A HIPPO'S VIEW: FROM MOLECULAR BASIS TO TRANSLATIONAL MEDICINE

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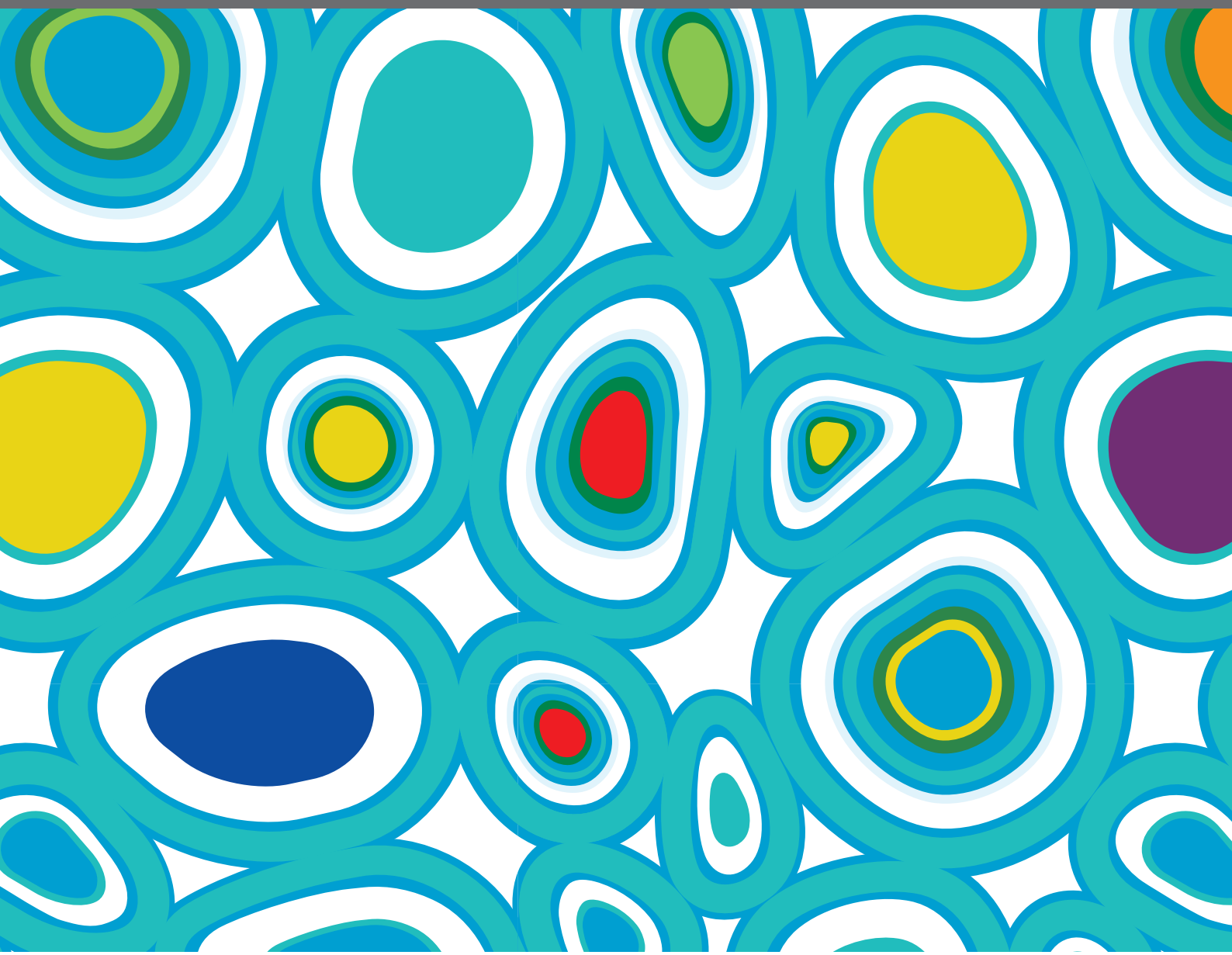

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A HIPPO'S VIEW: FROM MOLECULAR BASIS TO TRANSLATIONAL MEDICINE

Topic Editors:
Zhaocai Zhou, Fudan University, China
Zengqiang Yuan, Chinese Academy of Medical Sciences and Peking Union Medical College, China
Wanjin Hong, Agency for Science, Technology and Research (A*STAR), Singapore
Wenqi Wang, University of California, Irvine, United States

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Table of Contents

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Editorial: A Hippo's View: From Molecular Basis to Translational Medicine

Zhaocai Zhou[1], Zengqiang Yuan[2]*, Wanjin Hong[3]* and Wenqi Wang[4]**

[1] State Key Laboratory of Genetic Engineering, Zhongshan Hospital, School of Life Sciences, Fudan University, Shanghai, China, [2] The Brain Science Center, Beijing Institute of Basic Medical Sciences, Beijing, China, [3] Institute of Molecular and Cell Biology, Agency for Science, Technology, and Research (A*STAR), Singapore, Singapore, [4] Department of Developmental and Cell Biology, University of California, Irvine, Irvine, CA, United States

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Editorial on the Research Topic

A Hippo's View: From Molecular Basis to Translational Medicine

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Ana Cuenda,
Consejo Superior de Investigaciones Científicas (CSIC), Spain

****Correspondence:***
Zhaocai Zhou
zczhou@sibcb.ac.cn
Zengqiang Yuan
zqyuan@bmi.ac.cn
Wanjin Hong
mcbhwj@imcb.a-star.edu.sg
Wenqi Wang
wenqiw6@uci.edu

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Initially identified in *Drosophila*, the major components of the Hippo pathway including the core kinase cascade, downstream effector and nuclear transcription factor are evolutionarily conserved, resulting in extensive studies from many investigators in the past decades. The Hippo pathway is now recognized as a key player in organ size control *via* primarily stimulating programmed cell death and restricting cell proliferation (Halder and Johnson, 2011; Yu et al., 2015; Zheng and Pan, 2019).

The Hippo pathway regulates cell proliferation, survival and differentiation in response to a wide range of extracellular cues including growth factors, mitogenic hormones, metabolic inputs, and perceived physical signals from cell microenvironment, suggesting its crucial role in normal physiology. Moreover, Hippo signaling dysregulation has been linked to various human diseases like developmental anomalies, impaired immunity, cancer development, cancer metastasis and drug resistance. Therefore, elucidating the molecular basis of Hippo pathway regulation and underlying mechanism will not only provide novel insights into many fundamental processes in physiology, but also foster the development of therapeutic strategies for translational medicine. Here, we prepare a special Research Topic issue to provide an overview of the up-to-date research findings on this exciting and burgeoning filed.

While various intracellular and extracellular signals have been discovered to regulate the Hippo pathway, the current focus in the field is how these signals converge on the Hippo pathway for growth control and tissue homeostasis.

In this issue, Cai et al. reviewed how the Hippo pathway effectors YAP and TAZ are modulated under different mechanical cues. Given the fundamental roles of YAP and TAZ in mechanotransduction, the authors discussed the potential pathological roles of YAP/TAZ in several human diseases involving mechanical cues, such as pulmonary hypertension, atherosclerosis, cardiac hypertrophy, fibrosis, musculoskeletal disorder, and cancer.

Calcium (Ca^{2+}) functions as an essential intracellular messenger in a number of cellular signaling events (Clapham, 2007). Wei and Li summarized the molecular mechanisms for the Ca^{2+}-mediated regulation of the Hippo pathway, underscoring the important role of Ca^{2+}-mediated actin reset (CaAR) in this process. Uncovering additional effectors for Ca^{2+} signaling with relevance to the Hippo pathway will enrich our understanding of the Hippo pathway regulation from a new perspective.

The tissue architecture-related cell polarity, cell-cell junctions and cell-extracellular matrix (ECM) interaction are known regulators of the Hippo pathway. Using *Drosophila* wing discs as a model, Wang et al. identified *Wallenda* (*Wnd*) (MAP3K13 in human) as a new player in the cell polarity-mediated Hippo pathway regulation. *Wnd* is involved in this process through nemo-like kinase, *Nmo* (NLK in human), and such *Wnd-Nmo* axis in regulating the Hippo pathway is shown conserved in evolution. Thus, it will be interesting to further examine whether dysregulation of these newly discovered regulators would cause cell polarity-associated human diseases like cancer.

Although the Hippo pathway kinase cascade transduces many upstream signaling events to YAP and TAZ, mechanisms that modulate YAP and TAZ activities independent of the Hippo kinase cascade do exist. In this Research Topic issue, Cho and Jiang authoritatively highlighted the recent findings of such "non-canonical" regulation for YAP and TAZ. These mechanisms highlight the complex regulation of the Hippo pathway, connect the Hippo pathway with other key cellular signaling events, and reveal novel therapeutic strategies for future investigations.

To resolve the complex regulation of the Hippo pathway, extensive efforts have been made to identify new Hippo pathway regulators. In line with it, Pipchuk and Yang described the application of luciferase-based biosensors to the Hippo pathway study. The authors discussed several assays using split luciferase complementation systems that have been successfully used for identifying new Hippo pathway regulators, highlighting the advantage of this luciferase-based biosensor method in studying real-time protein-protein interaction in live cells.

Although the Hippo pathway is known for its crucial role in organ size control, recent studies have extended it to other biological processes, such as embryogenesis, stem cell regulation and tissue regeneration. Here, Zhao et al. reviewed the emerging findings of the Hippo pathway in neural crest (NC) development. This work summarized the critical role of the Hippo pathway in many aspects of NC development, including NC initiation, migration, proliferation, survival and differentiation, as well as the NC-related diseases caused by the dysregulated Hippo signaling. In terms with organogenesis. Wu et al. described how the Hippo pathway acts in pancreatic development. This review highlighted the role of the Hippo pathway in progenitor cell maintenance and normal proper cell polarization/branching during pancreatic organogenesis and morphogenesis, which involve the crosstalk with several key developmental signaling pathways, such as Notch, Wnt, and PI3K-Akt.

Given the critical role of the Hippo pathway in numerous physiological processes, its dysregulation has been linked to many human diseases such as cancer. For example, the Hippo pathway is implicated to control cancer stem cell expansion and maintenance against cancer development (Park et al., 2018). To reveal the underlying mechanisms, Shen et al. investigated the Hippo pathway effector TAZ in breast cancer stem cells (BCSCs) and revealed the Cyclin D1-CDK4/CDK6 axis as downstream effector for the TAZ-driven breast tumorigenesis. This work suggests a possible vulnerability for BCSCs as well as a new opportunity for breast cancer therapy.

As for the cancer-related Hippo pathway dysregulation, He et al. systematically described the mutation and copy number abnormality for the Hippo pathway components in human cancer genome. Although the alterations of major Hippo pathway components in human cancers are rare, the impaired Hippo pathway may arise from the regulators of its core kinase cascade and/or nuclear transcriptional complex.

By examining The Cancer Genome Atlas (TCGA), Gu et al. analyzed the Hippo pathway kinase *LATS2* in the isocitrate dehydrogenase1/2 (IDH1/2)-mutated low-grade glioma (LGG). Their findings confirmed the hypermethylation of *LATS2* promoter in IDH1/2-mutated LGG but failed to link this genomic alteration with YAP activation experimentally. Thus, the role of the *LATS2* hypermethylation in LGG development deserves further investigation.

Studies from multiple model systems have fully established the Hippo pathway as an evolutionarily conserved signaling cascade from unicellular organisms to humans (Sebe-Pedros et al., 2012). In the past years, tremendous efforts have been made to elucidate the mechanisms for the Hippo pathway regulation and function, providing a reliable molecular basis for further exploring diverse biological processes and human diseases. In this Research Topic issue, the 10 articles written by leading scientists in the field cover the updated research findings for the Hippo pathway from different aspects. Advances in biotechnology including gene editing and single-cell studies are in great progress to expand our insights into the nature of Hippo signaling, especially from the perspectives of cell-cell interaction and organ-organ communication. We anticipate more discoveries of this exciting pathway will be made in years to come, allowing us to unveil the mystery of organ size control and develop new approaches for treating human diseases.

AUTHOR CONTRIBUTIONS

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ORIGINAL RESEARCH
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Hypermethylation of *LATS2* Promoter and Its Prognostic Value in *IDH*-Mutated Low-Grade Gliomas

Yuan Gu[1†], Yu Wang[1†], Yebin Wang[1], Jiaqian Luo[1], Xin Wang[1], Mingyue Ma[1], Wei Hua[2], Ying Liu[3] and Fa-Xing Yu[1]*

[1] *Institute of Pediatrics, Children's Hospital of Fudan University and the Shanghai Key Laboratory of Medical Epigenetics, The International Co-laboratory of Medical Epigenetics and Metabolism, Ministry of Science and Technology, Institutes of Biomedical Sciences, Shanghai Medical College, Fudan University, Shanghai, China,* [2] *Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai, China,* [3] *Department of Pathology, School of Basic Medical Sciences, Fudan University, Shanghai, China*

Mutations in the enzyme isocitrate dehydrogenase 1/2 (*IDH1/2*) are the most common somatic mutations in low-grade glioma (LGG). The Hippo signaling pathway is known to play a key role in organ size control, and its dysregulation is involved in the development of diverse cancers. Large tumor suppressor 1/2 (LATS1/2) are core Hippo pathway components that phosphorylate and inactivate Yes-associated protein (YAP), a transcriptional co-activator that regulates expression of genes involved in tumorigenesis. A recent report from The Cancer Genome Atlas (TCGA) has highlighted a frequent hypermethylation of *LATS2* in *IDH*-mutant LGG. However, it is unclear if *LATS2* hypermethylation is associated with YAP activation and prognosis of LGG patients. Here, we performed a network analysis of the status of the Hippo pathway in *IDH*-mutant LGG samples and determined its association with cancer prognosis. Combining TCGA data with our biochemical assays, we found hypermethylation of *LATS2* promoter in IDH-mutant LGG. *LATS2* hypermethylation, however, did not translate into YAP activation but highly correlated with *IDH* mutation. *LATS2* hypermethylation may thus serve as an alternative for *IDH* mutation in diagnosis and a favorable prognostic factor for LGG patients.

Keywords: low-grade glioma, Hippo pathway, *Lats2*, YAP, IDH1/2, isocitrate dehydrogenase

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Edited by:
Wenqi Wang,
University of California, Irvine, United States

Reviewed by:
Jianmin Zhang,
University at Buffalo, United States
Zhipeng Meng,
University of Miami, United States

****Correspondence:***
Fa-Xing Yu
fxyu@fudan.edu.cn

† These authors have contributed equally to this work

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INTRODUCTION

Mutations in isocitrate dehydrogenase 1 and 2 (*IDH1/2*), mainly Arg132 for *IDH1* and Arg140 and Arg172 for *IDH2*, occur in over 80% of low-grade glioma (LGG) (Parsons et al., 2008; Watanabe et al., 2009; Yang et al., 2012; Cancer Genome Atlas Research Network et al., 2015; Suzuki et al., 2015). While wild type IDH1/2 converts isocitrate to α-ketoglutarate (αKG), gain-of-function mutations of *IDH1/2* lead to the production and accumulation of oncometabolite R-2-hydroxyglutarate (R-2HG) (Zhao et al., 2009; Ward et al., 2010). R-2HG drives tumorigenesis by inhibiting αKG-dependent enzymes involved in epigenetic modifications, response to hypoxia, and other biological processes (Zhao et al., 2009; Figueroa et al., 2010; Chowdhury et al., 2011; Xu et al., 2011; Lu et al., 2012; Turcan et al., 2012).

The Hippo pathway consists of a kinase cascade and plays crucial roles in tissue homeostasis and tumorigenesis (Harvey et al., 2013; Yu and Guan, 2013; Moroishi et al., 2015; Yu et al., 2015; Patel et al., 2017; Luo and Yu, 2019). Hippo pathway activation results in the phosphorylation of core Ste20-like kinases MST1 and MST2 (MST1/2), which phosphorylate and activate large tumor suppressor 1/2 (LATS1/2) kinases. LATS1/2, in turn, phosphorylate and inactivate Yes-associated protein (YAP) and WW domain-containing transcription regulator protein 1(WWTR1, also known as TAZ), which function as transcription co-activators and serve as Hippo pathway downstream effectors by regulating expression of genes involved in cell proliferation, death, and differentiation. MST1/2 and LATS1/2 activity is further regulated by diverse regulators and upstream signals. Dysregulation of Hippo pathway has been associated with various cancers (Yimlamai et al., 2015; Yu et al., 2015; Zanconato et al., 2016; Gregorieff and Wrana, 2017; Zhang and Zhou, 2019). *LATS2* deficiency, for instance, has been studied in several cancers including glioma (Kawahara et al., 2008; Guo et al., 2017, 2019; Ye et al., 2017; Jin et al., 2018; Pan et al., 2018; Shi et al., 2018, 2019; He et al., 2019; Hsu et al., 2019).

A recent report from The Cancer Genome Atlas (TCGA) Research Network revealed that the promoter of *LATS2* is hypermethylated in almost all *IDH*-mutated LGG clinical samples but not in *IDH*-wild type samples (Sanchez-Vega et al., 2018). *LATS2* promoter hypermethylation in *IDH*-mutated LGG samples is expected to downregulate *LATS2* expression and subsequently activate YAP/TAZ and expression of downstream target genes. However, this hypothesis has not been systematically analyzed and experimentally tested. Here, combining TCGA data with our biochemical assays, we performed a network analysis of the status of the Hippo pathway in *IDH*-mutant LGG samples and determined its association with cancer prognosis.

RESULTS

Promoter Hypermethylation and Low Expression of *LATS2* in *IDH*-Mutant LGG

We examined *LATS2* methylation level and mRNA expression in LGG dataset from TCGA, and found that *LATS2* promoter was hypermethylated and *LATS2* mRNA was repressed in *IDH*-mutant LGG compared to *IDH*-wild type LGG (**Figures 1A,B**). Moreover, *LATS2* mRNA levels negatively correlated with methylation levels (**Figure 1C**). The differences in *LATS2* gene methylation were mainly located within the promoter region instead of gene body (**Figure 1D**). We also collected LGG specimens with or without *IDH*1/2 mutations, and measured *LATS2* promoter methylation using a methylation-specific PCR assay (Herman et al., 1996; Oh et al., 2015). Consistent with TCGA data, *LATS2* promoter methylation was significantly higher in *IDH*-mutant LGG (**Figure 1E**). It is worth noting that while *LATS2* promoter hypermethylation had been reported in another cancer with frequent *IDH* mutations, namely *IDH*-mutant acute myeloid leukemia (AML), it did not downregulate *LATS2* expression as it did in LGG (**Supplementary Figure 1A**), suggesting a different mechanism or role. Meanwhile, while *LATS1* was also hypermethylated, it was not downregulated as *LATS2* (**Supplementary Figure 2A**). Overall, our results indicate that *LATS2* is hypermethylated and repressed in *IDH*-mutant LGG.

Hippo Pathway Target Genes Are Not Activated by *LATS2* Deficiency in *IDH*-Mutant LGG

Given that LATS2 is a direct upstream regulator of YAP/TAZ, we examined the effects of *LATS2* knockdown on YAP activity. Using two independent siRNAs to target LATS2 in HEK293 cells, we observed that LATS2 knockdown significantly reduced YAP phosphorylation and increased target gene *CYR61* expression (**Figure 2A**). The same result was also observed in glioma cell lines (Guo et al., 2019; Shi et al., 2019). Hence, silencing LATS2 expression in HEK293 cells led to YAP activation.

Subsequently, we analyzed if Hippo pathway target genes were activated following *LATS2* downregulation in *IDH*-mutant LGG. Surprisingly, the association between Hippo pathway target gene expression with IDH mutation was weak (**Figure 2B**). For instance, the mRNA levels of *CTGF* and *CYR61* were reduced in *IDH*-mutant LGG samples, and correlation analyses showed a nearly negative correlation between Hippo pathway target gene expression and *LATS2* methylation (**Figure 2C**). Hence, it appeared that at least in *IDH*-mutant LGG, LATS2 downregulation did not translate to YAP activation and YAP-dependent gene expression.

Hippo Pathway Target Genes Are Universally Hypermethylated in IDH-Mutant LGG

The high methylation levels of *CTGF* and *CYR61* in *IDH*-mutant LGG suggested that Hippo pathway target genes were also regulated by methylation (**Figure 3B**). Indeed, a cluster analysis showed that most Hippo pathway target genes were hypermethylated in *IDH*-mutant LGG samples (**Figure 3A**). The methylation of these genes was comparable to that of *LATS2*, as indicated by a tight correlation between methylation levels of *LATS2* and those of *CTGF* or *CYR61* (**Figure 3B**). Thus, the nearly universal hypermethylation of Hippo pathway target genes may explain the ineffectiveness of *LATS2* hypermethylation in *IDH*-mutant LGG to affect YAP activation and target gene expression.

Low Expression of YAP/TAZ in LGG

We next analyzed the expression of YAP and TAZ in LGG. Interestingly, both *YAP* and *TAZ* were hypermethylated, and were expressed at lower levels in *IDH*-mutant LGG samples compared to *IDH*-wild type LGG samples (**Figures 4A–C** and **Supplementary Figure 3**). We then assessed YAP expression by immunohistochemistry (IHC) in LGG tumor specimens. Our IHC results indicated, however, that YAP expression was either absent or extremely weak in all LGG samples, regardless of *IDH* status. In contrast, YAP was highly expressed in glioblastoma (GBM), another common brain tumor (**Figure 4D**). This could be due to overall higher methylation and lower expression of

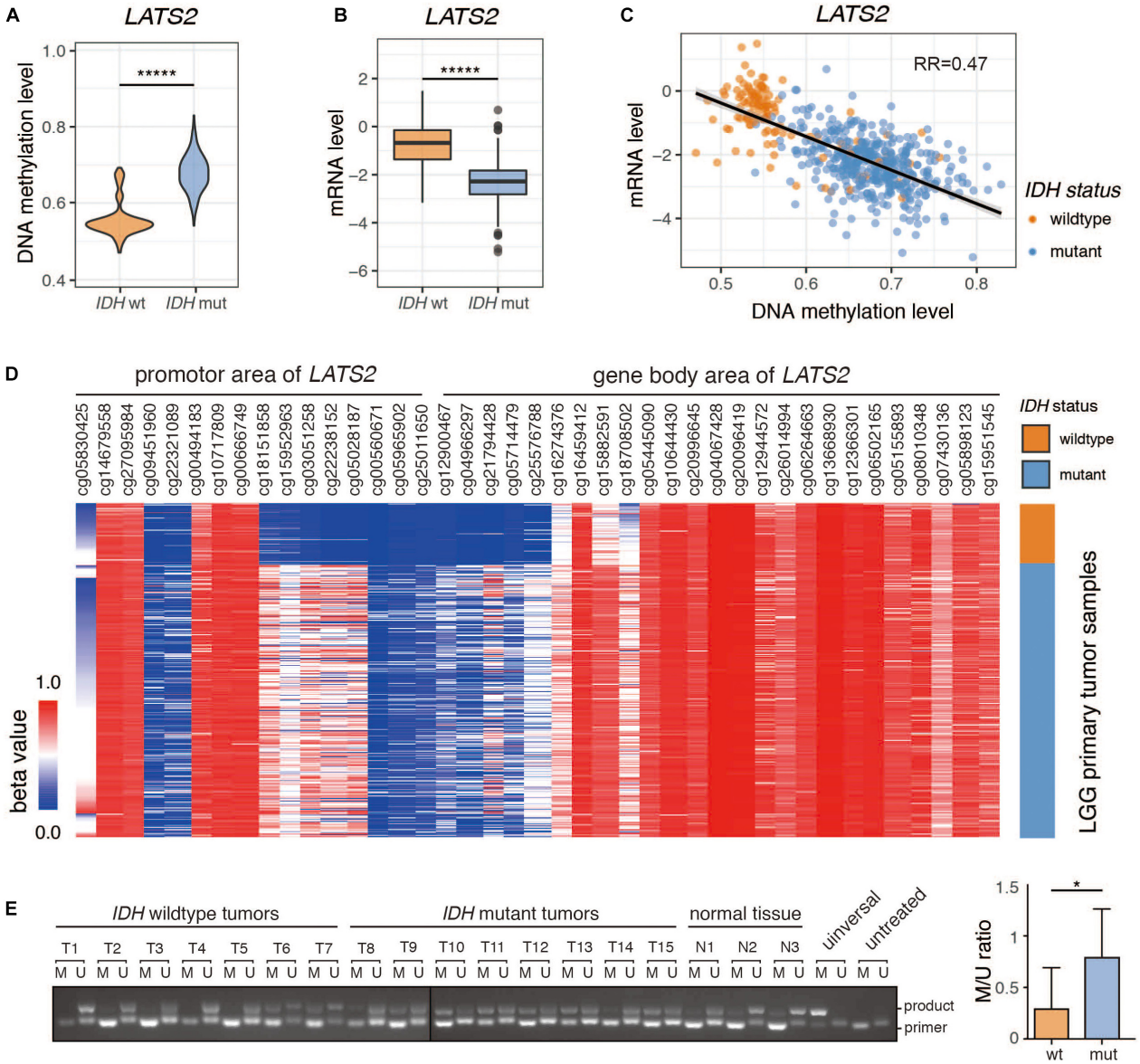

FIGURE 1 | Promoter hypermethylation and low expression of *LATS2* in *IDH*-mutant LGG. **(A)** *LATS2* methylation level is increased in *IDH*-mutant LGG. **(B)** *LATS2* mRNA level is decreased in *IDH*-mutant LGG. **(C)** Correlation between *LATS2* methylation level *and LATS2* mRNA level. RR indicates R squared value of linear regression. **(D)** Methylation level of different CpG islands in *LATS2* promoter and gene body area. **(E)** Methylation-specific PCR of LGG samples. M: methylation-specific primer; U: unmethylation-specific primers; universal: universally methylated genomic DNA control; untreated: untreated U87 cell genomic DNA; product: ~130 bp PCR products; primer: primer dimers. Quantitative result on the right. M/U ratio was calculated by comparing the bands from methylation-specific and unmethylation-specific primers of each sample. Mean and standard error were presented (*$p < 0.05$, *****$p < 0.000005$, *t* test).

YAP in LGG compared to GBM (**Figures 4E,F**), although it could not explain why YAP protein expression showed no significant difference between *IDH*-wild type and *IDH*-mutant LGG samples. Hence, it is possible that a posttranslational mechanism may account for low YAP protein levels in LGG. Intriguingly, we found that BTRC, an E3 ligase responsible for YAP degradation (Zhao et al., 2010), was dramatically upregulated in LGG compared to GBM (**Figure 4G**). On the other hand, several reported deubiquitinases for YAP (Li et al., 2018; Sun et al., 2019; Zhang et al., 2019; Pan et al., 2020; Zhu et al., 2020) were also upregulated (**Supplementary Figure 4**). Thus, further work is needed to dissect the mechanism(s) for the loss of YAP protein expression in LGG.

Dysregulated Expression of Multiple Hippo Pathway Genes in *IDH*-Mutant LGG

Since *LATS2*, *YAP*, and several Hippo pathway target genes were highly methylated in IDH-mutant LGG, we analyzed methylation

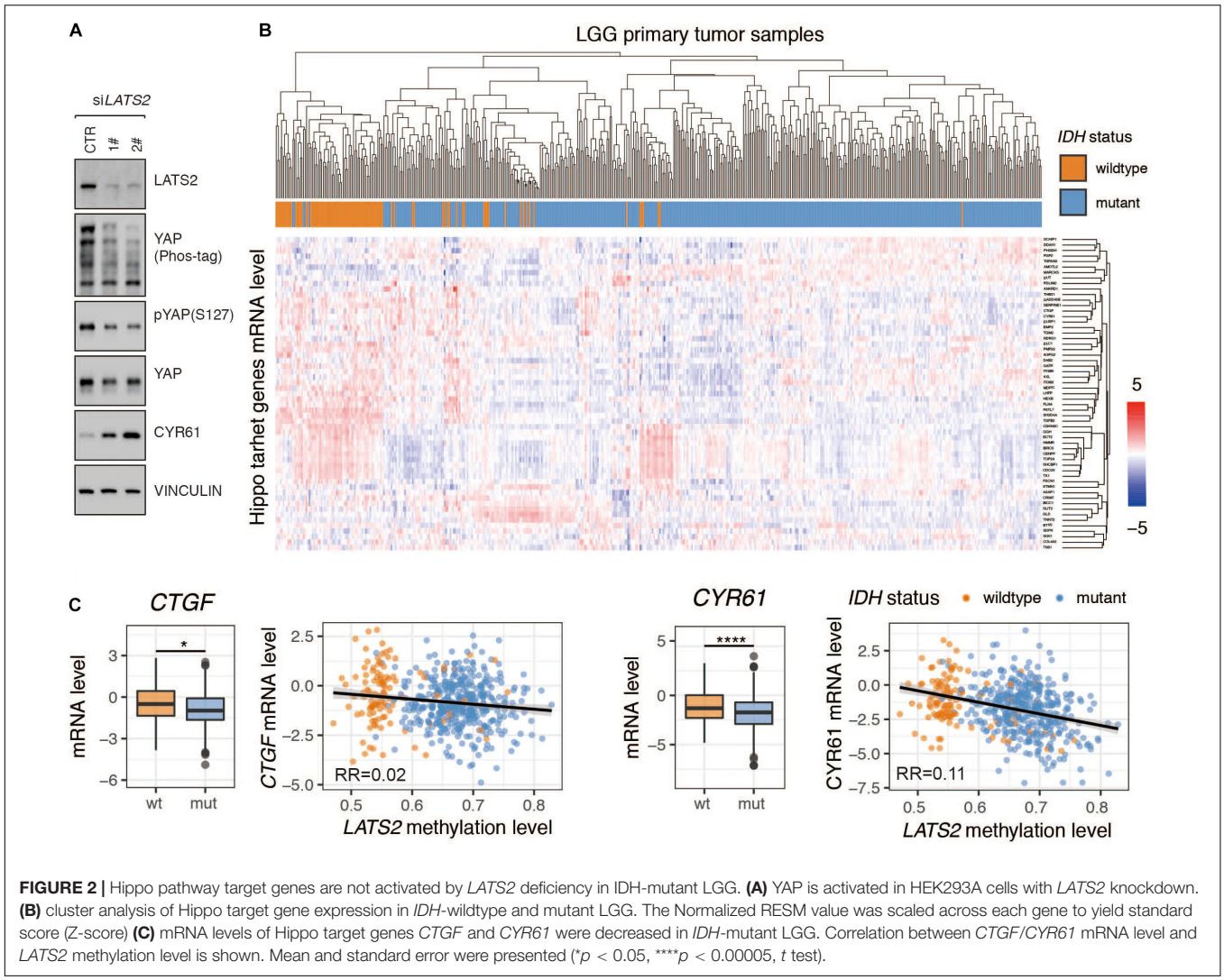

FIGURE 2 | Hippo pathway target genes are not activated by *LATS2* deficiency in IDH-mutant LGG. **(A)** YAP is activated in HEK293A cells with *LATS2* knockdown. **(B)** cluster analysis of Hippo target gene expression in *IDH*-wildtype and mutant LGG. The Normalized RESM value was scaled across each gene to yield standard score (Z-score) **(C)** mRNA levels of Hippo target genes *CTGF* and *CYR61* were decreased in *IDH*-mutant LGG. Correlation between *CTGF*/*CYR61* mRNA level and *LATS2* methylation level is shown. Mean and standard error were presented (*$p < 0.05$, ****$p < 0.00005$, *t* test).

and gene expression of known Hippo pathway components in LGG. We found that many of them are dysregulated in IDH-mutant LGG (**Supplementary Figures 5–8**, summarized in **Supplementary Figure 9**).

TEA-domain family proteins (TEAD1-4) are the major transcription factors that mediate functions of YAP/TAZ (Ota and Sasaki, 2008; Chen et al., 2010; Lamar et al., 2012; Lin et al., 2017; Holden and Cunningham, 2018). Notably, *TEAD2-4* were significantly downregulated in IDH-mutant LGG, while *TEAD1* showed a mild upregulation (**Figure 5A**). Low expression of TEAD genes was correlated with *LATS2* hypermethylation (**Supplementary Figure 5**).

Further, we observed that the expression of known upstream regulators of LATS1/2 were modulated in IDH-mutant LGG samples. For instance, the mRNA levels of Zyxin (ZYX), an inhibitor of LATS2 (Ma et al., 2016), were low in IDH-mutant LGG samples (**Figure 5B**). On the other hand, the expression of angiomotin like 2 (AMOTL2), an activator of LATS2 (Mana-Capelli and McCollum, 2018), was elevated in IDH-mutant LGG samples (**Figure 5C**). These changes may also play a role in restricting YAP/TAZ activity in IDH-mutant LGG by inducing activity of residual LATS1/2 (**Figure 5D**).

LATS2 Hypermethylation Is a Favorable Prognostic Factor in Overall LGG but Not in *IDH*-Wild-Type or Mutant Subgroups

Our results thus far indicated that the hypermethylation of *LATS2* in *IDH*-mutant LGG failed to activate YAP/TAZ activity. Next, we interrogated whether hypermethylation of *LATS2* could serve as a biomarker with clinical significance. In analyzing survival data of LGG patients, we found that *LATS2* hypermethylation is a strong favorable prognostic factor in LGG (**Figure 6A**). However, when we performed analysis separately in *IDH*-mutant patients, *LATS2* hypermethylation showed no prognostic significance in *IDH*-mutant subgroups (**Figure 6C**). In comparing the clinical features between high and low *LATS2* methylation groups, we uncovered several characteristics that varied between these two groups including *IDH1/2* status (**Supplementary Table 2**). As *IDH* mutation was a favorable prognostic factor of LGG

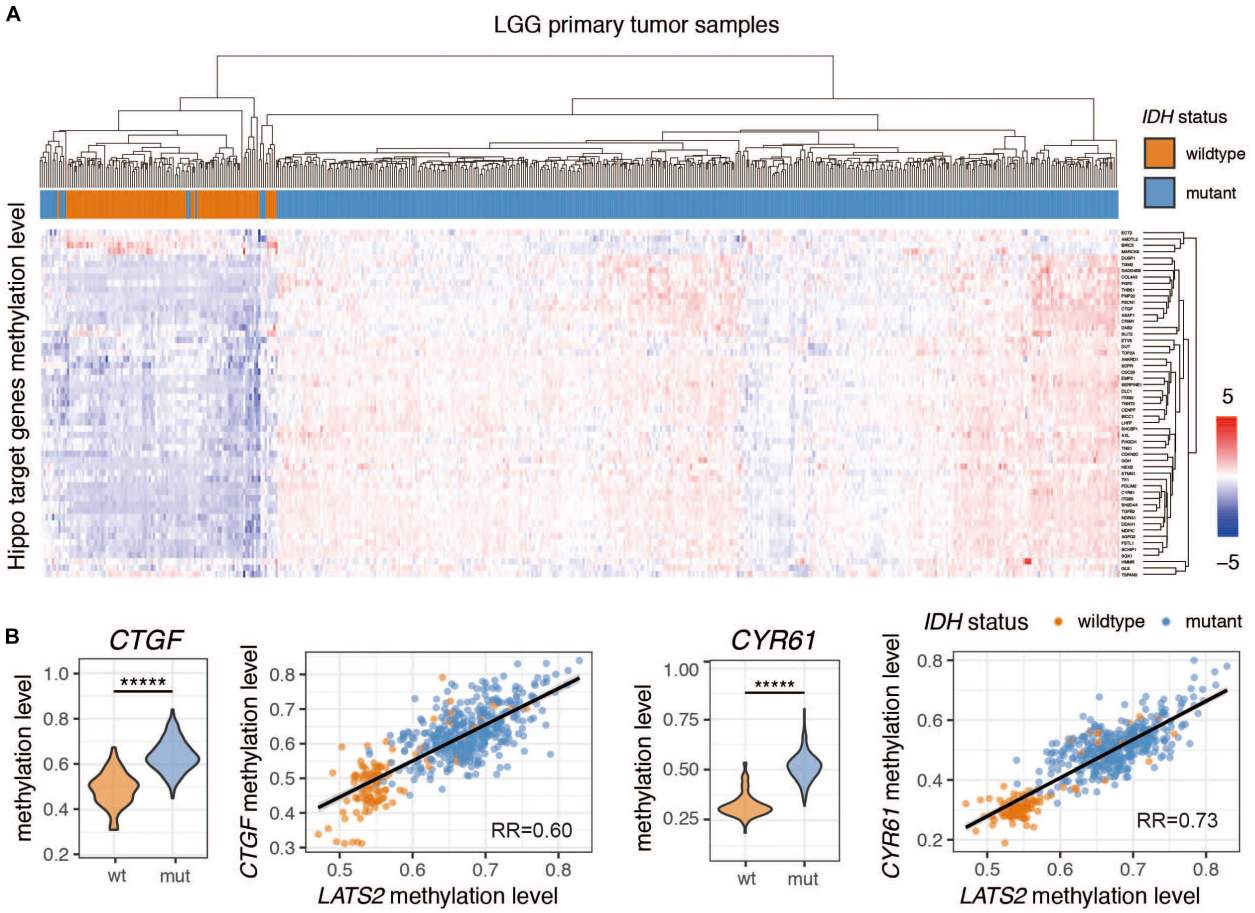

FIGURE 3 | Hippo pathway target genes are universally hypermethylated in *IDH*-mutant LGG. **(A)** cluster analysis of Hippo target gene methylation levels in *IDH*-wildtype and mutant LGG. The Normalized beta value is scaled across each gene to yield standard score (Z-score) **(B)** Methylation levels of Hippo target genes *CTGF* and *CYR61* are significantly increased in *IDH*-mutant LGG. Correlation between *CTGF*/*CYR61* methylation level and *LATS2* methylation level is shown. Compared by *t* test (*****$p < 0.000005$, *t* test).

(Vuong et al., 2019; **Figure 6B**), the prognostic significance of *LATS2* hypermethylation was likely due to its enrichment in *IDH*-mutant samples.

To further explore the prognostic value of *LATS2* methylation, we did Cox proportional hazard analysis of *LATS2* methylation (**Table 1**). We found *LATS2* methylation is a prognostic factor in overall LGG after adjusted for a series of covariates, but lost its prognostic value in either *IDH*-wildtype or mutant LGG subgroups, indicating its prognostic significance comes from correlation with *IDH* mutation instead of direct impact on Hippo pathway effectors.

LATS2 Hypermethylation Predicts IDH Mutation in LGG

Lastly, we determined whether *LATS2* methylation level may work as a biomarker of *IDH* status. Using CpG island cg03051258 in the promoter area of *LATS2* as an example, we applied beta value 0.1 as a threshold to identify *LATS2* hyper- and hypo-methylated samples, and IDH-mutant LGG was successfully enriched in the *LATS2* hypermethylated group. Using this approach, we could predict *IDH* mutation in LGG at the sensitivity of 0.95 and specificity of 0.97 (**Table 2**). Together, these data support that *LATS2* hypermethylation is a faithful biomarker of *IDH* mutations.

DISCUSSION

The Hippo pathway is known to play critical roles in cancer development, making this signaling network an area of high clinical interest. The TCGA Network project revealed that *LATS2* is commonly hypermethylated in *IDH*-mutant low-grade gliomas, prompting us to explore its role in LGG. Several groups have previously explored a role of LATS2 in gliomas (Guo et al., 2019; Shi et al., 2019). However, a systematic analysis to evaluate its effect on Hippo pathway in *IDH*-mutant LGG had not been carried out.

Our study found that *LATS2* promoter was hypermethylated while *LATS2* mRNA was repressed in *IDH*-mutant LGG samples. Unexpectedly, *LATS2* repression failed to activate

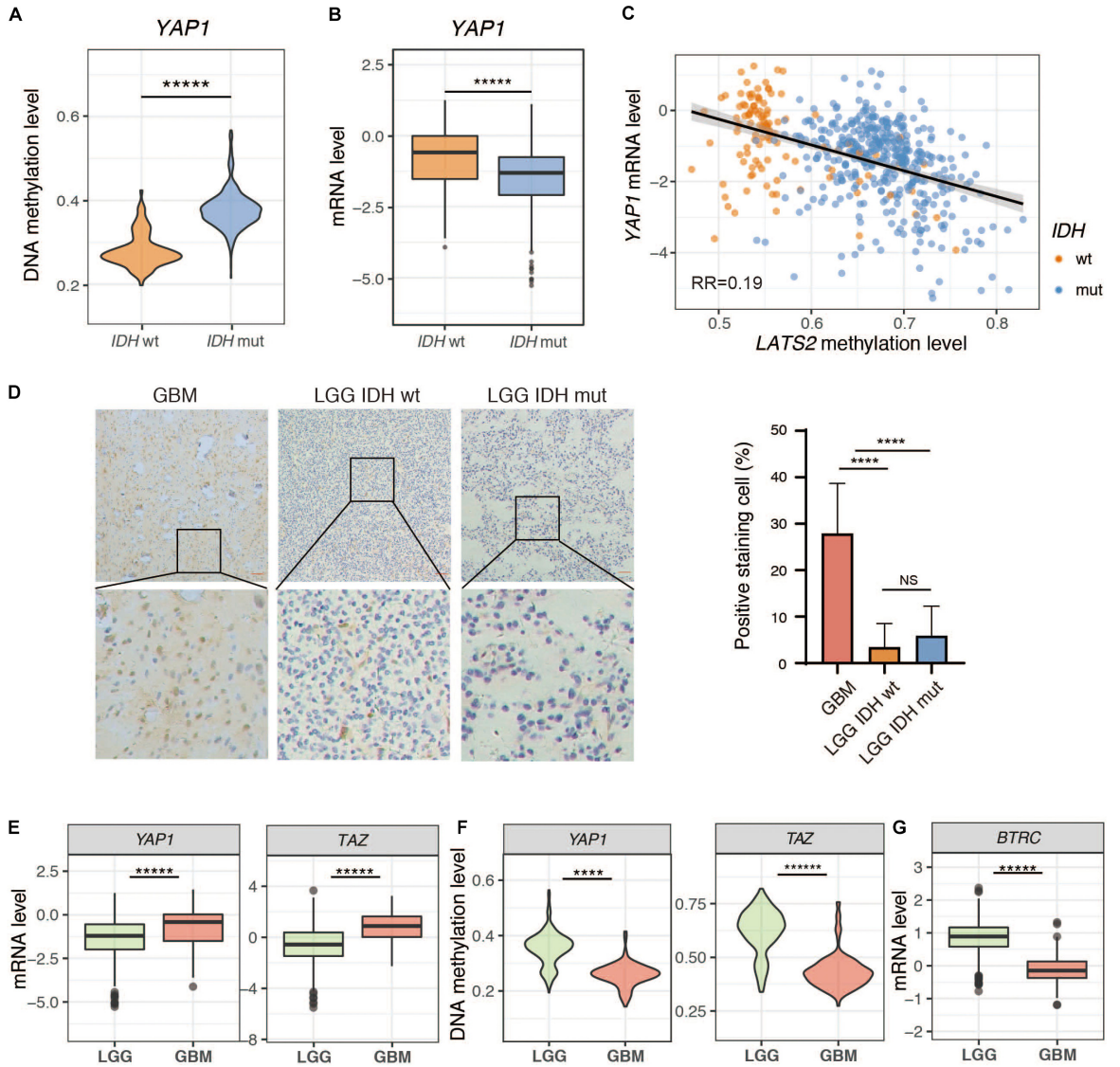

FIGURE 4 | Low expression of *YAP* in LGG. **(A)** *YAP* methylation is elevated in *IDH*-mutant LGG. **(B)** *YAP* mRNA level is reduced in *IDH*-mutant LGG. **(C)** Correlation between *YAP* mRNA level and *LATS2* methylation level. **(D)** IHC result of *IDH*-wildtype or mutant LGG samples and GBM samples. Left: Representative image; Right: Quantitative result. **(E)** *YAP* and *TAZ* mRNA levels in LGG and GBM. **(F)** *YAP* and *TAZ* methylation levels in LGG and GBM. **(G)** *BTRC* mRNA level in LGG and GBM. Mean and standard error were presented (****$p < 0.00005$, *****$p < 0.000005$, *t* test).

Hippo pathway target genes, as most of these genes were also hypermethylated in *IDH*-mutant LGG samples. The universal epigenetic changes caused by *IDH1/2* mutation could be a key point to understand this phenomenon. Oncometabolite *R-2HG* produced by mutated IDH1/2 inhibits the activity of αKG-dependent enzymes including DNA and histone demethylases (Yamane et al., 2006; Chowdhury et al., 2011; Ito et al., 2011; Xu et al., 2011; Turcan et al., 2012; Kohli and Zhang, 2013). In this way, *IDH1/2* mutation may cause genome-wide alterations in DNA methylation, including *LATS2*, Hippo pathway target genes, and additional Hippo pathway component genes.

It is interesting that YAP expression is high in GBM but is extremely low in all LGG regardless of *IDH* status. This could be a reflection of differentiation status and aggressiveness of tumors, because YAP is frequently activated in less differentiated and malignant cancers (Xu et al., 2009; Fullenkamp et al., 2016; Zanconato et al., 2016). Compared to GBM, LGG is usually well-differentiated and less malignant, and YAP may remain less active in LGG. Moreover, YAP is important in maintaining stemness of progenitor cells (Lian et al., 2010; Beyer et al., 2013; Li et al., 2013). Hence, the difference in YAP activity between GBM and LGG might be inherited from the status of respective cancer progenitor cells.

Along with low YAP expression, additional mechanisms may contribute to the lack of YAP activation in *IDH*-mutant LGG. For instance, the dysregulated expression of *ZYX* and *AMOTL2* may

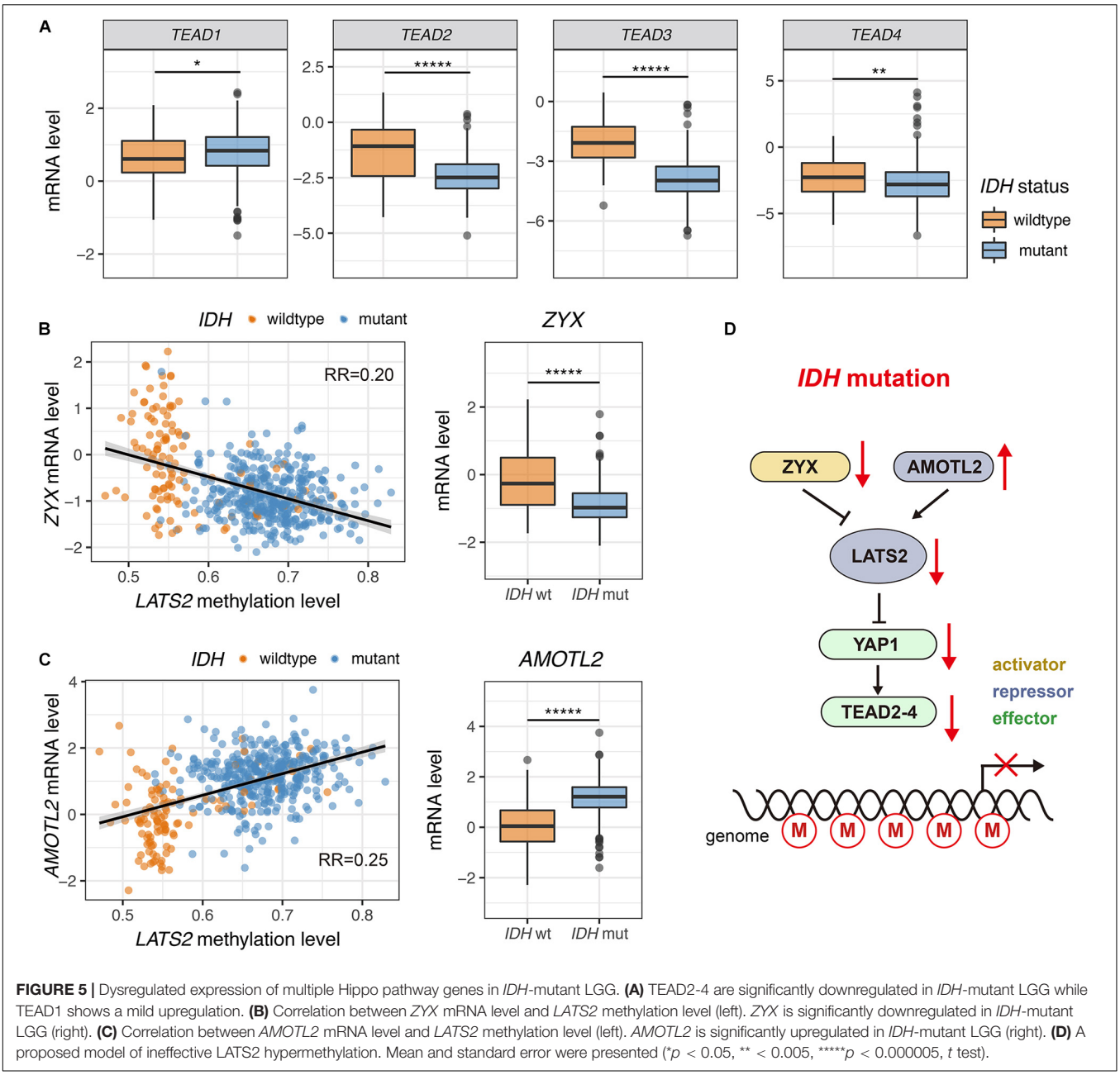

FIGURE 5 | Dysregulated expression of multiple Hippo pathway genes in *IDH*-mutant LGG. **(A)** TEAD2-4 are significantly downregulated in *IDH*-mutant LGG while TEAD1 shows a mild upregulation. **(B)** Correlation between *ZYX* mRNA level and *LATS2* methylation level (left). *ZYX* is significantly downregulated in *IDH*-mutant LGG (right). **(C)** Correlation between *AMOTL2* mRNA level and *LATS2* methylation level (left). *AMOTL2* is significantly upregulated in *IDH*-mutant LGG (right). **(D)** A proposed model of ineffective LATS2 hypermethylation. Mean and standard error were presented (*$p < 0.05$, ** < 0.005, *****$p < 0.000005$, *t* test).

inhibit LATS1/2 activity, while reduction of *TEAD2-4* expression may limit the transcriptional output of YAP.

Although *LATS2* hypermethylation was unable to activate YAP in *IDH*-mutant LGG, it displayed a strong correlation with *IDH1/2* mutation and could serve as a favorable prognostic factor for LGG patients. In addition, *LATS2* hypermethylation was a faithful biomarker of *IDH* mutations, and could potentially be used as an alternative for IDH mutation in diagnosis.

In conclusion, our study found *LATS2* promoter hypermethylation in *IDH*-mutant LGG samples which, surprisingly, did not translate into YAP activation, raising the role and involvement, if at all, of the Hippo pathway in the development of LGG. Meanwhile, *LATS2* hypermethylation showed a strong correlation with *IDH* mutation. Hence, *LATS2* hypermethylation can serve as an alternative for IDH mutation in diagnosis and a favorable prognostic factor for LGG patients.

MATERIALS AND METHODS

Data Collection and Processing

TCGA-LGG, TCGA-GBM, TCGA-AML RNA sequence level 3 normalized data, DNA Methylation Level 3 data, clinical data and somatic mutation data were downloaded from GDC Data Portal using package TCGAbiolinks in R (version 3.6.2) environment for further analysis (Colaprico et al., 2016). *IDH*-mutant samples

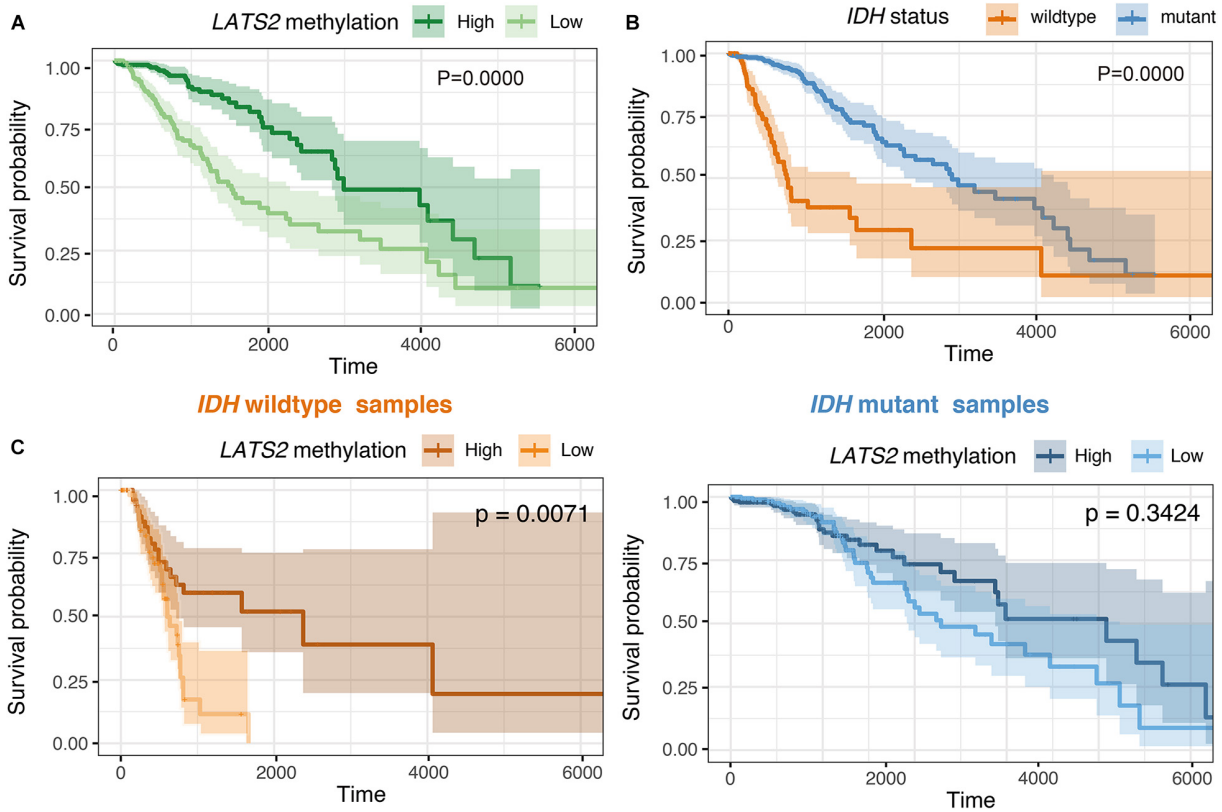

FIGURE 6 | *LATS2* hypermethylation is a favorable prognostic factor in overall LGG. **(A)** High *LATS2* methylation is a favorable prognostic factor in overall LGG. *P* value as indicated. **(B)** *IDH* mutation is a favorable prognostic factor of overall LGG; **(C)** High *LATS2* methylation level is a favorable prognostic factor in *IDH*-wildtype LGG but not in *IDH*-mutant LGG.

were composed of samples with IDH1 Arg132 or IDH2 Arg140 and 172 mutations. The level 3 expression data were normalized RMSE value. For each gene, we zero-centered expression data by calculating standard score (Z-score) between each individual sample. Comparison of gene expression between different tumor types were based on pan-cancer normalized expression data from UCSC Xena team (Goldman et al., 2020). Pre-process steps to yield level 3 methylation data (β-value) included background correction, dye-bias normalization. β-values ranged from zero to one, with zero indicating no methylation detected.

Statistical Analysis

The expression and methylation of Hippo-related genes were compared by *t* test or Mann-Whitney U test. The correlation between expression and methylation status was evaluated by fitting linear models. Survival data were analyzed by Kaplan-Meier analysis and Cox proportional hazard analysis. The Hippo pathway target genes (**Supplementary Table 1**). were determined according to RNAseq results of our Hippo element knock-out cell lines (data not shown). Hierarchical Clustering of each sample was done by calculating Euclidean distance matrix followed Pearson correlation analysis based on Hippo target features. All the analysis and image drawing (R package ggplot2) was done by R (version 3.6.2).

Patients Samples

This study enrolled patients with GBM (n = 8) and LGG (n = 12, including 5 *IDH* wildtype and 7 *IDH* mutant). Glioma frozen tissues and paraffin slides were obtained from Huashan Hospital, Fudan University, Shanghai, China. This study was approved by the Ethics Committee of the Huashan Hospital of Fudan University and informed consents were obtained from all participants. Specimens used for Methylation-specific PCR were taken at the time of surgical resection, snap−frozen in liquid nitrogen and stored at −80°C until use. Formalin-fixed paraffin-embedded (FFPE) tissues were used for immunohistochemical (IHC) staining.

Cell Culture and Gene Knock-Down by siRNA

HEK293A cells were cultured in DMEM (Corning) containing 5% FBS (Gibco) and 50 μg/mL penicillin/streptomycin (P/S). All cells were incubated at 37°C under 5% CO2. siRNAs were purchased from GenePharma and transfected into cells using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. The following siRNAs were used: si*LATS2* 1#: UACCAUAAAUACAAUCUUCTT (5′-3′), si*LATS2* 2#: CCGCAAAGGGTACACTCAATT (5′-3′). HEK293A cells

TABLE 1 | Cox proportional hazard model of *LAST2* methylation and expression in LGG patients.

	Overall		*IDH* wildtype		*IDH* mutant	
Variable	**Univariable HR (95%CI)**	**Adjusted* HR (95%CI)**	**Univariable HR (95%CI)**	**Adjusted* HR (95%CI)**	**Univariable HR (95%CI)**	**Adjusted* HR (95%CI)**
LATS2 methylation	**1.64e-05 (1.06e-6, 2.53e-4)**	**3.63e-04 (2.28e-5, 5.78e-3)**	**1.32e-05 (1.09e-8, 1.60e-2)**	2.72e-02 (2.39e-6, 309.82)	0.37 (1.99e-3, 68.60)	6.69e-03 (2.64e-5, 1.70)
IDH status wildtype	**4.80 (3.34, 6.90)**	**3.36 (2.18, 5.18)**				

**Adjusted for age, gender, neoplasm histologic grade, histological type, and tumor location. HR with p value < 0.05 is marked by bold formatting, CI denotes confidence interval.*

TABLE 2 | Methylation level of cg03051258 to predict *IDH* status.

cg03051258	***IDH* mutant**	***IDH* wildtype**	**Total**
beta value > 0.1	393	3	396
beta value < 0.1	19	88	107
Total	412	91	503

were seeded into 6-well plates and transfected the next day. 60 h later, cells were harvested for immunoblotting.

Immunoblotting

Cells were lysed in 1 × SDS loading buffer containing 50 mM Tris pH 6.8, 2% SDS, 0.025% bromophenol blue, 10% glycerol, and 5% BME. The concentration of total proteins was assayed by BCA method. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes, blocked with 5% non-fat milk in TBST for 1 h at room temperature. The membranes were washed with TBST three times for 5 min and then incubated with primary antibodies (4°C overnight) and HRP-conjugated secondary antibodies. ECL solution and image acquisition equipment (5200S Imager) were from Tanon Science & Technology Co., Ltd. The following primary antibodies were used: anti-LATS2 (CST, 1:1000, 5888S), anti-YAP (CST, 1:1000, 14074S), anti-pYAP (S127) (CST, 1:1000, 4911S), anti-CYR61 (Santa, 1:1000, sc-13011), and anti-vinculin (CST, 1:1000, 13901s).

Immunohistochemistry

Paraffin embedded tissue specimens were sectioned, dewaxed, and rehydrated. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) at 95–100°C for 20 min. Endogenous peroxidase activity was blocked by 3% H_2O_2 for 30 min. Sections were then blocked in 5% BSA for 1 h and incubated with primary antibodies overnight. After extensive washing, the sections were incubated with secondary antibodies at room temperature for 1 h. DAB solution was applied and hematoxylin was used for counterstaining. Anti-YAP (CST, 1:200) was used as a primary antibody. Staining results were visualized with Zeiss Axiocam 208 color. Quantification was conducted to measure the protein expression.

DNA Isolation and Bisulfite Conversion

Genomic DNA of glioma was isolated from frozen LGG tissues using DNA/RNA/protein Extraction Kit (DP423) (Tiangen, Beijing, China) according to the manufacturer's protocol. EZ DNA Methylation-Startup Kit (Zymo Research) was utilized to perform sodium bisulfite modification of DNA following the manufacturer's instructions. This converts cytosine residues to uracil in single-stranded DNA while leaving methylated cytosine unchanged.

Methylation-Specific PCR

The methylation status of *LATS2* was tested by Methylation-specific PCR utilizing both methylated and unmethylated specific sets of primers: 5′-GTT GGA GTT GTT GTT GGT TTC-3′ (forward) and 5′-CGA ATA TCC CAC TTA AAT CTA CG-3′(reverse) for methylated reaction (PCR products, 131 bp) and 5′-GTT GGA GTT GTT GTT GGT TTT G-3′ (forward) and 5′-AAA TAT CCC ACT TAA ATC TAC ACT-3′ (reverse) for unmethylated reaction (PCR product, 130 bp). PCR amplification was carried out on T-100 Thermal Cycler (Bio-Rad) using Taq DNA polymerase (Vazyme Biotech Co., Ltd., China) in a total volume of 10 μL. 5% DMSO was added to enhance the specificity and yield of PCR reactions. DNA samples were initial denatured at 95°C for 5 min, and was followed by 36 cycles of denaturing at 95°C for 30 s, annealing at 54°C (for methylated reaction) or 59°C (for unmethylated reaction) for 30 s, and extension at 72°C for 45 s. A final extension step at 72°C for 5 min was added for all reactions. Both positive and negative controls were included. Polymerase chain reaction products were subsequently electrophoresed on 2% agarose gels and visualized with image equipment from Tanon Science & Technology Co., Ltd.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://portal.gdc.cancer.gov.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Huashan Hospital of Fudan

University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YG and F-XY designed the study and wrote the manuscript. YG, YuW, YeW, JL, XW, MM, WH, and YL performed experiments and data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.586581/full#supplementary-material

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REVIEW
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Hippo-Independent Regulation of Yki/Yap/Taz: A Non-canonical View

*Yong Suk Cho[1] and Jin Jiang[1,2]**

[1] Department of Molecular Biology, UT Southwestern Medical Center, Dallas, TX, United States, [2] Department of Pharmacology, UT Southwestern Medical Center, Dallas, TX, United States

Initially identified in *Drosophila*, the Hippo signaling pathway has emerged as an evolutionarily conserved tumor suppressor pathway that controls tissue growth and organ size by simultaneously inhibiting cell proliferation and promoting cell death. Deregulation of Hippo pathway activity has been implicated in a wide range of human cancers. The core Hippo pathway consists of a kinase cascade: an upstream kinase Hippo (Hpo)/MST1/2 phosphorylates and activates a downstream kinase Warts (Wts)/Lats1/2, leading to phosphorylation and inactivation of a transcriptional coactivator Yki/YAP/Taz. Many upstream signals, including cell adhesion, polarity, mechanical stress, and soluble factors, regulate Hippo signaling through the kinase cascade, leading to change in the cytoplasmic/nuclear localization of Yki/YAP/Taz. However, recent studies have uncovered other mechanisms that regulate Yki/YAP/Taz subcellular localization, stability, and activity independent of the Hpo kinase cascade. These mechanisms provide additional layers of pathway regulation, nodes for pathway crosstalk, and opportunities for pathway intervention in cancer treatment and regenerative medicine.

Keywords: Hippo, Yap, Taz, CDK7, PRP4K, DCAF12, phosphorylation, ubiquitination

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***Correspondence:**
Jin Jiang
jin.jiang@utsouthwestern.edu

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INTRODUCTION

The regulation of cell growth, proliferation, and cell death is tightly controlled during embryonic development and adult tissue homeostasis not only by environmental cues such as morphogens, cytokines, hormonal signals, and nutrients but also by cell-intrinsic mechanisms. The Hippo signaling pathway, which was initially identified in *Drosophila*, has emerged as an evolutionarily conserved tumor suppressor pathway that regulates tissue growth and organ size in a wide range of species ranging from insects to humans (Pan, 2007; Zhang et al., 2009; Halder and Johnson, 2011). Deregulation of Hippo pathway activity has been implicated in many types of human cancer and other diseases (Yu et al., 2015; Zanconato et al., 2016; Zheng and Pan, 2019). Due to its critical role in developmental biology and human health, the Hippo pathway has been extensively studied over the past decade or so. Through genetic screen in *Drosophila* and RNAi screen in mammalian cells as well as proteomic and bioinformatic approaches, numerous pathway components have been identified that link the Hippo signaling to many upstream regulators and other signaling pathways. The rapid progress in the Hippo signaling field is also reflected by the numerous reviews on this topic. In this review, we focus on recent studies that reveal mechanisms that act in parallel to or

downstream of the core Hippo signaling pathway to modulate pathway outputs. We discuss how these findings inform us about new strategies for cancer treatment and regenerative medicine.

OVERVIEW OF THE CANONICAL HIPPO SIGNALING PATHWAY

The core Hippo signaling pathway (**Figure 1**) contains a kinase cassette: an upstream Ste20 family kinase Hippo (Hpo)/MST1/2, which exists in a complex with Sav/SAV1, phosphorylates and activates a downstream kinase Warts (Wts)/Lats1/2 that forms a complex with Mats/Mob (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003; Lai et al., 2005). Activated Wts/Lats1/2 in turn phosphorylates the Hippo pathway effector Yorkie (Yki) in *Drosophila* and Yes-associated protein (Yap)/Transcriptional activator with PDZ-binding motif (Taz) in mammals, resulting in its cytoplasmic retention by binding to the 14-3-3 protein (Huang et al., 2005; Dong et al., 2007; Zhao et al., 2007; Oh and Irvine, 2008; Zhang et al., 2008; Ren et al., 2010). When Wts/Lats1/2-mediated phosphorylation is compromised, Yki/Yap/Taz translocates into the nucleus where it binds to the Hippo pathway transcription factors Scalloped (Sd)/TEAD1-4 to regulate genes involved in the control of cell growth, proliferation, survival, and metabolism (Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008; Koo and Guan, 2018; Moya and Halder, 2018; Totaro et al., 2018). In the absence of nuclear Yki/Yap/Taz, Sd/TEAD binds Tgl/VGLL4 and functions as a default pathway inhibitor (Koontz et al., 2013; Jiao et al., 2014).

In several contexts, MAP4K family members Misshapen (Msn)/Happy hour(Hppy)/MAP4Ks act partial-redundantly with Hpo/MST12 to regulate Wts/Lats1/2 (Li et al., 2014, 2015; Meng et al., 2015; Zheng et al., 2015). Interestingly, in *Drosophila* adult intestine, Msn substitutes the function of Hpo and acts as a major upstream kinase for Wts in enteroblasts to regulate stem cell proliferation (Li et al., 2014). Hpo/MST1/2 and Msn are phosphorylated and activated by a conserved kinase Tao-1/TAOK that is recruited to Ex by Schip1 (Boggiano et al., 2011; Poon et al., 2011; Chung et al., 2016). A recent study revealed that enteroblasts in *Drosophila* adult intestine sense the mechanical force generated by food congestion to modulate the Hippo signaling and stem cell activity by regulating the membrane association of Msn and its phosphorylation by Tao-1 (Jiang, 2018; Li et al., 2018). Hpo/MST12 and MAP4Ks are negatively regulated by a large protein complex called STRIPAK that brings PP2A to dephosphorylate and inhibit these kinases (Bae et al., 2017; Zheng et al., 2017; Kim J.W. et al., 2020; Seo et al., 2020; Tang et al., 2020). A recent study suggests that the STRIPAK complex integrates multiple upstream signals to regulate Hippo signaling pathway (Chen et al., 2019).

Genetic studies in *Drosophila* have identified many upstream components that appear to play conserved roles in the Hippo signaling pathway, including the atypical protocadherin family members Fat/Fat1-4 and its binding partner Dachsous (Ds), the FERM domain-containing proteins Expanded (Ex)/Merlin (Mer)/NF2, Kibra (Kbr)/KIBRA, and Spectrin (**Figure 1**; Fulford et al., 2018; Misra and Irvine, 2018). A genetic modifier screen also identified a cell adhesion molecule called echinoid (Ed) that plays a unique role in *Drosophila* Hippo pathway by recruiting Hpo/Sav to the adherens junction (Yue et al., 2012). The Hippo pathway is also regulated by apical basal polarity complexes, cell junctions including adherens junction, tight junction (mammalian Hippo pathway only), mechanical signals, and soluble factors that activate GPCR pathway (**Figure 1**; Yu et al., 2015; Fulford et al., 2018). In most of the cases, these upstream regulators act either directly or indirectly to modulate the activity of the Hippo kinase cascade, leading to altered cytoplasmic/nuclear partitioning of Yki/Yap/Taz.

HPO KINASE CASCADE-INDEPENDENT REGULATION OF YKI/YAP/TAZ

Although most upstream signals regulate Hippo signaling through modulating the Hpo kinase cascade-mediated phosphorylation of Yki/Yap/Taz, mechanisms that regulate Yki/Yap/Taz activity independent of the core Hippo pathway do exist. For example, an early study indicated that direct interaction between Yki and Ex can sequester Yki in the cytoplasm in *Drosophila* (Badouel et al., 2009). Likewise, direct interaction of Yap with Angiomotin (Amot) also traps Yap in the cytoplasm of mammalian cells (Zhao et al., 2011). Furthermore, Tyr phosphorylation of Yap by Src family kinases can regulate Yap nuclear localization, stability, and activity (Rosenbluh et al., 2012; Taniguchi et al., 2015; Li et al., 2016). In this review, we focus on recent studies that uncover additional mechanisms that control Yki/Yap/Taz activity independent of the Hpo kinase cascade.

REGULATION OF YKI/YAP/TAZ BY OTHER CYTOPLASMIC SER/THR KINASES

Although Wts/Lats1/2-mediated phosphorylation of Yki/Yap/Taz provides a major mechanism that regulates the Hippo pathway effectors, Yki/Yap/Taz can also be regulated by other Ser/Thr kinases (**Figure 2**). For example, Nuclear Dbf2-related kinases, NDR1 and NDR2, which are structurally related to Lats1/2, can phosphorylate Yap on the same set of sites as Lats1/2 in the intestine epithelium (Zhang L. et al., 2015). MST4, which is closely related to MST1/2, binds and phosphorylates Yap at Thr83 to inhibit Yap nuclear import and activity independent of the canonical Hippo pathway (An et al., 2020). Deletion of MST4 in mice diminished Yap Thr83 phosphorylation, increased Yap activity, and promoted gastric tumorigenesis (An et al., 2020). Furthermore, loss of MST4 and YapThr83 phosphorylation is associated with poor prognosis of human gastric cancer (An et al., 2020). In response to inflammatory cytokine, TAK1 binds and phosphorylates Yap/Taz independent of Lats1/2 to promote Yap/Taz degradation, which alleviates the inhibition of NFκB, leading to the induction of matrix-degrading enzymes and subsequently cartilage degradation during osteoarthritis pathogenesis (Deng et al., 2018). On the other hand, binding of MK5 (also called MAPKAPK5 or PRAK) to Yap stabilizes Yap

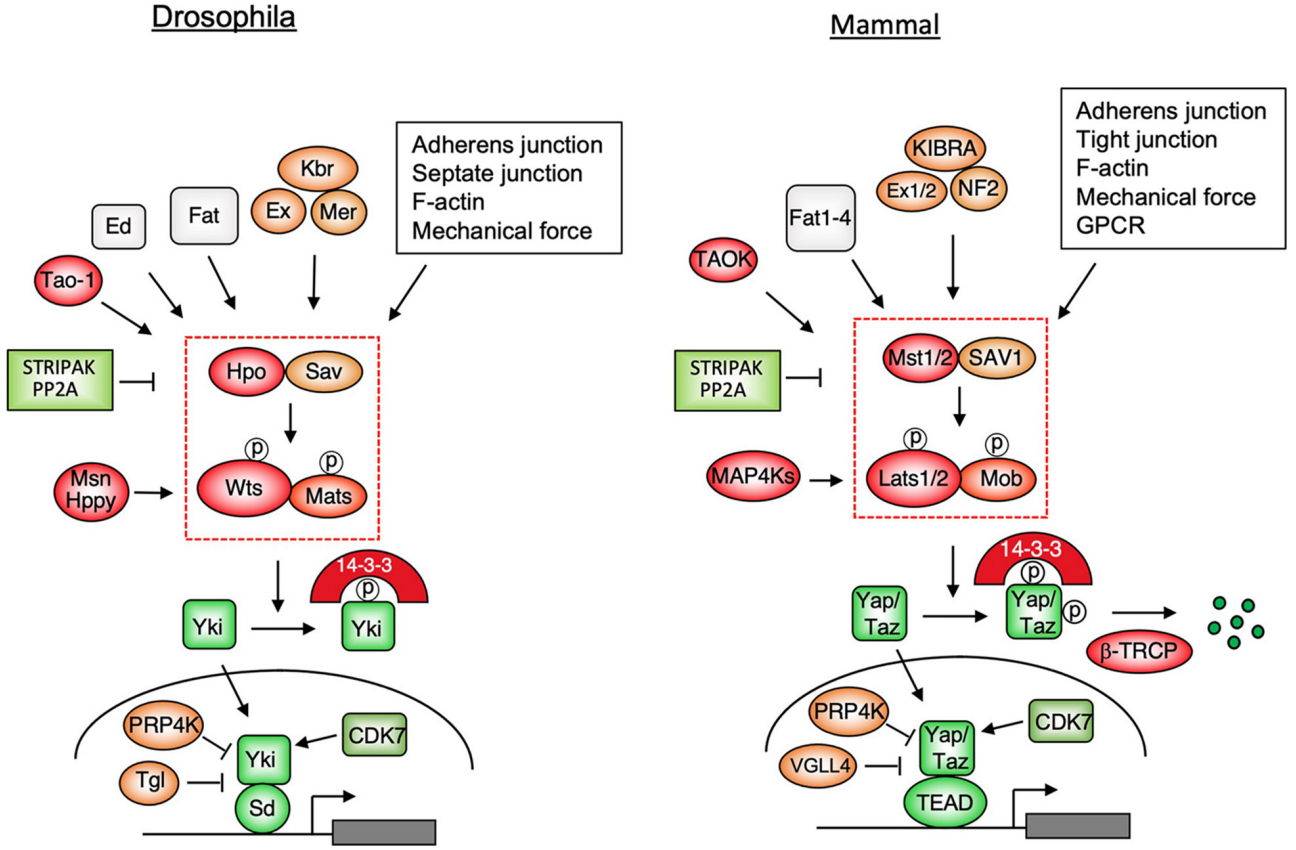

FIGURE 1 | Hippo signaling pathways in *Drosophila* and mammals. Multiple upstream signals act through the Hpo/MST1/2-Wts/Lats1/2 kinase cascade to control the subcellular localization and stability (for mammalian pathway only) of the pathway effectors Yki/Yap/Taz. When phosphorylation of Yki/Yap/Taz by the Hpo kinase cascade is compromised, these pathway effectors enter the nucleus and binds the transcription factors Sd/TEAD1-4 to regulate genes involved in cell growth, proliferation, survival, plasticity, and metabolism.

independent of Lats1/2, which is required for Yap-driven cancer progression (Seo et al., 2019). Two other studies revealed that in response to cellular energy starvation, AMPK can directly phosphorylate Yap on multiple sites including S61 and S94 to inhibit Yap activity at least in part by interfering with Yap–TEAD interaction (Mo et al., 2015; Wang et al., 2015). In addition, AMPK can also indirectly inhibit Yap by activating Lats (Mo et al., 2015). The regulation of Yap by AMPK appears to be evolutionarily conserved as AMPK, and its upstream kinase LKB1 restricts Yki activity in the *Drosophila* larval central nervous system in a manner independent of the Hpo-Wts kinase cascade (Gailite et al., 2015).

A recent study has revealed that the autophagy kinase Atg1/ULK1 acts in parallel to the Hpo-Wts cascade to restrict Yki activity and tissue growth (Tyra et al., 2020). Genetic experiments indicated that gain-of-function of Atg1 and its activator Acinus suppressed tissued overgrowth induced by Yki overexpression, while loss of Atg1 or Acinus increased Yki target gene expression and tissue growth. Biochemical studies demonstrated that Atg1 directly phosphorylated Yki on two Atg1/ULK1 consensus sites S74 and S97 to block its binding to Sd. Atg1-mediated phosphorylation of Yki is independent of Atg13, underscoring an autophagy-independent function of Atg1 in the regulation of Hippo signaling (Tyra et al., 2020). Atg1 is regulated by multiple pathways, including amino acid starvation that activates Atg1 in a Tor-regulated manner (Kim et al., 2011). Consistently, amino acid starvation increased Yki phosphorylation at S74 *in vivo* in a manner depending on Atg1 but independent of Atg13, suggesting that amino acid starvation could restrict Yki activity through activating Atg1. A previous study demonstrated that Tor is required for Yki to access to its target genes even after it enters the nucleus (Parker and Struhl, 2015). As Tor inhibits Atg1 (Kim et al., 2011), one possibility is that in the absence of Tor, Atg1 activity is elevated, leading to increased Yki phosphorylation at S74 and S97 and thus diminished Yki activity. It awaits to be determined whether Yap/Taz is also regulated by Atg1/ULK1 in response to nutrient deficiency in mammalian cells. Because YapS94, which is equivalent to YkiS97, is phosphorylated by AMPK in response to cellular energy starvation (Mo et al., 2015; Wang et al., 2015), phosphorylation at this conserved site could be a general mechanism to regulate Yki/Yap/Taz, which links nutrient starvation to growth inhibition.

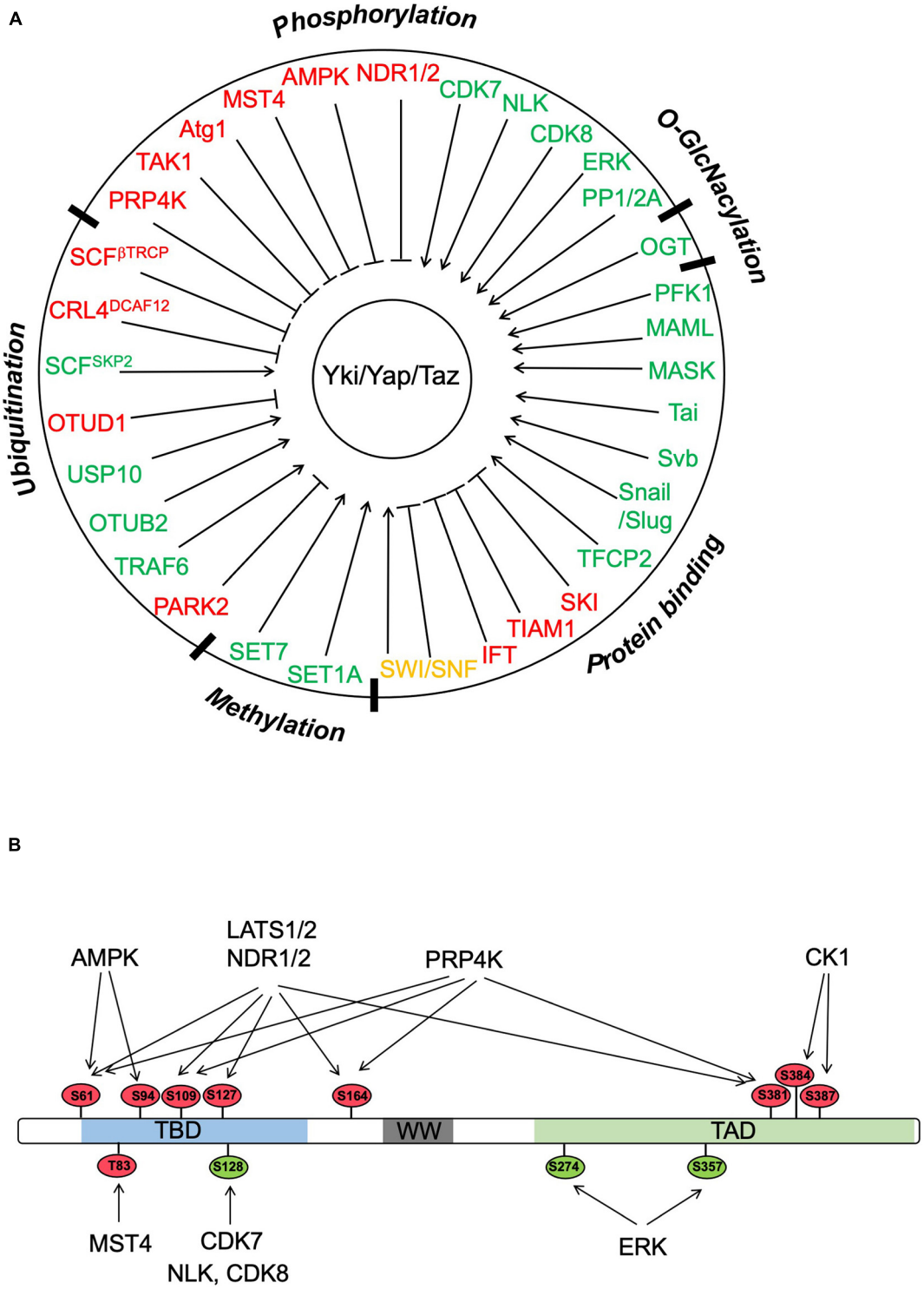

FIGURE 2 | Regulation of Yki/Yap/Taz by PTMs and protein–protein interactions. **(A)** Yki/Yap/Taz can be regulated by multiple posttranslational modifications (PTMs) including phosphorylation, ubiquitination, methylation, and O-GlcNacylation as well as by interacting partners. "Red" and "green" color codes indicate negative and positive regulators of Yki/Yap/Taz, respectively. Of note, SWI/SNF is color coded in "orange" because both negative and positive roles have been implicated depending on the context. **(B)** Yki/Yap/Taz activity can be regulated by multiple Ser/Thr kinases through phosphorylation of the indicated sites (based on Yap1). The phosphorylation sites are color coded with "red" and "green" indicating negative and positive effects on Yap activity, respectively.

The Hippo–Yap and the receptor tyrosine kinase ERBB2 signaling pathways are both required for heart regeneration after injury (D'Uva et al., 2015; Wang et al., 2018). A recent study uncovered a crosstalk between these two pathways in cardiomyocytes (CMs) in a heart failure mouse model (Aharonov et al., 2020). Transient overexpression of an activated form of ERBB2 in CMs induced an epithelial–mesenchymal transition (EMT)-like response to promoted cardiac regeneration (Aharonov et al., 2020). Through a combination of proteome and phospho-proteome analyses coupled with RNA-seq, Yap was identified as a critical mediator downstream of ERBB2 signaling in CMs (Aharonov et al., 2020). ERBB2 overexpression altered the mechanical state of CMs in part by enhancing the interaction of YAP with cytoskeletal and nuclear-envelope components (Aharonov et al., 2020). In addition, ERBB2 signaling promoted Yap phosphorylation at S352 and S274 (S367 and S289 in human Yap) through ERK, which is required for ERBB2 signaling-stimulated CM mitosis during heart regeneration (Aharonov et al., 2020).

REGULATION OF YKI/YAP/TAZ BY NUCLEAR KINASES

Although most of the regulatory events pertaining the control of Yki/Yap/Taz activity occur in the cytoplasm, a recent study identified a nuclear kinase, PRP4K, as a novel and conserved Hippo pathway component that directly phosphorylates Yki/Yap/Taz and excludes its nuclear localization (Cho et al., 2018). In a genetic modifier screen for genes whose loss of function modified the tissue overgrowth phenotype-caused Yki overexpression, Cho et al. (2018) found that RNAi knockdown of PRP4K enhanced, whereas overexpression of PRP4K, but not its kinase dead form, suppressed the eye overgrowth phenotype caused by eye-specific overexpression of Yki (*GMR > Yki*). Further genetic studies indicated that PRP4K acted downstream of Wts but upstream of Yki to regulate Hippo pathway target gene expression and tissue growth. Biochemical studies demonstrated that PRP4K phosphorylates Yki on a subset of Wts sites (S111 and S250), which leads to its nuclear exclusion and reduced interaction with Sd. The function of PRP4K in the Hippo pathway is evolutionarily conserved as PRP4K also acts downstream of Lats1/2 to phosphorylate Yap/Taz on a subset of Lats1/2 sites (excluding the YapS127/TazS89) to restrict their nuclear localization and interaction with TEAD. Hence, phosphorylation of Yki/Yap/Taz regulates their subcellular localization via two distinct mechanisms: phosphorylation of YkiS168/YapS127/TazS89 by Wts/Lats1/2 promotes the binding of Yki/Yap/Taz to 14-3-3, which sequesters Yki/Yap/Taz in the cytoplasm; phosphorylation of Yki/Yap/Taz on other Wts/Lats1/2 sites by Wts/Lats1/2 or PRP4K promotes their nuclear-to-cytoplasmic translocation through a 14-3-3 independent mechanism (Ren et al., 2010). Interestingly, high PRP4K expression correlates good prognosis in triple-negative breast cancer (TNBC) patients, suggesting that PRP4K may function as a tumor suppressor (Cho et al., 2018). Indeed, inactivation of PRP4K in a TNBC cell line MDA-MB-231 promoted cell growth and invasion (Cho et al., 2018). It would be interesting to determine whether and how the action of PRP4K is regulated in development, regeneration, or cancer. Because phosphorylation of Yki/Yap/Taz in the cytoplasm is dynamic, and dephosphorylation by several phosphatases including PP2A and PP1 leads to their nuclear translocation (Liu et al., 2011; Hu et al., 2017; Hein et al., 2019), PRP4K-mediated phosphorylation of Yki/Yap/Taz in the nucleus may provide a fail-safe mechanism to restrict aberrant pathway activity due to unchecked nuclear translocation of these pathway effectors.

In the same genetic modifier screen, Cho et al. (2020) identified the cyclin-dependent kinase 7 (CDK7) as a suppressor of the eye overgrowth phenotype caused by *GMR > Yki*. The effect of CDK7 on Yki-driven Hippo pathway target gene expression and tissue growth is independent of its role in cell cycle regulation or Pol II-mediated basal transcription but rather due to its ability to phosphorylate Yki in the nucleus and protect the nuclear Yki from ubiquitin/proteasome-mediated degradation independent of Wts (Cho et al., 2020). Further study demonstrated that CDK7 phosphorylated Yki on S169 and that a phospho-mimetic mutation (YkiS169D) rendered the mutant Yki insensitive to CDK7 inhibition (Cho et al., 2020). CDK7 plays a conserved role in mammalian Hippo pathway and can phosphorylate Yap/Taz on S128/S90 to stabilize the Hippo pathway effectors in the nucleus independent of Lat1/2 (Cho et al., 2020). Pharmacological inhibition of CDK7 by THZ1 rescued the liver overgrowth phenotype caused by MST1 and MST2 double knockout in mice and impeded Yap/Taz-driven tumor cell growth in xenografts (Cho et al., 2020). An independent study revealed a good correlation between CDK7 and Yap protein levels in malignant pleural mesothelioma (MPM) and showed that knockdown of CDK7 in MPM cells reduced Yap level, tumor cell migration and invasion, as well as tumor sphere formation (Miao et al., 2020). Taken together, these studies suggest that CDK7 could be an attractive drug target for Yki/Taz-driven cancers. It is interesting to note that NLK and CDK8 can also phosphorylate Yap at S128 to increase its activity and that phosphorylation of Yap by NLK is induced by osmotic stress (Hong et al., 2017; Moon et al., 2017; Zhou et al., 2018), suggesting that phosphorylation at this site could be regulated by multiple upstream inputs.

REGULATION OF YKI/YAP/TAZ BY UBIQUITINATION

Lats1/2 phosphorylates Yap/Taz on multiple sites including YapS127/TazS94 and YapS381/TazS311. While phosphorylation of YapS127/TazS94 restricts their nuclear access by promoting their binding to 14-3-3, phosphorylation of YapS381/TazS311 primes further phosphorylation by CK1 on adjacent sites, creating a docking site for the F-box protein β-TRCP (Liu et al., 2010; Zhao et al., 2010), which is a substrate recognition subunit of a family of modular E3 ubiquitin ligases containing SKP1-Cul1-F-box protein (SCF) complexes (Jiang and Struhl, 1998; Spencer et al., 1999). $SCF^{\beta\text{-}TRCP}$-mediated ubiquitination targets Yap/Taz for proteasome-mediated degradation (Liu et al., 2010;

Zhao et al., 2010). Hence, Lats1/2-mediated phosphorylation of Yap and Taz not only restricts their nuclear localization but also reduces their protein level.

While $SCF^{\beta\text{-}TRCP}$-mediated ubiquitination and degradation has not been shown to regulate Yki stability in *Drosophila*, a recent study identified a Cul4-RING E3 ligase (CRL4) complex as an evolutionarily conserved ubiquitin ligase that regulates Yki/Yap/Taz stability in the nucleus (Cho et al., 2020). RNAi-mediated knockdown of Cul4 as well as DCAF12, which serves as a substrate acceptor subunit in the CRL4 complex, promoted tissue growth driven by Yki (Cho et al., 2020). DCAF12 recruits the $CRL4^{DCAF12}$ complex to Yki/Yap/Taz, leading to their ubiquitination and proteasome-mediated degradation, whereas CDK7 phosphorylates Yki/Yap/Taz at S169/S128/S90 to inhibit $CRL4^{DCAF12}$ recruitment, leading to Yki/Yap/Taz stabilization in the nucleus (Cho et al., 2020). As a consequence, loss of DCAF12 rescued Yki instability and tissue growth defect caused by CDK7 inactivation (Cho et al., 2020). Hence, CDK7 safeguards Yki/Yap/Taz in the nucleus by protecting them from $CRL4^{DCAF12}$-mediated ubiquitination and degradation.

Hippo signaling could be regulated by the ubiquitin/proteasome pathway in a context-dependent manner. A recent study uncovered that PARK2, an E3 ubiquitin ligase implicated in Parkinson disease, could regulate Hippo/Yap signaling in esophageal squamous cell carcinoma (ESCC) (Zhou et al., 2020). Immunochemistry study revealed that PARK2 expression was low in human ESCC samples and reversely correlated with Yap expression, and TCGA data analysis indicated that high PARK2 expression correlated with good prognosis in ESCC patients (Zhou et al., 2020). PARK2 KO in ESCC cell lines increased Yap protein level, Hippo target gene expression, cell proliferation and invasion, and tumor progression in xenografts (Zhou et al., 2020). Mechanistically, PARK2 binds Yap and catalyzes its poly-ubiquitination at K90 (Zhou et al., 2020). Hence, PARK2 functions as a tumor suppressor in ESCC by targeting Yap for ubiquitin/proteasome-mediated degradation.

In addition to being targeted for degradation by ubiquitination, Yap can also be regulated by non-proteolytic ubiquitination that is catalyzed by the SCF E3 ubiquitin ligase complex containing SKP2 (SCF^{SKP2}) (Yao et al., 2018). In HEK293 cells cultured at low density, SKP2 promoted K63-linked polyubiquitination of Yap at K321 and K497, leading to increased Yap-TEAD association, Yap nuclear accumulation, and transcriptional activity (Yao et al., 2018). SKP2-mediated ubiquitination of Yap is reversed by OTUD1, a deubiquitinase that preferentially cleaves K63-linked polyubiquitin chain (Yao et al., 2018). Consistent with their opposing roles in the regulation of Yap, overexpression of SKP2 and knockdown of OTUD1 promoted cancer cell growth *in vitro* (Yao et al., 2018). Furthermore, high SKP2 is associated with poor whereas high OTUD1 with good prognosis in breast cancer patients (Yao et al., 2018). It remains to be determined whether SKP2/OTUD1-mediated Yap regulation plays any role in development, regeneration, and tumorigenesis by *in vivo* study.

A recent study has demonstrated that, in macrophages, IL-1 induces Yap nuclear localization and protein stability by TRAF6-mediated K63-linked poly-ubiquitination of Yap at K252, which disrupts the interaction between Yap and angiomotin (Liu et al., 2020). Macrophage Yap is upregulated in both patients and mouse atherosclerotic lesions, and myeloid-specific overexpression of Yap in mice promoted the development of atherosclerosis, suggesting that interfering of Yap activation could be a therapeutic opportunity for atherosclerosis (Liu et al., 2020).

REGULATION OF YKI/YAP/TAZ BY DEUBIQUITINATION

Protein ubiquitination is a reversible process, and a poly-ubiquitin chain on a substrate can be removed by deubiquitinating enzymes (DUBs). An *in vivo* DUB cDNA screen identified OTUB2 as an enhancer of cancer metastasis (Zhang et al., 2019). OTUB2 promoted cancer stemness and metastasis by deubiquitinating and stabilizing Yap/Taz in a manner independent of Lats1/2 (Zhang et al., 2019). Interestingly, OTUB2 is sumoylated on Lys 233, which promotes its association with Yap/Taz via a conserved but previously uncharacterized SUMO-interacting motif (SIM) in Yap/Taz (Zhang et al., 2019). This sumoylation-mediated interaction is essential for Yap/Taz deubiquitination by OTUB2. As a consequence, sumoylation-deficient OTUB2 and SIM-mutated Yap exhibited diminished metastasis-promoting activity (Zhang et al., 2019). Furthermore, OTUB2 sumoylation is stimulated by EGF and oncogenic KRAS, which is essential for EGF and KRAS to stabilize Yap/Taz (Zhang et al., 2019). In breast cancer patients, there is a good correlation between the levels of KRAS and the levels of OTUB2 sumoylation as wells as the levels of Yap/Taz protein expression (Zhang et al., 2019). Hence, OTUB2 sumoylation represents a novel mechanism that links the oncogenic EGFR-RAS pathway to Yap/Taz activation and a potential therapeutic target for cancer treatment. It remains to be determined whether OTUB2-mediated regulation of Yap/Taz is involved in development and tissue regeneration.

In a search for DUBs that could regulate Yap/Taz-mediated transcriptional luciferase reporter, 8XGTIIC, in cultured liver cancer (HepG2) cells, Zhu et al. (2020) found that knockdown of USP10 had the strongest effect on 8XGTIIC expression among the 98 DUBs tested. They found that USP10 interacted with stabilized Yap/Taz by reverting their ubiquitination (Zhu et al., 2020). As a consequence, inactivation of USP10 promoted YAP/TAZ ubiquitination and proteasome-mediated degradation, and inhibited hepatocellular carcinoma cell growth both *in vitro* and in xenografts (Zhu et al., 2020). In hepatocellular carcinoma patient samples as well as in chemical-induced mouse liver cancers, USP10 expression positively correlated with YAP/TAZ abundance, and high USP10 expression correlated with poor prognosis in hepatocellular carcinoma patients (Zhu et al., 2020). Taken together, this study revealed a role of USP10 in liver cancer by promoting Yap/Taz stability and suggested a potential new strategy for therapeutical intervention.

REGULATION OF YAP BY METHYLATION

In addition to being regulated by phosphorylation and ubiquitination, Yap/Taz can also be regulated by other posttranslational modifications such as methylation (**Figure 2**). Two studies revealed that Yap subcellular localization could be regulated by mono-methylation, yet by different lysine methyltransferases (Oudhoff et al., 2013; Fang et al., 2018). Oudhoff et al. (2013) found that knockout of SET-domain-containing lysine methyltransferase 7 (SET7) in mouse intestinal epithelial cells led to increased frequency of cell proliferation per crypt accompanied by increased Yap nuclear localization and Hippo target gene expression. Consistent with this *in vivo* finding, Yap failed to translocate to the cytoplasm in SET7 KO MEFs grown at high density although phosphorylation of Yap by Lats1/2 at S127 was not affected (Oudhoff et al., 2013). Set7 formed a complex with Yap in the cytoplasm in MEFs grown at high density and promoted mono-methylation of Yap at K494 (Oudhoff et al., 2013). Furthermore, YapK494R failed to localize to the cytoplasm in MEFs grown at high density even though it exhibited normal phosphorylation at S127 (Oudhoff et al., 2013). These observations suggest that SET7-mediated mono-methylation Yap at K494 is required for cell density-mediated cytoplasmic localization of Yap, although the underlying mechanism remains unknown.

In contrast to Yap K494 mono-methylation that promotes cytoplasmic retention of Yap, another study found that mono-methylation of Yap at K342 by methyltransferase SET1A promoted nuclear retention of Yap activity by blocking its nuclear export (Fang et al., 2018). Using mass spectrometry analysis and a rabbit polyclonal antibody that specifically recognized a mono-methylated site on Yap, Fang et al. (2018) found that lysophosphatidic acid (LPA) could stimulate Yap mono-methylation at K342 in cancer cell lines. By screening a panel of methyltransferases, the authors identified SET1A as the only methyltransferase that catalyzed Yap K342 methylation in cancer cells (Fang et al., 2018). LPA treatment and low cell density enhanced the interaction between Yap and SET1A and consequently increased Yap K342 mono-methylation (Fang et al., 2018). SET1A-mediated Yap K342 methylation enhanced Yap nuclear localization, Yap-TEAD transcriptional activity, and tumor growth (Fang et al., 2018). Mechanistically, SET1A-mediated Yap K342 methylation inhibited the association between Yap and the nuclear export receptor CRM1 and blocked Yap nuclear export, leading to its nuclear retention (Fang et al., 2018). Interestingly, tissue microarray-base immunohistochemistry study of human lung adenocarcinoma and colorectal cancer (CRC) revealed a good correlation between high SET1A expression with high YAP expression and K342 methylation (Fang et al., 2018). Analysis of TCGA database indicated that SET1A is highly expressed in a number of types of cancer including lung, colon, and breast cancer and that a high SET1A expression is associated with poor outcome in lung and gastric carcinomas (Fang et al., 2018). Taken together, this study suggests that SET1A-mediated Yap methylation may play an important role in tumorigenesis and thus provides an attractive drug target for cancer treatment.

REGULATION OF YAP BY O-GLCNACYLATION

O-linked β-N-acetylglucosamine (O-GlcNAc) is a sugar attachment to Ser/Thr hydroxyl moieties on proteins localized in cytoplasm or nucleus, and protein O-GlcNAcylation is regulated by multiple metabolic nutrients including glucose (Slawson et al., 2010). Two independent studies identified Yap O-GlcNAcylation as a mechanism that regulates Hippo pathway outputs in response to altered glucose metabolism (Peng et al., 2017; Zhang et al., 2017a). Both groups found that O-GlcNAc transferase (OGT) interacts with and O-GlcNacylates Yap and that O-GlcNAcylation reduces Yap binding to, and phosphorylation by, Lats1/2, leading to increased Yap activity and Yap-driven tumor growth (Peng et al., 2017; Zhang et al., 2017a). Intriguingly, Peng et al. (2017) found that Yap Thr 241 is the main Yap O-GlcNAcylation site, whereas Zhang et al. (2017a) identified Ser109 as the major site. Mutating either Yap Thr241 or Ser109 to Ala to block O-GlcNAcylation increased the phosphorylation of the Yap mutants by Lats1/2 and consequently reduced their activity (Peng et al., 2017; Zhang et al., 2017a). It is possible that both sites can be O-GlcNAcylated, but the relative contribution of each site may vary depending on cell types. Zhang et al. (2017a) found that OGT is a transcriptional target of Yap–TEAD, uncovering a positive feedback between Yap and global cellular O-GlcNAcylation. Indeed, in a tissue microarray analysis of over 200 liver cancer samples, a statistically significant positive correlation between Yap expression and global O-GlcNAcylation was observed (Peng et al., 2017). Taken together, these studies suggest that Yap O-GlcNAcylation links glucose abundance to Hippo signaling activity and tumorigenesis and could be a potential therapeutic intervention point for cancer treatment.

REGULATION OF YKI/YAP/TAZ BY PROTEIN–PROTEIN INTERACTION

In addition to posttranslational modifications, Yki/Yap/Taz subcellular localization, stability, and activity can be modulated by interacting proteins (**Figure 2**). Early studies have demonstrated that binding of Yki/Yap/Taz to 14-3-3 after their phosphorylation by Wts/Lats1/2 or binding of Yap/Taz to Amot promotes cytoplasmic sequestration of these Hippo pathway effectors (Ren et al., 2010; Zhao et al., 2011). A recent study has unraveled a non-canonical role of intraflagellar transport (IFT) complex B proteins (IFT88, IFT55, and IFT20) in the regulation of Hippo/Yap during cardiogenesis independently of primary cilia (Peralta et al., 2020). IFT proteins form a complex with Yap and AMOTL1 to restrict Yap nuclear localization and activity, and this mechanism plays a key role in restricting the formation of the proepicardium and the myocardium in both zebrafish and mouse embryos (Peralta et al., 2020).

Although Yki contains an N-terminal non-canonical nuclear localization signal (NLS) that binds importin α1 to mediate its nuclear import (Wang S. et al., 2016), binding of Yki/Yap/Taz to the Mask family proteins (Mask in *Drosophila* and ANKHD1/2 in mammals) modulates their nuclear import through a canonical

NLS in the Mask proteins (Sidor et al., 2019). Another recent study reported that Mastermind-like (MAML) 1 and 2 binds and promotes Yap/Taz nuclear localization and activity depending on MAML NLS (Kim J. et al., 2020). Interestingly, Yap1-MAML2 fusion events leading to constitutive nuclear localization and activation of the fusion proteins were frequently found in a type of benign skin tumor called poroma and its malignant counterpart porocarcinoma (Sekine et al., 2019). Hence, interacting with multiple binding partners regulates nuclear/cytoplasmic localization and activity of the Hippo pathway effectors.

Once in the nucleus, the activity of Yki/Yap/Taz can be further modulated by other interaction partners. Although several studies have revealed that glucose metabolism can regulate Hippo signaling output though AMPK-mediated phosphorylation or OGT-mediated O-GlcNAcylation of Yap, another study showed that increased glucose metabolism and reprogramming toward aerobic glycolysis in cancer cells can upregulate Yap/Taz target gene expression through phosphofructokinase (PFK1), a key enzyme that regulates glycolysis. PFK1 binds TEAD and promotes Yap/Taz interaction with TEAD (Enzo et al., 2015). In addition, a transcriptional signature associated to aerobic glycolysis correlates with elevated YAP/TAZ activity and is predictive of poor prognosis in breast cancer patients (Enzo et al., 2015). Interestingly, the function of PFK1 is conserved in *Drosophila* where it is required for Yki-driven tissue overgrowth (Enzo et al., 2015).

In *Drosophila*, the ecdysone (Ec) receptor coactivator Taiman (Tai) interacts with Yki to enhance Yki-drive tissue growth and intestinal stem cell proliferation (Zhang C. et al., 2015; Wang C. et al., 2016). Interestingly, the Hippo/Ec pathway cooperativity through the formation of Yki–Tai complex drives a distinct pro-growth transcriptional program including germline stem cell factors whose expression is normally suppressed in developing somatic cells (Zhang C. et al., 2015). Another study showed that the transcription factor Shavenbaby (Svb) is expressed in *Drosophila* renal/nephric stem cells and is required for their maintenance during adulthood by physically interacting with Yki to promote the expression of the inhibitor of apoptosis DIAP1 (Bohere et al., 2018).

In mammalian skeletal stem cells (SSCs), the zinc finger transcription factors Snail and Slug promote stem cell proliferation and differentiation through Yap/Taz (Tang et al., 2016). Deletion of Snail/Slug diminished SSC proliferation and blocked osteogenesis both *in vitro* and in mice (Tang et al., 2016). Snail/Slug forms a complex with Yap/Taz to activate a set of Yap/Taz/TEAD target genes that control SSC proliferation, whereas Snail/Slug forms a complex with Taz/Runx2 to promote the expression of Runx2 target genes involved in osteogenesis (Tang et al., 2016). Mechanistically, the extended SNAG domains of Snail/Slug, which recruit chromatin-modifying enzymes critical for transcriptional repression, mediate interactions with the YAP/TAZ WW domains, and these interactions not only stabilizes Yap/Taz by preventing their interactions with Lats1/2 and thus phosphorylation by Lat1/2, but also promotes Yap/Taz transcriptional activity in the nucleus by increasing their promoter occupancy (Tang et al., 2016). Hence, SSCs employ Snail/Slug–YAP/TAZ complexes to control stem cell function. It would be interesting to determine whether similar mechanisms are utilized by other stem cells to regulate their function.

In a proteomic screen, Zhang et al. (2017b) identified the transcription factor TFCP2 as a binding partner of Yap in liver cancer cells. Loss of TFCP2 attenuated, while gain of TFCP2 enhanced, Yap-driven liver growth (Zhang et al., 2017b). Mechanistically, TFCP2 interacts with the WW domain of Yap through a PSY motif, and this interaction enhances Yap binding to TEAD in addition to increasing Yap stability by preventing it from β-TRCP-mediated ubiquitination (Zhang et al., 2017b). TFCP2 and Yap co-regulated a number of Yap–TEAD target genes important in Yap-driven tumorigenesis (Zhang et al., 2017b). Tissue microarray analysis revealed a statistically significant positive correlation between YAP and TFCP2 in liver cancer samples (Zhang et al., 2017b), consistent with the notion that TFCP2 cooperates with Yap to stimulate liver malignancy.

While many Yap/Taz/TEAD-binding proteins promote its activity, others inhibit Yap/Taz activity in the nucleus. For example, Ski, the transforming protein of the avian Sloan–Kettering retrovirus, can inhibit Taz transcriptional activity by binding to TEAD and recruiting the transcriptional co-repressor NCoR1 (Rashidian et al., 2015). Another study showed that TIAM1, a guanine nucleotide exchange factor specific for RAC1, can shuttle between cytoplasm and nucleus, and that nuclear TIAM1 binds TAZ/YAP and blocks its interaction with TEADs, leading to inhibition of TAZ/YAP target genes involved in EMT, cell migration, and invasion (Diamantopoulou et al., 2017). As a consequence, TIAM1 knockdown increased Yap/Taz activity and CRC cell migration and invasion and high nuclear TIAM1 in clinical specimens associates with increased CRC patient survival (Diamantopoulou et al., 2017).

A recent study revealed that the SWI/SNF chromatin remodeling complex is a mechano-regulated inhibitor of Yap/Taz (Chang et al., 2018). The SWI/SNF complex components, such as ARID1A, are frequently inactivated in a wide range of human cancers. By identifying the nuclear factors that interact with YAP/TAZ using chromatin immunoprecipitation followed by mass spectrometry, Chang et al. (2018) identified several components of the SWI/SNF complex in association with Yap/Taz. They further demonstrated that the SWI/SNF complex interacts with Yap/Taz through ARID1A, which blocks the Yap/Taz–TEAD association and hence Yap/Taz transcriptional activity (Chang et al., 2018). As a consequence, loss of SWI/SNF promotes Yap/Taz-driven tissue growth and tumor formation (Chang et al., 2018). Interestingly, the association of Yap/Taz with the SWI/SNF complex is regulated by mechanical cues. At high mechanical stress, nuclear F-actin binds the ARID1A–SWI/SNF complex, thereby preventing its association with Yap/Taz and allowing the formation of Yap/Taz/TEAD complex (Chang et al., 2018). Hence, TEAD competes with the SWI/SNF to bind Yap/Taz, which is favored by high mechanics. This study suggests that oncogenic activation of Yap/Taz not only requires genetic or epigenetic events that increase nuclear Yap/Taz level but also requires genetic or mechanical influence to remove the inhibitory function of the ARID1A–SWI/SNF complex.

Intriguingly, another recent study has revealed that ARID1A endows a permissive chromatin state that promotes Yap to access its target genes involved in hepatocyte-to-progenitor conversion during liver injury and regeneration (Li et al., 2019). Hence, the function of ARID1A in Hippo–Yap signaling is complex and context dependent.

CONCLUSION

Although many upstream signals regulate Yki/Yap/Taz activity through the Hpo kinase cascade, an increasing number of studies have uncovered other mechanisms that regulate Yki/YAP/Taz subcellular localization, stability, and activity independent of the Hpo kinase cascade. It is highly anticipated that more new mechanisms will be unraveled in the near future by ongoing studies in many labs around the world. These new mechanisms will provide additional layers for pathway regulation, nodes for pathway cross talks, and opportunities for pathway intervention. It is worth noting that many of the mechanisms uncovered so far relied heavily on *in vitro* culture systems, and their physiological relevance needs to be established by genetic studies in model organisms. Other mechanisms such as regulation of Yap methylation by SET1A and regulation of Yap ubiquitination by PARK2 have been derived from studies using cancer cell lines and xenograft models, leaving unclear whether these mechanisms play a role during development and whether they are evolutionarily conserved. Validating these mechanisms using more relevant disease models is important for harnessing these and other mechanisms for therapeutical intervening to treat Yap/Taz-driven cancer and to facilitate tissue repair and regeneration.

AUTHOR CONTRIBUTIONS

YC and JJ wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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Wallenda-Nmo Axis Regulates Growth via Hippo Signaling

Xianping Wang[1,2,3†], Hui Liang[1,2,3†], Wenyan Xu[2,3,4] and Xianjue Ma[1,2,3]*

[1] Key Laboratory of Growth Regulation and Translational Research of Zhejiang Province, School of Life Sciences, Westlake University, Hangzhou, China, [2] Westlake Laboratory of Life Sciences and Biomedicine, Hangzhou, China, [3] Institute of Biology, Westlake Institute for Advanced Study, Hangzhou, China, [4] Key Laboratory of Structural Biology of Zhejiang Province, School of Life Sciences, Westlake University, Hangzhou, China

Both Hippo signaling pathways and cell polarity regulation are critical for cell proliferation and the maintenance of tissue homeostasis, despite the well-established connections between cell polarity disruption and Hippo inactivation, the molecular mechanism by which aberrant cell polarity induces Hippo-mediated overgrowth remains underexplored. Here we use *Drosophila* wing discs as a model and identify the Wnd-Nmo axis as an important molecular link that bridges loss-of-cell polarity-triggered Hippo inactivation and overgrowth. We show that Wallenda (Wnd), a MAPKKK (mitogen-activated protein kinase kinase kinase) family member, is a novel regulator of Hippo pathways in *Drosophila* and that overexpression of Wnd promotes growth via Nemo (Nmo)-mediated Hippo pathway inactivation. We further demonstrate that both Wnd and Nmo are required for loss-of-cell polarity-induced overgrowth and Hippo inactivation. In summary, our findings provide a novel insight on how cell polarity loss contributes to overgrowth and uncover the Wnd-Nmo axis as an essential additional branch that regulates Hippo pathways in *Drosophila*.

Keywords: Wnd, Hippo signaling, Nmo, growth, *Drosophila*

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Zengqiang Yuan,
Peking Union Medical College, China

Reviewed by:
Ana Carmena,
Institute of Neurosciences of Alicante (IN), Spain
Jurgen Muller,
University of Bradford, United Kingdom

****Correspondence:***
Xianjue Ma
maxianjue@westlake.edu.cn

† These authors have contributed equally to this work and share first authorship

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INTRODUCTION

Proper control of cell proliferation is fundamental for correct organ development, disruption of which would cause tumorigenesis. *Drosophila* imaginal discs, the precursor of the adult organ during the larval stage, are a powerful model to study tissue growth, and numerous signaling pathways essential for cell proliferation have been uncovered using this system (Gokhale and Pfleger, 2019; Gou et al., 2020), including the Hippo pathway, a key regulator of cell proliferation and organ size (Pan et al., 2018; Zheng and Pan, 2019). The core components of the Hippo signaling pathway consist of serine/threonine kinases Hippo (Hpo) and Warts (Wts) and the transcriptional coactivator Yorkie (*Yki*) (Justice et al., 1995; Xu et al., 1995; Harvey et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003; Huang et al., 2005). Upon phosphorylation by Hpo, Wts is activated and subsequently phosphorylates *Yki* to restrict its nuclear entrance, thereby preventing the transcription of pro-proliferative target genes, including *expanded* (*ex*), *bantam* (*ban*), and *Cyclin E* (*cycE*) (Pan, 2010; Snigdha et al., 2019; Zheng and Pan, 2019). Recent advances on this evolutionary conserved pathway have revealed comprehensive roles of Hippo signaling in regulating a wide range of biological functions, ranging from cell adhesion and mechanical tension to regeneration and immune surveillance (Fallahi et al., 2016; Chen, 2019; Ma et al., 2019; Zheng and Pan, 2019).

Cell polarity maintenance is essential for tissue homeostasis, and dysregulation of apical-basal polarity has been linked to various developmental disorders and cancer (Wodarz and Nathke, 2007). Studies in *Drosophila* have revealed that several apical-basal cell polarity modules regulate tissue growth via the Hippo pathway, including lethal-2-giant larvae (Lgl)-atypical protein kinase C (aPKC), Crumbs (Crb), and Scribbled (Scrib)/Disc large (Dlg) (Richardson and Portela, 2017). We recently identified the E3 ubiquitin ligase POSH (Plenty of SH3s) as a key regulator of Hippo pathway, which integrates the signal from the cell polarity protein Crb to negatively regulate Ex (Expanded)-mediated Hippo activation in *Drosophila* (Ma et al., 2018). We also identified Wallenda (Wnd), a member of mitogen activated protein kinase kinase kinase (MAPKKK) family, as an essential downstream component that links loss-of-polarity-induced cell invasion phenotype to JNK pathway activation (Ma et al., 2016). Although recent studies on *MAP3K13*, the human homolog of Wnd, have revealed its tumor promoting roles in head and neck squamous cell carcinoma (HNSCC) and hepatocellular carcinoma (HCC) patients (Edwards et al., 2017; Zhang et al., 2020), the mechanism by which Wnd/MAP3K13 regulates tissue growth remains underexplored.

In this study, we uncovered Wnd as a novel regulator of Hippo signaling. Our results showed that overexpression of Wnd induces cell proliferation in the *Drosophila* wing imaginal disc via inactivating the Hippo signaling. Knockdown of *wnd* impedes *Rho1* expression or loss-of-polarity induced Hippo inactivation. We further identified Nemo (Nmo) as an essential downstream mediator of Wnd in regulating Hippo signaling. Our data demonstrate that Wnd-Nmo represents a new axis that bridges cell-polarity-loss-induced growth and Hippo pathway inactivation in *Drosophila*.

RESULTS AND DISCUSSION

Wnd/MAP3K13 Negatively Regulates Hippo Signaling

Our previous studies have revealed that both *Rho1* and Wnd are essential for tumorigenesis by inducing JNK signaling mediated cell death and cell invasion in wing imaginal discs (Ma et al., 2015b, 2016). Interestingly, we noticed that *Rho1*-induced actin accumulation could only be impeded by depletion of Wnd but remained unaffected by complete inhibition of JNK signaling, suggesting that Wnd has additional roles other than the JNK pathway activation. To further explore the role of Wnd in regulating tumorigenesis, we examined the potential role of MAP3K13 (human homolog of Wnd) through data mining using several open-access online servers. We found that copy number variants (CNV) of *MAP3K13* are dysregulated in various human cancers (**Figure 1A**), especially in lung squamous cell carcinoma (LUSC) and ovarian serous cystadenocarcinoma (OV). Accordingly, transcripts of *MAP3K13* are significantly up regulated in LUSC and OV (**Figure 1C**) and the high expression level of *MAP3K13* is correlated with low survival probability in endometrial cancer patients (**Figure 1B**). Together, these results indicate that *MAP3K13* is a potential oncogene.

Next, we tried to further address the function of Wnd in tissue growth control *in vivo* using *Drosophila*. Given that ectopic *wnd* expression can induce the up-regulation of *wingless* (*wg*) and *cycE* (Ma et al., 2016), two known downstream target genes of the Hippo pathway, we tested the possibility that *wnd* could regulate the Hippo pathway in *Drosophila*. We examined the transcriptional change of two additional Hippo signaling reporters, *ban* and *ex* (Cho et al., 2006; Thompson and Cohen, 2006), and found that upon Wnd overexpression under *ptc* promoter along the anterior-posterior boundary of wing imaginal discs, an obvious up-regulation of *ban-lacZ* was seen (**Figures 1F,F′**), compared with the wild-type control (**Figures 1E,E′**). Given that ectopic expression of Wnd also leads to apoptosis as reported previously (Ma et al., 2015b), we inhibited apoptosis simultaneously by co-expression of *P35* with *wnd* and observed a stronger up-regulation of *ban-lacZ* (**Figures 1G,G′**). Similarly, overexpression of *wnd* by *hh-Gal4* in the posterior region of wing imaginal discs significantly up-regulated *ex-lacZ* level (**Figures 1H,I**). More importantly, we also observed strong nuclear *Yki* localization in heat-shock-induced *wnd* expressing clones compared with endogenous control outside the GFP positive clones (**Figures 1J–J′′**). Furthermore, our data mining results suggested that the up-regulation of *MAP3K13* in LUSC is positively correlated with increased expression of *MYC*, *BIRC5,* and *EDN1* (**Figure 1D**), three known target genes downstream of YAP/TAZ (Choi et al., 2018), indicating a potential conserved role of *MAP3K13* in regulating Hippo pathway in human cancers. Interestingly, we also observed an increased level of cytoplasmic *Yki*, suggesting that there is an overall increase of the cellular *Yki* amount, which we could not explain at this point, and for which further investigation is required to dissect the underlying mechanism. Therefore, we conclude that Wnd enhances *Yki* nuclear localization and up-regulates Hippo pathway target genes.

Wnd Acts Downstream of Cell Polarity Loss-Rho1 Axis

Our previous study revealed a physical interaction between Wnd and the Rho GTPase *Rho1* in regulating cell invasion (Ma et al., 2016), and we identified *Rho1* as an important regulator of Hippo signaling-mediated growth (Ma et al., 2015a). These clues raised the possibility that Wnd and *Rho1* are somehow linked in the regulation of the Hippo pathway. Therefore, we investigated whether Wnd is also required for *Rho1*-mediated Hippo inactivation in *Drosophila*. Overexpression of *Rho1* with *P35* by *ptc-Gal4* in wing imaginal discs induces obvious cell proliferation and enhanced *Yki* nuclear localization (**Figures 2B,B′**), both of which are significantly impeded by knockdown of *wnd* (**Figures 2C,C′**). Consistently with this, we found that compared with the wild type control (**Figures 2D,D′**), knockdown of *wnd* also reduces *Rho1* overexpression-induced *ban-lacZ* up-regulation (**Figures 2E–F′** and **Supplementary Figure 2**), suggesting that Wnd is required for Rho1-induced Hippo signaling inactivation.

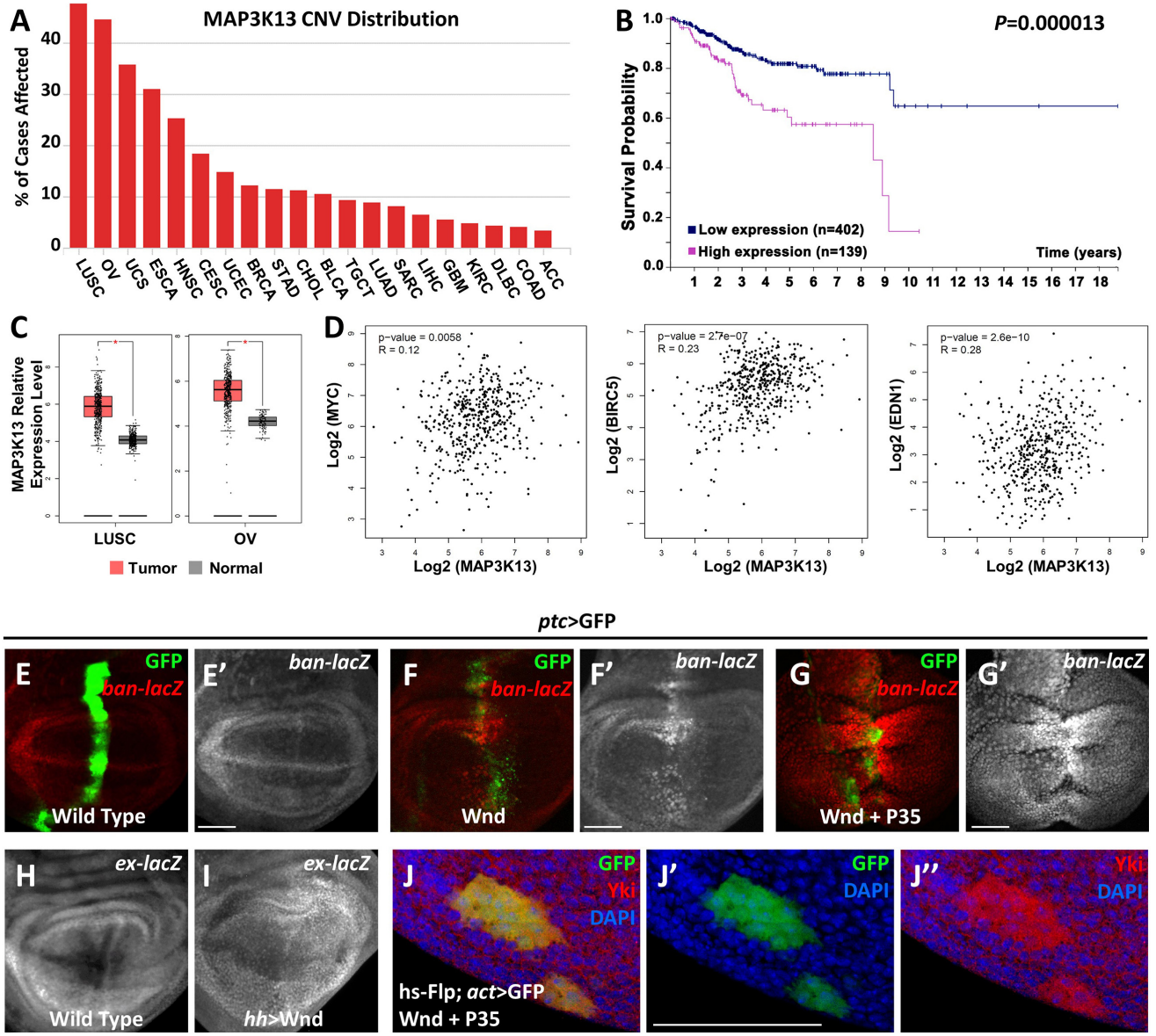

FIGURE 1 | MAP3K13/Wnd activates expression of Hippo pathway target genes. **(A)** The distribution of copy number variation (CNV) of *MAP3K13* across different cancer types. **(B)** Analysis of the prognostic significance of high and low expression level of *MAP3K13* in endometrial cancer. **(C)** Box plots showing the relative expression level of *MAP3K13* in lung squamous cell carcinoma (LUSC) and ovarian serous cystadenocarcinoma (OV). **(D)** Expression correlation between *MAP3K13* and *MYC*, *BIRC5*, or *EDN1*, three target genes downstream of the transcriptional co-activators YAP/TAZ, in LUSC. **(E–J'')** Wing imaginal discs from third instar larva are shown. Compared with wild type controls **(E,E')**, expression of Wnd for 2–3 days **(F,F')** or co-expression of *wnd* and *P35* **(G,G')** driven by *ptc*-Gal4 (GFP positive area) elevates *ban* expression level. Expression of Wnd in the posterior region of wing disc elevates *ex* expression level **(I)**, compared with wild type controls **(H)**. **(J–J'')** *Yki* nuclear localization is increased in heat shock-induced clones expressing *wnd*. Scale bars, 50 μm.

Loss of *scrib* has been reported to upregulate *Yki* target genes (Verghese et al., 2012). To test whether Wnd is also required for *scrib*-loss induced Hippo inactivation, we monitored *ex-lacZ* level in wing imaginal discs. Knockdown of *scrib* by *dpp*-Gal4 induces evident upregulation of *ex-lacZ* (**Figures 2H,H'**), in comparison with the wild type control (**Figures 2G,G'**), while knockdown simultaneously of *wnd* reduces the *ex-lacZ* level to a certain degree (**Figures 2I,I'**). Additionally, knockdown of *scrib* by *nub*-Gal4 leads to significant overgrowth of the wing pouch region (**Figure 2J**), which is also inhibited by *wnd* knockdown (**Figures 2K,L**). In summary, these results indicate that Wnd acts downstream of cell polarity-loss-induced Hippo inactivation.

Nmo Genetically Acts Downstream of *Wnd*

Next, we further dissected the potential mechanism by which *wnd* overexpression inactivates Hippo signaling. As a *MAPKKK*,

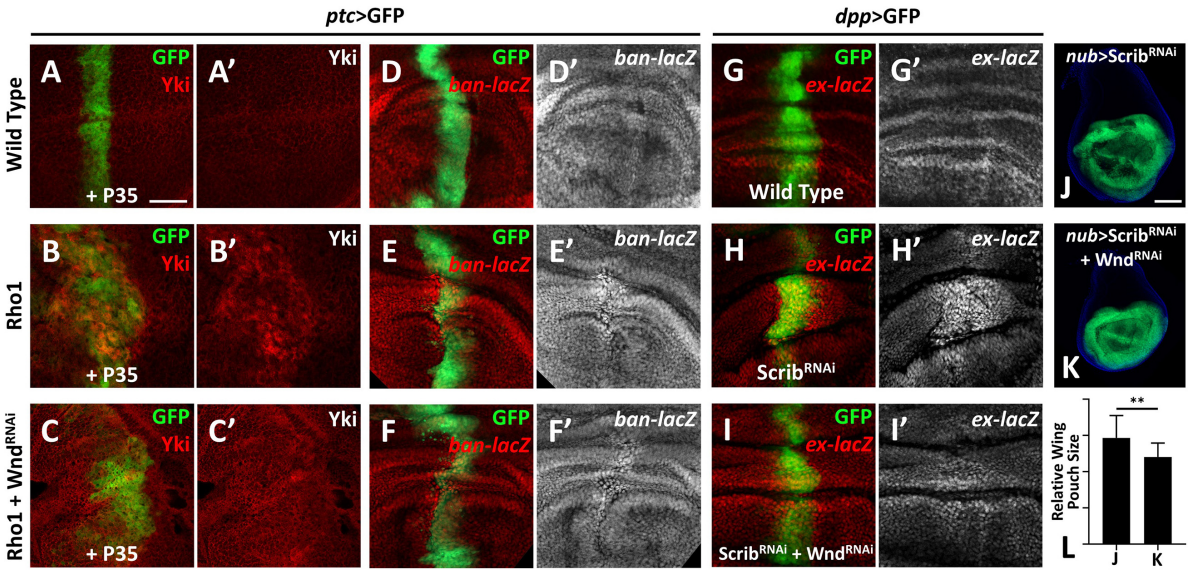

FIGURE 2 | Wnd is essential for *Rho1* activation and cell polarity loss-induced Hippo inactivation. **(A–K)** Wing imaginal discs from third instar larva with genes or RNA is expressed in GFP positive area are shown. **(A–C′)** Compared with wild type controls **(A,A′)**, expression of *Rho1* together with *P35* enhances Yki nuclear localization **(B,B′)**, which is rescued by knocking down *wnd* **(C,C′)**. **(D–F′)** Compared with wild type controls (D,D′), expression of *Rho1* induced *ban* upregulation **(E,E′)** is suppressed by knocking down *wnd* **(F,F′)**. **(G–I′)** Compared with wild type controls **(G,G′)**, *scribble* (*scrib*) knockdown upregulates *ex* transcription **(H,H′)** and this is impeded by *wnd* knockdown **(I,I′)**. **(J)** Knocking down *scrib* in the wing pouch region causes tissue overgrowth **(J)**, which is suppressed by *wnd* knockdown **(K)**. Statistical analysis of **(J,K)** is shown in **(L)**. Scale bars for **(A–I′)**, 50 μm; for **(J,K)**, 100 μm. $^{**}P < 0.01$. (A Student *t*-test was used to calculate statistical significance, mean + *SD*, $n = 15$).

logically, Wnd may activate certain protein kinase(s) that are known to genetically interact with Hippo signaling. However, the classical known kinases of the Hippo pathway are all negative regulators of *Yki*, suggesting that Wnd might act through a non-canonical kinase target to regulate the Hippo pathway. The only exception known so far is a MAPK family protein named Nmo, a serine/threonine protein kinase essential for cell death, planar cell polarity, neuronal function, and circadian clock regulation (Mirkovic et al., 2002; Merino et al., 2009; Yu et al., 2011; Collu et al., 2018). It is recently reported that Nemo-Like Kinase (*NLK*, human homolog of Nmo) phosphorylates YAP and subsequently positively regulate its transcriptional activity (Moon et al., 2017). It is worth noting that both Wnd and Nmo are essential for the overgrowth phenotype of neuromuscular junction (NMJ) caused by a mutation in a ubiquitin E3 ligase named Highwire (Collins et al., 2006; Wu et al., 2007; Merino et al., 2009), indicating a potential genetic interaction between *wnd* and *nmo*. Therefore, we examined whether Nmo is genetically involved in Wnd-mediated *Yki* activation.

Given that *wnd* overexpression induces cell death by activating JNK pathway, we co-expressed *P35* to inhibit Wnd-induced cell death. Overexpression of *P35* alone by *ptc*-Gal4 leads to no change on Wg level comparing to the wild type (**Figures 3A–B′**), iwhile co-expression of *wnd* and *P35* and observed a significant up-regulation of *wg* expression along the *ptc* stripe (GFP positive) (**Figures 3D,D′**), which was dramatically reduced by simultaneously knocking down *nmo* (**Figures 3E,E′**). Strikingly, we found that *nmo* knockdown not only reduced the nuclear *Yki* accumulation induced by co-expression of *wnd* and *P35*, but also caused sharp decrease of *Yki* protein level (**Figures 3I–J′′**), whereas knockdown of *nmo* alone did not cause obvious phenotypes (**Figures 3C,C′,H–H′′**, and **Supplementary Figure 4**). Since the increased nuclear *Yki* localization causally leads to cell proliferation, we also examined cell proliferation by PH3 staining. We found that though there is no significant difference of proliferation rate in *P35* overexpression and *nmo* knockdown wing discs compared with the wild type control (**Figures 3P–R′,U**), the increased proliferation caused by *wnd* and *P35* co-expression was significantly suppressed by *nmo* knockdown (**Figures 3S–U**). Consistent with genetic interactions between *wnd* and *nmo* in *Drosophila*, we found that upregulation of *MAP3K13* in LUSC and OV are positively correlated with increased expression of NLK (**Supplementary Figure 1**). Yki level reduction would induce apoptosis by downregulating the expression of *diap1* (Huang et al., 2005). Compared with controls (**Figures 3K,K′′**), overexpression of *P35* (**Figures 3L,L′′**) or knockdown of *nmo* (**Figures 3M,M′′**) has no significant changes on apoptosis. However, we also observed non-autonomous apoptosis when Wnd and *P35* are co-overexpressed (**Figures 3N,N′**), which is commonly seen when JNK signaling is hyperactivated (Uhlirova et al., 2005; Perez-Garijo et al., 2013; La Marca and Richardson, 2020). Surprisingly, we found that Nmo inhibition caused a shift toward a strong autonomous apoptosis increase in the *wnd* and *P35* co-expression wing discs (**Figures 3O,O′**). Given that loss of *Yki* activity facilitates cell death (Huang et al., 2005; Liu et al., 2016), we speculate that in the Wnd and *P35* co-expressing wing disc, *nmo* knockdown leads to the downregulation of *Yki* level and therefore increases apoptosis.

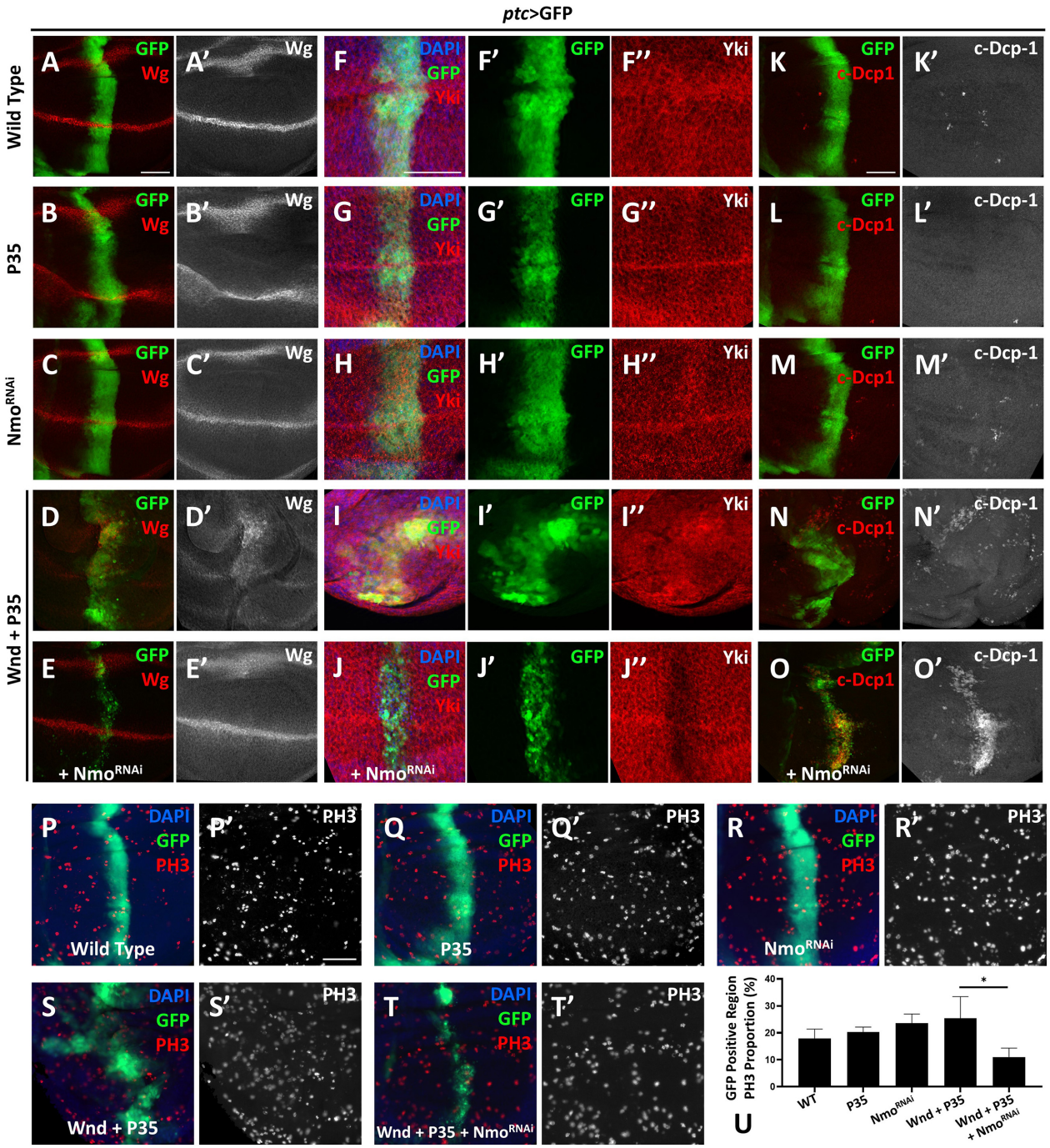

FIGURE 3 | *Nmo* acts genetically downstream of *wnd*. Wing imaginal discs from third instar larva with genes or RNAi expressed in GFP positive area driven by *ptc*-Gal4 are shown. **(A–E′)** Compared with wild type **(A,A′)** or *P35* expression alone control **(B,B′)**, co-expression of *wnd* and *P35* elevates Wingless (Wg) level **(D,D′)**, which is rescued by knocking down Nmo **(E,E′)**, while *nmo* knockdown alone shows little effect **(C,C′)**. **(F–J′′)** Compared with wild type **(F–F′′)** or *P35* expression alone control **(G–G′′)**, co-expression of *wnd* and *P35* enhances *Yki* nuclear localization, which is completely rescued by *nmo* knockdown **(J–J′′)**, while Nmo knockdown alone has little effect **(H–H′′)**. **(K–O)** Compared with wild-type controls **(K,K′)** or *P35* expression alone controls **(L,L′)**, co-expression of Wnd and *P35* induce non-autonomous apoptosis (shown by c-Dcp-1 staining) throughout the wing disc **(N,N′)**. Although *nmo* knockdown alone does not affect apoptosis rate **(M,M′)**, upon Nmo knockdown under Wnd and *P35* co-expression background, fewer apoptosis signals are found outside GFP positive region while severe apoptosis is detected in an autonomous manner **(O,O′)**. **(P–T′)** PH3 staining images are shown to demonstrate cell proliferation. Compared with wild type **(P,P′)** or *P35* expression alone control **(Q,Q′)**, co-expression of *wnd* and *P35* elevates cell proliferation rate **(S,S′)**, which is suppressed by knocking down *nmo* **(T,T′)**, while *nmo* knockdown alone does not show significant difference **(R,R′)**. Scale bars, 50 μm. **(U)** Statistic analysis of the relative PH3 + number in panel P–T. $^*P < 0.05$ (unpaired *t*-test with Welch's correction was used to calculate statistical significance mean + *SD*, $n \geq 4$).

Taken together, these data indicate that Nmo is an essential downstream effector of Wnd in regulating Hippo signaling.

Nmo Is Essential for Impaired Cell Polarity or Rho1-Induced Hippo Inactivation

The data described above show that Nmo is required for Wnd-induced *Yki* activation, and Wnd is required for Rho1 activation and cell polarity-loss-induced Hippo inactivation. Next, we asked whether Nmo is also required for *scrib* loss and *Rho1* activation-induced tissue growth and Hippo inactivation. Expression of *Rho1* in the posterior region of wing discs under the control of *hh*-Gal4 autonomously up-regulates *ex* transcription and induces tissue overgrowth (**Figures 4A–B″**), which are significantly reduced by co-expression of *nmo* and RNAi (**Figure 4C″** and **Supplementary Figure 3**). Consistently, we found that reducing Nmo activity significantly impeded *scrib*. *RNAi* induced *ex-lacZ* upregulation (**Figures 4D′,E′**). Together, these results indicate that Nmo is an essential regulator of impaired cell polarity and *Rho1*-induced Hippo inactivation in *Drosophila* wing disc.

In summary, our data here revealed a novel role of Wnd in promoting cell proliferation through inhibiting the Hippo pathway activity. We further demonstrated that Nmo, a MAPK family kinase, is required for the regulation of the Hippo pathway downstream of Wnd (**Figure 4F**). As a MAPKKK, Wnd is orthologous to both MAP3K13 and MAP3K12 in humans. Studies of these two proteins mainly focus on neuronal development, which is also the main topic of Wnd-related studies, while several studies on MAP3K13 also revealed its relation to tumor development (Han et al., 2016; Edwards et al., 2017; Chen et al., 2018; Wlaschin et al., 2018; Zhang et al., 2020). In this study, we genetically linked Wnd to the Hippo pathway for the first time and provided *in vivo* evidence that Wnd promotes cell proliferation in some contexts. Nmo (NLK) is involved in diverse developmental and cellular processes and was recently identified as a positive regulator of *Yki*/YAP activity (Moon et al., 2017; Daams and Massoumi, 2020). Despite its sequence characteristics as a MAPK, no MAPKK has been reported to phosphorylate NLK, and in contrast it can auto-phosphorylate itself. It is possible that at least in *Drosophila*, a Wnd-Nmo axis may exist. Interestingly, in accordance with our findings, a recent study identified both Wnd and Nmo in the same genetic screen, aiming to reveal the underlying mechanisms of Alk (Anaplastic lymphoma kinase) oncogenic signaling (Wolfstetter et al., 2020). It is also noteworthy that we found *wnd* and *P35* overexpression-induced growth phenotype can be significantly suppressed by co-expression of *wts* (**Supplementary Figure 5**), indicating that an additional regulating mechanism may exist downstream of Wnd. Therefore, we cannot exclude the possibility that Wnd-Nmo could act on *Yki* via the inactivation of Wts or other kinase(s), and further investigation is required to dissect the underlying mechanism of Wnd-mediated Hippo pathway regulation. Taking advantage of the *Drosophila* model, our findings here suggest the exciting prospect that similar mechanisms may exist in human cancer progression, and further investigation is required to elucidate the potential link between MAP3K13-*NLK* axis and polarity loss induced YAP/TAZ activation in tumorigenesis.

MATERIALS AND METHODS

Drosophila Strains

Drosophila stocks were reared on standard media at 25°C unless otherwise indicated. For **Figures 1F–I**, *tub*-Gal80ts was used, flies were first raised at 18°C to restrict Gal4 activity for 5–6 days, then shifted to 29°C for 2–3 days to inactivate Gal80ts. The following strains were used for this study: *ptc*-Gal4, *UAS*-GFP, *UAS*-p35, *ban-lacZ*, *ex-lacZ*, *UAS*-Rho1 (#7334), and *UAS*-wnd.*RNAi* (#27525) were obtained from the Bloomington Stock Center. *UAS-scrib.RNAi* (v27424) and *UAS-nmo.RNAi* (v3002) were collected from the Vienna *Drosophila* Resource Center. *UAS*-Wts was a gift from Shian Wu (Nankai University, Tianjin, China), *UAS*-Wnd (Collins et al., 2006) was a gift from Aaron DiAntonio (Washington University, St. Louis, MO).

Clonal Analysis

Flp-out ectopic expression clones in **Figure 1J** were generated by crossing *UAS*-Wnd; *UAS-p35* with y w *hs*-FLP; *act* > y^+ > Gal4, *UAS*-GFP. Clones were induced at the second instar: heat shock for 10 min at 37°C 48–72 h after egg laying (AEL), dissection were performed 36 h after clone induction.

Immunohistochemistry

Third instar larvae were dissected to collect their wing imaginal discs. Tissues were fixed with 4% formaldehyde and then washed with PBST (PBS + Triton, 1,000:3) for 5 min for 3 times at room temperature (RT). Block in PBST with Goat Serum (Solarbio SL038, 1:10) at RT for 30 min, then treat with 1st antibody at 4°C overnight. The following antibodies were used: rabbit anti-*Yki* (gist from Duojia Pan, 1:500); mouse anti-β-Gal (Promega, 1:500); mouse anti-wingless (DSHB 4D4, 1:100); rabbit anti-cleaved Dcp-1 (CST 9578, 1:100); Rat anti-Ci (DSHB 2A1 1:50); rabbit anti-Phospho-Histone H3 (Ser10) (CST 9701, 1:200). Next, wash the tissue with PBST for 10 min for 3 times at RT, then treat with the second antibody at RT for 2 h. Wash with PBST for 5 min for 3 times. For experiments involving nuclear localization of *Yki* and for **Figure 4**, additional DAPI staining was conducted by treating the tissue with DAPI (Beyotime C1,002) in PBST (1:1,000) at RT for 10 min. Finally, move the tissue onto a slide glass and mount with mounting media with DAPI (VECTASHIELD H-1,800) and then covered by cover glass.

Imaging and Analysis

Wing discs were imaged with the Zeiss Axio Observer microscope. Image J (Fiji) was used to count PH3 the number of positive cells. All statistical analyses were performed using GraphPad Prism 8. The experiments are repeated for at least 4 times except for **Figures 3F–G″**, which is repeated for 3 times. Selected pictures are representative ones. Adobe Photoshop (22.1) was used to process and adjust images. Data were statistically analyzed by the Student *t*-test, the unpaired *t*-test

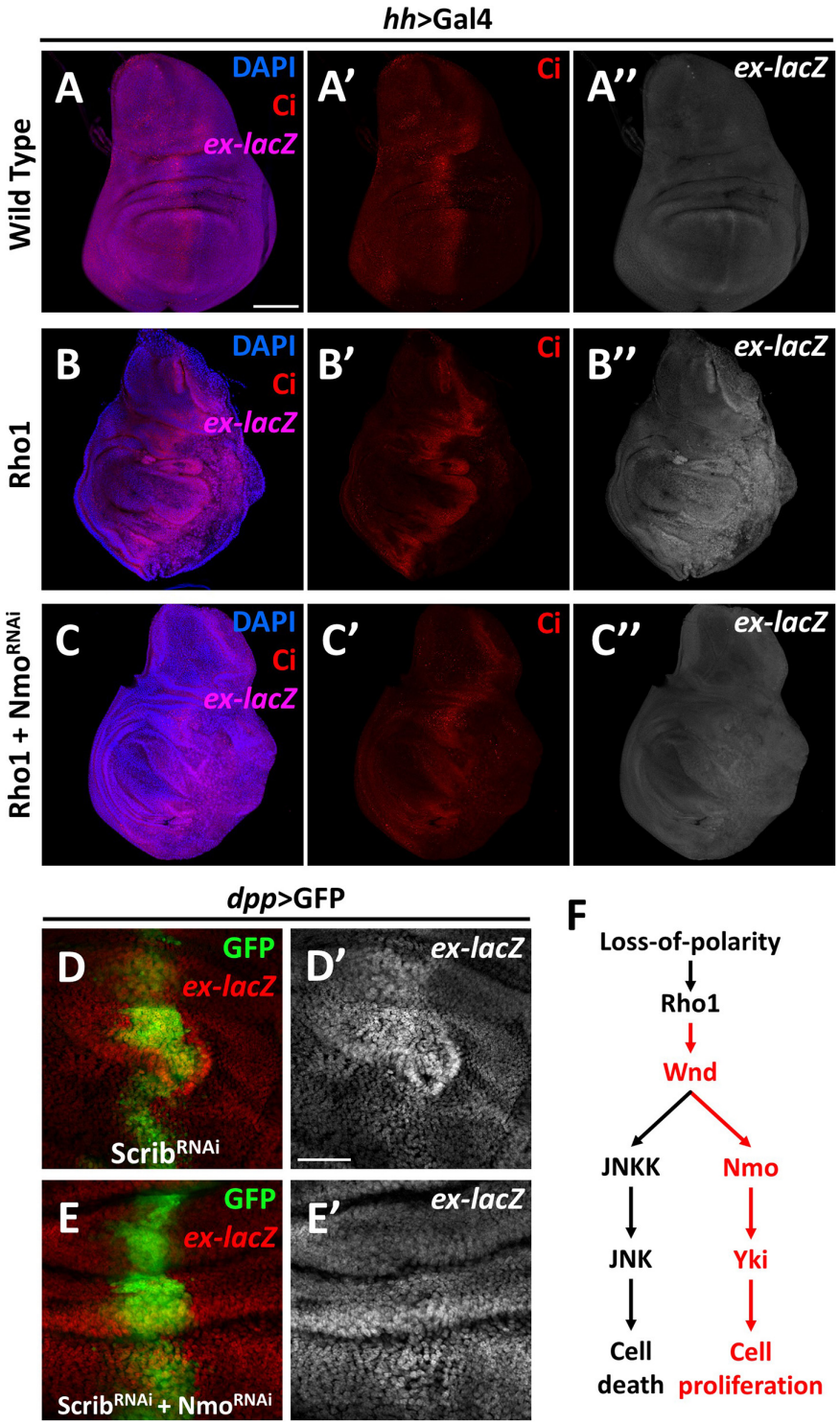

FIGURE 4 | Nmo is essential for loss-of-cell polarity induced growth and *Yki* activation Wing imaginal discs from third instar larva are shown. **(A–C′′)** Gene or *RNAi* is expressed driven by *hh*-Gal4 in the posterior region (opposite to ci-stained zone). Compared with wild type control **(A–A′′)**, expression of *Rho1* elevates *ex-lacZ* expression level **(B–B′′)**, which is suppressed by *nmo* knockdown **(C–C′′)**. **(D–E′)** RNAi lines are driven by *dpp*-Gal4 in the GFP positive region. *scrib* knockdown caused *ex* transcription upregulation **(D,D′)** is rescued by *nmo* knockdown **(E,E′)**. **(F)** A schematic model showing the regulation of Hippo signaling by the Wnd-Nmo axis. Scale bars, 50 μm.

with Welch's correction, or the Kruskal-Wallis test, showed in bar graph as mean + *SD*.

Database Analyses

CNV analysis in **Figure 2A** was processed on the Genomic Data Commons Data Portal[1], a robust data-driven platform for access to cancer data for analysis, based on The Cancer Genome Atlas (TCGA) database. Only gain-of-number results are shown. Prognostic analysis in **Figure 1B** was conducted through The Human Protein Atlas[2], a program aiming to map all the human proteins. This platform automatically found three cancer types that show significant difference in prognosis under low or high MAP3K13 expression level. The selected endometrial cancer result is a representative one. Expression analysis in **Figure 1C** and correlation analysis in **Figure 1D** was carried out by using the GEPIA[3], an interacting web server to analyze RNA sequencing data provided by TCGA and Genotype-Tissue Expression (GTEx). We choose LUSC and OV for expression analysis and LUSC for correlation analysis because these two cancer types are the top two hits in the CNV analysis.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: The Cancer Genome Atlas (TCGA) (https://tcga-data.nci.nih.gov/tcga/; dbGaP accession number: phs000178.v1.p1).

[1] https://portal.gdc.cancer.gov

[2] https://www.proteinatlas.org

[3] http://gepia.cancer-pku.cn/index.html

AUTHOR CONTRIBUTIONS

XM conceived the study. XW, HL, and WX performed the experiments. XW, HL, and XM analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.658288/full#supplementary-material

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MINI REVIEW
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Using Biosensors to Study Protein–Protein Interaction in the Hippo Pathway

*Alexander Pipchuk and Xiaolong Yang**

Department of Pathology and Molecular Medicine, Queen's University, Kingston, ON, Canada

The Hippo signaling network is dependent on protein–protein interactions (PPIs) as a mechanism of signal transduction to regulate organ size, cellular proliferation and differentiation, tumorigenesis, and other cellular processes. Current efforts aim to resolve the complex regulation of upstream Hippo components or focus on identifying targeted drugs for use in cancer therapy. Despite extensive characterization of the Hippo pathway interactome by affinity purification mass spectrometry (AP-MS) and other methodologies, previous research methods have not been sufficient to achieve these aims. In this review, we describe several recent studies that make use of luciferase-based biosensors as a new approach to study the Hippo Pathway. These biosensors serve as powerful tools with which to study PPIs both *in vitro* using purified biosensor proteins, and in real time in live cells. Notably, luciferase biosensors have excellent sensitivity and have been used to screen for upstream kinase regulators of the Hippo pathway. Furthermore, the high sensitivity and stability of these biosensors enables their application in high throughput screening for Hippo-targeted chemotherapeutics. Finally, we describe the strengths and weaknesses of this method relative to AP-MS and discuss potential future directions for using biosensors to study Hippo signaling.

Keywords: Hippo pathway, biosensor, luciferase, protein–protein interaction, NanoBiT, NanoLuc

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University of California, Irvine, United States

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Xu Li,
Westlake University, China
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University of Nebraska Medical Center, United States

***Correspondence:**
Xiaolong Yang
yangx@queensu.ca

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INTRODUCTION

The Hippo Pathway

The Hippo pathway is an evolutionarily conserved signaling cascade that plays central roles in human physiology and disease (Pan, 2010; Ma et al., 2019). This signaling pathway has been connected to a wide variety of processes, including development (e.g., organ size control, early embryogenesis, skeletal development) (Kegelman et al., 2020; Wu and Guan, 2021), tissue

Abbreviations: AP-MS, affinity purification mass spectrometry; BRET, bioluminescence resonance energy transfer; BRET^n, BRET platform developed by Mo et al. that uses NanoLuc as a luciferase donor; CLuc, C-terminal segment of firefly luciferase in a split luciferase system; FLuc, firefly luciferase; LgBiT, large luciferase constituent of NanoLuc for use in split luciferase complementation system; NanoBiT, NanoLuc binary technology i.e., a split luciferase system derived from NanoLuc; NanoLuc, a luciferase enzyme derived from *Oplophorus gracilirostris*; NLuc, N-terminal segment of firefly luciferase in a split luciferase system; PPI, protein–protein interaction; SmBiT, Small luciferase constituent of NanoLuc for use in s split luciferase complementation system; YAP15, 15 amino acid segment of the YAP1 protein containing the consensus LATS phosphorylation site at serine-127.

homeostasis (regeneration, fibrosis, cell proliferation, cell death, differentiation, and stem cell renewal) (Kim et al., 2019; Cao et al., 2020; Dey et al., 2020), mechanotransduction (Meng et al., 2016; Misra and Irvine, 2018; Ma et al., 2019; Zhang et al., 2020), cardiovascular development and disorders (cardiomyocyte proliferation and cardiac injury) (Moya and Halder, 2019; Chen et al., 2020), diabetes (e.g., insulin/glucose metabolism, β-cell function) (Ardestani and Maedler, 2018; Ardestani et al., 2018), neurodegenerative disease (neuronal apoptosis) (Sahu and Mondal, 2020a,b), and cancer progression and therapy (tumorigenesis, angiogenesis, metastasis, immune response, and drug resistance) (Visser and Yang, 2010; Lai et al., 2012; Zhao and Yang, 2015, 2019; Janse van Rensburg and Yang, 2016; Yeung et al., 2016; Taha et al., 2018; Wu and Yang, 2018; Azad et al., 2019).

The canonical Hippo pathway (**Figure 1**), first identified in *Drosophila* and later in mammals (Xu et al., 1995; St John et al., 1999), is depicted as two kinases that act in series to regulate downstream co-activators of transcription. In mammals, upstream regulators activate the MST1/2 kinases, which in turn mediate phosphorylation of the LATS family of kinases (Chan et al., 2005). The MST1/2-LATS interaction is facilitated by the SAV1 and MOB1 adaptor proteins (Bae and Luo, 2018). Phosphorylation of LATS results in its activation and subsequent phosphorylation of two paralogous transcriptional coactivators: YAP and TAZ (YAP/TAZ). Phosphorylated YAP/TAZ is sequestered in the cytoplasm and is unable to associate with the TEAD family of transcription factors in the nucleus to direct transcription of Hippo-associated genes. Notably, the YAP/TAZ-TEAD interaction has been identified as a promising drug target for molecular therapeutics (Liu-Chittenden et al., 2012; Johnson and Halder, 2014; Zhou et al., 2015; Wu and Yang, 2018).

Regulation of the Hippo pathway extends far beyond the canonical kinase cascade (Yu and Guan, 2013). Several MAP4K family proteins (Meng et al., 2015), NF2/merlin (Hamaratoglu et al., 2006; Zhang et al., 2010; Yin et al., 2013), angiomotin (Zhao et al., 2011; Li et al., 2015), kibra (Yu et al., 2010), and expanded (Hamaratoglu et al., 2006) have also been established as influential regulators of Hippo signaling. Adding further complexity, the Hippo pathway is engaged in crosstalk with other signaling cascades at the level of YAP and TAZ (Piccolo et al., 2014; Totaro et al., 2018; Zhao et al., 2018), and several mass spectrometry analyses have revealed an extensive Hippo pathway interactome (Couzens et al., 2013; Kwon et al., 2013; Wang et al., 2014). Overall, the large network of dynamic protein–protein interactions (PPIs) in the Hippo pathway has made it difficult to study using traditional techniques.

Luciferase Biosensors

Bioluminescence is the process whereby light is produced and emitted by a living organism. This phenomenon, which is distinct from fluorescence, famously occurs in fireflies and many species of marine animals. At the chemical level, the light-emitting reaction is catalyzed by a luciferase enzyme in the presence of its substrate, a luciferin. The light produced can be easily measured by a photometer, allowing for non-invasive, real time monitoring of cellular processes. As such, luciferase-based technologies hold a broad range of utility in cancer and molecular biology research, with applications for use as a reporter of gene expression, a marker of cellular proliferation, *in vivo* tumor imaging, and more (Badr and Tannous, 2011; Xu et al., 2016).

Additionally, split luciferase systems allow for the development of complementarity assays to measure protein-protein interactions (**Figures 2A,B**; Azad et al., 2014). In principle, a luciferase enzyme is divided into two component parts, abolishing its activity. Each of the two luciferase constituents can be fused onto two interacting proteins. Upon PPI between these recombinant proteins, the luciferase components reform a functional enzyme capable of emitting light in the presence of its substrate (**Figures 2A,B**). Therefore, these systems enable non-invasive, real time monitoring of PPIs and provide several key advantages over previous methods of PPI detection.

Our lab has recently developed several split luciferase systems to study PPIs within the Hippo pathway. Using the same luciferase-based technology, another group has developed a separate Hippo pathway biosensor platform that differs mechanistically from our system but may be used for many of the same purposes. In this review, we describe how these systems were designed and what they have since been used to accomplish. We discuss the advantages and disadvantages of these systems over other methods of studying PPIs, and, importantly, explore future directions for the application of this technology to study Hippo signaling.

BODY

Firefly Luciferase-Based LATS Biosensor

Historically, split luciferase biosensors have been derived from enzymes present in firefly or renilla species. In 2018, we presented research that made use of a firefly luciferase (FLuc)-based LATS biosensor to identify VEGFR as an upstream regulator of Hippo signaling (Azad et al., 2018). For this work, an N terminal segment of FLuc (called NLuc, consisting of amino acids 1-416) was fused with a 15 amino acid segment of the YAP protein (YAP15, consisting of residues 120-134). YAP15 contains the consensus LATS substrate identification sequence (HxH/R/KxxS/T; H-histidine; R-arginine; K-lysine; S-serine; T-threonine) and a critical LATS1/2 kinase phosphorylation site, S127 (Zhao et al., 2007; Hao et al., 2008). Upon S127 phosphorylation by LATS, YAP associates with 14-3-3 proteins in the cytoplasm and is prevented from binding to nuclear transcription factors (Zhao et al., 2007). As such, the C-terminal segment of FLuc (CLuc) was cloned onto 14-3-3.

Upon phosphorylation of NLuc-YAP15 ("A") and association with CLuc-14-3-3 ("B"), the luciferase constituents reform a functional enzyme that emits light in the presence of the luciferin substrate (**Figure 2A**). The emitted light intensity can be measured by a luminometer to quantify protein–protein

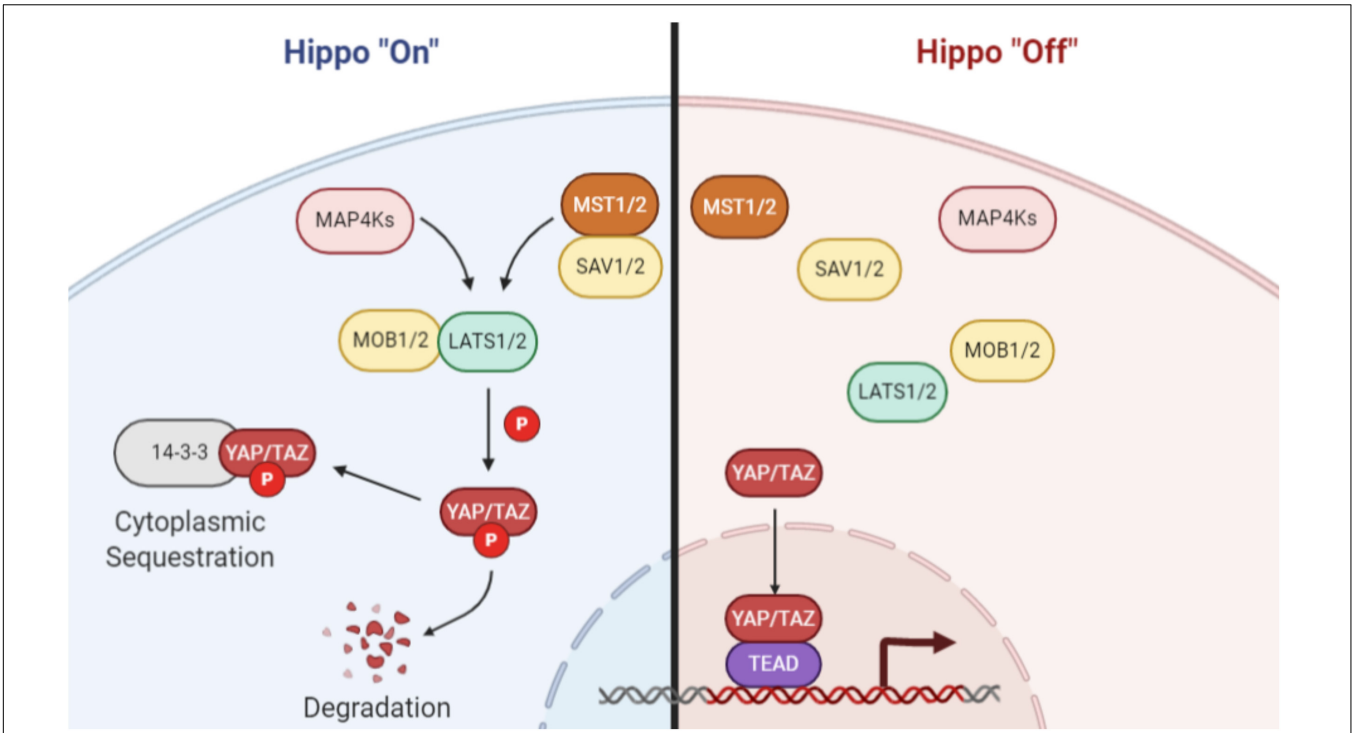

FIGURE 1 | Canonical Hippo signaling. When Hippo signaling is activated, MAP4 kinases or the MST1/2 kinases phosphorylate and activate the LATS family of kinases. The MST/LATS interaction is facilitated by MOB and SAV adaptor proteins. Activated LATS kinases subsequently phosphorylate the YAP and TAZ paralogs, which then associate with 14-3-3 proteins and are sequestered in the cytoplasm or degraded. When Hippo signaling is inactive, YAP/TAZ are not phosphorylated and are free to translocate to the nucleus, where they act as coactivators of transcription through interaction with TEAD family and other transcription factors. Image created with BioRender.com.

interaction between YAP15 and 14-3-3. Thus, the NLuc-YAP15 and CLuc-14-3-3 fusion proteins, when co-expressed, constitute a biosensor for LATS activity. This LATS biosensor responded as expected to the overexpression or inhibition of upstream Hippo pathway components, showing increased bioluminescent activity when co-transfected with MST, and decreased luminescent activity upon inhibition of the Hippo pathway. The LATS biosensor was further validated by mutating 3 distinct residues on the consensus LATS binding motif (H, R, and S), all of which abolished luminescent activity and S127 phosphorylation. Significantly, the LATS biosensor was also used to monitor LATS activity in vivo in a xenograft tumor mouse model. Overall, the validation procedures convincingly established the LATS biosensor as an accurate method to assess LATS kinase activity both in vitro and in vivo through quantitation of YAP and 14-3-3 interaction.

Furthermore, the FLuc LATS biosensor was used to screen for upstream kinase regulators of the Hippo pathway (Azad et al., 2018). In this approach, the LATS biosensor components were transfected into a cell line that was subsequently exposed to 80 distinct kinase inhibitors. The results of the screen revealed 6 kinase inhibitors that activated the biosensor, some of which targeted kinases that had previously been established as Hippo pathway regulators. Ultimately, VEGFR was identified as a novel upstream LATS regulator. Mainly, this study demonstrates the application of split luciferase systems as a powerful tool to study complex signaling networks and facilitate the discovery of novel regulators.

Improving the LATS Biosensor Using NanoLuc Binary Technology

We improved upon the LATS biosensor in a subsequent iteration using NanoLuc binary technology (NanoBiT). NanoLuc luciferase is derived from the deep-sea shrimp *Oplophorus gracilirostris* and holds several advantages over firefly or renilla based technologies (Hall et al., 2012; England et al., 2016). NanoLuc is roughly three-fold smaller than firefly luciferase, potentially limiting steric inhibition of PPIs in the context of a split luciferase assay. Also, this luciferase is ATP-independent and, importantly, shows improved thermal stability at 37°C. Finally, NanoLuc is over 100-fold brighter than other luciferase enzymes, enabling the development of more sensitive assays. In the NanoBiT system, NanoLuc luciferase is split into two components: the 18 kDa Large BiT (LgBiT) and the 1.3 kDa, 11 amino acid Small BiT (SmBiT; **Figure 2B**). Similar to other split luciferase systems (**Figure 2A**), these components have been extensively engineered such that their association is dictated by the interaction of the target proteins to which they are attached (Dixon et al., 2016). In other words, the NanoBiT constituents SmBiT and LgBiT do not associate unless they are brought into proximity of each other by PPI between the target proteins. This

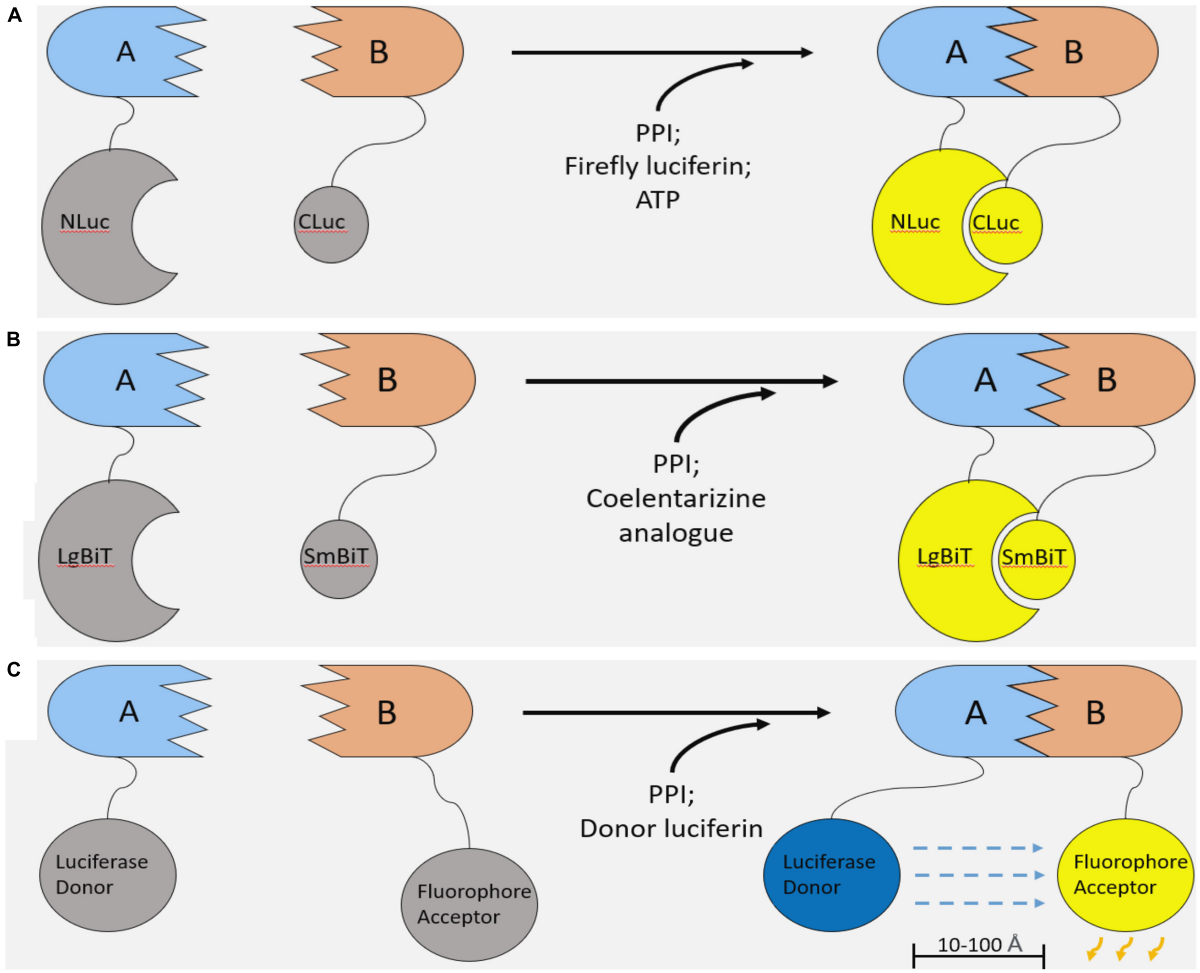

FIGURE 2 | Luciferase-based methods of studying protein–protein interaction. **(A)** Firefly Luciferase complementation system. Two components of the firefly luciferase are fused with two interacting proteins. Upon PPI between the fusion proteins, luciferase complementation occurs, and, in the presence of a luciferin substrate and ATP, light is emitted. **(B)** NanoBiT system. The NanoBiT system is a split luciferase technology derived from a deep-sea shrimp. It is comprised of the 18kDa LgBiT and 1.3 kDa SmBiT. The main advantages of NanoBiT over other split luciferase systems are its small size, high sensitivity, and improved thermal stability. **(C)** Bioluminescence resonance energy transfer (BRET). Like split luciferase complementation assays, BRET is another mechanism of quantifying PPIs in real time. A functional luciferase donor and fluorophore acceptor are fused onto an interacting pair of proteins. Upon PPI, the fluorophore and luciferase are brought into proximity (10–100 Å). If the conditions are met for the luciferase to emit light (i.e., the presence of a luciferin and any necessary cofactors), the bioluminescent light will excite the fluorophore, which can emit light of a different wavelength.

allows for accurate and sensitive measurement without drastically altering the dynamics of a given PPI.

To improve upon the FLuc-LATS biosensor, the LgBiT constituent was fused with YAP15 (Protein "A"), and SmBiT was attached to 14-3-3 (Protein "B", **Figure 2B**; Nouri et al., 2019a). This NLuc-LATS biosensor indeed showed ∼150-fold increased luminescent intensity and improved thermal stability when compared to the FLuc-Lats biosensor. Following validation, the NLuc-LATS biosensor was then used to conduct an expanded, kinome wide high throughput screen for upstream kinase regulators. Of 560 compounds screened, 54 kinase inhibitors increased the bioluminescent signal more than two-fold compared to controls treated with DMSO. Like the FLuc-LATS biosensor screen, many of these compounds targeted kinases that had previously been established as Hippo pathway regulators, including EGFR (Fan et al., 2013), PI3K (Fan et al., 2013; Zhao et al., 2018), MAPK (Meng et al., 2015), and VEGFR inhibitors (repeating the findings from the FLuc-LATS biosensor screen). Ultimately, anaplastic lymphoma kinase (ALK) was identified and validated as a novel Hippo pathway regulator acting through LATS to affect YAP/TAZ activity (Nouri et al., 2019a).

NanoLuc-Based YAP-TEAD Biosensor and Small Molecule Screening

Following the development and application of both LATS biosensors, a second NanoBiT system was built to monitor the interaction between YAP and the TEAD family of transcription factors (Nouri et al., 2019b). Repressing the transcriptional

output of the Hippo pathway by inhibiting this interaction is a promising avenue for cancer therapy (Liu-Chittenden et al., 2012; Johnson and Halder, 2014; Zhou et al., 2015). Indeed, several peptides and small molecule inhibitors of the YAP/TEAD interaction have been identified as potential therapeutics (Liu-Chittenden et al., 2012; Pobbati et al., 2015; Song et al., 2018). However, none have advanced to clinical trials due to low stability *in vivo*, low cell permeability, or other drawbacks. As such, we developed a YAP-TEAD NanoBiT biosensor to enable high throughput screening for new small molecule inhibitors (Nouri et al., 2019b).

Similar to both iterations of the LATS biosensors, the YAP-TEAD biosensor made use of protein fragments based on structural insights of the YAP-TEAD interface (Li et al., 2010). Of note, extensive analysis was conducted to determine the optimal orientation of this biosensor. There are 8 possible orientations of a YAP-TEAD biosensor if the NanoBiT constituents are cloned onto the N or C-termini of the YAP and TEAD protein fragments (2 proteins—YAP and TEAD, by 2 luciferase constituents—SmBiT and LgBiT, by 2 termini). As such, 8 versions of the YAP-TEAD biosensor representing each of the distinct orientations were cloned. Ultimately, SmBiT-YAP and LgBiT-TEAD (SmBiT and LgBiT linked to the N-termini of YAP and TEAD, respectively) showed the highest luminescent signal. While most versions of the YAP-TEAD biosensor displayed an easily detectable luminescent signal, optimal placement of the luciferase constituents is an important consideration when using split luciferase systems.

After validation, the YAP-TEAD biosensor was used in a large-scale screen (2,688 compounds) for small molecule inhibitors of this interaction (Nouri et al., 2019b). Seventy-one compounds decreased the luminescent activity more than two-fold. Several follow-up screening protocols further refined the list of candidate YAP-TEAD inhibitors. The top hit from these secondary screens, celastrol, was validated as an anti-cancer agent in breast and lung cancer cell lines. Notably, secondary screening that did not make use of the NanoBiT biosensor was an important step in the validation procedures; some false positives were attributed to compounds that exert a non-specific quenching effect on the luciferase component of the biosensor, rather that inhibition of the PPI of interest. Nevertheless, a biosensor-based approach to enable high throughput drug screening proved to be extremely effective.

BRET and AP-MS to Study Protein–Protein Interaction in the Hippo Pathway

Bioluminescence resonance energy transfer (BRET) is a similar method of studying PPIs (Boute et al., 2002; Pfleger and Eidne, 2006). Like split luciferase systems, BRET relies on protein fusion with two binding partners of an interaction pair. In contrast, BRET involves the transfer of light from a functional luciferase donor to a fluorophore acceptor that emits light of a different wavelength (**Figure 2C**), rather than complementation of two non-functional luciferase components (Boute et al., 2002; Pfleger and Eidne, 2006). Overall, it is difficult to declare the superiority of one of these approaches over the other; both systems are highly sensitive and give real time information on PPIs.

Promega Corporation has developed a BRET system that also makes use of NanoLuc luciferase (Machleidt et al., 2015). A separate group has created a distinct NanoLuc-based BRET platform, dubbed BRET^n, that uses a different fluorophore (Mo et al., 2016). Following validation of BRET^n, this group applied their system in a high throughput screen for small molecule inhibitors of PRAS40 dimerization. Furthermore, the BRET^n platform was used to map out a small Hippo pathway interactome by cloning the donor (NanoLuc) and acceptor (a yellow fluorescent protein variant) in various configurations onto established Hippo pathway components (RASSF1, MST1, LATS2, YAP1, TEAD2, and 14-3-3) (Mo et al., 2016). The BRET^n system correctly identified several established Hippo pathway PPIs and proposed two novel PPIs: LATS2 homodimerization and RASSF1-LATS2 interaction. In essence, a network of biosensor fusion proteins was used to characterize the interactome of several Hippo pathway components.

Initial characterization of the Hippo pathway interactome was accomplished by affinity purification mass spectrometry studies from 3 separate groups in 2013 and 2014 (Couzens et al., 2013; Kwon et al., 2013; Moya and Halder, 2014; Wang et al., 2014). AP-MS relies on the purification and characterization of protein complexes bound to tagged 'bait' proteins. These 3 studies used established components of the canonical Hippo pathway as bait proteins to reveal an extensive interactome in both drosophila (Kwon et al., 2013) and humans (Couzens et al., 2013; Wang et al., 2014). Furthermore, Couzens et al. supplemented their AP-MS findings using a biotin labelling approach and investigated changes in the Hippo pathway interactome associated with phosphatase inhibition by okadaic acid (Couzens et al., 2013). Overall, these analyses provide an excellent overview of the Hippo PPI network.

Both AP-MS and split luciferase/BRET based methodologies provide unique insights and hold several key advantages over the other method. In general, AP-MS is better suited to large-scale characterization of interactomes to provide a high-level overview of a PPI network, whereas luciferase complementation or BRET can be used for more extensive study of individual PPIs. Mainly, this is because luciferase methods require molecular cloning or genome editing of both interaction partners, whereas AP-MS only requires tagging of 1 "bait" protein. Therefore, only one or a select few interactions can be studied easily with luciferase methods. While this does not allow for extensive or wholistic characterization of an interactome, a sensitive biosensor screen can be used to discover both direct and indirect upstream regulators of one (or a select few) high interest PPIs (Azad et al., 2018; Nouri et al., 2019a).

The main advantages of split luciferase or BRET methods are operational simplicity, high sensitivity, and the capacity to give real time information in live cells. AP-MS requires substantial expertise and familiarity with the pipeline for analysis, whereas luciferase techniques require only molecular cloning or genome editing and some specialized equipment to yield easily interpretable results. Consequently, split luciferase or BRET

techniques are more accessible. In addition, luciferase assays are better suited for thorough characterization of one, high-interest PPI because of their excellent overall sensitivity. Also, the ability to purify fusion protein biosensors is incredibly useful for high throughput drug screening. Finally, many luciferase substrates are permeable to the cell membrane. This allows for *in vivo* study of PPIs in a time and space dependent manner that accounts for cellular compartmentalization.

FUTURE DIRECTIONS, CURRENT LIMITATIONS, AND CONCLUSIONS

Both BRET and split luciferase complementation assays are powerful tools to facilitate future study of protein–protein interaction in the Hippo pathway. The recent development of NanoLuc luciferase, demonstrating extremely high sensitivity and improved thermal stability, has enabled the implementation of these systems for increasingly useful applications. Namely, for high throughput screening processes and for sensitive characterization of small protein network interactomes.

On the immediate horizon for future studies is the development of biosensors to monitor the interaction of other Hippo pathway PPIs of interest. For example, the LATS–ITCH interaction is a crucial regulator of LATS stability that plays an important role in proliferation of breast cancer cells, and is therefore an attractive drug target for molecular chemotherapeutics (Ho et al., 2011; Yeung et al., 2013). A NanoBiT-LATS/ITCH biosensor could be used to screen for small molecule inhibitors of this interaction for breast cancer therapy. Since the Hippo pathway also plays important roles in fibrosis, wound healing, and tissue regeneration, inhibition of core Hippo kinase activity may be desirable in the context of regenerative therapies (Moya and Halder, 2019; Dey et al., 2020). In particular, Hippo pathway inhibition to promote YAP/TAZ activity shows significant potential in activating heart repair mechanisms following myocardial damage (Xin et al., 2013; Leach et al., 2017; Zheng et al., 2020). Biosensor screens could also be used to facilitate the discovery of small molecules for this purpose. Also worth consideration, luciferase technologies may prove useful for studying the influence of dynamic mechanical cues on Hippo pathway activity. For example, YAP and TAZ signaling is altered in response to disturbed blood flow (Wang et al., 2016). A YAP/TAZ-TEAD or LATS biosensor could be a useful tool to study the influence of shear stress on Hippo pathway activity in the context of a dynamic, *in vitro* blood flow model, with implications for better characterization of the role of Hippo signaling in vasculature disease.

In addition, luciferase technology has promising applications for *in vivo* imaging of molecular processes. Recently, a firefly based split luciferase complementation assay was used to monitor GPCR signaling *in vivo* (Kono et al., 2017). Luciferase activity was responsive to inhibitors of the interaction being studied, meaning that split luciferase assays could potentially be used to assess the efficacy of targeted molecular therapeutics in pre-clinical animal models. Notably, the NanoBiT complementation system is not suitable for *in vivo* imaging due to its short emission wavelength of approximately 460 nm (Hall et al., 2012). This is where BRET based systems are preferred; many fluorophores have been engineered to fluoresce at a more red-shifted emission spectrum, which is better for penetrating tissue (Yuan et al., 2013). Hiblot et al. have recently developed a system of NanoLuc-BRET biosensors with a range of emission maxima from 480 to 680nm which could perhaps be applied for *in vivo* imaging (Hiblot et al., 2017).

Historically, the limitations of luciferase technologies have been low stability and large size. However, NanoBiT technology presents significant improvements in both of these areas while also showing improved sensitivity (England et al., 2016). Other than a low emission spectrum that is not optimal for *in vivo* imaging, the primary limitation of NanoBiT is its current cost. Furimazine, the NanoLuc substrate, is not generically available and therefore the cost of the NanoLuc platform is higher than other luciferase systems. Perhaps the discovery of new NanoLuc substrates will decrease the cost in the future. In addition, an important generic consideration for split luciferase assays is the requirement for fusion protein generation, introducing the potential for alteration of protein function or steric inhibition of the endogenous PPI. This limitation can be mitigated by using a small luciferase and by testing of different biosensor orientations. Furthermore, new luciferases or continued engineering of NanoLuc could potentially yield an even smaller enzyme.

In summary, this review presents an exciting new approach to studying protein–protein interaction in the Hippo pathway using luciferase-based biosensors. Namely, the main advantages of these systems are operational simplicity, high sensitivity, and the capacity to transmit reliable information in real time. These qualities lend themselves extremely well to high throughput screening processes, both for upstream kinase regulators and for targeted molecular therapeutics. Beyond their applications for screening, luciferase-based biosensors are convenient and easy to use. Future studies could look to apply this approach for expanded screens with novel biosensors, study of mechanical transduction, or for *in vivo* imaging.

AUTHOR CONTRIBUTIONS

AP wrote the manuscript. XY revised the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Hippo Signaling Pathway in Pancreas Development

*Yifan Wu[1,2], Pauline Aegerter[1], Michael Nipper[1], Logan Ramjit[1], Jun Liu[1] and Pei Wang[1]**

[1] *Department of Cell Systems and Anatomy, The University of Texas Health San Antonio, San Antonio, TX, United States,*
[2] *Department of Obstetrics, The Second Xiangya Hospital, Central South University, Changsha, China*

The Hippo signaling pathway is a vital regulator of pancreatic development and homeostasis, directing cell fate decisions, morphogenesis, and adult pancreatic cellular plasticity. Through loss-of-function research, Hippo signaling has been found to play key roles in maintaining the proper balance between progenitor cell renewal, proliferation, and differentiation in pancreatic organogenesis. Other studies suggest that overactivation of YAP, a downstream effector of the pathway, promotes ductal cell development and suppresses endocrine cell fate specification via repression of Ngn3. After birth, disruptions in Hippo signaling have been found to lead to de-differentiation of acinar cells and pancreatitis-like phenotype. Further, Hippo signaling directs pancreatic morphogenesis by ensuring proper cell polarization and branching. Despite these findings, the mechanisms through which Hippo governs cell differentiation and pancreatic architecture are yet to be fully understood. Here, we review recent studies of Hippo functions in pancreatic development, including its crosstalk with NOTCH, WNT/β-catenin, and PI3K/Akt/mTOR signaling pathways.

Keywords: Hippo, pancreas, development, Mst1/2, LATS1/2, YAP

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Wenqi Wang,
University of California, Irvine,
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Christopher Halbrook,
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Philippa Francis-West,
King's College London,
United Kingdom

****Correspondence:***
Pei Wang
wangp3@uthscsa.edu

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INTRODUCTION

Hippo signaling plays an important role in regulating cell proliferation and apoptosis; thus, it is thought to be a central regulator of organ size and tissue homeostasis. A complex network of biological processes has been shown to regulate the Hippo signaling pathway, including determinants of cell polarity and cell-cell junctions, factors mediating the activation of Hippo kinases, mechanotransduction, soluble factors acting through G-protein-coupled receptors (GPCRs) and Rho GTPases, and metabolic pathways mediating YAP/TAZ nuclear localization (mevalonate pathway) or their binding to TEAD factors (glucose metabolism and aerobic glycolysis) (Maugeri-Sacca and De Maria, 2018). These pathways are all important for organogenesis, asserting the essential roles of the Hippo pathway in the development of an organism (Zheng and Pan, 2019). Defective Hippo signaling can lead to pancreatic dysfunction such as defects in both embryogenesis and postnatal development. This review aims to outline the roles of the Hippo signaling pathway in pancreatic development.

The pancreatic developmental process begins with two outpouchings arising from the distal foregut endoderm, forming into the dorsal and ventral pancreatic buds. Meanwhile, the organ must properly form acinar cells, ductal cells, and five different endocrine cell lineages. Pancreatic tubulogenesis is a complex process that must be regulated by a network of transcription factors and signaling pathways (Jennings et al., 2015; Fujitani, 2017). As an essential organ for metabolic regulation, dysfunction of the endocrine pancreas causes serious chronic metabolic diseases,

such as diabetes mellitus. On the other hand, damage to the exocrine pancreas can lead to pancreatitis and pancreatic cancer. Possible treatment strategies for these debilitating diseases include the generation and transplantation of pancreatic endocrine cells, reprogramming the original pancreatic cells (Zhou and Melton, 2018), or reducing inflammation to subsequently revert metaplastic cells back to normal cells. Understanding how the pancreas develops with correct organ size, composition, and architecture will provide insights into these diseases. In this review, we summarize recent knowledge of the role of Hippo signaling in pancreatic development, discuss the importance of crosstalk with other signaling pathways, and explore future directions of investigation.

AN OUTLINE OF PANCREATIC DEVELOPMENT

The mammalian pancreas is a dual-function organ that is essential for proper digestion and subsequent energy consumption. The exocrine pancreas is known to produce digestive enzymes (lipases, proteinases, and amylases), which are secreted by acinar cells and delivered to the small intestine by a branched ductal network. The endocrine pancreas is key to the maintenance of glucose homeostasis through five kinds of hormone-producing cells (Shih et al., 2013). These endocrine cells cluster in the islets of Langerhans and include α-, β-, δ-, PP-, and ϵ- cells that synthesize glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin, respectively. Pancreatic development has been reviewed in detail (Benitez et al., 2012; Bastidas-Ponce et al., 2017; Larsen and Grapin-Botton, 2017). Here, we provide a brief review of pancreatic development to help contextualize the role of Hippo pathway in it.

Early pancreatic organogenesis can be divided into two transitional stages: the primary transition occurs during E9.0-E12.5 while the secondary transition occurs during E13.5-E16.5 (**Figure 1A**; Bastidas-Ponce et al., 2017). The primary transition is marked by the specification and proliferation of pancreatic progenitors, accompanied by some development of glucagon-producing cells. Importantly, the number of multipotent pancreatic progenitors developed during the primary transition correlates with final organ size (Seymour, 2014). Prior to the onset of the primary transition (E8.5-E9.0), *Pdx1* (pancreatic and duodenal homeobox 1) expression marks the pre-pancreatic endoderm. In a *Pdx1*-null pancreas, the dorsal bud develops normally until approximately E10.5, is smaller by E11.0, and fails to grow past E11.5, while the ventral bud never invaginates (Hale et al., 2005). *Pdx1* also regulates pancreatic tubulogenesis and E-cadherin expression (Marty-Santos and Cleaver, 2016). Lastly, *Pdx1* can negatively regulate the expression of pancreatic ductal cell-specific keratin 19, which leads to the inhibition of the ductal differentiation program within the pancreatic endocrine compartment, particularly in β cells (Deramaudt et al., 2006). Because of these influential roles, *Pdx1* is often termed a "master regulator" of whole pancreatic development (**Figure 1A**; Vinogradova and Sverdlov, 2017).

Specification and differentiation of pancreatic cells during the primary transition is further guided by a complex network of additional transcription factors (Arda et al., 2013). One such transcription factor, *Ptf1a*, is expressed in the early pancreas, directing the expansion of multipotent progenitor cells (MPCs) along with the specification of acinar cells, and is finally restricted to acinar cells (Jin and Xiang, 2019). *Sox9*, which is also required for maintenance of the pancreatic progenitor cell pool, is critical in gene regulation as well (Seymour et al., 2007; Seymour, 2014). Within the human fetal pancreas, *Sox9* is important for the expression of *Ngn3* and other molecular markers of endocrine cell differentiation (McDonald et al., 2012).

During the secondary transition of pancreatic organogenesis, all five hormone-producing endocrine cells begin to develop, and amylase-producing acinar cells arise from the extending tip epithelium. During this transition, basic helix-loop-helix transcription factor neurogenin 3 (*Ngn3*) drives bipotent pancreatic progenitor cells toward the endocrine cell fate (Gradwohl et al., 2000; McGrath et al., 2015). Furthermore, biphasic expression of *Ngn3* correlates with the "first" and "second" transitions, which encompass two distinct waves of embryonic endocrine differentiation (Villasenor et al., 2008). Not only is *Ngn3* a master regulator of pancreatic islet differentiation and regeneration, but it also initiates stepwise delamination of differentiating endocrine cells during pancreatic development (Rukstalis and Habener, 2009; Gouzi et al., 2011). Lastly, achieving high levels of *Ngn3* expression is a critical step for endocrine commitment from multipotent pancreatic progenitors (Wang et al., 2010; Magenheim et al., 2011).

A "third" transition has been proposed by some researchers, which occurs from E16.5-E19. During this time, endocrine cells migrate and cluster into islets of Langerhans while acinar cells further expand. However, the signaling pathways guiding the formation of the islets of Langerhans are not fully understood. Starting from the secondary transition, committed endocrine cells leave the ductal epithelium, migrate into the surrounding mesenchyme to coalesce into proto-islets, and finally develop into functional islets of Langerhans. The process is regulated by the spatiotemporal activities of various signaling factors along with coordinated cell dynamics, such as the crosstalk between endothelial and mesenchymal cells (Bastidas-Ponce et al., 2017).

As β cells exit the epithelial progenitor cell layer, they acquire some mesenchymal characteristics (Cole et al., 2009). Cdc42-mediated tubulogenesis controls cell specification by providing the correct micro-environment, and it links actin dynamics to pancreatic β cell delamination and differentiation (Kesavan et al., 2009; Kesavan et al., 2014). Snail2/Slug, a known inducer of epithelial to mesenchymal transition (EMT) and cell movement, plays a vital role in endocrine cell delamination and migration as well (Rukstalis and Habener, 2007; Lee et al., 2011). The transcription co-repressor Grg3/Tle3 promotes the delamination of endocrine progenitors along with β cell differentiation (Morris and Machesky, 2015). During β cell development, EphB3 can mark delaminating endocrine progenitors and help define the timeframe of endocrine differentiation (Villasenor et al., 2012).

Besides the formation of different cell types, the developmental mechanisms controlling the morphogenesis

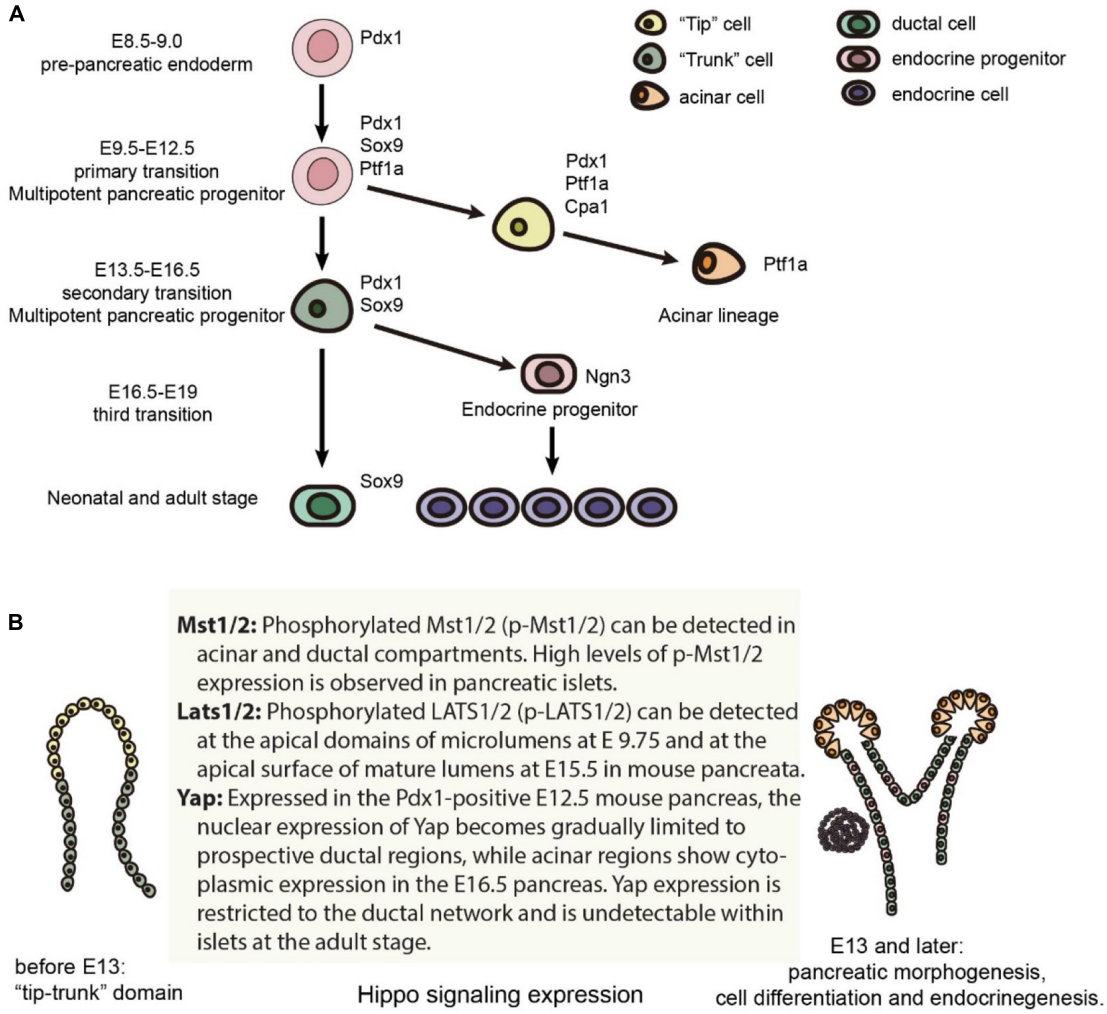

FIGURE 1 | Mouse pancreas development and the expression of Hippo components. **(A)** Major cell lineages in the pancreas and the master regulators of each lineage. **(B)** The expression of Hippo components in the mouse pancreas.

of the pancreatic epithelium are also essential (Shih et al., 2013). When the pancreatic buds develop, their morphogenesis generates a highly branched, tree-like tubular epithelial network. The process includes epithelial stratification, cell polarization, microlumen formation and fusion, and finally gives rise to a luminal plexus to be remodeled into a complex network. From E11.5 onward, the mouse pancreatic epithelium consists of MPCs that progressively segregate into tip or trunk domains and are allocated to acinar or bipotent endocrine/duct progenitor cell fates, respectively (Larsen and Grapin-Botton, 2017). The cells in the tip domain express Ptf1a and Nr5a2, while the trunk cells express Sox9, Nkx6.1, Hnf1b, and Pdx1. Several signaling pathways such as Notch, EGFR and RhoA regulate the tip-trunk pattern along with tubulogenesis (Murtaugh et al., 2003; Afelik et al., 2012; Azizoglu et al., 2017; Lof-Ohlin et al., 2017). Over the past decade, efforts have aimed to pinpoint the molecular mechanisms governing pancreas development and organogenesis. However, much remains to be discovered, particularly regarding the cellular processes that coordinate the morphogenesis of this complex organ (Bastidas-Ponce et al., 2017).

AN OVERVIEW OF THE HIPPO SIGNALING CASCADE

The Hippo signaling pathway is a highly conserved kinase cascade that was initially characterized in *Drosophila*, and most components have since been found to have multiple orthologs in mammals. The pathway regulates diverse cellular processes, including proliferation, differentiation, cell survival, and organ size (Gomez et al., 2014; Mo et al., 2014). In mammals, the Hippo pathway consists of a kinase cascade of the Ste-20-like protein kinases MST1 and 2, which phosphorylate large tumor suppressor 1 and 2 (LATS1/2). Once phosphorylated, LATS1/2 are activated and can phosphorylate the main effectors of the

Hippo pathway, the transcription coactivators Yes-Associated Protein 1 (YAP), and Transcription co-activator with a PDZ-binding motif (TAZ). Following phosphorylation, YAP and TAZ are either sequestered in the cytoplasm or degraded in a ubiquitin-proteasome-dependent manner. When LATS are inactive, unphosphorylated YAP and TAZ translocate to the nucleus to initiate transcription and induce the expression of genes regulating proliferation, differentiation, and apoptosis. Although YAP and TAZ have some redundant functions, studies of mammary and kidney organogenesis have shown that the inactivation of either gene produces very different phenotypes (Piccolo et al., 2014; Skibinski et al., 2014), emphasizing their distinct roles and the need to further delineate their functions in other tissues.

Neither YAP nor TAZ have a DNA-binding domain, so they require other transcription factors to exert control on gene expression. Transcription enhancer associate domain (TEAD) 1-4 have been shown to interact with YAP and TAZ to mediate downstream gene expression (Santucci et al., 2015), including genes involved in cell growth and proliferation (e.g., DNA replication, mitosis, and chromosome organization) as well as stem cell identity and tissue architecture (e.g., cytoskeleton, extracellular matrix) genes (Holden and Cunningham, 2018). The various target genes of YAP-TEAD result in a diverse set of functions of the Hippo signaling pathway including development, organ size control, tissue homeostasis, cell ploidy, innate immunity, miRNA biogenesis, atherogenesis, and tumorigenesis (Kim and Jho, 2018).

A number of studies have revealed that the Hippo pathway is a critical player in the development of many different organ systems, including the heart, lung, brain, and liver (Dai et al., 2017; Wu et al., 2017; Nantie et al., 2018; Wang et al., 2018; Noce et al., 2019; van Soldt et al., 2019). These studies indicate that the Hippo pathway has distinct functions under different physiological and pathological conditions, highlighting the importance of investigating this pathway in a tissue- and context-specific manner. Thus, the Hippo signaling pathway and its transcription effectors YAP/TAZ have emerged as key regulators of numerous developmental decision-making processes.

RECENT ADVANCES IN UNDERSTANDING THE HIPPO PATHWAY IN PANCREAS DEVELOPMENT

Recent studies have identified that the Hippo signaling pathway and its effectors are vital for pancreatic development and function. It influences not only embryonic pancreatic development but also several pancreatic diseases, including acute and chronic pancreatitis, pancreatic cancer, and diabetes mellitus. Multiple Hippo pathway genes have been shown to be expressed in the developing pancreas (**Figure 1B**). These genes have since been investigated through selective deletion at embryonic and adult stages using different pancreatic-specific Cre lines in genetically engineered mice models (George et al., 2012; Gao et al., 2013; Braitsch et al., 2019; Liu et al., 2019). Some Hippo components have also been investigated in human pancreatic cells differentiated from human pluripotent stem cells (Zhang et al., 2013; Cebola et al., 2015; George et al., 2015; Mamidi et al., 2018; Rosado-Olivieri et al., 2019).

Utilizing genetically engineered mouse models (GEMM) is the primary method by which various components of the Hippo pathway have been studied within the context of pancreatic development. It is also essential to use specific Cre or Cre-ER lines to achieve pancreas-specific gene modification with spatial and temporal control. There are over seventy pancreatic Cre driver lines directed by over thirty different gene promoters (Magnuson and Osipovich, 2013). For example, 11 Cre or Cre-ER driver lines have been generated using different DNA fragments of the Pdx1 promoter, including Pdx1$^{Cre-early}$ and Pdx1$^{Cre-late}$, which enable Cre to be expressed at different time points during mouse pancreatic development. Thus, we must consider the specific Cre driver lines utilized when discussing knockout studies of Hippo components and their resulting phenotypes.

MST: The Mammalian Ste20-Like Kinases 1 and 2

MST1 and 2 kinases were the first components of the Hippo pathway to be investigated in pancreatic development. Phosphorylated Mst1/2 (p-Mst1/2) can be detected in the acinar and ductal compartments of a developing mouse pancreas, and much higher levels of p-Mst1/2 expression are found in the pancreatic islets (George et al., 2012). A *Cre/loxp* system was used to delete Mst 1 and 2 (Mst1/2) within the pancreatic epithelium, ($Pdx1^{cre}Mst1^{-/-}Mst2^{fl/fl}$, DKO) and ($Pdx1^{cre}Mst1^{fl/fl}$ $Mst2^{fl/fl}$, DKO) (George et al., 2012; Gao et al., 2013) in early development. The Pdx1 Cre lines used in these two studies were notably different; Gao et al. (2013) utilized $Pdx1^{early}$ while the $Pdx1^{Cre}$ line that (George et al., 2012) used has not been characterized in detail. Nonetheless, the major resulting phenotypes were similar: de-differentiation of acinar cells to ductal-like structures, immune infiltration, and auto-digestion. Typical characteristics of pancreatitis were also observed postnatally. Although pancreatitis phenotypes occurred early in *Mst1/2* null mice (around P14), tumor formation was not observed at 1 year of age (George et al., 2012).

Although the phenotypes found from these two papers were similar, different mechanisms for the results are discussed. The first difference surrounds the paradox as to how increased cell proliferation within the exocrine cells is associated with reduced pancreatic mass. Gao et al. (2013) suggest that the loss of *Mst1/2* and subsequent de-differentiation of acinar cells triggers an upregulation in cell death. Conversely, George et al. (2012) found that there are no changes in cell apoptosis, and they propose that the reduction in pancreatic mass is instead the result of activation of auto-digestion. Specifically, *Mst1/2* DKO mice fail to form a highly branched ductal network, which causes digestive enzymes to be released into the surrounding tissue and initiate auto-digestion. In addition, Gao et al. (2013) propose that acinar cell development is not affected by the loss of *Mst1/2*, because acinar-related transcription factors, Mist1, Ptf1a, RBP-JL, and Lrh1, are found to have normal expression at P0. The two papers also consider different reasons for immune infiltration.

Gao et al. (2013) suggest that acinar de-differentiation precedes cell death and pancreatitis, and that loss of *Mst1/2* promotes leukocyte invasion. George et al. (2012) report that acinar to ductal metaplasia is the result of immune infiltration, but they do not propose the molecular mechanisms for the initial onset of pancreatitis.

The second difference between the two papers is in regard to the endocrine compartment. Gao et al. (2013) found that the loss of *Mst1/2* leads to an increase in the ratio of α/β cells. George et al. (2012) reported changes to the islet architecture in that β cells were not surrounded by α cells, but the α/β ratio stayed the same. Numerous single insulin positive cells were found throughout the Mst1/2 KO pancreas instead of the normal pattern of central β cells surrounded by α cells. Neither of these two papers reported precise time points during the discussion of endocrine cell development. Despite these differences, they both conclude that the overall function of the islets is not affected by the loss of *Mst1/2*. Both papers also demonstrate that the expression of YAP is undetectable in endocrine cells, even in the DKO offspring from E16.5. This suggests that YAP is regulated in a Hippo-independent manner during pancreatic endocrine development.

Through analysis of gene ontology of differentially expressed genes in DKO pancreas versus control, Gao et al. (2013) suggest that the Hippo pathway may first lead to changes in cell shape and adhesion, followed by effects on cellular identity. In their *Mst1/2* DKO mice model, the pancreas exhibited deregulation of genes involved in integrin signaling and cell adhesion (Gao et al., 2013). Although both studies display YAP stabilization through low levels of YAP phosphorylation in DKO mice, only Gao et al. (2013) used a genetic method to demonstrate that the phenotype of DKO pancreas was largely rescued by deleting one copy of the YAP gene. They also showed that overexpression of a constitutively activated form of YAP in the pancreas mimics the *Mst1/2* null phenotype. Altogether, these papers delineate the functions of MST1/2 in pancreatic development and suggest that YAP is the downstream factor responsible for the pancreatic defects (George et al., 2012; Gao et al., 2013).

LATS: Large Tumor Suppressor 1 and 2

Pancreatic progenitor epithelial cells give rise to acinar, ductal, and endocrine lineages, coinciding with branching and tubule development. The process of organ morphogenesis includes epithelial stratification, cell polarization, microlumen formation and fusion, and formation of a luminal plexus to be remodeled into a complex tubular network. It is a complicated developmental process that requires multiple transcription factors and signaling pathways to govern it. However, we still do not have a complete understanding of how these processes are carried out. Nonetheless, it has been proposed that LATS1/2, the downstream kinases of MST1/2, function in pancreatic morphogenesis.

In the early developmental stage, phospho-LATS1/2 localize to the apical domains adjacent to mucin-1^{+} (MUC1^{+}) microlumens in the normal pancreatic bud at E9.75, and are expressed at the apical surface of mature lumens at E15.5 (Braitsch et al., 2019). Following the early embryonic deletion of *Lats1/2* using Pdx1^{early} Cre (Gu et al., 2002), the pancreas lacks all differentiated cell types including the acinar, ductal and endocrine cells. The loss of pancreatic cell identity occurs before and during the secondary transition. At E10.75, there are no changes in expression levels of PDX1 and PROX1, but there are fewer NGN3^{+} cells. Knockout pancreata do not initiate branch formation at E11.5, and MUC1 expression is reduced at E12.5, which leads to the loss of cell polarity and subsequent failure of epithelial expansion/branching. Progressive luminal hyperfusion, increased lumen size, and loss of the normal ductal plexus have also been observed. When studying cell shape, increases in the ratio of apical to basal width leads to failure of apical constriction, which is vital for microlumen formation. Abnormal expression of Vimentin within epithelial cells can also be found at E11.5. However, the cells remain E-cadherin positive, suggesting incomplete EMT. Partial EMT is indicated by unchanged expression of most EMT transcription factors. At E13.5, the mutant pancreas remains small and rounded, which shows that early pancreatic morphogenesis requires a properly functioning Hippo signaling pathway.

It was found that further deleting *Yap1&Taz* in *Lats1/2* null mice (*Pdx1Cre*^{early}; *Lats1*$^{f/f}$; *Lats2*$^{f/f}$; *Yap1*$^{f/f}$; *Taz*$^{f/wt}$) largely rescues the phenotype, suggesting that LATS1/2 regulate pancreatic differentiation via YAP1&TAZ (Braitsch et al., 2019). It was found that loss of *Lats1/2* upregulates the expression of *Vnn1*, which leads to activation of ROS and NFκB signaling pathways. However, blocking the NFκB signaling pathway does not rescue the defects of pancreatic cell differentiation, indicating that activation of the NFκB pathway may be a secondary effect. It is suggested that LATS1/2 suppress the activation of NFκB and EMT via YAP (**Figure 2A**). Lastly, RNA-seq analysis indicates that cell adhesion molecules and tight junction pathways are upregulated, which may be a compensatory response to the defects of EMT, loss of apicobasal polarity, and altered cell shape (Braitsch et al., 2019).

Interestingly, the deletion of *Lats1/2* in adult pancreata has shown very distinct phenotypes (Liu et al., 2019). Our lab used the *Ptf1a*$^{Cre-ER}$ system to delete *Lats1/2* specifically in acinar cells at the adult stage. The knockout mice displayed a very severe pancreatitis-like phenotype, which differs from the phenotype seen in both *Mst1/2* knockout studies. Subsequent analysis found that deletion of *Lats1/2* did not directly affect acinar proliferation and apoptosis. Instead, *Lats1/2* null acinar cells produced cytokines, such as CTGF and SPP1, which directly activated pancreatic stellate cells and consequently induced fibrosis and immune cell infiltration. This research suggests that Hippo signaling plays critical roles in acinar-stromal communication, which promotes the proliferation and metaplasia of acinar cells. Additionally, the phenotype can be rescued by further deletion of YAP and TAZ. These findings underscore the mechanism through which epithelial acinar cells can mount an inflammatory response within the pancreas (Liu et al., 2019). Disruption of Hippo signaling directly contributes to the activation of stromal cells through upregulation of inflammation and fibrosis-associated genes in acinar cells (**Figure 2B**). However, it will be important to fully explore the direct targets of YAP/TAZ and their contributions to the immune cell recruitment.

Developmental-stage deletion of *Mst1/2* produces a phenotype similar to what is seen in an adult-stage deletion of *Lats1/2*,

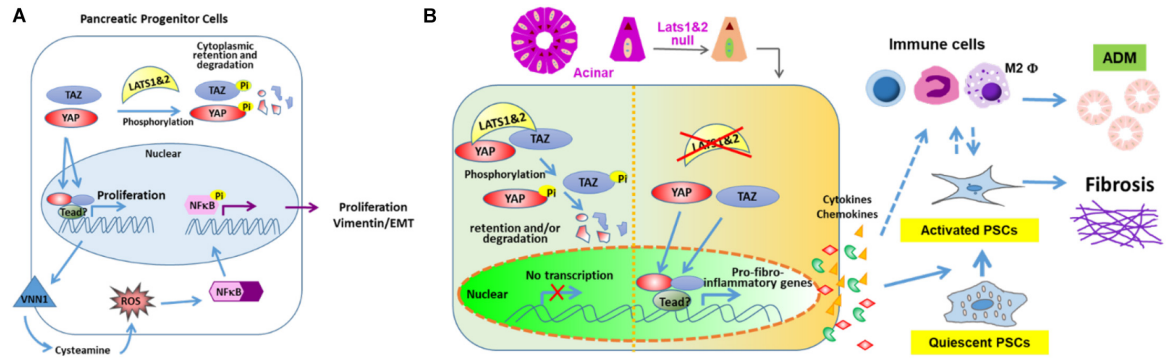

FIGURE 2 | LATS 1 and 2 function in pancreas. **(A)** LATS 1/2 phosphorylate YAP/TAZ to indirectly suppress NFκB and aberrant EMT initiation to allow proper pancreatic morphogenesis. **(B)** LATS 1/2 control the intrinsic fibroinflammatory program in adult pancreatic acinar cells through inhibition of YAP/TAZ.

but differs from a developmental-stage deletion of *Lats1/2*, even though both used same Pdx1^{early} Cre (George et al., 2012; Gao et al., 2013; Braitsch et al., 2019). The different phenotypes in these two knockout mice models suggest that, during pancreatic development, LATS1/2 may have other upstream regulators such as GPCR signaling, AMPK, or MAP4K (Hong et al., 2016), and further study will be required to reveal their identities. Although MST1/2 have yet to be deleted in adult acinar cells, the results will most likely phenocopy what has been seen in a LATS1/2 deletion, as it should also result in YAP/TAZ hyper-activation. Nevertheless, both MST1/2 and LATS1/2 affect pancreas development through YAP, making YAP the center of Hippo signaling pathway during development.

YAP/TAZ

In the developing pancreas, progenitors show high levels of proliferation, followed by exiting the cell cycle and differentiating into different cell types. Here, it is important to maintain a balance between self-renewal and specification of daughter cells. It has been thought that the Hippo pathway contributes to this balance and overall organogenesis by regulating growth and the time at which progenitors exit the cell cycle (Halder and Johnson, 2011). As one of the most important effectors of the Hippo signaling pathway, YAP expression is robust, which coincides with the high levels of proliferation during the primary and secondary transition stages (George et al., 2015). However, in the pancreatic endocrine lineage, YAP expression is turned off at the RNA level rather than by the canonical Hippo pathway control, and the downregulation of YAP expression consequently correlates with a decrease in endocrine cell proliferation (George et al., 2015). Zhang et al. report that YAP1 is a target gene of miR-375, which acts on the 3' UTR of YAP1 mRNA to decrease its mRNA and protein levels. Similar to silencing YAP1 by shRNA, the proliferation of pancreatic progenitor cells is inhibited significantly by forced expression of miR-375 (Zhang et al., 2013). Whether the suppression of YAP/TAZ in endocrine lineage cells is through microRNA or epigenetic regulation requires further investigation.

During early pancreatic development, the two outpouchings of distal foregut endoderm consist of multipotent epithelial progenitors that branch and specify into the trunk domain of bipotent pancreatic progenitors (bi-PPs), which further differentiate into both duct and endocrine lineages, and to the tip domains that give rise to acinar cells. YAP transgenic mice models (*tet-YAP1*^{S127A}*;Pdx1*$^{tTA/+}$, referred as YAPtg) in which constitutively active human YAP1 is expressed at E12.5 (Mamidi et al., 2018) have shown that YAP1 target genes including Cdc20, Ctgf, Birc5, and Snai2, are upregulated during pancreatic development. The expression of SOX9, HNF1β, MUC1, and E-cadherin are also upregulated, suggesting that overexpression of YAP drives bi-PP cells toward ductal lineage *in vivo*. Meanwhile, the expression of pancreatic progenitor markers (*Pdx1* and *Nkx6.1*), acinar markers (*Cpa1* and *Amy2A5*) and endocrine markers (*Ngn3* and *Ins1*) are lower in the YAP1tg embryonic pancreas compared with controls. In addition, normal branching and tip-trunk patterning is perturbed at E12.5. Altogether, the phenotypes indicate that YAP1 regulation is important for mid-embryonic pancreas development (Mamidi et al., 2018).

There has been an increase in research focused on identifying the extrinsic and intrinsic signaling mechanisms that govern cell fate, with activation of the Hippo signaling pathway occurring via sensors of cell density (Mamidi et al., 2018). Specifically, the F-actin-YAP1-NOTCH mechanosignaling axis, which ultimately controls the fate of bipotent pancreatic progenitors, is initiated by the interaction of the extracellular matrix with integrin α5. Cell spreading positively regulates YAP1 activity by forming actin bundles, which then drives progenitors toward a ductal fate. Conversely, endocrine specification is driven by cell confinement negatively regulating YAP1, dissociating actin bundles (Mamidi et al., 2018). Altogether, the Hippo signaling pathway helps to maintain the state of progenitors and promotes development of ductal lineage during the second transition in a YAP-dependent manner.

At E16.5, YAP becomes gradually limited to prospective ductal and acinar regions. Conversely, endocrine cells lack detectible YAP expression (George et al., 2015). The loss of YAP in endocrine development is independent of canonical Hippo signaling, and the regulation occurs at the transcription level. The reduction of YAP mRNA and proteins coincides with decreased proliferation of endocrine cells (George et al., 2012).

There are two possible mechanisms to explain how the Hippo signaling pathway drives cell proliferation. The first mechanism proposes that Hippo signaling regulates cell proliferation and apoptosis via a YAP-TEAD complex, which may directly activate genes governing proliferation. Using human embryonic pancreata and embryonic-stem-cell-derived progenitors, the regulatory landscape of *in vivo* and *in vitro* MPCs has been investigated (Cebola et al., 2015). Through analysis of RNA-seq and ChIP-seq data, it has been found that TEAD factors are vital components of the combination of transcription factors that activates both stage and lineage-specific pancreatic MPC enhancers. Additionally, TEAD1 is a core component of pancreatic progenitor cis-regulatory modules (CRMs). The role of TEAD and YAP in pancreatic development was determined using chemical and genetic inhibitors to disrupt TEAD/YAP complexes, which subsequently led to the reduction of pancreatic epithelial proliferation. These findings indicate that YAP-TEAD directly regulate the outgrowth of pancreatic progenitors. During these experiments, SOX9 was the proliferative mediator of TEAD and YAP in early pancreatic development (Cebola et al., 2015). The second possible mechanism of Hippo influence on cell proliferation considers the crosstalk between the Hippo signaling pathway and other proliferative signaling, including Notch, WNT/β-catenin, and PI3K/Akt/mTOR signaling. These relationships will be further discussed in the next section.

In addition to TEAD1 regulating the proliferation of pancreatic progenitors, Lee et al. studied TEAD1 function in β cells using two β cell specific Cre drivers, Rip^{Cre} which results an early constitutive gene deletion from E15.5, and Mip^{CreER} which deletes genes at the time of tamoxifen administration (Lee et al., 2020). The mice with TEAD1 deleted at E15.5 developed early diabetes at 5 weeks of age. Further experiments revealed that deletion of TEAD1 increases the number of β cells in the active phases of the cell cycle in a cell-autonomous way. Mechanistically, Lee et al. found that TEAD1 activates $p16^{INK4a}$ in adult β cells to maintain proliferative quiescence. TEAD1 also activates the transcription of critical genes required for maintaining mature β cell function. These data indicate that TEAD1 controls, directly or indirectly, the gene regulatory network critical to maintain β cell functional competence and proliferative quiescence (Lee et al., 2020). Whether the function of TEAD1 in β cells is independent of Hippo pathway remains unknown. Several studies have shown that β cells do not express YAP; however, the expression of TAZ has been found in β cells (Lee et al., 2020). Thus, it will be interesting to find out if deletion of TAZ phenocopies deletion of TEAD1 in β cells.

THE CROSSTALK BETWEEN HIPPO SIGNALING AND THE NOTCH, WNT/β-CATENIN, AND PI3K/AKT/MTOR PATHWAY IN THE PANCREAS

The Hippo signaling pathway influences pancreatic cell identity and morphogenesis development by regulating several other signaling pathways. These pathways have been shown to play essential functions during pancreatic organogenesis.

The Notch signaling pathway has effects on determination of cell fate and normal pancreatic architecture development. During the early stage, Notch signaling helps to maintain proliferation and prevent premature differentiation of pancreatic progenitors into ductal and endocrine cells. Notch signaling regulates pancreatic development through an expression level-dependent manner, instead of a simple "on or off" modality (Murtaugh et al., 2003; Fujikura et al., 2006; Afelik and Jensen, 2013; Li et al., 2015). The Notch pathway affects endocrine linage development by regulating expression of SOX9 and NGN3 via a complex network (Seymour et al., 2007, 2008; Shih et al., 2012). Hes1, one of the target genes and effectors of Notch signaling during pancreatic development, has been found to be a key player in the differentiation of endocrine cells by suppressing Ngn3 expression (Lee et al., 2001). Notch signaling promotes expression of Sox9 to activate expression of Ngn3, which initiates the development of endocrine cells. However, high levels of Notch signaling also induces Hes1 expression, repressing Ngn3 and consequently blocking endocrine cell fate determination. Thus, Notch signaling governs cell fate determination and pancreatic patterning through the actions of Sox9 and Hes1 (Seymour et al., 2007, 2008; Shih et al., 2012). Other studies have found that overexpression of YAP can upregulate Hes1 and Notch1 in YAP1tg pancreata at E15.5, resulting in a phenotype with an expanded ductal compartment. However, this phenotype can be partially rescued by blocking Notch signaling. ChIP-seq analysis has shown that YAP1 acts as an activator of Hes1 transcription to indirectly suppress NGN3, while YAP1-TEAD4-HES1 directly represses the transcription of Ngn3 by specifically binding to the *NGN3* promoter (Gao et al., 2013; Mamidi et al., 2018). Thus, YAP acts as both a transcription activator and repressor during endocrine development (**Figure 3**).

The WNT/β-catenin signaling pathway also controls pancreatic specification and patterning during different stages, specifically through its effects on proliferation of pancreatic progenitors (Hendley et al., 2015; Scheibner et al., 2019; Sharon et al., 2019). Deletion of WNT related genes in early stages has been found to reduce the MPC pool, which subsequently decreases the amount of both exocrine and endocrine cells (Afelik et al., 2015). Further, the WNT/β-catenin signaling pathway regulates pancreatic epithelial morphogenesis through influencing cell-cell adhesion and regulating the Notch signaling pathway. George et al. suggest that the mechanism causing increased proliferation in the *Mst1/2* null pancreas may be activation of both Wnt/β-catenin and mTOR signaling. They found that *Mst1/2* null pancreata had increased expression of β-catenin, along with β-catenin dephosphorylated to an "active" form, which increased cell proliferation. Expression levels of Wnt signaling target genes, including c-Myc and Tcf1, were also increased in *Mst1/2* null pancreata (Gao et al., 2013). Based on analysis of the regulatory landscape of *in vivo* and *in vitro* MPCs, the enrichment of non-canonical WNT regulators such as FZD2, SFRP5, CELSR2, and VANGL2, suggests an evolutionarily conserved signaling mechanism operating within early pancreatic development (Cebola et al., 2015). It has been

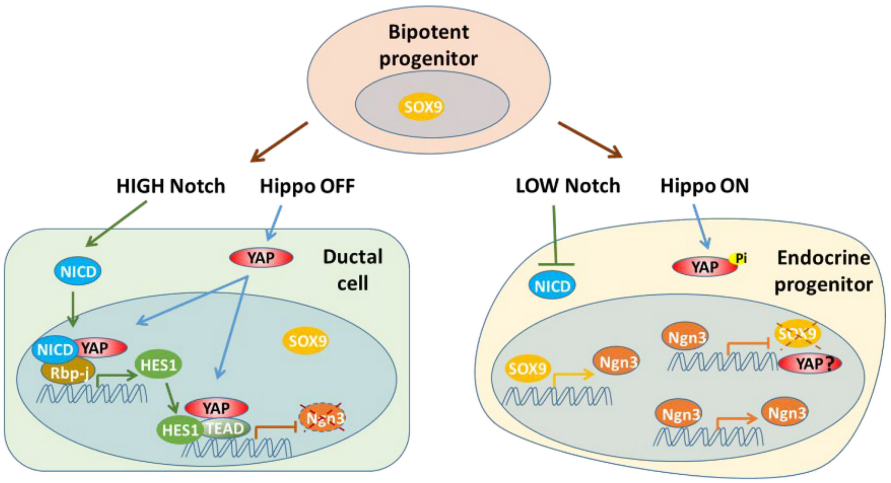

FIGURE 3 | YAP acts as both a transcription activator and repressor with Notch signaling pathway during endocrine development. YAP may be suppressed at transcription level in endocrine progenitor cells.

shown that LATS1/2 have vital functions to sustain WNT signaling via a YAP-dependent but TEAD-independent pattern in intestinal stem cells (Li et al., 2020). Although this mechanism has not been investigated within the developing pancreas, we expect that the same signaling regulation occurs due to the similarities between pancreatic and intestinal organogenesis.

The mammalian target of Rapamycin (mTOR) integrates signals from both nutrients and growth factors (Elghazi et al., 2017). Inhibition of PKA or mTOR promotes Ngn3-driven β cell regeneration in human T1D islets (Cheng et al., 2017). Within a *Mst1/2* null pancreas, there is an upregulation in expression of phosphorylated S6 and 4E-BP1, indicating that deletion of *Mst1/2* leads to an increase in proliferation via activation of mTOR signaling (George et al., 2012). Akt, an upstream positive regulator of mTOR signaling, has been found to be robustly activated within YapS127A-transduced islets, which have YAP constitutively activated. When treated with mTOR inhibitor rapamycin, the proliferation of β cells which is induced by overexpression of YAP can been blocked in human islets, suggesting that mTOR signaling may regulate mature β cell proliferation downstream of YAP (George et al., 2015). Nonetheless, whether the mTOR signaling pathway is involved in Hippo function during early pancreatic development remains elusive.

CONCLUSION AND FUTURE PERSPECTIVE

The Hippo signaling pathway is an essential regulator of pancreatic development, directing cell differentiation and organ morphogenesis. Upstream components of YAP include cell-cell contact and the interaction of the extracellular matrix with integrin α5. However, whether these biological processes influence the function of the Hippo pathway during pancreatic development remains unclear. Components and pathways downstream of YAP include the YAP-TEAD complex and crosstalk with other signaling pathways including Notch, Wnt/β-catenin, and mTOR. Although great progress has been made toward uncovering the mechanisms through which Hippo governs pancreatic development, there are still several components of the pathway that warrant further study.

The upstream kinases of the Hippo pathway, MST1/2 and LATS1/2, have been studied in early pancreatic development. Deletion of either set of kinases stabilizes YAP and TAZ, enabling them to translocate to the nucleus and regulate proliferation, differentiation, and apoptosis. However, *Mst1/2* and *Lats1/2* have only been deleted with Pdx1^{Cre} during early pancreatic development, so how these kinases function in the development of specific cell lineages remains largely unknown. Thus, using other Cre lines, such as Ngn3^{Cre} or Mip^{Cre} (mouse insulin promoter), to study these components in a lineage-specific manner will provide a more comprehensive understanding of the extent to which the Hippo signaling pathway contributes to pancreatic organogenesis.

Pancreatic mesenchymal cells play pivotal roles in pancreas development, mainly via the FGF (fibroblast growth factor) signaling pathway (Lv et al., 2019). Research using single-cell RNA sequencing, *in situ* hybridization, immunofluorescence staining, and genetic lineage tracing, has found that the mesenchymal cells can be identified as 10 transcriptionally distinct populations. Pathway analysis of genes expressed by cells within each population has indicated that Hippo signaling may have important functions within at least some of these cells, suggesting a need for further focus on studying the link between Hippo signaling and the regulation of mesenchymal cells during pancreatic development (Byrnes et al., 2018).

Pancreatic cancer is the third most common cause of cancer-related deaths in the United States. Disruption of Hippo signaling has been found to be associated with pancreatic cancer via promoting pancreatic tumor development and progression, even without mutant Kirsten RAS (KRAS) (Kapoor et al., 2014). Hippo

disruption and YAP/TAZ upregulation promotes tumorigenesis through EMT, activation of pancreatic stellate cells, recruitment of immunosuppressive cells, and subsequent resistance to gemcitabine, which is the standard chemotherapeutic agent in pancreatic cancer. The involvement of YAP/TAZ in pancreatic cancer development has been intensively reviewed by others as well (Rooman and Real, 2012; Zhang et al., 2014; Ying et al., 2016; Jiang et al., 2018; Ansari et al., 2019; Eibl and Rozengurt, 2019).

Diabetes mellitus becomes largely intractable once there is loss of β cell function and mass. The Hippo signaling pathway is a key regulator of final organ size as it regulates the balance between cell proliferation and apoptosis. Thus, it has been considered as a potential therapeutic target for increasing β cell proliferation without a change in function (Ardestani and Maedler, 2018). Overexpression of active YAP has been shown to induce β cell proliferation within isolated human islets, but it has no effect on β cell function and functional identity genes. On the other hand, YAP is downregulated in Ngn3 positive endocrine progenitor cells and remains low. We scrutinized the transcriptome of human β cells for expression levels of Hippo pathway components. The data showed that YAP mRNA is low, 0.5 FPKM (Fragments Per Kilobase of transcript per Million mapped reads), TAZ is tenfold higher than YAP at 5.9 FPKM, LATS1 at 10.4, LATS2 at 0.3, MST1 at 2.4, and MST2 at 3.35 (Nica et al., 2013). A study on the deletion of MST1 in mice has shown that MST1 is a critical regulator of β cell apoptosis and functions through direct phosphorylation of PDX1, and that this is a Hippo-independent function (Ardestani et al., 2014). Whether other components of the Hippo pathway play roles in β cell development and function requires further investigation.

The Hippo signaling pathway plays critical roles in maintaining organ size, making it an important pathway to consider manipulating for regenerative medicine (Moya and Halder, 2019). Rosado-Olivieri et al. demonstrated that inhibition of YAP can enhance endocrine progenitor differentiation and result in the generation of improved insulin-secreting cells derived from stem cells (Rosado-Olivieri et al., 2019). On the other hand, even though YAP is not expressed in endocrine cells, two labs have tried to use its pro-proliferation ability to expand insulin producing beta cells (George et al., 2015; Yuan et al., 2016). Here, overexpression of an active form of YAP has been found to greatly induce β cell proliferation in adult human islets. Both labs showed that islets with high levels of YAP expression retain normal gene expression and insulin secretion. However, these experiments must be repeated with islets *in vivo* before attempting to rescue diabetes in human patients. Depending on when YAP is manipulated, inhibiting or activating YAP can both lead to the generation of more insulin-producing cells. It will be interesting to test whether inhibiting YAP during the progenitor stage followed by activating YAP in mature cells will increase the number of insulin-producing cells derived from stem cells (Panciera et al., 2016).

In conclusion, the Hippo signaling pathway has profound impacts on pancreatic development at multiple levels, and its continued roles in tissue homeostasis and tumorigenesis warrant further research into this complex network. Thus, a better understanding of the Hippo pathway's various stage-dependent contributions within both the developing and mature pancreas will provide insightful knowledge, which can one day be incorporated into the generation of new regenerative and oncologic therapies.

AUTHOR CONTRIBUTIONS

YW and PW were responsible for contemplating the concept. YW, PA, MN, LR, JL, and PW were responsible for drafting and editing the manuscript. PW was the guarantor of this work. All authors critically revised and approved the final version of the manuscript.

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REVIEW
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Mechanoregulation of YAP and TAZ in Cellular Homeostasis and Disease Progression

Xiaomin Cai[1], Kuei-Chun Wang[2] and Zhipeng Meng[3,4]**

[1] Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China, [2] School of Biological and Health Systems Engineering, Arizona State University, Tempe, AZ, United States, [3] Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, Miami, FL, United States, [4] Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, FL, United States

Biophysical cues, such as mechanical properties, play a critical role in tissue growth and homeostasis. During organ development and tissue injury repair, compressive and tensional forces generated by cell-extracellular matrix or cell-cell interaction are key factors for cell fate determination. In the vascular system, hemodynamic forces, shear stress, and cyclic stretch modulate vascular cell phenotypes and susceptibility to atherosclerosis. Despite that emerging efforts have been made to investigate how mechanotransduction is involved in tuning cell and tissue functions in various contexts, the regulatory mechanisms remain largely unknown. One of the challenges is to understand the signaling cascades that transmit mechanical cues from the plasma membrane to the cytoplasm and then to the nuclei to generate mechanoresponsive transcriptomes. YAP and its homolog TAZ, the Hippo pathway effectors, have been identified as key mechanotransducers that sense mechanical stimuli and relay the signals to control transcriptional programs for cell proliferation, differentiation, and transformation. However, the upstream mechanosensors for YAP/TAZ signaling and downstream transcriptome responses following YAP/TAZ activation or repression have not been well characterized. Moreover, the mechanoregulation of YAP/TAZ in literature is highly context-dependent. In this review, we summarize the biomechanical cues in the tissue microenvironment and provide an update on the roles of YAP/TAZ in mechanotransduction in various physiological and pathological conditions.

Keywords: YAP, TAZ, the Hippo pathway, mechanotransduction, ECM stiffness, stretch, flow shear, contact inhibition of cells

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Edited by:
Wenqi Wang,
University of California, Irvine, United States

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Vijaykumar S. Meli,
University of California, Irvine, United States
Jung-Soon Mo,
Ajou University, South Korea

****Correspondence:***
Kuei-Chun Wang
kuei-chun.wang@asu.edu
Zhipeng Meng
zxm282@miami.edu

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INTRODUCTION

The fate of individual cells is shaped and determined by both biochemical and biophysical factors in cellular microenvironments (Discher et al., 2005; Bonnans et al., 2014; Yang et al., 2014; Kumar et al., 2017). While much is known about cell signaling and functional consequences in response to biochemical cues, such as nutrients, growth factors, and hormones, the impacts of biophysical modulations on tissue growth and homeostasis in health and diseases are understudied. With developments in advanced engineering substrates and apparatus to better mimic mechanics of microenvironments in native tissues, biophysical factors, such as matrix stiffness, cell-cell contacts,

and local hemodynamic forces, have been appreciated for their roles in regulating cellular functions and phenotypes (Lampi and Reinhart-King, 2018; Mohammadi and Sahai, 2018; Maurer and Lammerding, 2019; Sheetz, 2019; Yang et al., 2019). However, our knowledge is rather limited for how cells sense the changes of microenvironments and transduce such mechanical stimuli to biochemical signaling cascades governing cellular responses.

The Hippo pathway, since it was identified in *Drosophila melanogaster* less than two decades ago (Harvey et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003), has been extensively studied and now regarded as a master regulator of organ development, regeneration, and carcinogenesis, *via* integrating extrinsic and intrinsic cues that reshape cellular transcription programs (Pan, 2010; Meng et al., 2016; Moya and Halder, 2019; Dey et al., 2020). The core of the mammalian Hippo pathway is a kinase cascade consisting of the Mammalian STE20-like kinase 1/2 (MST1/2) and the Large tumor suppressor kinase 1/2 (LATS1/2). When cells are exposed to growth-inhibiting signals, MST1/2 phosphorylate LATS1/2 at their hydrophobic motif and thus activate LATS1/2, which in turn phosphorylate and inactivate two transcription factors Yes-associated protein (YAP) and Transcriptional coactivator with PDZ-binding motif (TAZ) by inducing their cytoplasmic retention and protein degradation. When the Hippo pathway is inactivated by growth-promoting signals, YAP and TAZ are dephosphorylated and thus located in the nucleus, where they bind to the TEAD family of transcription factors and drive transcription programs that promote cell proliferation, mobility, and stemness. In addition, novel Hippo kinases (e.g., MAP4Ks), accessory proteins (e.g., SAV1, NF2, and MOB1/2), and tuning machineries (e.g., the STRIPAK-PP2A complex) have been shown as indispensable components, among dozens of many other peripheral regulators that finely tune the pathway (Misra and Irvine, 2018; Ma et al., 2019; Zheng and Pan, 2019), for relaying environmental signals to the core of the Hippo pathway.

There have been many comprehensive reviews on the Hippo pathway regulation and its biological roles as cited above. In this review, we will focus on the molecular mechanisms by which the Hippo pathway is modulated by mechanical cues in microenvironments (**Figure 1**). Furthermore, we will summarize the recent progress on how mechanoregulation of the Hippo pathway contributes to human disease development.

GENERAL ROLES OF MECHANICAL SIGNALS IN CELL BEHAVIORS AND FATE DECISION

Cells and tissues perceive microenvironmental physical forces that are generated both internally and externally. Internal forces are mainly generated by cytoskeleton dynamics (endogenous forces), while external forces result from both environment and neighbor cells (applied forces) (Chen, 2008). The resulting mechanical cues, including tissue stiffness, stretch, and shear stress, modulate cell behaviors, such as cell proliferation, spreading, migration, and differentiation. The process through which biophysical forces are sensed and converted into biochemical signals that elicit responses is termed *mechanotransduction* (Ingber, 2006; Dasgupta and McCollum, 2019). This process was typically divided into mechanosensing (the act of sensing a mechanical stimulus), mechanotransmission (the act of transmitting such a stimulus to signaling events), and mechanoresponse (the functional response of cells to the mechanical stimulus) (Hoffman et al., 2011).

Mechanosensing is usually initiated at the cell surface, where plasma membrane receptors, their associated proteins, and the plasma membrane itself sense and propagate mechanical cues to trigger signaling cascades that generate mechanoresponses. Integrins, G protein-coupled receptors, Enzyme-linked Receptors (i.e., Receptor Tyrosine Kinase), and Ion Channels can be all such "mechanosensors" (Chen et al., 2017). Forces applied to these receptors or on/through their ligands change configurations of the receptors, leading to enzymatic reactions and/or cytoskeleton remodeling. Our understanding of membrane-associated mechanosensors has been greatly improved in the past decade. For example, Integrins and Piezo channels, evolutionarily conserved in mammalian cells, are mechanosensitive, characterized by the activation of corresponding downstream effectors and regulation of their biological functions in response to mechanical stimuli (Kechagia et al., 2019; Xiao, 2020). Besides the membrane-associated receptors, mechanical forces can be directly transmitted to the nucleus, the envelope of which responds to mechanical compression or stretch to alter transport of transcription factors and other nucleus-cytoplasm shuttling proteins, as well as remodel nucleoskeleton (Dahl et al., 2008; Wang et al., 2009; Enyedi et al., 2016; Elosegui-Artola et al., 2017; Hoffman et al., 2020).

It was recognized a long time ago that mechanical signals during development dynamically control gene transcription programs to determine cell fate and organ growth (Discher et al., 2005; Engler et al., 2006). However, it was then unclear how these mechano-responsive transcription programs are regulated by the mechanotransduction initiated by membrane proteins and cytoskeletons. Emerging efforts have been made to elucidate the signaling cascades that link membrane mechanosensors to the nuclear transcription machinery. One such "missing" signaling cascade that has been increasingly appreciated is the Hippo pathway, though many details of mechanoregulation of this pathway remain yet to be determined.

YAP AND TAZ ARE AT THE CENTER STAGE OF MECHANOTRANSDUCTION

Two landmark studies from the Piccolo group and the Sasaki group (Dupont et al., 2011; Wada et al., 2011) published in 2011 opened a new avenue for us to understand how mechanical cues modulate the activities of nuclear factors that dictate transcription programs to control cell behaviors and fate. Their studies demonstrated for the first time that YAP and TAZ, the Hippo pathway effectors, are mechanotransducers to relay cytoskeletal tension to nuclei and to regulate cell functions, and more importantly, they are functionally indispensable for

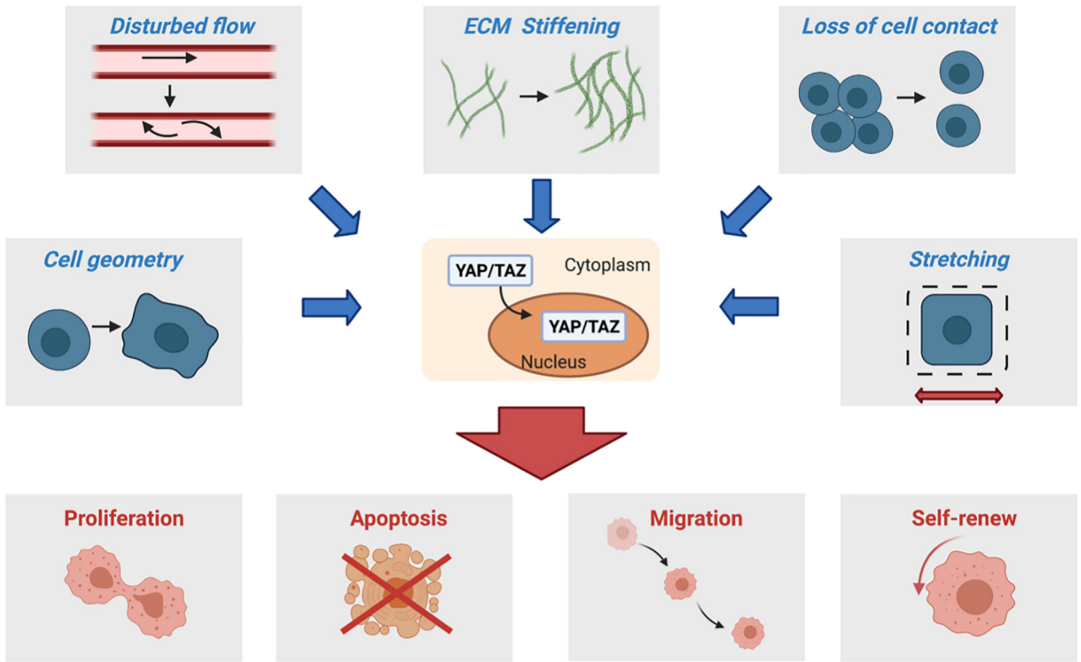

FIGURE 1 | Changes in the biophysical environments, such as those resulting from ECM stiffening, loss of cell contact, disturbed flow, cell spreading, and stretching, promote nuclear translocation of YAP/TAZ and thus activate their transcription programs for cell proliferation, survival, migration, and self-renew, among others.

the biological outputs of mechanical cues. Thereafter, new nuclear factors (i.e., β-catenin, Twist) and new mechanosensitive signaling molecules, such as RAP2 and MAP4K, have been identified (Benham-Pyle et al., 2015; Wei et al., 2015; Li et al., 2018; Meng et al., 2018), thus greatly advancing our mechanistic understanding of mechanotransduction and the Hippo pathway regulation. In the following session, we will summarize the most updated knowledge of how each type of mechanical cues regulates YAP and TAZ and the biological context(s) for the corresponding regulation (**Figure 2**).

Extracellular Matrix (ECM) Stiffness

The mechanical microenvironment of cells is largely determined by their neighbor cells and surrounding extracellular matrix (Discher et al., 2005; Paszek et al., 2005; Engler et al., 2006; Wells, 2008). The difference in ECM composition leads to unique three-dimensional networks in various tissues and determines the rigidity or elasticity of substrates where cells habituate and grow. This physical characteristic of the microenvironment profoundly influences cell phenotypes, not only their morphology but also internal cytoskeleton organization and trafficking (Myers et al., 2011; Wong et al., 2020). To investigate the effect of ECM stiffness on cell functions, engineered hydrogels interfacing with ECM proteins, such as fibronectin and collagens, have widely been used as substrates to grow cells *in vitro*. As a result, ECM stiffness has been shown to regulate cell growth, migration, and differentiation, among many other important cell behaviors.

It was first shown by the Piccolo group that increased ECM stiffness promotes nuclear localization of YAP and TAZ and upregulation of their target genes (Dupont et al., 2011). Though the underlying molecular mechanisms were not fully uncovered then, they demonstrated that actin cytoskeleton tension resulting from manipulations of cell spreading and substrate rigidity constitutes the key link between ECM stiffness and YAP/TAZ activation. High ECM stiffness promotes cell spreading and subsequently leads to actin cytoskeleton tension, which in turn results in nuclear translocation of YAP/TAZ with a to-be-defined mechanism. One key player that relays stiffness signals to YAP/TAZ is RhoA GTPase, which can sense ECM stiffness through focal adhesion and in turn promote actin polymerization and stress fiber formation. Actin cytoskeleton can regulate YAP and TAZ through both Hippo-dependent and -independent pathways (Dupont et al., 2011; Wada et al., 2011). On one hand, the Hippo pathway can be inactivated by actin polymerization and stress fiber formation at high ECM stiffness, and activated by relaxed actin cytoskeletons at low ECM stiffness. Phosphorylation and localization of YAP/TAZ are thus modulated by ECM stiffness through the Hippo pathway (Codelia et al., 2014; Li et al., 2018; Meng et al., 2018). On the other hand, the ARID1A-SWI/SNF complex binds to YAP/TAZ and thus prevents the interaction between YAP/TAZ and TEAD. Polymerized nuclear actin, under high ECM stiffness, binds to ARID1A-SWI/SNF complex, subsequently relieving its sequestration of YAP/TAZ (Chang et al., 2018).

To date, our mechanistic understanding of how ECM stiffness regulates YAP/TAZ remains incomplete as emerging players have been reported. ECM stiffness can be sensed by cells through focal adhesions, as cells use integrin to pull integrin ligands in the ECM. In addition to common integrin ligands such as collagens and fibronectin, an ECM proteoglycan, Agrin, also

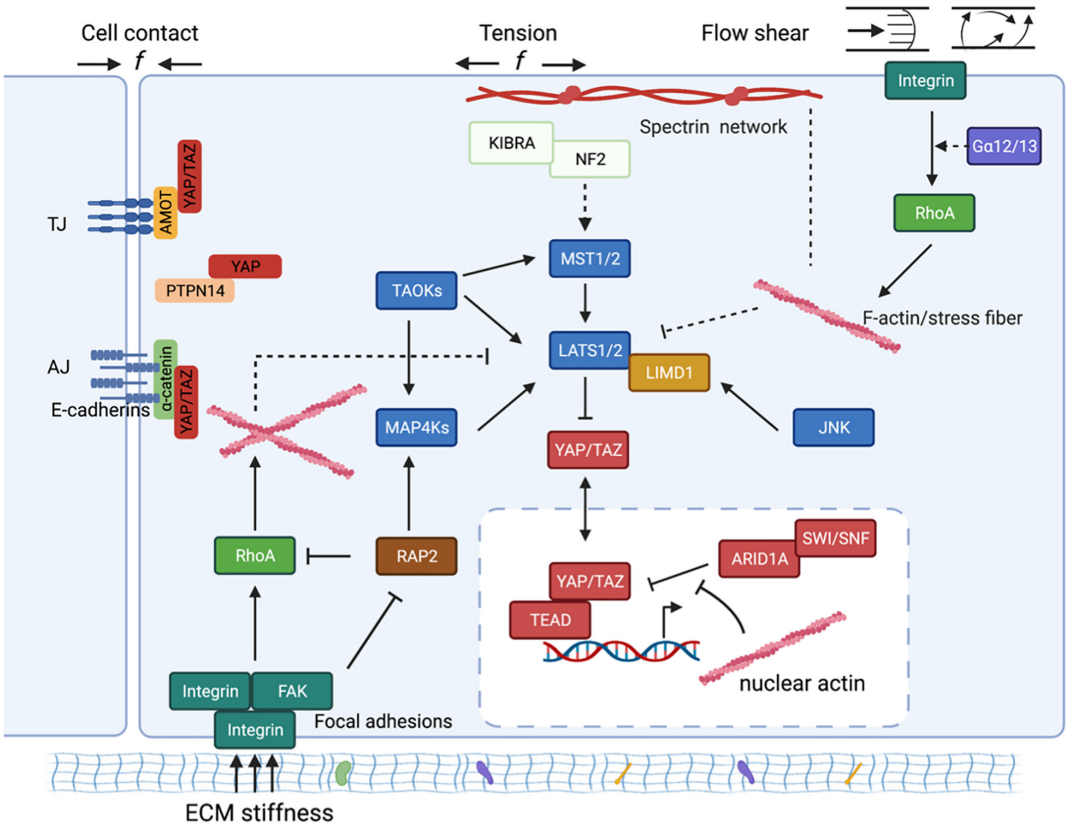

FIGURE 2 | The schematic diagram of molecular mechanisms by which mechanical cues regulate YAP/TAZ. Mechanical cues, such as cell-cell contact, ECM stiffness, externally applied mechanical stretch, and flow shear, control YAP/TAZ activity through both Hippo-dependent and -independent pathways. The core kinase cascade consisting of MST1/2 and LATS1/2, as well as novel Hippo kinases such as MAP4Ks, respond to the mechanical cues to modulate phosphorylation and localization of YAP/TAZ. Mechanical cues can also bypass these kinases and act through both cytoplasmic and nuclear actins to modulate YAP/TAZ localization. (1) In response to cell-cell contact, AMOT directly binds to YAP and thus sequesters YAP at tight junctions regardless of YAP phosphorylation status. AMOT can simultaneously act through NF2/Merlin to activate the MST1/2-LATS1/2 kinase cascade to induce phosphorylation and inactivation of YAP. Adherens junction (AJ) protein E-cadherin, upon cell confluence, *trans*-dimerize and subsequently inactivate YAP/TAZ through the MST1/2-LATS1/2 kinase cascade. Cell-cell contact inhibition also promotes direct interaction between PTPN14 and YAP, and thus leads to cytoplasmic translocation of YAP. (2) ECM stiffness sensed by focal adhesion promotes actin polymerization and stress fiber formation. The Hippo kinase cascade is inactivated by actin polymerization and stress fiber formation at high ECM stiffness. On the other hand, polymerized nuclear actin, under high ECM stiffness, binds to ARID1A-SWI/SNF complex, subsequently relieving its sequestration of YAP/TAZ. In addition, the stiffness-regulated GTPase RAP2 directly activates MAP4K4/6/7 as well as inhibits Rho GTPases, leading to LATS kinase activation and YAP/TAZ inhibition. Stiffness-activated JNK can phosphorylate LIMD1, which directly binds to LATS1/2 and reduces their kinases activities, thus activating YAP/TAZ. (3) Mechanical stretch or tension also acts through actin cytoskeleton to modulate YAP/TZ activities. Spectrin, a cytoskeletal protein, serves as a key linker that connects the cellular tension-sensing system to the Hippo regulation network. (4) Flow shear patterns and speeds differentially regulate activity of the Hippo kinase cascade in endothelial cells *via* an integrin–Gα12/13–RhoA axis.

act as an extracellular mediator of matrix mechanotransduction to regulate YAP/TAZ *via* integrins, its specific membrane receptor LRP4, and the actin cytoskeleton (Chakraborty et al., 2017; Chakraborty et al., 2020). Similarly, matricellular protein thrombospondin-1, acts through integrin-1 and the Hippo pathway, but not actin cytoskeleton, to activate YAP/TAZ (Yamashiro et al., 2020). Besides signaling through integrin/focal adhesion, plasma membrane protein Caveolin-1 (CAV-1) also play a critical role in mechanoregulation of YAP/TAZ (Moreno-Vicente et al., 2018). CAV1 loss promotes the interaction between YAP and the 14-3-3 protein YWHAH in an F-actin-dependent but Hippo-independent manner.

Besides membrane proteins, signaling transducers like small GTPases and protein kinases also mediate stiffness signals to the Hippo pathway. Small GTPases, such as RhoA and RAC1, have been demonstrated for their roles as molecular switches for stiffness sensing (Bae et al., 2014). Another GTPase, RAP2, also senses stiffness cues from focal adhesions (Meng et al., 2018; Yamashiro et al., 2020), as ECM stiffness modulates the GDP/GTP loading status of RAP2. Active/GTP-loading RAP2 activates non-canonical Hippo kinases MAP4K4/6/7 by direct binding to their hydrophobic motif leading to LATS activation and YAP/TAZ inhibition. JNK is also an important mediator of stiffness sensing for YAP/TAZ regulation in epithelial cells (Codelia et al., 2014). Stiffness-activated JNK can phosphorylate LIMD1, which directly binds to LATS1/2 and reduces their kinases activities, eventually leading to activation of YAP/TAZ.

Static and Cyclic Stretch

Almost all adherent cells, *in vitro* or *in vivo*, experience physical forces transmitted between cells and across tissues, and one of the most common forces in animals is tension caused by mechanical stretch. For example, when cells undergo tissue growth or disease progression, the increase in ECM stiffness generates a static tension that stretches cells. Such kind of stretch forces, as previously discussed, modulate cell behaviors *via* a YAP/TAZ-dependent mechanism. In addition to ECM stiffening, stretching forces from external sources, such as tissue mechanical strain and artificial cell stretching devices, are able to regulate cell functions through the interplay of the Hippo pathway (Driscoll et al., 2015; Fletcher et al., 2018). Moreover, recent research has linked YAP dysregulation to malfunction of the cardiovascular system caused by pressure overload (Byun et al., 2019), suggesting a critical regulatory role of the Hippo-YAP/TAZ pathway in cells in response to various magnitudes of stretch force.

Static stretch activates YAP/TAZ by suppressing capping and severing proteins of F-actins (Aragona et al., 2013). Additionally, the stretch force applied to the nucleus may decrease the mechanical restriction of nuclear pores, thus facilitating nuclear transport of YAP/TAZ (Elosegui-Artola et al., 2017). The linker of nucleoskeleton and cytoskeleton (LINC) complex, which transfers cytoskeletal strain to the nucleus, is a key player for mechanical stretch to activate YAP/TAZ in this process (Driscoll et al., 2015; Koushki et al., 2020).

Cyclic stretch has also been reported to activate YAP/TAZ by suppression of the Hippo pathway (Codelia et al., 2014), even in cells on soft matrices (Cui et al., 2015). In Drosophila, the physiological mechanical strain can drive activation and nuclear localization of YAP/TAZ homolog Yki in *Drosophila* follicular epithelium to promote cell proliferation, *via* inactivating the LATS1/2 homolog Warts (Fletcher et al., 2018). The novel Hippo kinase Msn plays a critical role in sensing mechanical stretch in *Drosophila* gut. Mechanical stretch dissociates Msn from the plasma membrane and thus prevents phosphorylation and activation of Msn by the Tao kinase, which in turn activates expansion of *Drosphila* gut stem cells and leads to intestinal hyperproliferation (Li et al., 2014, 2018).

In mammals, cyclic stretch is known to act through thrombospondin-1/RAP2 in blood vessel cells to activate YAP and promote vascular remodeling (Yamashiro et al., 2020). Thrombospondin-1 acts *via* integrin αvβ1 to form focal adhesions and promotes nuclear shuttling of YAP by inactivating the RAP2 GTPase, results in vascular remodeling in response to the pulsatile blood flow and pressure. It is worth mentioning that externally applied mechanical stretch and ECM stiffening shared many mechanosensing machineries, such as RAP2 and Msn/MAP4Ks, to regulate YAP/TAZ activities. It would be important to understand how these universal machineries work in concert with cell-specific mechanosensors (e.g., VEGFR2 in endothelial cells) to control mechanoresponses of cells in future.

Cell-Cell Contact

Cell-cell contact inhibition is the phenomenon that cells avoid proliferating as they achieve convergence in monolayers. During carcinogenesis, transformed cells slowly lose this character (Hanahan and Weinberg, 2011). Loss of contact inhibition allows the transformed cells to overcome physical restraint and in many cases contributes to cancer cell aggressiveness. Contact-dependent signaling is one of the first extracellular stimuli discovered to regulate the Hippo pathway (Zhao et al., 2007Zhao et al., 2008). Direct contact between normal cells activates the Hippo kinases, thus leading to phosphorylation and cytoplasmic retention of YAP/TAZ, triggering cell cycle arrest and/or autophagy (Pavel et al., 2018). In contrast, hypophosphorylation and nuclear localization of YAP/TAZ have been associated with loss of contact inhibition of cancer cells resulting from somatic mutations (Zhao et al., 2007; Zhang et al., 2010; Tranchant et al., 2017; Frank et al., 2018; Ouyang et al., 2020).

Various mechanisms have been proposed to explain how cell-cell contact inhibition modulates activities and localization of YAP/TAZ. These mechanisms can be roughly grouped into two types. The first one usually involves cis-interaction of cell membrane proteins at high confluence. For example, for tight junctions (TJs) form between cells, angiomotin (AMOT) complex at TJs is activated and transmit the antiproliferative signal from TJs to YAP via two independent mechanisms: AMOT can directly bind to YAP and thus sequester YAP at TJs, and/or AMOT can activate Merlin/NF2 to trigger LATS1/2-dependent YAP phosphorylation (Li et al., 2015). In addition to TJs, adherens junctions (AJs) protein E-cadherin, in confluent cells, *trans*-dimerize and subsequently stimulate MST1/2-LATS1/2 kinase cascade to inhibit activities of YAP/TAZ (Kim et al., 2011). PTPN14, a protein tyrosine phosphatase, can inhibit YAP transactivation activity through a direct interaction in response to cell confluence (Wang et al., 2012; Liu et al., 2013). The other type of mechanisms involves cell geometry and actin cytoskeleton remodeling. High cell density, as well as low ECM stiffness, reduces adhesive area and alters cell shape, which leads to inactivation of RhoA and subsequent reduction of stress fiber of actin cytoskeleton, which can inactivate YAP/TAZ through both Hippo kinases-dependent and -independent mechanisms (Aragona et al., 2013; Meng et al., 2018; Chang et al., 2018). Spectrin has been recognized as a key cytoskeletal protein that restricts YAP/TAZ activity in response to mechanical cues, such as cell-cell contact inhibition, through remodeling actin cytoskeleton particularly at cortical areas of cells. Loss of Spectrin proteins results in hyperactivation of YAP/TAZ, likely by elevating cortical myosin II activity, leading to cell over-proliferation even when cells confluence is reached in both mammals and *Drosophila* (Deng et al., 2015; Fletcher et al., 2015; Wong et al., 2015; Deng et al., 2020).

Fluid Shear Stress

Shear stress, a fluid frictional force, is another major mechanical stimulus maintaining tissue homeostasis. Indeed, one of the most studied cell types in mechanotransduction is vascular endothelial cells (ECs), lining in the innermost layer of blood vessels. ECs are constantly subjected to shear stress from blood flow, and it is well recognized that ECs are able to sense and respond to changes in flow direction, pulsatility, and magnitude of shear stress *via* mechanosensors and mechanosensitive signaling pathways.

As a result, endothelial phenotypes are highly associated with local blood flow patterns and distinct in different regions of the vascular tree. Three recent studies independently confirmed that flow patterns modulate endothelial phenotypes through regulation of YAP/TAZ activities: unidirectional laminar flow suppresses YAP/TAZ activities to keep ECs quiescent and inert to inflammatory cells, while disturbed oscillatory flow activates YAP/TAZ to promote a pro-proliferative and -inflammatory EC phenotype (Wang K. et al., 2016; Wang L. et al., 2016; Xu et al., 2016).

Mechanistically, the integrin–Gα13–RhoA axis was first reported to mediate the flow regulation of YAP/TAZ activities in ECs (Wang L. et al., 2016) and two recent studies revealed that disturbed flow acts through integrin α5β1 to induce YAP nuclear translocation and promote the pro-atherogenic responses, via c-Abl kinase and phosphodiesterase 4D5, respectively (Li et al., 2019; Yun et al., 2019). However, the mechanisms regarding how the flow-activated integrin signaling cascades crosstalk with Hippo kinases to modulate YAP/TAZ activities remain to be studied. In addition to the integrin-mediated mechanisms, it has been shown that short-term unidirectional laminar flow (15 dyne/cm^2 for 10 min) increases the nuclear localization of YAP in a LATS1/2-independent but an angiomotin-regulated manner (Nakajima et al., 2017). Last but not the least, caveolae, the plasma membrane microdomain, is known to sense shear stress signals and have been shown to relay such mechanical cues through the Hippo pathway to facilitate mechanoregulation of YAP/TAZ (Rausch et al., 2019). However, whether the caveolae-dependent mechanism mediates the flow regulation of endothelial phenotypes remains to be determined.

In addition to ECs, many other cell types, such as metastatic tumor cells and mesenchymal stem cells, are known to perceive shear stress stimuli and transduce the resulting biochemical signals in regulating cellular functions (Lee et al., 2017, 2018; Qin et al., 2019). In fact, the connection between shear stress and YAP was first reported in mesenchymal stem cells, where it was shown that exposure of mesenchymal stem cells to shear stress enhances YAP expression to promote their differentiation into chondrocytes (Zhong et al., 2013). More *in vitro* and *in vivo* studies are warranted to validate the roles of YAP/TAZ as mechanotransducers in modulating biological functions in the above-mentioned cell types.

MECHANOREGULATION OF YAP/TAZ IN HUMAN DISEASES

Mechanoregulation of YAP/TAZ plays a critical role in normal development and aging processes. For instance, it is known that physiological substrate stiffness directs human pluripotent stem cell specification by influencing cytoskeleton arrangement and intracellular tension through the YAP-TEAD complex (Pagliari et al., 2021). The behavior of limbal epithelial stem cells is strongly influenced by changes in corneal substrate stiffness, *via* the activation of YAP-dependent mechanotransduction pathways (Gouveia et al., 2019). Furthermore, an age-related stiffness drives YAP/TAZ-mediated pathogenic expression of ECM proteins, ultimately disrupting muscle stem cell fate (Stearns-Reider et al., 2017).

Emerging evidence connects YAP/TAZ dysregulation by mechanical cues to various human diseases. Although the underlying mechanisms remain to be defined in many cases, understanding of the Hippo pathway dysregulation by mechanical cues in human diseases potentially can provide us insights into new therapeutic strategies for these diseases (**Figure 3**).

Cardiovascular Diseases (CVDs)

The cardiovascular system is subjected to continuously shifting mechanical signals, including stretch, compression, distortion, and shear. Mechanotransduction profoundly influences the development of the cardiovascular system and the regulation of physiological functions (Garoffolo and Pesce, 2019). The roles of YAP/TAZ in CVDs have been well summarized in a recent review (Yu et al., 2020). Aberrant activation of YAP/TAZ contributes to a variety of cardiovascular conditions: atherosclerosis, pulmonary hypertension, myocardial hypertrophy, angiogenesis, restenosis, and myocardial fibrosis, while hypoactivation of YAP/TAZ is associated with aortic aneurysms, aortic dissection, reperfusion of myocardial ischemia, and myocardial infarction. Our review will solely focus on YAP/TAZ dysregulation in mechanical cues resulting from/in pulmonary hypertension, atherosclerosis, and cardiac hypertrophy.

Pulmonary Hypertension (PH)

Pulmonary hypertension (PH) refers to a pathophysiologic condition of increased blood pressure within the arteries of the lungs with many possible causes (Hoeper et al., 2013).

One feature of PH is remodeling of small pulmonary arteries caused by hyperproliferation of vascular smooth muscle cells (VSMCs) and myofibroblasts, leading to aberrant deposition of collagen and elastin and vascular ECM stiffening. A number of cross-sectional studies suggest that YAP/TAZ activation downstream of ECM stiffening is a key driver of PH (Bertero et al., 2015b, 2016). Vascular remodeling and stiffening activate YAP/TAZ, which then drive a transcription program that promotes ECM deposition and crosslinking and further enhances vascular remodeling and stiffening, thus constituting a forward feedback loop. Specifically, ECM stiffening activates YAP/TAZ in myofibroblasts, endothelial cells, and VSMCs and thus facilitates proliferation of these cells. Furthermore, YAP/TAZ in these cells activate genes involved in ECM synthesis (i.e., collagens) and crosslinking (i.e., lysyl oxidase) (Bertero et al., 2015b). In addition, YAP/TAZ also link mechanical stimuli to dysregulated vascular metabolism associated with PH. ECM remodeling controls the expression of glutaminase by activating YAP/TAZ, leading to activation of glutaminolysis and anaplerosis, fostering PAECs and PASMCs proliferation and migration. In mouse models, LOX inhibitors dampened nuclear YAP/TAZ and improved end-stage manifestations of PH (Bertero et al., 2016). Increased pulsatility and shear stress have been associated with YAP/TAZ activation in pulmonary vascular ECM remodeling and pulmonary adventitial myofibroblast proliferation. It is unknown, however, whether mechanical signals from increased

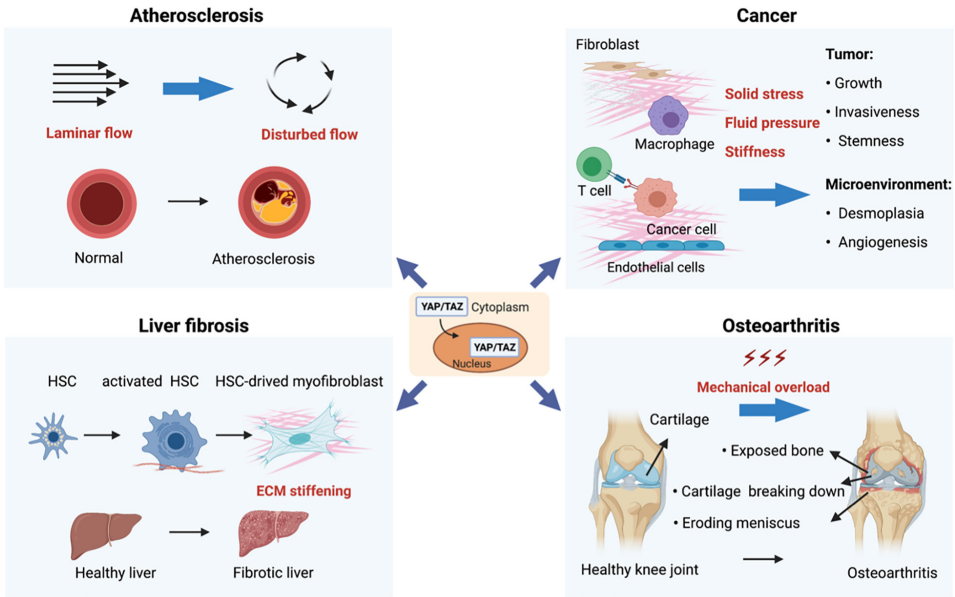

FIGURE 3 | Mechanoregulation of YAP/TAZ in human diseases. (1) Disturbed flow-activated YAP/TAZ is a key factor that promotes atherogenesis. (2) Mechanical properties in the tumor microenvironment (such as solid stress, fluid pressure, and stiffness) act through YAP/TAZ to regulate various aspects of tumor initiation and progression. (3) YAP/TAZ-mediated mechanoresponses strongly promote organ fibrosis. For instance, hepatic injuries and subsequent inflammatory responses activate quiescent hepatic stellate cells (HSCs), leading to activation and expansion of HSCs and accumulation of extracellular. (4) Osteoarthritis (OA) is mainly caused by mechanical overload, and YAP is both necessary and adequate to preserve cartilage homeostasis in OA.

pulsatility and shear stress alone will be sufficient to activate YAP/TAZ in the adventitial myofibroblasts in the absence of a rigid matrix (Thenappan et al., 2018).

Atherosclerosis

Atherosclerotic plaques develop preferentially at arterial bifurcations and high curvatures but not straight segments of arteries. The site-specific manner of lesion formation suggests that local hemodynamic forces acting on vessel walls play a critical role in exerting atheroprone or atheroprotective effects on vascular cells. Indeed, as discussed previously, disturbed flow, but not steady laminar flow, activates endothelial YAP/TAZ in ECs, leading to upregulations of pro-atherogenic (e.g., *VCAM1*) and YAP/TAZ-target genes (e.g., *CTGF* and *CYR61*) *in vitro* (Wang K. et al., 2016; Wang L. et al., 2016; Xu et al., 2016). In agreement with the *in vitro* findings, a higher level of YAP/TAZ activation was detected in atheroprone regions than in atheroprotective regions mouse aorta, suggesting the involvement of disturbed flow-activated YAP/TAZ in atherogenesis. In ApoE$^{-/-}$ mice, knockdown of YAP/TAZ expression decreases the pro-atherogenic phenotypes of vascular cells and attenuates lesion development in atheroprone regions of arteries. On the other hand, atherosclerosis is encouraged by overexpression of endothelial YAP, or systemic TAZ, or a constitutively active YAP/TAZ mutation (Wang L. et al., 2016; Li et al., 2019). Taken together, these studies reassuringly uncovered the importance of YAP/TAZ as mechanotransducers in vascular cells and atherogenesis.

Besides YAP/TAZ, hemodynamic forces activate a number of signaling pathways to regulate endothelial phenotypes associated with atherosclerosis, *via* other transcription factors, such as KLF2, KLF4, NRF2, HIF-1α, NF-κB, AP-1, and a few others (Niu et al., 2019). These transcription factors, collectively referred to mechanosensitive transcription factors (MSTFs), crosstalk with one another to regulate ECs upon exposure to hemodynamic forces, as well as control cellular responses to oxidative stress, inflammation, and metabolic programming. Understanding how YAP/TAZ work in concert with these MSTFs in endothelium homeostasis will be important for us to obtain a panoramic view of the mechanosensing signaling network in endothelial cells.

In addition to their roles in ECs, the phenotypic switch of VSMCs from contractile to synthetic phase is regulated by YAP/TAZ activities, in response to stretch or wall stress-induced vascular remodeling (Wang et al., 2018). Stretching VSMCs activates their PI3K-PDK1 signaling, which then prevents the Hippo kinase cascade from inactivating YAP/TAZ.

Cardiac Hypertrophy

Cardiac hypertrophy is an adaptive response to hemodynamic overload. In the beginning, cardiac hypertrophy is beneficial because it increases the number of contractile units and reduces the ventricular wall pressure to a normal level according to Laplace's law. However, as the adjustment is physically limited, cardiac hypertrophy will lead to heart failure (Nakamura and Sadoshima, 2018).

Experimental studies have supported the concept that mechanical cues, such as hemodynamic overload, predominantly affect cardiomyocytes (CMs) by stretching. Integrins and the cytoskeleton or sarcolemmal proteins (e.g., phospholipases, ion channels, and ion exchangers) are two main types of

mechanosensors, by which hemodynamic overload is coupled to intracellular signals responsible for the hypertrophic response (Ruwhof and van der Laarse, 2000).

Hemodynamic overload includes two forms: pressure overload and volume overload. Endogenous YAP is a crucial mediator of hypertrophy in response to volume overload, which regulates CM growth and survival in the adult mouse myocardium in response to myocardial ischemic injury (Del Re et al., 2013). It was believed that compensatory cardiomyocyte hypertrophy is at least partially regulated by Yap1 after chronic myocardial infarction and a Yap1 deficit impairs the growth response to stress in heart, contributing to worsened operation. In response to pressure overload, endogenous YAP is triggered in CMs through a RhoA-dependent mechanism. Heterozygous YAP depletion inhibited hypertrophy, but increased fibrosis and apoptosis, and decreased cardiac functions (Byun et al., 2019). These findings highlight YAP as a potential target for myocardial infarction and hypertension.

Organ Fibrosis

Inflammation and wound-healing process following tissue injury and/or an idiopathic disease induce profibrotic responses, including abnormal ECM synthesis and deposition by fibroblasts and tissue stiffening and thickening. Such changes in structural and mechanical properties of the tissue are usually irreversible and lead to the formation of scar tissue, also known as fibrosis. The physical features of fibrotic tissues, together with the unresolved inflammation, continuously stimulate fibroblasts, resulting in pathological accumulation of ECM components and permanent tissue damage (Rockey et al., 2015; Tschumperlin et al., 2018).

YAP/TAZ serve as critical mechanotransducers in fibroblasts, coordinating profibrotic responses in various tissues, such as hepatic fibrosis, pulmonary fibrosis, kidney fibrosis, cardiovascular fibrosis, and others (Liu et al., 2015; Kim et al., 2019). The initial increase in ECM stiffness activates YAP/TAZ in fibroblasts, encouraging the development of profibrotic mediators and excessive deposition of ECM components. This results in progressive tissue stiffening, thereby forming an activation feed-forward loop to promote tissue fibrosis (Bertero et al., 2015a; Liu et al., 2015). Moreover, YAP/TAZ act as a molecular link between fibrosis and cancer. Fibrotic ECM stimulates cell proliferation and changes cell polarity, thereby promoting tumor development and growth (Noguchi et al., 2018). Various studies have assessed the efficacy of inhibiting YAP/TAZ activity as a new therapeutic strategy to reverse fibrosis (Liang et al., 2017; Haak et al., 2019; Alsamman et al., 2020; Dey et al., 2020), and the results of those have unfolded a promise of YAP/TAZ-targeting therapies for organ fibrosis.

Musculoskeletal Disorders (MSDs)

MSDs refers to diseases that affect the muscles, bones, and joints, which mainly tendinitis, carpal tunnel syndrome, osteoarthritis (OA), rheumatoid arthritis (RA), etc.

Mechanical load has been shown to activate YAP by increasing the expression of nuclear accumulation of YAP. Hyperactivation of YAP by sustained mechanical overload or YAP overexpression alone is sufficient to induce skeletal muscle hypertrophy (Goodman et al., 2015; Iyer et al., 2019; Owens et al., 2020). Besides responding to mechanical load, YAP/TAZ play a crucial role in muscle cell stemness and myogenesis (Figeac et al., 2019; Zhang L. et al., 2019). However, more studies are required as their atrophic roles of YAP/TAZ in muscle cells have also been reported (Gnimassou et al., 2017), and more details about the connection between mechanical stimuli and YAP/TAZ signaling would further improve our understanding of the roles YAP/TAZ in mechanotransduction and muscle homeostasis.

OA is mainly caused by mechanical overload (Glyn-Jones et al., 2015), and it has been reported that YAP is both necessary and adequate to preserve cartilage homeostasis in OA (Deng et al., 2018; Zhang Q. et al., 2019). A few recent studies demonstrated that suppressing YAP activity is effective in attenuating OA progression (Gong et al., 2019; Thorup et al., 2020; Zhang et al., 2020). However, research is still lacking on how the mechanical forces, especially compression and hydrostatic pressure, regulate Hippo-YAP/TAZ pathway in chondrocytes in healthy cartilage and during OA development.

Cancer

The biophysical factors in the tumor microenvironment play a pivotal role in disease progression and treatment outcomes. Common physical features that hinder cancer treatment, including solid stresses, interstitial fluid pressure, stiffness, and tumor microarchitecture, have been nicely reviewed by Nia HT, Munn LL, and Jain RK very recently (Nia et al., 2020). It has long been known that the changes in tumor microenvironment trigger signaling pathways to fuel cancer progression, immune blockade, and cancer resistance to therapy. Among the mechanosensitive pathways, the dysregulation of the Hippo-YAP signaling plays a central role in tumor development as it has been connected to most of the physical features in tumors. Emerging efforts have been made to elucidate the molecular mechanisms by which mechanical properties of various tumor types act through YAP/TAZ to promote cancer pathology.

Loss of cell-cell contact inhibition and unchecked cell growth are hallmarks of cancer, and the Hippo pathway is known to mediate contact inhibition and cell proliferation. Indeed, dysregulation of the Hippo signaling and hyperactivation of YAP/TAZ not only abolish the cell cycle regulation, as a result of losing contact inhibition, but also promote the transformation of mammary epithelial cells (Zhao et al., 2007, 2008). As the tumor grows, the changes in mechanical properties of the microenvironment continuously contribute to cancer progression and malignancy. Numerous studies have documented that stiff substrates activate YAP/TAZ to increase primary cancer cell growth, migration, and chemotherapy resistance (Lin et al., 2015; Chakraborty et al., 2017; Foster et al., 2017; Meng et al., 2018; Santinon et al., 2018; Molina et al., 2019; Ghasemi et al., 2020; Liu et al., 2020; Qin et al., 2020). Furthermore, it has been shown that Ras signaling-mediated oncogenic transformation of normal cells requires a stiff and/or fibrotic microenvironment (Meng et al., 2018; Panciera et al., 2020).

Mechanistically, the ECM-activated YAP/TAZ regulates the expression of various cytoskeletal regulators in cancer-associated fibroblasts and increases intracellular isometric tension, thus forming a forward feedback loop to enhance the tumor microenvironmental rigidity and support cancer cell growth and invasion (Calvo et al., 2013; Foster et al., 2017). It is speculated that YAP/TAZ hyperactivation is required for cancer cells to overdrive the mechanical checkpoints for growth (Aragona et al., 2013). In addition to cancer cells and cancer-associated fibroblasts, ECM stiffening during tumor progression induces vascular cell growth and allows blood vessel infiltration, potentially through the Agrin-YAP-dependent mechanism (Chakraborty et al., 2020). Indeed, it has recently been shown that the increases in tissue rigidity at metastatic sites enhance cancer angiogenesis and elevate their resistance to anti-angiogenesis therapy (Shen et al., 2020).

Cancer cells migrate and metastasize in blood and lymphatic vessels. Therefore, it would be important to know the flow regulation of YAP/TAZ in cancer cells. It has been reported that fluid shear stress induces YAP/TAZ activation to promote cancer cell migration and proliferation (Lee et al., 2017, 2018; Qin et al., 2019), suggesting a causative role of YAP/TAZ activation in cancer metastasis. In this regard, modulation of Hippo-YAP/TAZ signaling may represent a novel strategy for anti-metastasis therapies. In addition, the role of YAP/TAZ-mediated mechanosensing in tumorigenesis may be more complex and multi-functions in context-dependent manners and therefore worth further exploring. For example, a recent study in mice showed that activation of the YAP/TAZ in peritumoral normal hepatocytes suppressed liver tumor growth *via* cell competition mechanism against tumor cells (Moya et al., 2019). It would be important to elucidate the exact roles of YAP-dependent mechanoresponses in physical interaction between normal and tumor cells in future studies.

CONCLUSION

Research in the last decade has established an indispensable role of the Hippo-YAP pathway in mechanobiology of tissue growth and homeostasis, although more research is still needed to finely define biological roles and molecular mechanisms for specific pathogenesis processes. Many studies have implicated that the Hippo pathway may serve as a signaling integration hub through interpreting both mechanical and biochemical cues. One recent study worth particularly mentioning is from Barry Thompson group. They reported that mechanically induced Yki nuclear shuttling requires growth factors-activated PI3K-AKT signaling in *Drosophila*. This coordination of the force-regulated Yki signaling and the PI3K-AKT signaling couples cell polarity and tissue mechanics to nutritional cues in tissue growth control (Borreguero-Muñoz et al., 2019). Future investigation into the crosstalk between YAP-mediated mechanotransduction and biochemical cues in the disease microenvironment will be crucial for us to obtain a more comprehensive knowledge of pathogenesis associated with dysregulated tissue mechanics. Moreover, it will be also important to understand how the Hippo-YAP pathway works in concert with other mechanosensing mechanisms (e.g., KLFs, MRTF-SRF, TWIST, and β-catenin) to generate a singular but context-specific mechanoresponse in cells. Furthermore, as industrial and academic efforts are emerging for developing YAP-targeting agents, testing such agents in animal models of diseases associated with mechano-dysregulation will likely lead to novel therapies.

AUTHOR CONTRIBUTIONS

XC, K-CW, and ZM worked together to conceive and draft the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Mutations and Copy Number Abnormalities of Hippo Pathway Components in Human Cancers

*Zhengjin He, Ruihan Li and Hai Jiang**

State Key Laboratory of Cell Biology, CAS Center for Excellence in Molecular Cell Science, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai, China

The Hippo pathway is highly conserved from *Drosophila* to mammals. As a key regulator of cell proliferation, the Hippo pathway controls tissue homeostasis and has a major impact on tumorigenesis. The originally defined core components of the Hippo pathway in mammals include STK3/4, LATS1/2, YAP1/TAZ, TEAD, VGLL4, and NF2. However, for most of these genes, mutations and copy number variations are relatively uncommon in human cancer. Several other recently identified upstream and downstream regulators of Hippo signaling, including FAT1, SHANK2, Gq/11, and SWI/SNF complex, are more commonly dysregulated in human cancer at the genomic level. This review will discuss major genomic events in human cancer that enable cancer cells to escape the tumor-suppressive effects of Hippo signaling.

Keywords: hippo deficiency, cancer formation, copy number abberation, gene mutation, cancer genome

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Haiwei Song,
*Institute of Molecular and Cell Biology (A*STAR), Singapore*

***Correspondence:**
Hai Jiang
hai@sibcb.ac.cn

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INTRODUCTION

The Hippo signaling pathway is highly conserved through evolution. The core components of the pathway were originally identified in *Drosophila*. Their orthologous genes in mammals were found later (Ma et al., 2019; Snigdha et al., 2019). A large number of studies have shown that the Hippo pathway controls organ size mainly by responding to cell contact and various mechanical signals. The Hippo pathway also responds to cell polarity and G protein-coupled receptor (GPCR) signals. Given that loss of contact inhibition is one of the major hallmarks of human cancer, dysregulation of the Hippo pathway, which enables cancer cells to overcome contact inhibition, should be common in human cancers.

Gene dysregulation in human cancer can occur at various levels, including gene mutation/copy number abnormality, DNA methylation, over/under-expression, and post-translational modifications. Comparing to other types of dysregulations, mutation, and copy number abnormality data are more tractable and concrete. Therefore, this review will focus on mutation and copy number abnormality of the Hippo pathway components in human cancers.

THE CORE COMPONENTS OF THE HIPPO PATHWAY IN CANCER

The originally defined core components of the Hippo pathway include neurofibromin 2 (NF2), serine/threonine kinase 3/4 (STK3/4, originally called MST1/2), large tumor suppressor kinase 1/2 (LATS1/2), Yes1-associated transcriptional regulator (YAP1), tafazzin (TAZ), and TEA domain transcription factor (TEADS). When the Hippo pathway is activated by upstream signals, STK3/4

and Salvador Family WW domain containing protein 1 (SAV1) form a heterodimer through their C-terminal SARAH domain. Subsequently, STK3/4 phosphorylates LATS1/2, which then phosphorylates and inhibits the downstream substrate YAP1. Phosphorylated YAP1 is sequestered by 14-3-3 protein in the cytoplasm and/or degraded by the ubiquitination process.

When the Hippo pathway is inactivated, YAP1 is dephosphorylated and translocates to the nucleus, where YAP1/TAZ binds to TEAD, inducing target gene expression and promoting cell proliferation (**Figure 1A**). Vestigial-like family member4 (VGLL4) competitively inhibits the interaction of YAP1 and TEAD, providing another level of regulation on Hippo signaling output.

STK3/4 AND LATS1/2

In the Hippo signaling pathway, STK3/4 and LATS1/2 play antiproliferative roles and should act as tumor suppressors. However, according to the Catalogs of Somatic Mutations in Cancers (COSMIC) database[1], genetic dysregulation of these genes is rare for most types of human cancers. This may, in part, be explained by gene redundancy (Meng et al., 2015; Plouffe et al., 2016). For example, mutation or deletion of *LATS1* alone will not enable escape from the Hippo pathway's anti-proliferative effect since *LATS2* is still intact in cells. Therefore, single genetic events impacting *STK3/4* and *LATS1/2* will be insufficient to drive cancer formation. To fully destroy LATS1/2 activity, it will require four inactivating events to destroy both copies of *LATS1* and *LATS2*. Therefore, from a cancer development point of view, achieving YAP1 activation through inactivating Hippo kinases may not be an easy route.

In addition to gene redundancy, other possibilities may also have contributed to the low mutation rates of Hippo kinases in cancer. For example, (Moroishi et al., 2016) showed that although inactivation of *LATS1* and *LATS2* led to enhanced anchorage-independent cell growth *in vitro*, it also caused increased tumor immunogenicity and tumor regression *in vivo*. This highlights the dual functions of LATS1/2 in cancer and reflects the crucial role of Hippo kinases in regulating tissue homeostasis.

YAP1 AND TEADs

As the key component of the Hippo pathway, hyperactivation of YAP1 is widespread in cancers (Harvey et al., 2013; Mo et al., 2014) as evidenced from immunostaining of YAP1 in human cancer samples. These studies found that YAP1 is commonly enriched in the nucleus in tumors while residing in the cytoplasm in normal tissues. The percentage of cells with nuclear YAP1 staining in hepatocellular carcinomas, ovarian cancers, and non-small-cell lung cancers is 60, 15, and 65%, respectively (Harvey et al., 2013).

Yes1 Associated Transcriptional Regulator is not frequently mutated in human cancers. Although the YAP1 S127A activating mutant is commonly used in cellular studies and tumor models, there is no corresponding active point mutation of *YAP1* enriched in human cancer[2]. As discussed below, the increased activity of YAP1 in human cancers may be attributed to *YAP1* gene amplification and gene fusion as well as the dysregulation of other components of the Hippo pathway.

(Zender et al., 2006) first noticed that the chromosome 9qA1 region, containing *Yap1* gene, is amplified in mouse liver cancer cells. They also showed that overexpression of YAP1 can promote tumor formation. The corresponding amplified chromosome region in humans is 11q22 (Zender et al., 2006). This raised the possibility that the amplification of *YAP1* may contribute to cancer development. **Figure 1B** shows the gene amplification status in the 11q22 region, including *YAP1*. *YAP1* localized within the amplification peak region, suggesting for an oncogenic role. However, it is worth noticing that two adjacent genes, baculoviral IAP repeat containing 2/3 (*BIRC2/3*), are also amplified in human cancers. BIRC2/3 are anti-apoptotic proteins and promote cancer cell survival. It remains unclear whether the 11q22 amplicon utilizes all three genes to promote human cancer.

An interesting question is why *YAP1* amplification is not a more frequent event in human cancers. In the COSMIC database, the number of human cancer samples amplifying *YAP1* is 148. In comparison, such numbers for other major oncogenes are *MYC* = 968, *EGFR* = 514, *ERBB2* = 358, *SKP2* = 317, and *MDM2* = 324. Cellular studies demonstrated that YAP1 rapidly induces the expression of LATS2, thus forming a negative feedback loop that self-limits its activity (Moroishi et al., 2015). The YAP1 S127A mutant, which escapes negative regulation by LATS kinases, is more effective at inducing tumor and has been used in many cancer models (Zhang S. et al., 2017; Min et al., 2019; Smith et al., 2021). It is possible that, due to such a negative feedback mechanism, simple overexpression of wild-type YAP1 may not be sufficient to trigger long-lasting proliferative events, which may partially explain the relative lower frequencies of *YAP1* amplification in human cancers. On the other hand, it is possible that, similar to the case of LATS1/2 inactivation (Moroishi et al., 2016), YAP1 activity might also trigger additional events that negatively affect tumorigenesis.

Recent studies also identified *YAP1* gene fusion events in several kinds of rare cancers, such as supratentorial (ST) ependymoma (Pajtler et al., 2015, 2019), epithelioid hemangioendothelioma, cervical squamous cell carcinoma (Hu et al., 2018), endocervical adenocarcinoma (Antonescu et al., 2013), and other cancers (Picco et al., 2019; Sekine et al., 2019; Sievers et al., 2020). The fusion protein products of YAP1 in these cancers include YAP1-MAMLD1, YAP1-FAM118B, YAP1-TFE3, and YAP1-SS18. Szulzewsky et al. (2020) demonstrated that these fusion proteins are resistant to negative Hippo pathway regulation and stay in the nucleus. In addition, the fusion proteins of YAP1 are also more stable and escape from degradation.

As the final executor of Hippo signaling, the transcription factor TEAD has very low transcriptional activity without the binding of YAP1 (Ma et al., 2019). *TEAD* genes are not known to be localized in gene amplification peaks in human cancers.

[1]https://cancer.sanger.ac.uk/cosmic

[2]https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=YAP1

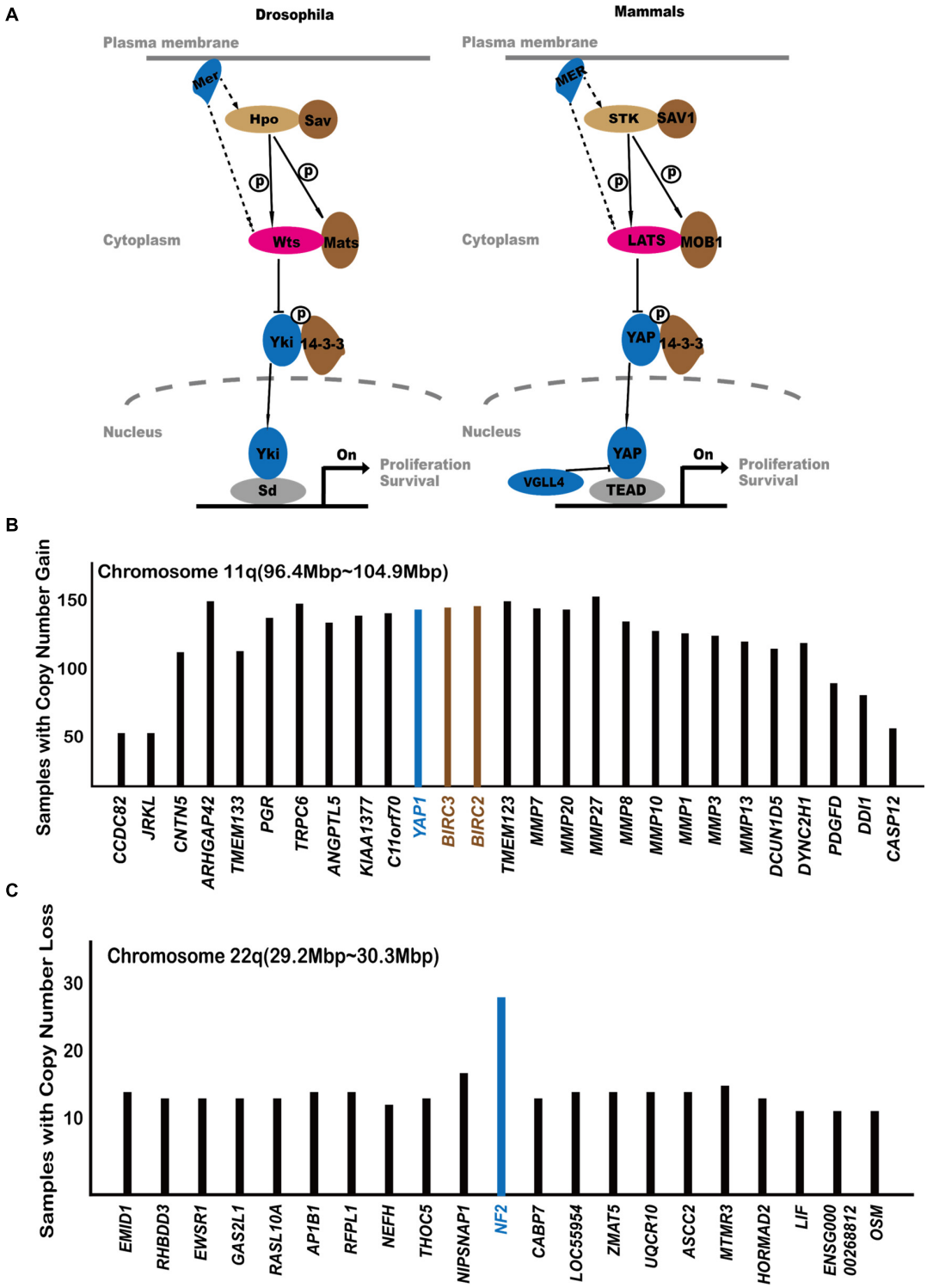


FIGURE 1 | The central players of the Hippo pathway. **(A)** The originally established core components of the Hippo pathway. **(B)** Analysis of COSMIC gene amplification data with regard to the 11q22 amplicon. Genes are arranged according to their locations on chromosome 11q22. The Y axis shows how many cancer samples in COSMIC database exhibit gene amplification of each gene. If a gene is located at the amplification peak position, it is more likely to be a cancer-driving event. **(C)** Analysis of COSMIC gene amplification data with regard to the 22q deleted region.

Mutations of *TEAD* genes are also rare in human cancer, and there are few literatures reporting the functional mutations of *TEAD* genes (Huh et al., 2019).

NF2

Neurofibromin 2 (*NF2*) is a well-established tumor suppressor gene. It encodes Merlin, ortholog of the *Drosophila* Merlin protein, which encodes a FERM domain-containing protein. Studies of mouse and tumor patients showed that the inactivation of *NF2* is an important cause of cancer. Hamaratoglu et al. (2006) firstly found that *NF2* inhibits tumor development by regulating the Hippo pathway. Many reports demonstrated that YAP1 is dephosphorylated and activated with the loss of *NF2*, whereas the proliferation effect of *NF2* loss can be eliminated by *YAP1* knockout (Zhang et al., 2010). Hong et al. (2020) found that the lipid binding ability of Merlin is critical for its function in activating the Hippo pathway, which further clarifies the function and mechanism of *NF2* (Yin et al., 2013). Several other mouse model studies also demonstrated that deletion of *NF2* promotes tumor development (Giovannini et al., 2000; Kalamarides et al., 2002).

Hereditary loss of function mutations of *NF2* causes type 2 neurofibromatosis, a disorder characterized by neoplastic growth in the nervous system (Yin et al., 2013). Somatic loss-of-function *NF2* mutations are also found in many other kinds of cancers such as mesotheliomas and bladder, thyroid, and skin cancer. The COSMIC database indicates that *NF2* mutations are highly enriched for nonsense mutations[3]. *NF2* is also located at a deletion peak in cancer samples (**Figure 1C**). This indicates that gene mutation and gene deletion are both common means of *NF2* loss of function in human cancers.

VGLL4

Vestigial Like Family Member 4 can inhibit organ overgrowth and cancer formation caused by YAP1 dysregulation in both human and *Drosophila* (Guo et al., 2013; Zhang et al., 2014). Jiao et al. (2014) found that VGLL4 competes with YAP1 to bind TEADs. Such an event will inhibit gene transcription by YAP1–TEADs and suppress cell proliferation (Zhang Y. et al., 2017).

Vestigial Like Family Member 4 has been described as a tumor suppressor in many cancers (Jiao et al., 2014; Zhang et al., 2014; Zhang Y. et al., 2017; Gallagher et al., 2020). For example, (Zhang et al., 2014) found that the VGLL4 expression level in mouse and human lung tumor specimen is significantly lower than in normal tissue. Overexpression of VGLL4 inhibits the progression of lung cancer in mice (Zhang et al., 2014). The findings of Jiao et al. (2014); Zhang Y. et al. (2017) in gastric cancer and breast cancer also support the idea that *VGLL4* inhibits tumor progression.

Although *VGLL4* is rarely mutated in human cancer, it is located at the short arm (3p) of chromosome 3, which is lost in many types of cancer (Williamson, 2002; Cancer Genome Atlas Research Network, 2013; Jonasch et al., 2020; Nidorf et al., 2020; Shaikh et al., 2020). The Cancer Genome Atlas (TCGA) Research Network found that around 90% of clear cell renal carcinoma patients exhibit loss of one or both copies of chromosome 3p. Chromosome 3p loss is also commonly observed in lung and stomach cancers. The rate of both 3p arm loss is lower in human cancer, typically around 10–20% in renal clear cell carcinoma. It is worth noticing that, through 3p loss, cancer cells also delete other important tumor suppressors such as *VHL*, *SETD2*,*BAP1*, and *PBRM1*. These events may also promote cancer development independent of *VGLL4*.

OTHER COMPONENTS OF THE HIPPO PATHWAY IN CANCER

In addition to the afore-mentioned core components of the Hippo pathway, several recent studies identified new regulators of Hippo signaling. Some of these new regulators are also prominently dysregulated in human cancers, providing additional routes for cancer cells to escape from Hippo signaling.

FAT1

FAT atypical cadherin 1 (FAT1) is a transmembrane protein, homologous to the tumor-suppressor genes *fat* and *kujelei* (also known as *fat2*) in *Drosophila*. By analyzing the TCGA database, (Martin et al., 2018) found that *FAT1* shows a high-frequency mutation in many types of cancer. The study revealed that the cytoplasmic domain of FAT1 can activate the Hippo pathway by recruiting Hippo components such as NF2, STK3/4, and LATS1/2 to the cell membrane, forming a "kinase signalome" (Martin et al., 2018). In addition, other researchers also found that FAT1 regulates the Hippo pathway by YAP1 and TAZ (Ahmed et al., 2015; Li et al., 2018). One recent study showed that deletion of *FAT1* in mouse epithelial cells induces epithelial–mesenchymal transition of epithelial cells and promote tumorigenesis in mice (Pastushenko et al., 2021).

According to the TCGA database, *FAT1* is frequently mutated in many types of cancer (Katoh, 2012; Ahmed et al., 2015; Zhang et al., 2016). *FAT1* exhibits 25.2 and 25.3% mutation rates in head and neck cancer and uterine corpus endometrial carcinoma, respectively, according to the TCGA database. Analysis of the COSMIC database indicated that *FAT1* is located at a gene deletion peak in tumor samples, further suggesting a tumor suppressor role for FAT1 (**Figure 2A**).

SHANK2

The SH3 and multiple ankyrin repeat domains (SHANK) protein family has been studied in the field of neuroscience mostly. As a scaffold protein, SHANK promotes synapse formation and balances synaptic transmission. In a recent study (Xu et al., 2020), found that the overexpression of SHANK2

[3]https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=NF2

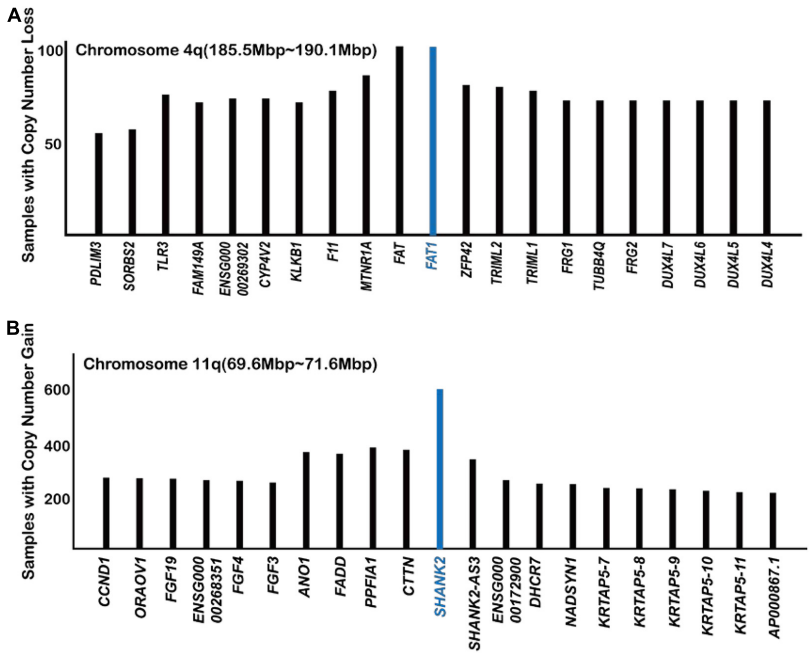


FIGURE 2 | Analysis of COSMIC gene copy number data with regard to FAT1 **(A)** and SHANK2 **(B)** (https://cancer.sanger.ac.uk/cosmic/).

ortholog in *Drosophila* suppresses Hippo signaling, causing an overgrowth of wings and eyes. In human cells, overexpression of SHANK2 also caused sustained YAP1 activity even at high cell density and promoted tumor formation in mice. SHANK2 inhibits the LATS1/2-mediated phosphorylation of YAP1 by competing for binding to rho guanine nucleotide exchange factor 7 (ARHGEF7), which is an activator protein of LATS1/2 (Heidary Arash et al., 2014). Analysis of the COSMIC database also showed that *SHANK2* is the most frequently amplified gene in the 11q13 amplicon (**Figure 2B**; Xu et al., 2020). It is worth noticing that the number of cancer samples with *SHANK2* amplifications way exceeds those with *YAP1* amplification, *FAT1* deletion, and *NF2* deletion (**Figures 1B**, **2**), indicating a previously unnoticed role of *SHANK2* as a major oncogene.

GQ/G11

The G protein subunit alpha Q (GNAQ) and G protein subunit alpha 11 (GNA11) genes encode Gq and G11 proteins, respectively, which play an essential role in GPCR signaling pathway. Yu et al. (2014) found that the mutant Gq/G11 can activate the YAP1 protein. In addition, inhibition of YAP1 can block the proliferation of Gq/G11 mutant cells These results imply that the pro-proliferation effect of mutant Gq/G11 depends on the function of YAP1.

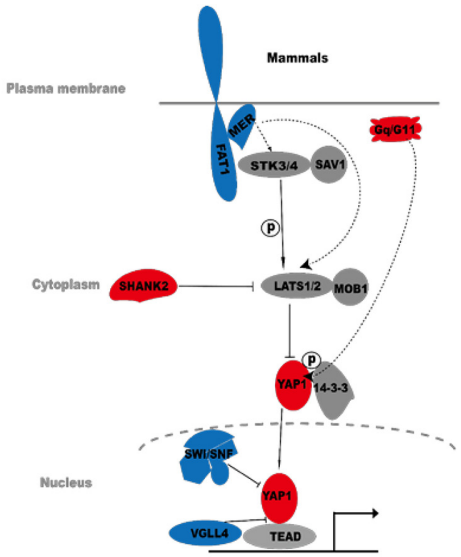
Previous studies (Van Raamsdonk et al., 2009, 2010) showed that overexpression of mutant Gq/G11 causes normal melanocyte transformation, whereas knockdown of *Gq/G11* blocks tumor formation in xenograft experiments. Data from the TCGA database indicated that the percentage of *GNAQ* and *GNA11* gene mutation is 50 and 43.8%, respectively, in uveal melanoma patients, suggesting a major involvement of the Hippo pathway for this type of cancer.

SWI/SNF

SWI/SNF is a multi-subunit ATP-dependent chromosome remodeling complex. The SWI/SNF complex plays key roles in regulating gene expression and tissue homeostasis. Mutations of the subunits of this complex are detected in a variety of human malignancies (Kadoch et al., 2013; Shain and Pollack, 2013; Lou et al., 2020).

Increasing evidence indicated that the SWI/SNF complex inhibits tumor development (Weissman and Knudsen, 2009; Wilson and Roberts, 2011; Shain and Pollack, 2013; Ribeiro-Silva et al., 2019). A large number of studies showed that the deletion of components of SWI/SNF promotes tumor development in mouse models (Klochendler-Yervin et al., 2000; Roberts et al., 2000; Glaros et al., 2007, 2008). In a recent study, (Chang et al., 2018) found that the ARID1A subunit of the SWI/SNF complex binds to and inactivates YAP1. Therefore, once the SWI/SNF complex is inactivated by various mutations in cancer cells, YAP1 will be released and promote carcinogenesis. In addition, the SWI/SNF complex may also inhibit cancer development by maintaining genome stability (Ribeiro-Silva et al., 2019). The statistical results of Ribeiro-Silva et al. (2019) indicate that mutations in genes encoding for SWI/SNF subunits are found in approximately 20% of all human cancers of various types. This may constitute one of the most frequent routes of Hippo dysregulation in human cancer.

A



B

	STK4	STK3	LATS1	LATS2	YAP1	NF2	VGLL4	FAT1	SHANK2	GNAQ	GNA11	BAF180	BRG1	BRM	ARID1A
AML	-	-	-	-	-	-	-	4.6	-	-	-	-	-	-	-
Bladder Urothelial Ca.	1.7	2.4	3.9	1.9	1.5	3.6	1.2	12.9	4.1	1.0	1.5	5.6	6.3	6.6	26.9
Breast Invasive Ca.	0.6	0.7	0.9	-	0.5	-	0.6	4.1	3.1	-	0.6	-	-	1.5	4.7
Cervical Squamous Ca.	1.7	1.4	5.5	2.8	2.1	3.8	3.1	10.0	4.8	0.7	1.7	4.5	5.9	3.5	8.3
Cholagioca.	-	-	-	2.0	2.0	3.9	2.0	-	-	3.9	-	21.6	3.9	-	17.7
Colon Adenoca.	3.3	3.5	6.0	4.8	1.3	3.3	3.0	17.3	11.3	2.8	2.3	6.0	9.3	6.5	16.5
Hematopoietic	1.4	-	0.9	-	1.3	1.4	0.5	6.1	3.4	1.7	0.3	-	-	-	-
DLBCL	-	2.7	-	2.7	-	-	-	13.5	13.5	2.7	-	2.7	-	2.7	10.8
Esophageal Ca.	2.7	2.7	1.6	2.7	2.2	-	1.1	5.4	9.8	0.5	0.5	3.3	7.6	2.2	10.3
GBM	0.5	0.5	1.8	1.5	-	1.3	0.5	-	3.1	0.5	0.3	-	-	2.8	-
Head&Neck Ca.	0.6	0.6	2.0	1.4	0.6	1.4	1.2	25.2	4.1	0.4	0.4	3.0	5.1	2.2	5.3
KIRC	-	0.6	-	-	0.6	1.8	-	-	-	-	0.3	43.5	4.2	-	4.8
KIRP	-	-	1.4	-	1.1	3.9	-	6.1	-	-	-	4.3	4.3	-	5.0
Liver Hepatocellular Ca.	-	0.6	1.9	-	-	1.9	-	-	2.8	0.3	0.3	-	3.0	2.5	8.8
Lung Adenoca.	1.6	1.2	3.4	3.0	0.5	1.1	-	12.5	4.8	1.1	0.7	2.7	9.4	4.1	6.7
Lung Squamous Ca.	1.4	1.4	6.1	1.6	1.0	1.6	0.6	16.4	4.7	0.8	0.2	3.6	4.9	3.2	7.7
Mesothelioma	-	-	2.4	9.8	-	24.4	-	-	-	-	-	3.7	-	-	-
Neuroblastoma	-	-	-	1.8	1.4	-	-	-	-	-	-	-	-	-	-
Ovarian Serous Ca.	0.9	0.5	2.3	-	0.5	1.2	1.2	6.0	4.4	-	1.2	-	4.1	2.1	-
Pancreatic Adenoca.	0.6	-	1.7	-	0.6	-	0.6	-	-	0.6	0.6	3.3	-	-	5.5
Prostate Adenoca.	0.6	-	-	-	-	-	0.4	-	-	-	-	-	-	-	-
Rectun Adenoca.	-	0.7	4.4	2.9	0.7	2.2	2.2	9.5	2.9	0.7	0.7	3.7	3.7	2.9	6.6
Sarcoma	-	-	-	3.0	-	1.3	0.4	3.8	5.5	-	0.4	-	-	2.1	-
SKCM	2.6	0.9	3.8	4.7	-	2.6	2.1	14.7	17.1	3.0	3.4	5.8	9.6	6.6	7.7
Stomach Adenoca.	1.4	2.7	5.0	5.0	1.1	1.8	1.6	11.1	7.5	1.4	1.6	8.4	8.0	7.7	27.1
TGCT	-	0.7	-	-	-	-	-	-	-	0.7	-	-	-	-	-
Thymoma	0.8	-	-	-	-	-	-	-	-	-	-	2.4	-	-	-
UCEC	10.0	8.9	10.6	14.0	9.3	5.7	4.9	25.3	18.9	6.4	4.0	12.6	15.3	13.6	46.6
Uveal MelanomaB	1.3	-	-	-	1.3	-	-	-	-	50.0	43.8	-	-	-	-

FIGURE 3 | Hippo components in human cancer. **(A)** Major cancer players of the Hippo signaling pathway. Genes in red are oncogenes. Genes in blue are tumor suppressors. **(B)** Percentage of genetic abnormalities of the indicated Hippo pathway components in human cancer. The analysis was performed on The Cancer Genome Atlas dataset. AML, acute myeloid leukemia; DLBCL, diffuse large B cell lymphoma; GBM, glioblastoma multiforme; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; SKCM, skin cutaneous melanoma; TGCT, testicular germ cell tumors; UCEC, uterine corpus endometrial carcinoma.

CONCLUSION

The Hippo pathway, by responding to cell density and maintaining cell–cell contact, is a crucial barrier for tumor development. When the Hippo pathway is dysregulated, cells will acquire the potential for uncontrolled proliferation, promoting cancer formation. Although mutations and/or copy number abnormalities directly impacting the core Hippo kinases are relatively rare in human cancers, cancer cells manage to escape from Hippo regulation by means of other upstream and downstream Hippo regulators, including FAT1, SHANK2, SWI/SNF, Gq/11, VGLL4, *etc*. In this review, we focused on Hippo dysregulation in human cancers at the genomic level. It is also worth noticing that other oncogenic events, for example, IDH1 mutation, can also affect the Hippo pathway components through gene hypermethylation (Gu et al., 2020).

Several recent studies also identified other important regulators of the Hippo pathway, including the RAP family of small GTPase, MST4, and others (Meng et al., 2018; An et al., 2020). These genes do not appear to be frequently dysregulated at the genomic level in human cancers, possibly due to gene redundancy or other reasons. For example, all three *RAP2* genes (*Rap2A/B/C*) need to be simultaneously knocked out to cause YAP1 nuclear localization (Meng et al., 2018). Therefore, these genes are not included in this review. **Figure 3** summarizes the major cancer players of the Hippo pathway and their frequency of genetic dysregulation in various forms of human cancers. Such findings will help bring a clearer view of Hippo pathway

dysregulation in cancers as well as point to potential precision medicine approaches targeting YAP1 activity for cancer therapy.

For example, cancers that dysregulate the Hippo pathway may be more sensitive to suppression of YAP1 activity. On the one hand, the degradation of YAP1 may be a choice for cancer treatment. Proteolysis-targeting chimera (PROTAC) technology could provide us with a useful method for YAP1 degradation (Li and Song, 2020). On the other hand, VGLL4's ability to disrupt YAP1–TEADs interaction indicates that peptide mimics of VGLL4 could potentially be useful for suppressing cancers driven by Hippo pathway dysregulation. Newly identified oncogenes in the Hippo pathway such as Gq/G11 and SHANK2 may also represent potential PROTAC targets for cancer treatment.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript. ZH prepared Figures.

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Identification of TAZ-Dependent Breast Cancer Vulnerabilities Using a Chemical Genomics Screening Approach

He Shen[1], Yanmin Chen[1], Yin Wan[1], Tao Liu[2], Jianmin Wang[2], Yali Zhang[2], Lei Wei[2], Qiang Hu[2], Bo Xu[3], Mikhail Chernov[4], Costa Frangou[5] and Jianmin Zhang[1]**

[1] Department of Cancer Genetics and Genomics, Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States, [2] Department of Biostatistics and Bioinformatics, Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States, [3] Department of Pathology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States, [4] Department of Cell Stress Biology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States, [5] Molecular and Integrative Physiology Department, Harvard T.H. Chan School of Public Health, Boston, MA, United States

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Wenqi Wang,
University of California, Irvine,
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Hai-long Piao,
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Lorenzo Sempere,
Michigan State University,
United States

***Correspondence:**
Costa Frangou
frangou@icloud.com
Jianmin Zhang
Jianmin.zhang@roswellpark.org

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Breast cancer stem cells (BCSCs) represent a subpopulation of tumor cells that can self-renew and generate tumor heterogeneity. Targeting BCSCs may ameliorate therapy resistance, tumor growth, and metastatic progression. However, the origin and molecular mechanisms underlying their cellular properties are poorly understood. The transcriptional coactivator with PDZ-binding motif (TAZ) promotes mammary stem/progenitor cell (MaSC) expansion and maintenance but also confers stem-like traits to differentiated tumor cells. Here, we describe the rapid generation of experimentally induced BCSCs by TAZ-mediated reprogramming of human mammary epithelial cells, hence allowing for the direct analysis of BCSC phenotypes. Specifically, we establish genetically well-defined TAZ-dependent (TAZ_{DEP}) and -independent (TAZ_{IND}) cell lines with cancer stem cell (CSC) traits, such as self-renewal, variable resistance to chemotherapeutic agents, and tumor seeding potential. TAZ_{DEP} cells were associated with the epithelial to mesenchymal transition, embryonic, and MaSC signature genes. In contrast, TAZ_{IND} cells were characterized by a neuroendocrine transdifferentiation transcriptional program associated with Polycomb repressive complex 2 (PRC2). Mechanistically, we identify Cyclin D1 (CCND1) as a critical downstream effector for TAZ-driven tumorigenesis. Overall, our results reveal a critical TAZ-CCND1-CDK4/CDK6 signaling axis, suggesting novel therapeutic approaches to eliminate both BCSCs and therapy-resistant cancer cells.

Keywords: Hippo pathway, TAZ, breast cancer, stem cells, small molecule, Cyclin D, CDK4/6

INTRODUCTION

The genomic characterization of thousands of primary breast tumors has revealed considerable spatiotemporal heterogeneity within the tumors from individual patients, presenting a formidable challenge when it comes to diagnosis, prognosis, and developing effective breast cancer (BC) therapeutics. Two prevailing models have been proposed to account for tumor heterogeneity. In

the clonal evolution model, stochastic mutations in individual tumor cells serve as a platform for the adaptation and natural selection of the fittest variants. Cancer clone epigenetic and/or genetic diversification within tissue microenvironments confers phenotypic, behavioral, and functional differences among BC cells (Meacham and Morrison, 2013). In contrast, the cancer stem cell (CSC) model posits that a subset of cells in tumors can both self-renew and differentiate into diverse cancer cell hierarchies, which play a decisive role in tumor growth, progression, recurrence, and treatment resistance.

The importance of targeting CSCs [also known as tumor-initiating cells (TICs)] derives from the multiple clinical and experimental observations showing that CSCs have increased tumor seeding potential and are resistant to chemotherapeutic agents and ionizing radiation (Brooks et al., 2015; Vlashi and Pajonk, 2015). Extensive research has focused on discovering and characterizing CSCs at the origin of different tumors. Despite these efforts, the definition of CSCs remains largely operational and based on the functional assays that monitor their self-renewal and tumorigenic properties (i.e., the formation of tumor spheres *in vitro* and heterogeneous tumors at limiting dilutions *in vivo*). Nonetheless, CSC-enriched cancer cell populations can be isolated using cell-surface marker profiles. For instance, BC stem cells (BCSCs) were first identified as a $CD24^{-/low}/CD44^{+}$ population with an enhanced ability to initiate tumor growth when xenografted into immunocompromised mice (Al-Hajj et al., 2003). Subsequent studies have identified additional markers, such as Aldeflour, that measure the ALDG (aldehyde dehydrogenase) activity in mammary stem/progenitor cells (MaSCs) (Ginestier et al., 2007). Whether these markers are universally expressed in tumors, identify the same BC cell population, or correspond to therapeutic response remains unclear.

The ability of cells to adopt different and reversible identities is a phenomenon known as cellular plasticity. Dynamic interconversions between transitional epithelial and mesenchymal states predicate the intrinsic plasticity observed in mammary epithelial cells (MECs) in response to exogenous stimuli and microenvironment factors. Under such conditions, cellular plasticity serves as a tissue adaptation mechanism, but it can also predispose cells to malignant transformation and tumorigenesis. For example, epithelial-mesenchymal transition (EMT), where cells with an epithelial phenotype transition to display a mesenchymal phenotype while maintaining the capacity to reassume their epithelial state, is the best-known case of tumor cell plasticity (Kalluri and Weinberg, 2009; Chaffer et al., 2016; Brabletz et al., 2018). Notably, when EMT is aberrantly activated in cancer, cells gain attributes of stem cells that contribute to self-renewal capabilities and can differentiate to all cell types represented in the tumor (Mani et al., 2008). Although many studies have defined the association between EMT induction and acquisition of stemness, few have addressed the mechanisms by which EMT directly induces BCSCs and link these two cellular states.

Several pathways have been implicated in BCSC expansion and maintenance regulation, including Hedgehog, Hippo, TGF-β, Notch, and Wnt/β-catenin (Yousefnia et al., 2020). Hippo signaling is an evolutionarily conserved pathway that controls tissue and organ size by regulating cell proliferation, apoptosis, and MaSC self-renewal (Zheng and Pan, 2019). Hippo signaling involves a highly conserved core kinase cascade, including MST1/2, LATS1/2, and the transcriptional coactivators YAP (Yes-associated protein) and TAZ (WW domain-containing transcription regulator protein), which are downstream effectors of the pathway (Moroishi et al., 2015; Zanconato et al., 2016). Recent studies have highlighted YAP/TAZ's role in regulating MEC plasticity. For instance, the overexpression of TAZ can induce EMT (Chan et al., 2008; Lei et al., 2008; Yang et al., 2012). Furthermore, the transient expression of exogenous TAZ in primary differentiated mouse MECs can induce the conversion of a tissue-specific stem or progenitor cell state (Panciera et al., 2016). Similarly, the hyperactivation of YAP/TAZ gives non-BCSCs the ability to remain undifferentiated, self-renew, and metastasize (Cordenonsi et al., 2011; Kim et al., 2015).

Understanding the molecular mechanisms that underlie BCSC properties has been greatly hindered because of the difficulty in isolating rare and heterogeneous CSCs from bulk tumor tissues and propagating the cultures of the BCSCs *in vitro*. Here, we describe an experimental workflow that allows for the rapid isolation of these cells from mammary tumors with defined genotypes. Using this approach, we identify a TAZ-CCND1-CDK4/CDK6 signaling axis that is involved in BCSC self-renewal and propose that CDK4/6 inhibitors can serve as a potential therapeutic drug to target TAZ dependency in these cells.

RESULTS

Isolation of Tumor-Initiating Cells From Human BC Xenografts

To understand BCSC phenotypes and identify the pathways involved in their genesis and maintenance, we set out to establish a robust platform for enriching TICs from human tumor xenografts. We previously described a transplant model of BC in which constitutively active TAZ expression is controlled by the reverse transactivator system in a dox-dependent manner (Shen et al., 2019). Upon dox removal, most mammary tumors regressed; however, in a subcohort of mice, we also observed tumor growth that occurred independent of TAZ expression (**Figure 1A**). We reasoned that these mammary tumors contained TICs with stem markers and functional stem cell traits from the different oncogenic drivers required for tumor growth and progression.

We harvested TAZ-dependent (12 mice) and -independent mammary tumors (5 mice). Thereafter, for TICs, we procured samples from bulk tumor cells using mammosphere growth conditions, which rely on the fact that cells with stemness features preferentially respond to growth factors and grow in suspension as clonal non-adherent spherical clusters (Fillmore and Kuperwasser, 2008). Herein, we describe three genetically characterized cell lines derived from MCF10A-TAZ mammary tumors (**Supplementary Table 1**): TAZ-dependent cells (hereafter denoted as TAZ_{DEP}) and two TAZ-independent cell lines (TAZ_{IND}). As shown in **Figure 1B**, TAZ_{DEP} and TAZ_{IND}

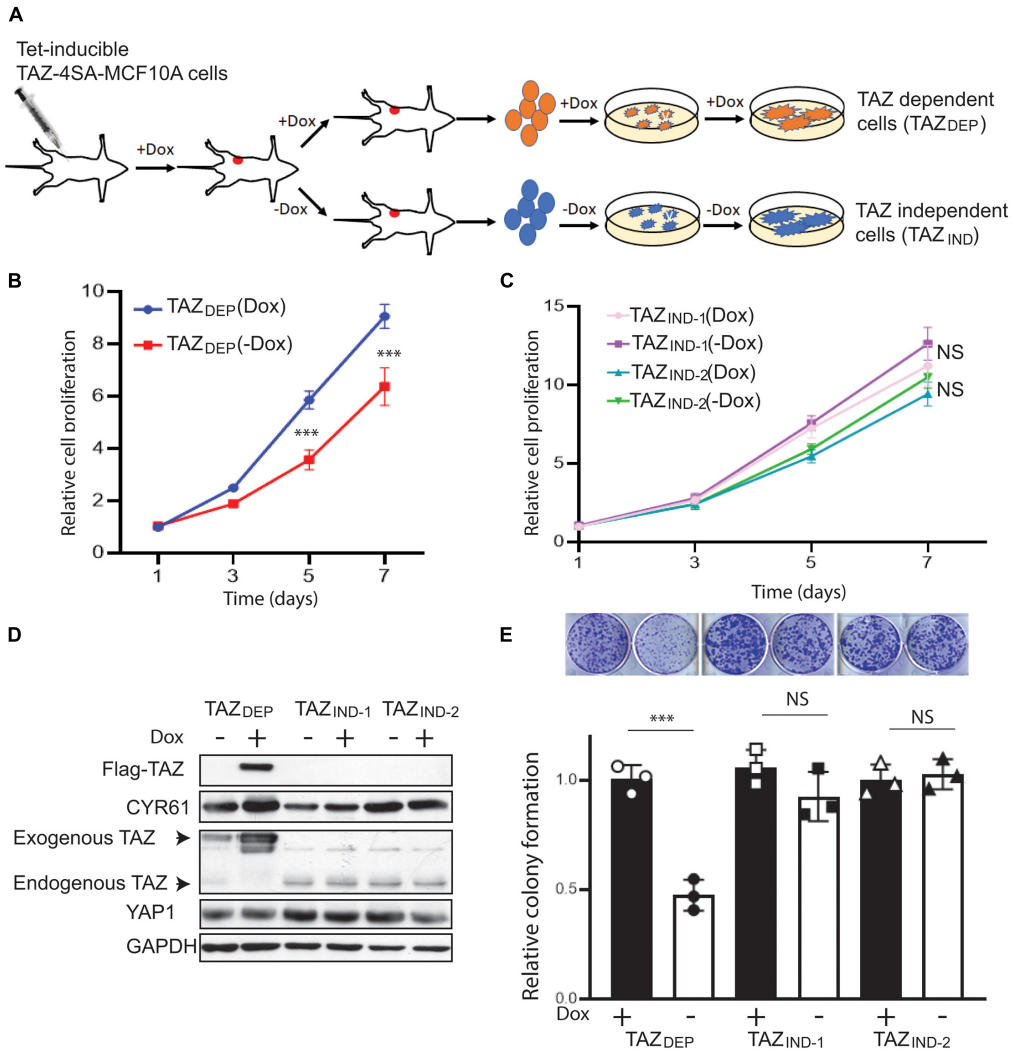


FIGURE 1 | Isolation of TAZ_{DEP} and TAZ_{IND} tumor-derived isogenic cells. **(A)** Schematic description of the preparation of TAZ_{DEP} and TAZ_{IND} tumor-derived isogenic cells. **(B)** Cell proliferation assay of TAZ_{DEP} cells in response with or without dox treatment (2 μg/ml). Data are shown as the mean ± SD. Unpaired two-tailed Student's *t*-test: $^{***}p < 0.001$. **(C)** Cell proliferation assay of TAZ_{IND} cells in response with or without dox treatment (2 μg/ml). Data are shown as the mean ± SD. Unpaired two-tailed Student's *t*-test: NS = not significant. **(D)** Exogenous (Flag-TAZ) and endogenous TAZ, CTGF, and YAP expression detected by immunoblotting. GAPDH was used as a loading control. **(E)** Representative images and quantification of colony formation of TAZ_{DEP} and TAZ_{IND} cells in response to or without dox treatment. Data are shown as the mean ± SD. Unpaired two-tailed Student's *t*-test: $^{***}p < 0.001$; NS = not significant.

cell proliferation rates were similar in the 2D culture. However, we observed a dramatic decrease in cell proliferation, viability, and long-term colony formation capacity for TAZ_{DEP} cells upon withdrawal of dox (**Figures 1B–E** and **Supplementary Figures 1A,B**). As expected, we did not detect high and sustainable TAZ expression in TAZ_{IND} cells (**Figure 1D**) because of inactivation or silencing of the transgene cassette *in vivo*.

Characterization of TAZ_{DEP} and TAZ_{IND} Tumor Cells

EMT is relevant to the acquisition and maintenance of stem cell-like characteristics and is sufficient for endowing differentiated MECs and BC cells with stem cell properties (Mani et al., 2008). Moreover, EMT can generate a spectrum of cellular states displaying mixed epithelial and mesenchymal features between these two extremes *in vitro* and *in vivo* (Celia-Terrassa et al., 2012; Kroger et al., 2019). We (among other studies) have previously shown that TAZ activation induced EMT in MCF10A cells (Lei et al., 2008; Li et al., 2015). Consistent with these findings, TAZ_{DEP} cells displayed mesenchymal morphologies (**Figure 2A**), whereas TAZ_{IND} cells maintained the cobblestone morphology characteristic of epithelial cells. To corroborate the observed changes in morphology, we examined changes in the expression of canonical markers of the epithelial and mesenchymal states. TAZ_{DEP} cells were associated with decreased E-cadherin protein expression and increased expression of mesenchymal markers such as fibronectin and vimentin, respectively (**Figure 2B**). The

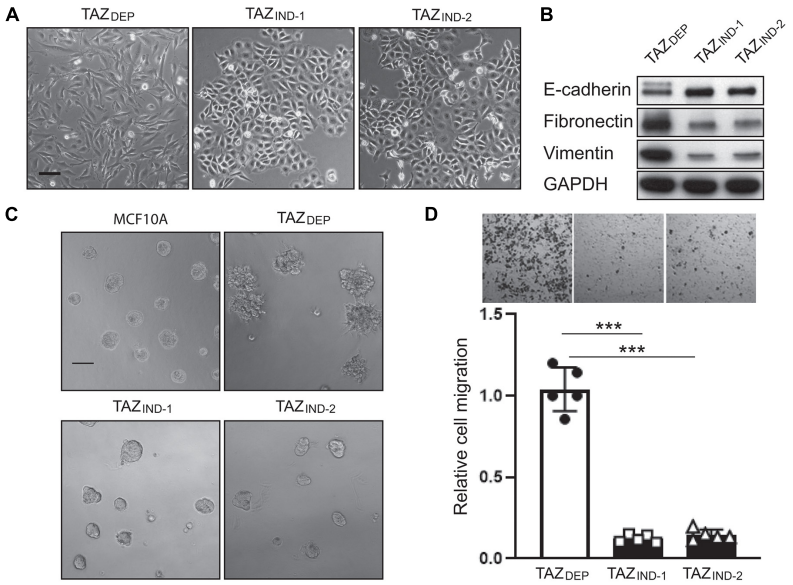


FIGURE 2 | TAZ_{DEP} cells undergo EMT. **(A)** Representative images of TAZ_{DEP} and TAZ_{IND} cell morphology in a 2D culture. TAZ_{DEP} cells grown in the presence of 2 μg/ml dox. Scale bar = 50 μm. **(B)** Immunoblotting detection of E-cadherin, fibronectin, and vimentin in TAZ_{DEP} and TAZ_{IND} cells. GAPDH was used as a loading control. **(C)** Representative images of MCF10A, TAZ_{DEP}, and TAZ_{IND} cells grown in a 3D culture. Scale bar = 100 μm. **(D)** Representative images and quantification of TAZ_{DEP} and TAZ_{IND} cell migration. Data are shown as the mean ± SD. Unpaired two-tailed Student's *t*-test: $^{***}p < 0.001$.

mesenchymal phenotype was partially reversed by the withdrawal of dox from TAZ_{DEP} cells, suggesting TAZ regulates cellular plasticity (**Supplementary Figures 1C,E**).

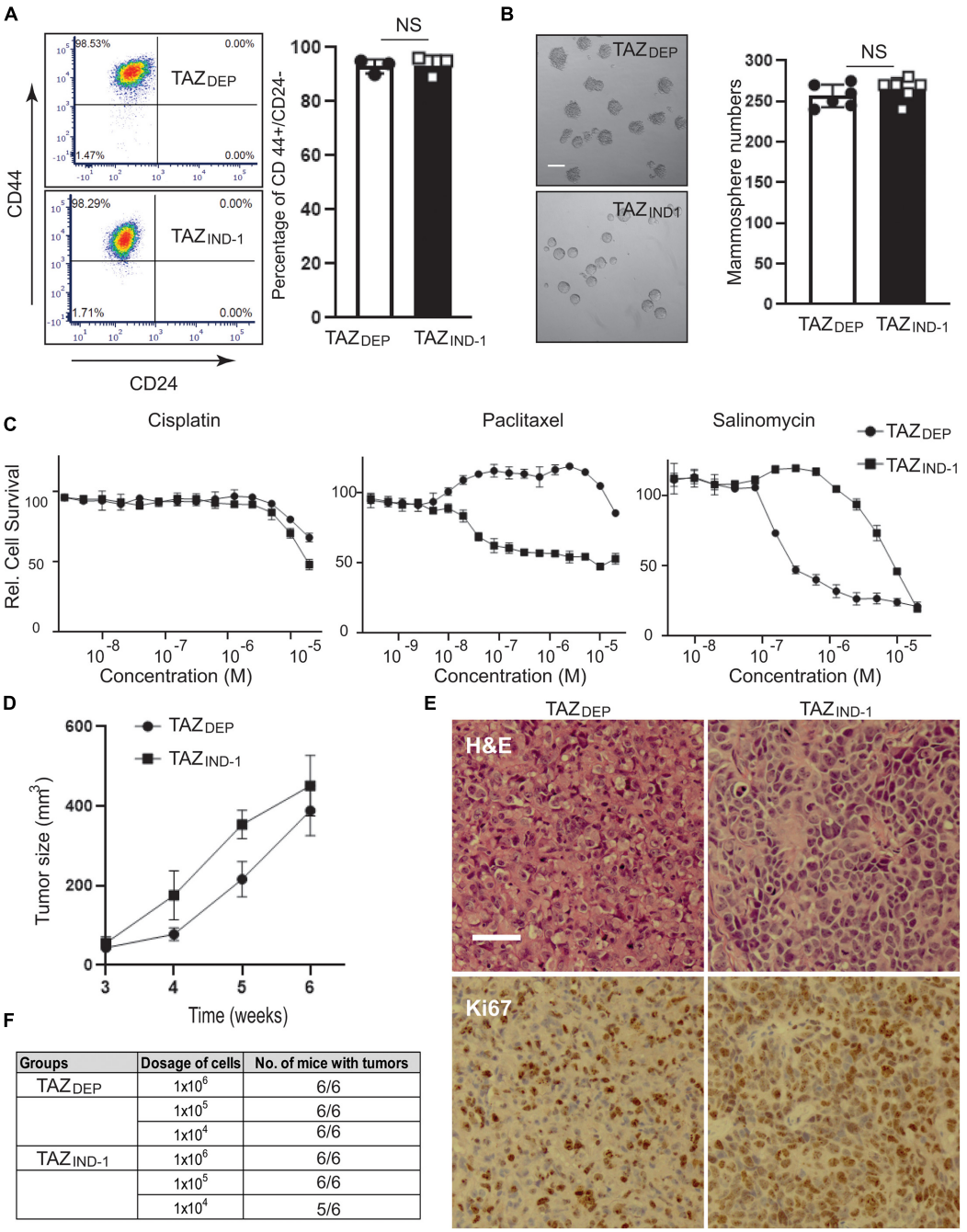
3D culture models allow for phenotypic discrimination between non-malignant and malignant MEC clones because they can recapitulate organotypic growth. For instance, transformed cells adopt various colony morphologies, including a loss of tissue polarity, a disorganized architecture, and the failure to arrest growth (De Angelis et al., 2019). With this in mind, we investigated non-malignant and tumor-derived mammary cell phenotypes and growth in a 3D context. As expected, MCF10A cells organized into polarized colonies with many of the morphological features of mammary acini (**Figure 2C**). TAZ_{DEP} cells formed enlarged acini with invasive (stellate) structures (**Figure 2C**). Dox withdrawal inhibited their growth in 3D culture (**Supplementary Figure 1D**). In contrast, TAZ_{IND} cells formed smaller round acini (**Figure 2C**). Consistent with these observations, cell migration potential—-as assessed by Boyden chamber assays—-was reduced in TAZ_{DEP} vs. TAZ_{IND} cells (**Figure 2D**). Together, these results reaffirm previous work that constitutive TAZ expression promotes an EMT program that enables TAZ_{DEP} cells to assume a mesenchymal cell phenotype, including enhanced migratory capacity and invasiveness (Chan et al., 2008). However, this occurs independently of BC cell proliferation.

Maintenance of BCSC Properties and Tumorigenic Potential

Although BC cell lines provide useful information about cancer biology, their adaptation to the *in vitro* environment and artificial selection pressures in tissue cultures result in biological properties that differ in essential ways from *de novo* tumor cells. BCSC phenotypes may be unstable, resulting in phenotypic reversion of cell surface markers and switching of the CSC phenotype (Visvader and Lindeman, 2012). In addition, most tumors and cell lines possess their own unique ratios of BCSC markers and populations. To investigate this possibility and gain insights into the nature of our experimentally derived BCSCs, we used multiple methods to identify BCSCs *in vitro* and *in vivo*. As shown in **Figure 3A**, a FACS analysis readily revealed a $CD44^{high}/CD24^{low}$ subpopulation in both TAZ_{DEP} and TAZ_{IND} cells (>95%) compared with MCF10A cells ($CD44^{low}/CD24^{low}$). Correspondingly, both TAZ_{DEP} and TAZ_{IND} cells were endowed with long-term self-renewal capacity, as measured by mammosphere assays (**Figure 3B**).

In addition to self-renewal, another characteristic of CSCs is their capacity to resist chemotherapy (Lai et al., 2011). Therefore, we treated MECs with two widely used BC chemotherapeutic drugs, Cisplatin and Paclitaxel. TAZ_{DEP} and TAZ_{IND} cells were more resistant (∼20-fold) than the control MCF10A cells to these drugs (data not shown). As shown in **Figure 3C**, TAZ_{DEP} cells are more sensitive to Paclitaxel treatment than TAZ_{IND} cells. Salinomycin, an antibacterial and coccidiostat ionophore, has been shown to efficiently suppress BCSC survival (Gupta et al., 2009; Zhou et al., 2013). Strikingly, TAZ_{DEP} cells are 100-fold more sensitive to Salinomycin treatment than TAZ_{IND} cells (**Figure 3C**), which is consistent with monovalent cation ionophores' potent and cytostatic effects against EMT-like cells and therapy-resistant cancer cells (Vanneste et al., 2019).

Anchorage-independent growth is one of the hallmarks of cancer. Thus, we examined the effect of TAZ-mediated



Groups	Dosage of cells	No. of mice with tumors
TAZ_{DEP}	$1x10^6$	6/6
	$1x10^5$	6/6
	$1x10^4$	6/6
TAZ_{IND-1}	$1x10^6$	6/6
	$1x10^5$	6/6
	$1x10^4$	5/6

FIGURE 3 | Conservation and BCSC features and tumor formation potential. **(A)** Representative images and quantification of $CD44^{high}/CD24^{low}$ cell population of TAZ_{DEP} and $TAZI_{ND}$ cells by FACS analysis. Data are shown as the mean ± SD. Unpaired two-tailed Student's *t*-test: NS = not significant. **(B)** Representative images and quantification of mammosphere formation of TAZ_{DEP} and TAZ_{IND} cells. Scale bar = 100 μm. Data are shown as the mean ± SD. Unpaired two-tailed Student's *t*-test: NS = not significant. **(C)** Cell survival was tested in response to the various concentrations of Cisplatin, Paclitaxel, and Salinomycin for TAZ_{DEP} and TAZ_{IND} cells. TAZ_{DEP} cells grown in the presence of 2 μg/ml dox. **(D)** 1×10^6 TAZ_{DEP} or TAZ_{IND} cells were injected into mammary gland fat pads of SCID mice ($n = 6$). TAZ_{DEP} cells injected into mice were fed with dox-containing chow (Bio-serv, NJ). Tumor growth rates were measured weekly by caliper. **(E)** H&E and Ki67 immunohistochemical (IHC) staining for TAZ_{DEP} -or TAZ_{IND} tumors. Scale bar = 50 μm. **(F)** Serial diluted (1×10^6, 1×10^5, 1×10^4) TAZ_{DEP} or TAZ_{IND} cells were injected into the mammary fat pads of SCID mice ($n = 6$). Tumor formation capability was recorded.

tumorigenic activity using anchorage-independent growth conditions as an *in vitro* readout of the malignant transformation of MECs. Specifically, we grew isogenic cell lines in soft agar for 10 days and quantified the number of colonies and size of the colonies formed. Both TAZ_{DEP} and TAZ_{IND} cells displayed similar growth properties in soft agar

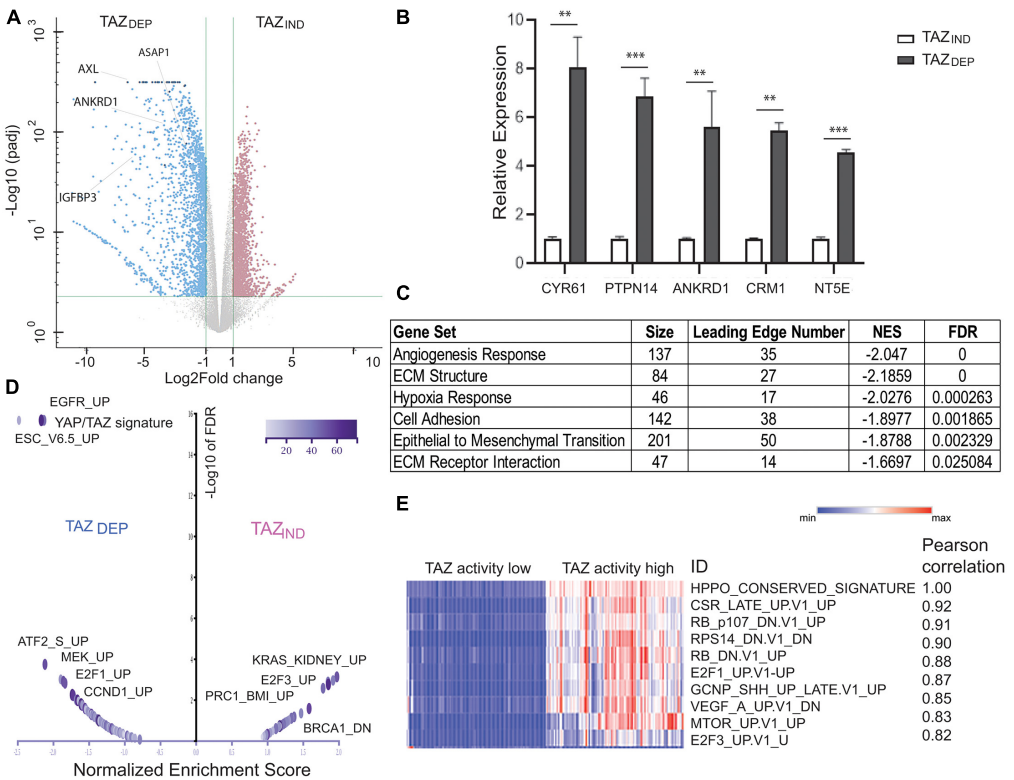
(**Supplementary Figure 1F**). Next, to confirm tumor formation potential *in vivo*, we injected TAZ_{DEP} or TAZ_{IND} cells into the mammary fat pad of $Prkdc^{scid}$ (SCID) mice. TAZ_{DEP} and TAZ_{IND} cells generated palpable tumors with similar penetrance and growth kinetics (**Figure 3D**). Histological analysis indicated that both TAZ_{DEP} and TAZ_{IND} cells formed a high-grade carcinoma with high proliferation potential (**Figure 3E**). However, TAZ_{IND} cells displayed neuroendocrine features, suggesting they may have undergone dedifferentiation (**Figure 3E**).

Finally, to better assess tumor-initiation capacity, we performed orthotopic transplantation of TAZ_{DEP} and $TAZI_{ND}$ cells in limiting dilution assays (**Figure 3F**). Our results show that our isogenic cell lines possessed > 30-fold higher tumor-initiating potential than bulk tumor cells. Furthermore, our data suggest that both TAZ_{DEP} and TAZ_{IND} cells display stable and heritable BCSC markers and functional characteristics, including the ability to form long-term tumors *in vivo*.

Transcriptome Analysis of TAZ_{DEP} and TAZ_{IND} Transformed MECs

Elucidation of the pathways that regulate the survival of BCSCs is important for the development of novel therapies. To gain a broader perspective of the underlying biological processes used by TAZ_{DEP} and TAZ_{IND} cells for the maintenance of their BCSC phenotypes, we performed an RNA-seq and over-representation analysis (ORA) using pathway annotations and GO term datasets (Jiao et al., 2012). We identified 1,854 significantly upregulated and 1,937 downregulated genes (twofold change, FDR $\leq$ 5%; **Figure 4A** and **Supplementary Table 2**). qRT-PCR analysis confirmed canonical TAZ target expression (Wang et al., 2018) highly expressed in TAZ_{DEP} cells (**Figure 4B**). TAZ_{IND} cells were enriched for MaSC and mammary cell signatures (**Supplementary Figure 2**). TAZ_{DEP} cells were enriched for cell-regulatory and growth processes, including epithelial cell proliferation, cell adhesion, cell-surface proteins, extracellular matrix (ECM) structure proteins, and the production of ECM-degrading enzymes. ECM cleavage and remodeling can promote cell movement, and profoundly influence the directed migration of BC cells. Hence, the deregulation of these biological processes may account for the different migration and invasive properties of TAZ_{DEP} vs. TAZ_{IND} cells.

As a complementary approach to ORA, we performed a gene set enrichment analysis (GSEA) (Subramanian et al., 2005). To expand our analysis beyond pathways, we also included MSigDB gene sets derived from different experiments, each of which



Gene Set	Size	Leading Edge Number	NES	FDR
Angiogenesis Response	137	35	-2.047	0
ECM Structure	84	27	-2.1859	0
Hypoxia Response	46	17	-2.0276	0.000263
Cell Adhesion	142	38	-1.8977	0.001865
Epithelial to Mesenchymal Transition	201	50	-1.8788	0.002329
ECM Receptor Interaction	47	14	-1.6697	0.025084

FIGURE 4 | Gene expression profiling for TAZ_{DEP} and TAZ_{IND} cells. **(A)** Volcano plot shows significant gene expression alterations between TAZ_{DEP} and TAZ_{IND} cells. **(B)** qRT-PCR experiment detection of canonical TAZ target expression in TAZ_{DEP} and TAZ_{IND} cells. Relative expression was normalized by GAPDH expression. Unpaired two-tailed Student's *t*-test: $^{**}p < 0.01$; $^{***}p < 0.001$. **(C)** Over-representation analysis (ORA) using pathway annotations. **(D)** Volcano plot summarizes the normalized enrichment score (NES) for MSigDB oncogenic signature sets with direction for TAZ_{DEP} vs. TAZ_{IND} cells. **(E)** Correlation analysis of TAZ_{DEP} and TAZ_{IND} modules using TCGA Breast Invasive Carcinoma patient (RNASeqv2 RSEM) gene expression data. Top 10 pairwise Pearson correlations between TAZ activation and oncogenic gene signatures are summarized.

represents genes whose expression is altered in response to a perturbation in a known cancer-associated gene (**Supplementary Figure 2**; Liberzon, 2014). YAP/TAZ target genes and EMT signatures were upregulated in TAZ_{DEP} cells (**Figures 4C,D**). Similarly, the Cyclin D (CCND1), RB/E2F, epidermal growth factor receptor (EGFR), and MAPK/ERK pathway signatures were also upregulated in TAZ_{DEP} cells (**Figure 4D**). Conversely, several PRC2 complex target signatures and neuron progenitor features were preferentially enriched in TAZ_{IND} cells (**Figure 4C** and **Supplementary Figures 2, 3**).

In order to determine the generality of our findings, we performed a correlation analysis between TAZ transcriptional activity and associated oncogenic pathways/signatures using gene expression data from TCGA BC patients (Ciriello et al., 2015). The top 10 most connected modules are dominated by genetic perturbations of RB and E2F genes, thereby supporting our approach for identifying biologically meaningful relationships (**Figure 4E**). Collectively, these results suggest that TAZ_{DEP} and TAZ_{IND} cells' phenotypic characteristics are manifested because they rely on different biological processes that contribute to their unique proliferation, survival, self-renewal, and differentiation properties.

A Small Molecule Library Screen Identifies Specific Vulnerabilities in TAZ_{DEP} and TAZ_{IND} Cells

Molecular alterations that confer phenotypic advantages to tumors can also expose specific genetic vulnerabilities (Lee et al., 2018). For example, cancers harboring translocations that form fusion transcripts, such as BCR-ABL, or mutations, such as BRAF or EGFR, depend on these gene products' activity for tumor maintenance. We hypothesized that our TAZ_{DEP} and TAZ_{IND} cells could be used to identify small molecules with anti-BCSC activity because they exhibit the CSC markers and functional characteristics that are stable in a 2D culture and, hence, are amenable to HT phenotype-based screens.

To investigate this possibility, we selected a library of ∼600 compounds that target essential cellular circuitry proteins and included FDA-approved drugs, preclinical agents, and small molecule pathway probes (**Figure 5A**). We annotated each compound with one or more mode of action (MoA) descriptors (**Supplementary Table 3**) using vendor compound catalogs, large-scale target annotation projects, and chemical databases such as DrugBank and the Therapeutic Target Database (TTD). We used this small molecule library to carry out viability screens to identify potent and selective cytostatic compounds for BC cells exhibiting a CSC-like phenotype dependent on oncogenic TAZ.

Most of the compounds had a modest effect on relative cell viability and/or growth (**Figure 5A**). The other hits showed a deviation from the negative controls and were categorized into three clusters: (1) hits cytotoxic to both TAZ_{DEP} and TAZ_{IND} cells; (2) hits preferentially cytotoxic to TAZ_{DEP} cells; and (3) hits preferentially cytotoxic to TAZ_{IND} cells. To identify TAZ_{DEP} cytotoxic compounds, we investigated compounds with a greater cytotoxic effect against TAZ_{DEP} vs. TAZ_{IND} cells (i.e., ratio $TAZ_{DEP}/TAZ_{IND} \leq 0.75$). Based on these criteria, 30 compounds showed a selective cytotoxic effect on TAZ_{DEP} cells and were selected for dose-response follow-up studies (**Figure 5B**).

As summarized in **Figure 5C**, TAZ_{DEP} cells were sensitive to most of these candidates (**Supplementary Table 4**). Multiple compounds were associated with known human cancer signaling pathways, including EGFR (Icotinib and WZ4002), RAS (6H050), and MEK (PD0325901 and Refametinib, and U0126-EtOH). Furthermore, we identified the small molecule inhibitors of metabolism (PFK15 and Pyrimethamine), cell cycle regulation (NSC 23766 and Palbociclib), DNA damage response (AZD2461, CRT0044876, and VE-8220), and epigenetic factors (Lomeguatrib, SGC0946, HLCL-61, and PFI-1) (**Figure 5C**). Notably, our chemogenomic analysis identified Cyclin D1 (CCND1)—- a well-recognized oncogene—-as a transcriptional target of TAZ. CCND1 is an activator of CDK4/6 kinases that promote the G1/S transition by inactivating RB (Malumbres and Barbacid, 2009). Palbociclib is a selective CDK4/6 inhibitor and was our top ranked hit meriting further investigation.

Identification of CCND1 as a Direct Target Gene of TAZ

The molecular mechanisms underlying TAZ-driven oncogenic transcriptional responses' specificity remain largely unknown (Zanconato et al., 2016). Because of the limitations in discriminating functional and incidental (secondary) gene expression, it is challenging to identify the genes directly regulated by TAZ solely from transcriptome profiling using a single (static) data point. Accordingly, to confirm CCND1 is a direct target of TAZ, we treated TAZ_{DEP} cells with dox for 3 and 6 days and/or removed dox for an extra 6 days. We harvested total RNA and performed a qRT-PCR analysis. CCND1 gene expression was significantly increased in response to TAZ activation but rapidly diminished upon dox removal (**Figure 6A**). In addition, CCND1 protein expression decreased in response to dox removal (**Figure 6B**).

It has been recently reported that the loss of FAT1 led to marked elevations in CDK6 through YAP/TAZ activation (Li et al., 2018). Therefore, we quantitated CDK6 protein expression in TAZ_{DEP} cells. We found CDK6 protein levels reduced in response to dox removal (**Figure 6C**). To further investigate whether TAZ regulates CCND1 gene expression, we perturbed TAZ in MDA-MB-231 and MDA-MB468 BC cell lines. As shown in **Figure 6D**, siRNA knockdown of TAZ reduced CCND1 expression in both triple-negative BC cell lines (**Figure 6D**). Furthermore, we detected increased CCND1 expression in LATS1/2-null cells (Hippo pathway kinases that negatively regulate TAZ) (Ma et al., 2021; **Figure 6E**).

TAZ lacks an intrinsic DNA-binding domain and is thought to exert its co-activator function by binding to target promoter sequences via interactions with many different transcription factors (TFs). Among these, the TEAD family members play a dominant role as primary mediators of TAZ-dependent gene regulation and tumor-promoting activity (Zhang et al., 2009). We used the LASAGANA Search 2.0 algorithm to analyze which TFs bind to the CCND1 promoter using matrix-derived models from JASPAR and TRANSFAC databases. We

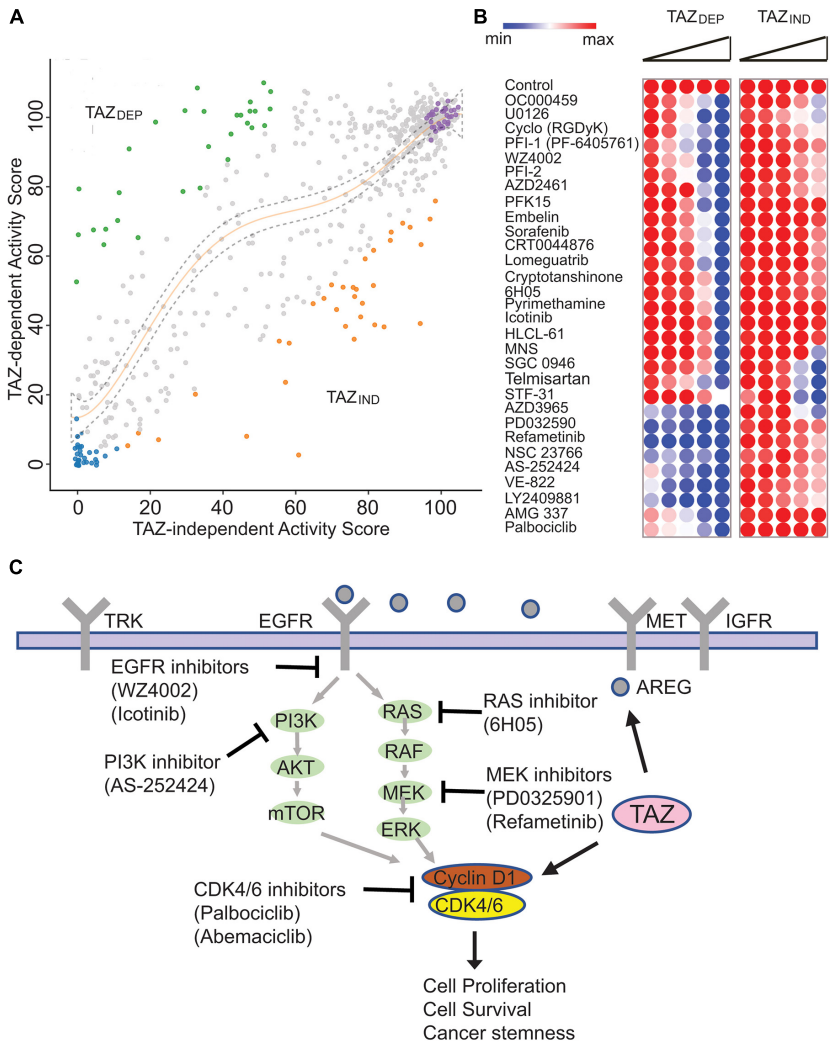


FIGURE 5 | Small molecule library screen identified isogenic cell line vulnerabilities. **(A)** Results of the small molecule screen representing the relative viability of TAZ_{DEP} (y axis) and TAZ_{IND} (x axis). Polynomial regression was used to identify non-linear relationships between TAZ_{DEP} and TAZ_{IND} cells. **(B)** Heatmap of TAZ_{DEP} and TAZ_{IND} cells in response to 30 compounds. Doses from low to high are 0.65, 1.25, 2.5, 5, and 10 μM, respectively. **(C)** Summary of TAZ_{DEP} small molecule hits and their predicted MoA.

found two regions with predicted TEAD tandem consensus motifs (5'-GGAATG-3'). To validate the in-silico analysis for TEAD-binding sites on the CCND1 promoter, we performed chromatin immunoprecipitation (ChIP) assays and showed TAZ directly bind to CCND1 promoter region (CYR61, a canonical transcriptional target of TAZ, was used as a positive control, and HBB served as a negative control) (**Figure 6F**).

The ability of D-type cyclin family members to activate CDK4 and CDK6 is the most extensively documented mechanism for their oncogenic actions (Knudsen and Witkiewicz, 2017). Selective CDK4/6 inhibitors, such as Palbociclib, Ribociclib, and Abemaciclib, have been developed. However, the *in vivo* functions of CDK4/6 inhibition are complex and extend beyond simply enforcing cytostasis. To confirm that CCND1 overexpression could be pharmacologically targeted/exploited, we investigated TAZ_{DEP} and TAZ_{IND} cell sensitivity to Abemaciclib. TAZ_{DEP} cells were more sensitive to Abemaciclib treatment than TAZ_{IND} cells (**Figure 6G**).

Overall, we have shown that TAZ positively regulates CCND1 expression by directly binding to its promoter region. Furthermore, the perturbation of the TAZ-CCND1-CDK4/CDK6 signaling axis led to the inhibition of TAZ_{DEP} cell proliferation, providing a rationale for its exploitation as a target in BCSC therapy.

DISCUSSION

The CSC model has been established as a cellular mechanism that contributes to phenotypic and functional heterogeneity in BC and other human tumors. The clinical applicability of the CSC concept to predicting patient responses remains a

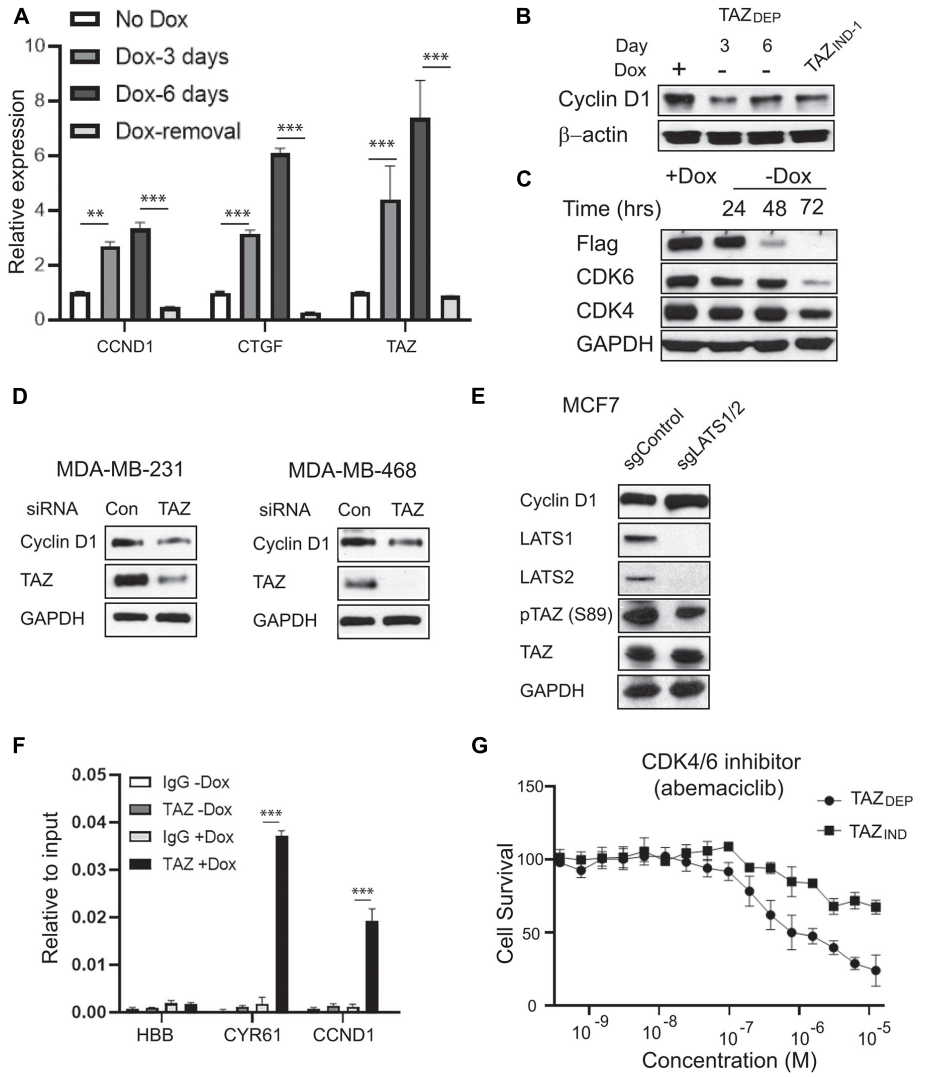


FIGURE 6 | Identification of CCND1 as a direct target of TAZ. **(A)** qRT-PCR experiment detection of CCND1, CTGF, and TAZ expression in TAZ_{DEP} cells in response to dox treatment or dox removal. Relative expression was normalized by GAPDH expression. Unpaired two-tailed Student's *t*-test: $^{**}p < 0.01$; $^{***}p < 0.001$. **(B)** Immunoblotting detection of CCND1 in response to dox withdrawal in TAZ_{DEP} cells and TAZ_{IND} cells. GAPDH was used as a loading control. **(C)** Immunoblotting detection of Flag-TAZ, CDK4, and CDK6 expression in response to dox withdrawal in TAZ_{DEP} cells. GAPDH was used as a loading control. **(D)** Immunoblotting detection of TAZ and CCND1 in siControl or siTAZ transfected MDA-MB-231 or MDA-MB-468 cells. GAPDH was used as a loading control. **(E)** Immunoblotting detection of CCND1, LATS1, LATS2, TAZ, pTAZ (S89) in sgControl or sgLATS1/2 cells. GAPDH was used as a loading control. **(F)** qPCR experiment detection of TAZ-ChIPed DNA for CCND1. CYR61 was used as positive control; HBB was used as negative control. Relative enrichment was compared with 2% of input DNA. Unpaired two-tailed Student's *t*-test: $^{***}p < 0.001$. **(G)** IC50 of CDK4/6 inhibitor Abemaciclib treatment. TAZ_{DEP} cells grown in the presence of 2 μg/ml dox.

fundamental biomedical question. As such, the delineation of critical genes and/or pathways that can distinguish CSCs from their normal counterparts may provide novel opportunities for therapeutic intervention and help overcome tumor heterogeneity and therapeutic resistance. In the current study, we describe a simple experimental workflow that allows for the rapid isolation of BCSCs from mammary tumors with defined genotypes.

The Hippo pathway plays a critical role in cell proliferation, survival, migration, tumorigenesis, and metastasis (Yu et al., 2015). The fact that deregulated Hippo signaling is essential for a tumorigenic subpopulation with stem cell properties raises the possibility that the therapeutic activation of Hippo signaling or a pharmacological blockade of its downstream effectors could improve current cancer treatment strategies. Indeed, we hypothesize that targeting the Hippo pathway is an effective CSC therapeutic strategy. Unfortunately, the direct pharmacological inhibition of oncogenic TAZ or YAP is challenging because these proteins have no known catalytic activity or function through engaging in domains that facilitate context-dependent protein–protein interactions with diverse upstream kinases, apical–basal cell polarity proteins, or transcription factors (Harvey et al., 2013; Hansen et al., 2015). Here, we employed an alternative strategy

to identify BCSC-specific druggable targets lying downstream of TAZ whose pharmacological perturbation influenced their tumor-initiating properties.

A systematic evaluation of drugs that specifically target BCSCs has been hindered because of the difficulty in isolating these cells from the bulk of tumor tissues or cell lines and the manipulations of pure populations ex vivo. Given the difficulty of targeting BCSCs therapeutically, we initially sought to characterize the transcriptional programs used by TAZ to confer CSC-related traits and predict cancer-specific vulnerabilities. Using a chemical genomics approach, we identified several candidate small molecules that target TAZ-driven cellular processes. For instance, we have previously demonstrated that amphiregulin, an EGFR ligand, is a direct target of TAZ (Yang et al., 2012). Consistent with these studies, we found TAZ_{DEP} is sensitive to the EGFR inhibitors WZ4002 and Icotinib, respectively. We also identified and validated small molecules that target the downstream effectors of multiple cancer-associated signaling pathways, such as the PI3K inhibitor AS-252424, the MEK inhibitor PD0325901, and Refametinib. Interestingly, MEK inhibitors can trigger YAP/TAZ degradation in a hippo-independent manner. We also identified multiple epigenetic targets, including PFI-1 (PF-6405761), a highly selective BET (bromodomain-containing protein) inhibitor for BRD4, which is part of the YAP/TAZ-TEAD transcriptional complex (Zanconato et al., 2018; Pobbati and Hong, 2020). Correspondingly, BRD4 inhibitors have been reported to inhibit YAP/TAZ pro-tumorigenic activity in several cells or tissue contexts and cause the regression of YAP/TAZ-addicted neoplastic lesions (Zanconato et al., 2018).

Of particular interest, we identified CCND1 and CDK6 as direct transcription targets of TAZ. CCND1 is one of the most commonly overexpressed genes in human BC and causes mammary tumors in transgenic mice (Sutherland and Musgrove, 2002). CCND1 activation promotes cell cycle progression through the phosphorylation of substrates, such as RB and transcription factors, with roles in proliferation and differentiation. CCND1 also performs additional functions to regulate gene expression in the context of local chromatin and promotes cellular migration and chromosomal instability. Notably, in our small molecule screen we found CDK4/6 inhibitors—Palbociclib and Abemaciclib—preferentially inhibited TAZ_{DEP} vs. TAZ_{IND} cell survival and proliferation. CDK4/6 inhibitors have demonstrated activity against HR^+ and $HER2^-$ BC (O'Leary et al., 2016). However, many patients exhibit primary resistance to CDK4/6 inhibition and do not derive long-term benefits from these agents. To the best of our knowledge, CDK4/CDK6 inhibitors and their MoA have not been extensively studied for TAZ-addicted tumorigenicity.

Although multiple small molecules have been identified, and further application of this method may enable the discovery of additional small molecules, some limitations merit discussion. First, all experiments were conducted using a limited set of TAZ_{DEP} and TAZ_{IND} cell lines, thereby potentially limiting the interpretability of our findings. Second, the overarching focus of our study was the discovery of TAZ_{DEP} vulnerabilities. Notably, BCSCs are phenotypically and functionally heterogeneous cells promoting tumor cell growth, progression, recurrence, and treatment resistance. As such, different oncogenic drivers will need to be investigated to determine the generality of our findings. Finally, targeting TAZ-dependence in BC cells by inhibiting CCND1-CDK4/CDK6-mediated cell cycle progression does not necessarily support the exclusive context to BCSCs and warrants further investigation.

In summary, our data reveal that TAZ-mediated tumor growth may lead to cellular plasticity and dedifferentiation. In addition, an oncogenomic analysis using pathway-specific probes identified that TAZ-expressing-driven cells are sensitive to CDK4/6 inhibitors and may be used as criteria for BC patient stratification, neoplastic growth, and anti-CSC therapy. More broadly, the ability to generate and characterize BC stem-like cells *in vitro* offers a cost-effective and scalable platform that can be perturbed with relative ease while also being compatible with high-throughput phenotype-based screens to reveal novel molecular vulnerabilities for BC therapeutics.

MATERIALS AND METHODS

Cell Line and Cell Culture

MCF10A cells have previously been described and were authenticated by STR profiling (Shen et al., 2019). MCF10A cells were cultured in DMEM/F12 media (Corning, NY) supplemented with 5% horse serum (Invitrogen, MA), 1% Pen/Strep, 20 ng/mL EGF (ProSpec, NJ), 0.5 μg/mg hydrocortisone, 100 ng/mL cholera toxin, and 10 μg/mL insulin. TAZ_{DEP} or TAZ_{IND} tumors were harvested and minced. Small tumor tissue pieces were digested by collagenase at a temperature of 37°C for 30 min. Single cell suspensions were grown in mammosphere growth conditions for 5 days. Sphere-forming cells were further digested trypsin and plated to 10 cm tissue culture dishes with MCF10A growth media. All cells were detected for being mycoplasma free. MDA-MB-231 and MDA-MB-468 cells were purchased from ATCC; MCF7- sgControl and sgLATS1/2 cells were kindly provided by Dr. Kun-Liang Guan (University of California San Diego). MDA-MB-231, MDA-MB-468 and MCF7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% Pen/Strep. All cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

$CD44^{high}/CD24^{low}$ Cell Population Detection by Flow Cytometry Analysis

TAZ_{DEP} or TAZ_{IND} cells were dissociated by trypsin and passed through a 35 μm filter, and cell pellets were prepared by centrifugation. After washing with 1X phosphate buffered saline (PBS) containing 0.5% fetal calf serum (FBS), the cells were counted. Then, 1×10^6 cells were resuspended in PBS/FBS and stained with APC antihuman CD44 and Brilliant Violet 421 antihuman CD24 antibody (Biolegend, CA) for 30 min on ice. Stained cells were washed and analyzed by flow cytometry.

Mammosphere Formation Assay

TAZ_{DEP} or TAZ_{IND} cells were grown in serum-free DMEM/F12 10 ng/ml recombinant human basic fibroblast growth factor (bFGF; Gold Biotechnology, MO) 1:1 media (Gibco, NY) supplemented with EGF (20 ng/mL) and B27 (2%) in ultra-low attachment six-well plates (Corning). The mammospheres were

allowed to grow for 5 days. Total mammospheres greater than 100 μm in diameter were counted under a microscope. Each experimental group was performed in triplicate.

Immunoblot Analysis and Antibodies

Cells were lysed in a RIPA buffer (Boston Bio-Products, MA) in the presence of protease and phosphatase inhibitors (Thermo-Fisher Scientific, NY). Protein concentration was determined using the Bradford protein assay. Here, 20–30 ug of protein was loaded and separated by SDS-PAGE; then, it was transferred onto PVDF membranes (EMD Millipore, MA). Membranes were blocked in 5% milk in TBS-T for 1 h at room temperature and incubated overnight at 4°C with primary antibodies. The membranes were washed and incubated with HRP-congregated antimouse or antirabbit secondary antibody (Bio-Rad, CA) for 1 h at room temperature. Proteins were detected using the ECL Western blotting substrate (Thermo-Fisher Scientific, NY). CDK4, CDK6, TAZ, YAP, E-cadherin, and vimentin antibodies were purchased from Cell Signaling Technology; Fibronectin antibody from BD Biosciences; Flag (M2) antibodies from Sigma-Aldrich; and anti-GAPDH from Ubiquitin-Proteasome Biotechnologies.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was harvested using Trizol Reagent (Life Technologies, NY) according to the manufacturer's instructions. cDNA synthesis and quantitative real-time PCR were performed. GAPDH was used as the internal control. The primer sequences were as follows:

TAZ-F: 5′-AGTACCCTGAGCCAGCAGAA-3′;
TAZ-R: 5′-GATTCTCTGAAGCCGCAGTT-3′;
CTGF-F: 5′-GGAAATGCTGCGAGGAGTGG-3′;
CTGF-R: 5′-GAACAGGCGCTCCACTCTGTG-3′;
CYR61-F: 5′-CACACCAAGGGGCTGGAATG-3′
CYR61-R: 5′-CCCGTTTTGGTAGATTCTGG-3′
CCND1-F: 5′-GTTCGTGGCCTCTAAGATGAAG-3′
CCND1-R: 5′-GATGGAGTTGTCGGTGTAGATG-3′
PTPN14-F: 5′-GGAAGTTGCAGAGGTAGATAGTG-3′
PTPN14-R: 5′-GGGAAAGGACAGCAGCTAAA-3′
ANKRD1-F: 5′-AGTAGAGGAACTGGTCACTGG-3′
ANKRD1-R: 5′-TGGGCTAGAAGTGTCTTCAGAT-3′
CRIM1-F: 5′-GCCCAGTGTGGTGAGATAAA-3′
CRIM 1-R: 5′-GCAGCCAGCGGGATTATTA-3′
NT5E-F: 5′-GGAGATGGGTTCCAGATGATAAA-3′
NT5E-R: 5′-CGACCTTCAACTGCTGGATAA-3′
GAPDH-F: 5′-GTGAAGGTCGGAGTCAACGG-3′
GAPDH-R: 5′-GAGGTCAATGAAGGGGTCATTG-3′
AXL-F: 5′-GTC CTC ATC TTG GCT CTC TTC-3′
AXL-R: 5′-GAC TAC CAG TTC ACC TCT TTC C-3′

Cell Proliferation, Cell Survival, Colony Formation

Three thousand cells were plated into 96-well plates with or without 2 μg/ml dox. The plates were harvested daily for 7 days. Ten microliters of resazurin (R&D Systems, MN) were added to each well and incubated for 4 h at 37°C. Fluorescence was read using 544 nm excitation and 590 nm emission wavelengths. The cell proliferation rate was calculated using the first day fluorescence read as the baseline.

For the drug treatment assay, the cells were plated into a 96-well plate. The next day, serial diluted (1:2; start with 20 μm) drugs were added and incubated at 37°C for 72 h. Resazurin assay was performed. Cisplatin, Paclitaxel, Salinomycin, and Abemaciclib were purchased from Tocris (MN).

For colony formation assay, cells were trypsin and counted. Approximately 200 cells/well were plated into a six-well plate and grown in present or absent of 2 μg/ml dox in 37°C about 8–10 days. Cells were washed with PBS and stained with 10% crystal violate for 2 h at room temperature. Images were taken, and colonies were counted. Each accumulation of more than 50 cells was counted as a positive colony. Each sample was performed in triplicate, and three independent experiments were performed.

For colony formation in soft agar, 0.5% agar containing cell grow media was plated into a six-well plate. 5×10^4 cells were suspended in a growth medium mixed with 0.4% agar and seeded into a base agar containing a six-well plate. Cells were incubated in the presence of or without 2 μg/ml dox in 37°C for 2 weeks. Colonies were stained with 0.02% iodonitrotetrazolium chloride (Sigma-Aldrich, MO) and photographed. Colonies larger than 50 μm in diameter were counted as positive for growth. Assays were conducted in triplicate in three independent experiments.

3D Morphogenesis and Mammosphere Formation

Four thousand cells were cultured in growth factor–reduced reconstituted basement membrane (Matrigel; BD Biosciences) in an eight-well Nunc™ Lab-Tek™ II chamber slide (Thermo-Fisher Scientific, NY) as described previously (Debnath et al., 2003). Cells were grown in the presence of or without 2 μg/ml dox in 37°C for 10 days. The cell lines were assayed in three independent experiments.

Transwell Migration Assays

Transwell migration assays were performed as previously described (Mussell et al., 2020). Briefly, 5×10^4 cells were plated on Transwell inserts (8 μm pore size; Corning, NY) in assay medium at 37°C for 24 h. The Transwell inserts were washed with PBS and wiped with a Q-Tip, fixed with 4% paraformaldehyde, and stained with 10% crystal violet for 2 h at room temperature. Migrated cell numbers were counted, and assays were conducted in duplicate in three independent experiments.

In vivo Tumor Growth and Immunohistochemistry

To start, 1×10^6 TAZ_{DEP} or TAZ_{IND} cells were injected into the mammary fat pad of 6–8-week-old female SCID mice. For serial dilution experiments, 1×10^6, 1×10^5, or 1×10^4 TAZ_{DEP} or TAZ_{IND} cells were injected into the mammary fat pads of 6–8-week-old female SCID mice. The SCID mice were bred at the Roswell Park Comprehensive Cancer Center (RPCCC). Tumor sizes were measured once a week using a caliper. Mammary tumor formations were also detected by the *In Vivo* Luminescence Imaging System. The care and use of the

animals were approved by the Institutional Animal Care and Use Committee of the RPCCC (Buffalo, NY).

For immunohistochemistry (IHC) staining, formalin-fixed paraffin embedded (FFPE) tissue blocks were sectioned 5 microns thick and subjected to hematoxylin and eosin (H&E) and IHC staining. The quality of the histomorphology of tumor samples was assessed by H&E staining. Antibodies against Ki-67 was obtained from Dako. Histomorphology and immunostaining results were interpreted by a board-certified pathologist (BX).

Small Molecule Library Screen and Validation

The small molecule library was purchased from Selleckchem (TX). Screening was performed by the Small Molecule Screen Shared Resource at RPCCC. TAZ_{DEP} or TAZ_{IND} cells were plated into 384-well plates and treated with 10 μM inhibitors 24 h later in duplicate. Resazurin (Sigma-Aldrich, MO) cell enumeration assay was performed after 72 h. Cell survival for each drug was compared with that of DMSO-treated controls.

For drug validation, 30 selected small molecules were serially diluted and dispensed with automated reagent dispensers into 384-well plates. Cell survival was measured after 72 h by resazurin assay.

RNA-Seq and ChIP Assay

For the RNA-seq analysis, RNA was extracted from 60% confluent monolayers of cells, as described above. The RNA samples were subjected to transcriptome sequencing (RNA-seq) with an Illumina HiSeq 2000 sequencer in the RPCCC genomic shared resource. Raw reads passed quality filter from Illumina RTA were mapped to the mm10 mouse reference genomes and corresponding GENCODE (v12) annotation databases using STAR two-pass algorithm (Dobin and Gingeras, 2015). The mapped bam files were further QCed using RSeQC (Wang et al., 2012), a quality control Bioconductor R package for RNASeq data, to identify potential RNASeq library preparation problems. From the mapping results, the read counts for genes were obtained by featureCounts from Subread (Liao et al., 2013). Transcript level quantification were generated using kallisto (Bray et al., 2016), an alignment free tool. Dara normalization and differentially expression analysis were performed using DESeq2 (Love et al., 2014), a variance-analysis package developed to infer the statically significant difference in RNA-seq data. Pathway analysis was done by GSEA (Subramanian et al., 2005) pre-ranked mode using ranked gene list based on test statistics from DE analysis against the hallmark (H) and the canonical pathways in MSigDB. The volcano plots were generated using Enhanced Volcano Bioconductor package and the heatmaps were generated using heatmap R package.

For the ChIP analysis, chromatin immunoprecipitation assays were performed using the SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads; Cell Signaling Technology, MA). Briefly, inducible TAZ-expressing MCF10A cells were given or withheld from dox treatment for 72 h. Cells were cross-linked, lysed, and sonicated to generate DNA fragments with an average size of 0.5 kb. Immunoprecipitation was performed using 5 μg antibody to IgG or TAZ (catalog number #4883; Cell Signaling Technology, MA), respectively. ChIPed DNA was subjected to real-time PCR.

The primer sequences were as follows:
CYR61-F: 5′-CACACACAAAGGTGCAATGGAG-3′
CYR61-R: 5′-CCGGAGCCCGCCTTTTATAC-3′
HBB-F: 5′-GCTTCTGACACAACTGTGTTCACTAGC-3′
HBB-R: 5′-CACCAACTTCATCCACGTTCACC-3′
CCND1-F: 5′-AAC TCG CTG GGC AAG TC-3′
CCND1-R: 5′-TAG GGA ATT CTG GGT CCT CA-3′

RNAi Assay

A mixture of four siRNAs (SMARTpool) targeting TAZ and non-targeting (control SMARTpool) siRNA were purchased from Dharmacon. RNAi transfection was performed according to the manufacturer's instructions. Cell lysate was harvested after 72 h RNAi transfection and followed by immunoblot.

Statistical Analysis

All statistical analyses of cell proliferation, cell migration, colony formation, FACS analysis, mammosphere formation, RT-PCR, and soft agar assay were performed with two-tailed Student's *t*-tests; data are expressed as mean ± SD.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Gene Expression Omnibus (GEO) repository, accession number is: GSE168672.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the RPCCC (Buffalo, NY).

AUTHOR CONTRIBUTIONS

CF and JZ designed the study and wrote the manuscript. HS, YC, YW, JW, YZ, LW, QH, TL, BX, and MC performed the experiments and data analysis. All authors contributed to the article and approved the submitted version.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.673374/full#supplementary-material

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Calcium, an Emerging Intracellular Messenger for the Hippo Pathway Regulation

Yiju Wei[1] *and Wei Li*[1,2]*

[1] *Division of Hematology and Oncology, Department of Pediatrics, Penn State College of Medicine, Hershey, PA, United States,* [2] *Department of Biochemistry and Molecular Biology, Penn State College of Medicine, Hershey, PA, United States*

The Hippo pathway is a conserved signaling network regulating organ development and tissue homeostasis. Dysfunction of this pathway may lead to various diseases, such as regeneration defect and cancer. Studies over the past decade have found various extracellular and intracellular signals that can regulate this pathway. Among them, calcium (Ca^{2+}) is emerging as a potential messenger that can transduce certain signals, such as the mechanical cue, to the main signaling machinery. In this process, rearrangement of the actin cytoskeleton, such as calcium-activated actin reset (CaAR), may construct actin filaments at the cell cortex or other subcellular domains that provide a scaffold to launch Hippo pathway activators. This article will review studies demonstrating Ca^{2+}-mediated Hippo pathway modulation and discuss its implication in understanding the role of actin cytoskeleton in regulating the Hippo pathway.

Keywords: Hippo pathway, calcium signaling, actin cytoskeleton, CaAR, protein kinase C, NEDD4L, Merlin

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Medha M. Pathak,
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****Correspondence:***
Wei Li
weili@pennstatehealth.psu.edu

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INTRODUCTION

The Hippo pathway is a conserved signaling network governing organ development and tissue homeostasis. Various diseases, such as distorted tissue regeneration and cancer, have been linked to dysfunction of this pathway (Zanconato et al., 2016; Ma et al., 2019). The Hippo pathway contains a core serine/threonine kinase cascade, which, in mammals, includes Mammalian Sterile 20-like kinase 1 and 2 (MST1 and MST2) as well as their substrates Large Tumor Suppressor kinase 1 and 2 (Lats1 and Lats2, denoted as Lats1/2 hereafter). Upstream signals could induce phosphorylation and activation of Lats1/2 (denoted as the Hippo pathway activation hereafter), which in turn phosphorylate and inhibit two paralogous transcriptional coactivators, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), by preventing their accumulation in the nucleus. Without this regulation, YAP/TAZ can accumulate in the nucleus and activate gene transcription largely through TEAD transcriptional factors.

Growth control or homeostatic signals emitted extracellularly or intracellularly can regulate YAP/TAZ through Lats1/2-dependent or -independent mechanisms. Previous studies have found that the Hippo pathway can respond multiple upstream signals, including mechanical forces, cell polarity and adhesion, soluble factors, as well as various cellular stress. The Hippo pathway regulation by these signals have been extensively reviewed recently (Ma et al., 2019). Ca^{2+} is a signaling messenger important for a variety of cellular functions. Recent studies found that Ca^{2+} can regulate YAP/TAZ in various situations and is an emerging signal for the Hippo pathway

regulation. This article will review the studies supporting this notion and discuss the potential mechanism underlying regulation of the Hippo pathway by Ca^{2+}.

Ca^{2+} SIGNALING ACTIVATES THE HIPPO PATHWAY

In an unbiased screen of 1650 compounds, Liu et al. found that an L-type calcium channel blocker, amlodipine, is able to inhibit survival of glioblastoma cells by suppressing YAP/TAZ (Liu et al., 2019). Instead of its known function as an L-type calcium channel blocker, amlodipine can increase intracellular Ca^{2+} level by enhancing store-operated Ca^{2+} entry (SOCE) (Liu et al., 2019; Johnson et al., 2020). The elevated intracellular Ca^{2+} level inhibits YAP/TAZ by activating the core kinase cascade of the Hippo pathway. In this process, inverted formin-2 (INF2)-mediated Ca^{2+}-induced actin remodeling drives accumulation of protein kinase C (PKC) beta II in an actin cytoskeletal compartment. Such translocation is critical for PKC beta II to activate Lats1/2 (**Figure 1**). The study suggested that Ca^{2+} is an intracellular cue that regulates the Hippo pathway. In line with this notion, knockout of two-pore channel 2 (TRC2), a Ca^{2+} channel responsible for Ca^{2+} releasing from acidic organelles (Calcraft et al., 2009), in metastatic melanoma cells increases YAP/TAZ activity (D'Amore et al., 2020). In these TRC2 knockout cells, expression of ORAI1, the plasma membrane Ca^{2+} channel responsible for SOCE, and PKC beta II was decreased. Overexpression of ORAI1 in TRC2 knockout cells reverses the effect of TRC2 depletion on YAP/TAZ target gene expression, suggesting that ORAI1 inhibition is responsible for TRC2 depletion-induced YAP/TAZ activation (D'Amore et al., 2020). In breast cancer cells, expression of secretory pathway Ca^{2+}-ATPase 2 (SPCA2) can inhibit the epithelial-to-mesenchymal transition. This is through increasing cellular Ca^{2+} level and expression of E-cadherin, which then promotes YAP phosphorylation through activating Lats1/2 (Dang et al., 2019).

In addition to responding to increased Ca^{2+} level caused by directly perturbating Ca^{2+} channels, the Hippo pathway regulation by mechanical force appears to involve Ca^{2+}. He et al. (2018) found that human fibrosarcoma HT1080 cells compressed by a microfluidic device can reduce RhoA activity through a Ca^{2+}-dependent manner. In this process, transient receptor potential cation channel subfamily V member 4 (TRPV4) is responsible for the mechanical compression-induced Ca^{2+} influx and RhoA inhibition. Along with the reduction of RhoA activity, YAP translocates to cytoplasm from the nucleus when cells are compressed. However, the YAP translocation is suppressed when Ca^{2+} is eliminated from the compressed cells (He et al., 2018). In human adipose derived stem cells, synchronized thermal and mechanical stimulation can increase intracellular Ca^{2+} level and inhibit YAP nuclear localization (Deng et al., 2020). Inhibition of Ca^{2+} influx reduces YAP phosphorylation and increases YAP nuclear localization. In a study of the relationship among patterns of mechanical stress, bioelectric field and proliferation, Silver et al. (2020) found that mechanical stress gradients in mammary epithelial tissues of defined geometry lead more YAP/TAZ nuclear localization in cells at the tissue periphery than the center region. This phenomenon was accompanied by an increase of Ca^{2+} level in cells at this region. Interestingly, when Ca^{2+} was chelated, YAP nuclear localization was no longer limited to cells at the tissue periphery, but also occurred in the tissue center region. How Ca^{2+} determines the specific pattern of YAP nuclear localization in the epithelial tissue is unclear.

In addition to the in vitro observations, regulation of the Hippo pathway by Ca^{2+} has been recently suggested in genetic studies of the human autosomal dominant polycystic kidney disease (ADPKD) and Drosophila wing epithelium development. ADPKD is caused by mutations in *PKD1* and *PKD2* genes. Their protein products PC1 and PC2, respectively, form a plasma membrane calcium channel complex (Koulen et al., 2002; Lemos and Ehrlich, 2018). It was shown that YAP is activated in ADPKD patients and the *Pkd1*-depleted mouse (Happe et al., 2011; Cai et al., 2018). Loss of YAP/TAZ is able to suppress cystogenesis (an ADPKD-associated symptom) in the ADPKD mouse model (Cai et al., 2018). These observations suggested that the PC1/2 calcium channel complex is involved in suppressing YAP/TAZ to ensure normal kidney functions. In Drosophila wing epithelium, simultaneous deficiency in both sarcoplasmic-endoplasmic reticulum ATPase (SERCA), an endoplasmic reticulum (ER) calcium pump, and Orai leads to increased tissue growth (Suisse and Treisman, 2019). Such tissue hypergrowth is accompanied by dislocation and loss of activity of Fat, a component of the Hippo pathway (Suisse and Treisman, 2019). The observation suggested that Ca^{2+} is required for the proper activation of the Hippo pathway during the wing epithelium development.

Overall, the above studies indicated that increase of intracellular Ca^{2+} level can inhibit YAP/TAZ activity, and that Ca^{2+} may be an intracellular messenger to transduce the mechanical cue to the Hippo pathway.

Ca^{2+} SIGNALING INHIBITS THE HIPPO PATHWAY

In human neural stem/progenitor cells, spontaneous Ca^{2+} transients were observed at the plasma membrane when cells grow on glass coverslips (Pathak et al., 2014). The Ca^{2+} transients require Ca^{2+} influx across the plasma membrane, and its magnitude positively correlates with the substrate stiffness. Because YAP preferentially localizes in the nucleus when cells grow on a stiff surface (Dupont et al., 2011), this observation suggested a connection between the Ca^{2+} transients and YAP nuclear localization. The traction force-induced Ca^{2+} transients and YAP nuclear localization require a stretch-activated ion channel Piezo1 because knockdown of Piezo1 eliminates both events (Pathak et al., 2014). This study suggested that mechanically activated Ca^{2+} influx through Piezo1 is required for YAP nuclear localization when cells grow on a stiff surface. The response of YAP localization to Ca^{2+} influx under this cellular mechanical circumstance appears to be opposite to those described above when cells are compressed (He et al., 2018; Deng et al., 2020). Notably, the mechanical force comes from the

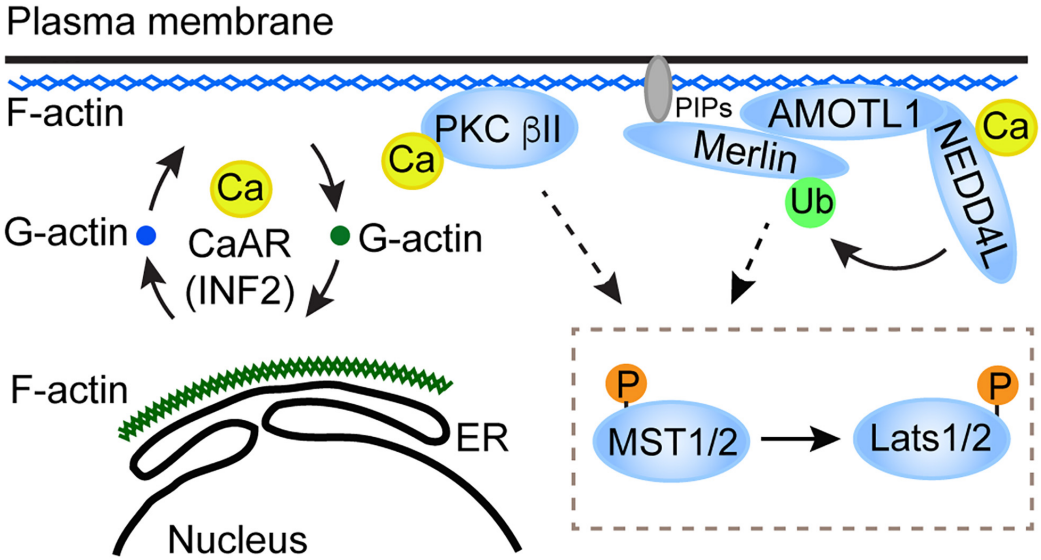

FIGURE 1 | A proposed model for the role of CaAR in the Hippo pathway activation. CaAR constructs a F-actin scaffold at the cell cortex. On the one hand, the F-actin scaffold recruits and activates PKC beta II; on the other hand, the F-actin scaffold can recruit the AMOTL1-NEDD4L E3 ligase apparatus, which then activates Merlin. Both of these two signaling effectors then activate the core kinase cascade in the Hippo pathway.

cell cortex when cells are compressed, whereas the force is applied to the cell basal membrane when cells grow on stiff surfaces. The distinct responses in these studies indicated that YAP regulation of Ca^{2+} at the different subcellular domains may be different. Although Ca^{2+} influx occurs in both situations, different Ca^{2+} channels are involved. Ca^{2+} signals initiated by these different channels may regulate the actin cytoskeleton through distinct effectors. Furthermore, the actin cytoskeleton rearrangement at different subcellular domains may have distinct impacts on YAP activity (see more discussion below).

In a study of how cholesterol induces hepatosteatosis transition to fibrotic non-alcoholic steatohepatitis (NASH), Wang et al. (2020) found that cholesterol can upregulate TAZ by inducing its dephosphorylation and stabilization in hepatocytes. This is through activating soluble adenylyl cyclase (sAC) by internalized cholesterol and in turn activating the cAMP-PKA signaling axis. PKA then induces ER Ca^{2+} release through activating inositol triphosphate receptor (IP3R), resulting in activation of RhoA, which can then activate TAZ through inhibiting Lats1/2. Ca^{2+} signals derived from ER perturbation can also regulate the Hippo pathway in *Drosophila* wing development. Ma et al. (2020) found that a loss of function mutation of Emei, an ER Ca^{2+} regulator, can synergize with Ras^{V12} to induce tumor growth through inhibiting the Hippo pathway. In this process, Emei mutation reduces Ca^{2+} level in ER and subsequently activates JNK. Perturbation of another ER Ca^{2+} regulator, SERCA, indicated that reducing ER Ca^{2+} can synergize with Ras^{V12} to promote tumor growth. Because preventing cytosolic Ca^{2+} from importing into ER by disrupting these ER Ca^{2+} importers may cause ER Ca^{2+} imbalance, which can trigger plasma membrane Orai Ca^{2+} channel through Stim to import extracellular Ca^{2+}, the Hippo pathway response in this case could be a consequence of increasing the cytosolic Ca^{2+} level. In this study, whether the cytosolic Ca^{2+} level is increased has not been directly tested. Notably, the aforementioned study reported that cells with SERCA and Orai double mutations in the wing epithelium also show an disrupted Hippo pathway (Suisse and Treisman, 2019), suggesting that SOCE induced by SERCA deficiency may not be required for suppressing the Hippo pathway. Therefore, it is possible that Ca^{2+} loss in ER and increase in the cytosolic compartment may regulate the Hippo pathway through different effectors. These studies emphasized that the Hippo pathway response to Ca^{2+} may be complex.

The complexity of the Hippo pathway regulation by Ca^{2+} was further demonstrated by a recent study through a real-time single-cell visualization of YAP subcellular localization and its target gene transcription dynamics (Franklin et al., 2020). In MCF10A human mammary epithelial cells, Franklin et al., observed a cycle of fast exportation and importation of YAP from the nucleus in response to the treatment of Ca^{2+} mobilizers, such as thapsigargin, ionomycin or ATP. Consistent with the previous observation (Liu et al., 2019), YAP exportation from the nucleus is induced by elevation of intracellular Ca^{2+} level in a Lats1/2- and PKC-dependent manner (Franklin et al., 2020). Interestingly, YAP re-enters the nucleus after the initial Ca^{2+} spike, and this nuclear re-entering can stimulate the expression of YAP target genes. Such Ca^{2+}-induced YAP localization-resets suggests that YAP activity may not be simply predicted by its nuclear or cytoplasmic localization, but rely on its nucleocytoplasmic shuttling (Franklin et al., 2020). Although this notion suggested that Ca^{2+}-induced YAP nuclear exportation leads to YAP activation upon its nuclear reentry, the canonical cytoplasmic YAP regulation, including tethering to 14-3-3 and ubiquitination-mediated degradation, may counteract the nuclear reentry and

contribute an inhibitory effect on YAP activity. These canonical cytoplasmic inhibitory effects may be even stronger for TAZ, because TAZ appears to be less stable than YAP and its expression was markedly decreased within 30 min after Ca^{2+} influx when LN229 cells are treated by thapsigargin or ionomycin (Liu et al., 2019). Therefore, the eventual impact of Ca^{2+} on YAP/TAZ may rely on the combination of these factors. The relative contributions of these factors may vary among different cells.

Overall, the above studies indicated that the response of YAP/TAZ to Ca^{2+} may be affected by the subcellular domains where Ca^{2+} signal is initiated and the interrelations among different regulatory modules.

MECHANISM OF THE HIPPO PATHWAY REGULATION BY Ca^{2+} AND ACTIN CYTOSKELETON

Ca^{2+} Effectors Involved in the Hippo Pathway Regulation

It is still unclear how Ca^{2+} signaling and the core Hippo pathway components are linked together. Conventional PKC is a Ca^{2+} effector that is required for Lats1/2 activation (Liu et al., 2019; Franklin et al., 2020). The role of PKC in the Hippo pathway regulation was also reported when the pathway responds to 12-O-tetradecanoylphorbol-13-acetate or G-protein-coupled receptors perturbation (Gong et al., 2015). Whether PKC directly phosphorylates Lats1/2 or other components required for Lats1/2 activation remains to be determined. Besides PKC, an E3 ubiquitin ligase, neural precursor cell-expressed developmentally downregulated protein four like (NEDD4L) could be another Ca^{2+} effector in the Hippo pathway regulation. Recently, Wei et al., found that elevation of intracellular Ca^{2+} level or loss of matrix attachment in human glioblastoma cells and mouse Schwann cells triggers ubiquitination of Merlin (Wei et al., 2020), an essential component in the Hippo pathway for Lats1/2 activation. This process is mediated by NEDD4L and a scaffold protein, AMOTL1 (**Figure 1**). The ubiquitination is required for Merlin to interact and activate Lats1 in response to upstream signals, including Ca^{2+}. Because Merlin-Lats1/2 interaction is important for Lats1/2 activation (Yin et al., 2013), Merlin ubiquitination appears to promote Lats1 activation through facilitating the interaction between Merlin and Lats1 (Wei et al., 2020). Currently, it is still unclear how Merlin ubiquitination can promote its interaction with Lats1. NEDD4L contains a C2 Ca^{2+}-binding domain and can be activated by Ca^{2+} (Goel et al., 2015). It was suggested that the C2 domain works as an auto inhibitor of NEDD4L, and that Ca^{2+} binding to the C2 domain disrupts the auto inhibitory function (Escobedo et al., 2014). It would be interesting to determine whether the C2 domain in NEDD4L is important for its function in Merlin activation in response to Ca^{2+}. Since both PKC beta II and NEDD4L have the C2 Ca^{2+}-binding domain and could be directly activated by Ca^{2+}, whether the two Ca^{2+}-activated events (activation of PKC beta II and Merlin) are interrelated or independent needs to be resolved.

CaAR Is Involved in the Hippo Pathway Regulation

The actin cytoskeleton appears to be a central mediator of various upstream biochemical and mechanical signals to YAP/TAZ through the core kinase cascade-dependent and -independent manners. How the cytoskeleton executes this role is unclear (Halder et al., 2012; Sun and Irvine, 2016; Fulford et al., 2018; Seo and Kim, 2018). Because of the apparent correlation between filamentous actin (F-actin) in stress fibers and YAP/TAZ activation, an inhibitory factors-sequestering model was proposed (Halder et al., 2012). This model suggested that stress fibers or other unknown F-actin networks associating with cell spreading serve as compartments sequestering YAP/TAZ inhibitory factors. Consistent with this model, disruption of stress fibers leads to Lats1/2 activation and YAP/TAZ inactivation (Halder et al., 2012; Sun and Irvine, 2016; Seo and Kim, 2018). During this process, the Merlin-Lats1 interaction (Yin et al., 2013) and the angiomotin-YAP interaction (Mana-Capelli et al., 2014) are enhanced. While Merlin and angiomotin provided clues to probe the regulation of Lats1/2-YAP/TAZ by F-actin, the detailed mechanism to reconcile their roles with the inhibitory factors-sequestering model remains elusive (Seo and Kim, 2018). Genetic studies in Drosophila found that the spectrin cytoskeleton at the cell cortex is required for the Hippo pathway activation (Deng et al., 2015; Fletcher et al., 2015; Wong et al., 2015). These studies suggested that certain cytoskeleton at the cell cortex is involved in activating the Hippo signaling. Notably, spectrin and actin are major proteins composing an integrated cytoskeleton network at the cell cortex (Holley and Ashmore, 1990). Therefore, it is plausible that loss of spectrin may interfere with the Hippo pathway activation through disrupting this cytoskeleton network.

Ca^{2+} can induce a characteristic actin rearrangement (Shao et al., 2015; Wales et al., 2016). In this process, the apical cortex actin transiently relocates to the perinuclear rim as well as ER, and rapidly reverts to the cortical distribution. Such actin remodeling could complete within 2 min of Ca^{2+} being increased and was called calcium-mediated actin reset (CaAR) (Shao et al., 2015; Wales et al., 2016). A formin family protein, INF2, is important for the actin polymerization during CaAR (Shao et al., 2015; Wales et al., 2016). In INF2-depleted glioblastoma cells, CaAR is disrupted and Ca^{2+}-induced phosphorylation of Lats1 and YAP is compromised, suggesting that CaAR is important for the Hippo pathway activation by Ca^{2+} (Liu et al., 2019). It was reported that PKC beta II is activated by binding to F-actin through its actin binding motif (Blobe et al., 1996). Therefore, Ca^{2+} might induce the association of PKC beta II with F-actin during CaAR. Consistent with this notion, subcellular fractionation revealed an increased amount of PKC beta II in the Triton X-100 insoluble actin cytoskeletal compartment in response to the increase of Ca^{2+} level in cells. Knockdown of INF2 eliminates PKC beta II from this actin compartment, suggesting that INF2-mediated actin assembly is required for such PKC beta II translocation (Liu et al., 2019). Collectively, these results suggested that INF2-mediated CaAR induces PKC beta II translocation to certain actin compartments and to

TABLE 1 | Ca^{2+} signaling regulates the Hippo pathway under various circumstances.

Source of the Ca^{2+} signal	Direction of Ca^{2+} change	Effect on YAP/TAZ	Stimulator	Signaling mediator	Biological model	References
ORAI1 activation	Increase	Inhibition	Amlodipine	PKC beta II	Human glioblastoma cells	Liu et al., 2019
SERCA inhibition	Increase	Inhibition	Thapsigargin	PKC beta II	Human glioblastoma cells	Liu et al., 2019
		Activation			Human mammary epithelial cells	Franklin et al., 2020
Ionophore	Increase	Inhibition	Ionomycin	PKC beta II	Human glioblastoma cells	Liu et al., 2019
		Activation			Human mammary epithelial cells	Franklin et al., 2020
Not examined	Increase	Activation	ATP	Unknown	Human mammary epithelial cells	Franklin et al., 2020
TRC2 knockout	unknown	Activation	N/A	ORAI1 Inhibition	Human melanoma cells	D'Amore et al., 2020
SPCA2 expression	Increase	Inhibition	N/A	E-cadherin activation	Human breast cancer cells	Dang et al., 2019
TRPV4 activation	Increase	Inhibition	Mechanical compression	RhoA inhibition	Human fibrosarcoma cells	He et al., 2018
Unknown	Increase	Inhibition	Thermal and mechanical stimulation	Unknown	human adipose derived stem cells	Deng et al., 2020
N/A	Decrease	Activation	Ca^{2+} chelator	Unknown	Human mammary epithelial	Silver et al., 2020
PC1/2 mutation	Not examined	Activation	N/A	Unknown	Mouse or human kidney	Happe et al., 2011; Cai et al., 2018
SERCA and Orai mutation	Not examined	Activation	N/A	Fat inactivation	Drosophila wing epithelium	Suisse and Treisman, 2019
Piezo1	Increase	Activation	Traction forces	Unknown	Human neural stem/progenitor cells	Pathak et al., 2014
IP3Rs	Increase	Activation	Cholesterol	RhoA activation	Mouse and human hepatocytes	Wang et al., 2020
Emei or SERCA mutation	ER Ca^{2+} decrease	Activation	N/A	JNK activation	Drosophila wing epithelium	Ma et al., 2020

activate the Hippo pathway. Future studies need to determine whether there is a connection between the actin compartment involved in CaAR-induced PKC activation and the spectrin-involved cytoskeleton network at the cell cortex. Notably, it was reported that thapsigargin- or ionomycin-induced Ca^{2+} influx can promote RhoA activation and increase actin stress fiber formation in human umbilical vein endothelial cells (HUVEC) (Masiero et al., 1999). Such phenomenon relies on specific matrix where the cells grow, because it occurs in cells plated on type IV collagen, but not on type I collagen. Experimental setting may explain the distinct actin filament rearrangements in response to Ca^{2+} in CaAR and this case. Alternatively, the difference may reflect the cellular response to Ca^{2+} at acute and adapted stages, respectively, because CaAR occurs within a couple of minutes (Shao et al., 2015; Wales et al., 2016), whereas stress fiber forms at 30–90 min after the treatments (Masiero et al., 1999). As the actin filaments change their subcellular locations and properties at different stages after Ca^{2+} influx, YAP/TAZ may change their behaviors in a dynamic manner. The functional consequence of YAP/TAZ in transcriptional control may therefore be an accumulative result of such dynamic changes of YAP/TAZ activities.

CONCLUSION AND OUTLOOK

Multiple studies in various circumstances have demonstrated that changes of intracellular Ca^{2+} level can regulate the Hippo pathway (**Table 1**). These findings suggested that Ca^{2+} could be an intracellular messenger for the Hippo pathway in responding to upstream signals, such as mechanical forces and soluble factors. Intriguingly, the Hippo pathway responses to Ca^{2+} appear to be variable when examined in different experimental settings. The distinct responses suggested that the Hippo pathway regulation by Ca^{2+} is complex, and that the Hippo pathway responses may be determined by the stages after the initial Ca^{2+} influx as well as the subcellular domains where Ca^{2+}

influx occurs. Therefore, it is important for the future study to examine these temporal and spatial factors when assessing the Hippo pathway responses as well as Ca^{2+} effectors involved in the Hippo pathway regulation. An emerging principle of Ca^{2+} regulation of the Hippo pathway is the involvement of actin cytoskeleton rearrangement, such as INF2-mediated CaAR and RhoA-mediated stress fiber formation. The transition among these actin filaments may modulate the effectors, such as PKC and NEDD4L, which are responsible for the Hippo pathway regulation (**Figure 1**). Further dissecting the involved actin cytoskeleton rearrangements as well as connections between the effectors and core Hippo pathway components would help us to understand how actin cytoskeleton reorganization and related upstream signals regulate the Hippo pathway.

AUTHOR CONTRIBUTIONS

YW and WL wrote the manuscript and approved the submitted version. Both authors contributed to the article and approved the submitted version.

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Hippo-Yap Pathway Orchestrates Neural Crest Ontogenesis

*Xiaolei Zhao, Tram P. Le, Shannon Erhardt, Tina O. Findley and Jun Wang**

Department of Pediatrics, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX, United States

Neural crest (NC) cells are a migratory stem cell population in vertebrate embryogenesis that can give rise to multiple cell types, including osteoblasts, chondrocytes, smooth muscle cells, neurons, glia, and melanocytes, greatly contributing to the development of different tissues and organs. Defects in NC development are implicated in many human diseases, such as numerous syndromes, craniofacial aberration and congenital heart defects. Research on NC development has gained intense interest and made significant progress. Recent studies showed that the Hippo-Yap pathway, a conserved fundamental pathway with key roles in regulation of cell proliferation, survival, and differentiation, is indispensable for normal NC development. However, the roles and mechanisms of the Hippo-Yap pathway in NC development remain largely unknown. In this review, we summarize the key functions of the Hippo-Yap pathway indicated in NC induction, migration, proliferation, survival, and differentiation, as well as the diseases caused by its dysfunction in NC cells. We also discuss emerging current and future studies in the investigation of the Hippo-Yap pathway in NC development.

Keywords: Hippo pathway, Yap and Taz, neural crest, migration, proliferation, differentiation

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Sally Ann Moody,
George Washington University, United States

***Correspondence:**
Jun Wang
jun.wang@uth.tmc.edu

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INTRODUCTION

Neural crest (NC) is a vertebrate-specific and migratory stem cell population. It originates from the dorsal margin of the neural tube (NT) and migrates along the anterior-posterior axis of the embryo following induction (Knecht and Bronner-Fraser, 2002; Sauka-Spengler and Bronner-Fraser, 2008). In accordance with the migratory path and function, the NC mainly involves cranial NC, trunk NC, vagal NC (containing cardiac NC), and sacral NC (Noisa and Raivio, 2014). Upon reaching their terminal location, the NC cells display their remarkable multipotency, generating numerous cell types, including osteoblasts, chondrocytes, smooth muscle cells, neurons, glia, and pigment cells, and contributing to the development of different tissues/organs in a region-specific manner (Ji et al., 2019). For example, cranial NC cells display a unique ability to be induced into osteoblasts and chondrocytes, forming the majority of the bone and cartilage of the head (Johnston, 1966; Cordero et al., 2011); cardiac NC cells contribute to the formation of the aortic arch and septation of the outflow tract (aorta and pulmonary arteries) (Kirby et al., 1983; Tomita et al., 2005); trunk NC cells produce neurons and glia of the peripheral nervous system and secretory cells of the endocrine system (Serbedzija et al., 1994); sacral NC cells contribute to the development of neurons and glia of the enteric nervous system (Le Douarin et al., 2008).

Abbreviations: NC, neural crest; NT, neural tube; EMT, epithelial-to-mesenchymal transition; dKD, double knock down; dCKO, double conditional knock out; RFP, Red Fluorescent Protein; RA, retinoic acid; DRG, dorsal root ganglia; NICD, Notch intracellular domain.

Defects in NC development lead to many congenital diseases, including craniofacial abnormalities, cardiovascular defects and neurological symptoms (Kirby et al., 1985; Bronner and LeDouarin, 2012). For example, Treacher Collins syndrome, a human congenital disorder caused by abnormal NC migration and differentiation, presents with mandibular hypoplasia and facial abnormalities (Sanchez et al., 2020). CHARGE syndrome is another NC formation defect-induced disorder characterized by a set of malformations including ocular coloboma, heart defects and choanal atresia (Okuno et al., 2017).

An intricate signaling network that controls NC development has been extensively studied, with great progress made in recent years. The signaling network, which includes Wnt, Sonic Hedgehog (Shh), Bmp, Fgf, Notch, and mechanical signaling, along with crosstalk among them, plays important roles in induction, specification, proliferation, migration, and differentiation of NC cells (Villanueva et al., 2002; Goldstein et al., 2005; Li et al., 2010; Manderfield et al., 2015; Chevalier et al., 2016). The Hippo-Yap pathway is also recently found indispensable for NC development (Gee et al., 2011; Hindley et al., 2016; Wang et al., 2016), but its role and related mechanisms are not well-described due to limited studies and warrant further investigation.

The Hippo signaling pathway is an evolutionarily and functionally conserved pathway involved in cell proliferation, survival, cell fate decisions and regeneration (Camargo et al., 2007; Sun et al., 2020). It contributes to the regulation of development, homeostasis, and regeneration of different tissues and organs, and its dysregulation gives rise to various diseases (Zhao et al., 2007; Lee et al., 2010; Wang et al., 2018; Zheng et al., 2020). For instance, family studies revealed that patients with heterozygous nonsense mutations in Yap1 displayed various defects including intellectual disability and orofacial clefting (Williamson et al., 2014). Anencephaly, one of the most common congenital diseases, was shown to relate with loss of UAK2 activity caused by decreased Hippo signaling via cytoplasmic Yap retention (Bonnard et al., 2020). In addition, the Cancer Genomic Atlas identified the Hippo signaling pathway as one of the 10 canonical signaling pathways frequently altered in human cancers (Sanchez-Vega et al., 2018). When Hippo signaling is on, upstream components of the Hippo signaling pathway Mst1/2 and Salv1 are phosphorylated, then activate the Lats1/2 kinases and subsequently promote the cytoplasmic degradation of the key downstream factors, Yes-associated protein (Yap) and its orthologous transcriptional coactivator with PDZ-binding motif (Taz) (Misra and Irvine, 2018). When Hippo signaling is off, Yap and Taz function as transcriptional co-activators and form a complex with transcription factors such as transcriptional enhancer activator domain (TEAD) family in the nucleus to mediate various biological activities of cells regulated by Hippo signaling (Hong and Guan, 2012; Varelas, 2014; Wang et al., 2018).

Recent studies indicated that the Hippo-Yap pathway is essential to NC development (Kumar et al., 2019; Bhattacharya et al., 2020), however, its role and related mechanisms are still largely unknown. In this review, we provide a research landscape on the Hippo-Yap pathway and its crosstalk with other signals in NC development shown in different experimental models (summarized in **Figure 1**) to provide a deeper insight into the mechanism of Hippo-Yap pathway in NC development and diseases. We also discuss the emerging areas of future studies in the Hippo-Yap regulation of NC-derived development, diseases and regeneration.

HIPPO-YAP PATHWAY IN NC CELL INDUCTION AND SPECIFICATION

Following gastrulation, NC is induced at the border between the neural plate and the non-neural ectoderm, known as the neural plate border. The induction process is orchestrated by a complex gene regulatory network that includes *Zic1*, *Gbx2*, *Pax3/7*, and *AP2* (Green et al., 2015). Among them, Pax3 and Zic1, direct upstream regulators of NC specifiers *Foxd3*, *Snail1/2*, *Twist1*, and *Tfap2b*, are both necessary and sufficient for NC cells induction (Plouhinec et al., 2014; Green et al., 2015).

Recently, there has been evidence that Yap plays a critical role in regulating *pax3* expression in the neural plate border zone in *Xenopus* embryos (Milewski et al., 2004; Gee et al., 2011). Yap gain-of-function could expand the $pax3^+$ domain surrounding the neural plate to give rise to NC precursors in *Xenopus*, but simultaneously inhibit later NC markers expression, including *Zic1* and *FoxD3*. These observations suggested that Yap holds these cells in a NC progenitor state. *Yap* loss-of-function completely abolished *pax3* expression and resulted in loss of $pax3^+$ NC progenitors (Gee et al., 2011). Concomitantly, a conserved Tead binding site was found in the enhancer loci of *pax3* in *Xenopus* and mice, and Tead bound to this site to activate *pax3* expression in NC (Degenhardt et al., 2010; Gee et al., 2011). Furthermore, Yap has also been shown to promote NC phenotype suggested by enriched gene expression of *SLUG, TWIST*, *AP2*, and *FOXD3*, in human pluripotent stem cells (PSC)-derived neural development studies (Hindley et al., 2016).

HIPPO-YAP PATHWAY IN NC CELL PROLIFERATION AND SURVIVAL

Beside the important roles of induction and specification, the Hippo-Yap pathway also takes part in the significant regulation of proliferation and survival of NC cells. Previous studies established that the Hippo-Yap pathway is essential in regulating various organ growth through the control of cell proliferation and survival (Cao et al., 2008; Heallen et al., 2011; Yu et al., 2015). In instances of absent Hippo activity, Yap and Taz are localized in the nucleus, where they bind to the transcription factors, such as Tead, to promote activation of genes for proliferation (Zhao et al., 2007). While some target genes rely on the interaction of Yap and Tead for gene regulation, Manderfield and colleagues observed that within NC cells, Pax3 can act as the DNA-binding moiety for Yap, allowing for activation of pathways favoring NC proliferation (Manderfield et al., 2014).

Double conditional knock out (dCKO) of *Yap* and *Taz* in NC of mouse embryos resulted in reduced proliferation and

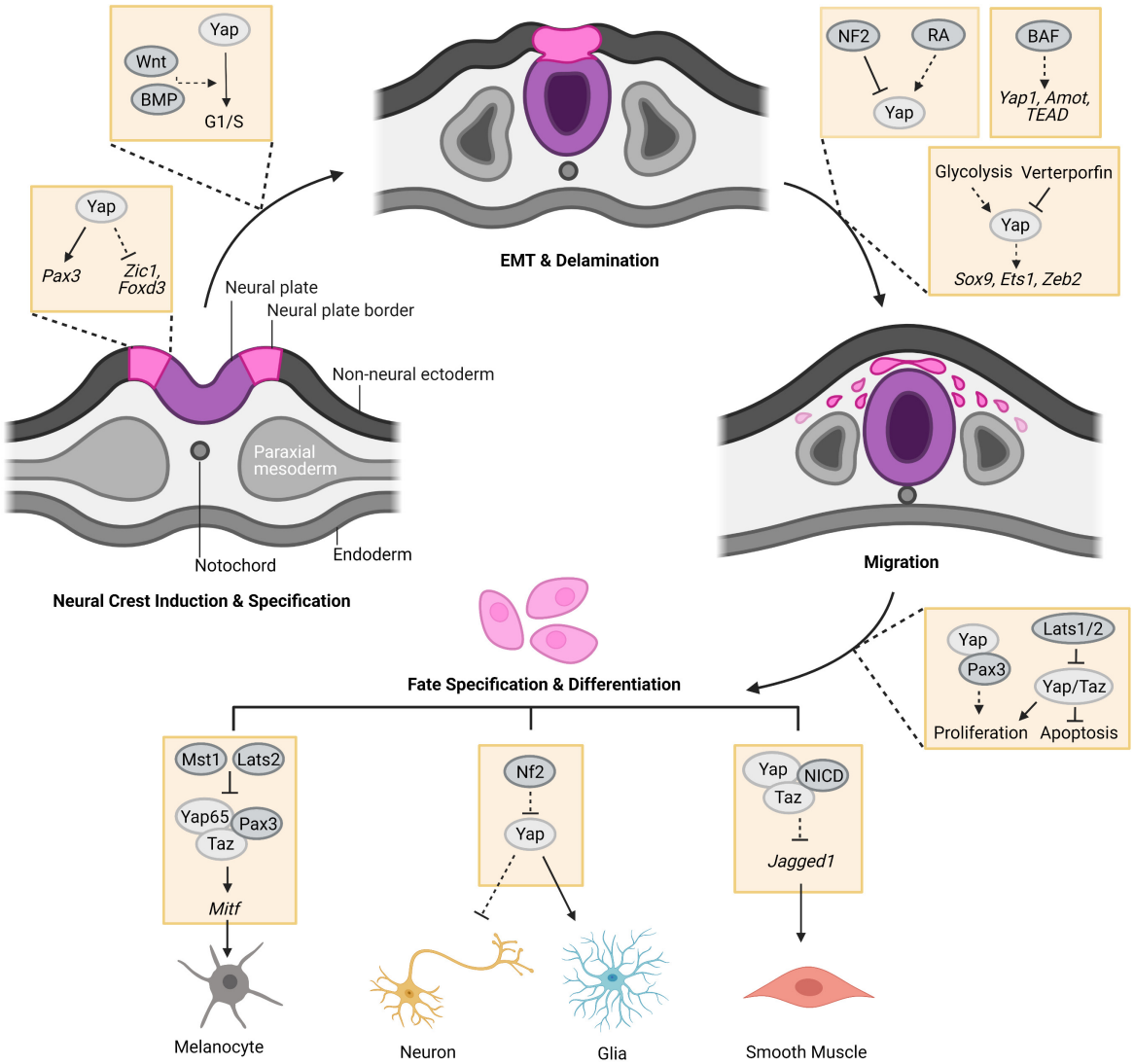

FIGURE 1 | The regulation of the Hippo-Yap pathway in neural crest (NC) development. At the stage of induction, Yap holds cells in NC progenitor state by regulating the expression of NC specifier genes, such as *Pax3*, *Zic1*, and *Foxd3*. After induction and specification, Yap potentially co-regulates G1/S transition with Wnt and BMP to promote pre-migratory NC cell EMT and delamination from the neural tube for migration. Meanwhile, glycolytic flux favors migration of NC cells through enhancing YAP1-TEAD1 interaction to drive the expression of EMT factors, such as *Sox9*, *Ets1*, and *Zeb2* in chick embryos. Hippo-Yap pathway may also play a role in the regulation of BAF to NC migration in mouse embryos. In addition, Yap and Taz were found to promote proliferation and survival of NC cells which could be inhibited by Hippo kinases, Lats1/2. During NC differentiation, Yap65/Taz form a complex with Pax3 to promote melanocyte differentiation of NC cells by upregulating *Mitf* expression, which could be repressed by Hippo kinases Mst1 and Lats2. Yap overexpression favors glia cell fate of NC cells. Accordingly, Nf2 loss-of-function boosts the cell fate of glia at the expense of neurons. The complex of Yap/Taz-NICD promotes smooth muscle cell differentiation of NC cells by inhibiting the expression of *Jagged1* (Created with Biorender.com).

increased apoptosis of the cells in NC-derived mandibular arch structures, at embryonic (E) 9.5 and E10.5 (Wang et al., 2016). Consistent with this observation, *Yap* and *Taz* double knockdown (dKD) O9-1 NC cells using siRNA showed reduced proliferation compared to the controls (Wang et al., 2016). In contrast, dKD of the Hippo signaling kinases Lats1/2 resulted in increased cell proliferation in O9-1 NC cells (Wang et al., 2016). In addition, Bi-Lin and colleagues observed that the loss of BAF155/170 in NC of mice embryos showed significantly decreased cell proliferation (at E10.5) and increased apoptosis (at E9.5 and 10.5) in the NT and pharyngeal arch area compared to control embryos (Bi-Lin et al., 2021). BAF complex component (Brg1) physically interacts with Hippo signaling components (Yap and Tead) in NC cells and potentially regulates many genes involved in cell proliferation and/or survival, such as *Axin2*, *Dll3*, and *Bcl10* (Bi-Lin et al., 2021). However, further studies are needed to delineate the roles of Hippo and BAF in NC proliferation and survival. Moreover, in avian embryos, the regulation of proliferation by Yap in pre-migratory NC cells was investigated by reducing *Yap* expression via electroporation of YAP shRNA construct (shYAP)

in the dorsal neural tube (Kumar et al., 2019). After 24 h of transfection, shYAP NC cells showed reduced phospho-histone $H3^+$ (pHH3) mitotic cells compared to control cells (Kumar et al., 2019). Altogether, Yap and Taz are essential regulators for proliferation and survival of NC cells demonstrated in both *in vivo* and *in vitro* analyses.

In contrast, the removal of *Yap* and *Taz* in NC cells (lineage labeled by tdTomato, RFP) resulted in similar proliferation rates of RFP-positive cells surrounding the third arch artery in E10.5 dKO mouse embryos compared to the controls (Manderfield et al., 2015). These inconsistent observations of Yap/Taz in proliferation rates could stem from underlying differences in NC and NC-derived cell types, a topic of interest for future studies.

HIPPO-YAP PATHWAY IN NC CELL EMT AND MIGRATION

After induction and specification, NC cells undergo epithelial-to-mesenchymal transition (EMT) which leads to cellular architecture remodeling and adhesive property changes, along with increased cell motility. NC cells delaminate from the dorsal NT and migrate extensively throughout the embryo to contribute to the development of various tissues and organs (Basch and Bronner-Fraser, 2006; Kulesa et al., 2010). A recent study demonstrated that Yap was actively expressed in pre-migratory NC of avian embryos, and loss or gain of Yap function inhibited or promoted NC migration, respectively (Kumar et al., 2019). Reports showed that G1/S transition in the pre-migratory NC is required for NC EMT (Burstyn-Cohen and Kalcheim, 2002; Kumar et al., 2019). In avian embryos, NC cells synchronously migrated from the NT during the S phase of the cell cycle, and specific repression of the G1/S transition inhibited NC EMT and delamination both *in vivo* and in explants (Burstyn-Cohen and Kalcheim, 2002). Notably, Deepak Kumar and colleagues found that *Yap* was activated and expressed in the pre-migratory NC of avian embryos and it stimulated G1/S transition to promote pre-migratory NC determination by crosstalk with Bmp and Wnt signaling (Kumar et al., 2019). G1/S transition was inhibited in pre-migratory NC cells when Yap activity was decreased, together with reduced proliferation and increased apoptosis, which may result in the failure of pre-migratory NC cells to delaminate and migrate from the NT (Kumar et al., 2019). Concomitantly, Bmp and Wnt activations were down regulated which indicated that the proliferation and migration of the pre-migratory NC cells may be co-regulated by Yap, BMP, and Wnt, however, further study is needed (Kumar et al., 2019). Likewise, in *Yap* morphants of zebrafish embryos, cranial NC cells, identified by positive crestin expression, were shown to have significantly reduced migratory abilities resulting in abnormal distribution to the developing cranium (Jiang et al., 2009).

Hippo-Yap signaling also interacts with retinoic acid (RA) signaling to regulate NC migration. A migratory NC phenotype of multiple human neural stem cell cultures was promoted in response to the treatment of RA and upregulation of *YAP* expression by siRNA-mediated knockdown of neurofibromatosis-2 (*NF2*), an upstream regulator of Hippo kinases (Hindley et al., 2016). Notably, a recent study demonstrated that in chick embryos, NC cells underwent extensive metabolic reprogramming, increasing glucose uptake to promote NC EMT and migration (Bhattacharya et al., 2020). Glycolytic flux was found to activate Yap/Tead signaling in NC cells by enhancing YAP1-TEAD1 interaction which drove the expression of EMT factors, such as *Sox9*, *Ets1* and *Zeb2*, by interacting with tissue-specific enhancers to promote NC delamination and migration (Bhattacharya et al., 2020). Inhibition of YAP1-TEAD1 interaction with verteporfin prevented NC migration (Bhattacharya et al., 2020). In addition, a recent paper by Bi-Lin and colleagues showed that BAF deficits resulted in NC cell migration deficiencies from the pharyngeal arches to the developing outflow tract (Bi-Lin et al., 2021). Furthermore, they found that BAF complex deficiencies in NC cells resulted in downregulation of the Hippo signaling pathway including *Yap1, Tead1, Amot, and Axin2* (Bi-Lin et al., 2021); but additional studies are needed to determine the interactions between BAF and Hippo signaling for NC migration. Based on current evidence, it suggests that the Hippo-Yap pathway benefits NC cell EMT and migration.

However, by using *Wnt1-Cre*, a NC specific driver, to create a dCKO of Yap and Taz in NC cells of mice embryos, Maderfield and colleagues found that the migration of NC cells to the pharyngeal arch arteries is intact and complete in E10.5 *Yap*/*Taz* dCKO embryos, as seen in control embryos (Manderfield et al., 2015). Serial sections of fate-mapped E10.5 *Yap*/*Taz* dCKO embryos displayed regions populated by NC cells in the presumptive facial mesenchyme, peripheral nervous system and enteric ganglia, demonstrating proper migration of NC throughout the embryo (Manderfield et al., 2015). These inconsistent results may be due to varying degrees of the Hippo-Yap dependency between model systems, or delayed delamination and migration of NC cells, while indicate that the role of the Hippo-Yap pathway in NC cell migration needs to be investigated further.

HIPPO-YAP PATHWAY IN NC FATE SPECIFICATION AND DIFFERENTIATION

NC is a multipotent stem cell population that can generate numerous cell and tissue types after delamination from the NT. However, there are various differentiation of preferences for different types of NC cells depending on the specific regions and surrounding signals. Recent studies displayed that the Hippo-Yap pathway was involved in fate specification and differentiation of NC cells. As mentioned above, Yap interacts with Pax3 to regulate NC induction in the NT border region in *Xenopus*, while in mouse embryo, the binding of Yap65/Taz and Pax3 is indispensable for melanocyte differentiation of NC cells in a Tead-independent manner (Manderfield et al., 2014). Yap65/Taz are Pax coactivators and their deficiency in pre-migratory NC cells downregulate the expression of Pax3 target gene, *Mitf*, a critical gene required for melanogenesis. The activations of Yap65/Taz-Pax3 complex can be inhibited by Hippo

kinases Mst1 and Lats2, revealing a role for Hippo signaling (Manderfield et al., 2014).

Neurons and glia in the dorsal root ganglia (DRG) are derived from NC cells. In mouse embryos, *Yap*/*Taz* are expressed in migratory NC cells, DRG progenitors and the glial lineage, but not the neuronal lineage (Serinagaoglu et al., 2015). In addition, *Yap* gain-of-function increases populations of DRG progenitors and glial cells (Serinagaoglu et al., 2015). *Nf2* loss-of-function boosts the cell fate of glia at the expense of neurons mirroring the phenotype of *Yap* gain-of-function further indicating the important roles of the Hippo-Yap signaling in NC derived neuron and glia specification.

Manderfield and colleagues found that the NC-specific deletion of *Yap*/*Taz* in mice led to vascular smooth muscle cell differentiation defects by inactivating the expression of *Jagged1*, a ligand and target of Notch signaling, by interacting with the Notch intracellular domain (NICD) (Manderfield et al., 2015). Our group also found vascular defects in *Yap*/*Taz* dCKO embryos (Wang et al., 2016). The *in vitro* O9-1 NC cells with *Yap* KO exhibited failure of smooth muscle differentiation due to Yap deficiency (Wang et al., 2016). A more recent paper showed defective smooth muscle differentiation of NC cells in pharyngeal arch arteries due to BAF155 or BAF155/170 KO in NC of E11.5 mouse embryos (Bi-Lin et al., 2021). Defective NC-derived palate mesenchyme formation was also demonstrated during secondary palate formation in BAF155 KO mouse embryos at E15.5 (Bi-Lin et al., 2021). Notably, BAF complex component (Brg1) physically interacted with Hippo signaling components (Yap and TEAD) in NC cells, potentially regulating many related genes although further studies are needed. Additionally, as a mechano-sensor of mechanical cues, Taz has been shown to promote mechanical tension-induced osteoblastic differentiation of mesenchymal stem cells (MSCs) in the rat cranial sagittal suture which are mainly derived from cranial NCCs by interacting with Rho-associated kinase (ROCK) (Li et al., 2020).

CONCLUSION AND PROSPECTIVE

NC is characterized as a transient and multipotent stem cell population that can differentiate into various cell types to contribute to the development of diverse organs and tissues, from peripheral nervous system to craniofacial skeletal system. It is well-known that environmental cues and transcription factors orchestrate induction, EMT, migration, proliferation, survival, and differentiation of NC cells. Thus, any small perturbation may open the door for diverse diseases caused by tissues/organs defects.

The Hippo-Yap pathway is a highly conserved pathway, regulating organ size, tissue homeostasis and regeneration through the regulation of cell stemness, proliferation, survival and differentiation. Dysfunction of the Hippo-Yap pathway has been linked to an increasing number of human diseases. Recent studies revealed that the Hippo-Yap pathway also plays important roles in NC developmental processes, including the induction, proliferation, survival, EMT, migration and differentiation of NC cells (summarized in **Figure 1**). However, our understanding about the subject is still in the early stages due to limited studies and numerous questions regarding the role and mechanisms of the Hippo-Yap pathway in NC development and disease. For example, NC cell migration is regulated by the Hippo-Yap pathway in avian and zebrafish embryos, as well as human NC cells. However, it is not as consistently demonstrated in mouse embryos. As mentioned above, using serial sections of fate-mapped E10.5 embryos, Manderfield and colleagues found intact and complete NC migration to the pharyngeal arch arteries in *Yap*/*Taz* dCKO embryos similar to controls (Manderfield et al., 2015). Similar observations of no gross migration difference in pharyngeal arches were also found in E10.5 and E11.5 mouse embryos with BAF155/170 dCKO, which have downregulation of a part of Hippo signaling including Yap1, Tead1 and Amot in NC cells (Bi-Lin et al., 2021). Interestingly, severe migration defects were found when NC cells migrated from pharyngeal arches to the developing outflow tract of the heart in E11.5 BAF155/170 dCKO embryos. These inconsistent findings indicate that the effects of the Hippo-Yap pathway on NC migration may be influenced by species, developmental stages and types of NC cells. The possibility remains that the varying degrees of Hippo-Yap dependency observed between model systems resulted in reduced and/or delayed NC migration in the mutants. Moreover, in mouse embryos, *Yap*/*Taz* loss-offunction in NC leads to early lethality of embryos with serious neural tube regression, craniofacial malformation and vascular defects (Manderfield et al., 2015; Wang et al., 2016; Wang, 2020)]. However, the mechanisms leading to embryo lethality with various defects mentioned above require further studies.

Mechanical signaling also plays important roles in NC migration. By performing mechanical and molecular manipulations, Barriga and colleagues found that, in *Xenopus laevis* embryos, mesoderm stiffening, mainly arising from cellular density, is necessary and sufficient to trigger NC migration, and changes in substrate stiffness can cause collective cell migration by promoting EMT *in vivo* (Barriga et al., 2018). Yap/Taz are mechanosensitive mediators of mechanical cues (Dupont et al., 2011; Wei et al., 2020; Yamashiro et al., 2020). It is an interesting field for further study whether different mechanical stimulation on Yap/Taz phosphorylation and cytoplasm/nuclei translocation are responsible for the effects of mesoderm stiffness on NC EMT and migration. Hippo-Yap pathway is essential for cell proliferation and tissue regeneration (Wang et al., 2018; Zheng et al., 2020). Notably, by using a genetically dissectible mouse model of mandibular distraction osteogenesis, Ransom and colleagues revealed that mechanical stimuli could promote jaw regeneration (Ransom et al., 2018). They found that newly formed bone is clonally derived from skeleton stem cells (SSCs) which are regulated by the focal adhesion kinase (FAK) signaling pathway that transduced mechanical signaling into SSCs during distraction osteogenesis of jaw regeneration. Their data also suggested that FAK stimulates SSCs to adopt an NCC-like state by expressing NCC-associated markers such as S100a4 and Plp1, and NCC specification markers including Twist1, Tfap2a and Sox10

during distraction (Ransom et al., 2018). In addition, a recent study found that mechanical stimulus activates FAK pathway, accompanied by down-regulation of Hippo-signaling during distraction osteogenesis (Song et al., 2018). This further suggest a role of the interaction between mechanical signals and the Hippo-Yap pathways in NC development and NC-derived tissue regeneration, which is surely also an interesting field for further studies.

Taken together, these findings indicate the critical roles of the Hippo-Yap pathway in NC development, while also highlight the significance of studying the Hippo-Yap pathway in NC-related human diseases. It is also important to figure out how environmental factors coordinate with Hippo signaling to orchestrate NC development or cause NC-related diseases. In summary, the Hippo-Yap pathway has pivotal functions in NC development and warrant further investigation in the mechanisms underlying NC-related diseases with the hopes of improving current therapeutic options.

AUTHOR CONTRIBUTIONS

XZ, TL, SE, TF, and JW wrote the manuscript. XZ, TL, and SE made the figure. All authors contributed to the article and approved the submitted version.

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