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Upstairs, downstairs: spatial regulation of Hippo signalling

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Cellular signalling lies at the heart of every decision involved in the development and homeostasis of multicellular organisms. The Hippo pathway was discovered nearly two decades ago through seminal work in *Drosophila* and rapidly emerged as a crucial signalling network implicated in developmental and oncogenic growth, tissue regeneration and stem cell biology. Here, we review recent advances in the field relating to the upstream regulation of Hippo signalling and the intracellular tug-of-war that tightly controls its main target, the transcriptional co-activator Yorkie/YAP.

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The Hippo core kinase cassette: Side A

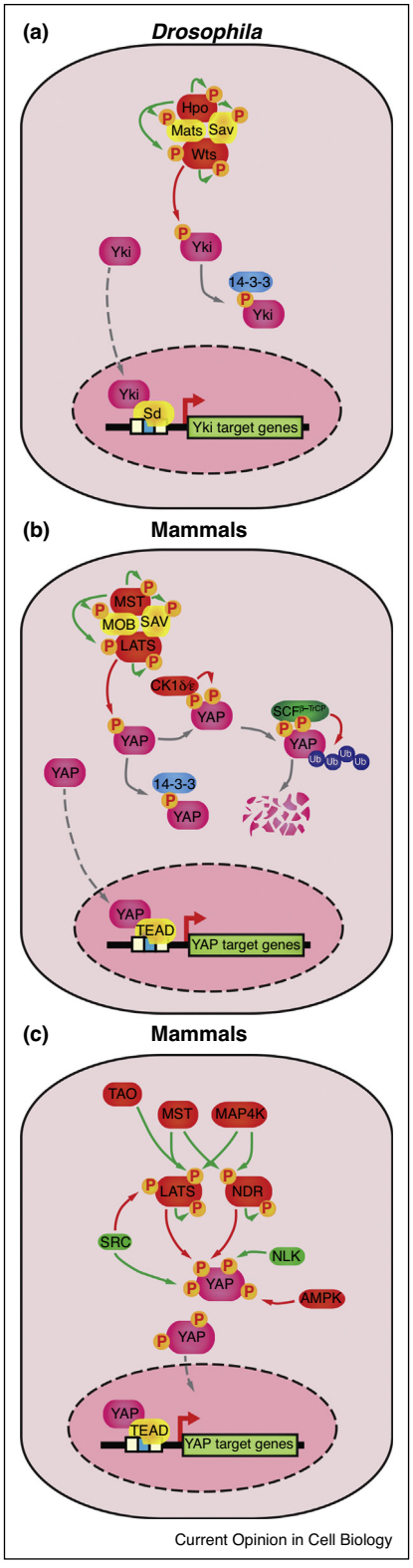
Genetic screens in *Drosophila* led to the identification of the main players involved in Hippo signalling and its role in tissue growth, regeneration and stem cell biology [1]. Together with elegant genetic and biochemical studies in mammalian systems, a picture emerged of the Hippo pathway core machinery, consisting of the serine/threonine kinases Hippo (Hpo; mammalian MST1/2) and Warts (Wts; LATS1/2) and their respective adaptor proteins Salvador (Sav; SAV1/WW45) and Mob as tumour suppressor (Mats; MOB1A/1B) [1]. Hpo and Wts function in a kinase cascade, which transduces multiple upstream signals (reviewed in [1,2]), ultimately leading to phosphorylation and inhibition of the pro-growth transcriptional co-activator Yorkie (Yki; YAP and TAZ in mammals) [1] (Figure 1a,b and Box 1). In both *Drosophila* and mammals, Wts/LATS-mediated phosphorylation leads to Yki/YAP cytoplasmic retention by 14-3-3 proteins and subsequent downregulation of downstream targets. In mammals, LATS phosphorylation can also prime YAP/TAZ for CK1δ/ε-mediated phosphorylation and β-TrCP-induced protein degradation [3,4]. Yki/YAP/TAZ target genes globally promote tissue growth and pluripotency (reviewed in [5]) and, for this reason, Yki and YAP/TAZ are tightly regulated by a complex machinery acting at multiple subcellular localisations. Here, we review new advances to our understanding of how this regulation is achieved.

Compartmentalisation of Hippo signalling: the apical membrane

The importance of apical–basal polarity and tissue architecture in Hippo signalling has been a long-standing paradigm in the field [6] (Figure 2). Recent *Drosophila* studies have confirmed that the sub-apical domain is a critical site for Yki inhibition and Hpo/Wts activation [7••]. Several upstream components of the *Drosophila* Hippo pathway are recruited to the apical cortex by transmembrane proteins such as the apical polarity determinant Crumbs (Crb), which recruits and controls the stability of the FERM domain protein Expanded (Ex) [8–12], Echinoid (Ed), which recruits Sav [13], and the giant cadherin Dachsous, which recruits the atypical myosin Dachs [14,15]. Thus, key Hippo signalling events occur at the apical membrane. Firstly, Sav is required for efficient membrane recruitment of Hpo [16•]. Secondly, the apically-localised FERM domain proteins Merlin (Mer) and Ex recruit Wts, which is activated through Hpo-mediated phosphorylation [7••,16•] and allosterically by its adaptor Mats [17•]. Thirdly, the WW domain protein Kibra recruits Mer, Sav and Hpo to a medial apical pool distinct from the sub-apical cortex where most of Kibra and Mer are localised [18•]. This Kibra:Mer medial pool acts independently of Ex and is negatively regulated by Crb [18•]. However, the relative importance of the medial and cortical pools for Hpo/Wts activation remains to be addressed [18•]. The apical membrane is also a key site of Wts repression, either via the LIM domain protein Ajuba, which traps Wts in an inactive complex at the adherens junction/apical domain boundary [7••,19], or by Dachs-induced and Zyxin-induced Wts degradation [20]. Finally, the apical domain has been proposed as a site of kinase-independent Yki activation, where Ex-mediated sequestration of Yki can be antagonised by Zyxin [21–23].

In mammals, CRB3 localises to the tight junctions, forming a complex containing the Hippo regulators AMOT, NF2/Merlin, Kibra and FRMD6 (an Ex-related protein)

Figure 1



Hippo signalling kinase cascades. **(a)** The *Drosophila* Hippo kinase cassette: Hpo phosphorylates and activates Wts, which in turn promotes Yki inhibition via phosphorylation of S168 and cytoplasmic retention through 14-3-3 binding. When Hippo signalling is inactive, Yki promotes gene expression through its interaction with the transcription factor Scalloped (Sd). **(b)** A similar pathway exists in mammals, with MST-mediated activation of LATS kinases resulting in YAP/TAZ inhibition. Unlike in flies, LATS also promotes YAP/TAZ degradation via the SCF-β-TrCP ubiquitin E3 ligase complex. YAP/TAZ promote gene expression via the interaction with TEAD transcription factors. **(c)** Updated view of the mammalian Hippo kinase cassette, including kinases that affect MST or LATS kinases, or that directly phosphorylate YAP/TAZ (see text for details). Yki/YAP/TAZ-inactivating or Yki/YAP/TAZ-activating kinases are respectively depicted in red or green. Green and red arrows indicate activating or inhibitory interactions, respectively.

Box 1 The Hippo core kinase cassette: side B

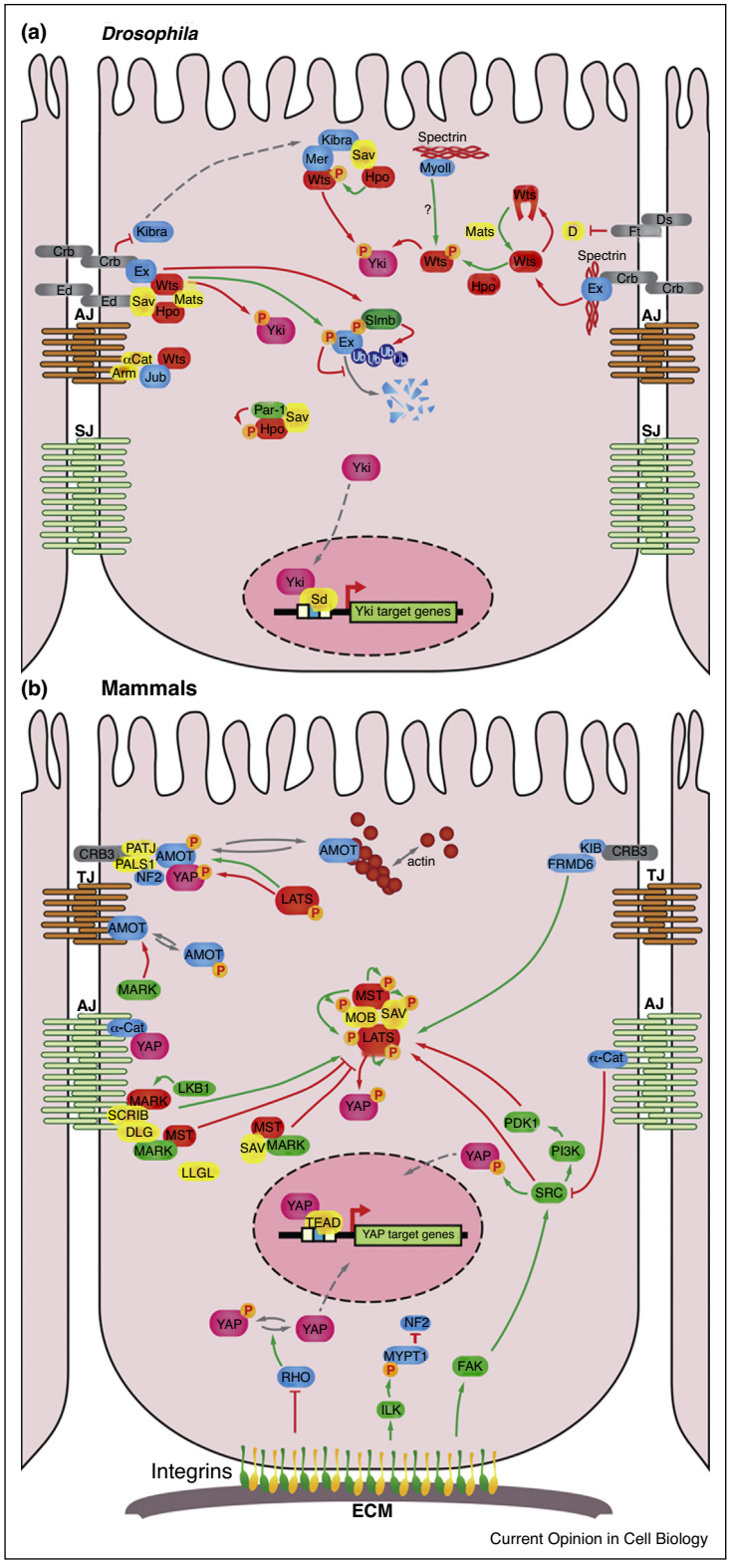
The initial picture of a simple Hippo core cassette has given way to a more complex reality following the discovery of multiple kinases that directly regulate Wts/LATS or Yki/YAP/TAZ (Figure 1c). Tao-1 was identified in *Drosophila* as a Hpo kinase, acting together with the scaffold Schip1, phosphorylating T195 within the activation loop, thereby promoting Hpo activation [108–110]. Interestingly, mammalian TAO kinases have recently been shown to act both upstream of MSTs and in parallel to directly phosphorylate LATS, raising the possibility that some YAP regulatory inputs could entirely bypass MST [111•]. Indeed, several Sterile 20 family kinases besides Hpo/MST are capable of phosphorylating the Wts/LATS hydrophobic motif, such as MAP4K1/2/3/5, MAP4K4/6/7 and their *Drosophila* orthologues Happyhour (Hppy) and Misshapen (Msn) [45•,47•,112•]. However, since *hppy* or *msn* loss-of-function phenotypes are less severe than *hpo* [112•], the precise physiological context of MAP4K-mediated Wts/LATS regulation remains to be thoroughly investigated.

NDR1/2 kinases are closely related to Wts/LATS and their activity is similarly regulated by MOB proteins (reviewed in [113]). Recent data has shown that NDR kinases directly phosphorylate YAP at S127 (the best characterised YAP site). In the mouse intestine, conditional loss of *NDR1/2* results in decreased YAP S112 phosphorylation (equivalent to human S127), increased YAP activity, hyperplasia and carcinogen-induced tumour formation. This suggests that, at least in the intestine, NDRs are bona fide YAP kinases [114•].

YAP is phosphorylated by a plethora of kinases other than LATS, including the Src family of tyrosine kinases ([61•,64,68•,115,116] — see below) and the non-receptor tyrosine kinase Abl [117,118]. Recently, the energy sensor AMPK has also been identified as a Yki/YAP inhibitory kinase [119,120•,121•,122•]. The atypical MAP kinase Nemo-like kinase (NLK) has been found to act as a YAP activator [123•,124•]. NLK phosphorylates YAP at S128, the residue directly adjacent to the crucial S127 site. Phosphorylation at S128 antagonises YAP:14-3-3 binding and promotes YAP nuclear localisation [123•,124•]. It remains unclear if S128 phosphorylation blocks phosphorylation at S127 and, despite the identification of osmotic stress as an inducer of S128 phosphorylation, it is not currently known if other stimuli regulate YAP in a similar manner or if there is crosstalk between NLK and other Hippo pathway components.

[24–26]. The angiomotin family (including AMOT and AMOTL1/2) can inhibit YAP function through direct binding [25,27–29], and by promoting LATS/NF2 association [30]. CRB3 promotes the AMOT:YAP interaction at junctions [25], as well as LATS-mediated YAP phosphorylation [31], thereby inhibiting YAP nuclear localisation. While initial observations showed that cytoplasmic AMOTs inhibit YAP function, subsequent reports have also proposed that AMOTs can positively regulate YAP in

Figure 2



Apical–basal polarity networks controlling Hippo signalling. Apical–basal signalling modules that regulate the Hippo pathway in *Drosophila* **(a)** and mammals **(b)**, whose mechanistic details are discussed in this review. AJ, SJ, TJ and ECM represent adherens junctions, septate junctions, tight junctions and extracellular matrix, respectively.

the nucleus [32–34]. Interestingly, post-translational modifications of AMOT influence its effect on YAP. AMOT is phosphorylated at S176 by LATS, resulting in its translocation to junctions, association with NF2 and YAP, and inhibition of YAP activity [35,36].

Thus, both in *Drosophila* and mammals, the Hippo pathway responds to cell–cell junctions via an apical NF2/Mer-containing complex. Indeed, Hippo signalling and NF2 are required for contact inhibition of growth in cell culture, which is thought to reflect an *in vivo* function as a sensor of tissue crowding [1].

The actin cytoskeleton and Hippo signalling

As well as sensing neighbours through cell adhesion molecules, cell junctions are associated with a robust contractile acto-myosin network, which maintains epithelial integrity, receives mechanical inputs, and also regulates Yki/YAP [37–41]. The mechanism(s) linking actin/mechanotransduction to Yki/YAP activity remains a source of debate. A number of reports suggest that cytoskeletal tension promotes YAP activity independently of LATS [38,42•,43,44]. On the other hand, F-actin disruption leads to increased LATS activity and YAP phosphorylation [41,45•,46]. Moreover, in flies, latrunculin-B treatment does not rescue Yki nuclear localisation in *wts* mutant cells [47•], and cytoskeletal tension blocks Wts activity via Ajuba and α-catenin, suggesting that acto-myosin may act upstream of Wts [19]. Actin/tension-mediated YAP modulation also involves AMOT, since binding of AMOT to F-actin prevents the AMOT:YAP interaction and releases YAP from inhibition [43,48–50]. AMOT:F-actin association is antagonised by LATS-mediated phosphorylation of AMOT [48–50] and F-actin disruption [43]. Inhibition of the AMOT:YAP interaction by shear stress-induced actin polymerisation links blood flow to blood vessel maintenance by YAP in developing zebrafish [51••].

Thus, LATS-dependent and LATS-independent modes of YAP regulation by actin appear to co-exist. However, actin cytoskeleton integrity is dominant in determining YAP activity, since for instance YAP mutants for the inhibitory LATS sites retain sensitivity to plating on soft substrates and treatment with actin depolymerisation drugs in cell culture [42•,52•]. The relative contributions of these mechanisms under physiological mechanical environments and tissue architecture is an important area for further research, particularly in light of a recent report suggesting that eliminating the basement membrane (and thereby tension at a global scale) in developing *Drosophila* imaginal discs does not alter Yki activity [53•].

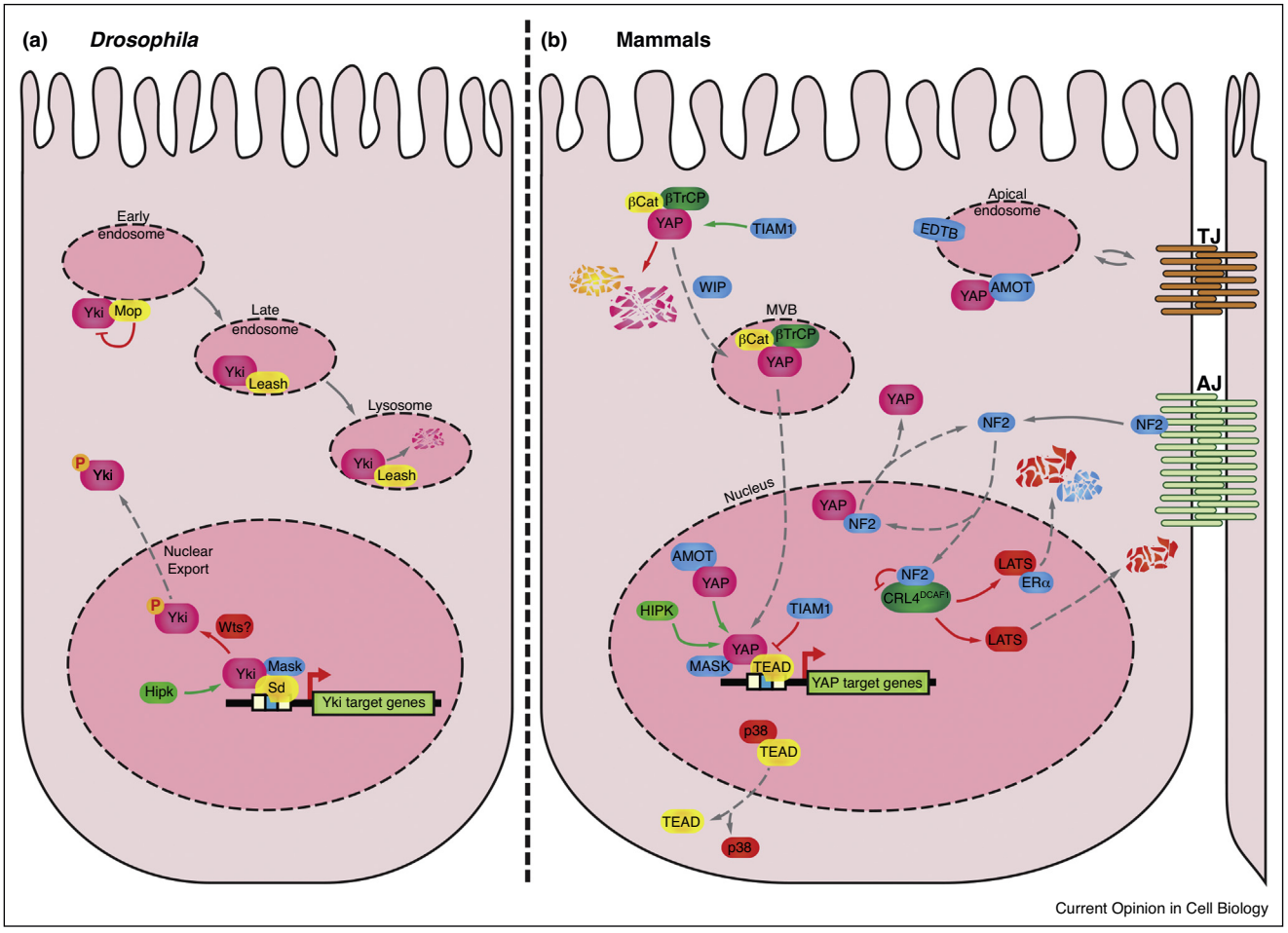
Compartmentalisation of Hippo signalling: basolateral membranes

The basal polarity complex comprised of Scrib, Dlg and Lgl, which is known to antagonise the function of apical determinants such as Crb also regulates Hippo signalling (reviewed in [54]) (Figure 2). Par-1/MARK kinases are crucial basolateral polarity regulators that have recently been associated with Hippo signalling regulation [55]. In *Drosophila*, Par-1 activates Yki by phosphorylating Hpo at S30, restricting its activity [56]. This mechanism is conserved, at least in MDA-MB-231 cells, where MARK4 phosphorylates and inhibits MST and SAV, preventing interaction with LATS [57]. In addition, MARK3 negatively regulates MST in a DLG5-dependent manner [58], and in pre-implantation mouse embryos, MARK2/3 inhibit AMOT junctional localisation, thereby positively regulating YAP activity [35]. However, the role of MARK kinases is complex, since MARK1/3/4 have also been described as YAP negative regulators, acting downstream of LKB1 to regulate Scrib localisation and Hippo kinase activity [59]. As MARKs interact with both Scrib [59] and Dlg [58], perhaps their differential binding partners determine whether they stimulate or inhibit YAP.

The integrin-containing focal adhesions (FAs) are the sites of cell attachment to the underlying extracellular matrix (ECM) and, as such, are key for the cell's ability to sense its mechanical environment. Integrin activation promotes YAP/TAZ nuclear accumulation and activation [60,61•,62]. YAP itself promotes FA gene expression, FA formation [63•], as well as ECM stiffening [64], thereby initiating a feed-forward loop that modulates the biophysical properties of tissues. Rho GTPases are downstream effectors of integrins that generally promote YAP/TAZ activity [62,65,66]. Integrins can also signal through Src family kinases to regulate YAP/TAZ [61•,67•]. FAK/Src signalling can activate YAP both by direct phosphorylation of three tyrosine residues (Y341, Y357 and Y394) [68•] and indirectly via inhibitory phosphorylation of LATS [67•,69•]. It is interesting to note that cell–cell junction signalling (YAP inhibition) and cell–ECM signalling (YAP activation) play antagonistic roles in YAP regulation, with the junctional protein αE-catenin acting as a mediator of this tug-of-war by antagonising integrin signalling [61•,68•,70]. Finally, integrins can inactivate Hippo signalling via integrin-linked kinase (ILK)-mediated phosphorylation of MYPT-PP1, which leads to NF2 inactivation [71]. The ECM proteoglycan Agrin has been reported to activate YAP both via ILK [72••], and by releasing YAP from an inhibitory complex with its receptor Dystroglycan [73••,74••]. Interestingly, Agrin can promote YAP-dependent cardiac regeneration upon injury and its disappearance during the first week of life is thought to cause the loss of regenerative potential in the adult compared with the neonatal mouse heart [73••,74••].

Recently, the spectrin cytoskeleton, a deformable actin-associated network, has been identified as a modulator of Yki function, potentially acting both apically and basally in different cell types [75•,76•,77•]. Several mechanisms

Figure 3



The role of the endosomal and nuclear compartments in Hippo signalling regulation. Endosomal network-associated and nuclear signalling events that regulate the Hippo pathway in *Drosophila* **(a)** or mammals **(b)**. See text for further details.

have been proposed, including regulation via Crb/Ex tethering [76•], through myosin II and cortical apical acto-myosin regulation [75•], or by regulating basal actin networks [77•].

Compartmentalisation of Hippo signalling: endocytic network and the nucleus

The endocytic network remains one of the least understood subcellular localisations involved in Hippo signalling (Figure 3). In *Drosophila*, Yki is regulated at endosomes by Myopic (Mop), the fly homologue of the endocytic regulator His-domain protein tyrosine phosphatase (HD-PTP). Mop interacts with Yki through a WW domain:PPxY interaction and negatively regulates a specific subset of Yki target genes [78]. A study of the Hippo pathway interactome revealed extensive connections to the endocytosis and vesicle trafficking machineries, and led to the identification of Leash, an α-arrestin molecule that controls lysosome-mediated Yki degradation [79]. However, whether Mop and Leash work concomitantly in the regulation of Yki remains unknown.

In MDCK cells, the endosomal membrane protein Endotubin (EDTB) promotes endosomal localisation of AMOT. At low cell density, EDTB inhibits AMOT:YAP binding, thereby promoting YAP function. At high density, EDTB interacts with recycling tight junction proteins, allowing AMOT to sequester YAP in endosomes and to block its activity [80]. Interestingly, AMOT proteins are degraded by the Nedd4 family of ubiquitin E3 ligases, which are prominent endocytosis regulators [81,82]. A recent report proposed that WASP-interacting protein (WIP) promotes YAP/TAZ stabilisation by sequestering the β-catenin destruction complex in multivesicular bodies [83•]. It is yet unclear if, like TOR (Target of Rapamycin), which senses amino acid availability via the lysosome-associated Ragulator [84], Hippo

pathway components associate with vesicular components in order to respond to external stimuli, or whether this represents a degradative route to prevent excess signalling.

Despite the fact that the primary site of Yki/YAP function is the nucleus, where it associates with TEA domain transcription factors, surprisingly little is known about the dynamics of its nuclear import/export. Yki nuclear localisation is regulated by Wts-mediated phosphorylation, which blocks Yki nuclear localisation by 14-3-3-dependent (S168 phosphorylation, with S127 fulfilling a similar function for human YAP) [85–87] and 14-3-3-independent mechanisms (S111 and S250 phosphorylation, which have been associated with Yki nuclear export) [88]. However, the effect of Wts/LATS-mediated phosphorylation on nuclear import/export rates has not been directly assessed.

Once nuclear, Yki/YAP is still likely subject to regulatory inputs. For instance, depending on its phosphorylation status, NF2 can localise to the nucleus, where it inhibits the $CRL4^{DCAF1}$ E3 ubiquitin ligase [89–91]. In the absence of NF2, $CRL4^{DCAF1}$ ubiquitylates and inhibits LATS, thus preventing YAP inhibitory phosphorylation [90]. Interestingly, cortical actin contractility dissociates NF2 from the cortex, allowing it to promote YAP/TAZ nuclear export via its NES (nuclear export sequence), providing a link between cell mechanics and NF2-mediated YAP/TAZ nuclear exclusion [92•]. As described above, localisation of AMOTs is dynamic and dependent on their phosphorylation status. The AMOT p130 splice variant binds YAP in the nucleus, thereby preventing LATS-mediated phosphorylation and promoting YAP function [34]. AMOT hypophosphorylation promotes its nuclear entry, where it facilitates the YAP/TEAD association, whereas phosphorylation at S176 induces junctional localisation and YAP inhibition [36]. In the mouse heart, AMOTL1 junctional localisation is controlled by FAT4. In the absence of FAT4, AMOTL1 enters the nucleus promoting YAP activity [93•]. Nuclear localisation of NF2, AMOT and LATS raises questions regarding the localisation of other Hippo components. MSTs can translocate to the nucleus in response to caspase cleavage [94,95]. However, to what extent the activity of the core kinase cascade occurs in the nucleus versus the cell cortex remains an open question.

YAP/TAZ nuclear function is also influenced by interaction with the TEAD family of transcription factors [96–101]. The RAC1 guanine exchange factor protein TIAM1 has recently been linked to YAP/TAZ regulation in the nucleus and cytoplasm. Nuclear TIAM1 inhibits YAP/TAZ binding to TEAD4, while cytoplasmic TIAM1 promotes YAP/TAZ degradation via the β-catenin destruction complex [102•]. TEAD nuclear localisation is antagonised by p38 MAPK, suggesting that YAP activity can be overridden by cellular stress even in the absence of core kinase activity [103•]. Therefore, YAP nuclear function requires not only low LATS-mediated phosphorylation but also the presence of nuclear TEAD [103•]. Finally, the RNA-binding protein MASK and the nuclear speckle-localised kinase Homeodomain-interacting protein kinase (Hipk) have been suggested to aid Yki/YAP function in the nucleus, though the mechanistic details are not yet elucidated [104–107]. Thus, the control of Yki/YAP nucleo-cytoplasmic shuttling dynamics, as well as the modulation of their activity in the nucleus remain little explored areas in need of investigation.

Conclusion

The demonstration that Hippo signalling plays crucial roles in development and disease resulted in a vast effort to study its regulation. As illustrated in this review, though many proposed molecular mechanisms suggest that components acting at different subcellular localisations link Yki/YAP activity to extracellular cues, no combined model has emerged as yet. Future efforts should address how cells integrate inputs stemming from distinct subcellular regions and whether a hierarchical relationship between these exists, ensuring Yki/YAP activity is tightly controlled. Further work should also address which of these mechanisms operate in different physiological contexts, and how vulnerabilities in this regulatory network are exploited during oncogenic transformation.

Conflict of interest

The authors declare that they have no conflicts of interest.

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