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YAP oncogene overexpression supercharges colon cancer proliferation

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Abbreviations: YAP, yes-associated protein; TAZ, transcriptional co-activator with PDZ binding motif

The transcriptional co-activator YAP is an evolutionarily conserved regulator of organ size and progenitor cell proliferation. YAP is overexpressed at high frequency in many common human cancers and can directly drive cancer development in mouse models. YAP abundance and nuclear localization are negatively regulated by the Hippo kinase cascade, which, in epithelia, is activated by physiological cell-cell contact. Recent work in intestinal epithelium has established that YAP is constitutively inhibited by the Hippo pathway and entirely dispensable for normal development and homeostasis. YAP serves only in a standby capacity; should cell-cell contact be abrogated, as after intestinal damage, the loss of Hippo input permits increased YAP abundance and nuclear residence. In turn, YAP cooperates with β-catenin to transactivate genes that promote stem cell expansion for epithelial repair. This interplay between overexpressed YAP and β-catenin also drives proliferation of colon cancer cells. The dispensability of YAP in normal intestine makes YAP's expression or outputs attractive targets for cancer therapy.

YAP is a WW Domain Transcriptional Coactivator Inhibited by the Hippo Kinase Cascade

The Yes-associated protein, YAP65 or YAP1, was discovered as a proline-rich phosphoprotein that bound to the SH3 domain of the c-Yes protein tyrosine kinase.[1] Analyses of YAP sequences from multiple species led to the identification of a novel 38–40 amino acid domain, the WW domain,[2] which was shown to bind the PPXY proline-rich motif present in the activation domains of many transcription factors.[3] YAP has multiple alternatively spliced isoforms, with the predominant isoform containing the WW domain as well as a C-terminal PDZ binding motif l.[4] A two-hybrid screen with the transcription activation domain of Runx1 (CBFA/AML1/PEBP2alpha) retrieved YAP through its WW domain, enabling the identification of YAP1 as a coactivator.[5] YAP was subsequently retrieved in complex with the nuclear protein TEAD2 and shown to bind and coactivate all four TEAD transcription factors through a conserved N-terminal YAP domain together with the WW domains.[6] Despite its nuclear function, the bulk of YAP polypeptide was found in the cytoplasm, associated with the phosphoserine binding protein 14-3-3.

The logic of a transcriptional coactivator residing predominantly in the cytoplasm was uncovered in 2005, when yorkie, the Drosophila ortholog of YAP, was identified as the crucial downstream target of the growth inhibitory Hippo pathway.[7] The core of the Hippo pathway consists of two protein (ser/thr) kinases, Hippo and Warts/Lats, which share a common scaffold, the protein Salvador. Hippo phosphorylates Warts and a small Mob1-like protein, Mats; phospho-Mats binds to the Hippo-phosphorylated Warts, promoting Warts autophosphorylation and activation. Elimination of any of these four components inactivates the pathway, resulting in a dramatic increase in organ size due to increased cellular proliferation and resistance to developmentally programmed apoptosis.[8,9] Seeking targets of Warts, a two-hybrid screen with the N-terminal noncatalytic segment of Warts/Lats retrieved yorkie, whose WW domain bound to a Warts PPXY motif. Overexpression of yorkie reproduced the proliferative/antiapoptotic phenotypes seen with loss of function of the core Hippo components; conversely, inactivation of yorkie expression completely suppressed the overgrowth phenotypes of Hippo, Warts, Mats and Salvador loss of function.[7] Thus, the critical function of the Drosophila Hippo pathway is the negative regulation of yorkie coactivation of the TEAD transcription factor, Scalloped.[10-12]

As regards the mechanism for yorkie inhibition, active Warts/Lats catalyzes the phosphorylation of yorkie at multiple HXRXXS motifs, enabling the binding of 14-3-3, which induces yorkie nuclear exit.[7,13,14] Mutation of certain of these yorkie phosphorylation sites, especially Ser168 (=Ser127 in human, Ser112 in mouse YAP) causes yorkie (or YAP) to become constitutively nuclear and permanently activated. Each of the core elements of the Drosophila Hippo pathway is conserved in mammals and present as multiple homologs (except for Salvador = WW45; Hippo = Mst1/Mst2; Mats1A/Mob1B; Warts = Lats1/Lats2; yorkie = YAP/TAZ; Scalloped = TEAD1–4). These mammalian Hippo components function redundantly in an analogous manner to the Drosophila pathway in some tissues and in vitro settings, although it is clear that there is additional complexity and context specificity of

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mammalian Hippo signaling. In this regard, it is worth noting that while this review will concentrate on YAP, emerging data have indicated that its closest homolog, TAZ, is under comparable regulation by the Hippo pathway,[15] and there are common themes in the control of progenitor cell proliferation[16,17] by both of these transcriptional co-activators.

YAP is an Oncogene that is Frequently Overexpressed in Common Human Cancers

The identification of yorkie as a proproliferative anti-apoptotic effector appeared to conflict with earlier reports attributing to YAP proapoptotic activity to stabilization and coactivation of the p73 transcription factor, a member of the p53 family. DNA damage results in the association of YAP with p73, which, together with PML and the p300 acetylase, can be found on the promoter of the proapoptotic gene p53AIP1.[18,19] YAP association with p73 appears to involve YAP tyrosine phosphorylation by cAbl kinase, and YAP contributes to cAbl-induced apoptosis consequent to DNA damage.[20] YAP tyrosine phosphorylation, putatively by Src family kinases, also enables YAP binding to Runx2, resulting in suppression of Runx2 transcriptional activity toward p21 cdki[21] while promoting transformation.[22] Thus, YAP is capable of being regulated by diverse upstream inputs that appear to elicit distinct and even opposing outputs, e.g., as an oncogene[23] or a tumor suppressor.[24] What then are YAP's physiologic functions in various contexts; what are the relevant upstream inputs, and by what mechanisms are each of these functions regulated? Key proproliferative antiapoptotic functions of nuclear YAP have been identified in stem cells, in development and in the regeneration of damaged tissues in the adult organism; each depends on YAP's function as a transcriptional regulator, and control of YAP function by regulation of its nuclear residence is a recurrent theme.

In 2007, it was shown that global transgenic overexpression of YAP(Ser127Ala) in mice leads to expansion of progenitor cells in multiple organs.[25] Inducible overexpression specifically in mouse liver produces overgrowth of adult hepatocytes that is reversible upon YAP withdrawal, but if sustained, this is followed by hepatocellular carcinoma (HCC).[26] Additional evidence of YAP's oncogenic capacity was the finding that the YAP gene is spontaneously amplified (together with cIAP1) in an experimental model of murine HCC.[27] Double elimination of the Hippo orthologs Mst1 and Mst2 from mouse liver results in an abrupt loss of YAP(Ser127) phosphorylation and enhanced YAP nuclear abundance accompanied by the immediate onset of hepatocyte proliferation, resistance to FAS-induced apoptosis and the subsequent emergence of multifocal HCCs and mixed HCC/cholangiocarcinomas.[28-31] Depletion of YAP from cells cultured from the Mst1/2-null HCCs results in an immediate cessation of growth and massive apoptosis.[28] Thus YAP is certainly an oncogene in murine liver.

Ample evidence has now established YAP as a human oncogene. Early on, a screen for gene copy number changes in cancers arising in Brca1-null mouse breast identified an amplicon that contained only YAP and is syntenic with the 11q22 amplicon frequently amplified in a variety of human tumors.[23] The 11q22 amplicon is usually quite large and contains many genes, including the BIRC family members 5 and 6; however, the ability of YAP overexpression in nontumorigenic breast cells to promote proliferation and induce epithelial-mesenchymal transformation provided strong evidence for YAP's oncogenic potential. Initial surveys of primary human tumors by immunohistochemistry[26,32] showed that YAP staining, which was low or undetectable in the majority of normal tissues, was markedly increased in nearly all breast, ovarian, lung, colorectal, pancreatic and prostate tumors, with high nuclear expression in nearly half. Yap is also overexpresed in approximately 60% of human liver cancers (usually intranuclear) as compared with the adjacent, nontumorous liver. When detectable in normal tissues, YAP was seen in the proliferative and/or regenerative compartments, e.g., colonic crypts, type II pneumocytes; in skin, YAP is abundant and intranuclear in the proliferative basal layer of keratinocytes. Normal breast exhibits strong cytoplasmic YAP staining in ductular epithelium and detectable cytoplasmic and nuclear YAP in the myoepithelial cells.[32] The biologic importance of YAP overexpression in cancer is indicated by its association with poorer outcomes; a correlation between YAP overexpression and decreased progression-free survival is reported with ovarian cancers[33] and with shortened survival in HCC,[34] non-small cell lung cancer[35] and esophageal squamous cell carcinomas.[36] Thus, overexpression of the YAP oncogene is a very frequent occurrence in many common human cancers and is associated with resistance to chemotherapy[30] and poorer survival. It has been consistently observed that depletion of YAP from cancer-derived cell lines of diverse origins that exhibit YAP overexpression radically reduces cell proliferation in vitro and often also engenders substantial apoptosis. This apparent dependence on YAP, when overexpressed, for viability and growth together with the ubiquity of YAP overexpression in human cancers suggests that interference with YAP expression or its outputs may have broad application in cancer therapy.

YAP Overexpression is Necessary to Drive Intestinal Proliferation. YAP at Physiologic Abundance is Not Sufficient, Even if Nuclear

Several recent observations in the intestinal epithelial compartment suggest that YAP may be an especially attractive therapeutic target. As noted above, YAP expression in mouse intestine is detected in crypts, the location of the stem cell compartment responsible for the renewal of the intestinal epithelium, which turns over completely every 4–5 d.[37] Conditional inactivation of the genes encoding the core Hippo components WW45 or Mst1 and Mst2 results in a drop in YAP phosphorylation, an increase in YAP abundance and an expansion of the crypt compartment. These responses are especially pronounced with Mst1/2 deletion;[38] there, YAP shows a marked increase in total and intranuclear abundance, accompanied by robust overproliferation of a stem cell-like population, and interruption of differentiation with the loss of all secretory cells. The Mst1/2-null colons develop polypoid lesions by 13 weeks age, by which time the mice are runted, and half have expired. The mice lacking intestinal expression of Salavador/WW45[39] also exhibit increased YAP

abundance and crypt expansion but to a much milder degree; nevertheless, by 13 months of age, the Salvador-deficient colons also show an average of 1–2 adenomas. The stem cell expansion, loss of differentiation and adenoma development depend on YAP, in that the conditional inactivation of a single YAP allele is sufficient to reverse these abnormalities completely. Perhaps more surprisingly, the conditional inactivation of both YAP alleles has no discernable effect on intestinal morphology;[38,39] thus, YAP is expressed in the intestinal stem cell compartment, and although capable of driving stem cell proliferation, YAP is kept largely or entirely inactive through the action of the Hippo pathway and is entirely dispensable for normal intestinal epithelial turnover. This situation differs from that in ES and iPS cells, where YAP is expressed and is required for maintaining pluripotency,[40] and from early embryogenesis, where YAP knockout results in defects in the elongation and cell proliferation of the embryonic axis and in development of the yolk sac vasculature and placenta. A plausible question, therefore, is why express YAP in the intestinal stem cell compartment if only to maintain it in an inactive state through the continuing activity of the Hippo pathway? Cai et al. suggest that YAP serves there as a ready reserve to support epithelial regeneration; they show that during the repair phase of a mouse colonic mucosal injury induced by dextran sodium sulfate, there occurs a marked increase in the abundance of the YAP polypeptide with little change in YAP mRNA. The induction of such an injury to the YAP-null colon is accompanied by a very poor reparative response and a high mortality rate. Thus YAP, although dispensable in normal intestinal epithelial turnover, is critical for the effective repair and regeneration of the injured colon. The role of YAP in other committed stem cell compartments is less well-described but may conform with this model. Thus YAP overexpression can drive proliferation of chick neural stem cells accompanied by increased cyclin D1; however, shRNA depletion of YAP in this compartment does not diminish cyclin D1 expression or BrdU incorporation.[41] An alternative mechanism of tissue regeneration is that which occurs after 2/3 hepatectomy in the rodent, where restoration of liver mass occurs by recruitment of adult hepatocytes into the cell cycle rather than from the putative liver stem cell compartment (i.e., oval cells);[42] the latter presumably contribute to liver regeneration when hepatocytes are injured and unable to proliferate.[43] YAP abundance increases 2–3 fold following 2/3 hepatectomy;[44] however, YAP's contribution in either hepatocyte-mediated or oval cell-mediated liver regeneration is not yet defined.

In the Mst1/2-null, YAP heterozygous intestine, YAP polypeptide abundance is close to levels seen in the wild-type intestine;[38] however, the residual YAP is underphosphorylated and predominantly intranuclear; nevertheless, intestinal epithelial differentiation and morphology is normalized. This finding indicates that the levels of YAP found in normal intestinal epithelia, even if underphosphorylated and intranuclear, are insufficient to drive the proliferation and inhibit the differentiation of the intestinal stem cell; YAP overexpression in this compartment is needed to recruit a proproliferative drive that is not engaged at normal YAP abundance. We propose that YAP overexpression in colon cancer (and perhaps other malignancies) is also crucial to YAP's ability to incite proliferative drive, and this is why depletion of YAP from those cancer cell lines that overexpress YAP is so effective in inhibiting proliferation.

Overexpressed YAP Synergizes with Beta Catenin to Drive Intestinal Stem and Tumor Cell Proliferation

As regards the identity of the proliferative driver engaged by high levels of YAP, in the case of the intestine, available evidence points to β-catenin.[38] The proliferation of intestinal stem cells during normal turnover is dependent on the classical Wnt pathway and β catenin transcriptional activity acting synergistically with Notch,[37,45] whereas Notch, independently of Wnt, negatively regulates epithelial differentiation.[46] The ability of transgenic overexpressed YAP(Ser127Ala) to expand the intestinal stem cell compartment and inhibit differentiation is greatly attenuated if YAP(Ser127Ala) is induced in the presence of γ-secretase inhibitors.[25] Mst1/2 deletion in the intestine[38] is accompanied by a small increase in the active dephosphorylated form of β catenin but a much stronger upregulation of the expression of the β catenin targets Lgr5 and Ascl2. Mst1/Mst2 deletion also activates Notch signaling, reflected by increased levels of the cleaved Notch intracellular domain (NICD) and of a variety of Notch target gene products. Notch activation is driven in part by increased expression of the Notch ligand Jagged, a β catenin target. A similar dependence of β catenin and Notch signaling on YAP is evident in colon cancer cell lines that overexpress YAP. Depletion of YAP from SW480 cells reduces β catenin-dependent transcriptional activity by over 80% and, in HCT116 cells, greatly decreases NICD levels and Notch transcriptional outputs, in both cases without affecting the level of nuclear β catenin. Thus YAP overexpression, both in the intestine and in colon cancers, drives β catenin and Notch signaling to promote proliferation of stem-like cells. Whether the ability of high levels of intranuclear YAP to enhance the β-catenin transcriptional efficacy is due to YAP binding to and coactivation of β-catenin/TCF transcriptional sites or to more indirect effects, e.g., via TEAD-mediated transcriptional outputs, is not known; however, in the developing mouse heart, YAP and β-catenin can be coprecipitated at regulatory sites in the sox2 and snail homolog 2 genes.[47]

YAP Polypeptide Degradation is Regulated by the Hippo Pathway

Given the link between upregulated expression of YAP and both tumorigenesis and epithelial regeneration, there is considerable interest in defining the mechanisms controlling YAP abundance under normal and pathologic conditions. Little information is available concerning YAP gene transcription (possible regulation by Sonic Hedgehog[48]), YAP mRNA translation and turnover; however, it is clear that the Hippo pathway is the major regulator of YAP polypeptide degradation. Hippo signaling mediates phosphorylation of YAP HXRXXS(381), a site not conserved in yorkie, creating a binding motif for casein kinase1δ/ε that catalyzes a processive phosphorylation, which creates a recognition site for

the ubiquitin ligase SCF$^{\beta TRCP}$.[49] The loss of these phosphorylations is the primary cause of enhanced YAP abundance in the Mst1/Mst2-deficient mouse intestine, in as much as YAP mRNA levels are not altered.[39] Diminished YAP degradation together with the loss of 14-3-3 mediated YAP nuclear exit, accounts for the massive increase in YAP action in the intestine and liver that occurs with deletion of Mst1/Mst2.[28] As regards human colon cancer, YAP mRNA levels average about 2-fold higher than those in normal colon, independent of stage, with the vast majority ranging from near normal to approximately 3-fold increased.[38] Whether this is sufficient to account for the high YAP polypeptide levels as assessed by IHC is not known. The state of YAP phosphorylation in colon cancer is yet to be evaluated; however, an initial survey of human hepatocellular carcinomas found evidence of diminished YAP phosphorylation, as compared with adjacent nontumorous liver, in approximately one-third of tumors as well as diminished phosphorylation of the specific Mst1/Mst2 substrate Mob1.[25] This points to a loss of the inhibitory upstream input from the kinase cascade.

Upstream Regulation of the Hippo Kinase Cascade and YAP Nuclear Residence

Control of YAP activity appears to be an important mechanism for maintaining appropriate organ size or progenitor cell pools in different organs. Thus, YAP regulation would be expected to be directly linked to sensors of these cell states. In this regard, the identity in mammalian tissues of the components of the kinase cascade upstream of YAP, of the major upstream inputs to the kinase cascade as well as an understanding of the extent to which YAP action is regulated by hippo-pathway phosphorylation as opposed to other inputs are all areas of considerable uncertainty. In the fly, Hippo and Warts are the only kinases currently recognized to operate upstream of yorkie, although the relative strength of hpo and warts LOF phenotypes varies in a tissue-dependent manner.[50] In the mouse, Mst1/Mst2 are clearly upstream components in mouse liver[28-30] and intestinal epithelia[38] but are entirely dispensable to YAP phosphorylation in mouse embryonal fibroblasts (MEFs),[28] mouse keratinocytes[51] and in the nontumorigenic MCF10A human breast cells.[52] In addition, preliminary evidence suggests that in mouse liver Lats1/Lats2 may not be the YAP kinase recruited by Mst1/Mst2.[28] As regards inputs upstream of the kinase cascade, genetic evidence in the fly has identified several cell surface and polarity-related protein complexes as regulators of YAP cellular localization, at least in part through modulation of the Hippo pathway. These upstream regulators include the atypical cadherins Fat and its transmembrane ligand, dachsous; the apical-basal polarity complexes Crumbs (Crb/PALS1/PATJ) and PAR (PAR3/PAR6/aPKC), the lateral Scribble complex (Lethal Giant Larvae/Scribble/Discs large) and the intracellular FERM domain proteins, Expanded and Merlin, acting independently and together with the WW domain protein, Kibra, which can associate directly with Salvador.[53-56] An important feature of these upstream elements is that their individual LOF phenotypes are much weaker than those of the kinase elements, suggesting that the activity of the kinase cassette integrates inputs from different cell-cell and positional sensors. In addition to regulating yorkie phosphorylation, the pathway components expanded[57,58] and warts,[58] through their PPXY motif, and hippo are each able to bind directly and retain yorkie in the cytosol in a phosphorylation-independent manner; the physiologic relevance of this mode of regulation is unknown, and the mammalian Expanded homolog FRMD6/Willin lacks the PPXY motif.[59]

In mammalian cells, cell-cell contact is also a major upstream input to YAP phosphorylation and nuclear localization.[60] Notably, when cultured MEFs reach confluence, LATS1/2 become phosphorylated and activated, resulting, in turn, in YAP phosphorylation and cytoplasmic retention. Similar processes appear operative in epithelial cells, and available data points to E-cadherin[52]/α catenin[51,61] as an important input, with little direct support thus far for other cell surface and polarity complexes. A variety of proteins in addition to WW45/Salvador have been shown to bind the kinase components and regulate their activity in a positive or negative manner, e.g., Mst1/Mst2 (e.g., the Rassf polypeptides[62,63]) and Lats1/Lats2 (e.g., Zyxin,[64] Kibra,[65-67] ASPP1,[68,69] LIM domain proteins[70]). YAP is also found bound to proteins (other than the Src family kinases cYes and cSrc) that modify its activity and localization. In mouse keratinocytes, phospho-YAP complexed with 14-3-3 is bound to α-catenin in the cytoplasm.[51,61] Depletion of α-catenin promotes dephosphorylation of YAP by PP2A, enabling YAP nuclear entry. Surprisingly, elimination of Mst1/Mst2 and depletion of Lats1/Lats2 from keratinocytes does not result in YAP dephosphorylation.[51] Angiomotin is another peripheral, tight-junction associated cytoplasmic protein that encodes a PPXY motif, binds and sequesters YAP (and TAZ).[71-73] Angiomotin also binds Mst1/Mst2 and Lats2, so in addition to YAP binding, Angiomotin may serve as a scaffold to promote YAP phosphorylation.[74] Depletion of Angiomotin in epithelial cell culture is accompanied by YAP dephosphorylation and a YAP-dependent transformation as well as loss of cell contact. The Neurofibromatosis type II (NF2) tumor suppressor encoding the mammalian ortholog of Drosophila Merlin also interacts with Angiomotin at cell junctions and has been linked to the negative regulation of YAP in vitro. However, the precise mechanisms by which NF2 may influence YAP activity and the relevance of YAP dysregulation for the phenotypes associated with NF2 inactivation remain under active study.[75] Overall, although knowledge concerning YAP regulation is enlarging rapidly, many gaps and open questions remain. It is clear that there will be considerable variation in the tissue-specific composition and organization of the regulatory apparatus upstream of YAP as well as context-dependent operation of the available signaling pathways.

In addition to this incomplete understanding of the processes controlling YAP activity in normal cells, the basis for YAP dysregulation in human cancers remains to be defined. Whereas amplification of the YAP1 locus, and loss-of-function mutations of WW45/SAV1 and MOB1 (and of NF2) have been described in some cancers, these genetic lesions appear relatively uncommon compared with the observed prevalence of YAP overexpression across many tumor types. Epigenetic silencing of multiple bona fide and putative Hippo pathway components have been

reported (e.g., LATS1/2, MST1/2, RASSF1, RASSF2, RASSF5/Nore1); however, their pathologic significance remains uncertain. Overall, an important area for future inquiry will be to establish systematically and definitely the relationship between genetic and epigenetic alterations in pathway components and aberrant YAP activity in human cancers.

Can YAP Overexpression be Exploited for Colon or Liver Cancer Therapy?

Regardless of whether the basis for YAP hyperactivation is defined in any given malignancy, the current data indicate that multiple cancers are dependent on sustained YAP transcriptional activity for both tumor initiation as well as maintenance, and thus, YAP may be a relevant therapeutic target. The most direct therapeutic strategy would be to deplete the YAP polypeptide using anti-sense RNA or, more likely, RNAi; only partial depletion would be required, and overshoot is unlikely to be problematic given YAP dispensability in the adult (at least in colon and liver). A discussion of RNA therapuetics is beyond the scope of this review. Manipulation of YAP's upstream inputs does not appear to offer attractive therapeutic approaches in view of the complexity and additive nature of the upstream inputs to the inhibitory kinase cascade and the multiplicity of other determinants of YAP nuclear localization. Interference with YAP's interactions with the relevant transcriptional partners also seems daunting, given the plethora of candidate YAP interactors, whose identity may differ from one cancer to another. Even in colon cancer, where β-catenin is clearly a YAP target, the identity of YAP's direct interaction partner is unknown. Nevertheless, because YAP transcriptional regulation requires its TEAD association and/or the WW domains, the latter interacting with PPXY-bearing targets, it may be feasible to identify small-molecule inhibitors of those interactions with sufficient specificity. Regarding transcriptional targets critical for oncogenic activity downstream of YAP, BIRC5/Survivin and the extracellular ligands connective tissue growth factor (CTGF)[76,77] and Amphiregulin,[78] appear relevant in certain contexts; however, the full spectrum of YAP targets and their contributions to tumorigenesis in vivo remain to be defined.

In conclusion, many strategies are being pursued in the effort to validate, identify and eradicate colon cancer stem cells.[79-81] The dispensability of the YAP oncogene for the viability and proliferation of normal hepatocytes and intestinal stem cells, together with its frequent overexpression in colonic, liver and other cancers, where it serves to drive proliferation and resist apoptosis, makes YAP an attractive new target in cancer therapy.

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