration (Fig. 3C-E). Furthermore, enhanced Scrib-RNAi-induced cell migration by yki overexpressing (Fig. 3F) was suppressed by src42A knockdown (Fig. 3G). Taken together, these results suggest Src42A is a critical downstream effector of Yki in tumor cell migration.

**Sd binds directly to the second intron of src42A**

Since Sd is indispensable for Yki-induced src42A expression, we next examined whether src42A is a direct transcriptional target of Yki-Sd complex. Sd recognizes a conserved DNA sequence, named as Hippo responsive element (HRE), to turn on gene expression [9]. We searched in the src42A gene and found three potential HREs (named as HRE-1, HRE-2, and HRE-3), of which HRE-1 is localized in exon region, while HRE-2 and HRE-3 are localized in the second intron region (Fig. 4A). To test which HRE is involved in regulating src42A expression, we performed luciferase analyses, in which Yki-Sd complex was used to drive luciferase expression from a DNA fragment containing one of these three HREs (Fig. 4A). Interestingly, only S3-Luc which contains HRE-3 responded to Yki-Sd (Fig. 4B). To check whether Yki-Sd directly binds to HRE-3, we carried out ChIP-qPCR and found that Yki indeed bound to HRE-3 in the presence of Sd (Fig. 4C). In addition, electrophoretic mobility-shift assays (EMSAs) confirmed the direct interaction between Sd and HRE-3 (Fig. 4D, E). To further validate this Sd binding site in vivo, we generated lacZ reporters, in which the lacZ sequence was inserted downstream of the DNA fragment containing one of these three HREs (Fig. 4A). S1-lacZ, S2-lacZ, and S3-lacZ were introduced into the same locus using the phiC31 integrase system. Compared with control disks (Fig. S5A, C), overexpression of yki was unable to activate S1-lacZ and S2-lacZ expression (Fig. S5B, D). However, Yki sharply turned on S3-lacZ expression (Fig. 4G), compared to the control disc (Fig. 4F).

After demonstrating that Sd directly binds to the third HRE, we sought to examine whether this HRE is essential for Yki to induce endogenous src42A expression. To this end, the Cas9-mediated genome editing was employed to delete the third HRE. Two guide RNAs (gRNAs) and Cas9 plasmid were injected into embryos (Fig. 5E). After screening, we identified a 241 bp deletion line of Drosophila, in which the third HRE together with part of the adjacent sequence were deleted without affecting the coding
sequence (Fig. S5F, G). The homozygous mutants (refers to del/del hereafter) could survive to adulthood and did not show any obvious developmental defects. Intriguingly, compared with wild type controls, del/del flies showed a decreased expression of src42A (Fig. 4H). In addition, overexpression of yki elevated Src42A protein in wild type background, but not in del/del src42A mutants (Fig. 4I–L). Together, our results reveal that Yki-Sd complex directly binds to HRE-3 to drive the expression of src42A. Src42A inhibits the Hippo pathway activity

The src42A gene encodes a non-receptor tyrosine kinase involved in multiple cellular processes, such as cell proliferation [37], cell adhesion [38], cell death [34], and regeneration [39]. Its mammalian orthologue SRC is the first identified oncogene [40]. Recently, several studies have shown that SRC is involved in mammalian Hippo pathway regulation. SRC inhibits the Hippo pathway through tyrosine phosphorylation of LATS1 (the Drosophila Wts homolog) to prevent LATS1 activation [41]. Besides, SRC activates the Rho/ROCK pathway to influence actin cytoskeleton, in turn suppress LATS activity [42]. In addition, SRC can also directly phosphorylate YAP to enhance its stability and transcriptional activity [43, 44]. Thus, we next explored whether Src42A regulates Yki activity to form a feedback loop in Drosophila. Compared with the control disc (Fig. 5A), knockdown of src42A slightly but detectably, decreased diap1-lacZ signals (Fig. 5B). Conversely, ectopic expression of a constitutive active form of Src42A (Src42A^{CA}) promoted diap1-lacZ expression (Fig. 5C). In addition, depletion of Src42A inhibitor, the C-terminal Src kinase (Csk) [45] also elevated diap1-lacZ level (Fig. 5D). These data show that Src42A is a positive regulator of Yki activity.

To elucidate the mechanism whereby Src42A regulates the Hippo pathway, we performed epistatic analyses. Compared with src42A RNAi alone (Fig. 5E), simultaneous knockdown of hpo failed to restore diap1-lacZ expression (Fig. 5F). However, knockdown of wts (Fig. 5G) or overexpression of yki (Fig. 5H) rescued src42A-RNAi-induced diap1-lacZ downregulation, suggesting that Src42A acts upstream of or parallel to Wts.

The Hippo pathway is a kinase cascade that ultimately leads to Yki phosphorylation and cytoplasmic retention [5]. Thus, the phosphorylation status of Yki protein can reflect the Hippo pathway activity [46]. Knockdown of src42A in S2 cells elevated phosphorylated Yki (pYki) level (Fig. 5I). However, we also observed that knockdown of src42A decreased Wts protein, without affecting Yki and Hpo protein levels (Fig. S6A). Considering the previous study has demonstrated that wts is a potential transcription target of Yki [28], it is acceptable that knockdown of src42A reduces Wts through inhibiting Yki expression.

Since the non-receptor tyrosine kinase Src42A/SRC plays its roles always through binding and phosphorylating substrates [47, 48], we expected that Src42A possibly interacted with a component of the Hippo pathway. To test this possibility, co-immunoprecipitation (co-IP) assays were carried out in Drosophila S2 cells. Consistent with above epistatic results, Src42A exclusively interacted with Wts (Fig. 5J, K), but not with other Hippo pathway proteins.
components (Fig. S6D–J). Furthermore, Src42A promoted Wts tyrosine phosphorylation (Fig. 5L). During Hippo signal transduction, the physical interaction between Wts and its adapter, Mob as a tumor suppressor (Mats), is a critical step for Wts kinase activation [49]. In agreement with the previous observation in mammalian cells [41], we found that the affinity between Wts and Mats was weakened by Src42A (Fig. 5M), indicating that the mechanism whereby Src42A/SRC regulates Hippo signaling...
through Wts/LATS1 is conserved from Drosophila to mammalian cells.

**SRC is a target of YAP and is critical for YAP-induced tumor cell migration**

Having shown that the Yki-Src42A module promotes tumor cell migration in Drosophila, we then tested whether a similar mechanism operates in human tumor cells. We chose human hepatocellular carcinoma (HCC) because our previous study has revealed that YAP protein is upregulated in HCC samples and closely linked to tumor progression [50]. To assess the expression of YAP and SRC in HCC, we first analyzed the microarray data from the Oncomine database (https://www.oncomine.org) and found that both YAP and SRC were highly expressed in HCC tissues compared with normal liver tissues (Fig. 6A, B) [51]. A co-expression analysis using protein atlas data (https://www.proteinatlas.org) showed that SRC mRNA levels positively correlated with YAP mRNA in HCC samples (Fig. 6C). To validate these bioinformatic results, we carried out RT-qPCR experiments and found that both YAP and SRC were indeed upregulated in HCC samples compared to the adjacent normal tissues (Fig. 6D).

To test whether SRC is a target of YAP in HCC cells, we first analyzed protein levels of SRC and YAP in six HCC cell lines. Both RT-qPCR and western blot results revealed that SRC positively...
Fig. 6  YAP promotes HCC cell migration through SRC. A Comparison of normal liver tissues \((N = 220)\) with HCC samples \((N = 225)\) showed upregulation of YAP mRNA in HCC samples. B The expression of SRC was increased in HCC samples. C Correlation analysis of SRC and YAP expression in HCC samples \((N = 360)\) revealed positive correlation between SRC and YAP. D RT-qPCR analyses showed upregulation of YAP and SRC expression in clinical HCC samples. E Immunoblots (IBs) analyses for protein levels of YAP and SRC in six HCC cell lines. PLC/PRF/5 cells showed minimum expression of SRC and YAP. Actin acts as a loading control. F Transfection of YAP increased SRC protein in PLC/PRF/5 cells. Actin serves as a loading control. Quantification was shown on the right. G RT-qPCR analyses showed YAP-dependent upregulation of SRC mRNA level in PLC/PRF/5 cells. AKD1, AREG, CTGF, and CYR61 are well-known YAP targets and used as positive controls. H Knockdown of YAP downregulated SRC protein level in PLC/PRF/5 cells. Actin serves as a loading control. Quantification was shown on the right. I Transwell analyses showed that YAP promoted PLC/PRF/5 cell migration, which was attenuated by SRC knockdown. Quantification was shown on the right. J The increased cell migration induced by YAP was suppressed by several SRC inhibitors in SK-Hep-1 cells. Quantification was shown on the right. Scale bars: 600 μm for all images.
correlated with YAP in different HCC cell lines (Fig. 6E and Fig. S7A). We then tried to manipulate YAP level in cultured HCC cells. In order to reduce the interference of endogenous YAP, PLC/PRF/5 cells with low YAP expression were selected for subsequent studies. In line with our idea, transfection of YAP substantially elevated both SRC protein (Fig. 6F) and SRC mRNA (Fig. 6G) in PLC/PRF/5 cells. Conversely, knockdown of YAP inhibited SRC expression (Fig. 6H). In mammalian cells, YAP activates target gene expression through its partner TEAD [5]. To test whether YAP turns on SRC expression through YAP-TEAD complex, we sought to silence TEAD. The mammalian genome contains four highly homologous TEAD family members: TEAD1-4. To overcome the functional redundancy, we attempted to block the activity of all TEAD factors by treating cells with verapamil (VP), a compound capable of blocking YAP-TEADs interactions [52]. YAP-SSA, the active form of YAP [53], was able to increase SRC protein level in HepG2 cells, which was attenuated by VP treatment (Fig. 5B7). Furthermore, we generated a mutant form of YAP, YAP-SSA-S94F, which failed to bind TEAD factors (Fig. 5C7). Compared with YAP-SSA, YAP-SSA-S94F was unable to increase SRC protein level (Fig. 5D7). The above results together suggest that YAP activates SRC expression through YAP-TEAD complex.

Moreover, we searched TEAD binding sites in SRC gene region and found three sites (HRE-1, HRE-2, and HRE-3) (Fig. 5E7). Intriguingly, all three HREs localized in the first intron (Fig. 5E7). We generated luciferase reporters, which respectively contain one HRE. The results showed that HRE-1-Luc and HRE-3-Luc were able to respond to YAP-SSA in both 293T cells (Fig. 5F7) and SMMC-7721 cells (Fig. 5G7). Consistently, ChIP-qPCR assay revealed that Myc-YAP-SSA could pull down HRE-1 and HRE-3, not HRE-2 in SMMC-7721 cells (Fig. 5H7). Taken together, these results suggest that YAP/TEAD activates SRC expression through direct binding the HRE-1 and HRE-3 degrons.

Given our *Drosophila* data demonstrating that Yki promotes tumor cell migration through its target Src42A, we next examined whether SRC is also involved in YAP-induced HCC cell migration. Using transwell assays, we found that YAP promoted PLC/PRF/5 cell migration, which was attenuated by SRC knockdown (Fig. 6I), indicating that YAP enhances tumor cell migration, at least partially through SRC. In fact, several inhibitors of SRC have been under preclinical anticancer experiments [54, 55]. We next examined whether these inhibitors are able to suppress YAP-induced HCC cell migration. Compared with control cells, transfection of YAP apparently promoted cell migration, which was blocked by treatment with SRC inhibitors, including PP2, Dasatinib (Dasa) and Saracatinib (Sara) (Fig. 6J). Taken together, our results show that SRC is a transcriptional target of YAP in HCC cells and SRC inhibitors are able to suppress YAP-induced tumor cell migration.

**DISCUSSION**

Over the past decade, the Hippo pathway effector YAP has been shown to be frequently activated in malignant tumors and has emerged as an important player in cancer initiation, progression and metastasis. The well-known function of YAP in promoting cell proliferation and inhibiting apoptosis has been linked to its potent role in driving the initiation and growth of tumors. In contrast, how YAP activation contributes to tumor metastasis is still poorly understood. In this study, we found that Yki directly induces the transcription of src42A, a homolog of human oncogene SRC, to promote cell migration, a key step of tumor metastasis. We went further to show that SRC is also a target of YAP and SRC induction by YAP contributes to the migration of HCC cells. Interestingly, Src42A/SRC is able to activate Yki/YAP, thus forming a feedforward loop to drive tumor progression. Taken together, our study identifies a conserved Yki/YAP-Src42A/SRC positive feedback loop promoting tumor cell migration and provides SRC as a potential therapeutic target for YAP-related malignancies.

Although it is clear that the Hippo pathway regulates growth and apoptosis, its role in cell migration is still unclear. Two previous studies showed that the Hippo pathway promotes the migration and extrusion of noncancerous cells through distinct mechanisms [56, 57]. Herein, we showed that the Hippo pathway restricts the activity of Yki to suppress the migration of tumor cells. Our results are consistent with the abundant evidence showing that Yki homolog YAP/TAZ promotes the migration and invasion of cancer cells [6, 18, 58]. Thus, whether Hippo signaling promotes or prevents cell migration might depend on cell transformation status. Interestingly, a recent study shows that YAP knockdown increases the migration of breast cancer cells [59]. Our results show that YAP promotes the migration of HCC cells. Thus, the role of YAP in cancer cell migration seems to be also context-dependent and requires more investigation.

So far, most of reported Yki transcriptional targets are proliferative or anti-apoptotic genes [28]. The key to elucidate the role of the Hippo pathway in cell migration is to identify migration-related targets of Yki. Here, we demonstrate that src42A is a novel transcriptional target of Yki, and provide strong evidence to support Src42A is a critical effector for Yki-induced tumor cell migration. Our conclusion is firmly supported by the identification of a HRE in the second intron of src42A gene and by showing that this HRE is not only essential but also sufficient for Yki to induce src42A expression. The SRC oncogene has been strongly implicated in the initiation and progression of various human cancers. SRC gene encodes a non-receptor protein tyrosine kinase that phosphorylates several downstream targets, including STAT3, FAK, and RAS to regulate many cellular processes such as cell growth, invasion, angiogenesis and migration [60]. Elevated SRC protein or kinase activity has been reported in many human cancers. Although it is clear that the activity of SRC is controlled by phosphorylation, myristoylation and partner interaction, how to upregulate its expression in tumor cells still remain unknown. Here, we reveal that SRC is a transcriptional target of YAP in HCC cells.

Strict regulation of the Hippo pathway activity is essential for animal development and adult tissue homeostasis [1, 2, 4, 5, 61]. To achieve this goal, multiple negative feedback regulatory mechanisms have been employed to ensure the homeostasis of the Hippo pathway. For example, in *Drosophila*, several components of the Hippo pathway, including Ex and Ds, are Yki transcriptional targets [12, 31]. In turn, Ex and Ds activate Hippo signaling to inhibit Yki activity to form negative feedback loops [62, 63]. In human ovarian surface epithelial cells, YAP activates its upstream inhibitory kinase LATS2 through inducing LATS2 transcription [64]. However, in this study, we instead identify a positive feedback loop to modulate the Hippo pathway. Yki activates src42A expression, and in turn, Src42A increases Yki transcriptional activity. We show that SRC is also a target of YAP in HCC. Several studies have demonstrated that SRC activates YAP in mammalian cells [41, 65], implying that this positive feedback mechanism is conserved in mammals. Although this study showed that Src42A activates Yki through phosphorylating Wts, we could not remove other mechanisms for Src42A regulating Yki. Our results suggest that through this positive feedback loop, Yki/YAP activity is amplified to drive tumor cell migration. It will be interesting to examine whether this positive feedback loop drives tissue growth in regeneration and tumorigenesis.

Although numerous studies have established an important role for the Hippo pathway in tumorigenesis, drugs targeting this pathway have been shown to be difficult due to a number of reasons. First, most of the key components of the Hippo pathway are tumor suppressors, which are not suitable targets for tumor therapy. Second, the downstream effector YAP is a transcriptional coactivator which is hard to be targeted by drugs. Third, YAP also plays an indispensable role in adult homeostasis and most of its known targets are necessary to maintain physiological cell growth
and apoptosis. Therefore, the search for tumor-related genes downstream of YAP, is urgent for drug development. Here, we show that the oncogene src42A/SRC is a downstream target of Yki/ YAP, and that SRC inhibitors are able to suppress YAP-induced tumor cell migration, providing SRC as an alternative target for Hippo-related cancer treatment. In addition to HCC, dysregulation of the Hippo pathway has been linked to various other types of cancers, such as colon cancer [66] and breast cancer [67]. It is thus worthwhile to examine whether targeting this YAP-SRC positive feedback loop also suppresses tumor cell migration in other types of malignancies.

**MATERIALS AND METHODS**

**Drosophila genetics**

UAS-GFP, hpo-RNAi, wts-RNAi, yki-RNAi, UAS-yki, Ayl-gal4, ApG4, ptc-gal4, En-gal4, and diap-1-lacZ had been described in our previous study [50]. src42A-RNAi, scrib-RNAi and UAS-lacZ were kindly provided from Dr. Lei Xue lab [34]. ex-lacZ (#11067, BDsk), csk-RNAi (#41712, BDsk), src42a-lacZ (#107235, KDsk), UAS-src42a (1009998, KDsk), UAS-src42a (1007747, KDsk) and src42a-RNAi (#101497, VDRC) were purchased from Bloomington Drosophila Stock Center (BDsk), Kyoto Drosophila Stock Center (KDSk) or Vienna Drosophila Stock Center (VDRC). Transgenic flies expressing equal Yki and Yki-src74F were generated by inserting UAS-attB-yki and UAS-attB-yki-src74F constructs into the 68A4 attP locus (#25710, BDsk) expressing phiC31 integrase.[33] To make lacZ reporter flies, we first amplified S1 (4501–5000), S2 (13201–13700) and S3 (19602–20000) sequences and respectively cloned into CPLE2N, an enhancer vector that contains the hsp70 basal promoter and lacZ reporter [9]. Then, these recombinant plasmids were introduced into 25C6 attP locus (#25709, BDsk) respectively cloned into CPLZN, an enhancer vector that contains the Vienna feedback loop.

**Immunostaining**

In brief, two gRNAs were designed using an on-line website (http://crispr.dbcls.jp/), and were inserted downstream of U6b promoter [68] to get U6b-gRNA-1 and U6b-gRNA-2 constructs. Then, these two U6b-gRNA constructs (250 ng/μl each) were co-injected with nos-Cas9 plasmid (500 ng/μl) into one-cell embryos. All G0 adult flies from injected embryos were crossed to balancer (Sp/CyO) females to get attB-S1-lacZ, attB-S2-lacZ and attB-S3-lacZ flies. To generate the third HRE site deleted src42a fly (src42a HRE−/−), Cas9-mediated genome editing technique was performed according to the previous described [68]. In brief, two gRNAs were designed using an on-line website (http://crispr.dbcls.jp/), and were inserted downstream of U6b promoter [68] to get U6b-gRNA-1 and U6b-gRNA-2 constructs. Then, these two U6b-gRNA constructs (250 ng/μl each) were co-injected with nos-Cas9 plasmid (500 ng/μl) into one-cell embryos. All G0 adult flies from injected embryos were crossed to balancer (Sp/CyO) females to get attB-S1-lacZ, attB-S2-lacZ and attB-S3-lacZ flies. To generate the third HRE site deleted src42a fly (src42a HRE−/−), Cas9-mediated genome editing technique was performed according to the previous described [68].

**RNA isolation and real-time quantitative PCR**

Cells, HCC samples and adult Drosophila heads were lysed in TRIzol (Invitrogen) for RNA isolation following standard protocols. One microgram RNA was used for reverse transcription by HsScript™ Q RT SuperMix with gDNA wiper (Vazyme) according to the instructions. Real-time quantitative PCR (RT-qPCR) was performed on Bio-Rad CFX96™ with ChamQ SYBR™ Color qPCR Master Mix (Vazyme). 2−ΔΔCT method was used for relative quantification. The primer pairs used were as follows: yki, 5′-TGG CGT ATT TNC ATT CTA C-3′ (forward) and 5′-CAG GTT GCT TGA GAG CAT C-3′ (reverse); Yki, 5′-TCT TCC TGT CTG CAG ACT A-3′ (forward) and 5′-GGA TTA CCG TAG ATC CTT-3′ (reverse); yki, 5′-GCC TTA CCG TAG ATC CTT-3′ (forward) and 5′-GGA CCG GAG CAT CGG TAA-3′ (reverse); and yki, 5′-GCC TTA CCG TAG ATC CTT-3′ (forward) and 5′-GGA CCG GAG CAT CGG TAA-3′ (reverse).

**Luciferase reporter assays**

For luciferase experiments, the sequences containing potential HREs were amplified and inserted into pGL3-Basic-Luc vector (71) to generate S1-Luc (4501–5000), S2-Luc (13201-13700), S3-Luc (19602-20000), HRE-Luc (16145-16645), HRE-2-Luc (17285-17785), and HRE-3-Luc (17744-18244) reporters. Dual luciferase reporter assays were carried out according to the previous described [30]. Drosophila S2 cells or 293T cells transfected with indicated plasmids. Forty-eight hours after transfection, cells were lysed with passive lysis buffer and luciferase activities were measured using a Dual Luciferase Assay Kit (Vazyme) according to the manufacturer’s instructions. In this assay, diaphorase S2 cells serves as a positive control. All luciferase activity data are presented as means±SD of values from at least three experiments.
**ChIP-qPCR assays**  
For chromatin immunoprecipitation (ChIP) assay, 52 cells were transfected with indicated constructs. Forty-eight hours after transfection, cells were harvested and fixed with 4% fresh-made formaldehyde for 10 min at room temperature. After washed with PBS, cells were lysed with lysis buffer supplemented with protease inhibitor cocktail (#GK10014, Gibiо) and PMSF (#93482, Sigma). Then, the chromatin was sheared by sonication for proper cycles at 4 °C. The sheared chromatin was subjected for immunoprecipitation with mouse IgG (Abclonal) or anti-Myc antibody. After immunoprecipitation, the beads were washed three times with lysis buffer and treated with reverse cross-linking buffer supplemented with proteinase K (#RT403, Tiangen) at 65 °C for 6 h. The immunoprecipitated DNA was quantified using qPCR. Primers used in ChIP-qPCR were as follows: diap1, 5′-GCC CGG CCT TCA CTA AAA AC-3′ (forward) and 5′-AAT TCT GTA AAC ATT TAA GG-3′ (reverse); S1, 5′-TATA AAT TAC GGT CTT TGT TG-3′ (forward) and 5′-AAT TAA GAC TGG AGT TG-3′ (reverse); S2, 5′-TTA AAC CCG TTC AAT TGT GG-3′ (forward) and 5′-GTG TGG GAG TTT TTT TTC AT-3′ (reverse); S3, 5′-CTT TAA TTA ATG ATG ATG-3′ (forward) and 5′-AAG TCG GGT TTC TGC ACA AA-3′ (reverse); HRE-1, 5′-CCC AAC AAT CTT ATC CTC AG-3′ (forward) and 5′-GAG CTT TGC TGG GCC CTC CT-3′ (reverse); HRE-2, 5′-CTG GAA CCC CCC GGG GAG GGA GG-3′ (forward) and 5′-AAG GAA GGA GCC GCC GGG GA-3′ (forward); HRE-3, 5′-CTA CAC CCA CCA CTC ATC TTC TTT CC-3′ (reverse) and 5′-GCG CAG GGC GCC ATC TAC CAG TGA TC-3′ (reverse). Data are presented as means ± SD of values from three experiments.

**Electrophoretic motility shift assays**  
Biotin-labeled S1, S2, and S3 probes were synthesized using PCR-mediated DNA amplification. Biotin was tagged on the upstream of forward primers. The competitor DNA was synthesized using biotin free primers to amplify E. coli (forward) and 5′-AAT TAC GGT CTT TGT TG-3′ (reverse); HRE-1, 5′-GCC CCG CCT TCA CTA AAA GTG-3′ (forward) and 5′-TTA AAC CCG TTC AAT TGT TC-3′ (reverse); HRE-2, 5′-GCC CCG CCT TCA CTA AAA GTG-3′ (forward) and 5′-TTC TAA TTA ATG ATG ATG-3′ (reverse). Primer sets instructions.

**Transwell assays**  
Transwell experiments were performed as described previously [66], PLC/PRF/S or SK-Hep-1 cells under indicated treatments were deprived of serum for 24 h before analyses. A total of 1 × 105 cells were seeded to the upper chamber in transwell inserts (BD Biosciences) with serum-free medium. After additional 48 h, the migrating cells adhered to the lower surface of filter were washed with PBS, fixed with 20% methanol for 20 min and stained with 0.1% crystal violet (Sangon Biotech). For SRC inhibitors treatment, cells were treated with PP2 (10 mM for 24 h, MedChemExpress) or Dasatinib (0.1 mM for 24 h, MedChemExpress) or Saracatinib (1 mM for 24 h, MedChemExpress) or Saracatinib (1 mM for 24 h, MedChemExpress) before seeding in transwell inserts (BD Biosciences) with serum-free medium. The density of IB band was measured by Image J software. Statistical significance was analyzed by Student’s t-test and *P < 0.05* was considered statistically significant. Where exact *P* values are not shown, statistical significance is shown as with ns, not significant, *P < 0.05*, **P < 0.01** and ***P < 0.001.

**Patient samples**  
Fresh-frozen primary HCC tissues and their paired normal samples were obtained from patients undergoing surgical resection at Zhuhai People’s Hospital (Zhuhai, China) after consent was obtained from the patients. None of the patients received any prior radiochemotherapy. For total protein extraction, place the equal amount tissues in tubes and grind the tissue with a plastic rod for 50–60 times. Cap the tube and incubate on ice for 15 min. Centrifuge at 12,000 rpm for 15 min. The supernatant was used to subject to western blot assay following standard protocols.

**Statistical analysis**  
The density of IB band was measured by Image J software. Statistical analysis was performed with GraphPad Prism software. The data shown in the Figures were representative of three or more independent experiments and were analyzed by one way student’s t-test, and *P < 0.05* was considered statistically significant. Where exact *P* values are not shown, statistical significance is shown as with ns, not significant, *P < 0.05*, **P < 0.01** and ***P < 0.001.

**DATA AVAILABILITY**  
All relevant data are available from the corresponding author upon reasonable request.

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ADDITIONAL INFORMATION

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