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Review

WW domain interactions regulate the Hippo tumor suppressor pathway

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The Hippo kinase pathway is emerging as a conserved signaling pathway that is essential for organ growth and tumorigenesis in *Drosophila* and mammalians. Although the signaling of the core kinases is relatively well understood, less is known about the upstream inputs, downstream outputs and regulation of the whole cascade. Enrichment of the Hippo pathway components with WW domains and their cognate proline-rich interacting motifs provides a versatile platform for further understanding the mechanisms that regulate organ growth and tumorigenesis. Here, we review recently discovered mechanisms of WW domain-mediated interactions that contribute to the regulation of the Hippo signaling pathway in tumorigenesis. We further discuss new insights and future directions on the emerging role of such regulation.
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The mechanisms controlling mammalian organ size have been the interest of scientists for a long time. During the last few years, immense progress has been made in deciphering these mechanisms and their implications in disease development, including cancer. The regulation of organ growth is controlled by the number of cell divisions and the rate of cell death. These processes regulate tissue homeostasis and maintain the proper function of organs. The recent discovery of the Hippo pathway as a key regulator of organ growth in fruit flies has generated deeper insights into the mechanism of organ size.[1,2] Moreover, deregulation of the Hippo pathway components in many different types of cancers furthers its critical role in tumorigenesis (reviewed in Zhao *et al.*[3]). Although significant progress has been made in understanding the core signaling cascade of the Hippo pathway, much less has been achieved in exploring the regulation of the pathway. Recently, much attention was given to the unusual abundance of WW modules and their interacting cognates within signaling molecules of the Hippo pathway.[4,5] This prevalence of WW domain-mediated complexes in the Hippo pathway perhaps facilitates its molecular analysis, aids in prediction of new pathway components and uncovers new mechanisms of regulation.

WW Domains

Many of the signaling proteins contain modular domains that facilitate protein-protein interactions, often through the recognition of specific and short peptide motifs in their binding partners. These interactions are mostly regulated by post-translational modifications, for example, phosphorylation. Specific protein-protein interactions can thereby control the subcellular localization, enzymatic activity and the assembly of multi-protein complexes, thus allowing the flow of information through signaling pathways. One such example is the WW domain modules' interactions.

WW domain, the smallest module that naturally occurs, consists of $\sim$35–40 amino acid residues, including two highly conserved tryptophan (W) residues separated by 20–23 amino acids in the polypeptide chain.[6–8] These two W amino acids give the domain its name, WW domain. Originally, WW domains were identified through detailed characterization of the Yes-associated protein (YAP) based on computer-aided analysis of imperfectly repeated sequences in the mouse isoform of YAP, and in yeast factor RSP5.[7,8] Functional screen of a cDNA expression library identified the first two putative WW domain ligands, WBP1 and 2.[9,10] To date, WW domains constitute five classes depending on the content of their cognate proline-rich binding motifs (PRM).[11–14] The most abundant type of WW domains are class-I WW domains, which bind to PPxY motifs, where P is proline, x is any amino acid and Y is tyrosine. Although WW domains within different proteins might have a very similar structure, they have differential binding to various ligands. Moreover, different WW domains falling in a tandem repeat manner have different

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Abbreviations: WBP1 and 2, WW domain binding protein 1/2; YAP, Yes-associated protein; PQBP1, polyglutamine tract-binding protein 1; MST1/2, mammalian STE20-like kinase 1/2; LATS1/2, large tumor suppressor, homolog 1/2; PRM, proline-rich binding motifs; Yki, Yorki; Sav, Salvador; Dchs, Dachsous; Ex, Expanded; Mer, merlin; TAZ, transcriptional coactivator with PDZ binding motif; EMT, epithelial-to-mesenchymal transition; CTGF, connective tissue growth factor; AMOTL1/2, angiomotin-like proteins 1 and 2; AMOT, Angiomotin; ASPP1/2, apoptosis-stimulating protein of p53 1 and 2; Dvl-2, dishevelled 2; RUNX2, runt-related transcription factor 2; ERBB4, erythroblastic leukemia viral oncogene homolog 4
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binding properties to different proteins, suggesting that WW domains bind to a vast repertoire of different proteins and that they might be part of complexes bridging blocks.[15–17]

WW domain-containing proteins appear to be very important in homeostasis as they occur in proteins involved in a wide array of biological processes including transcription, apoptosis, differentiation, splicing and ubiquitination. In fact, these domains gained their essential role after being shown to be involved in human diseases including, Liddle's syndrome of hypertension, where the WW domain ligand (PPxY domain) is deleted or mutated,[18,19] muscular dystrophy,[20,21] Alzheimer's,[22–24] Huntington's diseases,[25,26] Golabi-Ito-Hall syndrome of mental retardation, in which the binding of Y65C-mutated WW domain of polyglutamine tract-binding protein 1 (PQBP1) to its cognate proline-rich ligands is abrogated,[27] and more recently cancer.[3,28–30] Moreover, WW domain-containing proteins have gained further interest after being identified in the Hippo tumor suppressor pathway.

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.[16,17] One such important network that has received much attention in the last few years is the Hippo tumor suppressor pathway. The Hippo pathway is a highly conserved pathway that regulates tissue growth and organ size by regulating cell growth, proliferation, differentiation and apoptosis.[3,29] Inactivation or mutations of some components of the pathway were identified in different types of cancer.[3,29,31] The Hippo pathway is composed of a kinase cascade core that includes MST1/2 serine/threonine kinase (ortholog of Hpo), WW45 scaffold protein (Sav), MOB (Mts) and LATS1/2 kinases (Wts) (Figure 1). This kinase cascade is activated by a mechanism that is not yet fully established, although some proteins were identified to feed into the core Hippo kinase cassette-like Fat, Dachsous (Dchs), Kibra, Expanded (Ex), Merlin (Mer) and others (reviewed in Grusche *et al.*[32]). Activation of the core cascade leads to phosphorylation of YAP[33–35] and TAZ[36] (Yki in flies) leading to their sequestration in the cytoplasm, preventing their translocation to the nucleus and binding to TEAD transcription factor, thereby inhibiting transcription of downstream target genes implicated in proliferation, anti-apoptosis and epithelial-to-mesenchymal transition (EMT).[37]

A unique feature of the Hippo pathway is the high prevalence of WW domain-mediated complexes, defined recently as WW modularity of the Hippo pathway.[4] The WW domain containing proteins occur at different levels of the Hippo pathway. In the core components of the Hippo pathway in both *Drosophila* and mammals, the interactions are mediated via PPxY motifs and WW domains. In *Drosophila,* Hpo and Wts each contain PPxY motifs, and Sav contains two WW domains. In mammals, the core cassette also contains either PPxY/F motifs (Table 1), as in the case of LATS1/2 and MST1/2, or WW domains, as in case of WW45.[4] In addition, the nuclear effectors of the pathway, Yki in flies and YAP or TAZ in mammals, function through WW–PPxY interaction. Indeed, it has also been shown that the WW domains of YAP are crucial for YAP transcriptional co-activation function

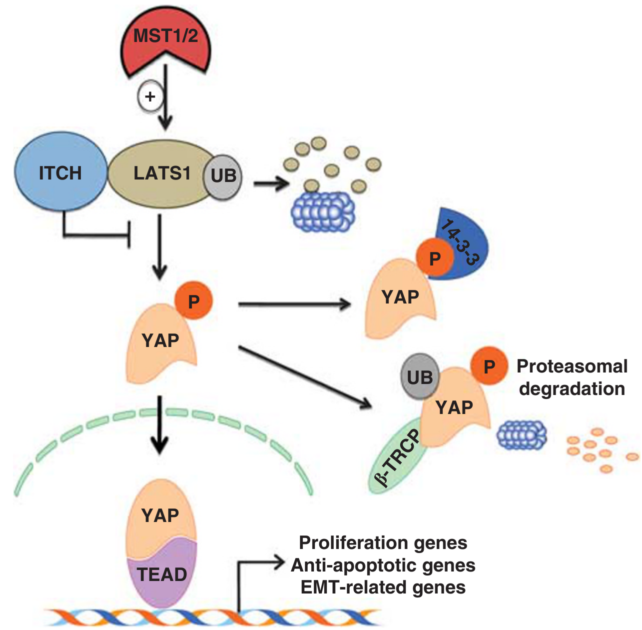

Figure 1 ITCH regulates the Hippo pathway by degrading LATS1. The E3 ubiquitin ligase ITCH interacts with LATS1 by WW domain–PPxY motif-dependent manner leading to ubiquitination and protaesomal degradation of LATS1. This results in reduced YAPS127 phosphorylation, thus less cytoplasmic sequestration by binding to 14-3-3 protein, reduced YAP protaesomal degradation mediated by β-TRCP E3 ligase and consequently enhanced YAP translocation to the nucleus to mediate YAP dependent co-activation of TEAD-responsive genes, including those implicated in proliferation, anti-apoptosis and EMT. Upon activation of the pathway, ITCH–LATS1 interaction is enhanced leading to more efficient degradation of LATS1 attenuating its phosphorylation activity of YAP. This functional association might have a role in fine-tuning the outcome of the Hippo pathway and could be deregulated in specific setting such as in tumorigenesis.

Table 1 Examples of WW domain and PPxY-containing proteins in the Hippo pathway

WW Domain proteins	
YAP1/2	1-WW and 2-WW[3,33–35,37,46]
TAZ	1-WW[29,36,59]
KIBRA	2-WW[38–40]
WW45 (SAV1)	2-WW[60,61]
ITCH	4-WW[42,43]
PPXY/F-containing proteins	
DCHS1/2	4-PPxF and 2-PPxF[a 4,32,62]
FT1/2	PPxY and PPxF[a 4,63–65]
CRB1/2	1-PPxY and 2-PPxF[a 4,66,67]
MST1/2	1-PPxF and 1-PPxF[a 4,60,68,69]
LATS1/2	2-PPxY and 1-PPxY[34,35]
WBP2	3-PPxY[47,48]
AMOT	2-PPxY[49,51]
AMOTL1/2	2-PPxY[50]
ASPP1/2	1-PPxY and 1-PPxF[a 4,52,53,70]
P73	1-PPxY[28,30,44,45]
ERBB4	3-PPxY[28,30,71–73]
SMAD1	1-PPxY[74]
RUNX2	1-PPxY[28,30,59,75]
DVL2	1-PPxY[76]

[a]PPxF motif was suggested by Sudol and Harvey[4] as a potential WW domain ligand based on *in vitro* results.

downstream of the Hippo pathway.[37] Not only do the core components or the downstream effectors contain WW domains but also several upstream regulators of the Hippo pathway, in both *Drosophila* and mammals, contain either WW or PPxY motifs. For example, the WW domain protein Kibra is a Hippo signaling component upstream of Hpo/MST and Merlin.[38,39] This modularity in the Hippo pathway might intend that this pathway is regulated by WW domain-containing proteins at different levels in the pathway, from the mediators down to the core components and effectors.

WW Domain Proteins Regulate Members of the Hippo Pathway

WW domains of kibra regulate Hippo pathway proteins. Recently, different reports have described growing evidence of a number of proteins that regulate the core components of the Hippo pathway. Some of these proteins can be broadly termed upstream Hippo pathway regulators and include proteins that signal via the atypical cadherin, Fat, which functions as a transmembrane receptor for the Hippo pathway.[32] Additionally, the Kibra–Expanded–Merlin complex links the apical membrane to the core of the pathway proteins and the apicobasal polarity proteins.[32] These upstream regulators make different physical interactions with the pathway to manipulate its functions. One example of these interactions is the WW domain–PPxY motif interaction induced by Kibra. Recently, it has been shown that different null mutants of the *Kibra* gene are associated with increased cell number leading to tissue overgrowth. On the other hand, Kibra overexpressing clones contain fewer cells than control clones associated with induced apoptosis.[40] Kibra functions primarily upstream of Mer and contributes to Mer-independent regulation of Yki activity. This effect on Mer seemed to be mediated by physical interaction of the two proteins. This interaction was found to be independent of the WW domains of Kibra.[40] On the other hand Ling Xiao *et al.*[41] showed that the Kibra WW domains are essential for Kibra–LATS interaction and regulation of LATS1/2 functions in the context of the mammalian Hippo pathway. Upon its expression, Kibra activates LATS1/2 as revealed by its increased phosphorylation, leading to increased phosphorylation of the ultimate effector of the pathway, YAP.[41] Not only was Kibra shown to enhance LATS function but it was also shown to be responsible for increased LATS2 protein levels. Kibra-LATS2 association increases LATS2 half-life, at least in part, by inhibiting LATS2 ubiquitination and its proteasomal degradation.[41] Implication of this functional interaction on tumorigenesis *in vivo* is still to be determined.

WW domains of ITCH regulates LATS1 stability. Recently, two reports identified the E3 ligase responsible for the proteasomal degradation of LATS1. The first, coming from our lab, identified ITCH as a WW domain-containing protein that regulates the stability of LATS1 using WW domain arrays.[42] These findings were confirmed later by another group that utilized SILAC (Stable Isotope Labeling with Amino Acids in cell culture).[43] Both articles came to the same conclusion, identifying LATS1 as a target of the E3 ligase ITCH (Figure 1). In our work, we demonstrated that ITCH, mostly via its first WW domain, interacts with the PPxY motifs of LATS1 and enhances its ubiqitination and proteasomal degradation.[42] Of note, ITCH interaction with LATS1 was increased upon activation of the Hippo pathway either by MST2 overexpression or by high-cell density culture. This interaction was associated with enhanced degradation of LATS1 and suggest that ITCH might specifically target the activated form of LATS1.[42] Expression of a kinase-dead mutant of MST2 (MSTD-KD), which is incapable of phosphorylating and activating LATS1, indeed rescued, at least in part, ITCH-mediated LATS1 degradation (Unpublished data, Salah and Aqeilan). Whether ITCH expression and/or function is affected by LATS kinases is still an open question. Collectively, this may suggest that ITCH might function as a fine-tuning regulator of the Hippo pathway under physiological conditions.

ITCH-mediated LATS1 degradation is also accompanied by reduced YAP phosphorylation on Ser127, mild YAP accumulation in the nucleus and increased co-activation function of TEAD-responsive genes.[42] As YAP phosphorylation has been shown to trigger its degradation by SCF-(βTRCP) E3 ubiquitin ligase, our results may suggest that ITCH expression might signal for YAP stabilization and TEAD co-activation.[42]

The findings by Salah *et al.*[42] further demonstrated that LATS1 degradation by ITCH enhances EMT in HeLa and MCF10A cells, phenocopying overexpression of YAP.[1,3] Increased levels of YAP-related EMT genes, including *CTGF* and *fibronectin*, and increased cellular migration and invasion are hallmarks of ITCH overexpression. Not only did the cells show more EMT phenotypes but also ITCH-manipulated cells are more tumorigenic both *in vitro* and *in vivo*. The findings of Ho *et al.*[43] also confirmed that ITCH negatively regulates LATS1 level and function as related to cell proliferation and apoptosis in the same way as demonstrated earlier.[42] Because ITCH, as an E3 ligase, targets many substrates,[44,45] it is possible to speculate that the phenotypes observed after ITCH overexpression are related to the regulation of the different targets in a given context. Nevertheless, these phenotypes were rescued, at least in part, in our settings when manipulating LATS1 expression, suggesting that LATS1 is a critical target of ITCH-mediated tumor growth and progression by regulating the Hippo pathway.

As different WW domain proteins may share common targets, it is likely to assume that changing the level, stability or subcellular localization of one WW protein would alter the function and outcome of WW domain targets, depending on the cellular context or the expression of the different proteins.[17,30] For example, p73 is a common ligand for ITCH and YAP. On one hand, ITCH degrades p73,[44] while on the other hand it leads to enhanced YAP translocation to the nucleus to promote TEAD-dependent transcription.[3] In addition, YAP is an important co-factor for p73-dependent transcriptional activity and exerts a tumor suppressor role in this context.[45] Therefore, ITCH overexpression might serve as a molecular switch between opposing YAP functions. Whether YAP relocates between p73/YAP targets and TEAD/YAP targets in response to ITCH is to be determined in future studies. It would also be necessary to determine whether targeted manipulation of WW domain proteins or their interacting partners in the Hippo pathway would tilt the outcome of organ size and/or tumorigenicity. As ITCH behaves as a proto-oncogene, it might also contribute to the observed downregulation of LATS1 levels in cancer, and possibly other components of the Hippo tumor suppressor pathway. In summary, these findings suggest that novel WW domains could regulate the core components of the Hippo pathway thereby affecting tumorigenesis and, perhaps, organ growth.

PPxY-containing proteins regulate effectors of the Hippo pathway. Another level where WW domains appear to regulate the Hippo pathway is on the level of the effectors, YAP and TAZ. Indeed, LATS proteins, via their PPxY motifs, have been shown to bind to WW domains of YAP leading to YAP phosphorylation, sequestration in the cytoplasm and inactivation.[33,34,46] This leads to reduce YAP-induced EMT phenotypes and is associated with reduced tumorigenicity.[1,34] In fact, it was shown that the WW domain of YAP has a critical role in inducing a subset of YAP target genes independent of, or in cooperation with, TEAD.[37] In addition, mutagenesis of the WW domains diminishes the ability of YAP to stimulate cell proliferation and oncogenic transformation.[37] In support of this notion, two recent papers showed that WW domain-mediated interaction with WBP2 is important for the phenotypes induced by both Yki[47] and TAZ.[48] In the first work, Zhang *et al.*[47] reported that Yki, via its WW domain, binds to the PPxY motifs of Wbp2. Importantly this interaction leads to increased Yki transcriptional co-activation function and is associated with Yki-driven tissue overgrowth. Knockdown of Wbp2 expression by RNAi in a *wts*-deficient background reversed the lethal overgrowth phenotypes in *wts* null organisms, suggesting that Yki function is mediated by Wbp2.[47] In mammalian cells, TAZ's WW domains' interaction with PPxY motifs of WBP2 suggested an indispensable role of WBP2 in TAZ transforming ability.[48] Although knockdown of WBP2 suppressed TAZ-driven transformation, its overexpression enhanced this transformation.[48]

Recently, the PPxY-containing Angiomotin (AMOT)-like proteins 1 and 2 (AMOTL1/AMOTL2) were identified as regulators of the downstream effectors of the Hippo pathway, YAP and TAZ.[49–51] Three articles highlight the significance of this interaction and shed light on the role of AMOT cell junction proteins in regulating YAP and TAZ function.[49–51] These proteins were found to specifically interact with YAP in a WW domain-PPxY motif-dependent manner. This interaction was found to be sufficient to sequester YAP and TAZ in the cytoplasm, independent of their phosphorylation status. Specifically, AMOT expression leads to YAP localization at the tight junction and cell membrane, preventing YAP nuclear translocation.[51] Moreover, it was shown that knockdown of AMOTL2 phenocopies YAP-induced EMT in MCF10A cells.[51] Considering this scenario, loss of tight junction-localized YAP and TAZ increased their nuclear localization and was accompanied by induction of YAP/TAZ target gene expression, and most importantly, transformation and loss of cell contact inhibition. Furthermore, AMOTL2 knockdown-dependent phenotypes were blocked by simultaneous knockdown of YAP and TAZ, demonstrating that the AMOT family proteins are new components of the Hippo pathway with tumor-suppressing potential, indicating a new mode of YAP and TAZ regulation.[51]

In a different manner, WW domain-PPxY motif interaction was involved in the regulation of the downstream effectors of the Hippo pathway by involving more than two proteins. For example, ASPP2 was shown to stimulate TAZ dephosphorylation, partly by promoting the interaction between TAZ and PP1; this function of ASPP2 requires the TAZ WW domain. ASPP2–TAZ interaction promotes TAZ nuclear localization and TAZ target gene expression.[52] In another example, it was shown that ASPP1 was able to inhibit YAP/TAZ interaction with LATS1, leading to enhanced nuclear accumulation of YAP/TAZ and YAP/TAZ-dependent transcriptional regulation. This results in YAP/TAZ activation and thus inhibits apoptosis, in part, through the downregulation of Bim expression, leading to resistance to anoikis and enhanced cell migration.[53]

Concluding Remarks and Future Directions

The unique feature of the Hippo pathway over other signaling pathways is its high modularity represented by the great prevalence of WW–PPxY interactions, which might strongly suggest that other WW domain and PPxY motif-containing proteins regulate, or are part of, the Hippo pathway. The study of WW domains and Hippo pathway in recent years further highlighted important aspects of WW domain protein signaling including dimerization capability, regulation of WW domain-PRM interaction and networking (reviewed in Sudol[5]). WW domains are present in a wide variety of cellular proteins including E3 ligases, co-activators, co-repressors and adapter proteins that could potentially regulate members of the Hippo pathway. Taking into consideration the important role of this pathway in tissue growth and homeostasis, further efforts should be invested in identifying new regulators and components of this pathway. The use of GFP-expressing tumor cells in fresh tissue or live animals shall facilitate better characterization of the Hippo pathway proteins and their role, both *in vitro* and *in vivo*, in tumor initiation and progression.[54–57] Expansion of this information may aid in developing new therapeutic strategies based on the WW domain interactions in this pathway. In fact, the design of inhibitors or activators of WW domain signaling complexes in the Hippo pathway could be facilitated by the considerable data available on the WW domain structure, the mechanism of interaction with its rigid ligands, and the complexes it forms.[58] Owing to the fact that the WW domain and its ligands' core motifs are relatively short, it might be possible to use small molecules that function as activators or inhibitors for the Hippo pathway signaling proteins; that is, small chemicals/peptides that inhibit YAP and TAZ oncogenic function. However, before thinking about therapeutic strategies based on WW domain interactions, further analysis of the WW domain-mediated complexes in the Hippo pathway must be elucidated to better design novel therapeutic strategies for malfunctions that involve the WW domain.

Conflict of Interest

The authors declare no conflict of interest.

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