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REVIEW

The regulation of Yorkie, YAP and TAZ: new insights into the Hippo pathway

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ABSTRACT

The Hippo pathway is a highly conserved signalling pathway that regulates multiple biological processes, including organ size control and cell fate. Since its discovery, genetic and biochemical studies have elucidated several key signalling steps important for pathway activation and deactivation. In recent years, technical advances in microscopy and genome modification have allowed new insights into Hippo signalling to be revealed. These studies have highlighted that the nuclear-cytoplasmic shuttling behaviour of the Hippo pathway transcriptional co-activators Yorkie, YAP and TAZ is far more dynamic than previously appreciated, and YAP and TAZ are also regulated by liquid-liquid phase separation. Here, we review our current understanding of Yorkie, YAP and TAZ regulation, with a focus on recent microscopy-based studies.

KEY WORDS: *Drosophila*, Hippo pathway, YAP, TAZ, Yorkie, Transcription

Introduction

Typically, signalling pathways transduce information from the extracellular space and neighbouring cells to the nucleus to regulate transcription. Many such pathways were first identified in *Drosophila*, and include the Hedgehog, Notch, Wnt and Hippo pathways. The Hippo pathway regulates multiple developmental processes, such as organ size and cell fate (Zheng and Pan, 2019; Meng et al., 2016). It is highly conserved throughout metazoans, and core elements also exist in metazoan ancestors; throughout this Introduction, we refer primarily to *Drosophila* proteins because, in most cases, key regulatory steps were first identified and/or are better understood in this organism. On first mention, we also provide the human orthologue of *Drosophila* proteins in brackets, although it must be noted that not all Hippo pathway proteins are obviously conserved between *Drosophila* and mammals.

The Hippo pathway responds to different cell biological properties, such as cell-cell adhesion, apicobasal cell polarity and mechanical forces (Fig. 1) (Halder et al., 2012). Accordingly, apical cell junctions and apical membranes have been identified as key sites of Hippo pathway regulation (Gaspar and Tapon, 2014). Here, proteins such as Merlin (NF2), Kibra (WWC1, WWC2 and WWC3), Expanded (no clear orthologue, although it shows homology to FRMD6), α-Spectrin (SPTAN1) and βH-Spectrin (SPTBN1) promote activation of the Hippo pathway core kinase cassette by poorly understood mechanisms. Cell polarity proteins, such as Crumbs (CRB1, CRB2 and CRB3) and L(2)gl (LLGL1 and LLGL2), also influence Hippo pathway activity, as do the planar cell polarity proteins Fat (FAT1, FAT2, FAT3 and FAT4) and Dachsous (DCHS1, DCHS2), which communicate via a range of cytoplasmic signalling proteins (Grusche et al., 2010).

The core cassette of the Hippo pathway (Fig. 1, Table 1) comprises the kinases Hippo [MST1 and MST2 (also known as STK4 and STK3, respectively)] and Warts (LATS1 and LATS2), and the non-catalytic adaptors Salvador (SAV1) and Mats [MOBKL1A and MOBKL1B (also known as MOB1B and MOB1A, respectively)] (Tapon et al., 2002; Harvey et al., 2003; Wu et al., 2003; Kango-Singh et al., 2002; Udan et al., 2003; Lai et al., 2005; Jia et al., 2003; Pantalacci et al., 2003). Hippo phosphorylates and activates Warts (Wu et al., 2003), and an additional kinase, Tao (TAOK1, TAOK2 and TAOK3), can phosphorylate and activate Hippo (Boggiano et al., 2011; Poon et al., 2011). Furthermore, the STRIPAK phosphatase complex can reverse the activation status of Hippo by dephosphorylating its activation loop (Ribeiro et al., 2010). Kinases that, like Hippo, belong to the Sterile 20-like family can also activate Warts, and these include Happyhour (MAP4K1, MAP4K2, MAP4K3 and MAP4K5) and Misshapen (MAP4K4, MAP4K6 and MAP4K7) (Meng et al., 2015; Zheng et al., 2015; Li et al., 2014). In human breast cancer cells, the basolateral polarity protein SCRIB is also capable of activating LATS1/2 by promoting formation of an activating complex with MST1/2 (Cordenonsi et al., 2011). In its active state, Warts can phosphorylate the transcriptional co-activator protein Yorkie (Yki) (YAP and TAZ); this phosphorylation dictates the balance between nuclear and cytoplasmic Yki (Dong et al., 2007; Oh and Irvine, 2008; Zhao et al., 2007).

When nuclear, Yki can connect to the genome via transcription factors, the best characterised of which is Scalloped (Sd) (TEAD1, TEAD2, TEAD3 and TEAD4) (Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008; Goulev et al., 2008). Yki and Sd and their mammalian orthologues (YAP and TAZ, and TEAD1-4, respectively) are thought to promote transcription by recruiting different complexes that modify chromatin state and transcription such as the Mediator complex, the SWI/SNF complex and the Trithorax-related complex (Hillmer and Link, 2019). Sd can also repress transcription and, in this role, it operates together with the Tondu domain protein Tgi (VGLL4) (Koontz et al., 2013; Guo et al., 2013), and the zinc finger protein Nerfin-1 (INSM1) (Vissers et al., 2018; Guo et al., 2019). A key factor that dictates whether Sd acts as a repressor or an activator appears to be the size of the nuclear pool of Yki: epithelial cells that lack Yki display dominance of Sd's repressor function (Koontz et al., 2013), whereas cells with hyperactive or supraphysiological Yki exhibit Sd-dependent transcriptional activation (Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008; Goulev et al., 2008). The size of the nuclear pool of Yki, in turn, is regulated by multiple factors, and these can

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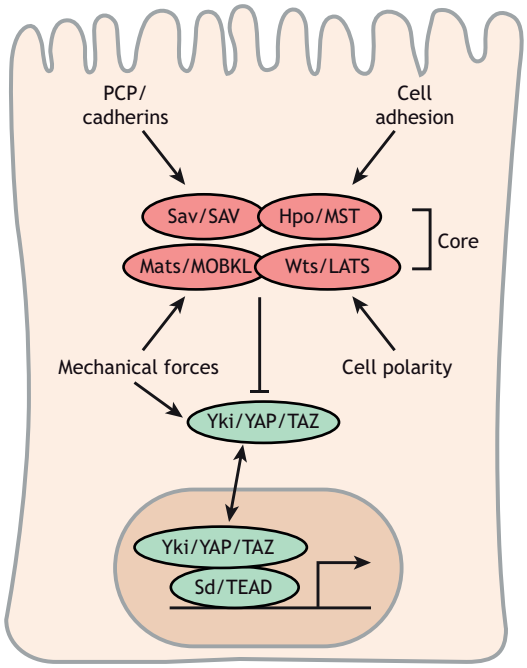

Fig. 1. A simplified depiction of the Hippo pathway. The Hippo pathway consists of more than 40 proteins and is regulated by different cell and tissue level properties, such as cell adhesion and cell polarity, as well as atypical cadherins (Fat and Dachsous in *Drosophila*) that also regulate planar cell polarity (PCP). These influence the activity of the core kinase cassette, which consist of the following proteins (note that *Drosophila* proteins are named first and human proteins in brackets): Hippo (MST1 and MST2), Salvador (SAV1), Mats (MOBKL1A and MOBKL1B) and Warts (LATS1 and LATS2). The key downstream effectors of the Hippo pathway are the transcription co-activator Yki (YAP and TAZ) and the partner transcription factor Sd (TEAD1, TEAD2, TEAD3 and TEAD4). Wts/LATS1/2 phosphorylate Yki/YAP/TAZ and thereby limit the nuclear pool of these proteins. Yki/YAP/TAZ rapidly transit between the nucleus and cytoplasm and the rate at which this occurs can be regulated at the level of both nuclear import and nuclear export. Mechanical forces that are mediated by the actin cytoskeleton can repress Yki/YAP/TAZ and this can be relayed via the core kinase cassette and also independently of it.

operate both via the Hippo pathway and independently of it. The best-characterised regulator of Yki subcellular localisation is Warts, which represses Yki activity by phosphorylating it on three serine residues (Dong et al., 2007; Oh and Irvine, 2008; Zhao et al., 2007). Phosphorylation of Yki at S168 (human YAP S127, TAZ S89) appears to be the most important phospho-regulatory site on Yki and triggers the association of Yki with 14-3-3 proteins (Dong et al., 2007; Oh and Irvine, 2008; Zhao et al., 2007). Warts and the predominantly nuclear kinase PRP4K also phosphorylate Yki at S111 and S250; these are less potent regulatory sites and do not appear to mediate association with 14-3-3 proteins (Johansen et al., 2009; Ren et al., 2010; Cho et al., 2018b).

Initially, association with 14-3-3 proteins was thought to stably sequester Yki/YAP/TAZ in the cytoplasm and thus prevent their access to the nucleus; this led to the conclusion that Hippo signalling is relatively static and operates in binary on and off states. However, several recent live-imaging studies have overturned this dogma, revealing that Yki/YAP/TAZ movement between the nucleus and cytoplasm is highly dynamic and, therefore, that control of Yki/YAP/TAZ localisation operates via an analogue mode, rather than being binary or digital (Manning et al., 2018; Elosegui-Artola et al., 2017; Ege et al., 2018; Kofler et al., 2018). Furthermore, microscopical analyses of Yki/YAP/TAZ have revealed additional layers of complexity in the behaviour of these proteins and the mechanisms by which they are regulated. In this Review, we describe key regulatory mechanisms of Yki, YAP and TAZ, with a focus on recent microscopical studies that have been performed in human cultured cells and *in vivo* in *Drosophila* organs. We focus on the dynamic control of Yki, YAP and TAZ nuclear-cytoplasmic shuttling and the role of nuclear import, nuclear export and Hippo-dependent and Hippo-independent regulatory mechanisms. We also discuss the recent discovery that the regulatory properties of YAP and TAZ are controlled by liquid-liquid phase separation.

The domain structures of Yorkie, YAP and TAZ

The Yki, YAP and TAZ proteins possess multiple conserved domains (Fig. 2), the best conserved of which are the eponymous TEAD-binding domain and the WW domains. Depending on the splice variant, Yki, YAP and TAZ can possess either one or two WW domains, and these have been reported to mediate interactions with a host of proteins that are either required for, or repress, Yki, YAP and TAZ activity. As the name suggests, the TEAD-binding domain facilitates interaction with TEAD1-4 (in humans) and Sd (in *Drosophila*), interactions that are essential for the ability of Yki, YAP and TAZ to regulate transcription (Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008; Goulev et al., 2008). Although the carboxy termini of YAP and TAZ also share high homology, this region has diverged in *Drosophila* Yki. YAP and TAZ each possess a coiled-coil domain in the carboxy terminus, a putative unstructured transactivation domain, and a short PDZ domain-binding motif (Fig. 2). Although the TEAD-binding domain is

Table 1. *Drosophila* and human Hippo pathway proteins

Category	*Drosophila* protein	Human homologue(s)
Core kinase cassette	Hippo	MST1 and MST2
	Salvador	SAV1
	Warts	LATS1 and LATS2
	Mats	MOBKL1A and MOBKL1B
Transcriptional regulators	Yorkie	YAP and TAZ
	Scalloped	TEAD1, TEAD2, TEAD3 and TEAD4
	Tgi	VGLL4
	Nerfin-1	INSM1
Upstream regulators	Merlin	NF2
	Kibra	WWC1, WWC2 and WWC3
	Expanded	FRMD6?
	Tao	TAOK1, TAOK2 and TAOK3
	Happyhour	MAP4K1, MAP4K2, MAP4K3 and MAP4K5
	Misshapen	MAP4K4, MAP4K6 and MAP4K7
	STRIPAK complex	STRIPAK complex
	Crumbs	CRB1, CRB2 and CRB3
	α-Spectrin	SPTAN1
	βH-Spectrin	SPTBN1
	Lethal Giant Larvae	LLGL1 and LLGL2
	Scribble	SCRIB
	?	AMOT, AMOTL1 and AMOTL2
	Fat	FAT1, FAT2, FAT3 and FAT4
	Dachsous	DCHS1 and DCHS2
	Mask	ANKHD1 and ANKRD17

Hippo pathway proteins discussed in this Review are listed. Not all proteins are obviously conserved between *Drosophila* and mammals, and these are indicated with a question mark.

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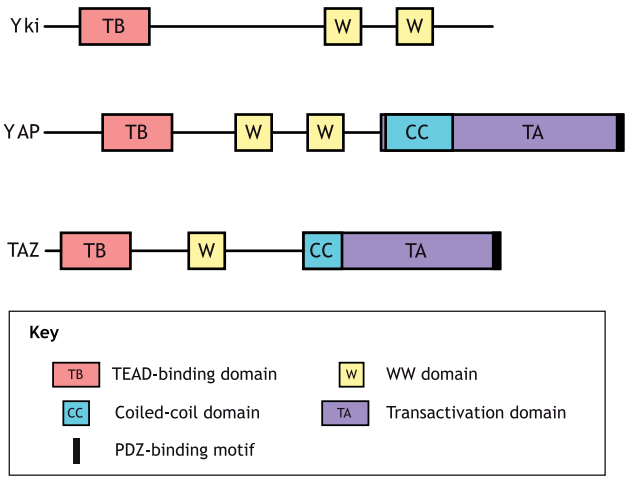

Fig. 2. Arrangement of protein domains in Yorkie, YAP and TAZ. Schematic of protein domains in the Yki, YAP and TAZ proteins. The different domains and motifs are: TEAD-binding domain (TB), WW domain (W), coiled-coil domain (CC), transactivation domain (TA) and PDZ-binding motif (black box at the carboxy termini of YAP and TAZ).

essential for Yki, YAP and TAZ function in almost all experimental settings, the precise functions of the WW domains and the carboxy terminal region are less clear. In *Drosophila*, the WW domains of Yki are required for its ability to promote tissue growth, whereas the carboxy terminal region is dispensable for this (Zhang et al., 2012, 2009; Oh and Irvine, 2009). In mammals, the YAP WW domains are required in some cells, but not others, for YAP-dependent functions and the same is true of the carboxy terminal region (Zhang et al., 2012; Zhao et al., 2009). The WW domains and carboxy terminal region of Yki, YAP and TAZ facilitate interactions with key transcriptional regulatory complexes and, in the case of YAP and TAZ, also mediate liquid-liquid phase separation (Cai et al., 2019; Lu et al., 2020) (discussed in detail later).

The dynamic shuttling of Yorkie, YAP and TAZ

Compartmentalisation of a eukaryotic cell into a nucleus and cytoplasm enables access to its genetic contents to be regulated, with the nuclear envelope acting as a semi-restrictive barrier between compartments. As proteins increase in size, the rate at which they passively diffuse through nuclear pores decreases (Timney et al., 2016). By altering the rate at which proteins are transported in and out of the nucleus, a cell can enrich proteins specifically in either the cytoplasm or the nucleus. For many proteins, compartmentalisation is linked to function; for instance, a transcription factor must be present within the nucleus to interact with the genome and directly impact gene expression.

Several studies have shown that Yki/YAP/TAZ are found in both the nucleus and cytoplasm and that they are actively transported between these cellular compartments. A common description of Hippo signalling's influence on Yki/YAP/TAZ evokes a relatively static system, whereby in a 'Hippo off' state, Yki/YAP/TAZ localise to the nucleus and activate target genes, and in a 'Hippo on' state, Yki/YAP/TAZ relocalise to the cytoplasm and are inactive. However, several recent studies have revealed a more dynamic view, whereby the majority of Yki/YAP/TAZ molecules constantly shuttle between the nucleus and the cytoplasm (summarised in Fig. 3 and Table 2). Although shuttling provides a mechanism for maintaining steady-state nuclear-cytoplasmic distribution (discussed in subsequent sections), it also enables cells to rapidly redistribute the bulk of Yki/YAP/TAZ from one compartment to another by altering rates of import and export, with apparent functional consequences, as we discuss below.

A number of studies have identified rapid redistributions of Yki/YAP/TAZ between compartments, and have shown that these events have functional transcriptional consequences. For example, in cultured rat intestinal epithelial cells, activation of YAP via G protein-coupled receptor stimulation induces a rapid but transient exportin 1 (also known as XPO1 and CRM1)-mediated redistribution of YAP from the nucleus to the cytoplasm, via LATS-mediated phosphorylation of YAP S127 and S397 (Wang et al., 2016a). Upon subsequent nuclear re-accumulation, YAP then increases target gene expression. Similar observations have recently been made using live-imaging approaches in human MCF10A cells, monitoring both YAP localisation and YAP-dependent transcription using the MS2-MCP system (Franklin et al., 2019 preprint). In this case, dramatic nuclear-cytoplasmic fluctuations occur sporadically in individual cells, but are also observed in larger clusters of cells and are regulated by the Hippo pathway kinases LATS1 and LATS2. Similar fluctuations are seen at the margins of cell monolayer wounds and can also be induced by calcium signalling (Franklin et al., 2019 preprint). A key conclusion of this study was that fluctuations in subcellular localisation are important for maintaining YAP in an active state, and that prolonged residence within the nucleus may lead to inactivation of YAP through post-translational modifications (Franklin et al., 2019 preprint). Further work will be required to determine whether such transient YAP relocalisation occurs *in vivo* and the functional importance of it. Additionally, it remains to be investigated how the rates of nuclear import and export are changed to drive the transient relocalisation of Yki/YAP/TAZ.

Regulation of Yki/YAP/TAZ nuclear import

In this and the following section, we discuss experiments that have demonstrated how nuclear import and export of Yki/YAP/TAZ are regulated in a wide range of contexts, both in cell culture and *in vivo*. As the breadth of these studies expands, it is becoming increasingly apparent that Yki/YAP/TAZ function is regulated at the level of subcellular localisation, stability and transcriptional competence, with many protein-protein interactions and post-translational modifications playing a part, seemingly to varying degrees in different cell types.

Consequently, at times it can be difficult to separate these processes. Where possible, we discuss nuclear import and export of Yki/YAP/TAZ separately, although in some contexts these processes appear to be tightly linked.

Live imaging of fluorescently tagged proteins both in mammalian cultured cells and in whole *Drosophila* organs has revealed that modulation of both nuclear import and export of Yki/YAP/TAZ are important for controlling their comparative nuclear/cytoplasmic pools. However, the relative importance of import versus export in determining steady-state localisation seems to be dependent on the cell type studied. This is clearly demonstrated in the *Drosophila* larval wing imaginal disc, where the rate of Yki nuclear import varies in different populations of cells across the tissue, leading to differences in subcellular localisation (Manning et al., 2018). Specifically, cells within the main wing disc epithelium exhibit a largely cytoplasmic localisation, whereas cells in the adjacent squamous peripodial epithelium have a higher level of nuclear Yki, owing to a higher rate of nuclear import. In this context, mutation of the Yki kinase gene *warts* leads to increased nuclear localisation of Yki, and this too is dependent, at least in part, on an increased rate of nuclear import (Manning et al., 2018). These studies used the XPO1 inhibitor leptomycin B (LMB), a commonly used compound in

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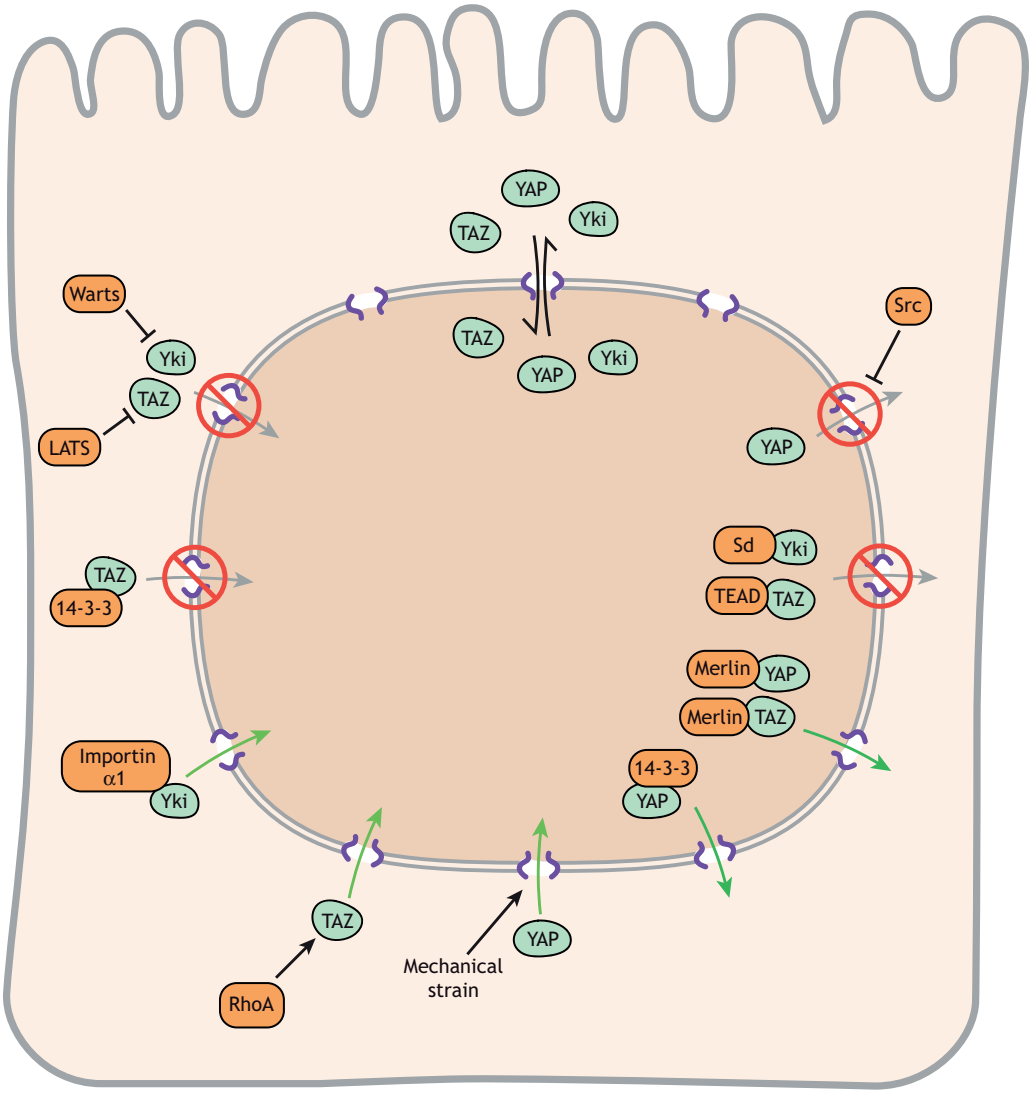

Fig. 3. The nuclear-cytoplasmic distribution of Yorkie, YAP and TAZ can be regulated at the level of both nuclear import and export. Schematic of a eukaryotic epithelial cell highlighting proteins with established functions in regulating Yki/YAP/TAZ shuttling through nuclear pores (shown in purple). Yki/YAP/TAZ continuously shuttle between the nucleus and the cytoplasm. Proteins that promote (green arrow) or inhibit (grey arrow with red circle) this shuttling are shown, with the arrow indicating the direction of transport affected. The rate of nuclear import of Yki and TAZ can be inhibited by Warts- and LATS-mediated phosphorylation, respectively, presumably through phosphorylation-stimulated interactions with 14-3-3 proteins. Importin α1 promotes the nuclear import of Yki through binding to its amino terminal NLS. RhoA activity drives nuclear import of TAZ via an NLS, and mechanical strain on a cell induces changes in nuclear pore structure that increase the rate of YAP nuclear import. Phosphorylation of YAP promotes interaction with 14-3-3 proteins and promotes nuclear export. Merlin promotes nuclear export of YAP and TAZ downstream of circumferential actin belt tension through direct binding and via Merlin NESs, whereas binding to TEADs masks the TAZ NES and inhibits export. Export of YAP is inhibited by the activity of the Src kinase and actomyosin.

studies of nuclear import and export. If a protein is continuously shuttled between the nucleus and the cytoplasm, and if its export is mediated by XPO1, acute blockade of nuclear export (using LMB treatment) and continuation of import results in subsequent accumulation of the protein in the nucleus. The rate of this accumulation can be measured as an indicator of nuclear import rate.

A challenge in studies of nuclear-cytoplasmic shuttling is to separate the contributions of active nuclear import and export from passive or unregulated movement across the nuclear envelope. This was addressed recently by increasing the size of TAZ by fusing it to a large protein tag, such that passive diffusion through nuclear pores is greatly reduced, and so the majority of transport can only occur via active means (Kofler et al., 2018). Many proteins that exhibit active nuclear import are known to contain a nuclear localisation signal (NLS) that directs them to the nucleus. Kofler et al. showed that TAZ is actively imported into the nucleus and that this is dependent on an NLS within amino acids 327-341, which fall within the transactivation domain of TAZ, hinting at further complexity whereby the shuttling and transcriptional activity of TAZ may be co-regulated (Kofler et al., 2018). However, this highly acidic NLS is unusual when compared with other known NLSs and is not reliant on RAN GTPase activity, which regulates the nuclear import of many proteins (Kofler et al., 2018). In addition, despite high conservation of the TAZ NLS amongst other species, including bees, sea urchins, sea slugs and human YAP, this NLS is not found in *D. melanogaster* Yki (Kofler et al., 2018). Instead, Yki nuclear import is mediated by an NLS contained within the first 55 amino acids, which mediate interaction with the nuclear import regulator

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Table 2. Nuclear import and export of Yorkie/YAP/TAZ is differentially regulated in different cell types

Protein	Cell type	Import	Export	Reference
Yki	*Drosophila* wing imaginal disc	Inhibited by Warts		(Manning et al., 2018)
Yki	*Drosophila* imaginal discs; S2 cells	Importin α		(Wang et al., 2016b)
YAP	Fibroblasts; cancer associated fibroblasts (CAFs)	Not regulated	LATS and XPO1 promote export; Src and actomyosin inhibit export; TEAD inhibits export in CAFs	(Ege et al., 2018)
TAZ	LLC-PK1 (porcine kidney); MCF7	LATS; RhoA (LATS independent)	XPO1; inhibited by TEAD binding	(Kofler et al., 2018; Chan et al., 2009)
YAP	MCF10A	Force-mediated deformation of nuclear pore		(Elosegui-Artola et al., 2017)
YAP, TAZ	MDCK		Promoted by Merlin (LATS independent)	(Furukawa et al., 2017)

Importin α1 (Wang et al., 2016b). As with YAP and TAZ, the Yki NLS is divergent from classical NLSs and hints that the diversity of NLS sequences may be wider than initially thought. Further insight into the regulation of Yki/YAP/TAZ import has been provided by recent experiments in *Drosophila* epithelial tissues, human cell culture and mouse epithelium, highlighting the importance of Mask proteins in regulating Yki/YAP/TAZ nuclear import (Sidor et al., 2019). This study revealed that *Drosophila* Mask, and its mammalian orthologues Mask1 (ANKHD1) and Mask2 (ANKRD17), contain both an NLS and a nuclear export signal (NES), and can mediate Yki/YAP/TAZ nuclear import by direct binding (Sidor et al., 2019).

Given that the mechanical properties of the cell and regulation of the actin cytoskeleton have been reported to influence the transcriptional activity of YAP and TAZ through both Hippo pathway-dependent and -independent mechanisms (Dupont et al., 2011; Zhao et al., 2012; Rauskolb et al., 2014), it is perhaps unsurprising that the cytoskeleton regulates the nuclear import of Yki/YAP/TAZ to some degree. For example, the actin regulator RhoA enhances nuclear import of TAZ independently of phosphorylation status (Kofler et al., 2018). Additionally, mechanical forces transmitted to the cytoskeleton from the extracellular matrix lead to deformation of the nucleus and nuclear pores, which in turn increases the rate at which YAP is imported into the nucleus (Elosegui-Artola et al., 2017). This phenomenon is dependent on the relatively 'flexible' nature of the YAP protein, and is also sensitive to protein size (Elosegui-Artola et al., 2017); thus, it remains to be seen whether this also regulates the localisation of other proteins with similar physical properties to YAP.

Regulation of Yki/YAP/TAZ nuclear export

The active export of proteins through nuclear pores counteracts nuclear import and ensures tight control of nuclear-cytoplasmic distribution. Several processes have now been characterised that mediate the nuclear export of Yki/YAP/TAZ, although their relative importance varies in different contexts, as discussed below. Active nuclear export of Yki/YAP/TAZ, mediated by XPO1, was initially identified by noting that inhibition of XPO1 with LMB induces the accumulation of Yki/YAP/TAZ in the nucleus (Dupont et al., 2011; Ren et al., 2010; Parker and Struhl, 2015; Wang et al., 2016a). There is evidence to suggest that, in some cell types, the export rate of YAP and TAZ is regulated to change steady-state localisation and activity. For example, YAP is predominantly cytoplasmic in normal fibroblasts, but it is more nuclear in cancer-associated fibroblasts; fluorescence loss in photobleaching (FLIP) experiments have shown that nuclear import of YAP is similar between these different cell types, implying that the control of YAP nuclear export is primarily responsible for the differential localisation (Ege et al., 2018). Export in this context was found to be highly dependent on phosphorylation of the key LATS1/2-regulated serine residues of YAP, and it was further suggested that Src kinase activity, acting downstream of actomyosin, inhibits export of phosphorylated YAP (Ege et al., 2018). Src also modulates YAP activity within the nucleus through phosphorylation of Y357, which increases YAP transcriptional activity independently of localisation (Ege et al., 2018). This study did not identify a direct NES within YAP, although sequence analysis suggested that YAP contains an NES in amino acids 340-357 (in the vicinity of Y357), and an independent study indicated that the YAP NES mediates interactions with XPO1, and that its function is inhibited by SET1A-dependent methylation of YAP K342 (Fang et al., 2018). A putative NES has been identified in amino acids 2-60 of TAZ, which also contain the TEAD-binding domain (Kofler et al., 2018). The location of this NES may link TAZ localisation to its ability to regulate transcription effectively, as binding to TEAD masks the NES and thereby inhibits TAZ export (Kofler et al., 2018; Chan et al., 2009). Similarly, disruption of YAP-TEAD interaction leads to an increase in nuclear export in cancer-associated fibroblasts, although this interaction interestingly does not impact the export rate of YAP in normal fibroblasts (Ege et al., 2018). The reason for the observed discrepancy is unclear, but it highlights the theme that multiple factors regulate Yki/YAP/TAZ shuttling and that the relative importance of these can be cell and context dependent.

The membrane-cytoskeleton adapter protein Merlin also promotes the nuclear export of YAP and TAZ, acting downstream of mechanical tension and providing a further link between the actin cytoskeleton and Yki/YAP/TAZ shuttling. Specifically, it has been shown that Merlin-induced YAP and TAZ export in polarised MDCK cells is mediated by contraction of a circumferential actin belt, which is induced by high tension, and is independent of Hippo pathway activity (Furukawa et al., 2017). Merlin has also been demonstrated to bind to YAP and TAZ directly and mediate their export via three NESs within Merlin, which are conserved across species including in *Drosophila* (Furukawa et al., 2017). It will be interesting to see whether this mechanism of regulation operates *in vivo*, and which type of mechanical forces might be relevant for Merlin's ability to regulate YAP and TAZ subcellular localisation.

Overall, current evidence suggests that the relative importance of nuclear import and export in regulating Yki/YAP/TAZ localisation and function is highly cell type and context dependent. There are many mechanisms that regulate each of these processes but their relative importance seems to vary depending on cellular context. However, it is interesting to note several recurring themes on the regulation of Yki/YAP/TAZ subcellular localisation. These include

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the importance of the interaction between Yki/YAP/TAZ and Sd/TEADs (Wang et al., 2016b; Chan et al., 2009; Kofler et al., 2018), inhibition of Yki/YAP/TAZ import rate by Hippo signalling (Manning et al., 2018; Kofler et al., 2018), and processes downstream of the actin cytoskeleton that are able to regulate Yki/YAP/TAZ localisation via modulation of nuclear shuttling (Furukawa et al., 2017; Ege et al., 2018; Elosegui-Artola et al., 2017; Dupont et al., 2011).

The role of 14-3-3 proteins in Yki/YAP/TAZ regulation

14-3-3 proteins have diverse roles in development and commonly function through direct binding to regulate the localisation of other proteins. In this section, we discuss the function of 14-3-3 proteins in regulating the localisation of Yki/YAP/TAZ. 14-3-3 proteins were initially linked to regulation of Hippo signalling when they were found to bind to phosphorylated Yki/YAP/TAZ (Ren et al., 2010; Dong et al., 2007; Oh and Irvine, 2008). This suggested a model in which Warts/LATS1/2-mediated phosphorylation of Yki/YAP/TAZ leads to their interaction with predominantly cytoplasmic 14-3-3 proteins and, hence, stable sequestration of Yki/YAP/TAZ in the cytoplasm. There is much compelling data from biochemical, genetic and cell biological studies to suggest that 14-3-3 proteins are important for regulating the localisation and function of Yki/YAP/TAZ (Ren et al., 2010; Dong et al., 2007; Oh and Irvine, 2008), but, as we discuss below, their role and the importance of sequestration is a point of contention.

If binding of Yki/YAP/TAZ to 14-3-3 proteins leads to stable sequestration, one would expect this interaction to abrogate or reduce the rate of Yki/YAP/TAZ nuclear import. However, a mutant form of YAP lacking all inhibitory phosphorylation sites targeted by the Hippo pathway (YAP-5SA) was shown to be imported into the nucleus at the same rate as wild-type YAP, when measured using FLIP (Ege et al., 2018). Given that phosphorylation of these sites provides binding sites for 14-3-3 proteins (and therefore YAP-5SA is not able to bind 14-3-3 proteins), this argues against the model of cytoplasmic sequestration of YAP by 14-3-3 proteins and this interaction being a rate-limiting mechanism for YAP nuclear import. However, this contrasts to some degree with data indicating that TAZ-4SA (equivalent to YAP-5SA) accumulates in the nucleus to a greater extent than wild-type TAZ upon inhibition of export with LMB (Kofler et al., 2018). This could reflect a difference between YAP and TAZ, or may be due to the cell types studied, such that in some contexts a portion of YAP or TAZ is stably sequestered in the cytoplasm. Live imaging of endogenously fluorescently tagged Yki in *Drosophila* larval wing imaginal disc epithelia also shows that, although initially cytoplasmic in many cells, Yki accumulates in the nucleus on the scale of minutes after blocking nuclear export by LMB treatment. This indicates that Yki rapidly and continuously shuttles in and out of the nucleus *in vivo*, and that the majority of protein in the cytoplasm is not stably sequestered (Manning et al., 2018). Further experiments are therefore needed to determine whether this is also true in other tissues and, additionally, whether 14-3-3 proteins and cytoplasmic sequestration regulate Yki/YAP/TAZ shuttling *in vivo*.

A role for 14-3-3 proteins in regulating shuttling is supported by the finding that the export rate of YAP-5SA is greatly reduced compared with that of wild-type YAP (Ege et al., 2018), indicating that 14-3-3 proteins may operate primarily by promoting nuclear export. This is in addition to the finding that the binding of 14-3-3 proteins to TAZ reduces the potency of the TAZ NLS, indicating a mechanism by which this class of protein can increase the cytoplasmic pool of TAZ (Kofler et al., 2018). The binding of 14-3-3 proteins and TEADs to TAZ is also thought to be competitive; thus, the balance of these interactions plays an important role in establishing the steady-state localisation of TAZ and also determines its transcriptional activity (Kofler et al., 2018). This competitive interaction between 14-3-3 and the predominantly nuclear TEADs for TAZ binding also suggests that the proposed exclusively cytoplasmic role for 14-3-3 proteins in Yki/YAP/TAZ regulation is overly simplistic. Additionally, it has long been established in cell culture settings that 14-3-3 proteins themselves shuttle between the nucleus and the cytoplasm, and that interaction with binding partners in the nucleus is important for their function (Brunet et al., 2002; Hemert et al., 2004). Although it is possible that target protein binding prevents 14-3-3 proteins from shuttling, by increasing the size of the resulting complex (Kofler et al., 2018), it is also possible that the impact of 14-3-3 binding on Yki/YAP/TAZ localisation is due to changes in shuttling rates and an increase in transient cytoplasmic interactions rather than stable sequestration. An interesting open question concerns the relative importance of the different 14-3-3 isotypes (two in *Drosophila*, seven in vertebrates) in regulating Yki/YAP/TAZ localisation, as these isotypes display different localisations in cell culture (Hemert et al., 2004), and have clear functional differences *in vivo* (Acevedo et al., 2007).

Other proteins that regulate Yki/YAP/TAZ localisation and abundance

The subcellular localisation of Yki/YAP/TAZ has also been shown to be impacted by interactions with other proteins, most notably Angiomotin (AMOT) family proteins, although the influence of these interactions on shuttling rates remains to be fully explored. Binding of YAP and TAZ to AMOT (and the related proteins AMOTL1 and AMOTL2) appears to be independent of YAP and TAZ phosphorylation status (S127 and S89, respectively) and primarily promotes cytoplasmic localisation of YAP (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011). However, interaction with AMOT is also able to promote nuclear localisation of YAP in situations when AMOT is dephosphorylated at S176 (Moleirinho et al., 2017). AMOTs also regulate the localisation of Yap1 (zebrafish YAP) in the developing zebrafish vasculature following lumenisation; in this context, shear flow causes dissociation of cytoplasmic Yap1-AMOT complexes and increases the nuclear localisation and transcriptional activity of Yap1 (Nakajima et al., 2017).

Yki, YAP and TAZ are also regulated at the level of protein abundance by ubiquitin-mediated proteolysis by different mechanisms. In response to phosphorylation of carboxy terminal serine residues by LATS1/2 and CK1ε, both YAP and TAZ are targeted for proteasomal degradation, with TAZ being more labile than YAP (Kim and Jho, 2018). These residues are not conserved in Yki, but phosphorylation of Yki S169, which is juxtaposed to the major Warts phosphorylation site (Yki S168), by CDK7 was recently shown to protect Yki from proteasomal degradation by the E3 ubiquitin ligase $CRL4^{DCAF12}$ (Cho et al., 2020). This serine residue and CDK7-dependent regulatory mechanism appears to be conserved in human YAP and TAZ. In *Drosophila*, the Myopic phosphatase and alpha-arrestin family member Leash also directs lysosomal-mediated degradation of Yki (Verghese and Moberg, 2020), although it is unclear if this regulatory mechanism is conserved in mammals. Multiple deubiquitylating enzymes have also been implicated in fine-tuning the abundance of Yki, YAP and TAZ (Kim and Jho, 2018).

Phase separation of YAP and TAZ

Two very recent studies suggest that the ability of YAP and TAZ to regulate gene expression is influenced by their propensity to form

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phase-separated nuclear condensates (Lu et al., 2020; Cai et al., 2019). Phase separation of DNA sub-domains into liquid like droplets can either prevent (Larson et al., 2017) or promote (Boija et al., 2018) transcription. These condensates, considered a form of membraneless organelle, are formed through phase separation mediated by cooperative interactions between molecules that can compartmentalise biochemical reactions within cells (Alberti et al., 2019; Su et al., 2016). In some instances, such condensates have been proposed to generate transcriptional 'hubs' (Hnisz et al., 2017). They are distinct from other nuclear membraneless organelles, such as nucleoli, Cajal bodies and nuclear speckles (Mao et al., 2011; Zhu and Brangwynne, 2015). Similar condensates, which contain transcription factors such as Oct4 (Pou5f1), GCN4 and the oestrogen receptor, as well as core transcriptional components and regulators, such as RNA Pol II, Mediator and BRD4, have also been reported to modulate gene expression (Boija et al., 2018; Sabari et al., 2018; Lu et al., 2018; Cho et al., 2018a).

TAZ phase separation has been characterised using purified TAZ protein in human cells grown in culture, where it forms nuclear condensates containing important transcriptional regulators, including TEAD4, BRD4, Mediator and CDK9 (Lu et al., 2020). Mechanistically, TAZ phase separation is primarily mediated by its coiled-coil domain, and to a lesser extent by its WW domain, both of which are dispensable for direct interactions with key partners, such as TEADs (Lu et al., 2020). Importantly, TAZ phase separation is regulated by Hippo pathway activity: LATS2-mediated phosphorylation of TAZ negatively regulates its capacity to form nuclear condensates and activate gene expression (Lu et al., 2020). TAZ nuclear condensates are also regulated by other related kinases, NDR1 and NDR2 (also known as STK38 and STK38L), which can also phosphorylate TAZ. This suggests that Hippo signalling can regulate TAZ activity at multiple levels, not just by affecting the local level of TAZ in the nucleus (by changing the rate of TAZ nuclear-cytoplasmic shuttling and degradation), but also by affecting its ability to form phase-separated transcriptional hubs. However, it remains challenging to dissociate between these roles, as the local nuclear concentration of TAZ would likely impact its ability to form phase-separated condensates.

Phase separation of YAP has also recently been reported and linked to its role in gene regulation (Cai et al., 2019). Unlike TAZ, YAP does not readily phase separate into nuclear condensates in standard cell culture conditions (Lu et al., 2020). Under hyperosmotic conditions, the non-ionic crowder solutions polyethylene glycol (PEG) and sorbitol can trigger phase separation of YAP into both nuclear and cytoplasmic condensates in cultured cells and cause purified YAP proteins to form characteristic droplets *in vitro* (Cai et al., 2019). However, purified YAP proteins do not form these droplets in response to typical triggers of phase separation, including increased salt (NaCl), temperature or protein concentration (Cai et al., 2019; Lu et al., 2020), suggesting that YAP phase separation is specifically a hyperosmotic stress response.

Similar to TAZ condensates, induced nuclear YAP condensates contain TEAD1 as well as TAZ. Moreover, the local chromatin within these condensates is remodelled into a more open structure, with the condensates gradually becoming enriched in RNA Pol II, forming hubs of RNA production (Cai et al., 2019). Notably, YAP also forms large cytoplasmic condensates enriched in LATS1 and NLK, both of which act to regulate YAP nuclear translocation through phosphorylation, raising the possibility that these may be a sort of regulatory hub (Cai et al., 2019). Mechanistically, the molecular basis for phase separation of YAP and TAZ appears to be different. Using elegant domain-switching experiments, it was shown that the substitution of TAZ's coiled-coil domain with that of YAP's prevents TAZ phase separation and its ability to induce target gene expression (Lu et al., 2020). Instead, both nuclear and cytoplasmic YAP condensate formation rely on its disordered carboxy terminal transcription activation domain (TAD) (Cai et al., 2019). Beyond this, little is known about how YAP phase separation is regulated, for example whether it is linked to Hippo pathway activity. It is also difficult to ascertain the importance of YAP phase separation for target gene regulation, because deletion of its TAD may affect this function, independently of its role in phase separation (Yagi et al., 1999; Finch-Edmondson et al., 2016; Zhang et al., 2012).

There is therefore growing *in vivo* evidence for Yki/YAP/TAZ phase separation, although limited validation has been performed to investigate molecular mechanisms or to confirm its biological significance. Immunostaining of YAP in mouse kidneys has demonstrated that hyperosmotic medulla regions have more nuclear puncta than iso-osmotic cortical regions, in line with observations from cell culture studies (Cai et al., 2019). In *Drosophila* egg chambers, stretched follicle cells become enriched in granular nuclear Yki puncta (Sidor et al., 2019). In the future, it will be important to validate these descriptive assessments, for example by determining whether the puncta are sensitive to treatment with hexanediol, a chemical used to disrupt phase-separated condensates, and to see whether they contain important transcriptional regulatory components, such as RNA Pol II or CDK9. This would complement other studies that have shown how the carboxy terminal heptapeptide repeats in RNA Pol II mediate phase separation and are important for transcription elongation (Boehning et al., 2018; Lu et al., 2019; Lu et al., 2018). Furthermore, future work will be required to test whether the molecular mechanisms underlying Yki/YAP/TAZ phase separation, identified primarily using purified proteins and *in vitro* cell culture systems, are relevant *in vivo*.

Overall, the current evidence that phase separation regulates TAZ, YAP and Yki gene regulatory function is compelling, and deserves further investigation. This is compounded by recent studies showing how phase-separated nuclear condensates compartmentalise important transcription machinery, forming 'transcription factories' to drive gene expression (Boija et al., 2018; Sabari et al., 2018). Fundamentally, their existence may help us understand how cells overcome transcriptional stochasticity, as they could tune bursting behaviour by modifying the environment and frequency of interactions between specific loci and transcriptional machinery. In addition to promoting transcription, it is possible that phase separation of Yki/YAP/TAZ into nuclear condensates may insulate them away from the upstream regulatory components of the Hippo signalling pathway, many of which are cytoplasmic (Harvey et al., 2013). However, other studies have indicated that phase separation could be a mechanism of cellular buffering against gene expression noise (Klosin et al., 2020). It will therefore be important to determine whether all YAP and TAZ puncta function to promote transcription, or if some act to buffer against excessive interactions with chromatin to reduce heterogeneity in their levels and activity across a tissue.

Conclusions

Since their discovery as the key downstream mediators of the Hippo signalling pathway, much has been revealed regarding the biology of Yki/YAP/TAZ and how they regulate gene expression in a wide variety of developmental and disease contexts. The majority of current evidence indicates that the predominant mechanism by

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which Yki/YAP/TAZ are regulated is at the level of their nuclear-cytoplasmic distribution. Significant progress has been made in identifying the proteins responsible for this regulation, although we have only recently begun to piece together the complex dynamics of this process. Much of this progress has been due to the application of live-imaging approaches, which are essential for characterising dynamic processes. Ongoing advances in microscopy technologies are extending what is possible in terms of rapid and sensitive *in vivo* imaging, and the application of these technologies to the outstanding questions in the field of Hippo signalling are sure to bring many new and exciting insights. Such examples can be seen in recent studies applying elegant *in vivo* imaging to uncover novel dynamics and functions of Yap1 during lymphatics and blood vessel formation in zebrafish (Grimm et al., 2019; Nakajima et al., 2017). An important observation from current studies is that the regulation of Yki/YAP/TAZ shuttling depends on the cell type and developmental context being analysed, and so it seems that much can still be learned by assessing Yki/YAP/TAZ shuttling in a wider range of developmental settings. For instance, to what extent is regulation of shuttling important for the striking relocalisation of Yap in the mouse pre-implantation embryo, where nuclear or cytoplasmic localisation of Yap determines whether cells adopt the trophectoderm or inner cell mass fate (Nishioka et al., 2009)? Additionally, what role does deregulation of shuttling play in the oncogenic activity of YAP and TAZ? Oncogenic transformation of MCF10A breast epithelial cells leads to increased YAP shuttling and transcriptional activity (Franklin et al., 2019 preprint), but to what extent is this observed in human cancers?

Given that Yki/YAP/TAZ shuttle into the nucleus even when their predominant localisation is cytoplasmic, it will be important to determine the extent to which low levels of nuclear Yki/YAP/TAZ are able to elicit transcriptional responses, as in many settings even low levels may be sufficient. Additionally, there is emerging evidence that the rate of shuttling, independent of the total nuclear pool, may be important for the transcriptional function of YAP (Franklin et al., 2019 preprint), and that it is possible for Yki/YAP/TAZ to localise to the nucleus but be transcriptionally inactive (Parker and Struhl, 2015; Ege et al., 2018). Thus, it is overly simplistic to only assess nuclear or cytoplasmic levels of Yki/YAP/TAZ as a proxy for activity. Additionally, given that phosphorylated YAP is found within the nucleus (Ege et al., 2018; Wada et al., 2011) it will be interesting to determine the extent to which post-translational modifications of Yki/YAP/TAZ impact on transcriptional function beyond simply regulating localisation. An important precedent has been demonstrated here with the observation that phosphorylation of Y357 of YAP impacts transcriptional activity without greatly affecting localisation (Ege et al., 2018). As more layers of Yki/YAP/TAZ shuttling regulation are identified in tractable cell culture and *in vivo* systems, the next task is the identification of the relative importance of each factor in complex *in vivo* settings. This is especially important in the context of regulation of Yki/YAP/TAZ activity, which is known to be highly sensitive to, and dependent on, the extracellular environment.

Recent studies demonstrating the phase-separation properties of both TAZ and YAP are further reshaping our understanding of how these proteins function (Cai et al., 2019; Lu et al., 2020). They join a growing list of transcriptional components and regulators, including Mediator, BRD4 and RNA Pol II, that elicit this behaviour in order to facilitate target gene expression (Boija et al., 2018; Sabari et al., 2018; Boehning et al., 2018; Lu et al., 2018). Going forward, it will be essential to improve our understanding of the temporal dynamics of how Hippo pathway activity transduces its effects on Yki/YAP/TAZ phase separation in physiological settings. For example, what are the timescales of condensate formation and dissipation in response to signalling changes? What duration of time are condensates required for in order to change target gene transcriptional outputs effectively at a local level? Given that macromolecular crowding can elicit phase separation, future work should also explore whether there is a threshold level of Yki/YAP/TAZ required to nucleate condensate formation. Conceptually, this could link nuclear-cytoplasmic shuttling with phase separation-driven functional consequences, as altering the balance of Yki/YAP/TAZ levels within the nucleus would make condensate formation more or less favourable. The application of imaging techniques such as fluorescence correlation spectroscopy offer precise measurements of protein levels in living cells, and would enable researchers to test this hypothesis. Additionally, the field will also need to determine the extent to which phase separation of Yki/YAP/TAZ acts as a mechanism of cell buffering against excessive protein levels (Klosin et al., 2020).

Overall, these recent findings are highly significant within the Hippo field because they offer fresh insights into the behaviour and regulation of these crucial transcriptional regulatory proteins. The complex mechanisms that have been uncovered should spur on research that will further transform our understanding of the role of the Hippo pathway and Yki/YAP/TAZ in both development and disease.

Competing interests

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References

Acevedo, S. F., Tsigkari, K. K., Grammenoudi, S. and Skoulakis, E. M. C. (2007). In vivo functional specificity and homeostasis of Drosophila 14-3-3 proteins. *Genetics* **177**, 239-253. doi:10.1534/genetics.107.072280

Alberti, S., Gladfelter, A. and Mittag, T. (2019). Considerations and challenges in studying liquid-liquid phase separation and biomolecular condensates. *Cell* **176**, 419-434. doi:10.1016/j.cell.2018.12.035

Boehning, M., Dugast-Darzacq, C., Rankovic, M., Hansen, A. S., Yu, T., Marie-Nelly, H., Mcswiggen, D. T., Kokic, G., Dailey, G. M., Cramer, P. et al. (2018). RNA polymerase II clustering through carboxy-terminal domain phase separation. *Nat. Struct. Mol. Biol.* **25**, 833. doi:10.1038/s41594-018-0112-y

Boggiano, J. C., Vanderzalm, P. J. and Fehon, R. G. (2011). Tao-1 phosphorylates Hippo/MST kinases to regulate the Hippo-Salvador-Warts tumor suppressor pathway. *Dev. Cell* **21**, 888-895. doi:10.1016/j.devcel.2011.08.028

Boija, A., Klein, I. A., Sabari, B. R., Dall'agnese, A., Coffey, E. L., Zamudio, A. V., Li, C. H., Shrinivas, K., Manteiga, J. C., Hannett, N. M. et al. (2018). Transcription factors activate genes through the phase-separation capacity of their activation domains. *Cell* **175**, 1842-1855.e16. doi:10.1016/j.cell.2018.10.042

Brunet, A., Kanai, F., Stehn, J., Xu, J., Sarbassova, D., Frangioni, J. V., Dalal, S. N., Decaprio, J. A., Greenberg, M. E. and Yaffe, M. B. (2002). 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. *J. Cell Biol.* **156**, 817-828. doi:10.1083/jcb.200112059

Cai, D., Feliciano, D., Dong, P., Flores, E., Gruebele, M., Porat-Shliom, N., Sukenik, S., Liu, Z. and Lippincott-Schwartz, J. (2019). Phase separation of YAP reorganizes genome topology for long-term YAP target gene expression. *Nat. Cell Biol.* **21**, 1578-1589. doi:10.1038/s41556-019-0433-z

Chan, S. W., Lim, C. J., Loo, L. S., Chong, Y. F., Huang, C. and Hong, W. (2009). TEADs mediate nuclear retention of TAZ to promote oncogenic transformation. *J. Biol. Chem.* **284**, 14347-14358. doi:10.1074/jbc.M901568200

Chan, S. W., Lim, C. J., Chong, Y. F., Pobbati, A. V., Huang, C. and Hong, W. (2011). Hippo pathway-independent restriction of TAZ and YAP by angiomotin. *J. Biol. Chem.* **286**, 7018-7026. doi:10.1074/jbc.C110.212621

Cho, W.-K., Spille, J.-H., Hecht, M., Lee, C., Li, C., Grube, V. and Cisse, I. I. (2018a). Mediator and RNA polymerase II clusters associate in transcription-dependent condensates. *Science* **361**, 412-415. doi:10.1126/science.aar4199

Cho, Y. S., Zhu, J., Li, S., Wang, B., Han, Y. and Jiang, J. (2018b). Regulation of Yki/Yap subcellular localization and Hpo signaling by a nuclear kinase PRP4K. *Nat. Commun.* **9**, 1657. doi:10.1038/s41467-018-04090-2

Cho, Y. S., Li, S., Wang, X., Zhu, J., Zhuo, S., Han, Y., Yue, T., Yang, Y. and Jiang, J. (2020). CDK7 regulates organ size and tumor growth by safeguarding the Hippo pathway effector Yki/Yap/Taz in the nucleus. *Genes Dev.* **34**, 53-71. doi:10.1101/gad.333146.119

Cordenonsi, M., Zanconato, F., Azzolin, L., Forcato, M., Rosato, A., Frasson, C., Inui, M., Montagner, M., Parenti, A. R., Poletti, A. et al. (2011). The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells. *Cell* **147**, 759-772. doi:10.1016/j.cell.2011.09.048

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S. A., Gayyed, M. F., Anders, R. A., Maitra, A. and Pan, D. (2007). Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell* **130**, 1120-1133. doi:10.1016/j.cell.2007.07.019

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., Le Digabel, J., Forcato, M., Bicciato, S. et al. (2011). Role of YAP/TAZ in mechanotransduction. *Nature* **474**, 179-183. doi:10.1038/nature10137

Ege, N., Dowbaj, A. M., Jiang, M., Howell, M., Hooper, S., Foster, C., Jenkins, R. P. and Sahai, E. (2018). Quantitative analysis reveals that actin and Src-family kinases regulate nuclear YAP1 and its export. *Cell Syst.* **6**, 692-708.e13. doi:10.1016/j.cels.2018.05.006

Elosegui-Artola, A., Andreu, I., Beedle, A. E. M., Lezamiz, A., Uroz, M., Kosmalska, A. J., Oria, R., Kechagia, J. Z., Rico-Lastres, P., Le Roux, A.-L. et al. (2017). Force triggers YAP nuclear entry by regulating transport across nuclear pores. *Cell* **171**, 1397-1410.e14. doi:10.1016/j.cell.2017.10.008

Fang, L., Teng, H., Wang, Y., Liao, G., Weng, L., Li, Y., Wang, X., Jin, J., Jiao, C., Chen, L. et al. (2018). SET1A-mediated mono-methylation at K342 regulates YAP activation by blocking its nuclear export and promotes tumorigenesis. *Cancer Cell* **34**, 103-118.e9. doi:10.1016/j.ccell.2018.06.002

Finch-Edmondson, M. L., Strauss, R. P., Clayton, J. S., Yeoh, G. C. and Callus, B. A. (2016). Splice variant insertions in the C-terminus impairs YAP's transactivation domain. *Biochem. Biophys. Rep.* **6**, 24-31. doi:10.1016/j.bbrep.2016.02.015

Franklin, J. M., Ghosh, R. P., Shi, Q. and Liphardt, J. T. (2019). Concerted localization resets precede YAP-dependent transcription. *bioRxiv*, 539049. doi:10.1101/539049

Furukawa, K. T., Yamashita, K., Sakurai, N. and Ohno, S. (2017). The epithelial circumferential actin belt regulates YAP/TAZ through nucleocytoplasmic shuttling of merlin. *Cell Rep.* **20**, 1435-1447. doi:10.1016/j.celrep.2017.07.032

Gaspar, P. and Tapon, N. (2014). Sensing the local environment: actin architecture and Hippo signalling. *Curr. Opin. Cell Biol.* **31**, 74-83. doi:10.1016/j.ceb.2014.09.003

Goulev, Y., Fauny, J. D., Gonzalez-Marti, B., Flagiello, D., Silber, J. and Zider, A. (2008). Scalloped interacts with YORKIE, the nuclear effector of the hippo tumor-suppressor pathway in Drosophila. *Curr. Biol.* **18**, 435-441. doi:10.1016/j.cub.2008.02.034

Grimm, L., Nakajima, H., Chaudhury, S., Bower, N. I., Okuda, K. S., Cox, A. G., Harvey, N. L., Koltowska, K., Mochizuki, N. and Hogan, B. M. (2019). Yap1 promotes sprouting and proliferation of lymphatic progenitors downstream of Vegfc in the zebrafish trunk. *eLife* **8**, e42881. doi:10.7554/eLife.42881

Grusche, F. A., Richardson, H. E. and Harvey, K. F. (2010). Upstream regulation of the hippo size control pathway. *Curr. Biol.* **20**, R574-R582. doi:10.1016/j.cub.2010.05.023

Guo, T., Lu, Y., Li, P., Yin, M.-X., Lv, D., Zhang, W., Wang, H., Zhou, Z., Ji, H., Zhao, Y. et al. (2013). A novel partner of Scalloped regulates Hippo signaling via antagonizing Scalloped-Yorkie activity. *Cell Res.* **23**, 1201-1214. doi:10.1038/cr.2013.120

Guo, P., Lee, C. H., Lei, H., Zheng, Y., Pulgar Prieto, K. D. and Pan, D. (2019). Nerfin-1 represses transcriptional output of Hippo signaling in cell competition. *eLife* **8**, e38843. doi:10.7554/eLife.38843

Halder, G., Dupont, S. and Piccolo, S. (2012). Transduction of mechanical and cytoskeletal cues by YAP and TAZ. *Nat. Rev. Mol. Cell Biol.* **13**, 591-600. doi:10.1038/nrm3416

Harvey, K. F., Pfleger, C. M. and Hariharan, I. K. (2003). The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* **114**, 457-467. doi:10.1016/S0092-8674(03)00557-9

Harvey, K. F., Zhang, X. and Thomas, D. M. (2013). The Hippo pathway and human cancer. *Nat. Rev. Cancer* **13**, 246-257. doi:10.1038/nrc3458

Hemert, M. J. V., Niemantsverdriet, M., Schmidt, T., Backendorf, C. and Spaink, H. P. (2004). Isoform-specific differences in rapid nucleocytoplasmic shuttling cause distinct subcellular distributions of 14-3-3σ and 14-3-3ζ. *J. Cell Sci.* **117**, 1411-1420. doi:10.1242/jcs.00990

Hillmer, R. E. and Link, B. A. (2019). The roles of Hippo signaling transducers Yap and Taz in chromatin remodeling. *Cells* **8**, 502. doi:10.3390/cells8050502

Hnisz, D., Shrinivas, K., Young, R. A., Chakraborty, A. K. and Sharp, P. A. (2017). A phase separation model for transcriptional control. *Cell* **169**, 13-23. doi:10.1016/j.cell.2017.02.007

Jia, J., Zhang, W., Wang, B., Trinko, R. and Jiang, J. (2003). The Drosophila Ste20 family kinase dMST functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis. *Genes Dev.* **17**, 2514-2519. doi:10.1101/gad.1134003

Johansen, K. M., Cai, W., Deng, H., Bao, X., Zhang, W., Girton, J. and Johansen, J. (2009). Polytene chromosome squash methods for studying transcription and epigenetic chromatin modification in Drosophila using antibodies. *Methods* **48**, 387-397. doi:10.1016/j.ymeth.2009.02.019

Kango-Singh, M., Nolo, R., Tao, C., Verstreken, P., Hiesinger, P. R., Bellen, H. J. and Halder, G. (2002). Shar-pei mediates cell proliferation arrest during imaginal disc growth in Drosophila. *Development* **129**, 5719-5730. doi:10.1242/dev.00168

Kim, Y. and Jho, E.-H. (2018). Regulation of the Hippo signaling pathway by ubiquitin modification. *BMB Rep.* **51**, 143-150. doi:10.5483/BMBRep.2018.51.3.017

Klosin, A., Oltsch, F., Harmon, T., Honigmann, A., Jülicher, F., Hyman, A. A. and Zechner, C. (2020). Phase separation provides a mechanism to reduce noise in cells. *Science* **367**, 464-468. doi:10.1126/science.aav6691

Kofler, M., Speight, P., Little, D., Di Ciano-Oliveira, C., Szászi, K. and Kapus, A. (2018). Mediated nuclear import and export of TAZ and the underlying molecular requirements. *Nat. Commun.* **9**, 4966. doi:10.1038/s41467-018-07450-0

Koontz, L. M., Liu-Chittenden, Y., Yin, F., Zheng, Y., Yu, J., Huang, B., Chen, Q., Wu, S. and Pan, D. (2013). The Hippo effector Yorkie controls normal tissue growth by antagonizing Scalloped-mediated default repression. *Dev. Cell* **25**, 388-401. doi:10.1016/j.devcel.2013.04.021

Lai, Z.-C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L.-L. and Li, Y. (2005). Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. *Cell* **120**, 675-685. doi:10.1016/j.cell.2004.12.036

Larson, A. G., Elnatan, D., Keenen, M. M., Trnka, M. J., Johnston, J. B., Burlingame, A. L., Agard, D. A., Redding, S. and Narlikar, G. J. (2017). Liquid droplet formation by HP1alpha suggests a role for phase separation in heterochromatin. *Nature* **547**, 236-240. doi:10.1038/nature22822

Li, Q., Li, S., Mana-Capelli, S., Roth Flach, R. J., Danai, L. V., Amcheslavsky, A., Nie, Y., Kaneko, S., Yao, X., Chen, X. et al. (2014). The conserved misshapen-warts-Yorkie pathway acts in enteroblasts to regulate intestinal stem cells in Drosophila. *Dev. Cell* **31**, 291-304. doi:10.1016/j.devcel.2014.09.012

Lu, H., Yu, D., Hansen, A. S., Ganguly, S., Liu, R., Heckert, A., Darzacq, X. and Zhou, Q. (2018). Phase-separation mechanism for C-terminal hyperphosphorylation of RNA polymerase II. *Nature* **558**, 318-323. doi:10.1038/s41586-018-0174-3

Lu, F., Portz, B. and Gilmour, D. S. (2019). The C-terminal domain of RNA Polymerase II is a multivalent targeting sequence that supports Drosophila development with only consensus heptads. *Mol. Cell* **73**, 1232-1242.e4. doi:10.1016/j.molcel.2019.01.008

Lu, Y., Wu, T., Gutman, O., Lu, H., Zhou, Q., Henis, Y. I. and Luo, K. (2020). Phase separation of TAZ compartmentalizes the transcription machinery to promote gene expression. *Nat. Cell Biol.* **22**, 453-464. doi:10.1038/s41556-020-0485-0

Manning, S. A., Dent, L. G., Kondo, S., Zhao, Z. W., Plachta, N. and Harvey, K. F. (2018). Dynamic fluctuations in subcellular localization of the Hippo pathway effector yorkie in vivo. *Curr. Biol.* **28**, 1651-1660.e4. doi:10.1016/j.cub.2018.04.018

Mao, Y. S., Zhang, B. and Spector, D. L. (2011). Biogenesis and function of nuclear bodies. *Trends Genet.* **27**, 295-306. doi:10.1016/j.tig.2011.05.006

Meng, Z., Moroishi, T., Mottier-Pavie, V., Plouffe, S. W., Hansen, C. G., Hong, A. W., Park, H. W., Mo, J.-S., Lu, W., Lu, S. et al. (2015). MAP4K family kinases act in parallel to MST1/2 to activate LATS1/2 in the Hippo pathway. *Nat. Commun.* **6**, 8357. doi:10.1038/ncomms9357

Meng, Z., Moroishi, T. and Guan, K.-L. (2016). Mechanisms of Hippo pathway regulation. *Genes Dev.* **30**, 1-17. doi:10.1101/gad.274027.115

Moleirinho, S., Hoxha, S., Mandati, V., Curtale, G., Troutman, S., Ehmer, U. and Kissil, J. L. (2017). Regulation of localization and function of the transcriptional co-activator YAP by angiomotin. *eLife* **6**, e23966. doi:10.7554/eLife.23966

Nakajima, H., Yamamoto, K., Agarwala, S., Terai, K., Fukui, H., Fukuhara, S., Ando, K., Miyazaki, T., Yokota, Y., Schmelzer, E. et al. (2017). Flow-dependent endothelial YAP regulation contributes to vessel maintenance. *Dev. Cell* **40**, 523-536.e6. doi:10.1016/j.devcel.2017.02.019

Nishioka, N., Inoue, K.-I., Adachi, K., Kiyonari, H., Ota, M., Ralston, A., Yabuta, N., Hirahara, S., Stephenson, R. O., Ogonuki, N. et al. (2009). The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Dev. Cell* **16**, 398-410. doi:10.1016/j.devcel.2009.02.003

Oh, H. and Irvine, K. D. (2008). In vivo regulation of Yorkie phosphorylation and localization. *Development* **135**, 1081-1088. doi:10.1242/dev.015255

Oh, H. and Irvine, K. D. (2009). In vivo analysis of Yorkie phosphorylation sites. *Oncogene* **28**, 1916-1927. doi:10.1038/onc.2009.43

Pantalacci, S., Tapon, N. and Léopold, P. (2003). The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. *Nat. Cell Biol.* **5**, 921-927. doi:10.1038/ncb1051

Parker, J. and Struhl, G. (2015). Scaling the Drosophila wing: TOR-dependent target gene access by the Hippo pathway transducer yorkie. *PLoS Biol.* **13**, e1002274. doi:10.1371/journal.pbio.1002274

Poon, C. L. C., Lin, J. I., Zhang, X. and Harvey, K. F. (2011). The sterile 20-like kinase tao-1 controls tissue growth by regulating the Salvador-Warts-Hippo pathway. *Dev. Cell* **21**, 896-906. doi:10.1016/j.devcel.2011.09.012

Rauskolb, C., Sun, S., Sun, G., Pan, Y. and Irvine, K. D. (2014). Cytoskeletal tension inhibits Hippo signaling through an ajuba-warts complex. *Cell* **158**, 143-156. doi:10.1016/j.cell.2014.05.035

Ren, F., Zhang, L. and Jiang, J. (2010). Hippo signaling regulates Yorkie nuclear localization and activity through 14-3-3 dependent and independent mechanisms. *Dev. Biol.* **337**, 303-312. doi:10.1016/j.ydbio.2009.10.046

Ribeiro, P. S., Josué, F., Wepf, A., Wehr, M. C., Rinner, O., Kelly, G., Tapon, N. and Gstaiger, M. (2010). Combined functional genomic and proteomic approaches identify a PP2A complex as a negative regulator of Hippo signaling. *Mol. Cell* **39**, 521-534. doi:10.1016/j.molcel.2010.08.002

Sabari, B. R., Dall'agnese, A., Boija, A., Klein, I. A., Coffey, E. L., Shrinivas, K., Abraham, B. J., Hannett, N. M., Zamudio, A. V., Manteiga, J. C. et al. (2018). Coactivator condensation at super-enhancers links phase separation and gene control. *Science* 361, eaar3958. doi:10.1126/science.aar3958

Sidor, C., Borreguero-Munoz, N., Fletcher, G. C., Elbediwy, A., Guillermin, O. and Thompson, B. J. (2019). Mask family proteins ANKHD1 and ANKRD17 regulate YAP nuclear import and stability. *eLife* **8**, e48601. doi:10.7554/eLife.48601

Su, X., Ditlev, J. A., Hui, E., Xing, W., Banjade, S., Okrut, J., King, D. S., Taunton, J., Rosen, M. K. and Vale, R. D. (2016). Phase separation of signaling molecules promotes T cell receptor signal transduction. *Science* **352**, 595-599. doi:10.1126/science.aad9964

Tapon, N., Harvey, K. F., Bell, D. W., Wahrer, D. C. R., Schiripo, T. A., Haber, D. A. and Hariharan, I. K. (2002). Salvador promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell* **110**, 467-478. doi:10.1016/S0092-8674(02)00824-3

Timney, B. L., Raveh, B., Mironska, R., Trivedi, J. M., Kim, S. J., Russel, D., Wente, S. R., Sali, A. and Rout, M. P. (2016). Simple rules for passive diffusion through the nuclear pore complex. *J. Cell Biol.* **215**, 57-76. doi:10.1083/jcb.201601004

Udan, R. S., Kango-Singh, M., Nolo, R., Tao, C. and Halder, G. (2003). Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat. Cell Biol.* **5**, 914-920. doi:10.1038/ncb1050

Verghese, S. and Moberg, K. (2020). Roles of membrane and vesicular traffic in regulation of the Hippo pathway. *Front. Cell Dev. Biol.* **7**, 384. doi:10.3389/fcell.2019.00384

Vissers, J. H. A., Froldi, F., Schröder, J., Papenfuss, A. T., Cheng, L. Y. and Harvey, K. F. (2018). The Scalloped and Nerfin-1 transcription factors cooperate to maintain neuronal cell fate. *Cell Rep.* **25**, 1561-1576.e7. doi:10.1016/j.celrep.2018.10.038

Wada, K.-I., Itoga, K., Okano, T., Yonemura, S. and Sasaki, H. (2011). Hippo pathway regulation by cell morphology and stress fibers. *Development* **138**, 3907-3914. doi:10.1242/dev.070987

Wang, J., Sinnett-Smith, J., Stevens, J. V., Young, S. H. and Rozengurt, E. (2016a). Biphasic regulation of Yes-associated protein (YAP) cellular localization, phosphorylation, and activity by G protein-coupled receptor agonists in intestinal epithelial cells A novel role for protein kinase D (PKD). *J. Biol. Chem.* **291**, 17988-18005. doi:10.1074/jbc.M115.711275

Wang, S., Lu, Y., Yin, M.-X., Wang, C., Wu, W., Li, J., Wu, W., Ge, L., Hu, L., Zhao, Y. et al. (2016b). Importin α1 Mediates Yorkie nuclear import via an N-terminal non-canonical nuclear localization signal. *J. Biol. Chem.* **291**, 7926-7937. doi:10.1074/jbc.M115.700823

Wang, W., Huang, J. and Chen, J. (2011). Angiomotin-like proteins associate with and negatively regulate YAP1. *J. Biol. Chem.* **286**, 4364-4370. doi:10.1074/jbc.C110.205401

Wu, S., Huang, J., Dong, J. and Pan, D. (2003). hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with Salvador and Warts. *Cell* **114**, 445-456. doi:10.1016/S0092-8674(03)00549-X

Wu, S., Liu, Y., Zheng, Y., Dong, J. and Pan, D. (2008). The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. *Dev. Cell* **14**, 388-398. doi:10.1016/j.devcel.2008.01.007

Yagi, R., Chen, L.-F., Shigesada, K., Murakami, Y. and Ito, Y. (1999). A WW domain-containing Yes-associated protein (YAP) is a novel transcriptional co-activator. *EMBO J.* **18**, 2551-2562. doi:10.1093/emboj/18.9.2551

Zhang, L., Ren, F., Zhang, Q., Chen, Y., Wang, B. and Jiang, J. (2008). The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. *Dev. Cell* **14**, 377-387. doi:10.1016/j.devcel.2008.01.006

Zhang, X., Milton, C. C., Humbert, P. O. and Harvey, K. F. (2009). Transcriptional output of the Salvador/Warts/Hippo pathway is controlled in distinct fashions in Drosophila melanogaster and mammalian cell lines. *Cancer Res.* **69**, 6033-6041. doi:10.1158/0008-5472.CAN-08-4592

Zhang, X., Grusche, F. A. and Harvey, K. F. (2012). Control of tissue growth and cell transformation by the Salvador/Warts/Hippo pathway. *PLoS ONE* **7**, e31994. doi:10.1371/journal.pone.0031994

Zhao, B., Wei, X., Li, W., Udan, R. S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L. et al. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev.* **21**, 2747-2761. doi:10.1101/gad.1602907

Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., Lin, J. D., Wang, C.-Y., Chinnaiyan, A. M., Lai, Z.-C. et al. (2008). TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev.* **22**, 1962-1971. doi:10.1101/gad.1664408

Zhao, B., Kim, J., Ye, X., Lai, Z.-C. and Guan, K.-L. (2009). Both TEAD-binding and WW domains are required for the growth stimulation and oncogenic transformation activity of yes-associated protein. *Cancer Res.* **69**, 1089-1098. doi:10.1158/0008-5472.CAN-08-2997

Zhao, B., Li, L., Lu, Q., Wang, L. H., Liu, C.-Y., Lei, Q. and Guan, K.-L. (2011). Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein. *Genes Dev.* **25**, 51-63. doi:10.1101/gad.2000111

Zhao, B., Li, L., Wang, L., Wang, C.-Y., Yu, J. and Guan, K.-L. (2012). Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. *Genes Dev.* **26**, 54-68. doi:10.1101/gad.173435.111

Zheng, Y. and Pan, D. (2019). The Hippo signaling pathway in development and disease. *Dev. Cell* **50**, 264-282. doi:10.1016/j.devcel.2019.06.003

Zheng, Y., Wang, W., Liu, B., Deng, H., Uster, E. and Pan, D. (2015). Identification of happyhour/MAP4K as alternative Hpo/Mst-like kinases in the Hippo kinase cascade. *Dev. Cell* **34**, 642-655. doi:10.1016/j.devcel.2015.08.014

Zhu, L. and Brangwynne, C. P. (2015). Nuclear bodies: the emerging biophysics of nucleoplasmic phases. *Curr. Opin. Cell Biol.* **34**, 23-30. doi:10.1016/j.ceb.2015.04.003