CDK7 regulates organ size and tumor growth by safeguarding the Hippo pathway effector Yki/Yap/Taz in the nucleus

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Hippo signaling controls organ size and tumor progression through a conserved pathway leading to nuclear translocation of the transcriptional effector Yki/Yap/Taz. Most of our understanding of Hippo signaling pertains to its cytoplasmic regulation, but how the pathway is controlled in the nucleus remains poorly understood. Here we uncover an evolutionarily conserved mechanism by which CDK7 promotes Yki/Yap/Taz stabilization in the nucleus to sustain Hippo pathway outputs. We found that a modular E3 ubiquitin ligase complex CRL4DCAF12 binds and targets Yki/Yap/Taz for ubiquitination and degradation, whereas CDK7 phosphorylates Yki/Yap/Taz at S169/S128/S90 to inhibit CRL4DCAF12 recruitment, leading to Yki/Yap/Taz stabilization. As a consequence, inactivation of CDK7 reduced organ size and inhibited tumor growth, which could be reversed by restoring Yki/Yap activity. Our study identifies an unanticipated layer of Hippo pathway regulation, defines a novel mechanism by which CDK7 regulates tissue growth, and implies CDK7 as a drug target for Yap/Taz-driven cancer.

[Keywords: CDK7; CRL4; Cul4; DCAF12; Hippo; Taz; Yap; Yki; cancer; organ size]

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The regulation of tissue growth and organ size during development and regeneration depends on a delicate balance between cell proliferation and cell death, which is precisely controlled not only by environmental cues such as hormonal signals, cytokines, and nutrients, but also by organ-intrinsic mechanisms. The Hippo tumor suppressor pathway, which was initially discovered in Drosophila, has emerged as an evolutionarily conserved signaling pathway that regulates tissue growth and organ size in a wide range of species including humans (Pan 2007; Zhang et al. 2009; Halder and Johnson 2011). Not surprisingly, deregulation of Hippo pathway activity has been implicated in many types of human cancer and other diseases (Yu et al. 2015; Zanconato et al. 2016b; Zheng and Pan 2019).

The core Hippo signaling pathway contains a kinase cassette with an upstream kinase Hippo (Hpo)/MST1/2 phosphorylating and activating a downstream kinase Warts (Wts)/Lats1/2 (Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003). Activated Wts/Lats1/2 in turn phosphorylates the Hippo pathway effector Yorkie (Yki) in Drosophila and Yap/Taz in mammals, resulting in cytoplasmic retention of Yki/Yap/Taz via its interaction with 14-3-3 (Huang et al. 2005; Dong et al. 2007; Oh and Irvine 2008; Zhang et al. 2008; Ren et al. 2010). Various upstream signals act through Wts/Lats1/2-dependent and -independent mechanisms to promote translocation of Yki/Yap/Taz into the nucleus, where it binds to the Hippo pathway transcription factors Scalloped (Sd)/TEAD to regulate genes involved in cell growth, proliferation, survival, and metabolism (Wu et al. 2008; Zhang et al. 2008; Zhao et al. 2008; Koo and Guan 2018; Totaro et al. 2018; Moya and Halder 2019).

How Yki/Yap/Taz is regulated in the nucleus is still poorly understood, but recent studies revealed that phosphorylation by a nuclear kinase PRP4K restricts Yki/Yap/Taz nuclear localization whereas monomethylation of Yap by Set1A blocks its nuclear export (Cho et al. 2018; Fang et al. 2018). In addition, a recent study demonstrated...
that mechanical signals can promote Yap/Taz activation in the nucleus by dissociating it from a SWI/SNF inhibitory complex [Chang et al. 2018]. Here we identified CDK7 as a novel Hippo pathway component that phosphorylates and stabilizes Yki/Yap/Taz in the nucleus. We found that inhibition of CDK7 allows a modulatory E3 ubiquitin ligase CRL4CDAF12 to ubiquitinate nuclear Yki/Yap/Taz, and targets it for proteasome-mediated degradation, leading to down-regulation of Hippo pathway target gene expression, reduced organ size, and diminished tumor growth. Hence, CDK7 functions to safeguard nuclear Yki/Yap/Taz and serves as a promising drug target for Yap/Taz-driven cancer.

Result

Inactivation of CDK7/CycH/Mat1 suppresses Yki-driven tissue overgrowth

To identify additional Hippo pathway regulators, we conducted an in vivo RNAi screen to identify enhancers and suppressors of the tissue overgrowth phenotype caused by Yki overexpression in the Drosophila eye (GMR > Yki; GMR-Gal4/UAS-Yki) [Fig. 1A,B; Yue et al. 2012, Cho et al. 2018]. We initially screened transgenic RNAi lines targeting the Drosophila kinome and identified CDK7 as a suppressor of the eye overgrowth phenotype caused by GMR > Yki. Knockdown of CDK7 by three independent transgenic RNAi lines (UAS-CDK7-RNAi) lines: CDK7V10442, CDK7V103413, and CDK7V102304, suppressed the GMR-Yki phenotype in a similar manner [Fig. 1B,C, M; Supplemental Fig. S1G,H]. CDK7V10442-mediated suppression of the GMR-Yki phenotype was negated by coexpression of a wild-type CDK7 (CDK7WT) but exacerbated by a kinase-dead CDK7, CDK7DR [D137R] [Supplemental Fig. S1B–E]. Furthermore, expression of CDK7WT promoted, whereas CDK7DR inhibited, Yki-driven eye overgrowth [Fig. 1D,M; Supplemental Fig. S1F], indicating that the kinase activity is essential for CDK7 to promote Yki-driven tissue growth and that CDK7DR acts dominantly negatively to interfere with Yki activity. CDK7 RNAi did not suppress eye overgrowth caused by overexpression of a constitutively active form of insulin receptor (GMR > InRCA) or a constitutively active form of Sd (GMR > Sd-GA) [Fig. 1I–L], suggesting that CDK7 specifically modulates tissue growth driven by Yki. Consistent with this, CDK7 RNAi blocked GMR > Yki induced expression of a Hippo pathway target gene diap1-lacZ, while coexpression of CDK7WT enhanced it [Fig. 1A–D].

CDK7 is a transcriptional kinase and a subunit of the TFIIH complex that phosphorylates polymerase II (Pol-II) C-terminal tail (CTD) to regulate transcription [Fisher 2005]. In addition, CDK7 acts as a CDK activating kinase (CAK) to phosphorylate and activate other CDKs required for cell division [Fisher 2005]. However, the observation that CDK7 RNAi selectively suppressed eye overgrowth driven by GMR > Yki, but not by GMR > InRCA or GMR > Sd-GA, suggests that the growth suppression by CDK7 RNAi was unlikely to be a down-regulation of general transcription or cell cycle progression. Indeed, GMR > Yki-driven tissue overgrowth was also suppressed in a hypomorphic CDK7 mutant background (CDK7S164A/T170A) in which general transcription and cell cycle progression were unaffected [Fig. 1G,H,N; Laroche et al. 2001; Morishita et al. 2013]. Therefore, under the experimental conditions we used [RNAi and hypomorphic mutation], there was enough residual CDK7 kinase activity to support general transcription and cell cycle progression.

CDK7 forms a complex with cyclin H [CycH] and Mat1 that regulate its kinase activity and substrate recognition, respectively [Fisher 2005]. Knockdown of either CycH (CycHV104312; CycHBL57325) or Mat1 (Mat1V106760; Mat1BL57312) also suppressed Yki-driven eye overgrowth similar to CDK7 RNAi [Fig. 1E,F,M; Supplemental Fig. S1I,J], suggesting that the CDK7/CycH/Mat1 kinase complex is involved in modulating Yki-driven tissue growth. In contrast, knockdown of Xpd1 [Xpd1V106598; Xpd1BL57538], which recruits the CDK7/CycH/Mat1 complex to TFIIH, did not suppress eye overgrowth caused by GMR > Yki [Fig. 1M; Supplemental Fig. S1K,L], further supporting the notion that CDK7 can regulate Yki driven tissue growth independent of its role in basal transcription. Of note, knockdown of CDK7, CycH, or Mat1 posterior to the morphogenetic furrow in otherwise wild-type eye imaginal discs where cells exit cell cycle and undergo differentiation, did not cause a discernible phenotype [Supplemental Fig. S1M–O], again suggesting that reduction in CDK7 activity does not affect its house-keeping function.

CDK7/CycH/Mat1 regulates Hippo pathway target genes and organ size independent of Wts

CDK7 RNAi in wing imaginal discs using hedgehog(hh)-Gal4, which expressed Gal4 in the posterior compartment [Yue et al. 2012], diminished the expression of multiple Hippo pathway target genes including expanded [ex-lacZ], Diap1, and Wingless [Wg] as well as reduced the relative size of the posterior compartment in adult wings [Fig. 2A,B,E–G, J,K,N,O; Supplemental Fig. S2A–C′]. Coexpression of CDK7 restored the expression of ex-lacZ in hh > CDK7V10442 wing discs [Supplemental Fig. S2D–D′]. Similarly, knockdown of either CycH [hh > CycHV104312] or Mat1 [hh > Mat1V106760] also diminished the expression of Hippo pathway target genes and reduced the relative size of the posterior compartment in adult wings [Fig. 2C–E, H, J, L, M, P, Q, Q′]. Of note, the expression of Wg along the D/V boundary, which is under the control of Notch signaling, was not affected by knockdown of any component of the CDK7–CycH–Mat1 complex [asterisks in Fig. 2O–Q, cf. anterior vs. posterior compartment], suggesting that inactivation of CDK7 selectively affects the Hippo pathway under our experimental conditions.

To determine where CDK7 acts in the Hippo pathway, we carried out genetic epistasis experiments. Knockdown of Wts in the P compartment of wing imaginal discs [hh > WtsV106174] resulted in up-regulation of ex-lacZ and enlarged posterior compartment size [Fig. 2R; Supplemental Fig. S2E–E′]. Double knockdown of CDK7 and...
Wts (hh > Wts<sup>1106147</sup> +CDK7<sup>V10442</sup>) suppressed both the up-regulation of ex-lacZ and overgrowth of the posterior compartment (Fig. 2S; Supplemental Fig. S2F–F′′). In contrast, CDK7 RNAi did not affect the up-regulation of ex-lacZ caused by hh > Sd-GA (Fig. 2T,U). In conjunction with the observation that CDK7 RNAi also failed to suppress Sd-GA-driven tissue overgrowth (Fig. 1K,L), these results suggest that CDK7 regulates Hippo signaling upstream of Sd, but downstream from Wts. Wts inhibits Yki activity by phosphorylating Yki on Ser111, Ser168, and Ser250 with S168 phosphorylation playing a more dominant role. Indeed, mutating Ser168 (Yki<sup>S168A</sup>) dramatically increased Yki activity as indicated by the more dramatic overgrowth of eye caused by GMR > Yki<sup>S168A</sup> compared with GMR > Yki<sup>WT</sup> (Supplemental Fig. S3A,C). Nevertheless, GMR > Yki<sup>S168A</sup> driven eye overgrowth was suppressed by CDK7 RNAi [Supplemental Fig. S3B,D]. Similarly, the eye overgrowth phenotype caused by GMR > Yki<sup>S111A, S250A</sup> or GMR > Yki<sup>SSA</sup> [S111A, S168A, S250A] was also reduced by CDK7 knockdown [Supplemental Fig. S3E–H]. Hence, the activity of Wts-phosphorylation-deficient forms of Yki still depends on CDK7, consistent with the notion that CDK7 regulates Hippo signaling downstream from Wts.

**CDK7 regulates Yki stability in the nucleus**

The epistasis experiments conducted above suggest that CDK7 may regulate Hippo signaling at the level of Yki. Immunostaining of hh > CDK7<sup>V10442</sup> wing imaginal discs...
Yki mRNA level was not significantly altered in S2 cells treated with the proteasome inhibitor MG132 not only increased the basal level of Yki but also restored the Yki level in CDK7 RNAi cells [Fig. 3K,L], suggesting that knockdown of CDK7 leads to Yki degradation through the ubiquitination–proteasome (UPS) pathway.

Because CDK7 is a nuclear kinase, we asked whether CDK7 regulates Yki stability in the nucleus. Fractionation of S2 cells transfected with Myc-Yki and treated with CDK7 dsRNA indicated that CDK7 RNAi reduced the level of nuclear Yki to and lesser extent, the level of cytoplasmic Yki [Fig. 3M,M′]. Preventing nuclear localization of Yki by addition of a myristoylation signal [Myc-Yki-GFP] made it insensitive to CDK7 RNAi [Fig. 3N,N′], whereas forced nuclear localization of Yki by treating cells with a nuclear export inhibitor LMB resulted in reduction of nuclear but not cytoplasmic Yki [Fig. 3O,O′].

CDK7 regulates Yki stability and activity by phosphorylating Yki S169

The observations that CDK7 inactivation decreases nuclear Yki and Hippo target gene expression and that CDK7 acts downstream from Wts prompted us to determine whether CDK7 regulates Hippo signaling by directly phosphorylating Yki. CDK family kinases phosphorylate proline-based sites (S/T-P). Inspection of Yki protein sequence identified a total of six putative sites, among which only S169 is conserved between Yki and its mammalian homolog Yap and Taz [Fig. 4A]. In an in vitro kinase assay, GST fusion protein containing an Yki fragment from aa160 to aa181 was phosphorylated by immunopurified CDK7 fused to GFP (GFP-CDK7WT) but not by its kinase-dead version (GFP-CDK7RD) [Fig. 4B,C]. Mutating S169 but not S168 or S172 to Ala abolished such phosphorylation [Fig. 4B,C], suggesting that CDK7 could phosphorylate YkiS169.

To further characterize Yki phosphorylation by CDK7, we generated a phospho-specific antibody for YkiS169, pS169 [see the Materials and Methods]. In S2 cells, Myc-Yki was phosphorylated on S169 detected by the pS169 antibody also detected the phosphorylation of endogenous Yki. Because CDK7 is a nuclear kinase, we asked whether phosphorylation of Yki by CDK7 occurs exclusively in the nucleus. Western blot analysis indicated that Yki protein level was down-regulated [Fig. 3H], suggesting that CDK7 regulates Yki expression at the post-transcriptional level. CDK7 RNAi in S2 cells reduced the half-life of both transfected [Myc-Yki] and endogenous Yki [Fig. 3L], leading to diminished steady-state Yki protein expression [Fig. 3K,L]. Treating S2 cells with the proteasome inhibitor MG132 not only increased the basal level of Yki but also restored the Yki level in CDK7 RNAi cells [Fig. 3K,L], suggesting that knockdown of CDK7 leads to Yki phosphorylation by the ubiquitination–proteasome (UPS) pathway.

with an anti-Yki antibody revealed that CDK7 RNAi reduced the level of endogenous Yki in P compartment cells [Fig. 3A–B′]. Similar result was obtained by transfection of a GFP knock-in Yki, Yki:GFP [Fig. 3C–D′; Fletcher et al. 2018]. Expression of CDK7V10442 wing discs [Fig. 3G], Western blot analysis indicated that Yki protein level was down-regulated [Fig. 3H], suggesting that CDK7 regulates Yki expression at the post-transcriptional level. CDK7 RNAi in S2 cells reduced the half-life of both transfected [Myc-Yki] and endogenous Yki [Fig. 3L], leading to diminished steady-state Yki protein expression [Fig. 3K,L]. Treating S2 cells with the proteasome inhibitor MG132 not only increased the basal level of Yki but also restored the Yki level in CDK7 RNAi cells [Fig. 3K,L], suggesting that knockdown of CDK7 leads to Yki phosphorylation through the ubiquitination–proteasome (UPS) pathway.

The observations that CDK7 inactivation decreases nuclear Yki and Hippo target gene expression and that CDK7 acts downstream from Wts prompted us to determine whether CDK7 regulates Hippo signaling by directly phosphorylating Yki. CDK family kinases phosphorylate proline-based sites (S/T-P). Inspection of Yki protein sequence identified a total of six putative sites, among which only S169 is conserved between Yki and its mammalian homolog Yap and Taz [Fig. 4A]. In an in vitro kinase assay, GST fusion protein containing an Yki fragment from aa160 to aa181 was phosphorylated by immunopurified CDK7 fused to GFP (GFP-CDK7WT) but not by its kinase-dead version (GFP-CDK7RD) [Fig. 4B,C]. Mutating S169 but not S168 or S172 to Ala abolished such phosphorylation [Fig. 4B,C], suggesting that CDK7 could phosphorylate YkiS169.

To further characterize Yki phosphorylation by CDK7, we generated a phospho-specific antibody for YkiS169, pS169 [see the Materials and Methods]. In S2 cells, Myc-Yki was phosphorylated on S169 detected by the pS169 antibody [Fig. 4D]. The S169 phosphorylation signal of Myc-Yki was increased by cotransfection with wild-type (GFP-CDK7WT) but not kinase-dead (GFP-CDK7RD) CDK7 [Fig. 4D]. Mutating the S169 to Ala [Myc-YkiS169A] abolished the phospho-signal, confirming the specificity of the pS169 antibody. The pS169 signal of Myr-Myc-Yki was not affected by GFP-CDK7WT [Fig. 4D], supporting that CDK7 phosphorylates YkiS169 in the nucleus. The pS169 antibody also detected the phosphorylation of endogenous Yki in S2 cells, which was enhanced by CDK7 transfection but diminished by CDK7 RNAi [Fig. 4E], suggesting that CDK7 is a major kinase responsible for YkiS169 phosphorylation of the endogenous Yki.
When expressed in S2 cells, the steady-state level of Myc-Yki was decreased by S169A mutation (Myc-YkiS169A) but increased by S169D mutation (Myc-YkiS169D) (Fig. 4F). Unlike Myc-Yki, whose abundance was decreased by CDK7 RNAi but increased by CDK7 cotransfection, neither Myc-YkiS169A nor Myc-YkiS169D was responsive to CDK7 RNAi or overexpression (Fig. 4F). In a Sd-luc reporter assay (Zhang et al. 2008), Myc-YkiS169A exhibited lower, while Myc-YkiS169D exhibited higher transcriptional activity than Myc-YkiWT, and the activity of neither Myc-YkiS169A nor Myc-YkiS169D was affected by CDK7 RNAi (Fig. 4G).

To determine whether CDK7 regulates Yki activity and organ size by phosphorylating YkiS169 in vivo, we generated transgenic flies expressing UAS-YkiS169A or UAS-YkiS169D under the control of GMR-Gal4. We found that YkiS169D was more potent, while YkiS169A less potent, in driving eye overgrowth compared with YkiWT [Fig. 4H–J, Q]. Unlike GMR > YkiWT whose phenotype was modulated by altering CDK7 activity, the eye overgrowth phenotype caused by GMR-YkiS169A or GMR-YkiS169D was no longer modified by either RNAi or overexpression of CDK7 (Fig. 4K–Q). Taken together, these results demonstrate that CDK7 promotes Yki stability and activity by phosphorylating YkiS169.

Yki is degraded by CRL4DCAF12 in the absence of CDK7

The above results suggest that nuclear Yki is intrinsically unstable but phosphorylation by CDK7 on S169 increases its half-life and thus sustains the Hippo pathway output. Because the proteasome inhibitor MG132 could stabilize
Yki in CDK7 knockdown cells (Fig. 3K,L), nuclear Yki is degraded by the UPS pathway. To identify the E3 ubiquitin ligase(s) responsible for Yki degradation in the absence of CDK7, we carried out a genetic modifier screen using transgenic RNAi lines targeting Drosophila E3 ubiquitin ligases including the Cullin family of modular E3 ubiquitin ligases. We identified two independent UAS-Cul4-RNAi lines (v105668, v44829) that enhanced the eye overgrowth phenotype caused by GMR-Yki (Fig. 5A,B). Cul4 forms a multisubunit E3 ubiquitin ligase (CRL4) complex in which the DNA-damage binding protein 1 (DDB1) bridges Cul4 to multiple DDB1-Cul4–associated factors (DCAFs) that serves as substrate receptors [Angers et al. 2006; Lee and Zhou 2007]. We therefore screened RNAi lines that target Drosophila DCAFs and identified a UAS-DCAF12-RNAi line (v43758) that enhanced the GMR-Yki phenotype similar to UAS-Cul4-RNAi (Fig. 5C). In addition, we found that DCAF12 RNAi could rescue the phenotypes caused by CDK7 RNAi. For example, the suppression of GMR-Yki driven eye overgrowth by CDK7 RNAi was negated by DCAF12 RNAi (Fig. 5D–F).

Figure 4. CDK7 regulates Yki stability and Hippo signaling by phosphorylating YkiS169. (A) Diagram of Yki protein with the amino acid sequences surround YkiS169, YapS128, and TazS90 shown underneath. TBD, WW, and AD indicate the TEAD binding domain, WW domain, and activation domain of Yki, respectively. [B] GST-Yki fusion proteins containing the indicated wild-type or mutated Yki sequences. (C) In vitro kinase assay using immunopurified wild-type (WT) or kinase-dead (DR) GFP-CDK7 as kinase and the indicated GST-Yki fusion proteins as substrates. [D] Western blot analysis to examine S169 phosphorylation of the indicated Yki proteins expressed in S2 cells cotransfected with or without wild-type (WT) or kinase-dead (DR) GFP-CDK7 and treated with MG132. [E] S169 phosphorylation of the endogenous Yki in S2 cells transfected with or without wild-type GFP-CDK7, Luc dsRNA, or CDK7 dsRNA. [F] Western blot analysis of the indicated Myc-Yki proteins expressed in S2 cells cotransfected with Myc-CFP and with or without wild-type GFP-CDK7, Luc dsRNA or CDK7 dsRNA. [G] 3XSd2-luc reporter assay in S2 cells expressing the indicated Myc-Yki constructs and HA-Sd. [H–Q] Side view of Drosophila adult eyes [H–P] and quantification of the size of adult eyes [Q] of the indicated genotypes.
Figure 5. Yki is targeted for degradation by the CRL4<sup>DCAF12</sup> ubiquitin ligase. [A–I] Side view of adult eyes of the indicated genotypes. [J–M] Adult wings of the indicated genotypes. [N–O'] Late third instar wing imaginal discs of the indicated genotypes were immunostained to show the expression of Ci (red) and Yki (green). [P] Western blot analysis of late third instar wing discs of the indicated genotypes with Yki, pS169, and tubulin (tub) antibodies. [Q] Western blot analysis of Myc-Yki level in S2 cells transfected with luciferase (Luc) dsRNA (top), CDK7 dsRNA (middle), or CDK7 and DCAF12 dsRNAs (bottom) at the indicated time point following cycloheximide (CHX) treatment. Myc-CFP was used as loading control. Quantification of Myc-Yki at the indicated time point following cycloheximide (CHX) treatment is shown at the right. [R] Ubiquitination of Myc-Yki or Myr-Myc-Yki in S2 cells cotransfected with DCAF12, DDB1/Cul4, or DCAF12/DDB1/Cul4. [S] Ubiquitination of Myc-Yki, Myc-Yki<sup>S169A</sup>, or Myc-Yki<sup>S169D</sup> in S2 cells cotransfected with DCAF12/DDB1/Cul4 and with or without GFP-CDK7. [T] Ubiquitination of Myc-Yki in S2 cells transfected with Luc dsRNA, CDK7 dsRNA, CDK7/DCAF12 dsRNAs, or CDK7/DDB1/Cul4 dsRNAs and treated with or without MG132. [U] Western blot analysis of HA-DCAF12 coimmunoprecipitated with Myc-Yki, Myc-Yki<sup>S169A</sup> or Myc-Yki<sup>S169D</sup> from S2 cells transfected with Luc or CDK7 dsRNA and treated with MG132.
CDK7 stabilizes Yap/Taz by inhibiting DCAF12-mediated ubiquitination and degradation

We next determined whether CDK7 played a conserved role in the regulation of Yap/Taz stability. CDK7 RNAi resulted in a reduction of Yap/Taz level in both wild-type and Lats1/2 KO HEK293 cells (Fig. 6F,G), suggesting that CDK7 promotes Yap/Taz stabilization in a manner independent of Lats1/2, which is consistent with the finding in Drosophila. Serum depletion of HEK293 cells, which caused nuclear exclusion of Yap/Taz (Meng et al. 2015; Cho et al. 2018), made Yap/Taz less sensitive to CDK7 inactivation (Supplemental Fig. S4A). Fractionation of serum-depleted and control HEK293 cells revealed that CDK7 RNAi caused a reduction of Yap/Taz mainly in the nuclear fraction (Supplemental Fig. S4B,C), suggesting that CDK7 regulates the stability of nuclear Yap/Taz.

Western blot analysis using a phospho-specific antibody that recognized phosphorylated YapS128 (pS128) (Moon et al. 2017) revealed that overexpression of CDK7 promoted phosphorylation of coexpressed Flag-tagged Yap (Fg-Yap), whereas CDK7 RNAi inhibited S128 phosphorylation of both Fg-Yap and endogenous Yap in HEK293 cells (Fig. 6C–E). To determine the effect of S128 phosphorylation on Yap stability and activity, we generated Flag-tagged S128A and S128D Yap variants [Fg-YapS128A and Fg-YapS128D]. When expressed in HEK293 cells, Fg-YapS128A exhibited reduced, whereas Fg-YapS128D exhibited increased steady-state levels and transcriptional activity compared with wild-type Fg-Yap (Fig. 6F,G). Furthermore, both the protein level and transcriptional activity of either YapS128A or YapS128D were no longer affected by CDK7 RNAi (Fig. 6F,G). TazS90 is equivalent to YapS128 (Fig. 4A). We found that S90A decreased, whereas S90D increased, Taz protein stability and transcriptional activity in HEK293 cells (Fig. 6H,I). In addition, these mutations rendered Taz insensitive to CDK7 inactivation (Fig. 6H,I). Taken together, these results suggest that CDK7 promotes the stability and activity of Yap/Taz by phosphorylating YapS128/TazS90.

To determine whether DCAF12 is responsible for Yap/Taz ubiquitination and degradation when CDK7 is inactivated, we knocked down DCAF12 together with CDK7 by siRNA in HEK293 cells. DCAF12 RNAi increased the steady state level of Yap/Taz and suppressed the down-regulation of Yap/Taz caused by CDK7 RNAi (Fig. 6F,G). CDK7 RNAi increased Yap/Taz ubiquitination, whereas DCAF12 RNAi inhibited both basal and CDK7 RNAI-induced ubiquitination of Yap/Taz [Fig. 6L,M]. CDK7 RNAI increased the association of Yap/Taz with DCAF12 [Fig. 6N,O]. The S128A/S90A mutation increased, whereas the S128D/S90D mutation decreased the binding of DCAF12 to Yap/Taz [Fig. 6N,O] as well as ubiquitination of Yap/Taz [Fig. 6F,Q]. Taken together, these results demonstrate that DCAF12 mediates the ubiquitination and degradation of Yap/Taz and that CDK7-mediated phosphorylation of YapS128/TazS90 inhibits the binding of DCAF12, thus blocking the ubiquitination and degradation of Yap/Taz.
esophagus cancer line EC9706 [Mo et al. 2012; Zanconato et al. 2015; Chang et al. 2017]. We found that CDK7 knockdown by siRNAs reduced Yap level, Hippo target gene expression, cell growth, and invasiveness of these cancer cell lines [Supplemental Fig. S5]. Exogenous expression of a wild-type Yap partially rescued, whereas expression of YapSD128 more completely rescued Yap protein level, Hippo target gene expression, cell growth, and invasiveness of CDK7-depleted MDA-MB-231 cells [Fig. 7A–E], suggesting that CDK7 promotes cancer cell growth.
Figure 7. CDK7 regulates organ size and tumor progression through Yap/Taz. (A) Western blot analysis of Yap and CDK7 in MDA-MB-231 cells transfected with siControl or siCDK7 and infected with lentiviruses expressing the empty vector, wild-type Yap, or the S128D mutant. (B) Relative mRNA levels of the indicated Hippo target genes in the indicated MDA-MB-231 cells were measured by RT-qPCR. Data are means ± SD. (∗) P < 0.05; (∗∗) P < 0.01; (∗∗∗) P < 0.001 [Student’s t-test]. (C) Relative cell numbers of MDA-MB-231 cells treated with siControl or siCDK7 and infected with lentiviruses expressing wild-type or S128D Yap were measured by the WST-1 assay at different time points after infection. Data are means ± SD. (∗∗) P < 0.01; (∗∗∗) P < 0.001 (Student’s t-test). (D,E) Transwell invasion assay of MDA-MB-231 cells treated with siControl or siCDK7 and infected with lentiviruses expressing wild-type or S128D Yap. Data are means ± SD. (∗∗) P < 0.01; (∗∗∗) P < 0.001 (Student’s t-test). (F) Western blot analysis of Yap in MDA-MB-231 cells treated with THZ1. (G) Relative mRNA levels of the indicated Hippo target genes in MDA-MB-231 cells treated with THZ1. Data are means ± SD. (∗∗∗) P < 0.001 (Student’s t-test). (H) Tumor size of the indicated MDA-MB-231 xenografts in mice treated with vehicle or THZ1 at the indicated time after drug treatment. (I) Macroscopic images of tumors derived from the indicated MDA-MB-231 xenografts at the end of drug treatment. (J) Quantification of tumor weight of the indicated MDA-MB-231 xenografts at the end of drug treatment. Data are means ± SD. n = 7 mice for each group. NS not significant; (∗∗) P < 0.01; (∗∗∗) P < 0.001 [Student’s t-test]. (K) MST1/2 DKO mice were treated with vehicle or THZ1 (10 mg/kg) every other day starting at the age of 1 mo until 3 mo old. Representative macroscopic images of livers were shown. (L) Quantitative analysis of liver-to-body weight ratio. Data are means ± SD. n = 6 mice for each group. (∗∗∗) P < 0.001 (Student’s t-test). (M) Western blot analysis of control and THZ1-treated liver tissues of the indicated genotypes using the indicated antibodies. (N,O,Q) Liver cross-sections of the indicated genotypes treated with vehicle or THZ1 were subjected to immunostaining with anti-EPCAM, SOX9, F4/80, or Ki67 antibody. Scale bar, 100 μm. (P) Quantification of Ki67+ cells in liver sections of the indicated genotypes treated with vehicle or THZ1. Data are means ± SD. n = 5 sections for each group. (∗∗∗) P < 0.001 [Student’s t-test].
and invasiveness, at least in part, through stabilizing Yap. Of note, overexpression of YapWT or YapS128D in control MDA-MB-231 cells only slightly increased their growth and invasiveness [Fig. 7C−E] because MDA-MB-231 cells already have high Yap/Taz activity due to their loss of NF2 [Zanconato et al. 2015]. Treating MDA-MB-231 cells with a CDK7 pharmacological inhibitor THZ1 also reduced Yap protein level and Hippo target gene expression [Fig. 7F,G).

To determine whether CDK7 promotes tumor growth through Yap in vivo, we generated xenograft models of TNBC by transplanted MDA-MB-231 cells expressing pLVX-IRES-ZsGreen1 (vector) or pLVX-IRES-ZsGreen1-Yki-S128D (YAPS128D) into the mammary fat pads of nude mice. After tumors reached ~100 mm³, mice were treated with vehicle or THZ1 (10 mg/kg twice a day) continuously for 16 d. Tumor size was measured every 3 d during treatment and tumors were weighed at the end of treatment. Compared with vehicle treatment, THZ1 treatment dramatically slowed down the growth of MDA-MB-231 tumors [Fig. 7H−J]. Analysis of MDA-MB-231 tumors indicated that THZ1 reduced Yap level and Hippo target gene expression [Supplemental Fig. S6A,B].

Expression of Yki-S128D in MDA-MB-231 tumors resulted in higher levels of Yap protein and Hippo target gene expression compared with control tumors [Supplemental Fig. S6A,B] and rendered MDA-MB-231-YAPS128D tumors resistant to THZ1-mediated inhibition [Fig. 7H−J].

CDK7 inhibition reduces liver growth and tumor burden in MST1/2 DKO livers

To further investigate the role of CDK7 in Hippo-mediated organ size control and tumor growth, we turned to mouse liver in which both MST1 and MST2 were knocked out [MST1/2 DKO]. In mice, MST1/2 deficiency or Yap overexpression causes hepatocyte overproliferation, cirrhosis, and hepatocellular carcinoma (HCC) development [Dong et al. 2007; Zhou et al. 2009; Lu et al. 2010; Song et al. 2010]. In humans, ~50% of HCCs exhibited nuclear YAP staining, and high Yap activity correlated with poor survival after resection [Xu et al. 2009; Fitamant et al. 2015]. Genetic removal of one copy of Yap or Taz restored normal growth in the Hippo pathway mutant livers [Zhang et al. 2010; Fitamant et al. 2015], suggesting that reducing Yap/Taz level was able to attenuate liver overgrowth, slow down tumor formation, and progression caused by Hpo kinase deficiency.

Treating primary hepatocytes derived from MST1/2 DKO mice with THZ1 resulted in a reduction in Yap/Taz protein level and Hippo pathway target gene expression [Supplemental Fig. S7A], suggesting that inhibition of CDK7 could attenuate Yap/Taz activity in MST1/2 DKO hepatocytes. We next treated 1-mo-old MST1/2 DKO mice (Alb-Cre MST1−/−Mst2fl/fl) with THZ1 (10 mg/kg every other day) or vehicle for 2 mo. The protein level of YAP/TAZ as well as the expression of YAP/TAZ target genes SOX9, CTGF, and CYR61 in MST1/2 DKO livers were significantly down-regulated following THZ1 treatment [Fig. 7M; Supplemental Fig. S7B]. In addition, the expression levels of Wnt and Notch target genes were also reduced in THZ1-treated MST1/2 DKO livers compared with control group [Supplemental Fig. S7C], consistent with a previous study showing that both Wnt and Notch signaling activities were increased in MST1/2 DKO livers in a Yap/Taz-dependent manner [Kim et al. 2017]. Furthermore, THZ1 treatment significantly alleviated liver phenotypes caused by hepatic Yap activation, including increased organ size [Fig. 7K,L], expanded expression of oval ductal cell markers EPCAM and Sox9 [Fig. 7N], and hepatocyte proliferation [Fig. 7O,P] compared with vehicle-treated DKO mice.

Previous studies have shown that loss of MST1/2 in hepatocytes significantly enhanced macrophages infiltration and proinflammatory cytokine expression through Yap-mediated Mcp1(Ccl2) expression, and the infiltrated macrophages promoted liver tumor initiation and progression [Guo et al. 2017; Kim et al. 2018]. THZ1 treatment markedly reduced macrophage infiltration [Fig. 7Q], expression of proinflammatory cytokines [Supplemental Fig. S7D], and elevated Stat3 phosphorylation (p-Stat3) [Fig. 7M] caused by MST1/2 deficiency. Taken together, these results suggest that inhibition of CDK7 reduced liver size and tumor burden in MST1/2 DKO mice by down-regulating Yap/Taz.

Discussion

The evolutionarily conserved Hippo signaling pathway controls tissue growth and organ size in diverse species and its deregulation has been implicated in a wide range of human cancer. Indeed, a recent cancer genomic study reveals that the Hippo pathway is among the eight most mutated signaling pathways in human cancer [Sanchez-Vega et al. 2018]. In addition, activation of Yap/Taz has been implicated in drug resistance in cancer treatment [Kim and Kim 2017]. Hence, Yap/Taz is considered as an attractive drug target for cancer therapeutics, and understanding how Yki/Yap/Taz is regulated may provide important insight into cancer treatment. It has been well established that Yki/Yap/Taz is regulated mainly through its shuttling between the cytoplasm and the nucleus; phosphorylation by the Hippo kinase cascade sequesters Yki/Yap/Taz in the cytoplasm, whereas upstream signals leading to compromised Yki/Yap/Taz phosphorylation allows this Hippo pathway effector to enter the nucleus to activate Hippo target genes. Here we uncovered a new layer of Hippo pathway regulation consisting of a nuclear regulatory module: a Ser/Thr kinase CDK7 and an E3 ubiquitin ligase complex, CRL4DCAF12, which regulates Yki/Yap/Taz protein turnover and thus the duration of Yki/Yap/Taz protein expression (Supplemental Fig. S7E). We found that phosphorylation of Yki/Yap/Taz by CDK7 is required for CRL4DCAF12 recruitment, which leads to ubiquitination and degradation of Yki/Yap/Taz (Fig. 8A). An analogous mechanism has been observed in Hedgehog (Hh) signal...
transduction, in which casein kinase 1 (CK1) phosphorylates and protects the pathway transcription factor Ci/Gli from premature degradation by a nuclear E3 ubiquitin ligase CRL3HIB/SPOP, thereby sustaining Hh pathway activity (Shi et al. 2014).

We identified CDK7 as a genetic modifier of the eye overgrowth phenotype caused by Yki overexpression. Subsequently, we found that inactivation of CDK7 in developing tissues such as wing imaginal discs resulted in reduced organ size. Several lines of evidence suggest that CDK7 regulates tissue growth and organizes size by specifically targeting the Hippo pathway effector. (1) Inactivation of CDK7 did not suppress the tissue overgrowth phenotype caused by insulin pathway activation or by overexpression of a constitutively active and Yki-independent Sd [Sd-GA]. (2) Inactivation of CDK7 down-regulated Yki protein level, leading to reduced expression of Yki target genes. (3) The reduction in wing size caused by CDK7 inactivation was reversed by restoring Yki to wild-type level. (4) Yki-driven tissue overgrowth was suppressed in a CDK7 hypomorphic mutant background in which the general transcription and cell cycle progression were not affected. Hence, even though CDK7 functions as a CAK to regulate cell cycle progression and a component of TFIIH to phosphorylate the C-terminal tail of Pol-II to influence basal transcription, we believe that under our experimental conditions, these housekeeping roles of CDK7 were preserved by residual CDK7 activity due to incomplete inactivation of CDK7. Hence, our experimental approach—partial loss of function via RNAi in a genetic sensitized background [GMR > Yki]—can uncover pathway-specific role of genes (e.g., CDK7, Cul4, and PRP4K) with pleiotropic function (Cho et al. 2018).

Genetic epistasis experiments placed CDK7 downstream from Wts and upstream of Sd. Indeed, inactivation of CDK7 can suppress the elevated Yki activity in Wts-depleted wing discs and suppress tissue growth caused by Wts-phosphorylation deficient and constitutively active forms of Yki. Mechanistically, we demonstrated that CDK7 regulates Hippo signaling by phosphorylating Yki on S169 to protect nuclear Yki from premature loss. Using an antibody that recognizes Yki phosphorylated at S169 (pS169), we found that phosphorylation of endogenous Yki at this site was diminished but not completely abolished when CDK7 was inactivated in S2 cells or in wing imaginal discs. The residual phosphorylation at YkiS169 could be due to incomplete loss of CDK7 by RNAi or due to the presence of other kinase(s) such as Nemo-like kinase [NLK] that can also phosphorylate YkiS169 (Hong et al. 2017; Moon et al. 2017). We showed that blocking Yki phosphorylation at S169 [S169A] decreased, whereas the phosphomimetic mutation at this site [S169D] increased Yki stability and activity. Importantly, the stability and activity of these Yki variants were insensitive to either loss- or gain-of-CDK7 activity, demonstrating that CDK7 regulates tissue growth and organ size by modulating Hippo signaling through phosphorylating YkiS169.

Our genetic modifier screen also identified an E3 ubiquitin ligase complex [CRL4DCAF12] consisting of Cul4, DDB1, and DCAF12 as responsible for degrading Yki in the absence of CDK7. Strikingly, inactivation of DCAF12 suppressed the tissue growth defect caused by CDK7 inactivation by restoring Yki to wild-type level. Phosphorylation of YkiS169 by CDK7 reduced the binding of DCAF12 to and CRL4DCAF12-mediated ubiquitination and degradation of Yki, providing a molecular mechanism
CDK7 safeguards nuclear Yki/Yap/Taz

by which CDK7 protects nuclear Yki. We provided evidence that the CDK7/CRL4DCAF12 regulatory module identified in Drosophila plays a conserved role in the mammalian Hippo signaling pathway by modulating the stability and activity of Yap/Taz independent of Lats1/2. We demonstrated that CDK7-mediated phosphorylation of YapS128, and likely TazS90, increased the stability and activity of Yap/Taz by inhibiting CRL4DCAF12-mediated ubiquitination of Yap/Taz. Pharmacological inhibition of CDK7 in M5T1/2 DTO liver decreased Yap/Taz protein level and transcriptional activity and reversed the overproliferation phenotype caused by Hippo signaling deficiency. Hence, CDK7-dependent Yap/Taz stabilization many represent an Achilles heel in the Hippo signaling pathway that can be explored therapeutically. It remains to be determined whether Yap/Taz phosphorylation by CDK7 is a regulated event under physiological and/or pathological conditions. Interestingly, recent studies reported that CDK7 is up-regulated in cancer and is associated with poor prognosis (Li et al. 2017; Jiang et al. 2019). On the other hand, many cancer cells have high levels of nuclear Yap/Taz without harboring identifiable Hippo pathway mutations (Zanconato et al. 2016a). Therefore, it would be interesting to determine whether up-regulation of CDK7 or down-regulation of CRL4DCAF12 could contribute to elevated nuclear Yap/Taz in cancer cells.

Hippo pathway mutations and Yap amplification that lead to increased Yap/Taz activity have been attributed to many types of human cancer, placing Yap/Taz as a prominent anticancer drug target; however, transcription factors and cofactors that do not possess enzymatic activity have been proved difficult to target and belong to the so-called "nondruggable class." Our finding that a Ser/Thr kinase CDK7 is required for Yap/Taz stabilization and activity in the nucleus raises an exciting possibility that targeting this Hippo pathway vulnerability could represent a new therapeutic strategy to combat cancers caused by Hippo pathway deregulation. Indeed, we found that depletion of CDK7 in several Yap/Taz-driven cancer cell lines including MDA-MB-231, HCT116, and EC9706 inhibited cancer cell proliferation and invasiveness. Furthermore, we found that pharmacological inhibition of CDK7 by a small molecule THZ1 blocked MDA-MB-231 tumor growth in Xenografts, and that this inhibitory effect was largely reversed by expressing a phospho-mimetic and CDK7-independent Yap variant (YapS128D).

Several recent studies demonstrated that THZ1 and its derivative THZ2 exhibited selective inhibitory effect on tumor growth in a number of preclinical cancer models including T-cell acute lymphoblastic leukemia (T-ALL) (Kwiatkowski et al. 2014), MYCN-driven neuroblastoma (Chipumuro et al. 2014), small cell lung cancer (SCLC) (Christensen et al. 2014), TNBC (Wang et al. 2015; Li et al. 2017), esophageal squamous cell carcinoma (Jiang et al. 2017), epithelial squamous cell carcinoma (Jiang et al. 2017), epithelial ovarian cancer (EOC) (Francavilla et al. 2017), diffuse intrinsic pontine glioma (DIPG) (Nagaraja et al. 2017), and HCC (Zhong et al. 2018; Tsang et al. 2019). While many cancer cell lines are sensitive to THZ1, others such as estrogen receptor positive breast cancer cells remained resistant, although the underlying mechanisms remain unknown (Kwiatkowski et al. 2014; Wang et al. 2015). It is thought that CDK7 inhibition affects cancer progression by interfering with the expression of genes associated with superenhancers, which are sensitive to reduced Pol-II CTD phosphorylation (Chipumuro et al. 2014; Wang et al. 2015). However, a previous study indicated that THZ1 could inhibit cancer cell growth at low doses that did not significantly affect Pol-II CTD phosphorylation (Kwiatkowski et al. 2014). In addition, CDK7 KO mice did not affect significantly Pol-II CTD phosphorylation likely due to the compensation by other kinases (Ganuza et al. 2012). Hence, CDK7 inhibition may affect superenhancer-associated gene expression through mechanisms other than or in addition to inhibiting Pol-II CTD phosphorylation. Interestingly, a recent study revealed that Yap/Taz occupied a large set of enhancers with superenhancer-like property in TNBC cells to mediate cancer transcriptional addiction (Zanconato et al. 2018). Our finding that inactivation of CDK7 destabilizes Yap/Taz provides an additional mechanistic and alternative explanation of why CDK7 inhibition tends to affect superenhancer-driven gene expression and suggests that Yap/Taz-driven cancers have become more "addicted" to CDK7 activity (Fig. 8B). Our finding also suggests that partial inhibition of CDK7 could preferentially affect Yap/Taz target genes and thus Yap/Taz-driven cancer growth without perturbing the general transcription and cell cycle progression in normal cells as we have demonstrated in Drosophila. In addition, we suggest that high Yap/Taz expression may serve as a biomarker for cancers sensitive to CDK7 inhibitors. It would be interesting to determine whether CDK7 could phosphorylate additional transcriptional factors/cofactors bound to superenhancers to influence gene expression in cancer.

Our finding that CRL4DCAF12 bind unphosphorylated Yap/Taz to target it for ubiquitination and degradation also has an important therapeutic implication. Small molecules that can promote the binding of the CRL4 family of E3s to oncogenic substrates have been explored as anticancer drugs (Fischer et al. 2014; Han et al. 2017). Therefore, searching for small molecules that can increase the binding of CRL4DCAF12 to Yap/Taz, especially the phosphorylated form of Yap/Taz, may provide a new strategy to identify anticancer drugs. On the other hand, inhibition of CRL4DCAF12-mediated degradation of Yap/Taz may enhance Yap/Taz activity in the nucleus, which could be beneficial for tissue regeneration.

Materials and methods

Drosophila genetics and transgenes

Fly culture and crosses were carried out according to standard procedures. The transgenic RNAi lines used were UAS-CDK7RNAi (VDRC#10442, VDRC#103413, BL#62304), UAS-CycH-RNAi (VDRC#104312, BL#34732), UAS-Mat1-RNAi (VDRC#104780, BL#57321), UAS-Xpd1-RNAi (VDRC#106998, BL#65883), UAS-Cul4-RNAi v105668, v44829), UAS-DCAF12-RNAi (VDRC#43758), UAS-Wts-RNAi (VDRC#106174). The
transgenes were used: UAS-Yki(WT), UAS-Yki(S169A), and UAS-Sd-GA [Zhang et al. 2008]; UAS-Yki(F111AS60A) and UAS-Yki(3SA) [Ren et al. 2008]; UAS-InR^{AC} [BL#8263]; UAS-GFP-CDK7 and UAS-GFP-CDK7^{D137R} were generated by P-element mediated transformation. UAS-Yki(WT), UAS-Yki(S169A) and UAS-Yki(S169D) were inserted at the 75B ballop [Bischof et al. 2007]. The Hippo pathway reporters were used: diap-GFP3.5, 3xSad-LacZ [Zhang et al. 2008]; ex-lacZ [Hamaratoglu et al. 2006]; diap-lacZ [th^{137A}] [Ryoo et al. 2002]; Yki-GFP [Fletcher et al. 2018]. The Gal4 drivers used were GM4-Gal4 [Freeman 1996], and hh-Gal4 [Zhang et al. 2008], MS1096 [Vidal et al. 2013]. The CDK7^{S164A/T170A} mutant flies were generated by P-element mediated transgene expression in S2 cells. HEK293A, MDA-MB-231, and MCF-7 cells were cultured in DMEM (Sigma-Aldrich) and transfected using Lipofectamine 2000 (Thermo Fisher Scientific, cat: 11668). After 48 h, the recombinant viruses were harvested for cell line infection. The recombinant viruses were infected into MDA-MB-231 cells using the standard method.

RNA interference

For RNAi experiments in S2 cells, dsRNAs were generated using the MEGAscript high-yield transcription kit [Ambion]. The following primers were used for generating the dsRNA targeting individual genes: CDK7 [5'-GAATTAATACGACTCACTATAGGGAGATTACAGGACTGACCTATGCTGAGGAGATGAACTTATGCTGCGAGTGC-3'] and [5'-GAATTAATACGACTCACTATAGGGAGATTACAGGACTGACCTATGCTGAGGAGATGAACTTATGCTGCGAGTGC-3']. CDK7 coding sequence of luciferase was used as a control. For RNAi in mammalian cells, siRNA was transfected into cells using Lipofectamine RNAi-MAX [cat:13778, Invitrogen] in antibiotics-free medium according to manufacturer’s instruction. Oligonucleotides of siRNA duplexes were purchased from Sigma as follows: siCDK7#1 [SASI_Hs_01_00214781], siCDK7#2 [SASI_Hs_01_00214781], siDCAF12 #1 [SASI_Hs_01_00216314], siDCAF12 #2 [SASI_Hs_01_00216315], siDCAF12 #3 [SASI_Hs_01_00216316], and siDCAF12 #4 [SASI_Hs_01_00216317]. The siControl used was UUCUCCGAACGUGUCACGUUTT.

Immunoprecipitation and Western blotting

For immunoprecipitation assay, cells were harvested and washed twice with PBS after transfection for 48 h and then lysed on ice for 30 min with lysis buffer containing 1% Triton X-100, 50 mM NaCl, 1% NaF, 0.1M NaVO₃, 1% NP-40, 10% glycerol, and 0.5M EDTA (pH 8.0). The cell lysates were incubated with protein A-Sepharose beads [Thermo Scientific] for 1 h at 4°C to eliminate nonspecific binding proteins. After removal of the protein-A beads by centrifugation, the cleared lysates were incubated with Myc [HA or Flag] antibody for 2 h or overnight. The complexes were collected by incubation with protein A-Sepharose beads for 1 h at 4°C, followed by centrifugation. The immunoprecipitates were then washed three times for 5 min each with lysis buffer and were separated on SDS-PAGE. Western blot was carried out using standard protocol. The cancer cells were lysed with RIPA buffer. Lysates were separated by electrophoresis on SDS-PAGE and electro-transferred to PVDF membrane. Mouse liver tissues or tumors were lysed in RIPA buffer containing protease inhibitor cocktail [Roche]. Lysates were clarified by centrifugation at 18,500 g for 30 min and subjected to nitrocellulose membranes [Life Technologies, Thermo Fisher Scientific]. After blocking with 5% BSA in TBS with 0.5% Tween-20 for 1 h, the membranes were probed with the corresponding antibodies overnight. Bound antibodies were visualized by ECL (EMD Millipore or Pierce, Thermo Fisher Scientific) using HRP-conjugated antibodies.

**Cell culture, transfection, and lentiviral production**

S2 [S2R+] cells were cultured in Schneider’s Drosophila medium [Life Technologies] with 10% fetal bovine serum [GE Healthcare], 100 U/mL penicillin [Life Technologies], and 100 mg/mL streptomycin [Life Technologies] at 24°C. Transfection of S2 cells was performed using calcium phosphate transfection kit [Specialty Media] following the manufacturer’s instruction. A ubiquitin-Gal4 construct was cotransfected with pUAST constructs to drive UAS transgene expression in S2 cells. HEK293A, MDA-MB-231, EC9706, and HC116 cells were obtained from the American Type Culture Collection [ATCC]. These cancer cells were cultured in Dulbecco’s modified Eagle’s medium [DMEM] [Life Technologies, 41965] supplemented with 10% fetal bovine serum [FBS, 10270, Life Technologies] and 1% penicillin/streptomycin. All cell lines were subject to cell line authentication via short tandem repeat [STR], which was performed via PowerPlex21 system and compared with STR data in ATCC. *Lats1/2*−/− HEK293 cells were obtained from Dr. Kun-Liang Guan [Meng et al. 2015]. HEK293 cells were cultured in DMEM [Sigma-Aldrich] containing 10% BCS [ATCC], and transfected using GenJet Plus in vitro DNA transfection kit [SignaGen]. For starvation experiments, HEK293A cells were cultured in DMEM [Sigma-Aldrich] without serum for 6 h 2 d after transfection. For lentivirus infection, Yap^{WT} and Yap^{S128D} were cloned into pLVX-ires-ZsGreen vector. The expression vectors and package vectors [PSPAX2 and PMDΔ2] were cotransfected into HEK293T cells with lipoparticle 2000 [Thermo Fisher Scientific, cat: 11668]. After 48 h, the recombinant viruses were harvested for cell line infection. The recombinant viruses were infected into MDA-MB-231 cells using the standard method.

**DNA constructs**

The pUAST constructs used were UAS-Myc-Yki [Zhang et al. 2008], UAS-Fg-Cul5, and UAS-Fg-DDB1 [Li et al. 2018]. Myr-Yki-GFP contained a myristoylation signal MGNNCKSKRQ and GFP tag at the N and C terminus, respectively. Myr-Yki-GFP contained a myristoylation signal at the N terminus of Yki. Yki(S169A) and Yki(S169D) were generated by PCR-mediated site-directed mutagenesis. Drosophila CDK7 CDNA clone was obtained from DGRG [Flybase]. CDK7^{D137R} [DI37R] variant was obtained from DGRC (Flybase). CDK7^{D137R} was generated by site-directed mutagenesis. The constructs were subcloned into the pUAST vector Digested with NotI and XbaI. pcDNA3.1 construct expressing Fg-Yap or HA-Taz [Cho et al. 2018] was pcDNA3.1 construct expressing human HA-CDK7 (Addgen). To generate HA-DCAF12 construct, DNA fragments encoding HA-DCAF12 were amplified by PCR and inserted between KpnI and EcoRI sites of the pcDNA3.1 vector. Fg-YapS128A, Fg-YapS128D, HA-TazS90A, and HA-TazS90D were generated by PCR-mediated site-directed mutagenesis. The constructs were subcloned into pcDNA3.1 for transient transfection experiments. To make GST-Yki fusion constructs, synthetic DNA oligos corresponding to the coding sequences for aa160–181 and their SA mutants [S168A, S169A, and S172A] were annealed and subcloned into pGE4Xt-1 vector digested with EcoRI and Xhol.
Immunostaining of imaginal discs was carried out as previously described [Jiang and Struhl 1995]. For immunostaining of cultured cells, cells were seeded on LAB-TEK chamber slides, transfected with indicated constructs, and stained using standard protocols. Liver tissues were perfused with ice-cold PBS and fixed in 4% paraformaldehyde for the preparation of cryosections. Sections were permeabilized in 0.5% Triton/PBS [PBST] for 5 min, followed by three washes in PBS. Sections were incubated in blocking buffer [1% BSA in PBST] for 1 h at room temperature and then incubated overnight at 4°C with the indicated primary antibodies. Sections were washed five times in PBS and then incubated with a secondary antibody for 1 h at room temperature. Donkey antiumouse Alexa fluor 488, donkey antirat Alexa fluor 594, or donkey antirabbit Alexa fluor 594 secondary antibodies (Life Technologies, Thermo Fisher Scientific) were used. Sections were washed 10 times in PBST and then mounted with mounting medium with DAPI [catalog H-1200, Vector Laboratories]. Immunofluorescence imaging was performed using a Zeiss 510 NLO META laser-scanning microscope.

Antibodies

Yki S169 phospho-specific antibody [pS169] was generated using IHSSRSRSPS [PASLQON] as antigen and affinity purified by Genemed Synthesis. Other antibodies used were rabbit anti-Yki [Li et al. 2015], Yap S128 phospho-specific antibody [pS128] [Moon et al. 2017], mouse anti-β-Gal [Promega], rabbit anti-β-Gal [Affinity Bioreagents], mouse anti-Wg [Developmental Studies Hybridoma Bank [DSHB]], rat anti-Ci, ZA1 [DSHB], rabbit anti-GFP [Invitrogen], mouse anti-Myc [Santa Cruz Biotechnology], rabbit and mouse anti-Flag [Sigma], mouse anti-HA [Santa Cruz Biotechnology], rabbit anti-Yap [Abcam], rabbit anti-CDK7 [Cell Signaling], mouse anti-Yap/TAZ [Santa Cruz Biotechnology, catalog sc-10119], mouse anti-TAZ [Santa Cruz Biotechnology, catalog sc-518026], mouse anti-ActinCell Signaling, catalog 3700], rabbit anti-Yap/Taz [Cell Signaling, catalog 8418], rabbit anti-p-Yap [Cell Signaling, catalog 4911], rabbit anti-p-STAT3 [Cell Signaling, catalog 9145], mouse anti-GAPDH [Sigma-Aldrich, catalog SAB1405848], rabbit anti-p-Histone3 [Cell Signaling, catalog 9701], goat anti-CTGF [Santa Cruz Biotechnology, sc-14940], goat anti-CYR61 [Santa Cruz Biotechnology, sc-8561], rabbit anti-SOX9 [EMD Millipore, catalog AB5535], rabbit anti-Ki67 [Invitrogen, catalog PA5-16785], rat anti-EPAC [DSHB, clone G8.8], and rat anti-F4/80 [AbD Serotec, catalog MCA497]. Antimouse and rabbit HRP-conjugated secondary antibodies were from GE Healthcare Life Sciences. Antigoat and antirat HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology, and Sigma-Aldrich, respectively.

Mouse lines, xenografts, and drug treatment.

The procedures for all animal experiments were reviewed and approved by the Institutional Animal Care and Use Committees of Harvard Medical School and University of Texas Southwestern Medical School. The Alb-Cre Mst1−/−Mst20/0 mouse lines have been described previously [Dong et al. 2007; Zhou et al. 2009; Song et al. 2010]. Both male and female mice were used, which did not show difference in this study. Mice were treated with THZ1 [APEXBIO, catalog no. A8882] by intraperitoneal injection at a concentration of 10 mg/kg every other day or in equivalent volumes of vehicle as a control. Mice received 28 injections during the 8-wk period starting at 1 mo of age. For TNBC xenograft experiments, MDA-MB-231 cells infected with control vector and Yap5128D lentivirus were FACS sorted, and 1 × 106 of each was suspended in 100 µL of PBS with 40% Matrigel [BD Biosciences] and injected into the fourth pair of mammary fat pads of 6-wk-old female NSG mice [purchased from the University of Texas Southwestern Medical Center animal breeding core facility]. Once the tumor size reached ~100 mm3, the animals for each cell line were randomly divided into two groups (seven mice/group) and treated with either vehicle [10% DMSO in D5W, 5% dextrose in water] or 10 mg/kg THZ1 [MedChemExpress, catalog no. HY-80013] through intraperitoneal administration twice daily. Growth in tumor volume was measured with digital calipers every 3 d. Tumor volumes were estimated using the formula V = length × width2 × 0.5. At the end of the studies, mice were sacrificed for tumor collection, tumors were weighed and frozen in liquid nitrogen for future analysis.

Hepatocyte isolation and treatment

Primary hepatocytes were isolated from Alb-Cre Mst1−/−Mst20/0 [DKO] mouse via collagenase-elastase perfusion according to the manufacturer’s protocol [Worthington Biochemical]. Briefly, mice were anesthetized with sodium pentobarbital solution [30 mg/kg i.p.] and their livers perfused with calcium- and magnesium-free HBSS [CMF-HBSS], followed by enzyme buffer solution [225 U/mL collagenase, 0.3 U/mL elastase, and 10 U/mL DNase]. The isolated mouse hepatocytes were then cultured in DMEM containing 10% FBS, l-glutamine, and penicillin-streptomycin in collagen-coated plates (Biocoat, BD). Primary hepatocytes were treated with THZ1 [500 nM] for 12 h, and cell lysates were subjected to Western blot analysis using indicated antibodies.

RT-qPCR

Total RNA was extracted using RNeasy Plus mini kit [Qiagen #74134], and cDNA was synthesized using the iScript cDNA synthesis kit [Bio-Rad]. RT-qPCR was performed using iQ SYBR Green system [Bio-Rad] and a Bio-Rad CFX96 real-time PCR system. 36B4 expression level was used as a normalization control. Primer sequences used were CTGF [CATCTTCGCTGTACCGTGT [F] and TTCCAGTCGCTAACGCCG [R], CYR61 [GGCGTTT CTTTCACAAGGCCG [F] and TGGAGCGCTCCTGTTT [R], AKR1D1 [GCCATGCCCTTCAAAATGCCA [F] and AGAA CTGTGCTGGAGAGACC [R], AJPUBA [TACCCAGCAAGAC TAACCG [F] and TACAGGTGGCAAGTATGCTC [R], and 36B4 [GGGACCTCGGAATCAACCT [F] and CCATACGAC CACAGCCTTC [R]].

Total RNA from mouse liver tissue was prepared using RNazol reagent [RNaZol RT, MRC] according to the manufacturer’s protocol. cDNA was synthesized from total liver RNA [1–2 µg] using high-capacity cDNA reverse transcription kit [Life Technologies, Thermo Fisher Scientific]. qRT-PCR was performed using SYBR Select Master Mix [Thermo Fisher Scientific] on a StepOnePlus thermal cycler from Applied Biosystems. Expression levels were always given relative to GAPDH. The following PCR primers for mouse samples were used: Ctgf [forward, CTGCACTGACACCGGCG [F] and CCATCTGACACCGGCG [R], Cyr61 [forward, GCTGACTGACACCGGCG [F] and AAGA CTGTGCTGGAGAGACC [R], Ajiub [TACCCAGCAAGAC TAACCG [F] and TACAGGTGGCAAGTATGCTC [R], and 36B4 [GGGACCTCGGAATCAACCT [F] and CCATACGAC CACAGCCTTC [R]].

Total RNA from mouse liver tissue was prepared using RNaZol reagent [RNaZol RT, MRC] according to the manufacturer’s protocol. cDNA was synthesized from total liver RNA [1–2 µg] using high-capacity cDNA reverse transcription kit [Life Technologies, Thermo Fisher Scientific]. qRT-PCR was performed using SYBR Select Master Mix [Thermo Fisher Scientific] on a StepOnePlus thermal cycler from Applied Biosystems. Expression levels were always given relative to GAPDH. The following PCR primers for mouse samples were used: Ctgf [forward, CTGCACTGACACCGGCG [F] and CCATCTGACACCGGCG [R], Cyr61 [forward, GCTGACTGACACCGGCG [F] and AAGA CTGTGCTGGAGAGACC [R], Ajiub [TACCCAGCAAGAC TAACCG [F] and TACAGGTGGCAAGTATGCTC [R], and 36B4 [GGGACCTCGGAATCAACCT [F] and CCATACGAC CACAGCCTTC [R]].
CTTACTGCTGCTG; reverse, GCCAGTGTATTCTTGACTGT, H11b (forward, CAACCACCTGATTATTCTCCATG; reverse, GATCCACACCTCCAGCCTGCA), EpCam (forward, CTTGAGATGACGAGGAGG; reverse, GACACCCACACTCTGACG), Sox9 (forward, CGACTCCTGACTATCA GA; reverse, AGACTGTGGTTCCACACTG), Gapdh (forward, ATCTGCTCAACCACTGCT; reverse, GGCCCATCACA GTCTTCTG), Tgfl-β (forward, GCCTAGTGGCTGCTTT TGA; reverse, GCCTGACTCCGGTTGATTC), Tgfl-β2 (forward, TGAGAGCAGCACACTA; reverse, AAACCT CC GATTTATGTTGTGT), Tgfl-β3 (forward, AGTGCTGCTGGA GGAGAGCT; reverse, GTGTTGCTGTTGTTG ATCTT), F4/80 (forward, CCCAGTGTCTTACAGTGG; reverse, GTGCCCCAGACTGATGCTT), c-Myo (forward, ATGCCGCC TCAACGTAACCTT; reverse, CGCAAACAGGATGGACA GCA), Acta2 (forward, ATGGCTCCAGGCTGTTTCTCT; reverse, GTGTTGCGACGCTTTTCTAC), and Gapdh (forward, ATCTGCGACCAAAGCT; reverse, GGCGG ATCCAGCTTCTTCTG.

In vitro kinase assay

In vitro kinase assay was carried out by incubating 25 μL of reaction mixtures containing 150 mM Tris-HCl (pH 7.5), 0.2 mM Mg2+/ATP, 1 μg of purified GST-Yki fusion proteins, together with appropriate amount of GFP-CDK7 or GFP-CDK7ΔN immunopurified S2 cells for 1.5 h at 30°C. The reaction was terminated by adding 2× SDS loading buffer. The resultant samples were load on SDS-PAGE and subjected to pIMAGO phospho-protein detection kit with fluoro-680 (Sigma)

Luciferase reporter assay

To measure the activity of Yki/YAP/TAZ, cells were seeded in 24-well plates and co-transfected with a Sd [Scalloped]-dependent luciferase reporter construct, 3XSk2- luc [Zhang et al. 2008], with pTK-Renilla and effector plasmids. The luciferase activities were analyzed using a dual-luciferase reporter assay kit (Promega) according to manufacturer’s instruction.

Ubiquitination assay

For Yki ubiquitination assay, S2 cells were treated with 50 μM MG132 [Calbiochem] for 4 h prior to harvest. Cells were lysed in lysis buffer [150 mM Tris pH 8.0, 100 mM NaCl, 10 mM NaF, 1 mM Na3VO4, 10% Glycerol, 1.5 mM EDTA]/1 for 30 min on ice. After centrifugation, SDS was added to the supernatants at 1% final concentration. After boiling for 3 min, the lysates were diluted 10-fold with lysis buffer and then subjected to immunoprecipitation and Western blot analysis. For Yap/Taz ubiquitination assay, HEK293T cells were lysed with lysis buffer containing 50 mM Tris HCl(pH 8), 100 mM NaCl, 10 mM NaF, 1 mM Na3VO4, 1% IGEPAI CA-650, 10% glycerol, 1.5 mM EDTA (1 mL for one 10-cm plate). After centrifugation to remove cell debris and nuclei, 225 μL of Supernatant was mixed with 25 μL 10% SDS and boiled for 3 min. One milliliter of the above lysis buffer was added prior to immunoprecipitation and Western blot analysis.

Cell proliferation assay

MDA-MB-231, EC9706, and HCT116 cells were seeded and transfected with 50 μM CDK7 siRNA or control siRNA. Twenty-four hours after transfection, cells were counted to ensure that the same number of cells were seeded into 96-well plates. Cell numbers were determined using WST-1 cell proliferation reagent (Sigma-Aldrich, cat: 501 5944001) at indicated time points.

Transwell invasion assay

Two-chamber plates (#3422, Corning) were used to perform the transwell assay. For the transwell assay, cells were transfected with 50 nmol/L siRNA or siControl. After 24 h, 1 × 105 control cells and CDK7 knocking-down cells were put into the chamber. To stimulate the invasion, complete medium was added to the bottom wells, while no FBS medium was added in the upper chamber. After 24 h of incubation at 37°C for invasion assay, cells in the upper chamber were carefully removed and the cells that had shuttled into the membrane were fixed and stained with Crystal Violet staining solution. Cellular quantification was analyzed in three fields with 100x magnification under a microscope. The quantification was based on cell-number counting in each vision.

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