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WW domain-containing proteins: Retrospectives and the future

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1. ABSTRACT

WW domains are protein modules that mediate protein-protein interactions through recognition of proline-rich peptide motifs (PRM) and phosphorylated serine/threonine-proline sites. WW domains are found in many different structural and signaling proteins that are involved in a variety of cellular processes, including RNA transcription and processing, protein trafficking and stability, receptor signaling, and control of the cytoskeleton. WW domain-containing proteins and complexes have been implicated in major human diseases including cancer as well as in major signaling cascades such as the Hippo tumor suppressor pathway, making them targets for new diagnostics and therapeutics. In this review, we discuss how WW domains provide versatile platforms that link individual proteins into physiologically important networks and the indispensible role of WW domain-containing proteins in biology and pathology, especially tumorogenesis.

2. INTRODUCTION

The function of a protein and its functional networks are determined by its amino acid composition, which encodes for unique modular domains. These domains mediate protein-protein interactions through the recognition of short peptide motifs in their binding partners. In many cases, these interactions control their partners' cellular signaling by modulating their activity, changing their subcellular localization, substrate specificity, assembly of multiprotein complexes, and modulating the function of an entire pathway in the cell. One such important domain is a small modular domain known as WW domain.

WW domains are found in many different signaling and structural proteins, often localized in the cytoplasm. Within these proteins, WW domains are joined to a number of distinct interaction modules (Figure 1), including phosphotyrosine (p-Y) binding domains (PTB)

Table 1. WW domain classes

Class	Recognized motif	Examples	References
I	L/ P)Pp(Y/poY)	YAP, WWOX, NEDD4, ITCH	(4, 13, 18, 22-24)
II	PPLPp	FBP-11	(159)
III	(p/f)P(p/g)PPpR and (P/f)PP(R/K)gpPp	FE65, FBP21	(17, 25)
IV	(poS/poT)P	PIN1	(17, 25, 27, 28)
V	(p/f)PPPPP	PRP40	(29)

L: leucine, S: serine, T: threonine, po: represents a phosphorylated residue, and lower-case letters represent favored but not highly conserved residues.

(e.g. in the FE65 protein) and FF domains (e.g. in CA150 and FBP11) (1, 2), as well as protein localization domains, such as C2 domain (e.g. in NEDD4 family proteins such as NEDD4 and ITCH) and pleckstrin homology (PH) domains (e.g. in PLEKHA5 and CAMGAP1) (3). WW domains are also linked to a variety of catalytic domains, including HECT E3 protein-ubiquitin ligase (in NEDD4 family proteins) (4), rotomerase/peptidyl prolyisomerase (e.g. in PIN1) (5), Rho GTPase-activating protein (e.g. in CAMGAP1) and short-chain dehydrogenases/reductases (SDR) / oxidoreductases (e.g. in WWOX) (6, 7). Consequently, WW domain-containing proteins are involved in a variety of cellular processes, including RNA transcription and processing, protein trafficking and stability, receptor signaling, and control of the cytoskeleton (8).

The WW domains attracted significant attention because of their presence in many proteins involved in signaling complexes that have been implicated directly or indirectly in several human diseases. Such diseases include Liddle's syndrome of hypertension, muscular dystrophy, Alzheimer's and Huntington's diseases (9), and cancer (10-12). In this review, we discuss the indispensible role of WW domain-containing proteins in biology and tumorogenesis.

3. IDENTIFICATION AND CLASSIFICATION OF WW DOMAIN

WW domains were identified through detailed characterization of the Yes-associated protein (YAP) using computer-aided analysis of imperfectly repeated sequences in the mouse isoform of YAP, and in a yeast factor RSP5 (13). To identify the WW domain ligand, Sudol *et al.* (14) performed a functional screen of a cDNA expression library and identified two putative WW domain ligands, WBP-1 and WBP-2 (14). These two ligands contained a proline-rich region that binds strongly and specifically to the WW domain of human YAP. This proline-rich region consisted of a five-amino-acid sequence, PPPPY (where P is proline and Y is tyrosine), which is perfectly conserved in the two ligands. By sequentially replacing each of these five positions with alanine for *in vitro* binding assays, a preliminary binding consensus of XPPXY (X, is any amino acid) was established (14).

Subsequent peptide-binding and substitution studies, as well as mutagenesis screens, have facilitated the identification of consensus binding sequences for a large number of WW domains. To date, there are five known classes of WW domains classified based on their ligand preferences (15-18) and are summarized in Table 1. Class I WW domains, recognize an (L/ P)Pp(Y/poY) motif (in which L represents leucine, po represents a phosphorylated residue, and lower-case letters represent favored but not highly conserved residues) (19, 20). In most cases, tyrosine phosphorylation of the terminal Y in PPxY motifs has been proposed to regulate their activity and hence affect their binding affinities to WW domains. For example, phosphorylation of tyrosine in the PPxY motif of beta-dystroglycan disrupts its interaction with the dystrophin WW domain-containing protein (21). Class I WW domains represent the largest group of WW domain-containing proteins and are present in a wide variety of signaling molecules such as YAP, ITCH and WWOX (4, 13, 18, 22-24). The second class of WW domains binds specifically to a PPLPp motif. An example of this class is the WW domain of the formin-binding protein, FBP-11 (25). The third class binds PR (R represents arginine) -rich motifs and can be further divided into two independent subclasses. One subclass recognizes sequences of the type (p/f)P(p/g)PPpR and binds, for example, to the WW domain of FE65. A second subclass recognizes sequences of (P/f)PP(R/K)gpPp (17, 26) and binds, for example, to the WW domain of FBP21. Members of the fourth class of WW domains, such as the PIN1 WW domain, bind proline residues preceded by phosphorylated serine or threonine (poS/poT)P (17, 27, 28). In general, phospho-threonine-containing peptides bind more strongly compared to phospho-serine-containing peptides (17). Finally, a fifth class, which includes the two tandemly repeated N-terminal WW domains of yeast PRP40 protein, binds uninterrupted polyproline sequences of the type (p/f)PPPPP, in which the first residue of the sequence must be hydrophobic (17). Interestingly, both WW domains of PRP40 also recognize class 1 and class 2 binding motifs (29).

3.1. WW domain structure

WW domain, consisting of about 40 amino acid residues, is the smallest and most compact globular structure known to occur naturally (Figure 2A). With the support of no disulfide bridges or cofactors, it forms a monomeric protein-binding module consisting of three-stranded antiparallel beta-sheet motif (30) (Figure 2B). The WW domain gained its name because of the presence of two highly conserved tryptophan (W) residues. These two conserved residues are separated by 20-23 amino acids in the polypeptide chain and are located on opposite faces of the twisted beta-sheet. A ‘‘hook’’ structure at the N-terminal region is stabilized by contacts between the N-terminal tryptophan and the proline of a highly conserved ‘‘Leu–Pro’’ motif, 3–4 residues preceding the N-terminal tryptophan. This cluster is part of an extended hydrophobic core that involves the C-terminal region of the WW domain (31, 32).

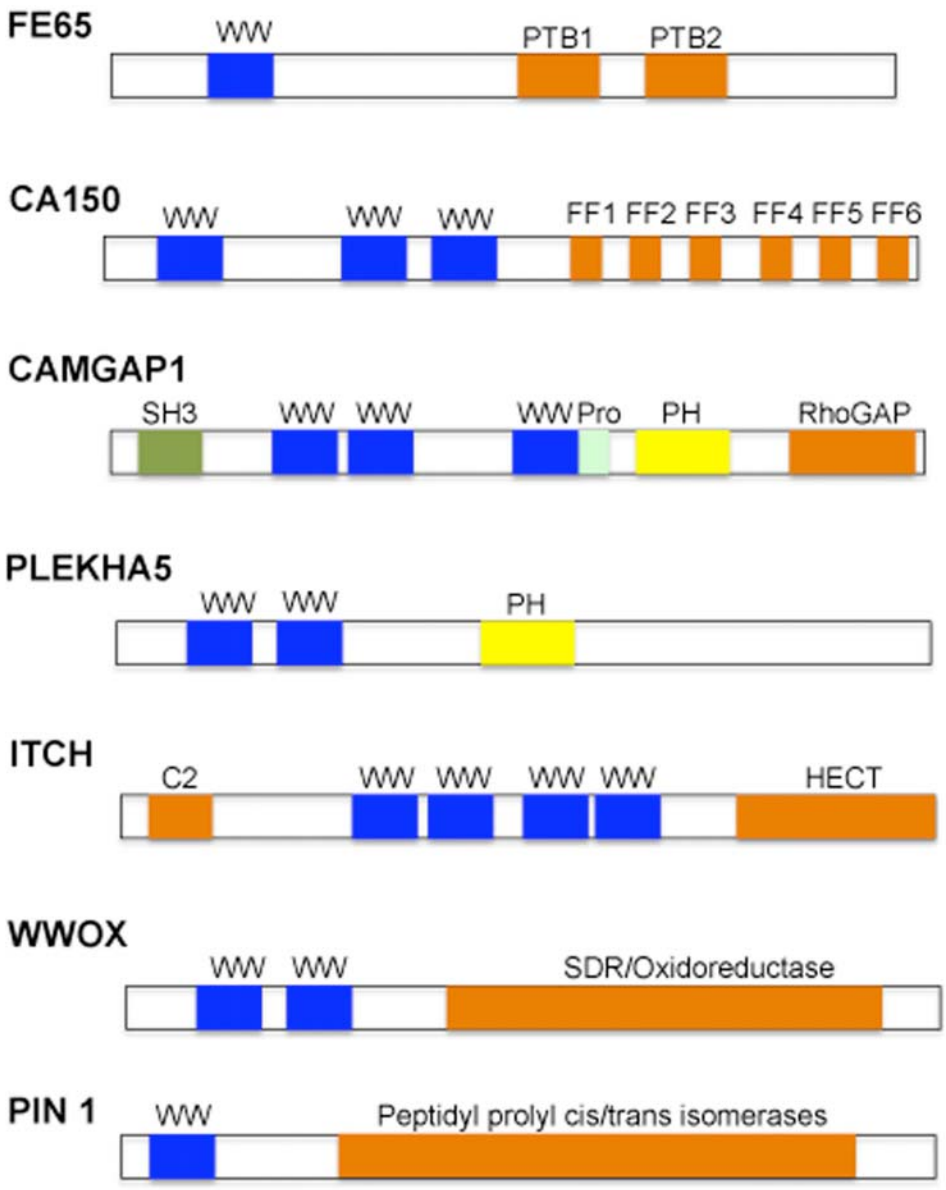

Figure 1. Presence of WW domains in different signaling and structural proteins. WW domains are joined to various interacting modules and catalytic domains. Phosphotyrosin binding domain (PTB) domains are important for protein–protein interaction found in proteins involved in numerous biological processes including signaling through cell-surface receptors and protein trafficking (1). FF domains present a variety of nuclear transcription and splicing factors, as well as the p190 family of RhoGAPs. With WW domains, FF domains may function in coupling the processes of transcription and splicing, as these WW domains can interact with essential splicing proteins (2). The Rho GTPase-activating proteins (RhoGAPs) are one of the major classes of regulators of Rho GTPases found in all eukaryotes that are crucial in cell cytoskeletal organization, growth, differentiation, neuronal development and synaptic functions. Recent studies have implicated them as specific negative regulators of Rho protein signaling pathways (7). Pleckstrin homology domains (PH) occur in a wide range of proteins involved in intracellular signaling or as constituents of the cytoskeleton (3). C2 domain is involved in binding phospholipids in a calcium dependent manner or calcium independent manner. HECT E3 protein-ubiquitin ligase domain found in this family of ubiquitin ligases leads to protein ubiquitination. This process affects protein stability, function and localization (4). Short-chain dehydrogenases/reductases (SDR)/oxidoreductases domain are enzymes that catalyze the transfer of electrons from one molecule to another utilizing usually NADP or NAD as cofactors and are involved in different cellular metabolic pathways (6). Peptidyl prolyl cis/trans isomerases are ubiquitous enzymes and have been shown to be involved in protein folding, protein translocation through biological membranes, and signal transduction (5).

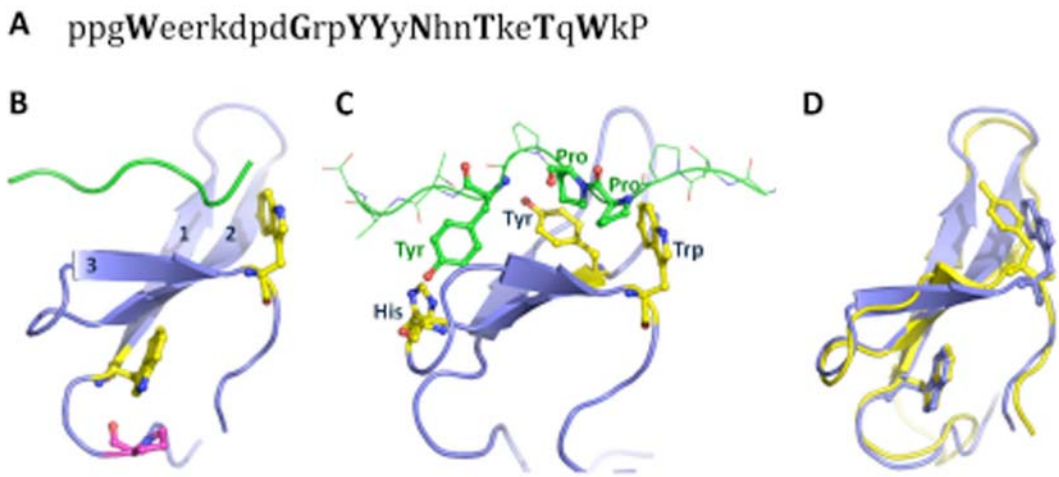

Figure 2. Model of a WW domain structure. (A) Consensus sequence of WW domain. Highly conserved amino acids are shown in bold upper case letters. (B) Cartoon presentation of the WW1 domain based on PIN1 crystal structure (PDB code 1F8A). The three beta-strands are numbered in sequence. The two conserved tryptophan (yellow) and proline (magenta) residues are shown in stick-ball presentation . The ligand is shown in green ribbon. (C) Presentation of a PPxY ligand (green) bound to WW1 of YAP65 (PDB code 1JMQ). Ligand Tyr (shown in green stick-ball) is coordinated by the WW1 His (shown in yellow stick-ball). Two Pro of the ligand (shown in green stick-ball) are fitted into a hydrophobic pocket formed by WW1 C-terminal Trp and Tyr. (D) Superimposition of atypical WW2 from WWOX (yellow, pdb code 1WMV) to the typical WW1 domain from Pin1 (blue, PDB code 1F8A) with an RMSD of 0.8Å. Conserved Trp and the atypical Tyr are shown in stick-ball presentation. Figure and superimposition were prepared by PyMOL (DeLano Scientific).

Proline-rich substrates bind to the hydrophobic surface formed by contacts between the C-terminal tryptophan on the third β-strand and a conserved aromatic residue, typically tyrosine or phenylalanine, on the second beta-strand. The ligand binding is coordinated by the C-terminal tryptophan which forms van der Waals contacts with the two conserved prolines of the PPxY ligand, whereas a conserved histidine coordinates the conserved terminal tyrosine of the ligand that fits into an extended hydrophobic pocket (33-35) (Figure 2C).

An atypical tyrosine substitution of the second conserved tryptophan classifies some WW domains as members of an atypical subfamily. Examples of such WW domains are the second WW domains (WW2) of the eukaryotic Salvador (SAV1) (36), the human MAGI1 (membrane associated guanylate kinase, WW and PDZ domain containing 1) (36), and the human WWOX (WW domain-containing oxidoreductase) (22). Two monomers of WW2 domain of SAV1 interact to form a beta-clam-like homodimer (36). Unlike SAV1, the WW2 of MAGI1 was found in the monomeric state. The inability of MAGI1 to form homodimers is caused by the presence of an Asp substitution in MAGI1 (corresponding to Ser in SAV1) (36). The larger side chain of Asp prevents MAGI1 dimerization probably by clashing with the conserved Glu immediately next to the N-terminal conserved Trp. Indeed, substituting the Asp with a Ser resulted in the dimerization of MAGI1 (36). Similar to MAGI1 WW2, NMR solution structure of the human WWOX WW2 shows that it is present in a monomeric form (pdb code 1WMV; (37)). Since the WW2 of WWOX also contains an Asp at the position of Ser of SAV1 WW2, similar to MAGI1, we suggest that this Asp is the reason for the observed monomeric state of the WWOX WW2. These atypical WW domains fold into a similar structure to that of the typical first WW (WW1) domain (Figure 2D).

3.2. Tandemness of WW domains

WW domains are found to occur in tandem repeats (Figure 1), raising questions whether these repeats are redundant or each one is specific for a certain target? Whether they work together in a synergistic fashion, or each one works independently? And if they are specific, then what factors would most likely contribute to increasing binding affinity and specificity of a WW domain? Answering these questions can lead to better understanding of the molecular mechanisms by which WW domain proteins ensure specificity and fidelity in cell signaling.

As for specificity, it seems that each of the tandem WW domains can be specific for a certain set of ligands. For example, most of the characterized WWOX targets bind to the WW1 domain of the protein and not to the WW2. In a recent proteomic mapping of human WW domains, it was shown that WW1 of WWOX binds 18 human proteins whereas WW2 interacts with 16 human proteins (38). Since some of the known ligands of WW domains of WWOX are common for the two domains while others are unique to each one, it is likely that many versatile proteins such as the tumor suppressing WWOX have evolved both tandem and non-tandem ways to target a large repertoire of proteins to control growth of cells in a precise way. Another example of specificity is the ITCH

protein which possesses four WW domains that differ in their ability to bind ligands (23, 39-41). Using mass spectrometry (MS), previous efforts have focused on investigating whether putative interacting proteins, which bind to WW2 of ITCH, can also bind to the other WW domains of ITCH. It has been shown that RNA polymerase II Large Subunit (LS), p68, and p25 subunits of mammalian Cleavage Factor I (CFIm) were pulled down by each of the four WW domains. On the other hand, Ewing Sarcoma (EWS) (a protein with an RNA recognition motif frequently rearranged with Ets family transcription factors in cancers such as Ewing's sarcoma (42)) was specifically precipitated by ITCH WW2 alone. In a recent study, Bellomaria *et al*. attempt to identify the determinants of the molecular interaction between p63 and ITCH (43). It was found that the residues close to the PPxY motif in p63 (I549) could also play an important role in mediating the binding affinity and specificity to WW-2 domain of ITCH (43). Thus, the isolated domains of the tandem WW domain arrays can show both common and selective binding suggesting that the tandem WW domains may act synergistically to bind common partners or, alternatively, may act as a scaffold to recruit specific proteins to individual WW domains (8).

Another issue in WW domain tandemness is the synergistic effect of binding to one domain on the binding of the other one. For example, in the case of suppressor of deltex (Su(dx)), *Drosophila* ortholog of ITCH, it appears that binding to one domain induces the folding of the other one to its native structure, enhancing its ability to interact with its target sites, and vice versa (44). Another example of binding synergism is the binding of YAP to receptor tyrosine-protein kinase, ErbB4. The first WW domain of YAP2 is primarily involved in recognition of PPxY motif(s) on ErbB4 (45). However, a sensitive functional assay of transcription has shown that the presence of an intact second WW domain of YAP2 enhances the biological function of the YAP2-ErbB4 complex (45).

In general, it has been suggested that WW domain tandem repeats could participate in bridging interactions by binding simultaneously to proline rich motifs (PRMs) in separate binding partners, thereby holding together complex interaction networks (29).

4. WW DOMAINS FUNCTION IN DIFFERENT BIOLOGICAL PROCESSES

Functional studies of WW domains revealed that novel WW domain-binding partners constitute many of the interacting proteins and are components of multiprotein complexes involved in molecular processes. Here, we summarize some of the main processes.

4.1. Transcription

Among several examples of WW domain complexes functioning in the cell nucleus, the best-documented one is the physical and functional complex formed between the WW domain of YAP and the PEBP2 (polyoma enhancer binding protein 2) transcription factor, in which the YAP WW domain acts as a transcriptional co-activator through interaction with a PPPY motif in PEBP2. It was shown that mutation of the PY motif in the activation domain of the DNA-binding subunit of PEBP2 (PEBP2 alpha) abolishes its transactivation function (46). YAP was also found to modulate the transactivation functions of steroid hormone-responsive target genes (47). YAP, together with WBP2, was shown to act as a coactivator for the transactivation pathways of estrogen and progesterone receptors thereby contributing to the regulation of female steroid hormone receptor function (47). Recently, the role of YAP in control of transcription/transactivation was highlighted in regulating genes involved in proliferation and apoptosis, and was implicated in the Hippo tumor suppressor pathway (below). Another example is the hematopoietic transcription factor (NF-E2), which contains two PPxY motifs, and can be recognized by WW domains of E3 ubiquitin ligases. Interaction of WW domain with the two PPxY motifs of NF-E2 is essential for transcriptional activation. This was demonstrated by the fact that NF-E2 mutants expressing one of the two PPxY motifs were found defective in their co-transcriptional activity.

Negative regulation of transcription by certain WW domains may also be possible. PQBP-1, a novel polyglutamine tract binding protein with a WW domain, was shown to inhibit activation of transcription by BRN2 (48). Another example is WWOX which alters transcription via sequestering its target transcription factors either in the cytoplasm (as in the case of c-JUN, AP2, ErbB4-ICD) or on the chromatin (as in the case of RUNX-2) (reviewed in (10, 11)). Intriguingly, some WW domain proteins can function as co-activators and co-repressors. TAZ, the YAP paralog, which contains a single WW domain, coactivates RUNX2-dependent gene transcription whileit corepresses PPAR-dependent gene transcription (49). Additionally, tyrosine phosphorylation controls RUNX2-mediated subnuclear targeting of YAP to repress transcription (50). The implications of regulating transcription by WW domain-containing proteins in apoptosis and differentiation were intensively studied (below).

4.2. Apoptosis

Apoptosis is a vital biological process that governs tissue development and homeostasis, and any defect in this process leads to disastrous effects, often in the form of disease or disorder including cancer. The role of different WW domain-containing proteins on apoptosis and tumorogenesis has been investigated in different contexts.

4.2.1. YAP-p73 functional role in apoptosis

One interesting WW domain-containing protein that appears to play a dual role in both apoptosis and transformation (below) is YAP. In the context of apoptosis, it was shown that by regulating p73 transactivation function, YAP is capable of inducing apoptosis. For example, following DNA damage, p73 interacts with YAP1 in the nucleus and associates with p300 and PML to induce pro-apoptotic gene transcription (51-53). Moreover, other studies have shown that YAP phosphorylation modulates its pro-apoptotic function in negative and positive manners. Upon phosphorylation by AKT, YAP is excluded from the

nucleus and can no longer enhance the transcription of pro-apoptotic genes via the p73 transcription factor. By contrast, Matallanas *et al* showed that upon FAS receptor stimulation and RASSF1A activation, phosphorylation of YAP by LATS1/2 stimulates its translocation into the nucleus, causing an increase in transcription of pro-apoptotic genes (51). In addition, it has been shown that c-Abl phosphrylates YAP upon DNA damage (54). This phosphorylated form of YAP activates the transcription of pro-apoptotic genes as a response to DNA insult. Additionally, YAP was shown to compete with the E3 ligase ITCH on interacting with p73, preventing its proteosomal degradation (54). By doing so, YAP1 prevents p73 disposal by the proteosome, leading to further transcription of pro-apoptotic genes, designating YAP as a pro-apoptotic protein and ITCH as a pro-survival one (below). Altogether, these observations indicate that depending on cell context and stimuli, different WW domain-containing proteins may respond in variable ways.

4.2.2. WWOX-p73 functional cross talk

We recently demonstrated that WWOX, via its WW1 domain, interacts with p73 to induce apoptosis in a p73-transcription-independent manner (55). We found that mutagenesis of Y487 of p73 beta abolished WWOX–p73 beta interaction, and that p73 isoform (p73 gammą lacking a PPPY motif, failed to bind WWOX. Furthermore, a mutation in Y33 in WWOX WW1 domain, but not Y61 in WW2 domain, abolished this interaction, indicating a specific association between the WW1 domain of WWOX and the PPPY motif of p73. Moreover, phosphorylation of Y33 by Src kinase enhanced WWOX–p73 interaction. Upon binding to WWOX, p73 is sequestered in the cytoplasm, whereas more p73 is translocated to the nucleus when WWOX is knocked down by a specific siRNA. Accordingly, we observed a significant decrease in p73-transactivation ability upon WWOX co-expression, as well as a decrease in p21 protein level, a p73 target gene. Intriguingly, this sequestration enhanced p73 proapoptotic activity; Saos2 cells coexpressing WWOX and p73 beta exhibited an increased sub-G1 fraction, compared with WWOX or p73 beta alone, indicating that p73 binding to WWOX increases apoptotic activity independent of p73 transcriptional activity. While p73-dependent apoptosis seems to be primarily regulated by its ability to transcriptionally activate proapoptotic p53 target genes (56), some studies have suggested transactivation-independent apoptosis (57, 58). Therefore, it is possible that WWOX enhances p73 cytoplasmic apoptotic function. Another possibility is that WWOX competes with other WW domain-containing proteins, such as ITCH, that bind and degrade p73 to potentiate or diminish p73 transcriptional and apoptotic activity (58). Indeed, we have found that WWOX inhibits coactivation of p73 by YAP, while expression of YAP2 did not affect this suppression. When WWOX is in the nucleus together with p73, it still inhibits its association with YAP and thus prevents its coactivation, indicating that the effect of WWOX expression is superior to that of YAP (55). Recently, a caspase-cleaved p73 fragment was demonstrated to localize to the mitochondria and enhance TRAIL-induced apoptosis (57). It is thus possible that following association with WWOX, p73 is cleaved in the cytoplasm and enhances transcription-independent apoptosis (55).

4.3. Differentiation

Differentiation is a vital biological process that governs tissue development and homeostasis, and any defect in this process leads to different diseases including cancer. Several WW domain-containing proteins have been shown to play a role in differentiation. Targeted ablation of murine *Wwox* gene led to postnatal lethality, however, by 3 weeks of age, mice developed focal lesions along the diaphysis of their femurs that resembles early osteosarcomas. Biochemical analysis of WWOX interacting proteins suggested that physical and functional association of WWOX with RUNX2, the key transcription factor specific for osteoblast differentiation, might contribute to the development of osteosarcoma in *Wwox*-deficient mice (11, 59). This association suppresses RUNX2 transactivation function. Interestingly, we observed impaired differentiation in osteoblasts isolated from *Wwox*-deficient mice, suggesting that osteosarcoma formation could be related to a differentiation defect in the osteoblast compartment. In fact, WWOX seems to be essential in regulating proliferation and maturation of osteoprogenitor cells during bone formation (11, 59). RUNX2 levels increased in *Wwox*-deficient mice both in clavaria and femur bones. Recently, we observed that whereas WWOX is deleted or attenuated in osteosarcoma cases, RUNX2 levels are highly elevated in the majority of samples (60) suggesting that these events are common in the development of human cancer.

Since WWOX seems to have a central role in osteoblast differentiation, its loss might promote development of osteosarcoma. Of note, RUNX2 is a target of other WW domain-containing proteins, including coactivators (such as TAZ (61)), repressors (such as YAP (50)) and ubiquitin ligases (such as SMURF2 (62)). TAZ is able to induce transcription of osteogenic genes by inducing RUNX2 activity which diverts mesenchymal stem cells from adipocyte differentiation by repressing the PPAR-γ transcription factor (63). In addition, TAZ was recently found to bind to SMAD-2/3-4 and regulate their activity by mediating their nuclear accumulation in response to TGF-beta (64) thus maintaining pluripotency while its loss leads to differentiation and loss of pluripotency markers (64). Therefore, deregulating the balance between the different WW domain adaptor proteins and RUNX2 may determine the functional outcome of RUNX2 expression thus regulating mesenchymal stem cells and osteoblast differentiation. Further delineation of WW domain proteins and RUNX2 association would uncover their functional cross-talk in osteoblast differentiation and osteosarcoma.

Another WW domain found to be involved in differentiation is the ubiquitin E3 ligase ITCH. It was shown that ITCH controls the function of TH2 cells by targeting Jun B that, following antigen exposure and in addition to other transcription factors, regulates naïve CD4 T cell differentiation into two distinct subsets of TH cells. Of note, serum concentrations of TH2-related

immunoglobulin G1 (IgG1) and IgE subclasses were much higher in Itch-deficient mice than in control littermates (65). These studies are consistent with ITCH being a modulator of TH2 differentiation through its ubiquitin E3 ligase function.

In the skin, ITCH can also modulate the epidermal keratinocyte differentiation program by targeting multiple substrates for protein ubiquitylation. For example, while the predominant p63 isoform in the epidermis, ΔNp63, is exclusively expressed in the basal proliferative compartment, Notch is mainly expressed in the spinous layer and ITCH is present throughout the epithelium (though ITCH mainly accumulates in the suprabasal cell layers). By regulating both ΔNp63 (66) and Notch (67) protein levels, ITCH could exert a role in governing epidermal stratification. ITCH would facilitate keratinocytes to exit the basal layer by shortening ΔNp63 half-life in the upper layers and fine-tuning Notch expression to promote the basal/spinous transition. This might, at least partially, be responsible for the increased epidermal thickness phenotype displayed by the Itchy mutant mice (reviewed in (24)).

A recent work correlated the WW domain containing protein YAP with embryonic stem (ES) cell differentiation. In this study, it has been shown that YAP protein levels decreased while its phosphorylated form increased, resulting in its inactivation during ES cell differentiation (68). Consistently, YAP is elevated during induced pluripotent stem (iPS) cell reprogramming. YAP knockdown leads to a loss of ES cell pluripotency, while ectopic expression of YAP prevents ES cell differentiation in vitro and maintains stem cell phenotypes even under differentiation conditions. Moreover, YAP binds directly to promoters of a large number of genes known to be important for stem cells and stimulates their expression. These data establish a critical role of YAP in maintaining stem cell pluripotency (68).

4.4. Splicing

The mechanism of RNA splicing, the removal of introns and joining of exons in a primary transcript, is quite complex and involves several small nuclear RNAs and their associated proteins consisting of large ribonucleoprotein complexes, called spliceosome. The WW domain of the prolyl isomerase (Ess1) binds the phosphorylated C-terminal domain (phospho-CTD) of the largest subunit of RNA Polymerase II. Analysis of phospho-CTD binding by other four WW domain-containing proteins indicates that splicing factor Prp40 and the RNA polymerase II ubiquitin ligase (Rsp5) can also bind the phospho-CTD. Domain dissection studies reveal that phospho-CTD binds to multiple locations in Prp40, including sites in both the WW and FF domain regions (69). Other studies demonstrated that WW domains might associate preferentially with the U2 small nuclear ribonucleoprotein and with splicing factors SF1, U2AF, and components of the SF3 complex (70). Altogether, these studies and many others suggest an essential role of the WW/FF domain-containing factors in pre-mRNA splicing that likely occurs in concert with transcription *in vivo*.

4.5. Ubiquitylation

Ubiquitylation of proteins targets them for degradation or other cellular fates, such as endocytosis, vesicular sorting, and histone modifications, and has been implicated in numerous human diseases (71). The specificity of the ubiquitination reaction is achieved by the E3 ubiquitin ligase (E3). Based on the sequence homology of their E2-binding domains, E3s can be generally classified into three subfamilies: (1) the homologous to E6-AP carboxyl terminus (HECT) domain-containing E3s; (2) the really interesting new gene (72) finger domain-containing E3s; (3) and the U box E3s. In the first group of E3s, the HECT domain associates with the E2 and provides the catalytic E3 activity (73), whereas the substrate specificity is dictated by protein-protein interaction, which account for their classification into three further subfamilies: (i) HERC E3s containing RCC1-like domains (RLDs); (ii) C2-WW-HECT E3s possessing WW domains (72); and (iii) SI-HECT E3s lacking either RLDs or WW domains (74). The C2-WW-HECT E3s likely represent the best characterized subgroup of HECT ligases. They consist of monomeric proteins with a common general modular architecture composed of an N-terminal protein kinase C (PKC)-related C2 domain, two to four WW domains that determine target specificity, and a C-terminal HECT domain (74). The C2-WW-HECT E3s are found in several subcellular locations, including the plasma membrane, early and late endosomal compartments, and lysosomes (75). Some family members can transiently enter the nucleus to target nuclear substrates for protein ubiquitylation (76, 77). The WW domains mediate ligase-substrate associations through interactions with a variety of PRM and proline-containing phosphoserine/phosphothreonine sequences of the protein substrate. For example, ITCH, a NEDD4-like E3 ubiquitin ligase, leads to degradation of c-JUN (78), p63 (66), p73 (79), and ErbB4-CYT1 (80) in a WW-PPxY dependent manner. Another example of WW domain containing E3 ubiquitin ligase involved in cancer is WWP1. WWP1 has been shown to modulate proteasomal degradation of p63 and regulate apoptosis and more recently to mediate degradation of ErbB4 in breast cancer cells (81-83). Characterization of specific E3 ligase inhibitors based on WW domain-PPxY complex structure might therefore contribute to regulating the stability of specific substrates and hence modulating their function in tumorogenesis.

5. WW DOMAIN PROTEINS IN TUMORIGENESIS

As mentioned above, WW domain proteins are involved in multiple signaling pathways and regulate the expression, localization and stability of their proline-rich containing targets. Therefore, deregulation of WW domain protein expression might have significant impact on cell growth and tumorigenesis. Indeed, genomic changes including deletion (as in the case of WWOX) and amplification (as in the case of YAP and ITCH) have been reported and shown to contribute to cancer transformation.

5.1. Molecular and cellular function of tumor suppressor wwox

WWOX spanning the second most-active common fragile site (reviewed in (84)) has been shown to behave as

a *bona fide* tumor suppressor. Aberrant expression of WWOX has been reported in various cancer cell types including breast, ovarian, prostate, gastric, hematopoietic, esophageal, hepatic, bladder and lung (11, 85). Work by Paige *et al.* has specifically shown that WWOX expression is altered in several tumor types (86). In an attempt to further explore the tumor suppressor behavior of *WWOX*, it has been shown that restoration of *WWOX* in cancer cell lines, harboring low expression of endogenous *WWOX*, results in inhibition of cell growth and suppresses tumorigenicity *in vivo* (10, 11). These findings also suggest that loss of WWOX expression is associated with a growth advantage for cancer cells and that restoration of *WWOX* in these cells sensitizes them to apoptosis. Indeed, targeted ablation of the *Wwox* gene in mice resulted in osteosarcomas (87). In addition, *Wwox*-heterozygous mice develop higher incidence of spontaneous lung and mammary tumors as compared to wild-type matched-littermate ((87) and unpublished data). Furthermore, *Wwox*-heterozygous mice significantly developed more lung tumors and lymphomas when treated with ethyl nitrosourea (ENU) and more forestomach tumors when treated with NMBA (reviewed in (87, 88). In some tumors that developed in the *Wwox*-heterozygous mice, WWOX expression was maintained, suggesting that haploinsufficiency of WWOX is enough to predispose to tumor formation, while in others the loss of WWOX was evident, suggesting loss of heterozygosity (87, 88).

On the molecular level, it appears that WWOX fulfills its tumor suppressor function by regulating cellular protein functions. WWOX, via its first WW domain, binds PPxY-containing proteins and sequesters them in the cytoplasm, suppressing their transcriptional transactivation functions. Examples of these proteins are p73 (55), AP2 alpha/gamma(89), ErbB4 (90), and c-JUN (91). Moreover, WWOX competes with other WW domain-containing proteins for binding to these interactor proteins; for example, WWOX outcompetes YAP for binding to the ErbB4 ICD and inhibits the YAP-induced ICD activity (90). In addition to sequestering the active ErbB4 fragment, WWOX also binds and stabilizes the full-length ErbB4 at the cell membrane (92). WWOX also regulates the Wnt-beta-catenin signaling pathway by preventing the nuclear import of the DVL protein (93). WWOX can also regulate function of RUNX2 (59), EZRIN (94) in WW-PPxY dependent fashion. Phosphorylation of WWOX at Tyr 33 in WW-1 of WWOX has been shown to mediate WWOX interaction with several key signaling proteins including p53, JNK1, MDM2 and Hyal-2 in a non-PPxY dependent manner (reviewed in (95, 96)). Recently, Fu *et al.* described a novel role of WWOX in NF-κB regulation and viral tumorigenesis (97). WWOX inhibits Tax-induced activation of the canonical, but not the non-canonical NF-κB pathway by blocking Tax-induced IKKα recruitment to RelA and subsequent RelA phosphorylation at serine 536. A point mutation in Tyr 33 of WWOX is unable to block the IKKα recruitment and RelA phosphorylation and therefore lose the ability to inhibit Tax-mediated tumorigenesis. The numerous interacting partners of WWOX suggest that it participates in multiple signaling pathways hence highlighting its indispensible role in cancer.

5.2. YAP in cancer

An interesting WW domain containing protein that appears to play a critical role in tumorigenesis is the major downstream effector of the Hippo pathway, YAP. In fact different convincing data support the notion that YAP is an oncogene. These include mapping of YAP to chromosome 11q22, a region commonly amplified in different human cancers including intracranial ependymomas, oral squamous cell carcinomas, medulloblastomas, lung, pancreas, cervix and ovary cancers (51). Beside its genomic amplification, YAP protein levels and its nuclear localization have been shown to be elevated in multiple types of cancer (12, 98-102). Knock-in mice overexpressing an inactive S127A mutant of YAP in hepatocytes display enhanced liver growth and HCC tumorigenesis (99). In addition, overexpression of YAP in transformed mammary epithelial cells (MCF10A) is sufficient to promote epithelial-mesenchymal transition (EMT), induce a proliferative advantage, inhibit apoptosis and induce anchorage-independent growth, characteristics associated with transforming oncogenes (103). On the other hand, different evidence showed that YAP acts as a tumor suppressor gene. For example, it was shown that the 11q22-23 is a site of frequent loss of heterozygosity in sporadic breast cancer (104), leading to decreased or lost YAP protein expression in breast cancers. Moreover, YAP knockdown in breast cell lines is associated with increased tumor growth in nude mice (105)**.** In addition, YAP has been shown to have proapoptotic functions in different cellular contexts (above). Both oncogenic and tumor suppressor functions of YAP might be explained by its subcellular localization. Phosphorylation of YAP by LATS1 sequesters it in the cytoplasm through 14–3–3 binding, leading to spatial separation from nuclear target transcription factors and target gene promoters, or leads to its degradation by the beta-TRCP E3 ubiquitin ligase (51). The interaction between LATS1 and YAP is dependent on YAP WW and LATS1 PPxY domains (106, 107). Whatever the mechanisms responsible for YAP regulation, they appear to be highly regulated by WW-PPxY interactions that regulate YAP phosphorylation and physical interaction with multiple binding partners that may explain the discrepancy in YAP functions.

5.3. ITCH as a proto-oncogene

One more WW domain-containing protein implicated in tumorigenesis and chemosensitivity is the ubiquitin E3 ligase ITCH. ITCH is a candidate human oncogene that is upregulated in different cancer types including anaplastic thyroid carcinoma (108), breast and ovarian carcinomas, as well as in some sarcomas as revealed by Oncomine database. In fact, it was demonstrated that depletion of ITCH by siRNA promotes apoptosis following chemotherapeutic drug treatment (109). In their work, Rossi *et al.* showed that cells with no functional p53 are more sensitive to ITCH depletion, indicating that changes in levels of ITCH may play an important role in the majority of cancers, where p53 is absent or mutated. Furthermore, reintroduction of ITCH in fibroblasts obtained from *Itch*-deficient mice results in reduced cell death upon DNA damage (109). Overall, these findings suggest that inhibition of ITCH potentiates the

effects of chemotherapeutic drugs and reveals the pharmacological potential of targeting ITCH for cancer therapy (109). A number of ITCH substrates that have been implicated in tumorogenesis and chemosensitivity have been identified, including c-JUN, JUN-B, p73, p63, ErbB4-CYT-1 and others (reviewed in (24)).

6. THE HIPPO TUMOR SUPPRESSOR PATHWAY

The fact that separate WW domains from the same protein, or closely related proteins, can have different specificities for protein ligands, and that a single polypeptide can bind multiple classes of WW domains through separate PRM, suggested that WW domains provide a versatile platform to link individual proteins into physiologically important networks (8). One such important network that has received much attention in the last few years is the Hippo tumor suppressor pathway, which regulates organ size by controlling cell proliferation and apoptosis (12, 102). In *Drosophila*, a kinase cascade composed of four tumor suppressor proteins forms the core of the Hippo signaling pathway. These tumor suppressor proteins are the Ste20-like kinase Hippo (Hpo) (110-114) and its regulatory protein Salvador (Sav) (115), the NDR family kinase Warts (Wts) (116, 117), and its regulatory protein Mats (118). The Hpo-Sav complex activates the Wts-Mats complex by phosphorylation (110, 119). Activated Wts-Mats complex then phosphorylates the oncoprotein Yki and thus inactivates it (120) by excluding it from the nucleus (99, 121). Yki oncoprotein normally functions in the nucleus as a coactivator for the TEAD/TEF family transcription factor Scalloped (122-124).

During cancer development and progression, the balance between cell death and proliferation is impaired. The role of Hippo pathway has been recently delineated and shown to be crucial in controlling cell growth and tumor suppression. For example, genetic mosaic screens in *Drosophila* identified the first component in the pathway *wts* which encodes a nuclear Dbf-2-related family protein kinase (116, 117)**.** Mutation of *wts* leads to robust tissue overgrowth (117). In another screen for *Drosophila* mutations that result in tissue overgrowth, salvador (115) was identified, a gene that promotes both cell cycle exit and cell death. Elevated Cyclin E and DIAP1 levels are found in mutant cells, resulting in delayed cell cycle exit and impaired apoptosis. Salvador contains two WW domains and binds to the Warts protein kinase and exerts its function by restricting cell numbers by functioning as a dual regulator of cell proliferation and apoptosis (reviewed in (115))**.**

The Hippo pathway components are highly conserved from *Drosophila* to mammals, and mammalian homologs of Hpo (MST1/2), Sav (WW45), Wts (LATS1/2), and Yki (YAP) constitute an analogous kinase cascade (99, 100, 125). In both *Drosophila* and mammals the core components of Hippo pathway interact with each other via PPxY motif-WW domain interactions (Figure 3). While the *Drosophila* Hpo and Wts contain PPxY motifs, Sav contains two WW domains. Comparable to this, the mammalian orthologs in the core cassette also contain either PPxY/F motifs, as in the case of LATS1/2 and MST1/2, or WW domains, as in case of WW45. Not only do the core components of the Hippo pathway contain PPxY/WW domains, but also the nuclear effector of the Hippo kinase cassette, YAP or TAZ, function through WW-PPxY interaction. Indeed, it has also been shown that the WW domains of YAP are crucial for YAP transcriptional coactivation function downstream to the Hippo pathway (126). Moreover, several upstream regulators of the Hippo pathway, in both *Drosophila* and mammals, also contain either WW or PPxY motifs (reviewed in (127)). For example, recently, it was shown that the WW domain protein Kibra is a Hippo signaling component upstream of Hippo and Merlin. Kibra acts synergistically with Expanded, and physically interacts with Merlin (128).

Deregulation of key members of the Hippo pathway in cancer, including YAP as mentioned above, has been demonstrated in several studies. The tumor suppressor LATS1/2 promoters are hypermethylated and their mRNA is downregulated in soft tissue sarcoma, astrocytoma, and breast cancer (105, 129-131), and their activity is reduced in medulloblastome (132). In addition the *MOB1* gene is mutated in human melanoma and mouse mammary gland carcinoma cell lines (118) and its mRNA levels are downregulated in human colorectal and non-small-cell lung cancer (133, 134) Importantly, genetic studies in mice have unequivocally demonstrated MST1/2 as tumor suppressors. The germline *Mst1−/−Mst2+/−* mice mainly developed hepatocellular carcinoma (HCC) due to *Mst2* loss of heterozygosity (135). Moreover, tissue-specific ablation of both *Mst1* and *Mst2* in the liver leads to massive HCC (135-137). Strikingly, 70% of human HCC samples examined show markedly reduced MST1/2 activity, as determined by MOB phosphorylation, and most are also confirmed by loss of the cleaved, presumably active, form of MST1 (135). It appears that not only the core components of the Hippo pathway are attenuated in cancer but that the upstream regulators and downstream effectors are as well. As an example, the upstream regulators NF2 and FAT4 are either lost or mutated in different types of cancer (reviewed in (102, 139).

The prevalence of WW domain-mediated complexes in the Hippo pathway should facilitate its molecular analysis, aid prediction of new pathway components, and identify other proteins that could regulate the pathway in a positive or negative manner (127). Based on this knowledge, we have recently mapped several new WW domain proteins that may associate with LATS1. For example, ITCH, via its WW domains, binds PPxY motifs of LATS1, catalyzes its ubiquitination and stimulates its proteasomal degradation (Figure 3) (140). Importantly, ITCH-mediated degradation of LATS1 was associated with enhanced cell growth, induction of epithelial-mesenchymal transition, and increased tumorigenicity (140). Further analysis of WW-PPxY interaction should reveal novel molecular regulators of the Hippo pathway functional networks and its role in normal and disease states. We expect that identification of new components of the Hippo pathway will reveal new levels of regulation and roles in tumorigenesis.

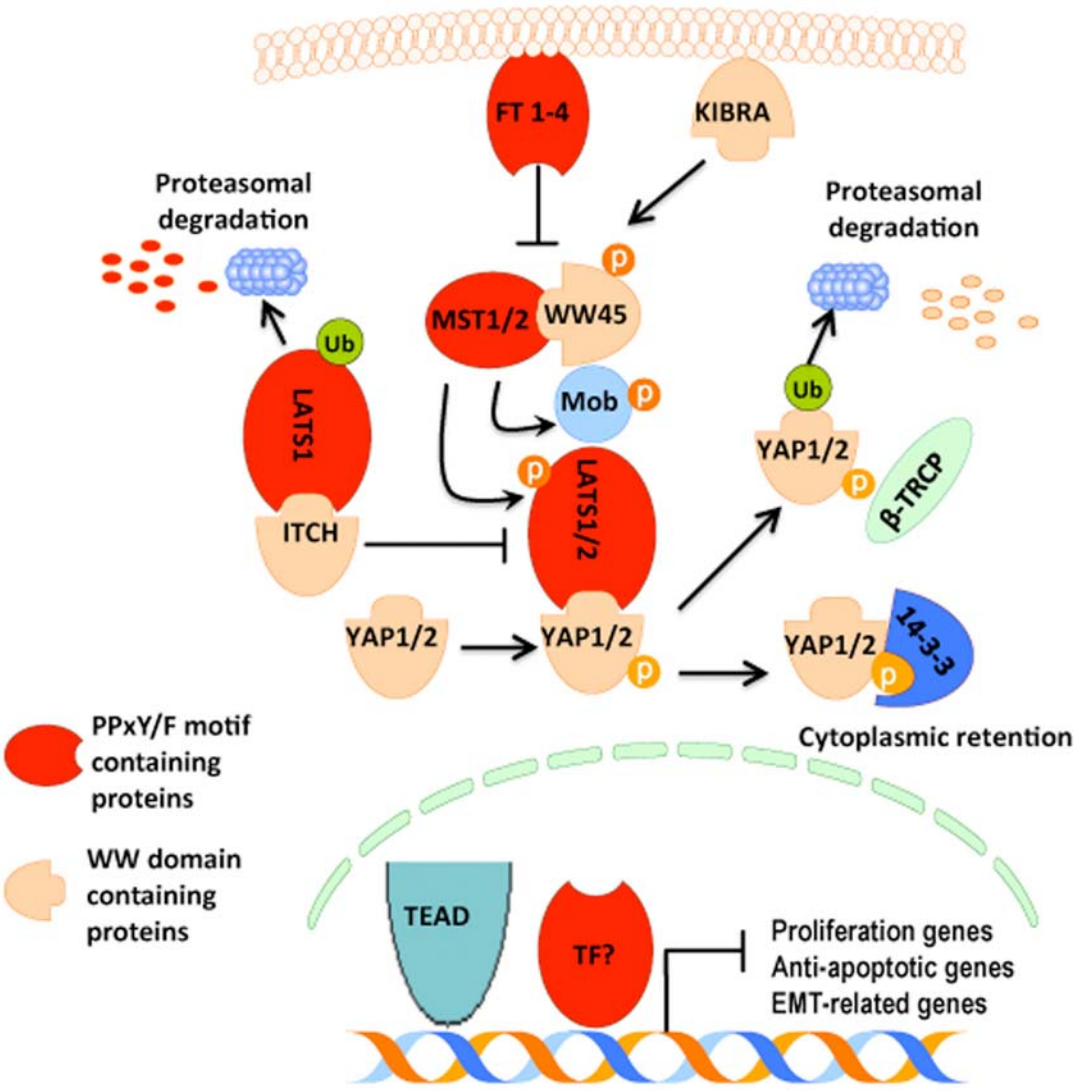

Figure 3. High prevalence of WW domain and WW domain-interacting proteins in the Hippo tumor suppressor pathway. Activation of the Hippo pathway, for example due to high cell-cell contact, drives a kinase cascade leading to the inactivation of YAP. This in turn inhibits YAP co-activation function on downstream target genes involved in pro-proliferation, anti-apoptosis and EMT. Some of the known upstream regulators of the Hippo pathway contain either WW domain/s or PPxY domain/s like KIBRA (128) and FT1-4 (139) respectively. The core cassette of the Hippo pathway including WW45, MST1/2, MOB and LATS1/2 is rich of WW-PPxY interactions. The Hippo pathway effectors, YAP and TAZ, also contain WW domains that play indispensable roles in their co-activation functions and hence regulating organ size and tumorigenesis.

7. WW DOMAIN PROTEINS IN OTHER DISEASES

Besides their involvement in cancer, WW domain proteins are also implicated in several other diseases. We summarize here some of these abnormalities that were reviewed elsewhere (13). These include (i) Liddle's syndrome which results from PPxY mutations in β and γ subunits of the amiloride-sensitive epithelial sodium channel. Here, the PPxY motif is imperfectly degraded by the E3 ubiquitin protein ligase NEDD4, thus leading to a sodium imbalance and subsequently high blood pressure (141, 142); (ii) Rett syndrom which is a dominant neurological disorder caused by loss-of-function mutations of methyl-CpG-binding protein 2 (MeCP2). These result in its impaired interaction with WW domain protein FBP11, hence leading to mental retardation in males (143); (iii) Alzheimer's disease (AD) which is associated with TAU hyperphosphorylation in paired helical filaments (PHFs) (144-146). Phosphorylation on a serine or threonine that precedes proline (pS/T–P) alters the rate of prolyl isomerization and creates a binding site for the WW domain of the prolyl isomerase PIN1 (147). PIN1 specifically isomerizes pS/T–P bonds and regulates the function of mitotic phosphoproteins (147). It was shown that PIN1 can bind TAU contributing to its hyperphosphorylation (148) leading to the formation of

neurofibrillary tangles (NFTs) in the neurons of AD patients. Recent evidence has suggested that downregulation of WWOX in the neurons of AD hippocampi is associated with TAU phosphorylation (149); (iv) Huntington's disease which results from impaired huntingtin isoform interaction with distinct WW domain proteins that bind normal and mutant huntingtin in extracts of HD lymphoblastoid cells (143). Among these proteins, are the spliceosome related (HYPA/FBP-11 and HYPC) and the transcription factor (HYPB) proteins (150, 151); and (v) Muscular dystrophy which refers to weakened muscles and hardship in movement. This is caused by an impaired function of the dystrophin glycoprotein complex that mediates connections between muscle cells and their surrounding cellular structure. The most C-terminal PPxY motif in beta-dystroglycan has been established as a binding site for dystrophin WW domain (21, 152, 153).

8. CONCLUDING REMARKS AND FUTURE PERSPECTIVE

WW domains and their interactions play important roles in different biological processes involved in cellular homeostasis, response to stress conditions and pathology. It is obvious that WW domain proteins do not always act as single proteins, but appear to be part of complexes that can form a complicated pathway with distinct functions in the cell. These complexes and pathways seem to function in a tissue type and cell context dependent manner. Since WW domains and their ligands have been implicated in several major human diseases, the identification and characterization of more WW domain-containing proteins and their cognate ligands, as well as the complexes they involve will have an impact on diagnostics, drug discovery and ultimately on strategies to control these diseases. In fact, the considerable data now available on WW domain structure, the mechanism of interaction with its rigid ligands, and complexes it forms, should facilitate the design of inhibitors or activators of signaling complexes of these domains. Moreover, it is possible that human syndromes involving mutations in the WW domain and the core motif of its ligand can be treated by gene therapy and by small molecules. This is because WW domain and its ligands' core motifs are relatively short (~38 and 5 residues, respectively). Using FDA approved drugs, a recent computational analysis of drug interactions with peptide-binding-domains predicted that the cardiac glycoside digitoxin, which has antiproliferative effects on tumor cell lines (154), has a very strong binding affinity for WW domain modules (155). This antiproliferative effect of digitoxin has been speculated to inhibit interaction of WWOX and TNF receptor death-associated domain (TRADD), and of TNF receptor complex formation (156). Another compound that appears to bind class I WW domain is etoposide phosphate (155), an antineoplastic agent which is thought to interfere with DNA topoisomerase and might have a secondary action by interfering with the JNK stress response pathway (157). The authors raised the possibility that etoposide might also modulate WW signaling and block JNK-WWOX interaction (158) that leads to the inhibition of the pro-apoptotic function of WWOX (157). Although it is important to assume a therapeutic potential of utilizing WW-PRM as targets, we believe that it is still simplistic to consider specific therapies in this regard, especially if we take into consideration the fact that different WW domain containing proteins (that have WW domains of the same class) have antagonistic functions and that a single protein has different roles in different cellular contexts or cell types. Add to this, tandem domains of the same class in a single protein may have different specificities towards different ligands. In conclusion, further analysis of the dynamics and the factors that determine binding specificities of the different classes of WW domains is needed to better design novel therapeutic strategies for malfunctions that involve the WW dominome.

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