Control of proliferation and cancer growth by the Hippo signaling pathway

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Abstract

The control of cell division is essential for normal development and the maintenance of cellular homeostasis. Abnormal cell proliferation is associated with multiple pathological states, including cancer. While the Hippo/YAP signaling pathway was initially thought to control organ size and growth, increasing evidence indicates that this pathway also plays a major role in the control of proliferation independent of organ size control. In particular, accumulating evidence indicates that the Hippo/YAP signaling pathway functionally interacts with multiple other cellular pathways and serves as a central node in the regulation of cell division, especially in cancer cells. Here recent observations are highlighted that connect Hippo/YAP signaling to transcription, the basic cell cycle machinery, and the control of cell division. Furthermore, the oncogenic and tumor suppressive attributes of YAP/TAZ are reviewed which emphasizes the relevance of the Hippo pathway in cancer.

Introduction

A tight control of cell division is essential for the maintenance of homeostasis in adult organs and tissues. Several cellular mechanisms exist to regulate and fine tune cell proliferation during physiological growth and regeneration. Failures in one or more of these control mechanisms can result in unchecked cell divisions and, eventually, cancer development (1). A number of cellular pathways have been implicated in the regulation of cell division. In particular, the importance of the so-called "Hippo" signaling pathway in the control of proliferation was discovered a little more than 10 years ago in Drosophila (2, 3). Since then, it has become evident that the Hippo signaling pathway is involved in a plethora of cellular functions, ranging from organ size control, cellular differentiation, and metabolism (4–7). However, the control of cell proliferation – mainly by negatively regulating the pathway's downstream effectors YAP and TAZ – remains one of its central functions (8). In this review we will provide a detailed overview of the role of the Hippo pathway in the control of proliferation and summarize recently published data that shed light on the molecular basis for Hippo-mediated cell cycle control. Finally, we will discuss the

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implication of these findings for our understanding of the role of Hippo signaling in cancer and for the development of novel tumor therapies.

The canonical Hippo/YAP signaling pathway - a brief overview

The first big steps in our understanding of Hippo pathway function were made in flies. While many pathway functions are conserved between flies and mammals (9), for historic reasons, mammalian and Drosophila orthologues differ in their respective names. With few exceptions, we will use here the mammalian nomenclature and focus on mammalian systems. This should by no means make the major advances in the Drosophila field less important.

The backbone of the Hippo signaling pathway consists of a kinase cascades that works to control the activity of the downstream effectors YAP and TAZ. The upstream kinases of the Hippo signaling pathway, MST1 and MST2, work together with the adaptor protein SAV1/WW45 to phosphorylate and activate LATS1 and LATS2. The activated LATS kinases together with MOB1 then phosphorylate the YAP and TAZ effector proteins (Figure 1). This phosphorylation event results in the nuclear exclusion of YAP/TAZ mediated by 14-3-3 proteins and, ultimately, their cytoplasmic degradation (5, 10). However, not all cytoplasmic YAP/TAZ is degraded and the phosphorylated, cytoplasmic proteins can associate with protein complexes of other pathways such WNT or TGF-β signaling to modify signaling through these pathways (11, 12). Nevertheless, the main functions of YAP and TAZ can be attributed to their unphosphorylated, nuclear state, where the proteins function as transcriptional co-factors that can interact with several transcription factors. Their major transcriptional binding partners are members of the TEAD/TEF transcription factor family (TEAD1-4) that are believed to mediate the majority of pro-proliferative and oncogenic functions of YAP and TAZ (13).

Apart from so-called "canonical" Hippo signaling, several cellular components independent of the classical pathway can control YAP and TAZ. Importantly, the differential regulation is likely tissue specific (14). Particularly, the requirement of all kinases of the core Hippo kinase cascade in the control of YAP and TAZ activity does not seem to be conserved in all mammalian cell types. While MST1/2 control the phosphorylation of YAP in the liver, the function of the upstream Hippo kinases is dispensable for YAP activation in mouse embryonic fibroblasts, keratinocytes, and possibly also melanocytes (15–17). Furthermore, the functional requirement of either YAP or TAZ varies between different cell types. While many studies specifically highlight the role of YAP in tumorigenesis – or consider YAP and TAZ as functionally redundant – at least in some cancers such as breast cancer, melanoma, or glioblastoma, TAZ may play a more important role (18–20).

Cellular functions of Hippo signaling

In many tissues, YAP and TAZ regulate key cellular functions that include – but are not limited to – proliferation control, inhibition of apoptosis and promotion of metastasis (2, 3, 18, 21). By negatively regulating oncogenic YAP and TAZ activities, the kinases of the Hippo pathway work as important tumor suppressive molecules. In many cancers, Hippo signaling is dysfunctional and activation of YAP and/or TAZ is observed, with high levels

of nuclear proteins; in addition, direct genetic mutations in Hippo pathway genes are not very frequent, with the exception of *NF2* (reviewed in (22)). This unique mutation pattern for *NF2* could either suggest that loss of NF2 function results in additional oncogenic effects beyond inactivating Hippo signaling; alternatively, there may be an optimal level of altered Hippo pathway activity to promote cancer development and losing NF2 may achieve this oncogenic level in cancer cells. Activation of Hippo/Yap signaling can also be achieved through alterations in many of the pathways that functionally interact with Hippo signaling (see below), which again may lead to just enough perturbation in the pathway to be oncogenic. Nevertheless, more and more genetic and genomic alterations are being discovered in Hippo pathway members, including amplification of the YAP gene (23, 24) or gene fusion events involving multiple pathway members (25); it is also possible that a number of epigenetic events lead to silencing of tumor suppressive elements in the pathway (26).

Aside from its role in cancer, the physiological role of Hippo signaling is no less important: by being involved in the maintenance of tissue specific stem cells, tissue regeneration, wound healing as well as in the control of differentiation (6, 27–33), this pathway is a major player in the control of embryonic development and tissue homeostasis. YAP deficient mouse embryos die at E8.5 with multiple developmental defects (34). Knock-out of Mst1/2 or Sav1 results in liver overgrowth and the development of hepatocellular carcinoma (35, 36), a phenotype that is also observed in transgenic mice overexpressing YAP (4, 37). However, organ size control only presents one outcome of deregulated Hippo signaling. The direct effects of unleashed YAP/TAZ activation are tissue and often cell-type specific, but the control of cell proliferation remains a common theme. Interestingly, progenitor cell compartments seem to be especially sensitive to the loss of Hippo control mechanisms, including in the liver (36, 37), the intestine (37, 38), the nervous system (39), the skin, and the lung (40). Several studies further indicate that YAP and TAZ are involved in the maintenance of a stem cell phenotype and can inhibit cellular differentiation (6, 27, 32, 41, 42). Key functions for the Hippo signaling in the biology of stem/progenitor cells include the regulation of cell cycle and interactions with other signaling pathways (e.g. Hedgehog, Wnt, or Notch) (see below). In addition, this control of stem cells by Hippo/YAP signaling can be achieved in a cell-intrinsic manner but also by controlling the stem cell niche, as has been described in flies (43) and in mammals (44).

Keeping the balance between promotion of physiological proliferation and prevention of unrestricted cell divisions that lead to cancer development is a key function of Hippo signaling and makes it a promising therapeutic target in many human pathologies. For instance, promotion of downstream YAP or TAZ activity could be useful to promote tissue regeneration; in contrast, strategies to activate of Hippo signaling or inactivate YAP/TAZ may help treat certain cancers (Figure 2).

Regulation of Hippo signaling

The core Hippo kinase cascade functions as a central hub that relays input from the "outside world" of the cell and translates it into specific cellular responses by controlling the activity of downstream YAP/TAZ and by interacting with other pathways such as Wnt/β-catenin,

Notch, AKT or Hedgehog signaling (45–50). While the regulation by extracellular receptors is shared by many signaling pathways, Hippo signaling seems to be especially sensitive to input from mechanical cues. How signals from beyond the cell's borders feed into the Hippo signaling cascade has been extensively studied over the recent years and is reviewed in detail elsewhere (14). Below, we will provide an overview of the most important regulators.

The long quest to find receptors that directly activate or inactivate Hippo signaling only recently led to the identification of G-protein coupled receptors (GPCRs) as regulators of this pathway (51, 52). GPCRs are integral membrane receptors that are activated by external ligands whose binding leads to activation of the alpha-subunit of one or more of the four sub-classes of G proteins $G_{\alpha i/0}$, $G_{\alpha 12/13}$, $G_{\alpha s}$, and $G_{\alpha q/11}$. In the regulation of Hippo signaling, activation of GPCRs linked to $G_{\alpha s}$ results in the phosphorylation of LATS and therefore inactivation of YAP. On the other hand, activation of $G_{\alpha 12/13}$-coupled GPCRs is correlated with inhibition of LATS and activation of YAP (51). Additionally, a role for mutated $G_{\alpha q/11}$ in uveal melanoma carcinogenesis mediated by YAP has been reported (53). How GPCR signaling transduces into activation or inactivation of YAP is not well understood, but at least for some GPCRs these effects seem to be mediated by Rho GTPases and/or alterations in F-actin polymerization (51, 52, 54). Targeting this large class of cell surface receptors may be a promising approach to modify Hippo pathway activity in cancer or regeneration.

Other membrane receptors that have been shown to regulate YAP expression and proliferation are the receptor tyrosine kinases EGFR and ERBB4. Treatment with EGF leads to EGFR-mediated activation of PI3K and PDK1, which connects to the Hippo pathway through the adaptor protein SAV1/WW45. PDK1 activation triggers the dissociation of the Hippo core kinases from the scaffolding protein SAV1/WW45 and results in inactivation of LATS, dephosphorylation and nuclear translocation of YAP and expression of YAP target genes (55, 56). Interestingly, one of the YAP targets identified is the EGFR ligand amphiregulin (AREG), providing a positive feedback loop between YAP and EGFR signaling that can work to amplify proliferative signals from both signaling pathways and also trigger cell proliferation in a non-cell-autonomous manner (57). Another member of the EGR receptor family, ERBB4, has been shown to trigger YAP activation and expression of YAP target genes through direct interaction of its soluble intracellular domain with YAP and possibly also TEAD1 in the nucleus (58). Most likely, future research will identify additional receptors that feed signals into the Hippo signaling cascade. Manipulating these receptors to specifically alter Hippo signaling will then present the next big challenge.

In addition to receptor-mediated regulation, the Hippo signaling pathway receives multiple "mechanical" inputs from the cell surface as well as from within the cell (reviewed in (59, 60)). The mechanical cues involved in the regulation of Hippo signaling include proteins that bind to intercellular junctions such as tight and adherence junctions as well as components of the cytoskeleton, including F-actin, microtubules, actomyosin, and possibly centrosomes (61–66). Specifically, the interactions with centrosomes may also be relevant to the regulation of cell cycle progression by Hippo signaling. It is highly likely that these biomechanical regulators are also involved in the promotion of oncogenic YAP and TAZ activity in cancer. It is tempting to speculate that changes of the extracellular matrix found

in cancer (67, 68) might influence cell proliferation through mechanical regulation of Hippo pathway effectors (59, 69). Additionally, components of the cytoskeleton are often deregulated in cancer cells, which could possibly contribute to the activation of YAP/TAZ in carcinogenesis. In cancer-associated fibroblasts, matrix stiffening enhances YAP activation – which in turn promotes expression of cytoskeletal regulators involved in remodeling of the extracellular matrix (69). Additionally, the connection of intercellular junctions-associated proteins to the Hippo pathway seems to be essential for the maintenance of apical-basal cell polarity (14). Several well-established upstream regulators of Hippo signaling such as Merlin, Kibra, Expanded, the Angiomotin proteins, E-cadherin and α-catenin are known to associate with tight and adherence junctions or other cell polarity complexes (16, 70–73). Alterations in this regulatory network may be linked to the disruption of cell polarity that is linked to cancer progression and invasiveness (74).

One of the best-studied upstream regulators of the Hippo signaling pathway is the apical membrane protein NF2/Merlin – a well described tumor suppressor inactivated in many cancers (75). In mammals as well in flies, NF2/Merlin can interact with the Hippo signaling pathway on multiple levels (reviewed in (75)). Importantly, Merlin directly binds and recruits the LATS kinases to the plasma membrane to promote their phosphorylation and activation by MST1/2 and SAV1 (76). An additional mechanism to directly regulate LATS activity is mediated by phosphorylated Merlin that inhibits the tumorigenic effects of the E3 ubiquitin ligase CRL4-DCAF1 in the nucleus (77). Bound by Merlin, CRL2-DCAF1 is not able to ubiquitinate and inactivate LATS1/2 to promote YAP activity (78). Furthermore, Merlin can bind to other cell membrane and junction proteins such as Angiomotin and α-catenin and probably influences their interaction with the Hippo signaling kinases (reviewed in (75)). Interestingly, tumor development upon NF2 inactivation in the liver and other tissues is suppressed by heterozygous deletion of YAP, supporting the idea that NF2/Merlin inhibits proliferation by repression of YAP activity (79, 80).

The Angiomotin family of proteins AMOT (Angiomotin), AMOTL1 and AMOTL2 interact with tight junction proteins as well as the actin cytoskeleton and play an important role in the maintenance of cell polarity. With exception of the AMOT-p80 variant, all members of Angiomotin protein family can directly bind to YAP and TAZ and inhibit their activity, either by recruitment to the extranuclear environment or by inducing YAP/TAZ phosphorylation (72, 81, 82). Interestingly, several recent reports show that AMOT proteins themselves are targets of the LATS1/2 kinases (83, 84). Phosphorylation by LATS1/2 negatively regulates actin binding of Angiomotins and increases their ability to inhibit YAP (82, 84), providing an additional mechanism for LATS kinases to negatively regulate downstream YAP. Another connection between Hippo signaling and intercellular junctions is the adherence junction protein α-catenin, which is able to directly bind and inhibit phosphorylated YAP by facilitating its sequestration in complex with 14-3-3 proteins (16). This mechanism restricts proliferation in several different cells types and seems important to mediate the tumor suppressor activity of α-catenin (12, 16, 85).

Other cell junction proteins or proteins involved in the maintenance of apical–basal polarity such as SCRIB, PTPN14, LIN7C, PATJ, and MPDZ or E-cadherin, Crumbs, ZO1, ZO2,

NPHP4, and LKB1, respectively, interact with the Hippo signaling cascade on different levels (reviewed in (14) and (86)).

Sensing inputs from neighboring cells, the extracellular matrix, or the cytoskeleton is key to trigger several cellular responses, but most importantly one decision – to grow and divide or to remain quiescent. Clearly, integration of these signals plays a key role in the activity of the Hippo signaling pathway. Failures in the translation of these cues from the outside world can be deleterious to the maintenance of tissue integration and furthermore underline the importance of tight control mechanisms in this process.

The many nodes that connect to Hippo signaling

In addition to the multitude of upstream inputs to Hippo signaling there are several pathways that directly interact with the more downstream components of the signaling cascade to modify the output of Hippo signaling.

The most well-investigated partner of the Hippo pathway is the Wnt signaling pathway. Binding of WNT ligands to their receptors in the cell membrane results in phosphorylation of the β-catenin degradation complex. The phosphorylation of this complex upon Wnt activation, however, leads to stabilization of β-catenin and its translocation into the nucleus, where it associates with TCF transcription factors to promote the expression of WNT/β-catenin target genes (87). A large body of data now indicates that cytoplasmic YAP and TAZ can inhibit Wnt pathway activation – mainly through interaction with the β-catenin destruction complex.

A first study that gave evidence for a connection between Wnt and Hippo signaling showed that phosphorylated, cytoplasmic TAZ binds to DVL, a member of the β-catenin degradation complex. TAZ binding inhibits the phosphorylation of DVL by CK1δ/ε upon Wnt activation, thereby stabilizes the β-catenin destruction complex and prevents the translocation of β-catenin into the nucleus (46). Interestingly, the β-catenin degradation complex also seems to be involved in the degradation of TAZ in the cytoplasm, as it mediates the interaction of phosphorylated TAZ with its ubiquitin ligase β-TrCP (88). Compelling evidence also indicates that both YAP and TAZ are integral components of the β-catenin destruction complex and are required for the overgrowth phenotype in APC deficient intestines (11). In addition, phosphorylated YAP/TAZ can directly bind to β-catenin to suppress its nuclear translocation in colorectal cancer cells (89). A third mechanism by which cytoplasmic YAP can inhibit Wnt signaling output is by restricting DVL activity independent of the β-catenin degradation complex, likely through inhibition of the transcriptional co-activator function of DVL (44). This latter mechanism seems to be highly important to counterbalance activated Wnt signaling in regenerating intestinal stem cells, but also has implications in carcinogenesis as re-expression of previously silenced YAP can inhibit cell proliferation in xenograft models of an aggressive subtype of human colorectal carcinomas (44). These findings indicate that YAP might have important tumor suppressive functions under certain conditions that are related to activated Wnt/β-catenin signaling. However, the role of YAP in cancers with activated β-catenin is not fully understood and likely highly context dependent: in a screen in β-catenin-activated cancer

cell lines, YAP was essential for tumorigenicity (90). Mechanistically, YAP binds to the transcription factor TBX5 in a complex with β-catenin to induce the transcription of pro-oncogenic and anti-apoptotic target genes independent of TCF transcription factors. The oncogenic activity of YAP may require nuclear translocation and likely relies on tyrosine phosphorylation by the YES protein kinase independent of canonical Hippo signaling (90). A similar interaction was observed in mice with cardiac deletion of Sav, where increased nuclear YAP was associated with cardiac overgrowth that was dependent on Wnt signaling (45). In chromatin immunoprecipitation assays, both YAP and β-catenin were shown to bind to common targets, such as *Snai2* and *Sox2*, to enhance their transcription and contribute to increased proliferation in cardiomyocytes (45). The contradicting findings on the interplay between YAP/TAZ and Wnt signaling show that multiple factors might influence the outcome observed in Wnt-activated cells. To understand the mechanisms that divert YAP/TAZ activity towards tumor suppression or oncogenesis presents a major challenge and will be of high importance for therapeutic targeting of Hippo signaling.

Long before LATS kinases were identified as the main regulators of YAP/TAZ activation, AKT was shown to phosphorylate YAP at Ser127 resulting in nuclear exclusion and degradation by 14-3-3 proteins similar to the effect observed after LATS-mediated phosphorylation (91). A recent publication indicates that YAP is able to promote PI3K-AKT signaling in cardiomyocytes by direct transcriptional activation of the catalytic PI3K-subunit p110β (92). This activation of AKT signaling is associated with increased survival and proliferation in cardiomyocytes and can at least in part explain the promotion of cardiac regeneration by YAP (93) that is counteracted by overexpression of Mst1 resulting in dilated cardiomyopathy (94). Recently, a crosstalk between mTOR and YAP has been described in TSC1/2 mutant cells. In this system, hyperactive mTOR results in activation of YAP – most likely in an autophagy-dependent manner – to promote proliferation and tumor formation (95).

There is strong evidence that the Notch pathway is activated by YAP through direct transcriptional targeting of *JAG1* in the liver (27, 50). Additionally, high activity of YAP in Mst1/Mst2-deficient intestinal epithelial cells correlates with activation of Notch signaling (96). While these finding indicate that Notch signaling is downstream of Hippo/YAP, the two pathways can also act in concert by regulating expression of CDX2 in the trophectoderm (97). Notch signaling can also inhibit the Drosophila homologue of the YAP/TAZ-TEAD complex – Yki/Sc – under specific conditions (98). Therefore, the interaction between Hippo and Notch signaling is likely context-dependent and further research is needed to investigate the interaction between these two pathways.

A connection between Hedgehog signaling and YAP has been identified in medulloblastoma and neural precursors, where Sonic hedgehog (SHH) promotes nuclear accumulation of YAP and results in increased proliferation (49). On the other hand, activation of SHH inversely correlates with the expression of nuclear YAP in pancreatic adenocarcinoma (99). *Gli2*, a major transcriptional mediator of Hedgehog signaling, has been identified as a transcriptional target of YAP/TEAD in neural precursors (49), while YAP can bind to and inhibit GLI transcription factors in fibroblasts resulting in decreased expression of Hedgehog pathway targets (99). Importantly, Hippo upstream kinases as well as YAP/TAZ

are involved ciliogenesis of the primary cilium (100, 101), an organelle of the plasma membrane that is an important determinant of Hedgehog signaling (and other signaling pathways). These findings indicate that the interaction between Hippo and Hedgehog signaling is highly complex and tissue- as well as context-dependent.

In summary, the downstream effectors of Hippo signaling interact with many key pathways that play important roles in cell proliferation and also oncogenesis. In many cases these interactions might contribute to the amplification of the pro-proliferative output of YAP/TAZ signaling. As this pathway activation often seems to be dependent on the transcriptional activity of YAP/TAZ, it might be targetable by specific inhibition of YAP/TAZ-TEAD-mediated transcription. However, some interactions seem to function outside canonical Hippo signaling. Their role in cancer as well as possible means to target these interactions to inhibit proliferation or promote regeneration remain to be investigated.

Fine-tuning YAP/TAZ activity

The most important regulators of YAP/TAZ are LATS kinases. By phosphorylation at one or more serine residues LATS1/2 negatively regulate the transcriptional activity of YAP and TAZ. In addition to LATS1/2, other proteins such as AKT and JNK can mediate YAP serine phosphorylation (91, 102). Phosphorylated YAP/TAZ proteins are retained in the cytoplasm, where they are targeted for degradation by 14-3-3 protein. Interestingly, YAP methylation by SET7 also results in cytoplasmic retention and decreased YAP transcriptional activity, a mechanism that seems to be of relevance in the maintenance of tissue homeostasis in the intestine (103). Another level of YAP regulation is mediated by tyrosine phosphorylation by YES, SRC, or c-ABL kinases (90, 104–106). However, the biological effects of tyrosine phosphorylation seem to be context dependent and can divert YAP transcriptional activity towards expression of pro- or anti-apoptotic genes, respectively (90, 104).

Since phosphorylation presents such an important determining factor of YAP/TAZ activity, it is highly likely that one or more proteins exist that control their dephosphorylation. Recently, PP1A and PP2A have been identified as phosphatases of YAP and TAZ that promote nuclear translocation and expression of target genes (16, 107, 108). Further research is needed to determine the *in vivo* relevance of PP1A, PP2A, and other Hippo pathway phosphatases, including those interacting with kinases in the pathway (109–111). The search for phosphatases in the Hippo pathway is an area of active investigation that will give novel insights into the mechanisms regulating Hippo activity. Ultimately, inhibition of oncogenic phosphatases in the Hippo pathway could also provide new therapeutic options.

Transcriptional control of proliferation

The range of biological functions of the YAP and TAZ effector proteins we know about is ever expanding. YAP and TAZ can bind to several different transcription factors, each likely initiating transcriptional programs that contribute to the functional diversity of active YAP/TAZ (14). In general, transcription factor binding requires nuclear translocation of dephosphorylated YAP and TAZ. Upon phosphorylation by LATS as well as other kinases, YAP and TAZ are retained in the cytoplasm and become transcriptionally inactive, resulting in decreased expression of their target genes.

The main binding partners of transcriptionally active YAP and TAZ in the nucleus are TEA domain (TEAD) transcriptions factors (112). They mediate the majority of proliferative and oncogenic functions of YAP and TAZ (13, 113, 114). The TEAD family of transcription factors consists of four highly homologous proteins in humans and mice. TEAD1 is expressed in most in adult tissues, while the expression of the other members of the protein family varies considerably between different tissues and developmental stages (115). All TEADs share almost identical DNA-binding domains and seem to bind to the same DNA binding motif with comparable affinity (115). However, binding to different TEAD proteins may explain at least some of the tissue-specific outcomes that are observed upon YAP/TAZ activation.

Overexpression studies lead to the identification of an array of YAP and TAZ transcriptional targets, including *CTGF, CYR61, BIRC5, AREG*, and others. These genes are considered as canonical target genes and are used as a read-out of YAP/TAZ activity (116, 117). Additionally, YAP/TAZ or TEAD target genes signatures have been identified *in vitro* and *in vivo* in different cells and organs (4, 5, 118). However, these gene signatures rely on overexpression studies and only identify upregulated genes in general that might not represent direct transcriptional targets. Additionally, a substantial number of identified target genes seem to be expressed in a tissue specific fashion. Additional ChIP-seq analyses in different tissue contexts are required to identify transcriptional programs directly regulated by YAP/TAZ-TEAD complexes.

Among the known transcriptional targets of YAP/TAZ and TEAD, several pro-proliferative target genes have been identified. However, the profound proliferation response upon activation of downstream Hippo effectors suggests that a larger transcriptional program exists to promote cell division. A study in p53-deficient breast cancer suggested a link between deregulated RB/E2F activity and YAP1 (119), but the molecular basis for the synergy between E2F transcription factors and YAP remained unknown until recently.

In quiescent cells, the Retinoblastoma (RB) family proteins – RB, p107, and p130 – bind to and inhibit activating E2F transcription factors to restrict cell cycle entry. This G1/S restriction point is released upon phosphorylation of RB family proteins by the Cyclin dependent kinases CDK4 and CDK6. Phosphorylated RB dissociates from activating E2F proteins that can now initiate the transcription of a plethora of target genes to trigger S phase entry and govern the transition through subsequent phases of the cell cycle (120, 121). Deregulation of RB/E2F activity is observed in the large majority of human cancers, either by loss or mutation of RB or by other means that interfere with functional retinoblastoma signaling such as inactivation of Cyclin dependent kinase inhibitors (122, 123). In addition to cell cycle control, the RB pathway is involved in the regulation of stem cell maintenance and differentiation. Inactivation of RB can lead to the expansion of stem cell populations and to lineage specific differentiation defects (124), and at least to some extent these functions seem to depend on E2F activity.

Aside from the functional similarity between E2F and YAP/TAZ mediated transcription – that is without doubt shared by other pro-proliferative transcription factors – the promoters of at least some genes have been reported as targets of both E2F and TEAD transcription

factors, such as *BIRC5* (Survivin) (125) and *c-MYC* (4, 126, 127). We and others have shown that E2F and YAP/TEAD coordinately regulate a transcriptional program in cell cycle control in mammalian cells (23, 128, 129), a function that is conserved in Drosophila (130) (Figure 3A).

YAP/TAZ-dependent gene transcription in oral squamous cell carcinoma cells is enriched for cell cycle genes controlled by E2F, suggestive of a common mechanism of these transcription factors to overcome cell cycle checkpoints (129). In hepatocytes, unrestricted E2F activity in mice with hepatic inactivation of all three RB family members leads to cell cycle entry. However, inactivation and downregulation of YAP and TEAD1 counteracts the proliferative response in this model, is associated with reduced expression of E2F target genes and ultimately induction of complete cell cycle arrest (128). In pancreatic cancer, where activation of YAP can compensate for loss of oncogenic KRAS to maintain tumor cell proliferation independent of the KRAS signaling cascade, YAP/TEAD2 and E2F1 coordinately bind to several cell cycle associated target genes in YAP activated tumors (23). Importantly, YAP-driven proliferation is abolished in the presence of inactive E2F (23).

Mechanistically, several genes specifically upregulated in YAP activated tumors exhibit binding sites for both YAP/TEAD2 and E2F1. Binding of E2F1, YAP and/or TEAD2, respectively, to the same promoter fragments of canonical cell cycle genes such as *Mcm3, Mcm6, Cdk1*, and *PolA1* could be shown by chromatin immunoprecipitation *in vivo* and *in vitro* (23, 128) (Figure 3A). Additionally, bioinformatics analysis revealed that transcriptional start sites of YAP targets in the liver (4, 5) overlap with E2F ChIP-seq peaks in more in 60% of genes (128). Promoters of genes specifically upregulated in YAP-activated pancreatic cancer are enriched for E2F motif containing gene signatures (23). However, it remains to be investigated if E2F and YAP/TEAD directly bind to each other in the coordinate regulation of cell cycle genes or if they are at least part of a common regulatory complex. An alternative explanation for the synergy of YAP/TAZ and E2F is that YAP/TAZ-TEAD in conjuncture with AP-1 bind to more distant enhancers of E2F–regulated cell cycle genes to promote proliferation (131). Independent of the direct molecular mechanism, the common activation of pro-proliferative target genes by E2F and YAP/TEAD transcription factors is likely to play a central role in the oncogenic function of the YAP transcriptional co-factor.

A recent study in endothelial cells showed that YAP might play in role in the regulation of S-phase entry (132). The authors identified several differential regulated genes in YAP KD cells that are part of the replication machinery, such as *CDC6, CDT1, MCM4*, and *MCM10* (132). The possibility of direct YAP binding to the promoter of these genes as well as the transcription factors involved remains to be investigated. Interestingly, a number differentially regulated S-phase genes also present E2F target genes in line with E2F requirement for S-phase progression (133). Beyond S-phase entry and progression, there is increasing evidence that the Hippo pathway is involved in the regulation of G2/M-phase (134). However, these functions seem to be independent of the Hippo downstream effectors YAP and TAZ and mainly mediated by MST1/2 and NDR kinases (reviewed in (134)). Additionally, LATS2 seems to be required for mitotic progression and cytokinesis (135). Some of the cell cycle functions of the Hippo pathway may also be linked to interactions

with transcriptional complexes such as the DREAM complex (dimerization partner, RB-like, E2F and multi-vulval class B) (136) and related complexes involved at various steps of cell cycle progression. In particular, LATS2 has been shown to phosphorylate DYRK to promote the assembly of the DREAM repressor complex at promoters of E2F regulated cell cycle genes (136) – providing an additional level of cell cycle control by upstream Hippo kinases (Figure 3B).

In summary, an important role of YAP/TAZ induced proliferation might be transcriptional activation of cell cycle genes in a TEAD- and maybe also E2F-dependent fashion. However, further research is needed to understand the interplay between the cell cycle regulation machinery and Hippo pathway components.

In addition to direct control of the cell cycle, YAP and TAZ can probably influence cell proliferation less directly by transcriptional regulation of several pathways that promote growth, cell divisions and other pro-oncogenic functions. In the liver, a complex of YAP and TEAD4 directly targets *JAG1* and *NOTCH2* genes for transcriptional activation (27, 50). Recent data indicates that YAP-mediated activation of Notch signaling can differentiate mature hepatocytes into cells with characteristics of liver progenitor cells and propagate their expansion (27). In hepatocellular carcinoma, activation of YAP directly targets Notch signaling and is correlated with shorter survival times in human patients (50). In addition to Notch signaling, YAP as well as TAZ can activate the EGFR signaling axis by targeting the promoter of the amphiregulin (*AREG*) gene (57, 137). In breast cancer cells this activation contributes to cell proliferation and migration independent of EGF (137). Thus, many transcriptional targets of YAP and TAZ work together to promote proliferation by directly activating genes important in cell division and simultaneously targeting other pro-proliferative signaling pathways such as Notch and EGFR to sustain a robust proliferative response.

Transcriptional regulation by YAP/TAZ beyond TEAD

In addition to TEADs, several other transcriptional binding partners of YAP and TAZ have been identified. Interestingly, binding to transcription factors other than TEADs seem to mediate functions different from proliferation in many cases.

Both activated YAP and TAZ have been reported to bind to SMAD proteins to promote signaling from TGF-β and BMP pathways, adding another level to promote proliferation and oncogenic signaling (12, 138). On the other hand, phosphorylated and transcriptionally inactive YAP/TAZ can bind and retain SMAD2/3 proteins in the cytoplasm, inhibit their transcriptional activity, and induce their sequestration (12). Recently, YAP has been shown to interact with the AP-1 transcription factor family member FOS to promote epithelial-mesenchymal transition (EMT) and metastasis (139). In models of KRAS driven lung and colorectal cancer, activation of YAP mediates resistance to inactivation of oncogenic KRAS, highlighting its role in tumor plasticity. Mechanistically, both KRAS and YAP converge on the transcription factor FOS to activate transcriptional targets that are involved in EMT, such as *Vimentin, Slug*, and *Snail* (139).

While nuclear YAP/TAZ play important roles in oncogenic proliferation and EMT, they can also trigger expression of pro-apoptotic genes to counteract tumorigenesis. In DNA-damage, YAP can interact with and stabilize p73 to enhance the apoptosis response (140). The interaction with p73 is greatly amplified by YAP tyrosine phosphorylation that is induced by c-ABL upon DNA damage (105). This mechanism presents an important system to divert YAP transcriptional control away from proliferation in the context of DNA damage. Interestingly, YAP is downregulated in several hematological malignancies with activated DNA damage signaling and high nuclear c-ABL kinase activity (141). Restoration of YAP activity in these tumor cells by inhibition of the upstream Hippo MST1 kinase is sufficient to drive them into apoptosis (141). Therefore, YAP phosphorylation by the Hippo core kinase cascade may not only exert tumor suppressive functions, but may be oncogenic under certain conditions (Figure 2). In addition to upstream Hippo kinases, AKT can phosphorylate YAP on Ser127 to inhibit the pro-apoptotic synergy between YAP and p73 (91, 142).

Apart from the control of oncogenic signaling, YAP and TAZ play a role in differentiation in several organs. While both transcriptional co-factors generally seem to drive cells towards a more stem cell like phenotype (6, 18, 143), they also can induce lineage specific differentiation. TAZ interacts with RUNX2 to co-activate RUNX2-dependent gene transcription and direct mesenchymal stem cells towards osteogenic differentiation (144). At the same time, TAZ inhibits PPARγ-dependent gene expression and adipogenic differentiation (144). Interestingly, similar effects have been reported for phosphorylated RB that can bind to RUNX2 to promote osteogenic differentiation and can inhibit PPARγ-driven adipogenic differentiation in binding to E2Fs (145), presenting another example for functional convergence of Hippo and RB/E2F signaling (Figure 3C). Besides RUNX2, several other developmental transcription factors are bound by the Hippo signaling downstream effectors. YAP and TAZ both co-activate PAX3, a transcription factor important in the development of the neuronal crest (146), as well as TBX5, which initiates transcriptional programs in heart development (147). Additionally, a transcriptional complex of β-Catenin-YAP-TBX5 is required for the maintenance of β-Catenin driven cancers and can bind to pro-proliferative target genes independent of TEAD transcription factors (90). In the intestine, YAP/TAZ cooperate with KLF4 in promoting differentiation into goblet cells (38). In addition to direct binding of transcription factors involved in development and differentiation, a complex of YAP and TEAD2 has been reported to influence the binding of HNF4α and FOXA2 to enhancers of specific target genes in embryonic liver (148). While this YAP/TEAD2-induced target gene switch likely involves direct binding to DNA, it remains unclear to date if the YAP/TEAD complex might part of a larger transcriptional complex with FOXA2 or HNF4α.

It is highly likely that the number of developmental transcription factors identified as binding partners of YAP and TAZ will be expanding in the future. Even from what we know to date it is obvious that the Hippo downstream effectors have a key role in development to promote differentiation into distinct cellular lineages dependent on the transcriptional binding partner.

Targeting Hippo signaling

An increasing amount of data highlights the important role of Hippo signaling in the control of proliferation, including unrestricted proliferation found in cancer. Inhibiting oncogenic YAP and TAZ to control proliferation is therefore a promising approach in cancers that show increase activity of these transcriptional co-factors. Additionally, YAP has been shown to be a critical mediator of Ras-driven cancer and to mediate resistance to suppression of RAS and RAF signaling and could therefore be used in combination therapy with inhibitors of these oncogenic pathways to prevent or break drug resistance (23, 139, 149, 150).

Several different mechanisms are known to activate YAP and TAZ in human cancers to promote proliferation, including genetic amplification of YAP or genetic inactivation of upstream Hippo regulators such as *LATS1, SAV1, NF2*, or GPCRs (reviewed in (151)). Additionally, input signals from other pathways (e.g. Wnt) are likely involved in deregulation of Hippo signaling. However, all these different effects on Hippo signaling converge onto one specific outcome: activation of YAP and/or TAZ to promote tumorigenesis. It therefore seems logical to target these downstream effectors directly to inhibit tumor development. While YAP/TAZ bind to different transcription factors, their interaction with TEADs is likely the most important one in carcinogenesis as it promotes proliferation as well as metastasis (13, 114, 118, 152). Efforts to find specific inhibitors of YAP/TAZ-TEAD function have led to the identification of verteporfin. The compound was identified in a drug screening approach and scored together with other porphyrins in inhibiting the interaction between YAP and TEAD (114). Most importantly, verteporfin also inhibits proliferation and liver overgrowth *in vivo* in mouse models with either YAP overexpression or inactivation of upstream *Nf2*/Merlin resulting in disinhibition of endogenous YAP (114). An anti-tumorigenic effect of verteporfin was shown *in vitro*, where the drug was able to block tumor growth in uveal melanoma cells exhibiting activated YAP (53). Therefore, verteporfin could present a promising agent in the treatment of cancers dependent on high YAP activity. However, as a drug used as a photosensitizer in photodynamic therapy for macular degeneration the phototoxic properties of verteporfin are likely to limit its application in a therapeutic setting. The development of alternative compounds that specifically inhibit the interaction between YAP/TAZ and TEAD is therefore of high interest in cancer therapy. In this line, several alternative approaches were described recently. Engineered YAP-like peptides are able to disrupt the YAP-TEAD interaction *in vitro* (153). However, their efficiency to target the transcriptional complex *in vivo* remains unclear to date. Another option to target YAP-TEAD induced transcription might be by mimicking the interaction between vestigial-like proteins (VGLL) and TEADs. As alternative binding partners for TEAD transcription factors that compete with YAP for the same binding sites VGLL proteins counteract the activity of the Hippo downstream effectors at least in some tissues. VGLL4 has recently been identified as a tumor suppressor in lung cancer, where the transcriptional co-factor negatively regulates YAP-TEAD activity (154). A peptide mimicking VGLL4 function was reported to inhibit growths of gastric cancer cells *in vitro* and *in vivo* and to restrain tumor development in an *H. pylori* mouse model of gastric cancer (155). Even though the effects to VGLL4 might be tissue specific

and need to be evaluated in other cancers, this novel peptide could present a promising option to inhibit YAP-TEAD driven transcription in cancer.

Direct inhibition of YAP by genetic targeting was effective in an *in vivo* HCC model with deregulated Hippo signaling: in *Mst1/2* conditional knock-out mice, small interfering RNA-lipid nanoparticles targeting YAP were able to significantly reduce tumor burden in comparison to controls (156). While this method did not specifically inhibit the YAP/TEAD interaction, transcriptional programs that are likely dependent on TEAD were significantly altered in this model, including E2F target genes (23, 128), as well as targets of HNF4α and FOXA2 (153).

In addition to direct inhibition of YAP and TAZ transcriptional activity, modifying upstream Hippo signaling could present another approach to restrict – or activate – YAP/TAZ function. The identification of specific receptors that regulate Hippo signaling such as GPCR and EGFR (51, 56, 58) offers novel opportunities to functionally target this pathway. However, these receptors are mostly not pathway-specific and the complexity of signaling networks that are influenced for example even by a single member of the GPCR receptor family might limit this therapeutic approach. Additionally, dependent on the cellular context, the outcomes of YAP/TAZ inhibition might vary. This is of high importance in cancers where the transcriptional co-factors also might have a tumor suppressive role (Figure 2), such as in some hematological malignancies where YAP promotes apoptosis (141). Additionally, the role of YAP in breast cancer remains controversial and the protein might act as a tumor suppressor possibly in the presence of additional genetic changes (157, 158). In the intestine, YAP can function to restrict the expansion of intestinal stem cells and to inhibit the growth of colorectal carcinoma xenografts (44). However, from what we know to date, these tumor suppressor functions may not depend on the interaction between YAP/TAZ and TEAD. Instead, they do at least in part depend interaction with other transcription factors such a p73 in the apoptotic response (141) or KLF4 and RUNX2 in the induction of differentiation (38, 144). Additionally, the tumor suppressive activity can be independent of any transcriptional activity at all and stem from functions specific to cytoplasmic YAP (44). Given the multitude of interactions of Hippo signaling with different pathways and the diverse functions of YAP/TAZ it seems to be the safest and most predictable approach to target the interaction with TEAD transcription factors to inhibit proliferation and other oncogenic effects in cancer. However, further studies have to show the efficiency and feasibility of this approach in a clinical setting.

It is also worth considering the possibility to inhibit Hippo pathway function to enhance the activity of YAP and TAZ. Applications for this approach include c-ABL-driven malignancies, to promote the induction of an apoptotic response dependent on a YAP/TAZ interaction with p73 (141) as well as targeting Hippo signaling functions beyond cancer. This is of special interest in organs where high YAP/TAZ activity is associated with tissue growth and regeneration such as the heart (45, 159) or the liver (160, 161). Specific receptors such as GPCRs are the most promising targets to dampen the activity of upstream Hippo kinases: at least *in vitro*, GPCR ligands such as LPA promote the activity of YAP and TAZ (51). However, a large number of different GPCRs exist and the effects on the Hippo pathway differ in quality dependent on the class of GPCR as well as in quantity (51).

Therefore, further research is needed to identify specific and ligands to effectively inhibit Hippo signaling upstream of YAP/TAZ – especially *in vivo*. Even if we succeed to successfully induce YAP/TAZ activation, the pro-oncogenic side effects associated with any drug that promotes proliferation should be considered carefully – even when the treatment is only given for a short period of time.

Conclusion

There has been an explosion of reports on the Hippo/YAP pathway in the past few years. These studies have brought this signaling pathway to the forefront of cancer research. Similar to other key cancer pathways, the Hippo/YAP pathway is complex and coordinates multiple cellular functions, including proliferation, cell death, and differentiation. This plethora of functions and recent evidence strongly indicate that the Hippo/YAP pathway is a central player in tumor plasticity and how cycling cells interact with their environment. Superficially, the Hippo/YAP pathway seems to serve similar functions to other central pathways such as the Rb/E2F pathway. However, accumulating evidence points to unique functions for this pathway, even though the basis of this specificity is still not fully understood. A key aspect of the Hippo/YAP pathway may be its ability to sense and mediate unique extra-cellular signals, including mechanical forces. The Hippo/YAP pathway can be both tumor suppressive and oncogenic, and it will be key in the future to better understand the tissue-specificity of action of this pathway, as well as cell intrinsic and non-cell autonomous effects on cell proliferation. This will be crucial before anti-cancer therapies targeting this pathway can be implemented. However, early success repurposing a drug such as verteporfin to regulate the Hippo pathway suggests that, in the future, this pathway is "druggable" and may thus expand therapeutic options in cancer patients.

Acknowledgments

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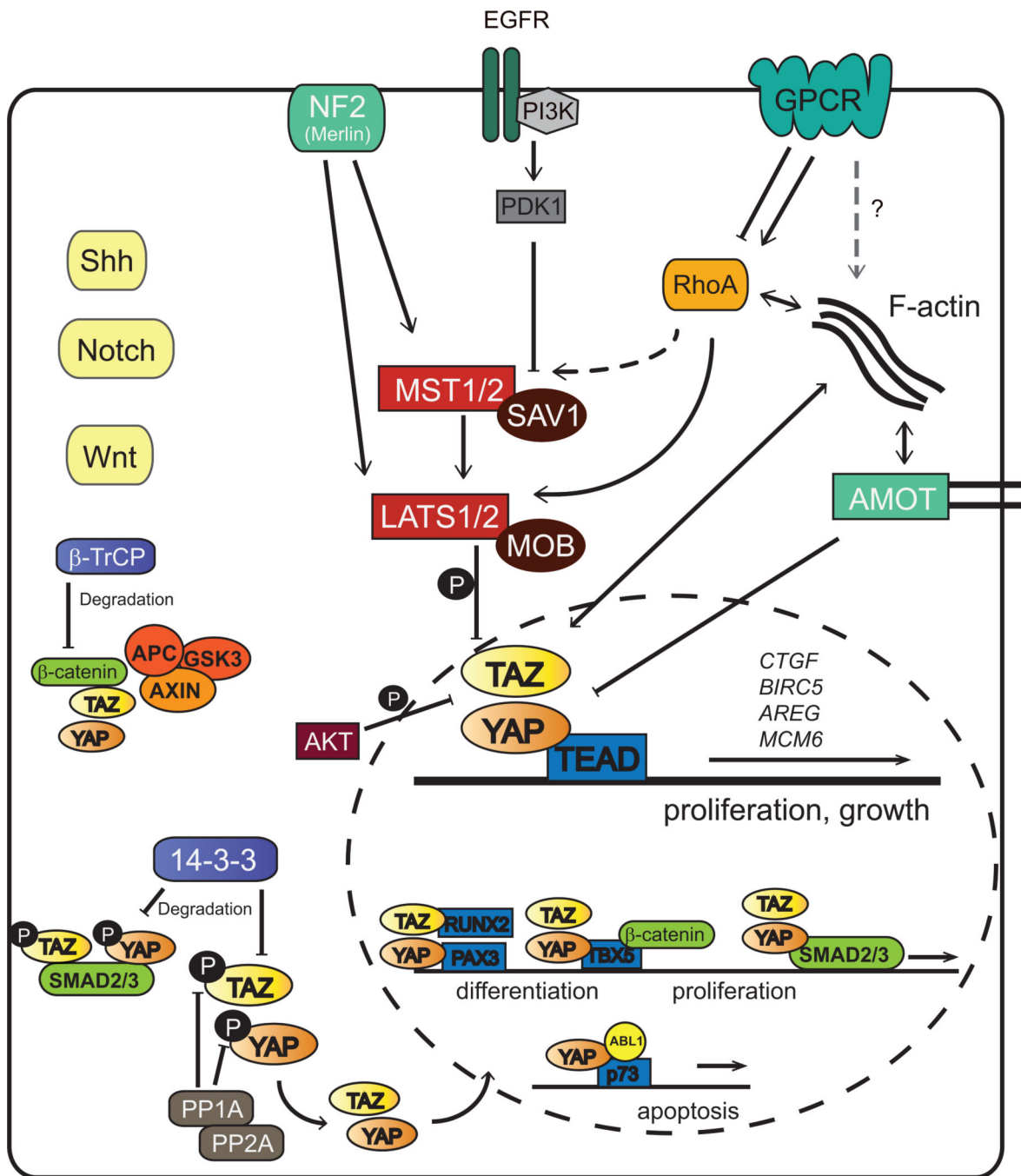
Figure 1.

An overview of the regulation of transcription by YAP/TAZ transcriptional co-factors and the regulation of YAP/TAZ activity in mammalian cells. YAP/TAZ are downstream mediators of numerous signaling pathways in cells, including from the cell surface. Their transcriptional targets have been involved in the regulation of proliferation and growth, but also cell death and differentiation.

Oncogenic YAP/TAZ

Tumor suppressive YAP/TAZ

Brain:
YAP functions as an oncogene in medulloblastoma, glioblastoma, and meningioma

Breast:
TAZ promotes drug resistance in breast cancer therapy. Both YAP and TAZ are linked to increased proliferation and metastasis in breast cancer

Lung:
YAP expression correlates with survival in non-small cell lung cancer and synergizes with Kras to promote EMT

Liver:
Inactivation of Hippo signaling and activation of YAP both induce liver overgrowth and cancer development

Bone:
Up-regulation of YAP downstream of deregulated Hedgehog signaling contributes to carcinogenesis in osteosarcoma

Skin:
YAP and TAZ enhance proliferation and metastasis in melanoma

Breast:
YAP has been shown to promote anoikis and to inhibit metastasis in breast cancer

Intestine:
Inhibition of colorectal cancer through inhibition of Wnt signaling by cytoplasmic YAP

Blood:
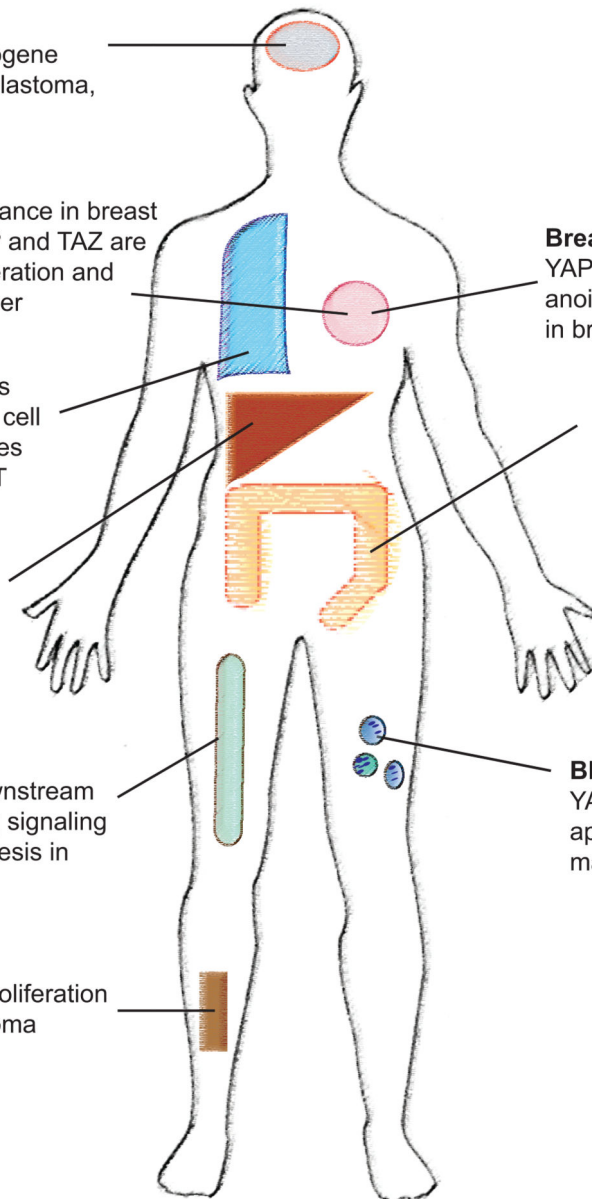YAP promotes ABL1-induced apoptosis in hematologic malignancies

Figure 2.
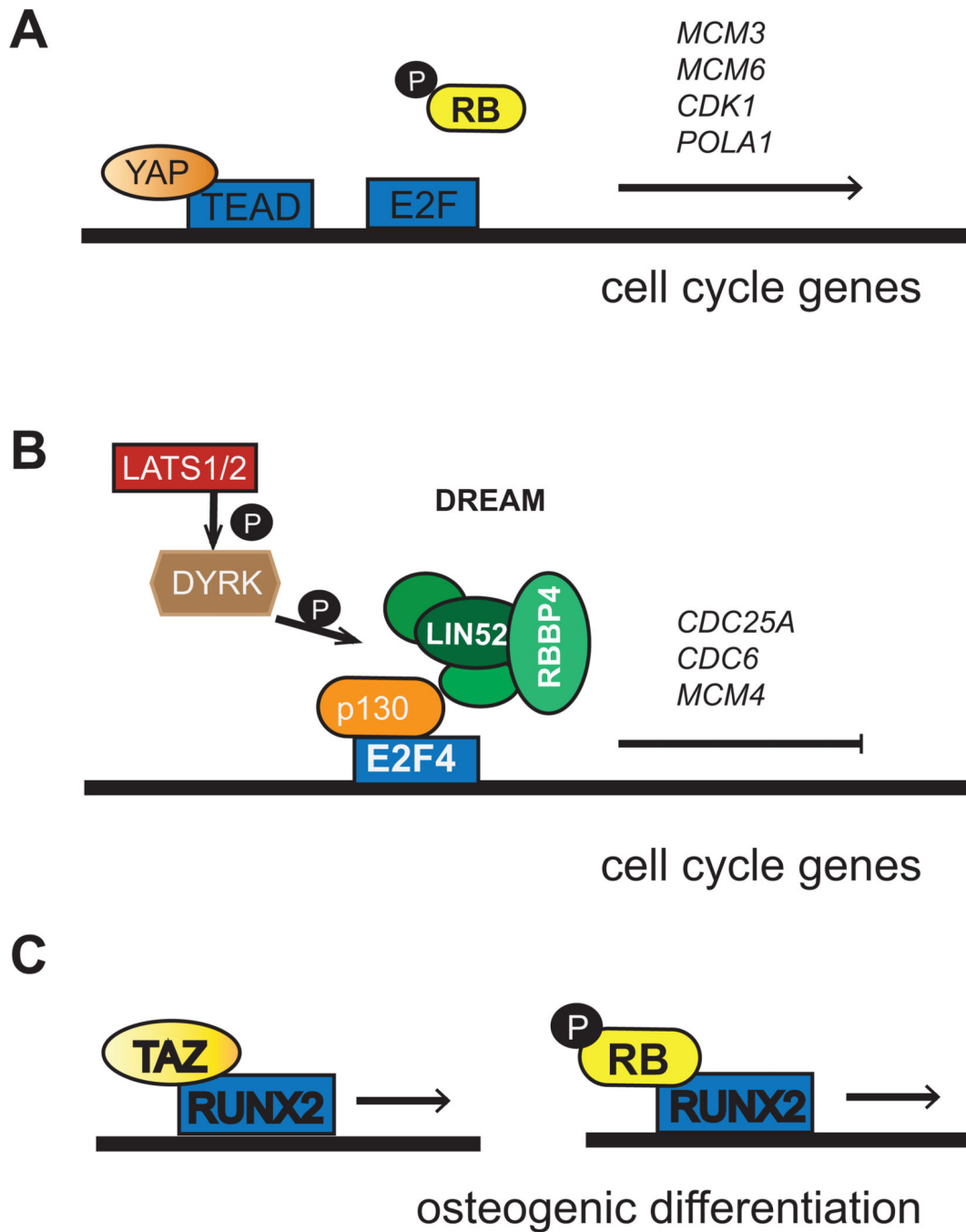Oncogenic and tumor suppressive functions of YAP and TAZ in human cancer (86, 151, 162). See text for details.

A

MCM3
MCM6
CDK1
POLA1

cell cycle genes

B

DREAM

CDC25A
CDC6
MCM4

cell cycle genes

C

osteogenic differentiation

Figure 3.
Interconnections between the Hippo and RB/E2F pathways. (**A**) Common regulation of cell cycle genes by YAP/TAZ-TEAD and activating E2F transcription factors. (B) Negative regulation of cell cycle gene transcription by LATS1/2 through activation of the repressive DREAM complex. (**C**) Regulation of osteogenic differentiation by TAZ and RB through activation of RUNX2 transcription factors.