

## Chapter 4

# YAP1 Uses Its Modular Protein Domains and Conserved Sequence Motifs to Orchestrate Diverse Repertoires of Signaling

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**Abstract** Yes-associated protein 1 (YAP1) is a potent oncogenic protein and is one of the two main effectors of the Hippo tumor suppressor pathway. Originally, YAP1 cDNA was isolated by screening expression libraries for proteins that associate with SH3 domains of Yes and Src protein-tyrosine kinases. Subsequently, YAP1 was shown by homology searches or functional assays to encode multiple protein–protein binding modules including a WW domain, a PDZ domain-binding motif, and TEAD-interaction domain (TID) as well as a transcriptional activation domain (TAD). The TID region encodes a major regulatory phosphorylation site, Serine 127, which plays a critical role in regulating the subcellular localization of YAP1. The TAD region contains a putative coiled-coil region, whose function is unknown, and a tyrosine phosphorylation site that is the subject of intense study. Through reductionistic approaches of molecular and cellular biology, we have gained insight into the detailed function of most of the individual domains, motifs, and selected phosphorylation sites of YAP1. Here, we review how these YAP1 domains act in concert to regulate cell contact inhibition as well as a balance between cell proliferation and apoptosis. Given the mounting evidence that many parameters of malignant cancer progression are driven by Hippo-regulated pathways, understanding the details of how YAP1 regulates signaling is of paramount importance in designing effective strategies to control the oncogenic function of YAP1.

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## 4.1 Introduction

YAP1 (**Y**es-associated **p**rotein **1**) is a potent oncogene and is one of the two main effectors of the Hippo tumor suppressor pathway (reviewed by Pan 2010). Originally, YAP1 cDNA was isolated by screening expression libraries for proteins that associate with SH3 (**S**rc **H**omology **3**) domains of Yes and Src protein-tyrosine kinases (reviewed by Hong and Guan 2012). Subsequently, homology searches or functional assays have shown YAP1 to encode multiple protein–protein binding modules including a WW domain (Tryptophan–Tryptophan domain), a PDZ-BM (**P**ost synaptic density-96, **D**iscs Large, **Z**onula Occludens-1 domain-**b**inding **m**otif), and TID (**T**EA domain-containing factor-**i**nteraction **d**omain), as well as a TAD (**t**ranscriptional **a**ctivation **d**omain) (reviewed by Sudol and Harvey 2010). The TID region encodes a major regulatory phosphorylation site, S127 (Serine 127), which plays a critical role in regulating the subcellular localization of YAP1. The TAD region contains a putative coiled-coil (C) domain, whose function is unknown, and a tyrosine phosphorylation site that is the subject of intense study. Through reductionistic approaches of molecular and cellular biology, we have gained detailed insight into the detailed function of most of the individual domains, motifs, and selected phosphorylation sites of YAP1. In this chapter, we review how these YAP1 domains act in concert to regulate cell contact inhibition as well as a balance between cell proliferation and apoptosis. Given the mounting evidence that many parameters of malignant cancer progression are driven by Hippo-regulated pathways, understanding the details of how YAP1 regulates signaling is of paramount importance in designing effective strategies to control the oncogenic function of YAP1.

## 4.2 YAP1 Discovery and Its Modular Structure

YAP1 was first identified by virtue of its ability to associate with the SH3 domain of Yes and Src protein-tyrosine kinases (Sudol 1994). The chicken YAP1 gene was shown to encode a protein of 65 kDa that was easily precipitated from normal chicken embryo fibroblasts as a phospho-serine-rich protein. No traces of threonine or tyrosine phosphorylation were detected in the phospho-amino acid analysis of the precipitated YAP1. A PxxP (P—Proline, x—any amino acid) consensus motif for binding to SH3 domains was found in the middle of the YAP1 molecule and it was confirmed as a functional motif that is involved in YAP1–Yes kinase interaction

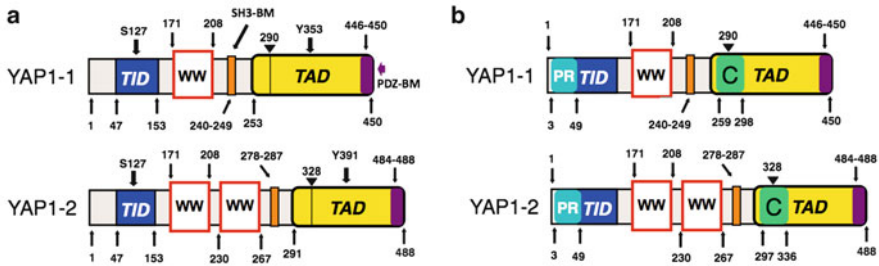
in vitro binding assays (Sudol 1994). When the mouse and human orthologs of the YAP1 gene were cloned, a repeated and inserted block of semi-conserved 38 amino acids was identified within the amino terminal half in the mouse YAP1 protein (Bork and Sudol 1994; Sudol et al. 1995). Since this repeated block also showed significant sequence similarity to repeats in other proteins, including that of human Dystrophin and yeast Rsp5 E3 ubiquitin ligase, it was suspected that it might represent a modular protein domain, such as the SH2 and SH3 domains (Bork and Sudol 1994; Pawson 2004). Computer-aided homology searches of all known protein sequences confirmed the suspicion, and this block was provisionally named the WW domain, after the presence of two conserved tryptophans spaced 20–21 amino acids apart (Bork and Sudol 1994; Sudol et al. 1995). Shortly after the initial publication, two other groups also reported the same domain as the WWP motif (Andre and Springael 1994) and the Rsp5 repeat (Hofmann and Bucher 1995). The WW domain became a *bona fide* modular protein domain when it was shown that it mediated protein–protein interactions with ligands containing Proline-Proline-any-amino-acid-Tyrosine (PPxY) consensus motifs (Chen and Sudol 1995) and when the structure of the domain in complex with its ligand peptide was elucidated (Macias et al. 1996).

Apart from the WW domain, the modular structure of YAP1 contains a Proline-rich region (PR) at the very amino terminus, which is followed by a TID region. Next, following a single WW domain, which is present in YAP1-1 isoform, and two WW domains, which are present in the YAP1-2 isoform, there is the SH3-BM (SH3 binding motif) (Ren et al. 1993) (Fig. 4.1b). Following the SH3-BM is a TAD region which contains a coiled-coil (C) domain and a PDZ binding motif (PDZ-BM) (Fig. 4.1b). The two major isoforms of human YAP1, namely YAP1-1 and YAP1-2, are generated by differential mRNA splicing of exon 4, which encodes the second WW domain (Fig. 4.1b) (Sudol et al. 1995).

Below, we will briefly discuss the individual domains and regions of YAP1 protein, describing in detail how they were originally identified and what is known about their functions. We will try to present an integrated picture of how they function in concert to regulate cell contact inhibition as well as the fine balance between proliferation and apoptosis. In particular, we will emphasize the versatility and plasticity of YAP1 WW domains.

### 4.3 The Role of TEAD Interacting Domain, TID

The family of four transcription factors known as TEAD factors was shown to require YAP1 for its transcriptional activity (Vassilev et al. 2001). At the amino terminal region of TEADs resides a TEA domain that recognizes GGAATG elements in the promoter region of target genes (Anbanandam et al. 2006). Many of these genes robustly regulate cell proliferation, such as *AXL* and *CTGF*, or inhibit apoptosis, such as *BIRC5* (Zhao et al. 2009; Zhang et al. 2008). The protein



**Fig. 4.1** (a) Schematic structure of YAP1 isoforms depicting functional regions, modular domains, domain binding motifs, and major serine and tyrosine phosphorylation sites. At the amino terminal region of YAP1, there is a TEAD interaction domain (TID), shown in *blue*, which was originally delineated for the mouse YAP1 protein in an *in vitro* protein-binding assay. This arbitrarily chosen region of YAP1 amino terminus corresponds to amino acids 32–139 in mouse YAP1 and to residues 47–153 in human YAP1. Within the TID domain, there is a major Serine phosphorylation site S127 (*black arrow*) that functions as 14-3-3 protein-binding site. YAP1-1 and YAP1-2 isoforms differ by the number of WW domains (depicted as *red boxes*). The length of the WW domain of YAP1 is estimated as 38 amino acids based on the degree of sequence similarity among proteins containing WW sequences. Also, the length of the WW domain was discerned from the differentially spliced exon 4 in human YAP1-2 isoform, which encodes the second WW domain and is 38 amino acids a long (Bork and Sudol 1994). The PxxP consensus motif for SH3 domain binding PVKQPPPLAP is located between WW domain and TAD domain and indicated as *orange rectangle*. TAD domain (shown in *yellow*) was delineated in a GAL4-based transactivation assay using a mouse YAP1 carboxy-terminal fragment encompassing amino acids 276–472, where 472 L residue is the last coding amino acid of the mouse YAP1 isoform. This arbitrarily chosen region of YAP1 carboxy-terminus is termed TAD domain and corresponds to residues 253–450 in human YAP1-1 and to residues 291–488 in human YAP1-2. Within the TAD domain at position 290 in YAP1-1 and position 328 in YAP1-2, there is a region where additional sequences are spliced in various isoforms of YAP1 gene. YAP1 is tyrosine phosphorylated on Y357 in one of the YAP1-1 isoforms within TAD that contained a spliced in sequence at position 290. In the isoforms YAP1-1 and YAP1-2 without splicing in the TAD region, the Y357 site (SSYSVPRT) corresponds to Y353 and Y391, respectively. The last 5 carboxy-terminal residues—FLTWL (shown in *dark violet*) represent PDZ domain binding motif. (b) Schematic structure of YAP1 isoforms depicting two additional regions whose function is unknown. At the amino terminal region, YAP1 harbors Proline-rich region (PR) between amino acids 3–49, shown in *light blue*. Within the TAD domain there is a coiled-coil domain (C), shown in *light green*, which was predicted by Coils 2 program available via SMART resource as the following sequence: GSNSNQQQQMRLQQLQMEKERLRLKQQELLRQELALRSQL that is located at position 259–298 in YAP1-1 and 297–336 in YAP1-2. Curiously, the C domain covers the 290 and 328 positions, which are sites of spliced in sequences in some of the YAP1 isoforms. These spliced in sequences may disrupt the C domain structure and function

complex between YAP1 and TEAD2 factor was originally characterized by the DePamphilis laboratory at the NIH (Vassilev et al. 2001). In their detailed study, the amino-terminal region of YAP1, containing the TID region, was shown to interact physically and functionally with the carboxyl-terminal region of TEAD2. This finding had important ramifications for the emerging Hippo pathway because it connected cytoplasmic signals of serine-threonine kinases and its effector YAP1 with a transcriptional program. Specifically, the *Drosophila* ortholog of mammalian TEAD,

Scalloped, was quickly shown as a major transcription factor for the *Drosophila* ortholog of YAP1, Yorkie (Yki) (Zhang et al. 2008). The growth-promoting and oncogenic activities of YAP1 and Yki were shown to require complex formation with TEAD and Scalloped, respectively.

The structure of YAP1 in complex with TEAD factor was solved at high resolution and serves as a subject of intense structure–function analyses and drug discovery efforts (reviewed by Sudol et al. 2012). The disruption of this complex by small molecules should help in controlling cancers that harbor amplification of the YAP1 gene, such as hepatocellular carcinomas. The TID is composed of two  $\alpha$  helices and a hydrophobic linker, which together form a clip-like structure that is accommodated by three major sites of interaction on TEAD protein (Chen et al. 2010; Li et al. 2010; Tian et al. 2010). The interface between TID and TEADs is druggable and a recent report provides a successful example of a small molecule inhibitor that attenuates the YAP1-TEAD complex (Liu-Chittenden et al. 2012).

### 4.3.1 Serine 127 as a Critical Regulatory Site

Intriguingly, within the TID domain there is a major site of regulatory phosphorylation at S127 (Fig. 4.1b), which was first mapped by the Downward laboratory (Basu et al. 2003) in a screen for B/AKT protein kinase substrates that associate with 14-3-3 proteins when phosphorylated. Although the AKT kinase is not the major kinase that phosphorylates YAP1 on S127, and the pro-apoptotic function of YAP1 used as a biological “read-out” by the team of Julian Downward is observed for YAP1 only under conditions of stress, this study was critical in showing that a single site of serine phosphorylation can promote the cytoplasmic localization of YAP1 via 14-3-3 proteins, and therefore dramatically curtail its function as a nuclear transcriptional co-activator. There are no solid reports on S127 phosphorylated YAP1 being detected in the nucleus; however, if the phospho-S127-YAP1 would find its way to the nuclear compartment, it would not be able to form TEAD/YAP1 transcriptional complex because of steric hindrance.

#### 4.3.1.1 WW Domain Partners Play Diverse Roles

The Hippo tumor suppressor pathway is highly enriched with proteins that cross talk via their WW domains and PPxY motif ligands (Sudol and Harvey 2010). None of the other signaling pathways that we know at present utilize WW domain complexes to the extent seen in the Hippo pathway.

The protein partners of the YAP1 WW domains, which are part of the Hippo signaling pathway or which network from cross talking pathways can be divided into two groups. One group is represented by LATS (large tumor suppressor) kinases, members of the AMOT (Angiomotin) family and PTPN14 (protein-tyrosine phosphatase of non-receptor type number 14), which act to retain YAP1 in the

cytoplasm (Hao et al. 2008; Oka et al. 2008, 2012; Chan et al. 2011; Zhao et al. 2011; Wang et al. 2011; Huang et al. 2012; Liu et al. 2012). The other group of WW domain ligands is represented by transcription factors, such as the RUNX (Runt domain-containing proteins) family of transcription factors, p73 pro-apoptotic factor and the SMAD (SMA—after small protein, and MAD—after mothers against decapentaplegic protein) factors (Yagi et al. 1999; Strano et al. 2001; Ferrigno et al. 2002). In addition, a transcriptional regulator WBP2 (WW domain binding protein 2) also binds to YAP1 WW domains (Chen et al. 1997) to regulate gene expression in a phosphorylation-dependent manner (see below). Although most of the ligands of YAP1 WW domains contain the PPxY motif, some of them employ additional noncanonical sequences for efficient complex formation, such as those used by certain SMADs (Massague 1998; Mauviel et al. 2012). A very interesting study, which illuminated the versatility of YAP1 WW domains, was recently completed by the Macias and Massague laboratories (Alarcon et al. 2009; Aragon et al. 2011). They showed that activation of TGF  $\beta$ /BMP (transforming growth factor  $\beta$ /bone morphogenetic proteins) pathway induced SMAD1 binding to the YAP1-WW domain via its PPxY motif and an additional phosphorylated linker sequence, resulting in translocation to the nucleus and the regulation of target genes. The phosphorylated linker of SMAD1 is located in close proximity to the PPxY motif, and two CDKs (cyclin-dependent kinases), CDK8, which curiously harbors a conserved PPxY motif as well, and CDK9, which have been implicated as potential linker kinases (Alarcon et al. 2009; Aragon et al. 2011). In contrast, the complex of YAP1 with inhibitory SMADs, such as SMAD7, seems to be constitutive and could be phosphorylation-independent (Ferrigno et al. 2002). The ability of YAP1 WW domains to recognize phosphorylated and non-PPxY-containing motifs indicates the tremendous plasticity of the WW module in reaching a variety of partners to orchestrate diverse signaling events.

The YAP1-1 and YAP1-2 isoforms, which differ in their number of WW domains seem to signal differently. For example, YAP1-1 does not bind p73 factor and cannot induce apoptosis when HEK293 cells are stressed by low serum conditions (Oka et al. 2008). Moreover, YAP1-1 does not interact with Angiomotin-Like-1 (AMOTL1) protein while YAP1-2 does. Also AMOTL1 has the ability to inhibit YAP1-2 function by preventing its nuclear localization (Oka et al. 2012). More work needs to be done to characterize the differences between YAP1-1 and YAP1-2 isoforms in order to address a potential dominant negative activity of YAP1-1.

#### 4.4 SH3 Binding Motif

The SH3 BM of YAP1 was shown to be required for YAP1–Yes-SH3 domain complex formation in *in vitro* binding assays. A 22-amino acid peptide, which spanned the PxxP motif of YAP1, was shown to compete with YAP1 binding to purified Yes-SH3 fragments. Little has been done on the SH3 BM because initially YAP1 could not be shown to be tyrosine phosphorylated in cells grown under standard

conditions. However, the Ito laboratory reported that YAP1 deleted of its SH3 BM was less active in GAL4-based transcription activation assay (Yagi et al. 1999), suggesting that SH3 domain-containing proteins could modulate the transcriptional function of YAP1. In addition, a recent report shows that YAP1 recruits the c-Abl kinase to phosphorylate the Nedd4-2 ubiquitin ligase, suggesting that the SH3 BM of YAP1 might be involved in this process (Skouloudaki and Walz 2012). With several recent reports of YAP1 being phosphorylated by non-receptor protein-tyrosine kinases, and interacting with a protein-tyrosine phosphatase (see below), it is expected that the role of the SH3 BM in the function of YAP1 will be revisited.

## 4.5 Transcription Activation Domain, TAD

The Ito laboratory was the first to show that YAP1 is a strong transcriptional co-activator, rivaling even herpes simplex virus VP16 known for its promiscuous transactivation ability (Yagi et al. 1999). YAP1-2 employs its WW domains to recognize and form a stable complex with PPxY motif in Polyoma enhancer binding protein 2  $\alpha$  (PEBP2 $\alpha$ ), one of the RUNX family transcription factors, which regulates transcription of the osteocalcin gene. Subsequent studies that characterized YAP1 complexes with p53 binding protein 2 (p53 BP2) and the carboxy-terminal fragment of ErbB4 also revealed that YAP1 has the ability to act as a transcriptional co-activator (Españel and Sudol 2001; Komuro et al. 2003). The TAD of YAP1 was mapped within the carboxy-terminal half of the YAP1 protein (see Fig. 4.1b) (Yagi et al. 1999). It is important to note that the boundaries of TAD in YAP1 were chosen arbitrarily and that the actual functional region of TAD may be shorter than the one that was originally delineated. Within the TAD region, there are three important elements: the C domain, a Tyrosine 357 (Y357) phosphorylation site and PDZ-BM.

### 4.5.1 Coiled-Coil Domain

Even though the C domain of YAP1 was identified a decade ago, its function remains unknown (Mohler et al. 1999). It is likely that through the C domain, YAP1 forms complexes with other proteins, especially nuclear proteins, which have cognate coiled-coil regions. Perhaps via such complexes, the transactivation function of YAP1 could be finely regulated. One of the potential candidates for the coiled-coil-mediated interaction with YAP1 is c-Jun that harbors a coiled-coil domain itself and also has a conserved PPxY motif for additional contact with YAP1 via the WW domain (Einbond and Sudol 1996; Kanai et al. 2000; Hu and Li 2002; Gao et al. 2006). The proposal of the existence of YAP1-c-Jun complex is supported by a short report suggesting that some of the functions of YAP1 are indeed c-Jun-dependent (Danovi et al. 2008). There could also be cytoplasmic proteins that contain coiled-coil domains and interact with the C domain of YAP1 to modulate its activity (see below).



Curiously, several YAP1 isoforms, which have been characterized so far (Sudol et al. 1995; Komuro et al. 2003; Muramatsu et al. 2011) contain spliced insertion sequences within the C domain of YAP1. We suggest it will be interesting to explore these isoforms to see if their cytoplasmic and nuclear functions differ from the isoforms with intact C domains.

#### **4.5.2 Tyrosine 357 Site**

Originally, three publications raised the possibility that YAP1 could be phosphorylated on tyrosine in some signaling scenarios (Zaidi et al. 2004; Levy et al. 2008; Tamm et al. 2011). The Stein laboratory reported that YAP1 tyrosine phosphorylation is necessary for its interaction with Runx2 transcription factor as well as for its subsequent nuclear trafficking (Zaidi et al. 2004). Moreover, inhibition of the Src and Yes kinase activity reduced the tyrosine phosphorylation of YAP1, resulting in the dissociation of the endogenous Runx2-YAP1 complexes (Zaidi et al. 2004). A second publication from the Shaul laboratory reported that in response to DNA damage, YAP1 is phosphorylated by c-Abl kinase on Y357 and this modification stabilizes YAP1 protein. Furthermore, p-Y357 YAP1 has higher affinity for the p73 transcription factor and this association results in increased expression of p73-dependent and pro-apoptotic target genes (Levy et al. 2008). The third publication came from the Anneren laboratory reporting that Yes kinase binds and phosphorylates YAP1 and activates the YAP1-TEAD2-dependent transcriptional program underlying the self-renewal process of mouse embryonic stem cells (Tamm et al. 2011).

Phosphorylation of YAP1 on tyrosine has become a subject of intense investigation. Recently, a novel regulatory mechanism for the YAP1 oncogenic function by PTPN14 was reported (Huang et al. 2012; Liu et al. 2012). YAP1 and PTPN14 form a complex through direct interaction between the PTPN14 PPxY motifs and YAP1 WW domains (Huang et al. 2012; Liu et al. 2012). PTPN14 contains two conserved structural elements: an amino terminal FERM domain (band 4.1-ezrin-radixin-moesin family of adhesion molecules) and a carboxy-terminal PTP (protein-tyrosine phosphatase) domain. The subcellular localization of the PTPN14 protein depends on a variety of factors, such as cell type, cell-matrix adhesion status (Ogata et al. 1999a), presence of serine phosphorylation (Ogata et al. 1999b), and cell confluence (Wadham et al. 2000; Wyatt and Khew-Goodall 2008). YAP1 is a direct substrate of PTPN14, and PTPN14 inhibits the transcriptional co-activator function of YAP1 by retaining it in the cytoplasm. As expected, the knockdown of PTPN14 leads to nuclear retention of YAP1 as well as to an increase in the YAP1-dependent cell migration (Liu et al. 2012). This finding is in agreement with a previous publication reporting a potential tumor-suppressive function of PTPN14 in colon cancer (Wang et al. 2004). Taken together, these data revealed a new mechanism by which the function of YAP1 might be regulated.



In addition to YAP1, PTPN14 is reported to mediate the dephosphorylation of tyrosine residues in an adherens junction protein,  $\beta$ -catenin (Smith et al. 1995; Wadham et al. 2003). Also, it has been recently shown that YAP1 interacts with adherens junction protein  $\alpha$ -catenin in the epidermis (Schlegelmilch et al. 2011; Silvis et al. 2011). Binding of the YAP1/14-3-3 complex to  $\alpha$ -catenin stabilizes this complex and inhibits the access of PP2A to YAP1 (Schlegelmilch et al. 2011). Furthermore, the Crumbs polarity complex, which includes PALS1, PATJ, MUPP1, LIN7C, and AMOT proteins, interacts with YAP1 and relays cell density information by promoting YAP1 phosphorylation and its cytoplasmic retention, as well as by suppressing the TGF- $\beta$  signaling (Varelas et al. 2010). These data strongly suggest that there must be extensive cross talk among adherens junction and polarity complex proteins that involve PTPN14 and YAP1 (Mauviel et al. 2012). Intriguingly, a recent report from the Stocker laboratory (Poernbacher et al. 2012) demonstrated that *Drosophila* Pez (the ortholog of PTPN14) interacts with KIBRA, a WW domain-containing upstream component of the Hippo signaling pathway (Kremerskothen et al. 2003), and functions as a negative upstream regulator of Yki (the fly ortholog of YAP1) in the regulation of *Drosophila* intestinal stem cell proliferation. Therefore, it is tempting to predict that PTPN14 not only directly interacts with and regulates YAP1 function, but may also regulate YAP1 by indirect interaction with KIBRA.

Although YAP1 was first identified as a Src and Yes kinase-associated protein (Sudol 1994), it is only recently that evidence exists that Src family kinases can phosphorylate YAP1 on Y375 within the TAD region (Huang et al. 2012; Liu et al. 2010; Levy et al. 2008).

### 4.5.3 PDZ Binding Motif

Screening of random peptide libraries with various PDZ domains resulted in the identification of a number of PDZ ligand sequences including peptides that terminated with the amino acid sequence TWL, the very sequence of the YAP1 carboxy-terminus (Wang et al. 1998). Aided with this data, the Milgram laboratory documented that YAP1 contains a functional PDZ-BM and forms complexes with a PDZ domain of SLC9A3R1 (solute carrier family 9, subfamily A member 3 regulator 1) protein (Mohler et al. 1999). At present, we know of another protein, ZO2 (Zona Occludens 2), that uses its first PDZ domain to form functional complexes with YAP1 and is critical in regulating nuclear localization of YAP1 (Oka and Sudol 2009; Oka et al. 2010). ZO2, like YAP1, has a propensity to shuttle between the cytoplasm and nucleus in a cell density-dependent manner (Balda et al. 2003) and has been shown to regulate nuclear transport of another protein LASP-1 (LIM and SH3 domain protein 1) via its SH3 domain (Mihlan et al. 2012). A YAP1 deletion mutant lacking the FLTWL motif in the PDZ-BM is unable to enter the cell nucleus, implicating PDZ domain-containing proteins in controlling this critical regulatory function.

## 4.6 Examples of Concerted Actions

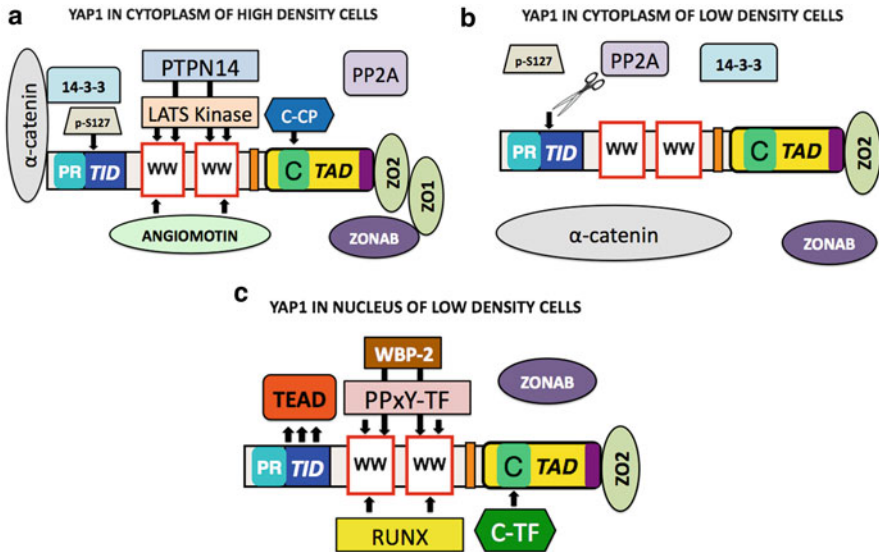
The Hippo pathway plays a critical role in regulating cell contact inhibition. (Hong and Guan 2012). The subcellular localization of YAP1 is cell density-dependent. In sparsely populated cultures, YAP1 tends to accumulate in the nucleus and promotes cell proliferation. Whereas in densely populated cultures, YAP1 localizes in the cytoplasm, rendering it incapable of directly regulating transcription. The modular domains and sequence motifs of YAP1 drive an assembly of multicomponent protein complexes, which differ between growing or contact-inhibited cells.

In high-density cultures, YAP1 forms signaling complexes with several cytoplasmic proteins, which apart from their enzymatic or other functions, have the ability to physically sequester YAP1 in the cytoplasm (Fig. 4.2a). Examples of such proteins are the WW domain-interacting LATS kinases, PTPN14 phosphatase and three members of the AMOT family of tight junction proteins (AMOT, AMOT-Like-1, and AMOT-Like-2) (Hao et al. 2008; Oka et al. 2008, 2012; Chan et al. 2011a; Zhao et al. 2011; Huang et al. 2012; Liu et al. 2012). In addition, another tight junction protein, ZO2, uses its first PDZ domain to complex with YAP1 via its PDZ-BM (Oka et al. 2010). As ZO2 and ZO1 are able to heterodimerize, it is expected that at least a subpopulation of YAP1-Angiomotin-ZO2-ZO1 tetrapartite protein complexes localize to tight junctions, not only as sensors of cell-to-cell contacts but also as anchors that prevent YAP1 from nuclear entry.

Although little is known about cytoplasmic proteins that interact with the C domain of YAP1, we speculate here that ROCK1 (**R**ho-associated, **c**oiled-coil domain-containing protein **k**inase **1**) together with Rho GTPase regulates YAP1 through mechano-sensing signals, and may also function as a cytoplasmic anchor (Dupont et al. 2011). Moreover, in epidermal cells, an adherens junction protein,  $\alpha$ -catenin, facilitates the interaction of S127 phosphorylated YAP1 with 14-3-3 proteins, preventing access of PP2A to dephosphorylate YAP1, and also further stabilizing the cytoplasmic localization of YAP1 (Schlegelmilch et al. 2011).

Several regulatory loops of positive feedback that further promote cytoplasmic localization of the YAP1 protein and enhance the maintenance of tight junctions were uncovered recently. For example, AMOTL2 was shown to activate LATS2 kinase activity, thereby enhancing the phosphorylation of YAP1 on the regulatory S127 site, promoting its cytoplasmic localization (Paramasivam et al. 2011). Also, YAP1 was recently shown to protect AMOTL1 from Nedd-4.2 E3 ubiquitin-ligase-mediated-degradation by recruiting c-Abl kinase, which phosphorylates Nedd-4.2 on tyrosine residues, thereby negatively regulating its ligase activity (Skouloudaki and Walz 2012). These two examples of concerted action of YAP1 and its WW domain-interacting partners, AMOTL1, AMOTL2, and LATS2, reveal subtle molecular mechanisms that prevent YAP1 from nuclear entry while at the same time ensuring the integrity of tight junctions in densely populated cells (Fig. 4.2a).

In low-density cultures, YAP1 is dephosphorylated on S127 by the PP2A phosphatase and it subsequently translocates into the nucleus in complex with the ZO2 protein, which acts as a nuclear shuttle (Fig. 4.2b, c). The degradation of AMOTs



**Fig. 4.2** Three scenarios of YAP1 signaling via protein complexes: (a) In high-density cells, YAP1 is localized in the cytoplasm and forms complexes with selected cellular proteins. These complexes tend to sequester YAP1 in the cytoplasm. Activation of MST and LATS kinases renders YAP1 phosphorylated on S127, which creates a binding site for 14-3-3 protein that anchors YAP1 in the cytoplasm. In epidermal cells, an adherens junction protein,  $\alpha$ -catenin interacts with YAP1-14-3-3 complex, blocking PP2A access to dephosphorylate YAP1 on Serine 127, further stabilizing the complex. The interaction of WW domains with LATS kinases, PTPN14 phosphatase, and Angiomotin also tends to sequester YAP1 protein in the cytoplasm. The C domain of YAP1 may very well interact with cytoplasmic proteins that contain coiled-coil domain (CYT-CP). One potential candidate is ROCK1 (Rho-associated, protein kinase 1), which together with Rho-GTP-ase may mediate mechano-sensing signals to YAP1, independently from the canonical Hippo cassette (Dupont et al. 2011). ZO2 is known to bind to PDZ-BM of YAP1 and since ZO2 heterodimerizes with ZO1, they may also prevent YAP1 from nuclear localization. (b) In low-density cells YAP1 is dephosphorylated on S127 by PP2A phosphatase and it is ready to be translocated to the nucleus in complex with the ZO2 protein. In epidermal cells,  $\alpha$ -catenin can no longer maintain a complex with YAP1 and 14-3-3 to retain YAP1 in the cytoplasm. (c) YAP1 in the nucleus forms complexes with various transcription factors to regulate diverse transcriptional programs. Primarily it interacts with TEAD transcription factors to induce proliferative and anti-apoptotic genes. The WW domains of YAP1 will bind a number of transcription factors that contain PPxY motif (PPxY-TF) such as members of the RUNX family. In addition, WBP2 factor that is tyrosine phosphorylated by c-Src and c-Yes kinases positively regulates YAP-1-TEAD complex in promoting cell proliferation. The C domain may act in concert with WW domains of YAP1 and interact with coiled-coil domains of transcription factors (C-TF) such as c-Jun to regulate proliferation. The role of ZO2 and ZONAB in Hippo signaling is still not clear but theoretically intriguing (see text)

by WW domain-containing E3 ubiquitin ligases may release YAP1 from tight junctions. In epidermal cells,  $\alpha$ -catenin can no longer maintain a complex with YAP1 and 14-3-3 to retain YAP1 in the cytoplasm. In the nucleus, YAP1 forms complexes with various transcription factors to regulate diverse transcriptional programs. Nuclear YAP1 primarily interacts with TEAD transcription factors to induce both proliferative and anti-apoptotic genes. In addition to the YAP1-TEAD complex that engages the TID domain of YAP1, intact WW domains and TAD region are required to act in unison for YAP1 to mediate proliferation. The WW domains of YAP1 will assemble a number of PPxY motif-containing transcription factors (PPxY-TF) such as members of the RUNX family. In addition, WBP2, which is tyrosine phosphorylated by Src and Yes kinases, positively regulates the YAP-1-TEAD complex and enhances cell proliferation (Chen et al. 1997; Lim et al. 2011). The C domain of YAP1 may act in concert with its WW domains in order to interact with coiled-coil domain-containing transcription factors (C-TF) such as c-Jun to regulate proliferation. We also speculate that as the half-life of ZO-1 decreases in sparsely populated cells and the expression of its cognate transcription factor ZONAB (ZO-1-associated nucleic acid binding protein) increases (Balda et al. 2003), ZONAB will no longer be retained by ZO1 in tight junctions, but will preferentially localize in the nucleus to drive expression of proliferative genes as a Y-box transcription factor.

## 4.7 Hippo in the Context of Other Cancer Pathways

There is growing evidence that dysregulation of the Hippo pathway may contribute to other pro-oncogenic pathways. For example, a recent genetic link between the Hippo pathway and genes that control apico-basal polarity of epithelial cells, such as Scribble, DLG (Discs LarGe), and LLGL (Lethal Giant Large) (Enamoto and Igaki 2011) suggests that the loss of Hippo mediators contributes to epithelial-to-mesenchymal transition (EMT), a marker of cancer progression (Hanahan and Weinberg 2011). Similarly, the PAR family of polarity-regulating proteins is linked to Hippo pathway regulation (McCaffrey and Macara 2011).

Given that elevated YAP1 expression correlates with oncogenic progression in several human cancers, it is interesting to note that YAP1 expression can be regulated by miR-375, which itself is significantly downregulated in liver cancer (Liu et al. 2010).

The Hippo pathway may also promote oncogenic progression by controlling the differential expression of pro-oncogenic genes. For example, the YAP1 paralog, TAZ, promotes taxol resistance in breast cancer cells by inducing the expression of Cyr61 and CTGF (Lai et al. 2011). Indeed, the anticancer effect of the drug,  $\alpha$ -tocopheryl succinate, is due to the suppression of Hippo factors that normally, in complex with Foxo-family transcription factors, repress the expression of pro-apoptosis factors such as Noxa (Valis et al. 2011). A recent report (Xu et al. 2010) suggests that resistance of glioblastoma cells to cytotoxic chemotherapy-induced cell stress

pathways requires the suppression of Hippo signaling. It is interesting to speculate that Hippo pathway components such as LATS1 may normally regulate genotoxic responses by radical oxygen species (ROS) or DNA-damaging agents. This would correlate with the loss of LATS1 expression following oncogene-induced ROS (Takahashi et al. 2006) and the finding that LATS1-deficient cells suffer from premature senescence due to the inability to resolve their cytokinesis defects (Yang et al. 2004). Interestingly, RASSF1A (Ras Association Domain Family 1A) physically interacts with Hippo/MST2 and LATS1, promoting their phosphorylation, and cells deficient in RASSF1A suffer from cytokinesis defects (Guo et al. 2007).

The canonical Wnt/ $\beta$ -catenin signaling pathway is a critical regulator of cellular proliferation and its cross talks with the Hippo pathway (Clevers and Nusse 2012; Varelas and Wrana 2012). When the Wnt pathway is engaged, the  $\beta$ -catenin transcription co-activator translocates from the cytoplasm into the nucleus. Nuclear  $\beta$ -catenin associates with members of the T-cell factor/Lymphoid enhancer factor (TCF/Lef) family of transcription factors and together,  $\beta$ -catenin/TCF complexes drive expression of growth-promoting genes. Mutations in components of the Wnt/ $\beta$ -catenin signaling pathway are found in approximately 90 % of colorectal cancers and these mutations contribute to aberrant growth. While the Hippo pathway inhibits Wnt signaling in primary cardiomyocytes and in HEK293 cells (Heallen et al. 2011; Imajo et al. 2012), in colorectal cancer cells the relationship between these two pathways is different. An elegant study by Joe Avruch and colleagues found that deletion of Mst1/Mst2 kinases in the intestinal epithelium lead to accumulation of nuclear YAP1 and activation of  $\beta$ -catenin signaling (Zhou et al. 2011). Another study demonstrated that  $\beta$ -catenin/TCF complexes directly activate expression of the YAP1 gene in human colorectal cancer cells and that YAP1 was required for oncogenic properties of these cells (Konsavage et al. 2012). Together, these findings suggest that Hippo and Wnt/ $\beta$ -catenin signaling pathways may act in concert to drive colorectal carcinogenesis. It is tempting to speculate that perhaps YAP1/TEAD and  $\beta$ -catenin/TCF transcription complexes converge to activate a shared set of target genes. In support of this hypothesis, a search of 2,168  $\beta$ -catenin binding regions identified in a ChIP-Seq screen found that 397 contained coupled TEAD and TCF consensus DNA binding motifs (Greg Yochum, personal communication and Bottomly et al. 2010). Further experiments are required to determine whether all or a subset of these targets are controlled by Hippo and Wnt signaling and whether these targets could be exploited for diagnostic purposes.

## 4.8 Concluding Remarks

For clarity, we focused our discussion here almost exclusively on YAP1, one of the two main effectors of the Hippo tumor suppressor pathway, and omitted TAZ (WWTR1) which is a close paralog of YAP1. Many features discussed here for YAP1 are also relevant for TAZ; however, subtle structural and functional differences exist between these effectors and are being unraveled at a fast pace now.

We *deconstructed* YAP1 by dissecting its individual modular protein domains, and conserved binding motifs, which are the basic units of the canonical code of signaling. These modules are frequently called the Lego® blocks of Nature because they form a plethora of protein-to-protein complexes in a reiterated and combinatorial fashion, similar to a structure made of interconnecting Lego® blocks (Pawson 2004; Sudol 2004). The wide occurrence of these modules in YAP1, TAZ, and in other proteins of the Hippo network has facilitated a fast dissection of their function and their protein partners. These modules have helped the characterization of signaling steps that link cell density and junctional complexes to transcriptional programs. However, unlike Lego® blocks, modular protein domains and conserved motifs are embedded within host proteins and work in concert to transmit discrete signals. Several examples of this concerted action were discussed here to illuminate the intricacies of these processes. It seems that many parallel signals and positive feedback loops of regulation are acting together in a redundant fashion to maintain a specific state, such as contact-inhibited growth or vigorous proliferation under conditions of subconfluency. We hope that our discussion of YAP1 signaling via modules, motifs, and post-synthetic modifications provides insight into the vast repertoire of signaling processes that are used by the Hippo pathway.

The Hippo tumor suppressor pathway has quickly emerged in the past several years as a new signaling pathway that is directly relevant to human cancer. As discussed above, the pathway cross talks extensively with other pathways (Mauviel et al. 2011; Varelas and Wrana 2012) or with major cancer genes (Aylon et al. 2006, 2010), and understanding the details of signaling by both the canonical Hippo pathway and the extended Hippo network will be of paramount importance in designing new and effective strategies to control cancer.

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