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The Hippo Pathway and YAP/TAZ–TEAD Protein–Protein Interaction as Targets for Regenerative Medicine and Cancer Treatment

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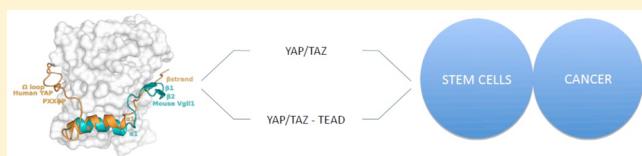
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ABSTRACT: The Hippo pathway is an important organ size control signaling network and the major regulatory mechanism of cell-contact inhibition. Yes associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) are its targets and terminal effectors: inhibition of the pathway promotes YAP/TAZ translocation to the nucleus, where they interact with transcriptional enhancer associate domain (TEAD) transcription factors and coactivate the expression of target genes, promoting cell proliferation. Defects in the pathway can result in overgrowth phenotypes due to deregulation of stem-cell proliferation and apoptosis; members of the pathway are directly involved in cancer development. The pharmacological regulation of the pathway might be useful in cancer prevention, treatment, and regenerative medicine applications; currently, a few compounds can selectively modulate the pathway. In this review, we present an overview of the Hippo pathway, the sequence and structural analysis of YAP/TAZ, the known pharmacological modulators of the pathway, especially those targeting YAP/TAZ–TEAD interaction.



INTRODUCTION

Coordination of cell proliferation and death is essential not only to attain an appropriate organ size during development but also to maintain tissue homeostasis during postnatal life. The mechanism by which multicellular organisms orchestrate the growth of their individual cells and the size of their organs is a longstanding puzzle in developmental biology that still remains to be resolved. Signaling pathways converting extra- and intracellular events into gene transcription are pivotal in cell number regulation.

Recently, a newly discovered and evolutionarily and functionally conserved signaling network, the “Hippo pathway”, has been shown to play a critical role in controlling organ size by regulating both cell proliferation and apoptosis.^{1–5} Initially, this pathway was discovered in *Drosophila melanogaster* by mosaic genetic screens, which proved to be a powerful tool in the elucidation of this molecular signaling. Each member of this pathway in *Drosophila* has a correspondent counterpart in mammals sharing a high analogy and homology level with it. Several genetic and biochemical studies gradually demonstrated that the *Drosophila* mutants for any of the signaling pathway components exhibit an overgrowth phenotype (as a *hippopotamus*), leading to the current model in which the Hippo pathway is the major regulatory mechanism of cell-contact inhibition.^{2–6} The terminal effector component of the Hippo pathway is a transcription coactivator, named Yorkie (Yki) in *Drosophila*. In mammals, Yes-associated protein (YAP) and its parologue transcriptional co-activator with PDZ-binding motif (TAZ) have been identified as

terminal effectors of the pathway.⁷ The final effect of the Hippo pathway on the Yki/YAP/TAZ proteins involves phosphorylation on specific serine residues to confine them in the cytoplasm for subsequent degradation. Consequently, Yki/YAP/TAZ cannot translocate to the nucleus, where they would bind to other proteins (including TEA domain proteins (TEADs) in mammals) and act as transcription coactivators, triggering the expression of cell proliferation-promoting genes.^{1,2,8}

Hippo signaling alterations are increasingly being associated with cancer development.^{4,9–11} It was observed that a significant percentage of patients affected by certain cancers, such as cancer of the liver, breast, or pharynx, harbor causative amplification or overexpression of the YAP gene; therefore, the use of YAP-inhibiting drugs could be tailored to the selected patients for optimal effects.¹² These clinical observations suggest that the pathway, particularly the YAP–TEAD complex, can be targeted to inhibit cancer or modulate proliferation. Because the YAP–TEAD complex corresponds to the final step of YAP activity, its targeted inhibition should diminish the potential side effects expected from targeting the upstream proteins of the pathway, which are more interconnected with other signaling networks. Therefore, the protein–protein interactions between YAP and TEAD emerge as the best candidate target for modulating the Hippo pathway with small molecules.

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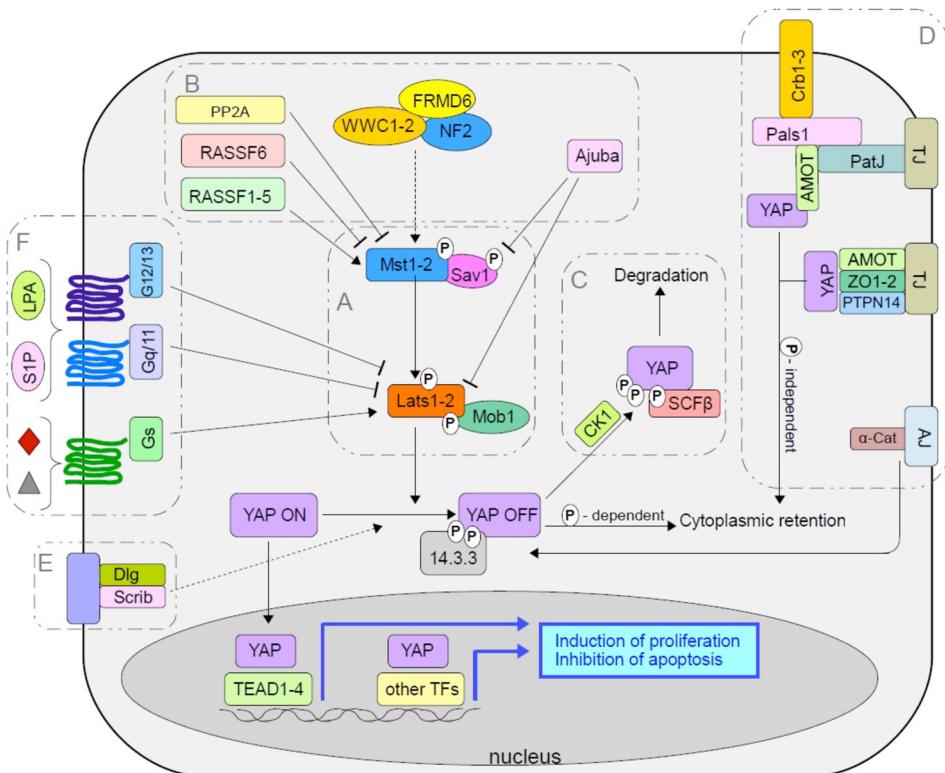


Figure 1. Hippo signaling pathway in mammals: the Hippo pathway consists of serine/threonine phosphorylation events leading to cell proliferation inhibition and the promotion of apoptosis via inhibition of the transcriptional coactivators YAP/TAZ. Interactors and regulators that play an important role in the mammalian Hippo pathway have been grouped into functional modules. Box A, core kinase cassette of the Hippo pathway; box B, regulators that act immediately upstream of the core kinase cassette; box C, phosphorylation-dependent degradation of cytoplasmic YAP; box D, regulation of YAP/TAZ by TJ- and AJ-related proteins; box E, regulators related to basolateral polarity complexes; box F, GPCR-related regulation. Pointed arrowheads represent activation, blunted arrowheads represent inhibition; dashed lines represent regulation mechanisms that are not fully elucidated (see text for detailed description).

Currently, there are no known compounds that can interfere in a consistent manner with the YAP–TEAD interaction. However, the YAP–TEAD complex structure has been resolved by X-ray crystallography, and mutagenesis studies have identified the amino acid residues crucial for forming the functional complex.¹³ This structural information could allow the design of new compounds directed to the target protein. Consequently, modulating YAP activity could possibly help regulate the self-renewing, proliferative potential and the fate of stem cells and could hopefully direct stem cell differentiation toward a specific cell-lineage. From this perspective, the following points should be considered: (i) the Hippo pathway is involved in cross-talk with several growth regulatory signaling pathways, (ii) not all tissue-specific progenitors are regulated by this pathway, and (iii) different sets of downstream target genes are regulated in a tissue-specific manner. Hence, the number of cross-linking signals could be very high, triggering a significant redundant and compensatory mechanism. Despite these limitations, a tissue-specific modulation of YAP activity could represent a powerful tool for future regenerative medicine applications. This review provides an overview of the Hippo signaling pathway, explaining its role in differentiated cells and stem cells throughout organ development. An emphasis will be placed on the importance of the YAP–TEAD complex as a drug target and on the possibility of using a medicinal chemistry approach to develop compounds that can interfere with stem cell fate, through this target, for future regenerative medicine applications.

BIOLOGY OF THE HIPPO PATHWAY

The Hippo Signaling Pathway and YAP/TAZ Regulation. The Hippo signaling pathway was initially described in *Drosophila*, where it gradually emerged as a major regulatory mechanism of contact inhibition during the growth of the cells.^{1–5} Numerous genetic and biochemical studies in *Drosophila* have led to an extensive characterization of the pathway: it consists of a series of serine/threonine phosphorylation events that lead to the inhibition of cell proliferation and the promotion of apoptosis via inhibition of the transcriptional coactivator Yki. In *Drosophila*, the pathway is initiated by transmembrane receptors belonging to the cadherin family (Ft, Ds): upon initiation of the pathway, a complex comprising Ex, Mer, and Kibra proteins is formed (Figure 1, box B, see Table 1 for correspondence between *Drosophila* proteins and their homologues in human), which in turn activates the core Hippo pathway kinase cassette (Hpo, Sav, Mats, and Wts, Figure 1, box A). The Hippo pathway activity can also be regulated by a variety of modulators, including components of tight junctions (aPKC, Par3, and Par6), adherens junctions (a-catenin), or apical–basal polarity protein complexes (Crb, PatJ, Std, Lgl, Scrib, and Dlg).^{1,2,9} When the core Hippo pathway kinase cassette is activated, the transcriptional coactivator Yki is phosphorylated on multiple sites, thereby creating a 14–3–3 binding site and resulting in cytoplasmic retention and inactivation of Yki. When the Hippo pathway is inactive, Yki bypasses the phosphorylation by Wts and enters the nucleus, where it binds and activates a transcription factor (Sd). This induces several downstream target

Table 1. Hippo Signalling Pathway Core Components and Modulators in *Drosophila melanogaster* and Their Homologues in Humans

<i>Drosophila melanogaster</i> proteins	human proteins	role in the pathway	effect on Yki/YAP
Modulators			
Ft (fat)	Ft1–4	in <i>Drosophila</i> , Ft initiates signaling upon binding to Ds (*) ^a	—
Ds (Dachsous)	Dchs1–2	in <i>Drosophila</i> , Ds binds to and activates Ft (*) ^a	—
Ex (expanded)	FRMD6 (Ex1) ^c	in <i>Drosophila</i> , Ex–Mer–Kibra complex activates Hpo (*) ^a	—
Mer (Merlin)	NF2	in <i>Drosophila</i> Ex–Mer–Kibra complex activates Hpo (*) ^a	—
Kibra	WWC1–2	in <i>Drosophila</i> , Ex–Mer–Kibra complex activates Hpo (*) ^a	—
Crb (Crumb)	Crb1–3	apical polarity complex with PatJ and Std/Pals1	—
PatJ	PatJ, MUPP1	apical polarity complex with Crb and Std/Pals1	—
Std (Stardust)	Pals1	apical polarity complex with Crb and PatJ	—
aPKC	aPKC	apical polarity complex with Par3 and Par6 (in <i>Drosophila</i>) (*) ^a	+
Baz (Bazooka)	Par3	apical polarity complex with Par6 and aPKC (in <i>Drosophila</i>) (*) ^a	—
Par6	Par6	apical polarity complex with Par3 and aPKC (in <i>Drosophila</i>) (*) ^a	—
Lgl	Lgl1–2	basolateral polarity complex	—
Scrib (Scribble)	Scrib	basolateral polarity complex (*) ^a	—
Dlg (Disc Large)	Dlg1–4	basolateral polarity complex (*) ^a	—
dRASSF	RASSF1–6	in <i>Drosophila</i> , dRASSF competes with Sav in binding to Hpo in mammals, RASSF1–5 activate Mst1–2 (except RASSF6 that inhibits Mst2)	—
Mts	PP2A	dephosphorylates Hpo/Mst1–2	+
Jub	Ajuba	interacts with Sav/Sav1 and Wts/Lats1–2	+
Dco	CK1 δ/ϵ	in <i>Drosophila</i> , Dco enhances Ft activation in mammals, CK1 δ/ϵ primes YAP for degradation	—
(#) ^b	AMOT, AMOTL1, AMOTL2	modulate YAP subcellular localization and/or phosphorylation	—
(#) ^b	ZO1–2	sequester YAP/TAZ in the cytoplasm	—
Pez	PTPN14	sequesters Yki/YAP/TAZ in the cytoplasm	—
α -catenin	α -catenin	stabilizes Yki/YAP complex with 14.3.3 proteins	—
Core Components			
Hpo (Hippo)	Mst1–2 ^d	phosphorylates and binds to Sav/Sav1; phosphorylates Mats/Mob1	—
Sav (Salvador)	Sav1	forms a kinase complex with Hpo/Mst1–2; the complex phosphorylates Wts/Lats1–2	—
Wts (Warts)	Lats1–2	forms a kinase complex with Mats/Mob1	—
Mats	MOBKL1A-B (Mob1)	forms a kinase complex with Wts/Lats1–2; the complex phosphorylates Yki/YAP/TAZ	—
Yki (Yorkie)	YAP, TAZ ^e	transcriptional coactivator	—
Sd (Scalloped)	TEAD1–4	transcription factor	—

^a(*) denotes not fully characterized function in human cells. ^b(#) denotes unknown homologues in *Drosophila melanogaster*. ^cHuman FRMD6 displays a relatively low sequence homology with *Drosophila* Ex. ^dHuman Mst1/2 have a C-terminal-located recognition site for caspases that is absent in *Drosophila* Hpo. ^eHuman YAP/TAZ have a C-terminal-located PDZ domain that is absent in *Drosophila* Yki.

genes, including promoters of cell growth (Myc and Ban), promoters of cell-cycle progression (E2F1, cyclin A, B and E), inhibitors of apoptosis (Diap1), and components of other important signalling pathways.^{2,8,9}

The Hippo pathway is highly conserved in mammals. The core kinase cassette components and downstream effectors of the *Drosophila* pathway all have specific homologues in mammalian cells: Mst1/2 (Hpo homologues), Sav1 (Sav homologue), Lats1/2 (Wts homologues), MOBKL1A and MOBKL1B (collectively referred to as Mob1; homologues of Mats), and YAP and its parologue TAZ (also called WWTR1; homologues of Yki) (Table 1).^{4,14} Relationships among Hpo, Sav, Wts, and Mats are also conserved among mammalian Mst1/2, Sav1, Lats1/2, and Mob1 (Figure 1, box A). As with the *Drosophila* Sav and Hpo proteins, the mammalian Sav1 protein interacts with and activates the serine–threonine kinases Mst1/2 through the SARAH domains present in both; however, the underlying mechanism is still unclear because experiments on transgenic or knockout mice and on immortalized mouse embryonic fibroblasts have produced discrepant results.¹² The autoactivation of Mst1 and 2, which occurs by phosphorylation on threonine residues within their activation domain, triggers the

phosphorylation and consequent activation of their direct substrates, Lats1/2.^{15,16} The latter forms a complex with Mob1, which is phosphorylated by Mst1/2, resulting in an enhanced Lats1/2–Mob1 interaction. The activated Lats1/2–Mob1 complex in turn phosphorylates YAP (on S127)/TAZ (on S89), preventing their nuclear translocation via binding to cytoplasmic 14–3–3 proteins. Phosphorylation of YAP at S127 (or of TAZ at S89) as a result of the activation of the core kinase cassette of the Hippo pathway is the most relevant and critical step in abrogating YAP nuclear localization and activity: whenever YAP is phosphorylated, then the expression of its target proliferation-related genes is downregulated, cell proliferation is inhibited, and apoptosis is induced. Mutation of S127 in YAP and subsequent disruption of the 14–3–3 binding site activates YAP, further confirming the inhibitory role of the phosphorylation on this specific residue: YAP S127 needs to be mutated in order for YAP to acquire a transforming potential in human Hek293 cells and in mouse NIH3T3 cells.^{17,18}

Homologues of *Drosophila* Ft, Ds, Ex, Mer, and Kibra do exist in mammalian cells (Ft1–4, Dchs1–2, FRMD6/Ex1, NF2, and WWC1/WWC2; Table 1 and Figure 1, box B); however, their functional significance in regulating the pathway is just beginning

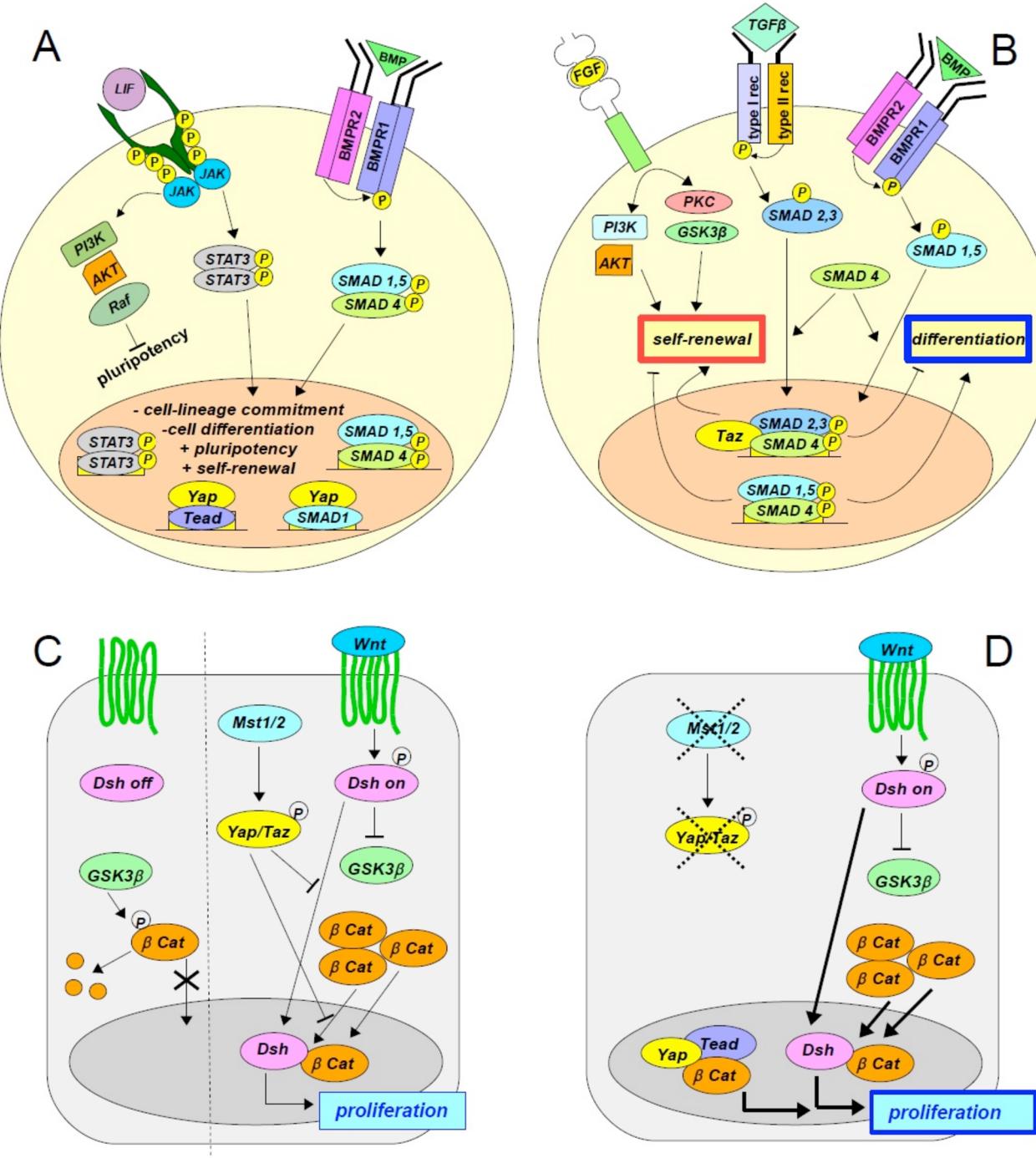


Figure 2. (A) Self-renewal and differentiation regulation in mouse ESCs: mESCs depend on the cytokine leukemia inhibitory factor (LIF) and signals from bone morphogenic proteins (BMPs) to reinforce the pluripotency network and block differentiation and progression to lineage commitment. The LIF pathway leads to the activation of JAK-STAT signaling, with STAT3 homodimers acting as transcription factors for major pluripotency and self-renewal genes; BMP-signaling determines the activation of Smad1/5–Smad4 transcription factors, targeting promoters for major stemness genes. (B) Self-renewal and differentiation regulation in human ESCs: the self-renewal and differentiation of hESCs depend on fibroblast growth factor (FGF) signaling and a balance between transforming growth factor- β (TGF- β) and BMP signaling. FGF signaling supports the self-renewal via protein kinase C (PKC) and phosphatidyl-inositol-3-kinase (PI3K). TGF- β signaling leads to Smad2/3 activation and the formation of Smad2/3–Smad4 heterodimers, which act as transcription factors promoting self-renewal and blocking differentiation. BMP signaling leads to Smad1/5 activation and subsequent Smad1/5–Smad4 heterodimers formation, promoting differentiation and inhibiting self-renewal: hESCs fate depends on the balance between the two pathways. (C) Wnt signaling in intestinal SCs: Wnt-OFF state (left), Wnt-ON state (right). (D) Hippo/Wnt signaling effects on intestinal crypt destiny: active YAP or Hippo ablation enhances Wnt signaling, increasing Dsh phosphorylation, and consequent β -catenin nuclear accumulation. Once in the nucleus, YAP interacts with β -catenin and TEAD to activate the expression of proliferation-related genes, and Dsh acts as a transcriptional cofactor to induce β -catenin target genes.

to be delineated, and further investigation is needed to characterize the steps that act immediately upstream of the core kinase Mst1–2 and that initiate the pathway in mammals.^{9,19} NF2, FRMD6, and WWC1/2 interact and promote activation of the Mst1/2–Sav1 complex by a mechanism that is not fully characterized.⁹

Although several modulators acting upstream of the core kinase cassette of the pathway identified in *Drosophila* have structural and functional homologues in mammals (Figure 1, box B), certain relevant differences have been highlighted. In contrast to dRASSF function in *Drosophila*, most mammalian RASSF homologues are scaffold proteins activating Mst1/2: RASSF1A, which has the highest homology with the fly protein dRASSF, activates the Mst1/2 kinases by preventing their dephosphorylation by PP2A, as demonstrated in numerous experiments in different cell contexts including human Hek293 and MCF7 cells and monkey COS7 cells.^{9,20,21} Another Ras effector family member, RASSF6, binds to Mst2 and antagonizes Hippo signaling.²² As in *Drosophila*, Ajuba (homologue of *Drosophila* Jub) physically interacts with Lats1/2 and Sav1, leading to the inhibition of the Hippo signaling activity in canine MDCK cells.²³ The presence of conserved *Drosophila* orthologues in the mammalian Hippo pathway may serve as a mechanism of redundancy that protects the organism against cancer-causing mutations.

At the nuclear level, YAP/TAZ associate with TEAD1–4 (homologues of *Drosophila* Sd) and stimulate the transcription of genes involved in the control of cell proliferation, differentiation, and development such as Myc,^{24,25} Gli2,²⁶ CTGF, and Cyr61.^{26–28} The TEAD family transcription factors are the main YAP/TAZ partners in the regulation of gene expression. Knockdown of TEADs or disruption of the YAP–TEAD interaction abolishes YAP-dependent gene transcription and substantially diminishes YAP-induced cell proliferation and oncogenic transformation, as demonstrated in independent experiments performed on mouse NIH3T3 cells, human Hek293 cells, and on mouse embryos.^{4,10,29} A mutation of TEAD1 T421, which forms a hydrogen bond with YAP, results in loss of interaction with YAP and leads to the human genetic disease Steinsson's chorioretinal atrophy.³⁰ Precise regulation of the YAP–TEAD interaction is therefore important in maintaining normal physiology.

Despite a major role for TEADs in YAP/TAZ function, other transcription factors are known to interact with the WW domains of YAP/TAZ, including Smad1, Smad2/3, RUNX, ErbB4, and p73 for YAP^{4,31} and RUNX, PPAR γ , Pax3, TBX5, and TTF-1 for TAZ (interactions identified in mesenchymal stem cells for RUNX and PPAR γ , in C2C12 mouse myoblast cell line for Pax3, in primary neonatal rat cardiac myocytes for TBX5, and in murine lung and thyroid cells for TFF-1).^{8,32–35} These transcription factors, but not TEAD, contain a PPXY motif that is considered important for recognition by YAP/TAZ. The interaction of YAP with Smad1 is important for maintaining the pluripotency of mouse embryonic stem cells (mESCs), mediated by the activation of the bone morphogenetic protein (BMP) transduction pathway (Figure 2A). Considering that BMP plays a role in mESC self-renewal and differentiation and that both BMP and Hippo pathways have the ability to control organ size, the regulated interaction of Smad1 and YAP could possibly mediate the cross-talk between these two networks.³⁶ Additionally, YAP and TAZ bind Smad2/3. In human embryonic stem cells (hESCs), Smad proteins are transcriptional modulators through which TGF- β family members regulate many developmental

events (Figure 2B). Particularly, the YAP–Smad2/3 interaction is believed to dictate the nuclear accumulation of Smad2/3 for subsequent transcription activation. In this manner, YAP regulates Smad nuclear localization and coupling to the transcriptional machinery.³⁷

In P19 mouse embryonic carcinoma cell line, YAP also interacts with p73, a p53 family pro-apoptotic transcription factor, to induce the expression of genes such as Bax, Puma, and PML.³⁸

Another YAP interaction partner is RUNX2, which stimulates the osteoblastic differentiation of mesenchymal stem cells (MSCs), to promote chondrocyte hypertrophy and to contribute to endothelial cell migration and vascular invasion in bone development. YAP interacts with the full-length RUNX2 protein, as well as RUNX2-responsive promoter regions; however, the effects of YAP on the expression of the RUNX2 target genes appear to depend on the promoter, namely on the cohort of other DNA-binding proteins and cofactors brought to the gene by specific DNA sequences and protein–protein interactions.^{39,40}

At the nuclear level, YAP can also coactivate genes under the transcriptional control exerted by ErbB4: ErbB4 is a tyrosine-kinase receptor protein which is proteolytically processed by membrane proteases in response to the ligand, resulting in the translocation of its cytoplasmic COOH-terminal fragment (CTF) to the cell nucleus. As demonstrated in experiments in human Hek293T cells and in monkey COS7 cells, YAP can associate with the cytoplasmic portion of the ErbB4 receptor and coactivate transcription mediated by CTF. Thus, the CTF of ErbB4 produced by γ -secretase cleavage, translocates, along with YAP, to the nucleus upon ligand stimulation, and YAP may act not only as a transcriptional coactivator for the CTF but also as a carrier protein for translocating the CTF from the membrane to the nucleus.⁴¹

Phosphorylation of YAP at S127 is crucial in promoting binding to the 14–3–3 proteins and subsequent cytoplasmic sequestration and inactivation of YAP; however, YAP phosphorylation can also induce its degradation (Figure 1, box C): experiments in human Hek293 cells and in mouse NIH3T3 cells have shown that Lats1/2 phosphorylates YAP at S381, which primes YAP for subsequent phosphorylation by another kinase, possibly casein kinase 1 (CK1 δ/ϵ). This might activate a phosphorylation-dependent degradation motif, termed phosphodegron. Subsequently, the E3-ubiquitin ligase SCF β –TRCP is recruited to YAP, leading to its polyubiquitination and degradation.¹⁷ YAP/TAZ can also be inhibited in a phosphorylation-independent manner through protein–protein interactions with transmembrane complexes-related proteins, resulting in YAP/TAZ cytoplasmic sequestration (Figure 1, box D). Recently, YAP/TAZ and angiostatin family proteins (AMOT) were shown to interact, resulting in YAP/TAZ inhibition through various mechanisms. AMOT was first identified as an “angiostatin binding protein” promoting endothelial cell migration and angiogenesis, and due to its angiogenic function, it has been implicated in tumor growth. However, the angiostatin-responsive migration-promoting functions are observed in the AMOT p80 splicing variant that does not bind YAP but not in the YAP-binding p130 variant. The AMOT family has three paralogues in humans and mice (AMOT, AMOTL1, and AMOTL2) and two isoforms generated by alternative splicing: the p80 isoform lacks 400 amino acids at the N-terminal present in the p130 isoform. YAP interacts with AMOT p130 alone because the p130 unique N-terminal region contains two PPXY motifs, which serve as binding partners for the YAP WW

Table 2. Known Effects and Mechanisms of Action of YAP in Different Stem Cell Types

stem cell type	YAP activation	YAP inactivation	mechanism of action
embryonic SCs	pluripotency support; hindering of differentiation; reprogramming process enhancement	loss of self-renewal and pluripotency	direct induction of important stemness-genes (i.e., Oct4, Sox2, Nanog)
skin SCs	thickening of epidermal layer; hyper-keratinization; tumor formation	epidermal hypoplasia	YAP overexpression drives the expansion of undifferentiated interfollicular SCs and progenitor cells; YAP inactivation drives the gradual loss of epidermal stem/progenitor cells and their limited capacity to self-renewal
intestinal SCs	ISCs proliferation and expansion of progenitor-like cells	loss of ISCs and degeneration of the intestinal epithelium	nuclear YAP enhances Wnt-signaling
neural SCs	neural progenitor cells expansion; expansion of cerebellar granule neural precursors; medulloblastoma		cytoplasmic YAP represses Wnt-signaling Shh-signaling induces the expression and nuclear localization of YAP in cerebellar granule neural precursors
liver SCs	liver hyperplasia; hepatocyte hyperproliferation	impaired liver function; hepatocyte apoptosis	YAP overexpression enhances proliferation of mature hepatocytes YAP inactivation induces enhanced hepatocyte apoptosis
cardiac muscle SCs	cardiomyocyte proliferation	myocardial hypoplasia	nuclear YAP enhances Wnt-signaling (both directly and via IGF-pathway)

domains. Mutation of the first PPXY motif, which is conserved in all three AMOT family members, significantly decreases the interaction with YAP. Mutation of the second PPXY motif, which is not conserved in AMOTL2, has little effect. The combined mutation of both PPXY motifs shows an effect similar to the mutation of the first PPXY motif. Therefore, the WW domains of YAP and the first PPXY motif of AMOT play major roles in the YAP–AMOT interaction.⁴² Studies conducted in various cell contexts, among which MCF10A human mammary epithelial cells, HeLa human cervical cancer cells, and U2OS human osteosarcoma cells have shown that AMOT can inhibit YAP/TAZ by various mechanisms (Figure 1, box D). Through physical interaction, AMOT recruits YAP/TAZ to various compartments such as tight-junctions and actin cytoskeleton, depending on the cellular localization of YAP/TAZ: AMOT binds to Pals and PatJ, thus forming the tight junction-related Crumbs cell-polarity complex, which sequesters the transcriptional coactivators YAP/TAZ at sites of cell–cell adhesion, preventing their nuclear localization.^{43–45} In addition, AMOT is present in protein complexes along with ZO-1/2 and PTPN14, which mediate the cytoplasmic retention of YAP/TAZ, independent of their phosphorylation status. ZO-2 was reported to consistently inhibit TAZ-mediated transactivation and YAP2-dependent induction of proliferation in human epithelial cells such as Hek293, MCF7, and MCF10A.^{46,47} AMOT family proteins additionally inhibit YAP/TAZ by promoting their inhibitory phosphorylation; however, it is unclear whether induction of YAP phosphorylation indirectly depends on the modulation of its subcellular localization. Other known membrane-related modulators of YAP/TAZ belong to adherens junctions (Figure 1, box D): α -catenin, an adherens junction component and a tumor suppressor, promotes YAP cytoplasmic localization and inhibits YAP activity by interacting with and stabilizing the YAP1/14–3–3 complex.^{48,49}

Dlg and Scrib proteins are associated with basolateral polarity complexes and increase YAP/TAZ phosphorylation; however, the molecular mechanisms underlying this process in mammalian cells are still poorly understood (Figure 1, box E).^{9,50}

Recent studies have highlighted the capability of G-protein coupled receptor (GPCR) signaling to regulate the Hippo pathway in a manner that is dependent on the specific G-protein coupled to the receptor (Figure 1, box F).^{11,51} This discovery arose from the observation that in multiple cell lines, YAP is

highly phosphorylated under serum starvation, whereas the addition of serum results in a rapid decrease in YAP phosphorylation. This suggested the presence of a serum component that activates YAP by inducing its dephosphorylation and nuclear localization. Further studies demonstrated that the active ingredient in serum was an amphiphilic molecule with an acidic group, such as lysophosphatidic acid (LPA) or sphingosine-1-phosphate (S1P). LPA and S1P act via activation of G12/13- or Gq/11-coupled receptor signaling, which leads to inhibition of Lats1/2 kinases and subsequent activation of YAP.^{51,52} In contrast, treatment of the same cell lines with epinephrine and glucagon increases YAP phosphorylation, via activation of the Gs-coupled receptors, which stimulate adenylyl cyclase (AC). This results in the accumulation of cAMP which in turn acts through activation of protein kinase A (PKA) to stimulate Lats kinase activity and inhibit YAP/TAZ.

Hippo Signaling Pathway and Stem Cells. Over the past decade there has been growing evidence that the Hippo pathway can affect tissue size by directly regulating stem cell (SCs) proliferation and maintenance. Numerous studies have investigated Hippo signaling in various stem cell populations and have revealed its crucial role in stem cell biology: in general, YAP overexpression or inactivation of any Hippo signaling pathway component promotes proliferation and prevents differentiation of different tissue-specific SCs (Table 2). However, not all tissue-specific progenitors are regulated by this pathway, and its manipulation leads to different effects depending on the specific cell-type considered. Consequently, the manipulation of Hippo signaling regulation may represent a possibility to influence stem cell commitment and differentiation toward a specific and unique cell lineage. Thus, the manipulation of the Hippo regulation mechanisms may represent a useful tool in clinical applications and regenerative medicine.

Embryonic Stem Cells (ESCs). Embryonic stem cells are isolated from the inner cell mass (ICM) of blastocysts. They are pluripotent stem cells because they have the ability to give rise to all cells and embryo tissues, except placental extraembryo tissue. Therefore, they represent the source of all tissues comprising the developing embryo, the fetus, and ultimately the adult organism.

ESCs depend on various signals for self-renewal: mouse-ESCs rely on the cytokine leukemia inhibitory factor (LIF) and signals from bone morphogenic proteins (BMPs), which reinforce the pluripotency network and block progression to lineage commit-

ment (Figure 2A), whereas human ESCs rely on fibroblast growth factor (FGF) signaling and a balance between transforming growth factor- β (TGF- β) and BMP signaling (Figure 2B).^{3,19,53} YAP and TAZ also play relevant roles in regulating ESC fate: the importance of YAP/TAZ during embryogenesis was uncovered when the transcription factor TEAD4 was found to be critical for the induction of Cdx2, which is a transcription factor required for the development of the trophectoderm lineage, the outer cells of the blastocyst stage embryo.^{54,55} However, TEAD4 expression is not restricted to these cells of the blastocyst. On the other hand, YAP is found in the nucleus of the outer cells, where it allows TEAD4-mediated Cdx2 transcription, whereas it is cytoplasmic in the ICM cells where Cdx2 is not expressed.⁵⁶ Although YAP is found in the cytoplasm in the ICM of mouse blastocyst, it is found in the nucleus of ESCs, which are derived from the ICM. As ESCs differentiate, YAP nuclear localization and protein levels diminish, and this is accompanied by increased Hippo pathway activity.⁵⁷

It has been demonstrated that YAP and TAZ play important roles in regulating ESC self-renewal and differentiation because they can interact with some of the downstream effectors of the main self-renewal stimulating pathways in mESCs and hESCs. YAP/TAZ contribute to maintaining mESC pluripotency in vitro by mediating the BMP-induced transcriptional program: the BMP signaling pathway leads to the activation of Smad1 transcription factor and the recruitment of YAP to Smad1 on target gene promoters, enhancing Smad1 activity (Figure 2A).³⁶ In hESCs, TAZ binds Smad2/3–Smad4 heterodimers in response to TGF- β stimulation, thus enhancing self-renewal and inhibiting differentiation (Figure 2B); TAZ depletion impairs Smad2/3–Smad4 accumulation in the nucleus and transactivation activity, and the cells tend to differentiate.^{37,50}

Loss of YAP or TAZ in mESCs and the conditional knockout of TAZ (but not YAP) in hESCs result in loss of self-renewal and ESC pluripotency; overexpression of YAP prevents differentiation in mESCs and results in increased reprogramming of fibroblasts to induced pluripotent stem cells (iPSCs) (Table 2).^{37,57} The latter are differentiated cells reprogrammed to an ESC-like state by inducing the activity of four transcription factors (Oct-4, Sox-2, Klf-4, and cMyc).^{57,58} iPSCs can self-renew, they are indistinguishable from ESCs in several ways, therefore, they are considered a valuable resource for cell or tissue replacement therapy. However, these data suggest that YAP activity enhances the reprogramming process, in conjunction with Oct4, Sox2, and Klf4. A possible mechanism may be via the activation of a YAP/TEAD2-gene expression program: YAP/TEAD2 directly bind to promoters and activate the transcription of important stemness genes such as Sox-2, Oct-4 (thus establishing a positive feed-back loop) and Nanog.⁵⁷

Skin Stem Cells (SSCs). To regenerate continuously and maintain its structural and functional integrity, the skin relies on the self-renewing abilities of epidermal SCs residing in the basal layer of epidermis. Asymmetric divisions in this SC compartment produce short-lived progenitor cells that stratify, leave the basal layer, and move up through the suprabasal layers to the outer surface of the skin as they terminally differentiate. YAP plays an important role in epidermal development and SC homeostasis.^{3,53} Recent studies have highlighted that YAP overexpression causes a severe thickening of the epidermal layer and that this hyperplasia is driven by the expansion of undifferentiated interfollicular SCs and progenitor cells; the knockout of YAP leads to an epidermal hypoplasia, and this phenotype has been attributed to the gradual loss of epidermal

stem/progenitor cells and their limited capacity to self-renew (Table 2).^{3,27} In the skin, YAP is not regulated by the canonical Hippo kinases, but rather by α -catenin, a component of adherens junctions (AJs), which is an upstream negative regulator of YAP (Figure 1, box D). These AJs could act as “molecular biosensors” of cell density and positioning according to the “crowd control molecular model”: sensing increased cell density leads to inhibition of SC expansion by inactivating YAP, whereas low basal cell density translates into nuclear YAP localization and cell proliferation.^{48,49}

Small Intestine Stem Cells (ISCs). The intestinal epithelium is one of the most rapidly regenerating tissues in the body, turning over completely every 4–5 days through the continual proliferation of intestinal stem cells located at the base of the crypt (crypt base columnar (CBC) cells, also known as Lgr5+ cells) and at the “+4 position” relative to the crypt bottom.⁵³ The major factor promoting the self-renewing capacity of Lgr5+ ISCs in mammals is the Wnt pathway. The driving force behind Wnt signaling is β -catenin. Generally, in a Wnt-unstimulated cell, glycogen synthase kinase-3 (GSK-3) phosphorylates the cytoplasmic pool of β -catenin and promotes its degradation through an ubiquitin-mediated proteasome pathway (Figure 2C, left). In a Wnt-stimulated cell, the Wnt receptor Frizzled activates Disheveled (Dsh), which in turn inhibits GSK-3 activity. On its inhibition, GSK-3 no longer phosphorylates β -catenin, which accumulates in the cytosol. Stable β -catenin subsequently enters the nucleus, forms a transcriptional complex with members of the Lef/Tcf family of DNA-binding proteins, and regulates downstream target genes (Figure 2C, right).⁵⁹ Recently, the Hippo pathway together with its final effector YAP have been found to be critical in balancing ISC self-renewal and differentiation. YAP is found in the nucleus of ISCs and some other crypt cells but is primarily cytoplasmic in the upper crypt and villi, where it is likely that Hippo targets YAP for phosphorylation and consequent inhibition.^{3,53} The phosphorylation of YAP/TAZ and their cytoplasmic localization counterbalance Wnt activity. Overexpression of active YAP or conditional knockout of Mst/Sav-1 expands progenitor-like cells and blocks differentiation (Table 2).^{60,61} The aberrant proliferation induced by unphosphorylated YAP in ISCs is in part or wholly due to the hyperactivation of Wnt signaling because of enhanced β -catenin transcriptional activity. Specifically, when YAP and TAZ are phosphorylated by the Hippo pathway and sequestered in the cytoplasm, phosphorylated YAP/TAZ interact with Dsh and β -catenin (Figure 2C, right). Cytoplasmic YAP inhibits the nuclear translocation of Dsh and β -catenin (through a mechanism which is distinct from the degradation pathway), whereas cytoplasmic TAZ inhibits the activity of the degradation-complex member casein kinase 1 (CK1), blocking Dsh phosphorylation. In the Wnt-ON state, if phosphorylated YAP/TAZ is lost, or Hippo signaling is ablated, cells undergo hyperactivation of Wnt signaling owing to increased Dsh phosphorylation and/or nuclear accumulation as well as additional nuclear β -catenin (Figure 2D). Once in the nucleus, Dsh acts as a transcriptional cofactor to induce β -catenin target genes in conjunction with another transcriptional cofactor cJUN.⁵⁸ In addition, YAP interacts with β -catenin and TEAD in the nucleus to activate the expression of proliferation-related genes. In conclusion, when the Wnt pathway is predominant, compared with phosphorylated YAP, Wnt produced by Paneth cells (components of the intestinal stem cell niche) and other sources is detected by ISCs in intestinal crypts: the ISCs divide and cells progress upward out of the crypt and begin to

differentiate. If YAP becomes overabundant in the cytoplasm of the crypt cells, Wnt signaling is repressed and the ISC niche is disrupted. This causes aberrant upward migration of Paneth cells and loss of ISCs. Because of the ISC loss, the intestinal epithelium degenerates.

Neural, Liver, and Cardiac Muscle Stem Cells. Neural progenitor cells reside along the subventricular zone in the developing vertebrate neural tube and are responsible for generating the myriad of cell types comprising the mature central nervous system. The conditional knockout of Mst/Lats or YAP activation expands neural progenitor cells in the neural tube.^{3,53,62} In the cerebellum, endogenous YAP is highly expressed in cerebellar granule neural precursors (CGNPs). YAP overexpression expands CGNPs in the cerebellum and leads to medulloblastoma. The CGNPs rely on sonic-hedgehog (Shh) signaling to expand and Shh signaling induces the expression and nuclear localization of YAP, which then drives the proliferation of these cells (Table 2).⁶³

The adult liver has a distinctive ability to rapidly regenerate following acute injury. The regeneration of the organ is dependent on the ability of hepatocytes and cholangiocytes (bile duct cells) to proliferate and on heterogeneous populations of transit-amplifying bipotential progenitor cells known as “oval cells” in rodents.⁶⁴ YAP overexpression leads to a dramatic but reversible liver hyperplasia, caused by an exacerbated proliferation of mature hepatocytes.⁶⁰ Conversely, conditional loss of YAP function leads to impaired liver function, primarily because of accelerated hepatocyte turnover due to enhanced apoptosis (Table 2). The conditional knockout of Mst1–2 leads to liver overgrowth, with mixed hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) phenotypes, whereas conditional knockout of Sav-1 leads to a similar liver overgrowth and development of HCC/CC mixed tumors but shows increased number of oval cells without concomitant hepatocyte expansion.^{64–66}

In contrast to that in tissues such as the liver, the role of Hippo signaling in the heart is just beginning to be delineated. Sav1/Mst2/Mst1–2 conditional knockout or YAP overexpression promotes cardiomyocyte proliferation, resulting in embryos displaying a cardiomegaly phenotype, whereas YAP conditional knockout leads to myocardial hypoplasia.^{53,67} YAP interacts with β -catenin in the nucleus to promote Wnt signaling (a well-known promoter of cell-stemness and proliferation in the heart), thus enhancing neonatal cardiomyocyte proliferation.⁶⁷ Moreover, YAP indirectly promotes Wnt signaling through the activation of the insulin-like growth factor (IGF) pathway (a potent signaling system that stimulates growth and blocks apoptosis in many different cell types), resulting in the inactivation of GSK3 β and consequently of the Wnt degradation complex (Table 2).⁶⁸

STRUCTURAL AND INHIBITION STUDIES

YAP/TAZ/Yki Domains Organization. The human YAP gene is located at 11q13; it can be transcribed into at least four isoforms (YAP1–4), which are generated by differential splicing of short exons located within the transcriptional activation domain of YAP: isoforms 1, 2, 3, and 4 have 504, 450, 488, and 326 residues in length, respectively, and their identity ranges between 53% and 96% (Figure 3).^{69–72} YAP was originally identified in chicken as an interacting protein of Yes protein tyrosine kinase. The interaction was shown to be mediated by the SH3-domain of the Yes protein and the proline-rich region (PVKQPPPLAP) of YAP (SH3 binding domain, Figure 3). Due to its size of 65 kDa, the chicken protein was referred to as YAP65

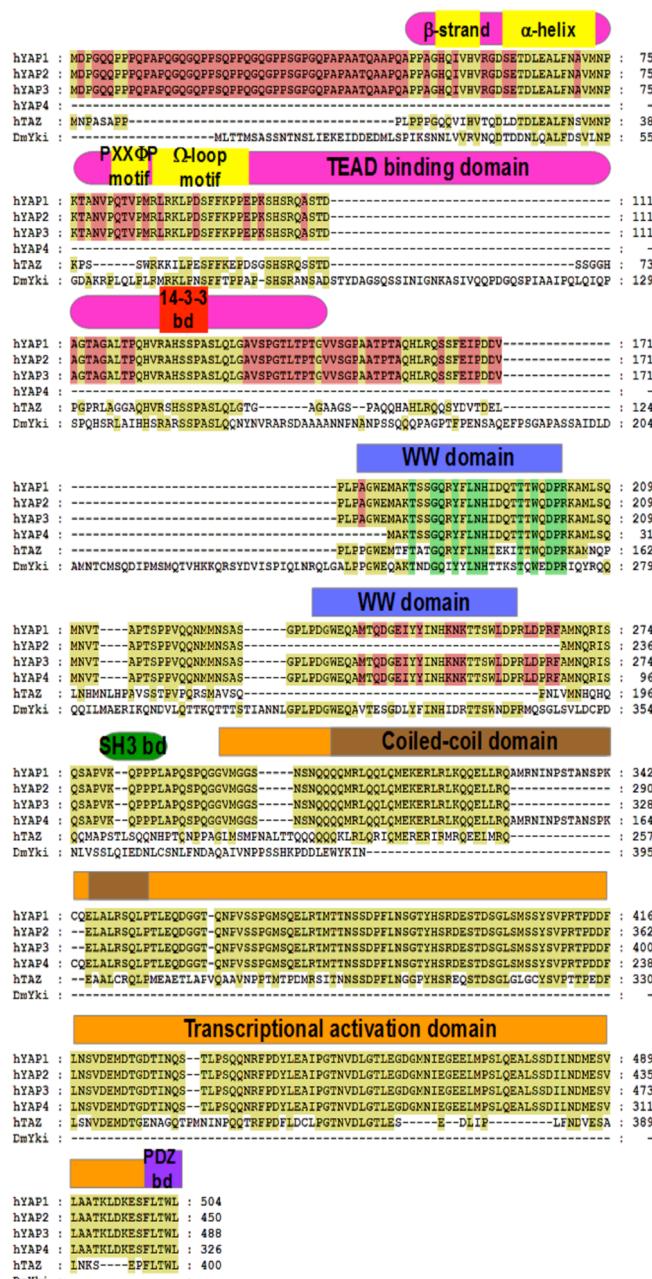


Figure 3. Alignment of the four isoforms of human YAP and human TAZ and *Drosophila* Yki. Identical residues are highlighted as follows: red if identical among 3 out of 6 sequences, yellow if identical among 4 out of 6 sequences, and green if identical among all the 6 sequences. Protein domains are indicated as follows: TEAD-binding domain in magenta (regions important for the YAP–TEAD interaction are highlighted in yellow), 14–3–3-binding domain in red, WW domains in blue, SH3-binding domain in green, transcriptional activation domain in orange, coiled-coil domain in brown, PDZ-binding domain in purple.

(Yes-associated protein of 65 kDa). The human and mouse homologues were identified by using the YAP65 cDNA to probe human and mouse cDNA libraries. During the course of sequence analysis by comparing YAP with other proteins, a conserved module was observed in several proteins of various species. This was named the WW domain to reflect the sequence motif containing two conserved and consistently positioned tryptophan (W) residues (Figure 3). Two consecutive WW domains are present in all isoforms except isoform 2, which has

only one WW domain. Isoform 3, containing 488 residues and two WW domains, is the most thoroughly studied isoform. These WW domains are important for YAP to interact with transcription factors containing the PPXY motif. The N-terminal region harbors the TEAD-binding domain, containing HXRXXS motifs (14–3–3 binding domain, Figure 3). Phosphorylation of S127 within this motif creates a binding site for 14–3–3 proteins and plays the most crucial role in determining YAP-cytoplasmic sequestration and inactivation. The C-terminus of YAP proteins exhibits strong transactivation property (transcriptional activation domain, Figure 3), and it contains a PDZ-binding motif (FLTWL, Figure 3) critical for nuclear translocation and binding to the PDZ domain of other regulator proteins such as ZO2. YAP proteins also contain a coiled-coil domain within the transcriptional binding domain (Figure 3).⁷¹

The TAZ gene can be transcribed into three variants, all of which have the same coding region for a protein having 400 amino acids in length (Figure 3). TAZ, also referred to as WWTR1 (WW domain containing transcription regulator 1), is homologous to YAP2 with 42% amino acid sequence identity and displaying similar domain organization but having only one WW domain (similar to YAP2 isoform). Biochemically, TAZ displays transcriptional coactivator function via interaction with PPXY-containing transcriptional factors through its WW domain. The C-terminal region is responsible for the transcriptional coactivation property. Similar to YAP, TAZ has a C-terminus with a PDZ-binding motif (FLTWL) and a N-terminal region, which harbors the TEAD-binding domain containing the HXRXXS-motifs with the S89, whose phosphorylation creates a binding site for the 14–3–3 proteins and results in TAZ inactivation (similar to S127 in YAP).

Both YAP and TAZ are homologous to fly Yki (Yorkie). Similar to YAP, Yki contains two WW domains. The N-terminal region of Yki shows the highest homology to YAP and TAZ, presenting the Scalloped (Sd)-binding domain which contains S111 homologous to YAP-S127 and TAZ-S89.

YAP–TEAD Complex: The Main Mediator of YAP/TAZ/Yki Transcriptional Function. TEADs in mammals and Sd in *Drosophila* are the major transcriptional factors mediating the biological outcome of YAP/TAZ and Yki, respectively. YAP was identified as a tight binding and major interacting protein for TEAD2 and was proposed to function as a general transcriptional coactivator for the TEADs transcriptional factors. There are four related family members (TEAD1–4) in mammals, whose identity ranges from 61% to 73% (Figure 4).⁷² The N-terminal regions of TEADs and Sd contain a conserved TEA domain involved in recognizing DNA elements such as GGAATG in the promoter region of target genes. The NMR structure of the TEA domain (PDB ID: 2HZD) revealed a three-helix bundle fold with the helix 3 containing a bipartite nuclear localization signal (NLS).⁷³ The C-terminal regions of TEADs and Sd interact with YAP/TAZ and Yki, respectively. Mutation of specific YAP and TEAD residues abolishes most, if not all, the formation of the protein–protein complex. Luciferase reporter assays in cells have moreover shown that the formation of the YAP–TEAD complex is required for its ability to promote transcription.^{13,71} A similar functional relationship between TEAD and TAZ has been demonstrated: the TAZ residues essential for interacting with TEAD are also essential to induce transformation and are well conserved in YAP and Yki. The putative YAP-binding domain of TEAD2 (YBD) in a crystal structure of TEAD (PDB ID: 3L15) adopts an immunoglobulin IgG-like fold with two β -sheets packing against each other to form a β -sandwich.⁷¹ The crystal

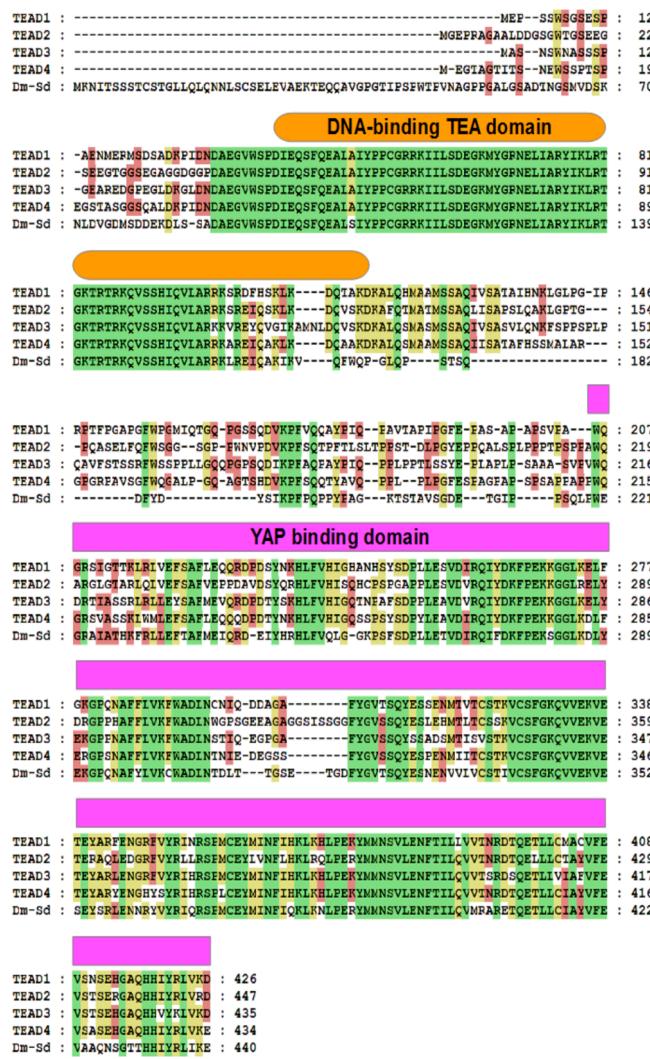


Figure 4. Alignment of the four isoforms of human TEAD. Identical residues are highlighted as follows: red if identical among 3 out of 5 sequences, yellow if identical among 4 out of 5 sequences, and green if identical among all the 5 sequences. Protein domains are indicated as follows: DNA-binding TEA domain in orange, YAP-binding domain in magenta.

structure of the complex between human TEAD1 and YAP (PDB ID: 3KYS) shed light on the structural features of the YAP–TEAD interactions.⁷³

As in the apo-structure, one β -sheet of TEAD contains five antiparallel strands, including β 1, β 2, β 5, β 8, and β 9, whereas the other contains seven parallel and antiparallel strands, including β 3, β 4, β 6, β 7, and β 10–12. In addition, the TEAD2–YBD contains two helix-turn-helix motifs. One helix-turn-helix motif consists of α A and α B and connects β 3 and β 4. This motif along with the β 2– β 3 loop encircles the C-terminal β 12 strand, forming an unusual pseudoknot structure. The second helix-turn-helix motif consists of α C and α D and connects β 9 and β 10. This motif caps the opening at one end of the β -sandwich. There are several surface-exposed residues that are identical in TEAD–YBD from all species. All these conserved residues form a contiguous surface on one face of TEAD2–YBD that contains β 7, α C, and α D and on the back of the strands β 4, β 11, and β 12, while the other face of TEAD2–YBD contains few conserved residues. A conserved tyrosine on the first face of human TEAD1–YDB (Y421, corresponding to Y442 in TEAD2) is

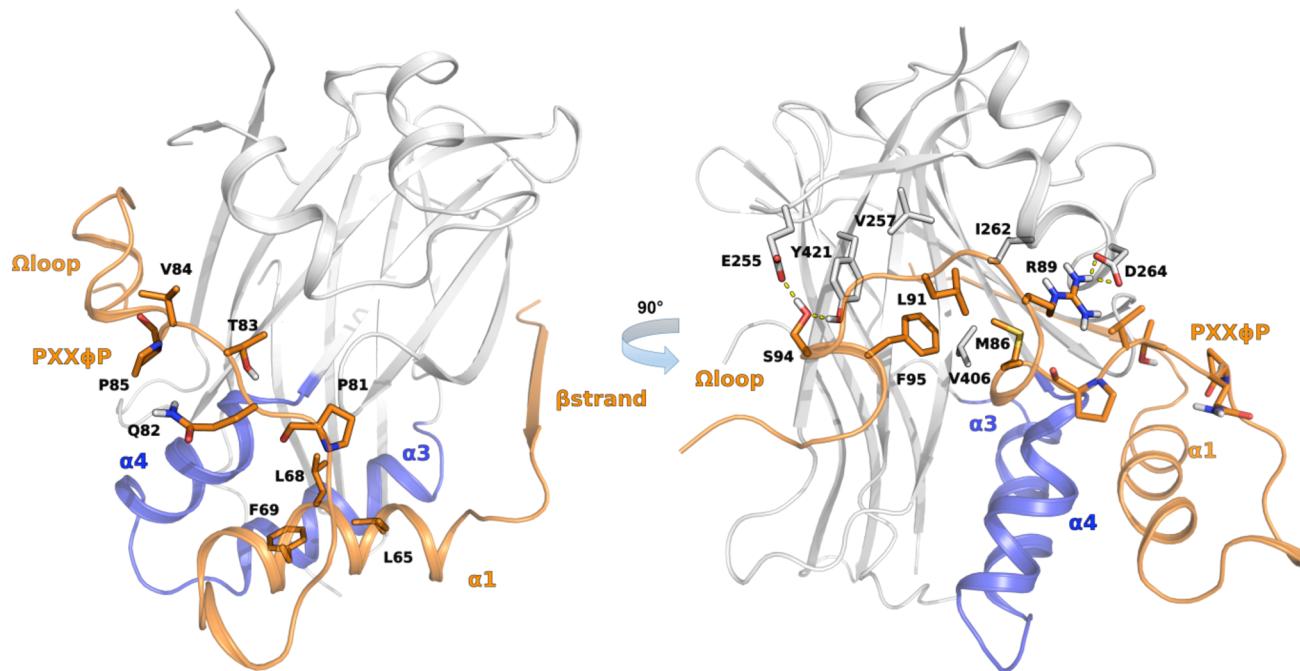


Figure 5. Protein–protein interactions between human YAP (orange ribbons; residues 50–100 from crystal structure 3KYS) and human TEAD (white ribbons; residues 200–426 from crystal structure 3KYS). Left panel: lipophilic residues on α -helix 1 (orange carbons) of YAP interact with the hydrophobic groove formed by the α -helix 3 and 4 of TEAD (blue ribbons); the PXXΦP links the α -helix 1 to the Ω -loop in YAP. Right panel (resulting from 90° rotation around y axis): the interaction between the YAP Ω -loop and TEAD is mediated through van der Waals interactions between hydrophobic residues and through hydrogen bonds highlighted by yellow dots (YAP residues, orange carbons; TEAD residues, white carbons).

mutated to histidine in patients with a rare eye disorder called Sveinsson's chorioretinal atrophy.³⁰ This mutation disrupts the YAP–TEAD interaction, thus hindering YAP-dependent induction of proliferation.

In the crystal structure, YAP surrounds TEAD with three major interaction interfaces. A β strand in YAP (residues 52–57) represents the first structural motif interacting with TEAD1 β through a series of H-bonds. The conserved hydrophobic residues (61–73) of helix α 1 in YAP represent the second contact area. A linker characterized by a PXXΦP motif (residues 81–85) connects the helix α 1 to the third interaction interface in YAP (Figure 3). This region is formed by an unusual twisted-coil structure (residues 86–100), also referred to as Ω -loop motif, which fits snugly within a hydrophobic site on the TEAD1 surface.¹³ YAP, TAZ, and Yki conserve, in different species, the residues necessary for interaction with TEAD and therefore for the growth-promoting activity of the YAP oncogene. In a crystal structure of mouse TEAD4–YAP (PDB ID: 3JUA), the major interactions of the last two interfaces are conserved, even if the Ω -loop is referred to as a second α helix (α 2), whereas the β strand has not been resolved.⁷⁴ It appears that all three sites of interaction act in concert to mediate the YAP–TEAD complex; β strand, helices α 1 and Ω -loop/ α 2 in YAP appear to contribute most significantly to the interaction with TEAD. In detail, the second interaction interface involves the helix α 1 of YAP and the helices α 3 and α 4 of TEAD, where the residues L65, L68, and F69 of YAP form the LXXLF motif, which interacts with the hydrophobic groove formed by the residues F329, Y361, F365, K368, L369, L372, V381, and F385 of TEAD; all residues involved in this contact area are highly conserved in YAP and TEADs (Figure 5, left panel). The PXXΦP-containing loop does not interact with TEAD, but the observation that mutation of a proline in this motif of Yki abolishes its binding to Sd⁷⁵ suggests that this loop may have a conformational role, allowing the

optimal arrangement of Ω -loop and helix α 1. Although TAZ lacks the PXXΦP motif, a computational model of its structure has suggested that its residues 24–56 can form both a α -helix and a Ω -loop and that these residues can reproduce the spatial arrangement of the α 1 and Ω -loop of YAP, respectively.^{72,74} The third interaction site is primarily mediated by the Ω -loop of YAP, which fits in the pocket formed by β 4, β 11, β 12, α 1, and α 4 of TEAD1. The interaction of the YAP Ω -loop with TEAD1 is chiefly mediated by hydrophobic interactions: M86, L91, and F95 of YAP establish van der Waals contacts with I262, V257, L297, and V406. This interaction is strengthened by the polar contacts of R89 and S94 of YAP with D264 and E254 and Y421 of TEAD, respectively (Figure 5, right panel).

Recently, the crystal structures of murine Vgll1 and Vgll4 complexed with TEAD4 have been elucidated, uncovering the important role of vestigial-like proteins 1–4 (Vg in *Drosophila*) as cotranscriptional factors. Similar to YAP and TAZ, Vgll proteins exert their function by binding the C-terminal region of TEAD proteins through the Tondu domain(s) (TDU). Mouse Vgll1–TEAD4 crystal structure (PDB ID: 4EAZ)⁷⁶ revealed that Vgll1 interacts with TEAD through two structural elements: the first interaction interface is composed by hydrogen bonds formed by a β strand of Vgll1 (β 2), interacting with β 7 of TEAD; the second interface is mediated by hydrophobic interactions between Vgll1 helix α 1 (TDU domain) and TEAD helices α 3 and α 4. As revealed by the superposition of the respective crystallographic complexes, mVgll1 and YAP bind to overlapping regions of TEAD, highlighting similarities and differences between the binding modes of the proteins. The β 2 strand of mVgll1 and the β 1 of YAP occupy the same region, whereas α 1 of mVgll1 is superposed to hYAP α 1, sharing a short hydrophobic motif (V41, F45, and A48 in mVgll1 and L65, F69, and V72 in hYAP) that interacts with the groove formed by TEAD α 3 and α 4 (Figure 6). In contrast, mVgll1 lacks the Ω -loop, which is

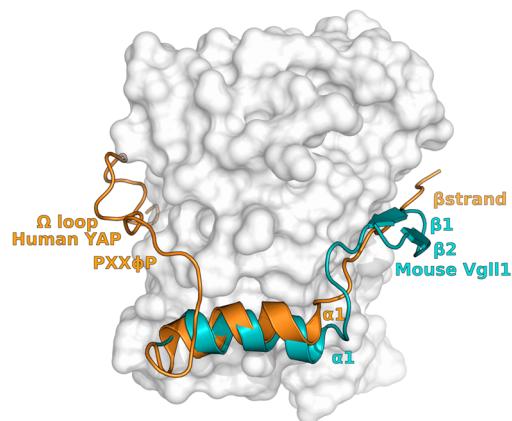


Figure 6. Comparison of the interacting conformations of human YAP (orange ribbon; residues 50–100 from crystal structure 3KYS) and mouse Vgll1 (cyan ribbons; residues 19–50 from crystal structure 4EAZ) with human TEAD (white surface; residues 200–426 from crystal structure 3KYS).

fundamental for YAP/TAZ binding to TEAD–YBD surface.¹³ Mutagenesis and TR-FRET experiments to describe the interaction modes of Vgll1 fragments and hTEAD⁷⁷ have revealed that the peptide fragment formed by β_2 and α_1 is fundamental for mVgll1 binding, showing nanomolar affinity for hTEAD4. This was unexpected, as YAP fragments missing the Ω -loop lack most of their affinity for TEAD.¹³ TR-FRET studies additionally showed that mVgll1-derived peptides can compete with hYAP, which supports the hypothesis that YAP and Vgll

have mutually exclusive effects on TEAD-dependent gene transcription.⁷⁷ This hypothesis was further supported by a recent study, which clarified the function of Vgll4 and reported the crystallization of the mVgll4–TEAD4 complex (PDB ID: 4LN0).⁷⁸ This crystal structure highlights striking differences between mVgll1 and mVgll4. Additionally, while Vgll1 has been shown to promote cancer progression, Vgll4 has been identified as a transcriptional repressor that inhibits YAP-induced overgrowth and tumorigenesis. On the basis of the rationale that the TDU region of Vgll4 is sufficient for inhibiting YAP activity and that most of the binding sites for Vgll4 and YAP do not overlap on TEAD, Ji and co-workers designed a peptide able to mimic Vgll4 activity, block overexpression of YAP target genes, and ultimately suppress tumor growth.⁷⁸ Indeed, the developed peptide, called Super-TDU, significantly inhibited YAP activity and gastric tumor growth both in vitro and in vivo. This provides clear support for the notion that Vgll4 acts as an antagonist of YAP and blocks YAP oncogenic activity at transcriptional level. On this basis, the development of Vgll4-mimicking peptides emerges as an alternative and promising therapeutic strategy against YAP-driven human cancers.⁷⁸ Notably, the same authors found that the downregulation of Vgll4 was correlated with the upregulation of YAP target genes and viceversa. Thus, as the YAP/Vgll4 ratio is markedly skewed in clinical samples of gastric tumor and well correlated with tumor progression, it has been highlighted as a prognostic marker for a personalized treatment.⁷⁸ Indeed, the YAP/Vgll4 ratio might be exploited for patient stratification to identify patients most likely to benefit from one or another strategic approach. For example, YAP

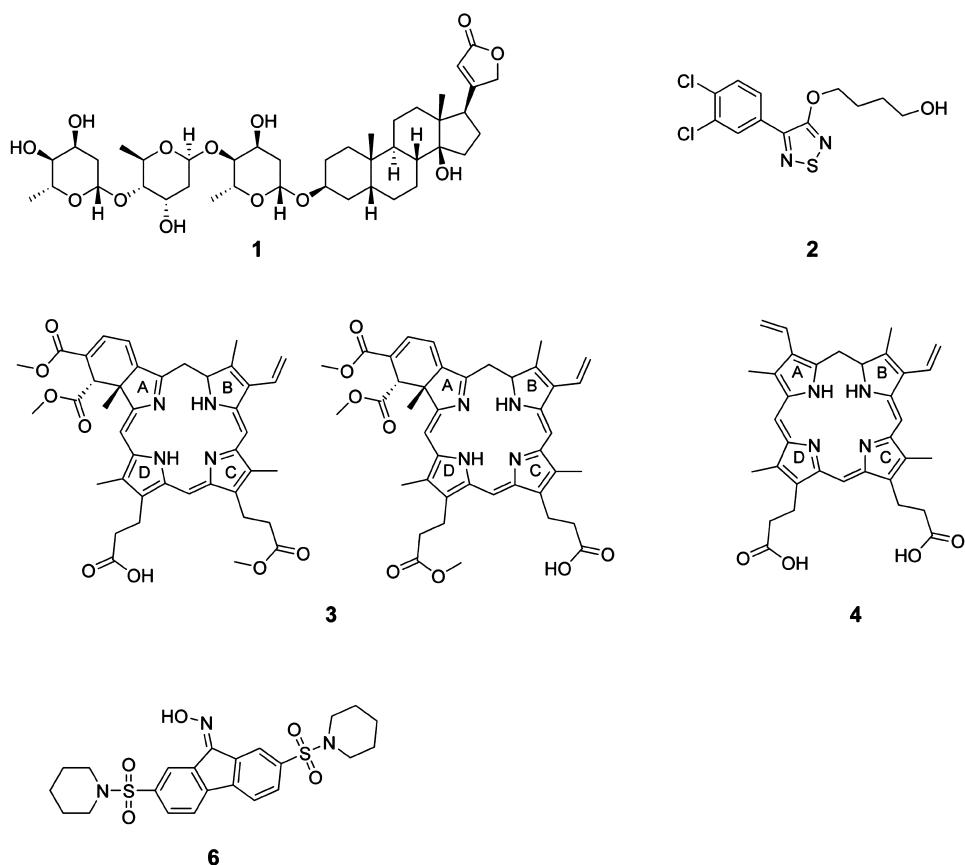


Figure 7. Chemical structures of small molecules interacting with Hippo pathway components are shown; compound 3 consists of a mixture of equally active regioisomers.

inhibitors would not work well in a cancer patient with rather low YAP/Vgll4 ratio.

Inhibition Strategies of YAP/TAZ/Yki. Considering the strict correlation between YAP hyperactivation and the outbreak of several malignancies, the modulation of its functions represents an innovative and promising approach to prevent and treat human cancers. Although the lack of information about the complete YAP structure prevents the application of a structure-based approach to discover novel compounds binding to YAP, the available structural data indicate that the YAP–TEAD complex could be a suitable target for developing new cancer therapeutics. In vitro binding and functional assays⁷¹ indicate that YAP–TEAD binding is mainly dependent on the interaction between YAP Ω-loop and the hydrophobic pocket formed by $\beta 4$, $\beta 11$, $\beta 12$, $\alpha 1$, and $\alpha 4$ of TEAD. This pocket is composed by a hydrophobic cavity surrounded by a number of polar residues, and it may, in principle, be addressed by small molecules. On the other hand, much bigger compounds would be required to bind at the same time a combination of more YAP–TEAD contact areas. Even if the design of small-molecule inhibitors of protein–protein interactions have traditionally represented a significant challenge, the availability of crystal coordinates for the YAP–TEAD complexes along with recent successes in structure-based design of protein–protein inhibitors allow envisioning of a more optimistic scenario for future accessibility to novel pharmacological tools interfering with YAP-mediated TEAD activation.

To identify small molecules that modulate YAP-dependent transcription by computer-aided drug design, Sudol et al. evaluated the possibility of targeting the WW domain, which recognizes proline rich motifs (PPXY), fundamental for YAP interaction with Lats and for its transcriptional activity.⁶⁹ Previously, the same group had predicted by virtual models that the cardiac glycoside digitoxin (**1** Figure 7) could be considered as a putative ligand at the WW domain of dystrophin.⁷⁹ **1** is used to treat cardiac arrhythmias, where its apparent mechanism of action involves modulating the activity of the sodium–potassium ATPase transporter pump. Moreover a growing number of evidence indicates that **1** may have anticancer activity, but the underlying mechanisms are still subject to study.⁸⁰ The potential affinity of **1** for the WW domain has emerged by a docking strategy developed to dock 287 FDA-approved small molecule drugs with 35 peptide-binding proteins, including 15 true positives. The selection of peptide binding domains included 20 cocrystal structures selected from the Protein Data Bank (PDB), representing a subset of eukaryotic linear motif (ELM) peptide binding domains complexed with peptides. Regarding the true positives selection, 14 cocrystal structures and one NMR model were selected from the PDB, with the requirements that they have a protein and a small molecule mimicking a natural peptide in the complex. A combined ligand and target normalization procedure was performed to improve the ability to rank true positives. The docking energy score was combined with a score based on the number of similar interactions formed between the compound and the native ligand. The 20 top ranking hits included 6 true positives, including **1**. Considering the similarities between YAP and dystrophin, Sudol et al. built a homology model of YAP WW domain and hypothesized that **1** may bind the portion recognizing the PPXY motif.⁶⁹ Thus, **1** was docked to the canonical hydrophobic groove within the WW domain of YAP. **1** could engage in an extensive network of intermolecular van der Waals and hydrogen bonding contacts with an array of residues,

such as Y188, L190, T197, and W199, lining the hydrophobic groove within the WW domain. Moreover, these residues are critical for the binding of PPXY ligands. However, other residues such as H192 and Q195 within the WW domain, which also play a key role in the binding of PPXY ligands, do not appear to be important for binding **1**. This suggests that **1** is unlikely to target all WW domains indiscriminately and that potential opportunities exist for the chemical modification of **1** to enhance its specificity toward a small group of WW-domains involved in regulating a specific signaling cascade such as the Hippo pathway. Currently, this is only a speculative hypothesis and no experimental data regarding the affinity of **1** for YAP are available.

Recent studies have highlighted the capability of G-protein coupled receptor (GPCR) signaling to regulate the Hippo pathway in a manner that is dependent on the specific G-protein coupled to the receptor.^{11,51} This discovery arose from the observation that in multiple cell lines, YAP is highly phosphorylated under serum starvation whereas the addition of serum results in a rapid decrease in YAP phosphorylation. This suggested the presence of a serum component that activates YAP by inducing its dephosphorylation and nuclear localization. Further studies demonstrated that the active ingredient in serum was an amphiphilic molecule with an acidic group such as lysophosphatidic acid (LPA) or sphingosine-1-phosphate (S1P). LPA and S1P act via activation of G12/13- or Gq/11-coupled receptor signaling, which leads to inhibition of Lats1/2 kinases and subsequent activation of YAP (Figure 1, box F).^{51,52} In contrast, treatment of the same cell lines with epinephrine and glucagon, which are known protein–kinase A (PKA) activators, increases YAP phosphorylation, via activation of the Gs-coupled receptors, which stimulate adenylyl cyclase (AC). This results in the accumulation of cAMP, an important second messenger with diverse physiological functions, including cell proliferation and differentiation. Thus, cAMP acts through protein kinase A (PKA) to stimulate Lats kinase activity and inhibit YAP/TAZ. Altogether, these data underline the possibility to modulate YAP/TAZ by a wide range of extracellular signals via GPCRs.

In a recently registered patent, Kung-Lian Guan et al. report the results of a HTS strategy based on a reporter assay in a mammalian cell culture system consisting of a luciferase reporter and a Gal4-fused TEAD transcription factor.⁸¹ A collection of small molecules was screened to search for compounds that change TEAD-dependent expression of the luciferase reporter. This approach led to the identification of compound **2**, an oxime derivative of 9H-fluoren-9-one bearing two piperidinyl-sulfonyl groups (C108, Figure 7). Although the direct involvement of YAP in the molecular mechanism of **2** has not been definitely assessed, the inventors claimed that this compound inhibits YAP-dependent cell proliferation by promoting YAP ubiquitination and its subsequent proteasome-mediated degradation. Cells treated with various doses of **2** in presence of MG132, a potent proteasome inhibitor, maintained the levels of endogenous YAP, proving that **2** reduces YAP protein amount by promoting its proteasome-dependent proteolysis. **2** also inhibits cell proliferation and retards the migration of multiple cancer cell lines in vitro, demonstrating its capability to inhibit YAP activity by decreasing the YAP protein levels in a cell-type-independent manner. Antitumor potential of **2** has also been evaluated in a xenograft mouse model, showing that **2** blocks melanoma and lung adenocarcinoma tumor growth and induces apoptosis in cancer cells.

Liu-Chittenden et al. set up a luciferase reporter assay to test the YAP-dependent transcriptional activity of a Gal4–TEAD4

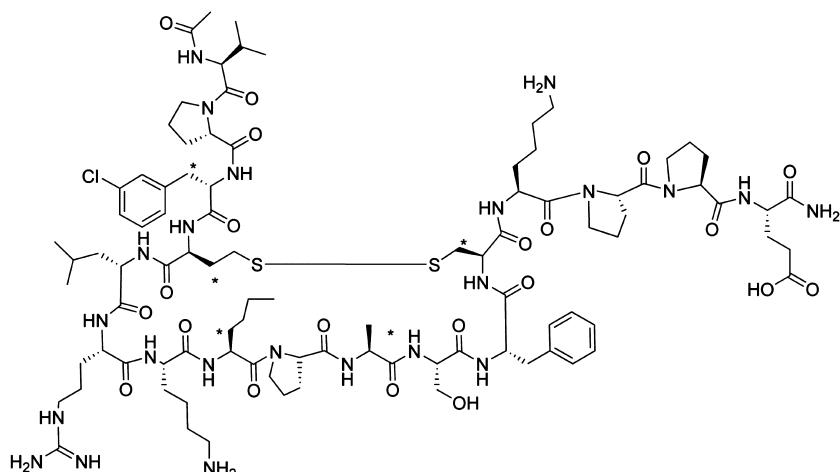


Figure 8. Chemical structure of **5** inhibiting the YAP–TEAD interaction. The peptide mimics the YAP Ω loop (residues 84–100); R87 and F96 were mutated to cysteine and homocysteine, respectively, to allow the formation of an internal disulfide bond; further modifications were performed to improve binding affinity to TEAD. α -carbons of mutated residues are marked by an asterisk.

assembly and screened the Johns Hopkins Drug Library to identify molecules that inhibit the YAP–TEAD interaction. Compounds **3** (verteporfin, VP, trade name Visudyne by Novartis) and **4** (protoporphyrin IX, PPIX) have been identified as top hits of the screening (Figure 7). **3** and **4** are compounds belonging to the porphyrin family, which are aromatic heterocyclic molecules composed of four modified pyrrole units, interconnected at their α -carbon atoms via methine bridges.²⁹ Co-immunoprecipitation assays revealed that both **4** and **3** inhibit the YAP–TEAD complex formation at 10 μM , whereas **3** showed >50% inhibition at 2.5 μM , showing a higher potency than **4**. **3** is used clinically as a photosensitizer in the photodynamic therapy of neovascular macular degeneration, where it is activated by laser light to generate reactive oxygen radicals that eliminate the abnormal blood vessels. As an inhibitor of the YAP–TEAD interactions, however, it does not require light activation. It was determined that **3** selectively binds YAP, thus altering YAP conformation and abrogating its interaction with TEAD in vitro. Moreover, it was also demonstrated that **3** inhibits the oncogenic activity of YAP in vivo: **3** suppresses the liver overgrowth resulting from either YAP overexpression or activation of endogenous YAP.²⁹ Recently, the effects of **3** without light activation have been evaluated on human retinoblastoma cell lines.⁸² This study shows that **3** determines inhibition of cell growth and viability and that it interferes with the YAP–TEAD proto-oncogene pathway in human retinoblastoma cells. These results are encouraging because **3** is a clinically applied drug with few side effects. Moreover, considering that in vivo results were obtained using an aqueous preparation in which **3** bioavailability is suboptimal, compared with the lipid-based formulation used in verteporfin, this strategy may represent an excellent therapeutic approach with minimal adverse effects.

Very recently, Zhang et al. made an important headway in the study of the YAP–TEAD complex, discovering **5** (Figure 8), a potent cyclic peptide, which inhibits the protein–protein complex by mimicking the Ω loop of YAP.⁸³ Truncation studies and an alanine scan performed on the TEAD-binding domain of YAP provided information about the optimal length of the synthetic peptide and advantageous mutations to obtain active parent peptides of YAP. Moreover, the authors optimized the

peptide by applying conformational constraints to the structure. As observed in the YAP–TEAD cocrystal structure, 3KYS, R87, and F96 of YAP are kept in close contact, within the Ω loop, by a cation– π interaction. The alanine scan study indicated that they are important for forming the complex, even if these residues are not involved in a direct interaction with TEAD surface. This suggested that R87 and F96 have a critical role in maintaining YAP in its active conformation. The authors thus applied a macrocyclization strategy, replacing R87 with homocysteine and F96 with cysteine and inducing the formation of a disulfide bond between the two new residues. The inhibitory activity of **5** was improved through several beneficial mutations: replacement of M86 with 3-Cl-phenylalanine, mutation of L91 to norleucine and of D93 to alanine resulted in a cyclic peptide with IC_{50} equal to 0.025 μM , nearly 1500-fold more potent than the parent YAP^{84–100} peptide fragment, which corresponds to the Ω loop (Figure 8). A computational model provided a rational explanation of the high potency of **5**, showing favorable van der Waals interactions between the 3-Cl-phenylalanine substituent and hydrophobic residues on TEAD surface. GST pull-down and functional assays revealed that **5** competes with the endogenous YAP protein, showing a high affinity for TEAD. Altogether, this study reveals not only a potent inhibitor of the YAP–TEAD interaction but also an exhaustive structure–activity relationship landscape for the YAP Ω loop. Additionally, an enhancement of the hydrophobic properties at M86, L91, and F95 of YAP represents a suitable strategy to improve the affinity of YAP-derived peptides for TEAD, and polar interactions between S94 of YAP and Y421 and E255 of TEAD are fundamental. This information can be advantageous for the design of small molecules inhibiting the YAP–TEAD complex.

■ CONCLUSIONS

Over the past few years, the Hippo pathway has emerged as a promising anticancer target, as revealed by several experimental lines of evidence, which indicate that targeting the Hippo pathway represents an effective strategy against oncogenic progression. It is becoming increasingly clear that the Hippo pathway can regulate SC proliferation and maintenance; therefore, its modulation may be therapeutically useful for tissue repair and regeneration following injury. Moreover, the complex

network of regulatory components within the Hippo pathway is being elucidated, and robust assays that can measure the activity of this pathway have been established. Altogether, these findings offer new possibilities for discovering useful pharmacological tools to further understand the precise role of the Hippo pathway components and simultaneously design novel small molecules that modulate the Hippo pathway. Although small molecules interfering with the Hippo pathway have been reported, the molecular information is scarce and incomplete. For example, sphingosine-1-phosphate (S1P) has been reported as an upstream potent activator of YAP, promoting YAP nuclear localization.⁵² As revealed by qRT-PCR mRNA expression profiling and siRNA transfection experiments, S1P receptor subtype has been identified as the responsible of YAP activity regulation by S1P. S1P2 is a GPCR coupled to G12/13 proteins, and interaction with S1P leads to the activation of Rho GTPases, which, in turn, promote YAP nuclear localization.⁵² A recently published patent reports the results of a HTS campaign that led to the discovery of inhibitors of the Hippo-YAP signaling pathway, including the fluorene derivative **2** in Figure 7, which should act as a G12/13 GPCR antagonist. Mechanistically, **2** promotes YAP degradation by increasing ubiquitylation. In addition, **2** inhibits cell proliferation in vitro and reduces growth of xenografted tumors in mice.⁸¹ Recently, compound **6** (C19, 4-(4,3,4-dichlorophenyl)-1,2,5-thiadiazol-3-yloxy)butanol, Figure 7) has been observed to inhibit the Hippo pathway by activating Mst/Lats kinase and promoting TAZ (but not YAP) phosphorylation and inactivation.⁸⁴ Furthermore, **6** markedly inhibited tumor growth in vivo. However, the molecular site of action for these compounds is not precisely characterized, which hampers structure-based discovery of new small molecules and their chemical optimization. Currently, YAP/TAZ could be considered a promising target for this aim, considering the availability of some structural information about their complex with TEAD. Although **5** has been recently discovered as being able to disrupt the YAP–TEAD complex formation, the only small molecule reported so far to directly inhibit the protein–protein interaction is **3**. Despite the interest regarding this compound, which is already being used as a drug, detailed structural information about its interaction with YAP or TEAD is still lacking, which signifies a pivotal role to (virtual) screening campaigns and/or fragment-based approaches for the discovery of new small-molecule inhibitors of YAP activation.

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Notes

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ABBREVIATIONS USED

A, alanine; AC, adenylyl cyclase; AJ, adherens junction; AMOT, angiotonin; aPKC, protein kinase C α ; BMP, bone morphogenic protein; CBC, crypt base columnar; CC, cholangiocarcinoma; CGNP, cerebellar granule neural precursors; CK1, casein kinase 1; Crb, Crumbs; CTF, C-terminal fragment; D, aspartic acid; Dlg, discs large; Ds, Dachsous; Dsh, Disheveled; ELM, eukaryotic linear motif; ESC, embryonic stem cell; Ex, expanded; F, phenylalanine; FDA, Food and Drug Administration; FGF, fibroblast growth factor; FRMD6, Ferm domain-containing protein 6; Ft, fat; GPCR, G-protein coupled receptor; GSK-3, glycogen synthase kinase-3; GST, glutathione S-transferase; H, histidine; HCC, hepatocellular carcinoma; Hpo, hippo; HTS, high-throughput screening; I, isoleucine; ICM, inner cell mass; IGF, insulin-like growth factor; IPSC, induced pluripotent stem cell; ISC, intestinal stem cell; K, lysine; L, leucine; Lats1/2, large tumor suppressor 1/2; Lgl, lethal giant larvae; LIF, leukemia inhibitory factor; LPA, lysophosphatidic acid; M, methionine; Merlin; Mer, Merlin; MOBKL1A/B, Mob1-like protein1 A/B; MSC, mesenchymal stem cell; Mst1/2, mammalian sterile 20-like 1/2; NF2, neurofibromin 2; NLS, nuclear localization signal; NMR, nuclear magnetic resonance; P, proline; Par3/6, partitioning-defective protein 3/6; PDB, Protein Data Bank; PKA, protein kinase A; PPAR γ , peroxisome proliferator-activated receptor- γ ; Q, glutamine; qRT-PCR, quantitative real-time reverse transcription PCR; R, arginine; RASSF, Ras association domain family member; RUNX, RUNT-related transcription factor; S, serine; S1P, sphingosine-1-phosphate; Sav, Salvador; SCF, Skp, Cullin, F-box containing complex; Scrib, scribble; Sd, Scalloped; Smad, Mothers Against Decapentaplegic, Drosophila, Homologue Of; SSC, skin stem cell; Std, Stardust; T, tyrosine; TAZ, transcriptional co-activator with PDZ-binding motif; TBX5, T-box 5; TDU, Tondu domain; TEAD, transcriptional enhancer associate domain transcription factors; TGF- β , transforming growth factor- β ; TJ, tight junctions; TR-FRET, time resolved fluorescence resonance energy transfer; TTF-1, thyroid transcription factor 1; V, valine; Vgll1/4, vestigial-like 1/4; W, tryptophan; Wnt, wingless-type MMTV integration site family member; Wts, warts; WWC1/2, WW, C2 and coiled-coil domain-containing 1/2; YAP, Yes associated protein; YBD, YAP-binding domain; Yki, Yorkie; ZO-1/2, zona occludens 1/2; β -TRCP, β -transducin repeat-containing protein

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