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REVIEW ARTICLE

YAPping about and not forgetting TAZ

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The Hippo pathway has emerged as a major eukaryotic signalling pathway and is increasingly the subject of intense interest, as are the key effectors of canonical Hippo signalling, YES-associated protein (YAP) and TAZ. The Hippo pathway has key roles in diverse biological processes, including network signalling regulation, development, organ growth, tissue repair and regeneration, cancer, stem cell regulation and mechanotransduction. YAP and TAZ are multidomain proteins and function as transcriptional coactivators of key genes to evoke their biological effects. YAP and TAZ interact with numerous partners and their activities are controlled by a complex set of processes. This review provides an overview of Hippo signalling and its role in growth. In particular, the functional domains of YAP and TAZ and the complex mechanisms that regulate their protein stability and activity are discussed. Notably, the similarities and key differences are highlighted between the two paralogues including which partner proteins interact with which functional domains to regulate their activity.

Keywords: cancer; cellular localization; functional regulation; gene transcription; Hippo signalling; phosphodegron; protein–protein interaction; stability; transcriptional coactivator with PDZ-binding motif; yes-associated protein

The Hippo pathway was initially discovered in *Drosophila* using elegant mosaic genetic screens to identify novel tumour suppressors. Subsequently, the mammalian counterparts were identified and Hippo signalling emerged as a crucial regulator of organ size that is evolutionarily conserved from flies to humans. Hippo signalling is now known to play a key role in cell proliferation and survival, stem cell regulation, tissue repair, regeneration and mechanotransduction, and is frequently dysregulated in human cancers (reviewed in ref. [1]). In addition, we now know that multiple signalling pathways and mechanotransduction cues converge on Hippo signalling to regulate its activity and function (reviewed in ref. [2]).

YES-associated protein (YAP) was identified in 1994 as a consequence of its interaction with the Src-homology 3 (SH3) domain of the YES tyrosine kinase [3]. The transcriptional coactivator with PDZ-binding

Abbreviations

αE, α epithelial; β-TrCP, beta-transducin repeat containing E3 ubiquitin protein ligase; AMOT, angiomotin; AMPK, AMP-activated kinase; AREG, amphiregulin; CK1, casein kinase 1; F-actin, filamentous actin; GSK3, glycogen synthase kinase-3; hnRNP, heterogeneous nuclear ribonuclear protein; LATS1 or 2, large tumour suppressor 1 or 2; MEF, mouse embryonic fibroblast; MOB1, Mps one binder kinase activator-like 1; MST1/2, mammalian STE20-like kinase 1 or 2; NDR, Nuclear Dbf2-related kinases; NFAT5, nuclear factor of activated T cells 5; NLK, Nemo-like kinase; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PP1A, protein phosphatase 1A; PP2A, protein phosphatase 2A; SH3, Src-homology 3; SOCS, suppressor of cytokine signalling; TAD, transcriptional activation domain; TAZ, transcriptional co-activator with PDZ binding motif; TEAD, TEA domain; VGLL4, vestigial like family member 4; YAP, YES-associated protein; ZO-2, zona occludens-2.

motif (TAZ, also known as WWTR1) was identified later as a coactivator of transcription with the ability to interact with PDZ domain proteins from which its name is derived [4]. It was not until the early 2000s however, with the emergence of the Hippo pathway in *Drosophila*, that it became apparent that YAP and TAZ were key components of mammalian Hippo signalling.

YES-associated protein and TAZ are transcriptional coactivators and function as the two principal effectors of mammalian Hippo signalling [5]. They possess similar structural features, bind to many of the same DNA-binding partners and are inactivated by active Hippo signalling [5]. Despite these similarities and evidence of functional redundancy, accumulating data suggest that YAP and TAZ have discrete functions mediated by different protein–protein interactions (Table 1). Moreover, they regulate overlapping and distinct sets of target genes [6] and have both shared and unique mechanisms of regulation. This review briefly overviews Hippo signalling and its role in growth. In addition, the functional domains of YAP and TAZ and the complex processes that regulate their protein stability and activity while highlighting similarities and key differences between them are reviewed.

Canonical Hippo signalling

Although the pathway was initially elucidated in *Drosophila*, for simplicity, we will only present the mammalian Hippo counterparts. The Hippo pathway (Fig. 1) functions to control YAP/TAZ activity. The canonical pathway comprises the mammalian STE20-like kinase 1 or 2 (MST1/2) and large tumour suppressor 1 or 2 (LATS1/2) serine/threonine kinases. These kinases interact with their respective adaptor proteins, namely, SAV1 (also called WW45) and Mps one binder kinase activator-like 1 (MOB1), to phosphorylate and consequently inactivate YAP and TAZ. YAP and TAZ bind to TEA domain (TEAD) proteins 1–4 (TEAD1–4) (Table 1) to drive the transcription of genes involved in cell proliferation and survival [7–16].

Upstream signals, initiated by cell–cell contact at high cell density, activate MST1/2-SAV1 complexes that consequently phosphorylate and activate LATS1/2-MOB1. In turn, LATS1/2-MOB1 phosphorylates YAP and TAZ on specific serine residues to generate one or more 14-3-3 consensus binding sites. Phosphorylated YAP and TAZ then bind to 14-3-3 proteins resulting in their cytoplasmic retention and supressed target gene transcription [4,17–20]. Additional serine phosphorylation by casein kinase 1 (CK1δ/ε) eventually leads to their degradation [21,22]. Conversely, at low cell density, MST–LATS kinase complexes are inactive, and unphosphorylated YAP and TAZ are free to enter the nucleus where they primarily bind to TEAD1–4 to activate target gene transcription [23–25].

Regulation of organ size

Early studies in *Drosophila* indicated that Hippo signalling has a key intrinsic role in restricting organ size. Inactivation of *hpo* (*MST1/2* orthologue), *sav* (*SAV1*) and *wts* (*LATS1/2*) genes or overexpression of *yki* (*YAP/TAZ*) in flies results in massive tissue overgrowth characterized by excessive cell proliferation and diminished apoptosis [8–13,16].

Fortuitously, many early studies of mammalian Hippo signalling were performed in the liver of genetically modified mice. Collectively, these studies confirmed that Hippo signalling is an evolutionarily conserved regulator of organ size [17,26–32] and also plays a major role in liver development, cell fate, progenitor homoeostasis, growth and tumorigenesis (reviewed in ref. [33]). Indeed, seminal papers from the Brummelkamp and Pan laboratories demonstrated that transgenic *YAP* expression in the livers of mice results in significant liver overgrowth that was evident within a few days, and 5–6 weeks of continual expression leads to a 4- to 5-fold enlargement of the liver [17,27]. It is worth noting that while Hippo/YAP signalling has a key role in regulating liver size in mice, its effect on organ size is not evident in all tissues. For example, *Mst/Mst2* deletion in newborn pups increases liver, stomach, heart and spleen size, but not the size of the lung, kidney, intestine, skeleton or limb [30]. It is also noteworthy that, at present, the effect of *TAZ* transgenic overexpression in mice has not yet been reported.

Consistent with these findings, it is not surprising that complete *Yap* deletion in mice is lethal, with severely runted embryos dying at embryonic day 8.5 [34]. In contrast, *Taz* null mice survive till adulthood with survivors exhibiting severe defects in the kidneys and lungs that are reminiscent of polycystic disease and emphysema respectively [35–37]. *Taz*-deficient mice were also slightly smaller and lighter compared to heterozygous and wild-type littermates, indicative of minor skeletal and muscle growth defects [35,38]. Not surprisingly, mice null for both *Yap* and *Taz* die very early, prior to implantation [39].

As a key regulator of growth and organ size, it is not surprising that numerous early studies identified Hippo signalling as a potent tumour suppressor

Table 1. YAP/TAZ partner proteins grouped by structural domain/motif. Lists the interacting protein and the main function associated with that interaction. p, phosphorylated; S, serine; Y, tyrosine; H, histidine; R, arginine; x, any amino acid.

Protein partner	Function	YAP/TAZ	Ref
Pro-rich domain			
hnRNP U	Attenuates coactivator function	YAP	[55]
TEAD-binding domain			
TEAD1–4	Facilitates target gene transcription by mediating DNA binding	YAP, TAZ	[23–25]
WW domain			
LATS-1/2	Phosphorylates HxRxxS sites in YAP/TAZ to promote cytoplasmic retention, inhibition of gene transcription and protein degradation	YAP, TAZ	[4,18–20,123]
p73	Enhances transcription of pro-apoptotic genes	YAP	[19,88,89,117]
RUNX1	Promotes osteocalcin transcription	YAP	[87]
RUNX2	Promotes osteocalcin transcription and represses PPARγ-dependent gene transcription	TAZ	[49,86]
	Src-pYAP^{Y433} [a] binds RUNX2 to suppress transcription	YAP	[115]
Smad1	Enhances pSmad1^{S206}-dependent transcription	YAP	[91,106]
Smad2/3–4	Required for nuclear accumulation of Smad2/3-4 complexes	TAZ	[92]
Smad7	Enhances Smad7-mediated inhibition of TGF-β signalling	YAP	[93]
PRGP2	May inactivate YAP by colocalizing to cell membrane	YAP	[180]
Pax3	Enhances Pax3 transcriptional activity	TAZ	[94]
ErbB4	Augments transcriptional activity of ErbB4 C-terminal fragment	Mainly YAP1-2 isoforms	[95]
ASPP2	Promotes PP1A-mediated dephosphorylation to increase protein stability and coactivator function	YAP, TAZ	[138,139]
AMOT, AMOT-L1, AMOT-L2	Sequestration of YAP/TAZ in cytoplasm and tight junctions to inhibit their coactivator function	YAP, TAZ	[147–149]
	AMOT-L1 opposes the function of ZO-2 to prevent YAP nuclear localization	YAP	[98]
AMOTp130	In serum starvation, forms trimeric complex with AIP4 and YAP to decrease YAP stability and activity	YAP	[150]
	Prevents LATS access to augment YAP nuclear localization and target gene transcription	YAP	[151]
Wbp2	Enhances TEAD-mediated gene transcription	YAP	[100,102]
β-catenin	Recruitment *via* GSK-3-pS-β-catenin[a] to AXIN1-destruction complex leads to YAP/TAZ cytoplasmic retention and inactivation	YAP, TAZ	[170,171]
	Recruits β-TrCP to the destruction complex to degrade β-catenin and TAZ	TAZ	[170,171]
Parafibromin (PF)	Unphosphorylated PF forms heterotrimeric complex with TAZ and β-catenin to costimulate their coactivator functions	TAZ	[104]
	pY-PF complex augments YAP coactivator function	YAP	[104]
RORα	Coactivates RORα transcription	YAP	[113]
SET1A	Methylates YAP^{K342} to block CRM1-mediated nuclear export	YAP	[142]
SH3-binding motif			
YES	Unknown	YAP	[3]
NCK, CRK	Unknown	YAP	[3]
SRC	Unknown	YAP	[3]
Coiled-coil domain			
YAP, TAZ	Mediates heterodimerization	YAP, TAZ	[63,110]
	Mediates homodimerization	TAZ	[63,110]
PDZ-binding motif			
ZO-1	Localizes at cell–cell junctions. Unlikely to facilitate YAP/TAZ nuclear import	YAP, TAZ	[120,121]
ZO-2	Facilitates YAP/TAZ nuclear import	YAP, TAZ	[120,121]
NHERF-2	Unknown	TAZ	[4]
HxRxxS sites			
14-3-3	LATS-pYAP$^{\mathbf{S127}}$ and pTAZ^{S89} [b] promote 14-3-3 binding, cytoplasmic retention and inactivation	YAP, TAZ	[4,18–20,123]
	In epithelial cells at high cell density, forms a tripartite complex with 14-3-3 and αE-catenin leading to its inactivation	YAP	[126,127]

Table 1. (Continued).

Protein partner	Function	YAP/TAZ	Ref
C-terminal phosphodegron			
β-TrCP	LATS and CK1δ/ε-pYAP$^{S381/S384}$ and pTAZ$^{S311/S314}$ [a] recruit βTrCP, leading to their degradation	YAP, TAZ	[21,22]
N-terminal phosphodegron			
β-TrCP	GSK-3-pTAZ$^{S58/S62}$ [a] recruits βTrCP to cause TAZ degradation	TAZ	[164]
pY^{316}			
NFAT5	Under conditions of hyperosmotic stress, pTAZ^{Y316} binds NFAT5 and suppresses its transcriptional activity	TAZ	[118]
Domain/motif not characterized			
Tbx5	Enhances Tbx5 transcriptional activity. Not mediated by TAZ WW domain	YAP, TAZ	[110]
	Tripartite complex with Tbx5 and β-catenin promotes β-catenin-mediated transcription	YAP	[181]
OGT	O-GlcNAcylation-YAP$^{S109/T241}$ decreases phosphorylation by LATS and increases YAP stability and activity	YAP	[173]
SOCS5/6	Promotes YAP degradation	YAP	[175]
TIAM1	In the cytosol, enhances recruitment to the β-catenin destruction complex. In the nucleus blocks association with TEADs to inhibit TEAD-mediated gene transcription	YAP, TAZ	[172]
	Enhances association with βTrCP in the β-catenin destruction complex causing their degradation	TAZ	[172]
CRM1	Mediates nuclear export	YAP	[142]

[a]Src-pYAP^{Y433} indicates Src kinase phosphorylates on YAP Y433. This format is used in this table. The kinase is listed before the phospho-substrate with residue, if known, in superscript. [b]Major LATS phosphosite for YAP and TAZ respectively.

pathway. Consistent with this, dysregulated expression of YAP and/or TAZ, or that of several upstream components of Hippo signalling, has been reported in over 20 different solid cancers, including hepatocellular carcinoma, medulloblastoma, glioma and ovarian, lung and colorectal cancers (reviewed in ref. [40]). Since this review focuses chiefly on YAP and TAZ structure and regulation, we will not summarize here the large volume of literature on this topic. For additional information, readers are directed to ref. [41–45].

YAP and TAZ – genes and mRNA

Despite the importance of the Hippo pathway in mammals and other eukaryotes, homologues of YAP/TAZ and TEAD factors have not yet been identified in yeast [46]. Transcriptional effectors of Hippo signalling seem to have emerged before the origin of metazoans since *Capsaspora owczarzaki*, a unicellular amoeboid, contains functional orthologues of YAP and TEAD-like factors [47]. Likewise, *Caenorhabditis elegans* has a YAP homologue known as YAP-1 [48]. The YAP and TAZ paralogues arose from a gene duplication event that occurred in vertebrates and their dynamic patterns of expression suggest that they have critical roles in organ development in frogs (*Xenopus tropicalis*) and zebrafish (*Danio rerio*) [49–52] through to mammals.

In humans, *YAP1* is located on chromosome 11 at position 11q22.1 while its paralogue *TAZ* is found on chromosome 3 at 3q25.1. *YAP1* comprises nine exons and through differential splicing, produces at least eight different isoforms of *YAP*1 that have been reported in a range of cell types and tissues [53]. As shown in Fig. 2, during YAP mRNA maturation, exons 4 and 6 are alternatively spliced, and in addition, exon 5 has an alternative 3′ donor splice site generating a 12 bp longer exon 5b. Combinations of these splicing events result in eight YAP isoforms. Interestingly, YAP is one of the more differentially spliced genes in canonical Hippo signalling [54]. Importantly, the functional effects of these splicing variations are partially characterized and will be discussed in greater detail below.

Human *TAZ* also comprises nine exons with 24 differentially spliced variants, eight of which are protein coding [54]. However, since several isoforms are non-full-length that lack either the N or C terminus, it remains to be established whether they are *bona fide* or experimental artefacts. At least three full-length *TAZ* transcripts are annotated by NCBI. All three transcripts comprise the same coding exons, exons 4–9, with the identical protein initiation and termination codons. Detailed analysis reveals that these transcripts differ in their 5′ untranslated regions. Variant 1

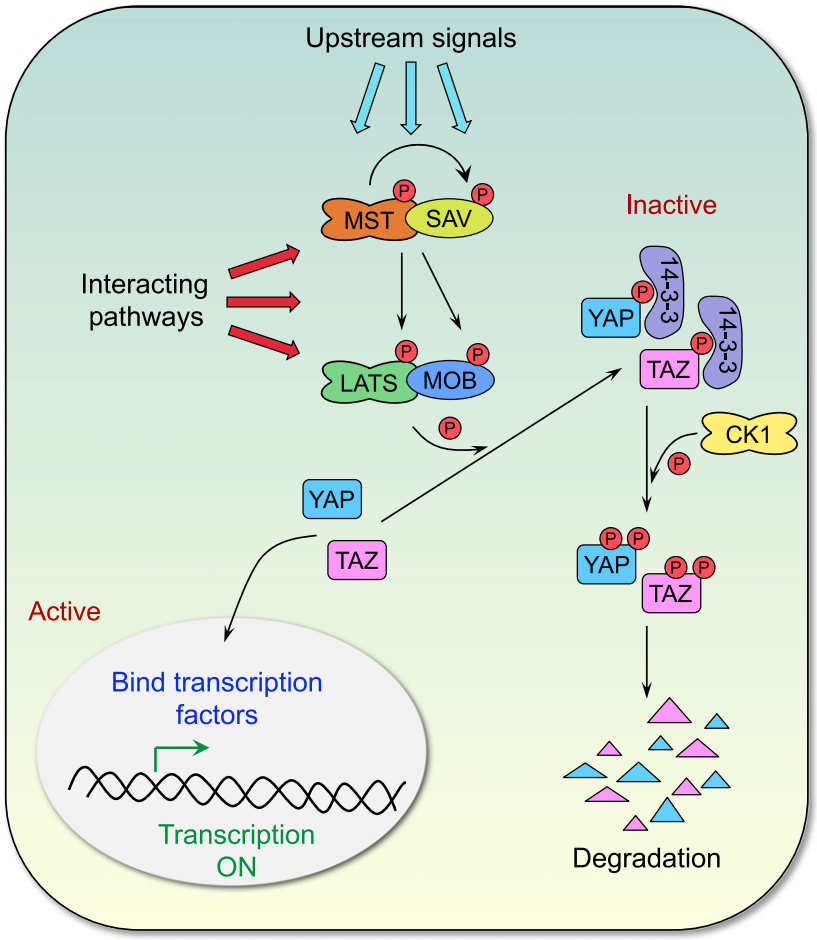

Fig. 1. Canonical Hippo signalling regulates YAP and TAZ. Cell–cell contact initiates upstream signals that lead to LATS1/2 kinase activation resulting in the phosphorylation of YAP and TAZ and their subsequent cytoplasmic retention bound to 14-3-3 proteins. Additional phosphorylation by CK1 kinase results in their degradation. Conversely, at low cell density, MST and LATS complexes are inactive, and unphosphorylated YAP and TAZ are free to enter the nucleus and bind to transcription factors, for example, TEAD factors, resulting in the transcription of genes involved in cell growth, survival, cell fate and homoeostasis. See text for additional detail.

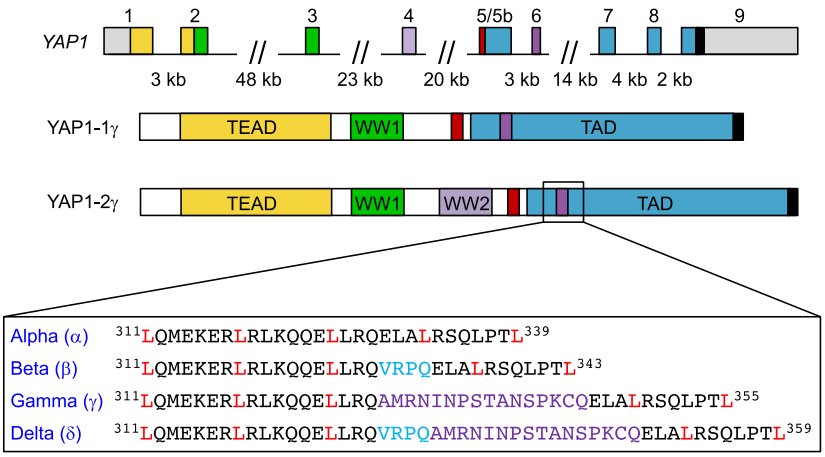

Fig. 2. Human YAP isoforms. Illustration of the *YAP1* gene with colours indicating which exon/s encode which functional domains. Note, introns and exons are not drawn to scale. Depicted are the YAP1-1γ and YAP1-2γ isoforms. The second WW domain encoded by exon 4 is alternatively spliced and occurs in *hYAP1-2* isoforms only. The red box represents the SH3-binding domain and the C-terminal black box represents the PDZ-binding motif. BOX: An alternative donor site in exon 5 (exon 5b) causes the insertion of VRPQ (light blue) residues in the transactivation domain (TAD) of β and δ isoforms. Exon 6 is differentially spliced and occurs in γ and δ isoforms and encodes 16 amino acids (purple) that insert into the same region of the TAD as the VRPQ residues. The critical leucine (L) residues that form the leucine zipper are shown in red. Numbers refer to the position of the indicated residues for YAP1-2 α, β, γ and δ isoforms.

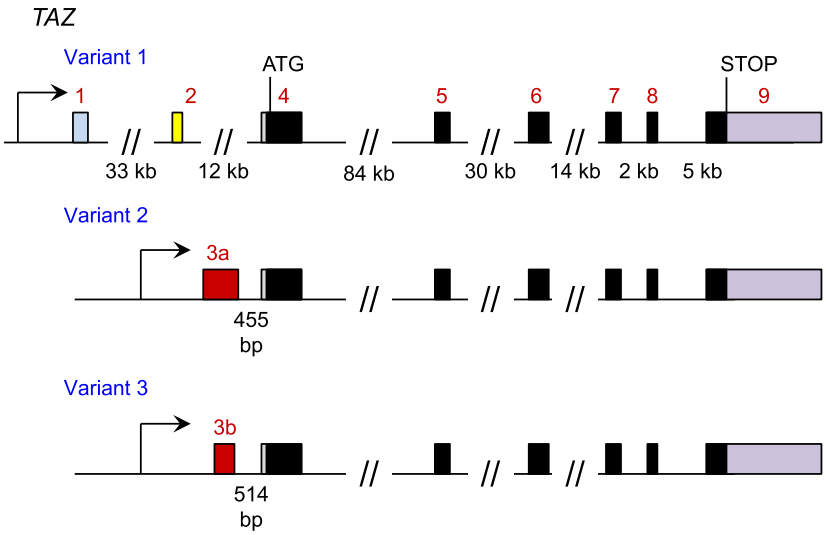

Fig. 3. Human TAZ transcript variants. Depiction of three full-length *TAZ* transcript variants showing their exonic structures. Exons 4–9 (black boxes) comprise all *TAZ* coding sequences. Note, introns and exons are not drawn to scale. Exon 1 (light blue) is approximately 45 kb 5′ to exon 3a/3b (red).

contains exons 1 and 2, while variants 2 and 3 instead contain either exon 3a or 3b, which have different transcription start sites and an alternative 3′ donor site (Fig. 3). Furthermore, exon 1 is approximately 45 kb 5′ to exon 3a/b. Thus, variants 2 and 3 are likely to be transcripts from a different promoter to that which transcribes variant 1.

YAP and TAZ – protein structure

YAP and TAZ are highly similar proteins and share many functional domains including WW, TEAD-binding, PDZ-binding and transcriptional activation (TAD) domains. Comparison of the most similar variants of human YAP and TAZ reveals 41% amino acid sequence identity and overall similarity of 53%. As noted, although several features are shared between YAP and TAZ, distinctions between the two are evident. However, these differing features of YAP and TAZ have not been well explored.

Pro-rich domain

A notable difference between YAP and TAZ is at their amino termini. The N terminus of YAP contains a proline-rich region (18 proline residues of 50) that is not present in TAZ. Heterogeneous nuclear ribonuclear protein U (hnRNP U), an RNA- and DNA-binding protein that regulates gene expression, specifically interacts with YAP's proline-rich region within the nucleus, and this association attenuates YAP's transcriptional activity [55]. Since TAZ lacks a proline-rich N terminus, the YAP–hnRNP U interaction suggests that it may uniquely regulate the nuclear function of YAP and target gene regulation.

TEAD-binding domain

YAP and TAZ do not harbour an intrinsic DNA-binding domain and therefore rely on their association with DNA-binding proteins to coactivate target gene transcription. In mammalian cells, YAP and TAZ primarily bind to TEAD transcription factors 1–4 [56] to drive gene transcription of several growth-promoting factors. Prominently, among these are the CCN family secretory proteins, CTGF and CYR61 [25] and also proteins such as AXL receptor tyrosine kinase [57], c-MYC and SURVIVIN [17].

YAP and TAZ interact with all four TEAD proteins *via* a novel N-terminal TEAD-binding domain [23,58]. The minimal TEAD-binding region is a stretch of 50 residues in YAP that encompasses several sequential structural elements: a β-strand (residues 50–60), an α-helix (residues 61–73), a linker region (residues 74–84) and upon binding, an omega (Ω)-loop structure (residues 85–99) [59]. Early work on YAP–TEAD structural complexes revealed extensive contacts between human YAP and TEAD1, with YAP residues 86–100 identified as most crucial for binding [60,61]. All YAP isoforms contain the PxxØP motif (where x = any residue and Ø = any hydrophobic residue) within the TEAD-binding domain (residues 81–85 in hYAP). The analysis of mouse Yap–Tead4 complexes revealed that the PxxØP motif forms part of the Ω-loop between two short helices and actually makes very few contacts with mTead4 [60]. Interestingly, TEAD1 interacts with an isolated YAP fragment (residues 86–100) lacking the PxxØP motif with reduced affinity, suggesting that the PxxØP motif is not essential for, but rather enhances TEAD interaction [61]. Similarly, mutating the PxxØP motif in hYAP abolishes TEAD4

interaction and also abrogates the ability of hYAP to transform MCF10A mammary cells [60] indicating that it has a critical role in TEAD4 binding and cellular transformation. These findings indicate that the PxxØP motif is important in TEAD binding and the discrepancies reported may be due to the full-length YAP protein being used in one study and an isolated YAP fragment in the other. Notably, the structures of YAP bound to TEAD-2 or -3 have not yet been solved. Once they have, it will be interesting to compare the role of hYAP's PxxØP motif in the interaction with TEAD-2 and -3.

Although YAP and TAZ bind TEAD proteins with essentially the same affinity, the mechanism by which they interact differs [59]. Available structures reveal that YAP interacts with TEAD through the α-helix and Ω-loop [60,61], and modelling suggests that individually these domains weakly interact with TEAD when not linked to each other. However, once they are connected by the linker, their overall affinity is greatly enhanced [59]. In contrast, the TAZ linker is shorter and does not have a PxxØP motif [62]. Consequently, there is no bend in the TAZ linker and this reduces the number of contacts with TEAD [59]. However, the isolated TAZ Ω-loop interacts with TEAD with an affinity almost fivefold higher than YAPs [59]. Recently, the crystal structure of mouse Taz–Tead4 complex was solved revealing two distinct binding modes [63]. The first shows one Taz molecule binding one Tead4 molecule to form a heterodimer that is similar to the observed Yap–Tead4 complex [60,61]. In the second, two Taz molecules straddle two Tead molecules in a heterotetramer conformation [63]. As noted, earlier TAZ can homodimerise *via* its coiled-coil domain and this ability may explain why Taz-Tead forms heterotetramers and Yap-Tead does not.

Like YAP and TAZ, many cancers also overexpress TEADs [64–71]. The importance of TEADs' interaction with YAP and/or TAZ in cancer is highlighted in the following examples. First, TEAD2-Δ1-111, which lacks its DNA-binding domain, suppresses YAP-induced proliferation [72]. Notably, overexpression of this TEAD2 mutant suppresses liver tumorigenesis and maintains normal liver growth [72]. Similar phenotypes are observed in HGC-27 gastric cancer cells transfected with TEAD4-DNA-binding mutants [73] and in *Drosophila* expressing a mutant Scalloped (TEAD orthologue) that also lacks its DNA-binding domain [74]. Furthermore, the TEAD1-Y421H mutation that impedes TEAD1–YAP interaction, severely retards tumour growth of BEL-7404 HCC cells in a mouse xenograft model [75]. Lastly, a similar dominant-negative mechanism was reported for a subset of lung and colon cancer patients with increased survival rates, due to elevated expression of a differentially spliced TEAD4-S isoform that lacks the DNA-binding domain, but retains its YAP-binding domain [76]. These observations are noteworthy in the context of YAP/TAZ function in cancer. Firstly, the cellular abundance of TEAD proteins is critical to mediate YAP/TAZ tumorigenesis. Secondly, tumorigenesis due to YAP upregulation can be inhibited by decreasing cellular TEAD levels using dominant-negative mutant proteins. Finally, targeting TEADs to prevent interaction with YAP and/or TAZ is a promising therapeutic strategy for YAP/TAZ-dependent tumours.

In support of the importance of YAP's interaction with TEADs, a key serine residue (S94) within YAP's TEAD-binding domain is critical for YAP interaction with TEADs. Notably, mutation of this residue to alanine diminishes YAP's interaction with TEADs and reduces YAP-induced gene transcription and cellular transformation [25]. Likewise, the analogous mutation in TAZ (S51A) abolishes its interaction with TEADs and reduces TAZ-induced gene transcription, cell growth, migration and epithelial to mesenchymal-like transition [24]. Furthermore, S97A mutation of *Drosophila* Yki diminishes its interaction with Scalloped and reduces growth *in vivo* compared to wild-type Yki [25].

Additionally, the transcriptional cofactor, Vestigial Like Family Member 4 (VGLL4), is a tumour suppressor and its low expression in a range of cancer cell types correlates with poor survival [77]. VGLL4 binds to TEAD proteins to competitively inhibit YAP–TEAD interaction to repress YAP target gene transcription [78,79]. VGLL4 inhibits lung cancer and breast cancer growth *in vivo* by preventing YAP–TEAD4 and YAP–TEAD1 interaction, respectively, to reduce cell viability, target gene transcription, cell proliferation and anchorage-independent colony growth [78,79]. The binding sites for VGLL4 and YAP on TEAD4 are overlapping and a peptide mimic designed to specifically antagonize YAP–TEAD interaction heralds great promise as an anticancer therapeutic [80]. The peptide mimic reduces the viability and proliferation of gastric cancer cells and reduces tumour growth and YAP–TEAD target gene transcription in gastric tumour mouse xenograft models [80]. It remains to be seen whether VGLL4 also represses TAZ–TEAD interaction and function.

WW domain/s

The cloning of chicken, mouse and human YAP led to the disclosure of a novel protein interaction

domain, the WW domain, so named because it contains two characteristic tryptophan (W) residues [81–83]. Its function was delineated following the identification of two putative WW domain ligands with homologous proline-rich domains referred to as the PY motif. The consensus PPxY-binding sequence was subsequently identified to contain two prolines (P) and a tyrosine (Y), where x represents any amino acid [84].

Elucidation of the WW domain was crucial to our understanding of the mechanism underlying numerous protein–protein interactions within the Hippo signalling network, since this pathway is rich in proteins containing WW domains and their PPxY ligands. The association of the Hippo regulator KIBRA with LATS [85] is mediated by WW domain–PPxY interactions, as is the interaction between LATS kinases and YAP [18,19]. Furthermore, several DNA-binding proteins also interact with YAP and/or TAZ *via* WW domain–PPxY interactions. These factors include RUNX1 (PEBP2α) and RUNX2 [49,86,87], p73 [19,88–90], Smad1 [91], heteromeric Smad 2/3–4 complexes [92], Smad7 [93], Pax3 [94] and ErbB4 [95].

YAP isoforms containing either one (YAP1-1) or two (YAP1-2) WW domains have been identified in humans. *YAP* exon 4 encodes the second WW (WW2) domain and it is differentially spliced during mRNA processing (Fig. 2). In contrast, only TAZ isoforms containing a single WW domain have been reported in mammals; however, a TAZ isoform with tandem WW domains occurs in fish [96], suggesting that additional isoforms may exist in mammals. The existence of tandem WW domain isoforms is significant since YAP/TAZ isoforms with two WW domains can bind to multi-PPxY partner proteins with approximately six-fold higher affinity [96]. Tandem WW domain–PPxY interactions maybe much more complex than suggested by Schuchardt *et al.* since tandem WW domains of YAP1-2 may negatively cooperate when binding to their cognate ligands [97]. However, it is likely that YAP isoforms harbouring two WW domains will bind interactors more tightly than isoforms with only one. This is supported by observations that ErbB4 interacts more strongly with YAP1-2 than with YAP1-1 [95], and that p73 and AMOT-L1 interact with YAP1-2 but not YAP1-1 [19,98]. Interestingly, YAP1-2 is a stronger co-activator than YAP1-1 [95,99]. The YAP WW domains can recruit both transcriptional repressors and enhancers, depending on the cell type and interaction with different DNA-binding proteins [100–103]. It is therefore conceivable that YAP1-2 isoforms may bind enhancers more tightly than YAP1-1 isoforms to account for their differing activities.

A recent novel finding further expands the role of the WW domain and underscores its importance in regulating YAP/TAZ function. YAP and TAZ bind to the nuclear protein, parafibromin *via* their WW domains, leading to their functional activation [104]. Interestingly, dephosphorylated parafibromin preferentially binds TAZ, while the tyrosine phosphorylated form preferentially binds YAP. Moreover, TAZ forms a heterotrimeric complex with β-catenin and parafibromin thereby co-stimulating both TAZ and β-catenin transcriptional activities [104].

As discussed, YAP and TAZ can partner with multiple proteins *via* the TEAD and WW domains. However, these associations do not necessarily occur in isolation and both are required for correct temporal and spatial regulation of YAP and TAZ activity. Indeed, YAP's WW domains work independently of, and in combination with, the TEAD-binding domain to regulate target gene transcription, with mutation of the WW domains reducing YAP's ability to stimulate cell proliferation and transform cells [103]. Furthermore, a tripartite complex of YAP/TEAD4/RUNX3 was identified with RUNX3 simultaneously interacting with both the YAP WW domain and TEAD4 (residues 101–125) to reduce YAP/TEAD4 transcriptional activity and colony formation in soft agar [105]. Notably, a RUNX3 mutant that occurs in gastric cancer neither binds TEAD4 nor suppresses YAP/TEAD4 activity, suggesting RUNX3 recruitment to the YAP–TEAD4 complex is vital to control YAP/TEAD4 activity and prevent tumour formation [105].

Worth mentioning is that in addition to PPxY-mediated interactions, YAP's WW domains can also bind phosphorylated serine residues [91,106]. YAP's WW1 domain binds Smad1 on S206 that has been phosphorylated by CDK8/9 cyclin-dependent kinases [91], which is required for BMP-mediated suppression of neural differentiation of mouse embryonic stem cells [106]. Subsequent phosphorylation of Smad1 by glycogen synthase kinase-3 (GSK3) inhibits YAP binding and recruits the ubiquitin ligase, Smurf1, *via* its own WW domains [91].

These findings highlight the importance and versatility of tandem WW domain proteins and expand our understanding of how WW domains mediate protein interactions. Moreover, they unveil a broader range of potential binding partners for WW domain-containing proteins, especially those with tandem WW domains, since they permit interactions not possible in proteins with only one domain. The versatility of WW domains to bind phosphoserine increases the possibility of novel YAP–TAZ interactions that remain to be identified.

SH3-binding domain

YAP was first identified bound to the YES tyrosine kinase *via* the YAP SH3-binding domain [3]. YAP also binds other SH3-containing proteins including the adaptor proteins, Nck and Crk, and Src tyrosine kinases [3]. Despite being the first *bona fide* characterized associations for YAP, scant information exists regarding the biological relevance of these interactions. Moreover, TAZ does not have an SH3-binding domain; therefore, it is hypothesized that an SH3-binding domain is not critical for YAP function in Hippo signalling.

Transcriptional activation domain

The C terminus of YAP is rich in serine, threonine and acidic amino acids and contains a strong TAD reminiscent of the herpesvirus transcription factor VP16 [87], and indeed is as potent as the VP16 TAD [99]. The YAP/TAZ TAD contains a coiled-coil motif characterized by a pattern of hydrophilic and hydrophobic residues that repeats every seven residues [107]. Specifically, YAP harbours a leucine zipper coiled-coil motif that is defined by five highly conserved leucine residues [108]. Although TAZ lacks the specific leucine zipper, it is still predicted to form a coiled-coil motif within its TAD [109]. This implies that the leucine zipper is not essential for transcription though it may enhance YAP activity by recruiting other leucine zipper-containing transcription factors. Interestingly, YAP and TAZ can heterodimerise, and TAZ can also homodimerise, *via* their coiled-coil domains [63,110] and, as described below, this accounts for the increased transcriptional potency of TAZ compared to YAP [63].

In addition to the alternative splicing of exon 4 that produces YAP1-1 and YAP1-2 isoforms, differential splicing also occurs between exons 5 and 9 that encode the TAD (Fig. 2) [53]. *YAP* exon 5 has an alternative splice donor site that generates an extended transcript (exon 5b) encoding four additional amino acids (VRPQ), while exon 6, which is alternatively spliced, encodes an extra 16 residues (AMRNINPSTANSPKCQ). Exons 5b and 6 are present in half of the *YAP* isoforms and the additional residues insert separately or together to disrupt the leucine zipper and generate the α, β, γ and δ isoforms [53]. Thus, by combining the differential splicing of exons 4, 5 and 6, eight distinct human YAP isoforms can be produced (Fig. 2). Notably, to date, only the equivalent α and γ YAP isoforms have been reported in the mouse (UniProtKB: P46938-1). Several Yap-ΔC isoforms that truncate before the TAD have also been reported in rat neuronal cells undergoing cell death induced by RNA-Pol II inhibition [111]. Although full-length Yap is also decreased in this model, the Yap-ΔC isoforms repress p73-mediated transcription in response to cisplatin treatment and slow apoptosis. Furthermore, when expressed as a transgene in the *Drosophila* eye, hYAP-ΔC preserved eye structure in models of neurodegeneration [111] and suggest that YAP-mediated transcription is necessary to cause apoptosis in these models. Yap-ΔC isoforms are also reported in a mouse model of amyotrophic lateral sclerosis and their expression decreases with disease progression and correlates with p73 activation and apoptosis [112]. Furthermore, transgenic expression of Yap-ΔC isoforms in a mouse transgenic model of spinocerebellar ataxia type 1 restored Yap/Yap-ΔC in RORα transcriptional complexes to normalize target gene transcription *in vivo* [113]. Collectively, these findings suggest that Yap-ΔC isoforms may be important in preventing neurodegenerative disease. It will be interesting to see whether future analyses of human tissue samples confirm the role of YAP-ΔC isoforms in these conditions. Interestingly, *TAZ* does not appear to undergo alternative splicing within exons 6–9 that encode the C terminus [53].

The full consequence of differential splicing of the YAP TAD is not yet fully understood. The additional residues found in the β, γ and δ isoforms interrupt the YAP leucine zipper and Gaffney *et al.* [53] predicted that this would alter transcriptional activity. Since the leucine zipper may not be essential for transactivation, the expression of YAP isoforms with variable TAD regions may subtly influence YAP's transcriptional activity to fine-tune target gene expression. In support of this, the silkworm (*Bombyx mori*) has four *Yki* (*YAP*) isoforms [114]. Alternative splicing of exon 3 results in one or two WW domains being expressed, while an alternative donor site in exon 5 leads to an insertion of 15 extra residues in the TAD in half the isoforms. Notably, the four different isoforms regulate several target genes to different extents [114]. Thus, the combination of one or two WW domains, with or without a TAD insertion, differentially affects transcriptional activity and is target gene dependent. Examination of several YAP C-terminal isoforms reveals that the β, γ and δ isoforms all retain transcriptional activity, though they were weaker compared to the α isoform [99]. Interestingly, the VRPQ residues in the β isoform reduce transcriptional activity in the context of the full-length protein, for example, hYAP1-1β versus hYAP1-1α, more so than when the isolated TADs were directly compared [99]. This

suggests possible interactions that require intact WW and leucine zipper domains are abrogated when the leucine zipper is disrupted. It remains to be determined whether similar results will be observed when the transcriptional activities of the remaining full-length YAP isoforms are examined.

In addition to disruption of the leucine zipper, the phosphorylation of tyrosine residue/s within the TAD also alters YAP and TAZ activity and/or protein binding. In osteoblasts, Yap tyrosine phosphorylation by Src/Yes kinase promotes its association with Runx2 leading to Runx2 suppression [115]. Conversely, inhibition of Src/Yes kinase decreases Yap tyrosine phosphorylation, and the dissociation of endogenous Yap–Runx2 complexes promotes Runx2-mediated transcription of *Osteocalcin* [115].

Similarly, c-ABL-mediated tyrosine phosphorylation within the YAP TAD alters its association with interacting proteins [116,117]. Under normal conditions, RUNX associates with YAP and this complex drives transcription of the E3-ubiquitin ligase, *ITCH* that degrades p73 [117]. In response to DNA damage, c-ABL phosphorylates YAP, which subsequently dissociates from RUNX thereby reducing *ITCH* transcription and causing p73 levels to rise [117]. Interestingly, tyrosine-phosphorylated YAP is more stable and associates with p73 to enhance transcription of pro-apoptotic target genes, for example, *BAX* [116]. Thus, DNA damage-induced phosphorylation of YAP by c-ABL influences YAP interactions and target gene transcription.

Finally, TAZ is phosphorylated in response to hyperosmotic stress, which is enhanced by activated c-ABL [118]. Phosphorylated TAZ associates with nuclear factor of activated T cells 5 (NFAT5), a major osmoregulatory transcription factor, and suppresses NFAT5 DNA binding and transcriptional activity to modulate the cellular response to hyperosmotic stress [118]. As these examples illustrate, the phosphorylation of residues within their TADs permits additional protein interactions to regulate and/or fine tune YAP/TAZ-mediated transcription.

PDZ-binding motif

YAP and TAZ must localize to the nucleus to exert their transcriptional effect, but do not harbour traditional nuclear localization signals. Instead, the PDZ-binding motif mediates their nuclear localization [4,119]. Specifically, the YAP PDZ-binding motif interacts with the first PDZ domain of the tight junction protein, zona occludens-2 (ZO-2), to mediate nuclear import [120]. This mechanism of interaction and nuclear localization was subsequently confirmed for TAZ [121]. Both exogenous TAZ and YAP can bind ZO-1 and ZO-2 with similar binding affinities; however, immunostaining of MDCK1 cells revealed that endogenous ZO-1 primarily localizes at cell–cell junctions while YAP and ZO-2 have similar cytoplasmic and nuclear staining [120,121]. This indicates that ZO-2 is most likely responsible for YAP nuclear localization. Interestingly, TAZ association with ZO-2 inhibits its transcriptional activity [121]. Although no mechanism was ascribed to account for this, it might have been a consequence of protein overexpression. The PDZ-binding motif is important for YAP's function. This is evident in the YAP 5SA gain-of-function mutant, which is not under control of canonical Hippo signalling [20], where the deletion of the PDZ-binding motif suppresses nuclear localization, reduces target gene transcription and impairs YAP-induced cellular transformation [122].

YAP and TAZ – protein regulation

Subcellular localization

Hippo signalling primarily functions to regulate the activity of YAP and TAZ by controlling their cytosolic/nuclear localization and *via* regulation of their protein stability (Fig. 1). Multiple LATS1/2 kinase consensus HxRxxS motifs within YAP and TAZ are critical for this regulation [4,18–20,123]. TAZ contains four of these motifs: S66, S89, S117 and S311, though S89 is recognized as the main phosphorylation site [4,123]. Similarly, YAP contains five LATS consensus sites: S61, S109, S127, S164 and S381 (in YAP1-2α) with S127 as the primary phosphosite [18–20]. Activation of LATS1/2 at high cell density leads to phosphorylation of these sites that results in their cytoplasmic retention, bound to 14-3-3 proteins, and inhibition of YAP/TAZ-mediated transcription [4,18,20,123]. The mutation of S89 or S127 to alanine creates a gain of function in TAZ and YAP, respectively, rendering both proteins resistant to inhibition by LATS1/2 and promotes their nuclear localization, target gene transcription and cell proliferation [18,20,123].

Interestingly, in addition to regulating YAP/p73 activity (reviewed earlier), ITCH ubiquitin ligase also degrades LATS1 [124]. The WW domains of ITCH and YAP compete with each other to bind LATS1, and ITCH–LATS1 interaction decreases YAP-S127 phosphorylation and promotes YAP nuclear localization to enhance cell proliferation, survival and tumorigenicity [124]. ITCH is frequently upregulated in metastatic breast cancer and is associated with lower

survival rates, and in MCF10A breast cancer cells, ITCH tumorigenicity is largely mediated by its effects on LATS and YAP [125].

Consistent with these observations, YAP2 overexpression in HEK293 cells cultured in low serum induces p73-mediated apoptosis, and under these conditions, YAP2-S127 phosphorylation by LATS reduces cell death, presumably by reducing p73-mediated transcription of pro-apoptotic genes [19]. Furthermore, in response to DNA damage, YAP binds p73 to enhance pro-apoptotic gene transcription and cell death [88,89]. Under these conditions, AKT (also known as protein kinase B, PKB) attenuates DNA damage-induced cell death by phosphorylating YAP-S127, which promotes 14-3-3 binding and cytoplasmic retention, thus reducing p73-mediated pro-apoptotic gene transcription and cell death [88]. Thus, phosphorylation of YAP-S127 is dependent on the cellular context and has a critical role in regulating YAP transcription of growth and/or survival genes.

In addition to regulation by LATS kinases, YAP binds to αE (α epithelial)-catenin, an adherence junction protein that mediates contact inhibition of epithelial cell proliferation. Association with αE-catenin results in YAP cytoplasmic sequestration, and in αE-catenin-null cells, YAP is constitutively nuclear (Fig. 4) [126]. Importantly, at high, but not low, cell density, YAP forms a tripartite complex with αE-catenin and 14-3-3 that requires YAP's WW domains (to possibly recruit protein kinases, e.g. LATS), to phosphorylate YAP-S127 [127]. *In vitro* binding results suggest that YAP and αE-catenin do not directly interact, rather 14-3-3 proteins 'bridge' their association and notably, YAP and 14-3-3 interaction is significantly weakened in the absence αE-catenin [127]. These findings suggest that αE-catenin mediates "crowd control" molecular circuitry in high-density cell–cell contact, at least in part by controlling YAP localization. In the epidermis, at high cell densities with increased numbers of adherens junctions, YAP is inactivated. Conversely, low cell density, as in a growing embryo or after wounding, promotes nuclear YAP and proliferation. Thus, when this molecular circuit is defective, such as αE-catenin deletion, 14-3-3 inactivation or YAP activation, hyperproliferation and tumours can arise. Although

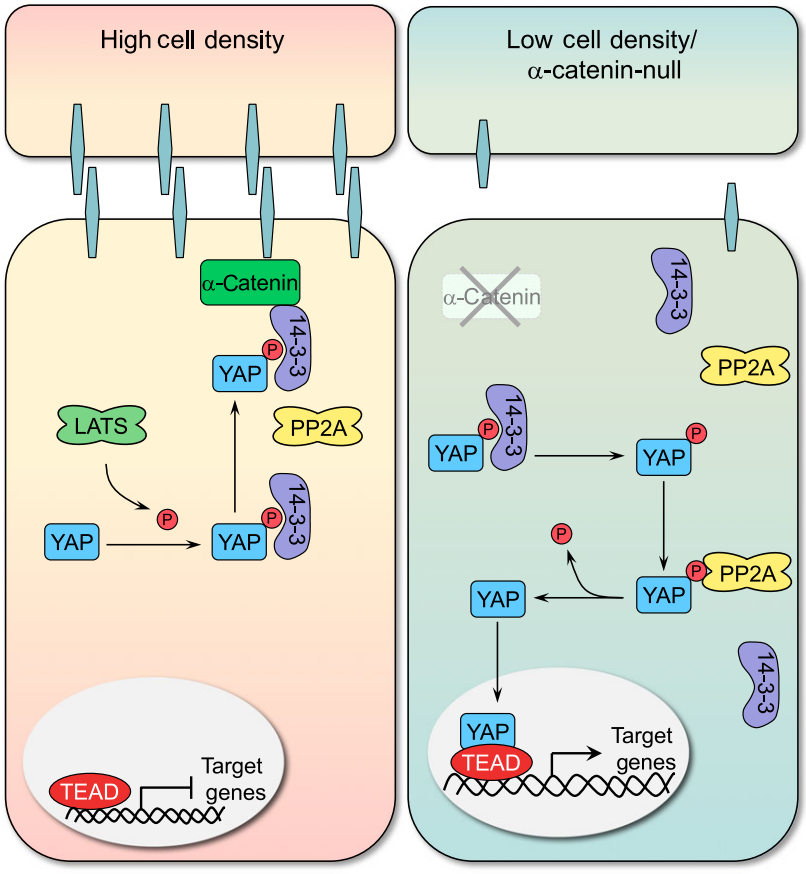

Fig. 4. Cell density, αE-catenin and phosphatases coordinate YAP/TAZ activity. At high cell density, active LATS kinase phosphorylates YAP resulting in cytoplasmic retention bound to 14-3-3 which bridges its association with αE-catenin in adherens junctions. The tripartite complex prevents YAP from being dephosphorylated by PP2A phosphatase. Conversely, at low cell density, the tripartite complex dissociates, permitting access and dephosphorylation of YAP by PP2A. Additionally, LATS kinase is inactive and no longer phosphorylates YAP. Together these effects result in YAP nuclear localization and activation of target genes. Notably, in *αE-catenin*-null cells, YAP is constitutively nuclear. Although not depicted here, TAZ is likely to be similarly regulated. See text for more detail. Model adapted from [127].

a similar role for αE-catenin in TAZ regulation has not yet been directly demonstrated, it is implicated since Taz and αE-catenin form a complex in mouse tissue [128], and the deletion of both *Yap* and *Taz* rescues the tooth defect seen in mice lacking *αE-catenin* [129].

Regulation by kinases

In addition to being phosphorylated by LATS kinases, YAP and TAZ are also phosphorylated and regulated by several noncanonical protein kinases that warrant mentioning. As mentioned previously and detailed below, phosphorylation by CK1δ/ε and GSK-3 kinases decreases the stability of YAP and TAZ. Also, Src and c-ABL tyrosine kinases phosphorylate YAP and TAZ on tyrosine residue/s within their TAD to regulate the Runx2, RUNX and NFAT5 transcriptional activities [115–118].

Nuclear Dbf2-related kinases, NDR1 and NDR2, are structurally related homologues of LATS1/2 and phosphorylate mouse Yap on S112, the equivalent of S127 in human YAP [130]. The ablation of NDR1/2 in mouse intestinal epithelium decreases S112 phosphorylation and increases total Yap abundance, rendering mice exquisitely sensitive to chemical-induced colon cancer [130]. The overexpression of or activation by osmotic stress of mammalian Nemo-like kinase (NLK) phosphorylates YAP on S128 [131,132]. This residue is juxtaposed to the major LATS kinase site, and phosphorylation of S128 by NLK reduces the phosphorylation at S127 and blocks the association of YAP with 14-3-3 proteins to enhance YAP nuclear localization and target gene transcription [131,132]. Interestingly, osmotic stress *via* NLK stimulates YAP nuclear localization even if it is phosphorylated on S127 [131]. These findings highlight that the activities of LATS and NLK together regulate YAP nuclear localization. The phosphorylation of TAZ by NLK has not yet been demonstrated.

Energy stress also regulates YAP activity *via* activation of the energy sensor, AMP-activated kinase (AMPK). Inhibitors of glucose and ATP production result in activation of AMPK and phosphorylation of YAP-S127 and TAZ-S89, YAP cytoplasmic retention and reduction in target gene transcription and reduce the oncogenic activity of YAP *via* S127 phosphorylation [133]. This study concluded that energy stress activates AMPK to phosphorylate AMOT proteins (see below), especially AMOTL1, to increase their stability and facilitate YAP-S127 phosphorylation by LATS kinases. In addition, AMPK was subsequently shown to also phosphorylate YAP mainly on S61 and S94 to inactivate YAP [134,135]. As indicated earlier, S61 is a LATS consensus site and S94 is necessary for interaction with TEADs; thus, the phosphorylation of either or both sites contributes to the inactivation of YAP. Similarly, the activation of AMPK in mouse fibroblasts and human retinal epithelial cells increases Taz/TAZ abundance and promotes their cytoplasmic retention; however, the direct phosphorylation of TAZ by AMPK was not demonstrated [136]. Collectively, these findings highlight a crucial link between energy metabolism *via* AMPK and YAP/TAZ activity.

Regulation by phosphatases

YAP and TAZ phosphorylation is reversible; protein phosphatases can dephosphorylate YAP/TAZ. *In vitro,* protein phosphatase 1A (PP1A) decreases YAP-S127 phosphorylation and 14-3-3 interaction to concomitantly increase YAP nuclear localization and transcriptional activity while the treatment of cells with okadaic acid, a PP1A inhibitor, reverses these effects [137]. Similarly, PP1A dephosphorylates TAZ at S89 and S311, promotes TAZ nuclear translocation and increases TAZ target gene transcription [138]. Interestingly, the p53-binding protein, ASPP2, augments TAZ transcriptional activity by facilitating TAZ and PP1A interaction and decreasing TAZ-S89 phosphorylation [138]. ASPP2 also regulates YAP and forms an apical–lateral polarity complex in epithelial cell tight junctions, acting as a scaffold to recruit PP1A and YAP to promote YAP-S127 dephosphorylation and consequent YAP activation [139].

The catalytic subunit of protein phosphatase 2A (PP2A) also binds and dephosphorylates YAP-S127 [127]. Interestingly, YAP association with the αE-catenin/14-3-3 complex prevents YAP interaction with PP2A and diminishes S127 dephosphorylation [127]. Furthermore, as mentioned earlier, the absence of αE-catenin weakens YAP/14-3-3 interaction and instead promotes YAP's association with PP2A [127]. Thus, in epidermal cells, αE-catenin plays a central role in regulating YAP nuclear localization by promoting 14-3-3 association and preventing PP2A binding (Fig. 4).

Regulation by alkylation

YAP is also regulated by alkylation (acetylation and methylation) of key residues. In liver cancer cells, YAP is acetylated (Ac-YAP) by p300/CBP acetyltransferases to reduce YAP activity [140]. Conversely, Ac-YAP is de-acetylated by SIRT1 to promote YAP nuclear localization and association with TEAD4 to support HCC cell growth [140]. Interestingly, in the

same study, YAP abundance was not significantly different in a panel of HCC tumours compared to normal samples [140]. However, expression of SIRT1 and the YAP-target gene, *CTGF*, was significantly increased in tumours, suggesting YAP de-acetylation by SIRT1 contributes to HCC tumorigenesis.

In addition to being acetylated, YAP is also methylated on specific lysine residues. Firstly, Set7 methyltransferase methylates YAP at K494 (hYAP1-2γ) [141]. Furthermore, in mouse embryonic fibroblasts (MEFs) grown at high cell density, Set7 expression promotes Yap cytoplasmic localization and reduces Yap target gene transcription [141]. Secondly, it was recently shown that SET1A interacts with the YAP WW domain to methylate YAP at K342 (hYAP1-2γ) [142]. In this study, the monomethylation of K342 by SET1A prevents CRM1-mediated YAP nuclear export resulting in YAP accumulation in the nucleus and promotion of cell proliferation and tumorigenesis. Moreover, YAP-K342 methylation mimic, $Yap^{K327M/K327M}$, 'knock-in' mice are more susceptible to colorectal tumorigenesis, and clinically, high levels of YAP-K342 methylation and SET1A predict poor prognosis and survival [142]. It is worth noting that K342 is encoded within exon 6 and therefore only present in the YAP-γ and -δ isoforms. Consequently, it is tempting to speculate that in the right cellular context, for example, high SET1A expression, the γ and δ isoforms are more tumorigenic than the α and β isoforms. Collectively, these studies show that alkylation adds another layer of complexity to control YAP nucleocytosolic shuttling and activity. To date, TAZ alkylation has not yet been reported.

Regulation by angiomotin

YAP and TAZ subcellular localization is additionally controlled by their WW domain–PPxY motif-mediated interaction with several angiomotin (AMOT) family members. AMOTs interact with the cytoskeleton and are part of the cell junctional complex [143–146]. The association with AMOTs results in YAP and TAZ sequestration in the cytoplasm and tight junctions and suppresses their transcriptional, growth and oncogenic transforming activities [147–149]. Subsequently, YAP was shown to form a tripartite complex with AMOT-L1 and ZO-2, and notably, the function of AMOT-L1 opposes that of ZO-2 to prevent YAP nuclear translocation [98]. Interestingly, in contrast to earlier work [147–149], this study demonstrates that AMOT-L1 specifically interacts with YAP1-2 and not YAP1-1. This indicates that YAP's tandem WW domains are indispensable for this interaction [98] and suggests that YAP isoforms that contain a single WW domain may not be regulated by AMOT proteins. These observations indicate the interaction of YAP with AMOT proteins is complex, and that discrepancies are potentially affected by experimental conditions and possible post-translational modification of either YAP or AMOTs. For example, serum deprivation induces LATS kinase phosphorylation and stabilization of the p130-isoform of AMOT (AMOTp130) [150]. This increases YAP association with both AMOTp130 and the E3 ubiquitin ligase, AIP4, to promote YAP degradation and thereby reduce YAP activity and cell growth [150]. However, in an alternative model, cytoplasmic AMOTp130 binds YAP, blocking access to YAP's WW domains and thereby preventing LATS-mediated YAP phosphorylation. The resultant increased nuclear YAP, in complex with TEAD1, is then bound by nuclear AMOTp130 to augment transcription of a subset of YAP-target genes that facilitate tumorigenesis [151]. Despite their differences, the above studies highlight that AMOT proteins have important role/s in regulating YAP and TAZ activity.

Regulation by actin cytoskeleton

As mentioned earlier, the activity of YAP and TAZ is also influenced by cell shape and mechanotransduction cues transmitted through cell–cell junctions and cell–matrix adhesions, and current data indicate that the actin cytoskeleton is a key mediator of YAP/TAZ regulation [152,153]. The induction of filamentous actin (F-actin) bundles by siRNA silencing of F-actin capping or severing proteins promotes YAP/TAZ nuclear localization and increases target gene expression [154]. Conversely, treatment of cells with actin disrupting agents (latrunculin A/B or cytochalasin D) promotes cytoplasmic retention and inactivation of YAP and TAZ, indicating that the actin cytoskeleton is required for nuclear localization [155–157]. Furthermore, latrunculin A/B promotes Yap and TAZ degradation in NIH3T3 and MCF10A cells respectively [155,158]. As mentioned above, the association with AMOTs results in YAP and TAZ inactivation in the cytoplasm and tight junctions [147–149]. AMOTs interact with F-actin and phosphorylation of AMOTs' actin-binding domain by LATS inhibits this association [159,160]. LATS phosphorylation of AMOTp130 increases with disruption of F-actin [159] and since YAP and F-actin compete for binding AMOTp130 [160], it is likely that F-actin disruption promotes YAP/TAZ cytosolic localization *via* their association with AMOTs. Furthermore, the recruitment of AIP4 E3 ubiquitin ligase to the AMOTp130 complex promotes YAP degradation [150,161]. Thus, it is possible

that disruption of the actin cytoskeleton regulates YAP/TAZ stability through AMOT and AIP4. Lastly, YAP overexpression in gastric cancer and glioma cells has recently been shown to regulate F-actin/G-actin dynamics to soften the cytoskeleton and promote a migratory/metastatic phenotype [162,163]. Thus, the interplay between the actin cytoskeleton and YAP/TAZ activity may have significant consequences for tumorigenesis and cancer progression.

Regulation of stability

YAP/TAZ phosphodegrons

In addition to controlling YAP and TAZ subcellular localization, subsequent work revealed that Hippo signalling also has a major role in regulating YAP and TAZ stability. Initially, the Guan laboratory identified a LATS phosphorylation site in YAP (S381) and TAZ (S311) that primes them for additional phosphorylation by CK1δ/ε on a proximal serine in YAP (S384) and TAZ (S314) [21,22]. Phosphorylation of this phosphodegron leads to recruitment of beta-transducin repeat containing E3 ubiquitin protein ligase (β-TrCP), a component of the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase complex that ubiquitinates YAP and TAZ for degradation [21,22]. Interestingly, the S384/S314 phosphodegron found in YAP and TAZ is not conserved in Yki [22]. This represents a divergence between *Drosophila* and mammals and adds an additional layer of complexity to the regulation of mammalian Hippo effectors. In addition, TAZ also contains a second phosphodegron within its N terminus that is not found in YAP. The phosphorylation of this phosphodegron (residues S58 and S62) by GSK-3 leads to TAZ degradation in response to the inhibition of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) [164].

Both CK1 and GSK-3 can phosphorylate "non-primed" sites; however, their preference is for "primed" substrates [165–167]. CK1 utilizes a phosphorylated recognition motif N terminus of the target site [168] while GSK-3 recognizes a phosphoserine or phosphothreonine four residues C terminus to the target site [169]. Consistent with this, CK1-mediated TAZ degradation depends on the initial phosphorylation by LATS kinases at S311, and mutation of either the LATS or the CK1 phosphosites significantly reduces TAZ and β-TrCP interaction, stabilizing TAZ [21]. Likewise, transfection of cells with a "kinase-dead" LATS mutant reduces TAZ and β-TrCP association, and using *in vitro* kinase assays, CK1ε was unable to phosphorylate TAZ without priming phosphorylation by LATS2 [21]. Notably, these critical serines (S381/384) are conserved in YAP, and the same group showed that dual phosphorylation by LATS and CK1δ/ε also mediates YAP degradation [22].

Using chemical PI3K and GSK-3 inhibitors as well as ectopically expressed AKT and GSK-3, Huang *et al.* [164] showed that GSK-3 destabilizes TAZ by phosphorylating residues S58 and S62 in response to PI3K inhibition. PI3K inhibition decreases Akt activity, an inhibitor of Gsk-3, effectively increasing Gsk-3 activity to promote phosphorylation and consequent degradation of Taz [164]. S66 was previously identified as a LATS phosphosite in TAZ [123], and it was speculated that S66 was the priming site for GSK-3. However, *in vitro* kinase assays and mutational studies revealed that neither LATS kinase nor S66 is required for GSK-3-mediated TAZ phosphorylation [164]. Conversely, mutation of residues S58/S62 to alanine enhances both TAZ stability and activity, indicating that GSK-3 does not require priming to phosphorylate and degrade TAZ.

YAP/TAZ recruitment to the destruction complex

In an alternative model, Azzolin *et al.* [170] reported that GSK-3 does not directly phosphorylate TAZ, but rather phosphorylates β-catenin that serves as a scaffold to recruit TAZ and promote its association with β-TrCP (Fig. 5B). Their conclusions were based on their findings that the mutation of S58 and S62 does not alter GSK-3-mediated TAZ degradation and that siRNA depletion of β-catenin stabilizes TAZ abundance [170]. Furthermore, escape of β-catenin from the destruction complex upon WNT signalling prevents TAZ degradation and leads to concomitant TAZ and β-catenin accumulation [170]. The same group later found that YAP and TAZ are required to recruit β-TrCP to the AXIN1-destruction complex to degrade β-catenin [171]. The WW domains of YAP and TAZ are not required for AXIN1 interaction though the precise mechanism remains to be identified. Notably, while YAP and TAZ are both recruited to the destruction complex, only TAZ is degraded [170,171]. The GSK-3 phosphodegron in TAZ could account for this discrepancy.

The discordance between these findings is possibly explained by the different cell types studied as Huang *et al.* [164] utilized mouse NIH3T3 fibroblasts and HeLa cells, whereas Azzolin *et al.* [170,171] used human MCF10A-MII premalignant breast cancer and HEK293 cells. This is supported by the observation that NIH3T3 and HeLa cells exhibit significant differences in the phosphorylation of TAZ N- and C-terminal phosphodegrons [164].

In support of Azzolin's model, TIAM1, a guanine nucleotide exchange factor, was recently shown to

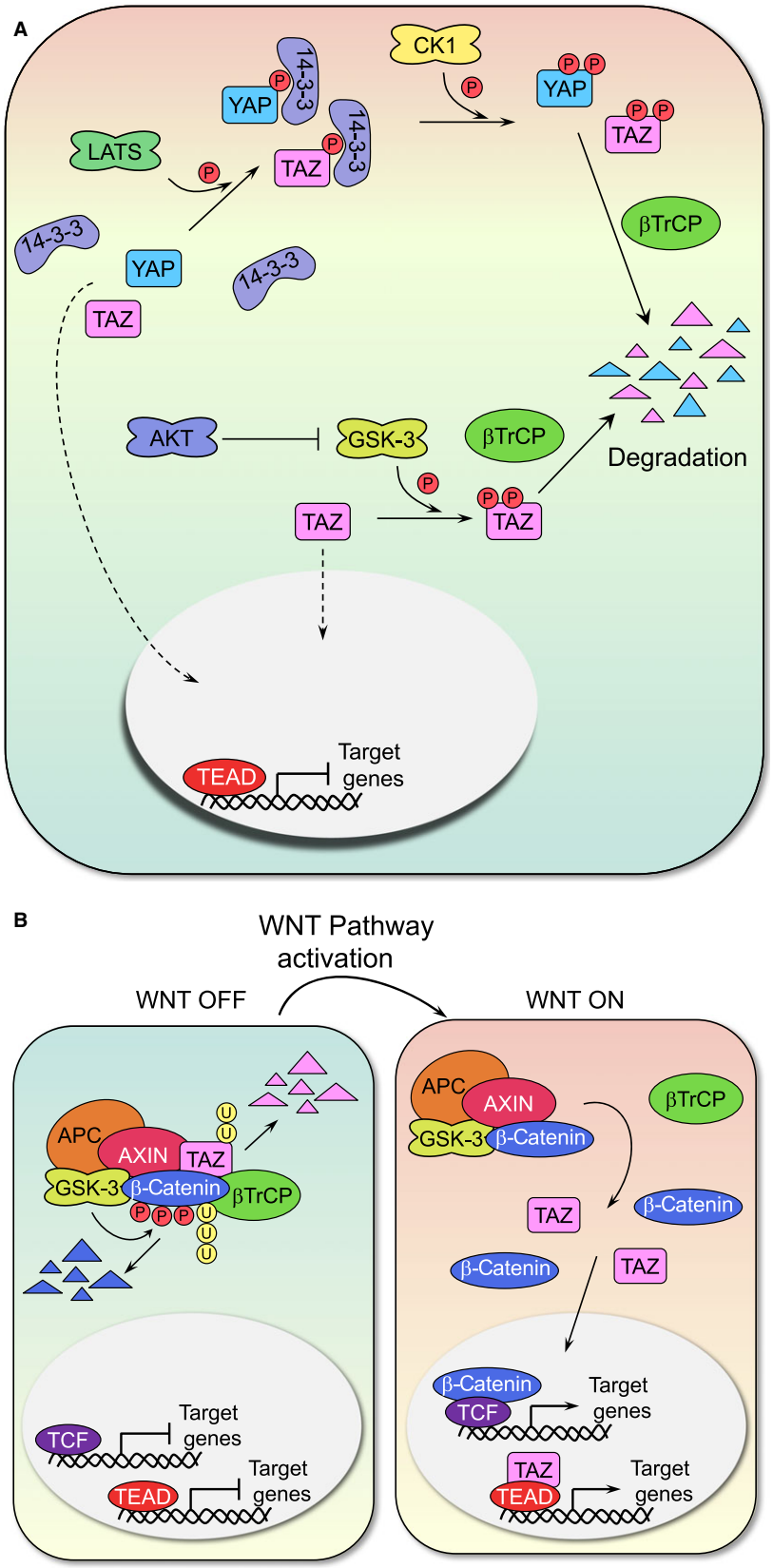

Fig. 5. Mechanisms of YAP and TAZ degradation. (A) Activation of LATS kinase at high cell density leads to YAP/TAZ cytoplasmic retention bound to 14-3-3. Additional phosphorylation of the C-terminal phosphodegron by CK1 results in β-TrCP recruitment and consequent degradation of YAP and TAZ. Additionally, GSK-3 directly phosphorylates the TAZ N-terminal phosphodegron which leads to β-TrCP recruitment and TAZ degradation. Note, GSK-3 is inhibited by AKT-mediated phosphorylation. (B) In the absence of WNT signalling (WNT OFF), GSK-3 phosphorylates β-catenin and serves to recruit YAP and TAZ to the AXIN-destruction complex resulting in their cytoplasmic retention and inactivation. YAP and TAZ further recruit β-TrCP to the complex which results in degradation of TAZ and β-catenin but not YAP. On WNT activation (WNT ON), β-catenin, YAP and TAZ are released from the complex whereupon they enter the nucleus to drive target gene transcription. Notably, the release of β-catenin and TAZ prevents their degradation and causes their accumulation in cells. For simplicity, only TAZ is depicted in this model. Model in (B) adapted from ref. [172].

antagonize colorectal cancer progression by inhibiting YAP and TAZ activity [172]. In the cytoplasm, TIAM1 localizes to the destruction complex and promotes TAZ degradation by enhancing its association with β-TrCP. While in the nucleus, TIAM1 inhibits YAP/TAZ activity by suppressing TEAD binding to reduce expression of YAP/TAZ target genes implicated in epithelial–mesenchymal transition, cell migration and invasion [172].

Recruitment of YAP and TAZ to the destruction complex also causes their cytoplasmic retention and inactivation (Fig. 5B). On WNT activation, low-density lipoprotein receptor-related protein 6 (LRP6) releases YAP and TAZ from the destruction complex and promotes their nuclear localization. Additionally, β-TrCP recruitment to the destruction complex is prevented by the release of YAP and TAZ, facilitating β-catenin accumulation and signalling [171].

Regulation by O-GlcNAcylation

Recently, it was shown that YAP undergoes O-GlcNAcylation in response to high glucose conditions. The attachment of β-N-acetylglucosamine (O-GlcNAc) to the hydroxyl group of YAP-S109 or T241 decreases YAP phosphorylation by LATS kinases in several cell types including ovarian, pancreatic and liver cancer cells [173,174]. Furthermore, O-GlcNAcylation modification reduces the interaction with β-TrCP ligase and consequently increases YAP stability (see below) [174]. The mutation of YAP-S109A or -T241A promotes cytoplasmic retention and decreases nuclear localization and target gene transcription [173,174]. Interestingly, global O-GlcNAcylation promotes and maintains cancer cell growth and tumorigenesis, potentially through targeting YAP, and mutation of YAP-T241A reduces colony and tumour growth of liver cancer cells *in vivo* [174]. It is worth noting that T241 is only present in YAP1-2 isoforms suggesting that, in some cell types, these isoforms may be more tumorigenic than YAP1-1 isoforms. Furthermore, OGT, NUDT9 and SLC5A3 genes were identified as YAP target genes and form a feedback loop to promote global O-GlcNAcylation and tumorigenesis [173,174]. Notably, the O-GlcNAcylation of TAZ has not yet been reported.

Other mechanisms

Other pathways also converge on Hippo signalling to regulate YAP/TAZ abundance. In colorectal cancer cells, RAS reduces suppressor of cytokine signalling (SOCS) 5/6 levels to control YAP turnover, and SOCS6 promotes YAP degradation *via* an Elongin B/C-Cullin-5 ubiquitin ligase complex [175]. Interestingly, a positive feedback loop involving upregulation of the YAP target gene amphiregulin (AREG) was required for activation of the EGFR pathway and cellular transformation by RAS. Furthermore, the EGFR/RAS/MAPK pathway inhibits LATS kinases *via* the phosphorylation of Ajuba proteins to reduce YAP phosphorylation and augment YAP activity [176]. Thus, RAS can act *via* LATS/β-TrCP- and Elongin B/C/Cullin-5-dependent mechanisms to reduce YAP turnover. The resultant increased YAP activity enhances RAS tumorigenicity by promoting EGFR signalling *via* an increase in AREG abundance.

Whilse both YAP and TAZ are complexly regulated by several distinct mechanisms, it is noteworthy that TAZ is more unstable than YAP. TAZ abundance is significantly decreased in HeLa and MCF10A cells following treatment with the protein synthesis inhibitor, cycloheximide [21]. Furthermore, in cycloheximide-treated U2OS and NIH3T3 cells, YAP's observed half-life was approximately twice that of TAZ [177,178]. Moreover, treatment of MCF10A and BT549 cells with the proteasome inhibitor, MG-132, significantly increases TAZ abundance [21], and in mouse NIH3T3 cells, MG132 treatment markedly increases Taz abundance without affecting Yap [177]. It is tempting to speculate why TAZ is more unstable than YAP. Perhaps, TAZ is biologically more potent than YAP and thus mechanisms evolved to tightly control TAZ levels. In support of this, *TAZ* siRNA treatment in H1299 cells has a greater effect on TEAD-mediated transcription than *YAP* silencing [63]. Similarly, TAZ overexpression causes greater TEAD transcription compared to YAP when tandem TEAD-binding sites were used in the reporter construct. These effects are possibly explained by the formation of TAZ-TEAD heterotetramers and thus additional TAZ molecules, compared with YAP-TEAD heterodimers in the DNA-bound complexes [63]. Conversely, a recent publication comparing YAP and TAZ activities indicates that YAP is more potent, at least in HEK293 cells [6]. Regardless, these discrepancies indicate that cellular context is an important factor when examining potency.

Another possibility is that the activity of YAP and TAZ need to be considered together as a unit and their combined activity is tightly controlled by distinct mechanisms in different cells. In support of this, the abundance of either YAP or TAZ can indeed affect the other. Treatment of MEFs, mouse liver progenitor cells and NIH3T3 cells with *Yap*-shRNA increases TAZ abundance. Conversely, YAP overexpression causes rapid TAZ disappearance, within 8–16 h, in these cells [177]. In NIH3T3 cells, YAP-induced loss of TAZ requires Hsp90 and GSK-3 activity but not LATS1/2 or CK1δ/ε activity.

Similar TAZ loss is observed when YAP is exogenously expressed in MCF-7 breast cancer, D645 glioblastoma and HeLa cervical cancer cells, and the ability to cause TAZ loss correlates with the overexpression of transcriptionally more potent YAP isoforms, for example, YAP1-2α [177]. Curiously, the ability of YAP to affect TAZ abundance was one-directional in all cell lines examined, as modulating TAZ levels with shRNA or overexpression does not affect YAP abundance [177]. In contrast to these findings, treatment of Hs68 normal fibroblasts, HLF and SK-Hep1 HCC, and SW620 colon cancer cells with *TAZ*-shRNA increases YAP abundance as well as nuclear YAP [179]. Interestingly, in this study, cell treatment with *YAP*-shRNA did not affect TAZ levels. Although the mechanism has not been fully elucidated, these findings highlight that the combined level of YAP and TAZ in cells is tightly controlled, and depending on the cellular context, either YAP or TAZ may be easier to regulate, especially TAZ since it is more unstable than YAP. Moreover, when performing functional studies and manipulating YAP and TAZ levels, for example, with shRNA, it is critical to examine the effects of both YAP and TAZ since, depending on cell type, either could affect the abundance and activity of the other paralogue.

Concluding remarks

The transcriptional coactivators YAP and TAZ are the principal effectors of canonical Hippo signalling and have understandably been the focus of a multitude of studies. As highlighted in this review, YAP and TAZ are multidomain proteins that interact with numerous regulatory proteins and DNA-binding partners, and their activities are controlled by a complex set of processes. The diverse functions of Hippo signalling and the multifaceted regulation of YAP and TAZ provides ample opportunity for therapeutic intervention using chemicals and small molecules to inhibit YAP and TAZ activity to treat disease. Indeed, there is strong pharmaceutical interest in developing inhibitors of YAP and TAZ, especially as anticancer therapeutics (reviewed in ref. [42]). The next decade heralds great promise and excitement and one wonders what new discoveries await.

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