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Review Article

Hippo and *rassf1a* Pathways: A Growing Affair

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First discovered in *Drosophila*, the Hippo pathway regulates the size and shape of organ development. Its discovery and study have helped to address longstanding questions in developmental biology. Central to this pathway is a kinase cascade leading from the tumor suppressor Hippo (Mst1 and Mst2 in mammals) to the Yki protein (YAP and TAZ in mammals), a transcriptional coactivator of target genes involved in cell proliferation, survival, and apoptosis. A dysfunction of the Hippo pathway activity is frequently detected in human cancers. Recent studies have highlighted that the Hippo pathway may play an important role in tissue homoeostasis through the regulation of stem cells, cell differentiation, and tissue regeneration. Recently, the impact of RASSF proteins on Hippo signaling potentiating its proapoptotic activity has been addressed, thus, providing further evidence for Hippo's key role in mammalian tumorigenesis as well as other important diseases.

1. Introduction

The Hippo pathway is a signaling pathway that regulates cell growth and cell death. It was discovered in *Drosophila melanogaster* as a pathway controlling organ size and of which mutations lead to tumorigenesis. This pathway is highly conserved, and its activation or repression could lead to the following most extreme outcomes: proliferation/transformation and death/tumor suppression. The Hippo pathway cross-talks with other signaling players such as Notch, Wnt, and Sonic hedgehog (Shh). It influences several biological events, and its dysfunction may possibly lie behind many human cancers. In this review, we discuss the complex data reported about *Drosophila* to date (schematic representation in Figure 1) and the human Hippo (schematic representation in Figure 2) pathways focusing on the relationship between the tumor suppression *rassf* protein family and the Hippo-like pathway in humans [1, 2].

2. The Hippo Signaling Network in *Drosophila*

Drosophila imaginal discs have facilitated molecular dissecting of signaling pathways controlling organ size during development. These imaginal discs allow to screen how organs grow several folds larger before differentiating into adult organs after proliferation in larval stages. By using the genetic analysis in *Drosophila*, Robin W. Justice and colleagues were the first to describe that loss of Wts (Warts), which encodes a kinase of Nuclear Dbf-2-related (NDR) family, results in a *Drosophila* phenotype characterized by tissue overgrowth [3]. Several years later many components of this pathway were characterized. Four tumor suppressors called Hippo (Hpo), Warts (Wts), Salvador (Sav), and Mats were established. These suppressors constitute the core linear kinase cassette of Hippo/Warts pathway whose products can affect proliferation without increasing apoptosis susceptibility [3–6] (Figure 1). Subsequent genetic screens identified at

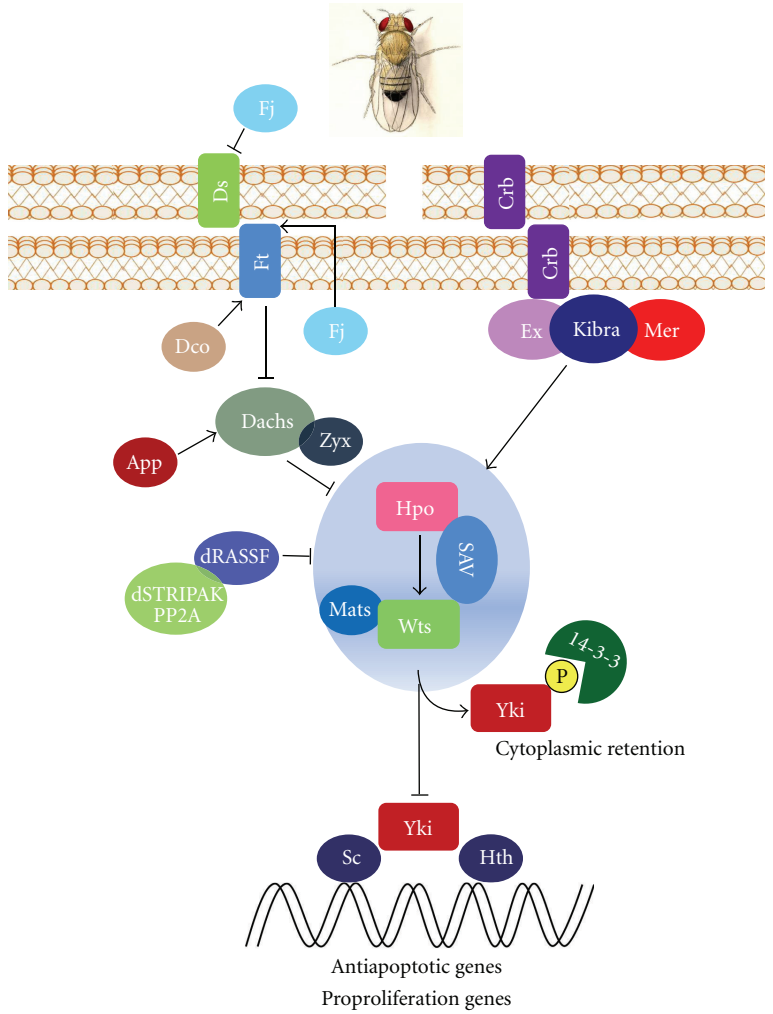

Figure 1: "Hpo signaling pathway in *Drosophila*." Schematic representation of Hippo kinases cascade and of its modulation by apical transmenbrame protein complexes.

least seven additional tumor suppressors whose biological functions converge on Hpo and/or Wts: the FERM domain proteins Merlin (Mer) and Expanded (Ex) [7–10], the protocadherins Fat (Ft) [11–14] and Dachsous (Ds) [15, 16], the CK1 family kinase Disc overgrown (Dco) [17, 18], the WW and C2 domain-containing protein Kibra [19–21], and the apical transmembrane protein Crumbs (Crb) [22–24]. All of these suppressors converge and act through a common downstream component, the transcriptional co-activator protein Yorkie (Yki) [25] (Figure 1). The mechanisms by which these upstream regulators signal towards the final player Yorkie are complex and are still focus of investigation. A great deal of evidence suggests that they work in a combinatorial or synergistic manner to regulate Hippo kinase activity.

2.1. The Apical Protein Complex: Kibra, Expanded, and Merlin. The molecular link between upstream regulators and the core complex has not yet been clarified in mammals nor in *Drosophila*. In 2006, Hamaratoglu and collaborators proposed Mer (Merlin) and Ex (Expanded) as potential upstream regulators of the Hippo pathway [9], proteins which contain a FERM (4.1/ezrin/radixin/moesin) domain. Both proteins are considered tumor suppressors which cooperate to control organ growth. Their function seems to be partially redundant. In fact, while single mutation of each gene results in increased tissue growth, mutations in both genes give rise to a more strongly affected phenotype [9, 10]. Kibra, a third component of this apical complex, has recently been found. This protein possesses a WW domain which facilitates the interaction with other members of the Hippo pathway, such as Wts. It further interacts with a C2 domain that consists of a phospholipid-binding motif through which Kibra is believed to potentiate its membrane association [19–21]. WW domains are 35–40 amino acid protein–protein interaction domains that are characterized by a pair of conserved Trp residues, which generally interact with Pro-rich sequence motifs [26]. WW domain-Pro motif interactions appear to be particularly common in the Hpo

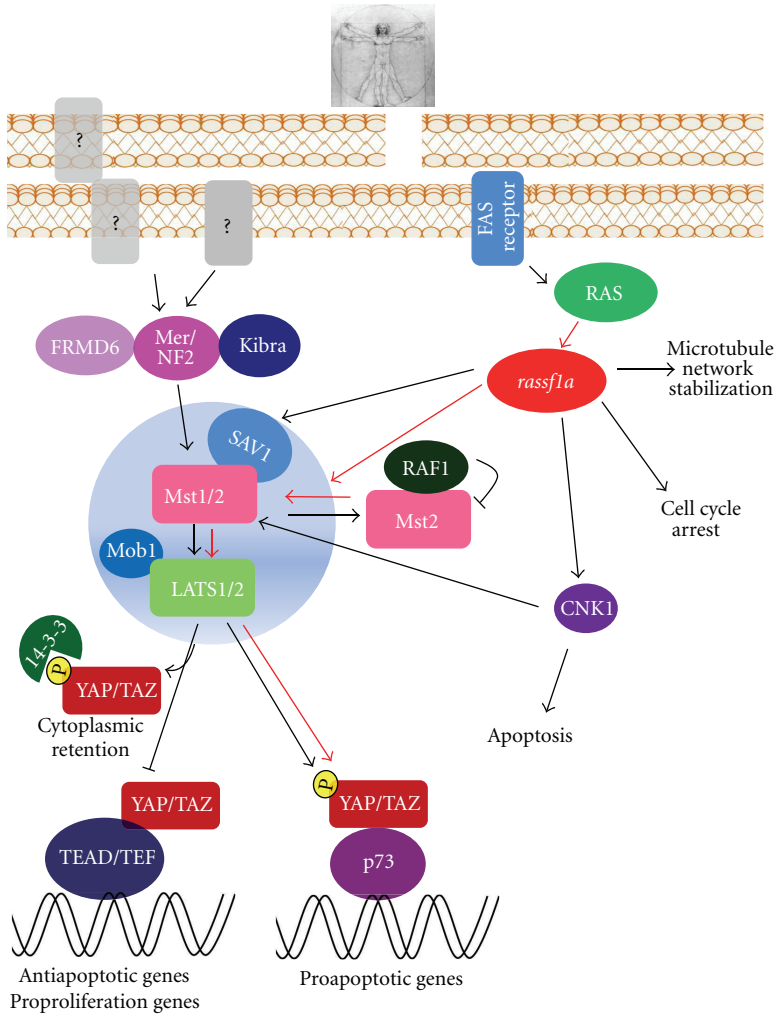

Figure 2: "Hpo signaling pathway in Mammals and the cross-talk with *rassf1a* signaling." Schematic representation of mammalian Hippo kinases cascade and interconnections between Hippo pathway and *rassf1a* protein signal. Red lines indicate the impact of *rassf1a* signaling in modulating activity of Hpo pathway components.

pathway. Three core components of Hpo signaling (Yki, Kibra, and Sav) contain WW domains, whereas three other components (Wts, Ex, and Hpo) hold PPxY motifs (reviewed in [27, 28]). While the formation of a ternary complex between Kibra, Ex, and Mer was observed, each protein was seen to localize to cellular membranes independently. Furthermore, it has been published that the Kibra-Mer-Ex complex is physically involved with the Hpo-Sav, constituting an apical protein complex required for associating the Hpo pathway to the cellular membranes [20, 21]. Studies on the Ex localization and function have led to the discovery of another important upstream regulator protein of Hpo, Crb (Crumbs) [22–24]. Crb is a transmembrane protein which normally localizes to the subapical membrane of epithelial cells that is responsible together with other apical complexes in *Drosophila* for organizing apical-basal polarity [29]. Crb binds to Ex through a short intracellular domain including a juxtamembrane FERM-binding motif (FBM). The FBM domain of Crb interacts with the FERM domain of Ex. This type of binding is necessary for Ex apical localization and stability. Furthermore, it has been published that Crb also works with Mer and Kibra [23]. The loss of Crb expression was shown to further determine a phenotype characterized by overgrowth, possibly to a lesser degree compared to the other members of Hpo signaling described until now [22–24]. Not long ago, this protein was proposed to have had an important function as a transmembrane receptor recognizing cell-cell contacts through Crb-Crb binding domains [22].

2.2. The Upstream Regulator: Transmembrane Protein Fat. The atypical cadherin FAT (Ft) was the first transmembrane protein shown to affect Hippo signaling. Fat is the first tumor suppressor gene isolated in *Drosophila*. In fact, the complete knock-out of the FAT protein induces death in *Drosophila* larvae with overgrown imaginal discs [11]. As previously mentioned, FAT is a large transmembrane protein, constitutively cleaved by unknown proteases. It contains

34 cadherin repeats in its extracellular domain, functioning as a receptor for Hippo signaling [12–14] as well as for planar cell polarity (PCP) [30, 31]. PCP is a mechanism through which cells orient themselves orthogonally to the apical-basal axis, as observed in the wing hairs of *Drosophila*, and the sensory hair cells in the inner ear of mouse. Notably, the mechanism by which FAT regulates Hippo signaling is different from the branch involving the ternary complex Ex-Mer-Kibra. Many lines of evidence suggest that the principal mechanism exerted by FAT is on the Wts function [18, 32]. Thus, FAT-Hpo signaling is genetically distinguishable, involved in Hippo pathway regulation of imaginal discs and neuroepithelial tissue, but not in other tissues such as ovarian tissue [14, 33, 34]. Many genes were reported to take part in this parallel mechanism together with FAT. First, Dachsous (Ds), an atypical cadherin which binds to FAT [15, 16]. FAT is regulated by an expression gradient of Ds [35, 36]. Four-jointed (Fj) is a kinase that typically localizes to the Golgi subcellular compartment and that phosphorylates the cadherin domains of FAT and Ds to mediate binding between these two proteins [37]. Another kinase responsible for FAT phosphorylation in its cytoplasmatic segment is a Casein I kinase, termed Discs overgrown (Dco) [17, 18]. The effective key mediator of FAT in the Hippo pathway seems to be Dachs, an unconventional myosin which antagonizes FAT, and whose activity is influenced by Approximated (App) [17]. App, in fact, antagonizes FAT signaling by modulating Dasch expression [38]. Another protein identified recently linked to the FAT branch in Hippo signaling is the LIM-domain protein Zyx102. It has been found to directly affect the core kinases of the Hippo pathway [39]. All of these components described above seem to be responsible for linking Hippo to extracellular stimuli [40].

Another so called "scaffold" protein that has been identified as a regulator of Hpo is called *Drosophila rassf* (*drassf*). This protein like its mammalian counterpart *rassf* can bind to Hpo through a conserved SARAH domain. But unlike in mammals, it hampers Hpo activity by competing with SAV to bind to Hpo [41] and by recruiting a Hpo-inactivating PP2A complex (dSTRIPAK) [42], thus showing a positive regulation of growth. Interestingly, Grzeschik and collaborators showed that the depletion of the *Drosophila* neoplastic tumor suppressor Lethal giant larvae (Lgl), which controls apical-basal cell polarity and proliferation, leads to upregulation of the Hippo pathway target Yki through a decreased phosphorylation and consecutively overproliferation of developing eyes, without affecting apical-basal polarity [43]. This mechanism is brought about by cellular mislocalization of Hpo and *rassf*. These both colocalize basolaterally leading to the deregulation of the Hippo kinase cascade, thereby preventing phosphorylation and inactivation of Yki. This concurs with data previously discussed wherein *rassf* is able to bind to Hpo precluding its interaction with SAV [41].

2.3. The Key Effectors of Growth Control: Hippo, Warts, Salvador, and Yorkie. Warts is crucial in the phosphorylation-dependent regulation of Yki [25, 44, 45]. Warts (Wts) encodes a Ser/Thr kinase of Nuclear Dbf-2-related (NDR) family. The activity of Warts is controlled through a series of phosphorylation events. Warts is directly phosphorylated by Hippo (Hpo), a member of the Sterile-20 family of Ser/Thr kinases, in a reaction that is facilitated by the Salvador protein [4, 5]. The fly protein Hippo (Hpo) is the first mediator of this pathway characterized by a kinase cascade. Wu and collaborators identified Hpo through analysing the phenotype of *Drosophila* Hpo mutants. Hpo is a kinase protein that regulates cell proliferation as well as apoptosis in *Drosophila.* In addition, it interacts, phosphorylates, and is activated by the WW domain-containing protein Salvador. Salvador (Sav) was described as a tumor suppressor gene, whose loss caused tissue overgrowth, similar to Wts loss of function. Tapon and collaborators were the first to observe, in 2002, that loss of Sav or Wts was strictly associated with increased expression of *cyc e*, a cell cycle progression regulator and *diap1*, an apoptosis inhibitor, thus, confirming these that two proteins' very important role in coordinating these two cellular processes [4]. Similar to Sav function on Hpo, Mats' role (Mob as tumor suppressor) which also belongs to the NDR family, as well as its kinase-like behavior binding to and potentiating Wts intrinsic activity, was described in 2005 [6]. Thus, Sav and Mats action as adaptor proteins, often termed scaffold proteins, both serve to potentiate Hippo signaling. Interestingly, it was also reported that Mats is a Hpo substrate. The latter phosphorylates Mats increasing its affinity for Wts binding, thus inducing potentiation of Wts kinase activity [46].

The downstream key regulator of Hpo signaling is Yorkie (Yki). It was identified in a yeast two-hybrid screen for Wts-binding protein, which is the final step in the Hippo pathway, driving its transcriptional regulation [25]. Yki is not a direct transcriptional factor because it does not possess its own consensus DNA-binding motif but is known as a potent transcriptional co-activator by cooperating with different DNA-binding proteins. Wts directly phosphorylates Yki at Ser 168, thus creating a binding site for 14-3-3 proteins which sequester Yki in the cytoplasm and prevent its nuclear import [44, 45]. In actual fact, the loss of Hippo signaling as well as mutations in 14-3-3 binding site for Yki was shown to produce strong nuclear accumulation, a common feature, coupled with aberrant activity of Yki [47]. Another two residues of Yki are believed to be targets of Wts phosphorylation (Ser111 and Ser250); however, little is known about the underlying mechanisms. As mentioned before, Yki cooperates with many DNA-binding proteins which act as transcription factors, potentiating their function. It is worth noting that some binding partners of Yki are the same kinases that function upstream to it in the Hippo pathway. Thus, through the PY (PPxY)-WW domain interactions, Yki is able to bind to Ex, Wts, and Hpo that sequester Yki at a cytoplasmatic level, independently from its phopshorylated state [48, 49]. Loss of Hippo signaling and consecutive aberrant Yki activation leads to deregulation of some gene class transcriptions. One class includes genes involved in cell survival and proliferation. One of the Yki partners, Scalloped (Sc), a member of TEAD/TEFs family, is responsible for Yki overexpression induced tissue

overgrowth [50, 51]. Another partner of Yki in *Drosophila* is Homothorax (Hth) that promotes cell survival and cell proliferation in eye development from eye imaginal discs [52]. Both Sc and Hth are able to bind a Hippo consensus DNA motif, termed Hippo response element (HRE), which is present in many Hippo target genes. Particularly, Sc together with Yki bind to the HRE present in a very well-known target gene, *diap1* [50], an apoptosis inhibitor, as mentioned above. Hth has only little influence on *diap1* transcription. It is very important in regulating the transcription of another Yki target, the growth promoting microRNA gene *bantam*. Other Yki targets in this class are the cell-cycle regulators *cyc e*, *e2f1* [4, 53], and *Drosophila* Myc (dMyc) whose expression seems to be positively regulated by Yki [54, 55]. Another important class is made up of components from other signaling pathways, such as ligands for Notch, Wnt, EGFR, and Jak-Stat pathways. In fact, other known Yki partners are believed to be Smad proteins [56]. This interaction appears to potentiate the transcriptional response to BMP/TGF-β signaling, addressing a possible crosstalk between Hippo and BMP/TGF-β pathway. Finally, a third class of Yki targets consisting of several proteins from its own Hippo cascade, such as Ex, Mer, Kibra, Crb, and Fj. These are downstream transcriptional targets of Yki [9, 17, 20, 57] and define a sort of positive feedback loop which characterizes most signal pathways.

3. The Hippo Kinase Signaling in Mammals

3.1. YAP and TAZ: Mammalian Effectors of Hippo Pathway. The Hippo pathway is highly conserved in mammalian systems. It was demonstrated that loss of function of mutant flies can be rescued by expressing their respective human counterparts [5, 6]. These data strongly correlate with the importance of Hippo signaling in controlling organ size, tumorigenesis as well as the insurgence of other important diseases in mammals. The ortholog human counterparts of core kinases Hpo and Warts are represented by the pro-apoptotic MST1/2 and LATS1/2 kinases [58, 59] (Figure 2). One ortholog exists for the adaptor protein Sav, termed WW45 or SAV1, and the other two orthologs for Mats are termed MOBKL1A and MOBKL1B (referred to as Mob1). These proteins form a conserved kinase cassette that phosphorylates and inactivates the mammalian Yki homologs YAP and TAZ [25, 47, 60] in response to cell density. This cell density-dependent activation of the Hippo pathway is required in contacting inhibition of cultured mammalian cells [47]. Similar to *Drosophila* Hippo signaling, all the mammalian components of the Hippo pathway clearly show tumor suppression activity. In fact, transgenic overexpression of YAP [61, 62] and liver-specific knockout of *Mst1/2* or *Sav1* [63–66] induce abnormal liver expansion in terms of size, and eventually hepatocellular carcinoma formation (HCC). YAP was initially identified as a 65 kDa binding partner of *c*-Yes from Sudol and collaborators [67]. YAP is a transcriptional co-activator of many transcription factors via its own WW-domain (reviewed in [68]). The TEAD/TEF family of transcription factors, whose homolog is represented by Sc in *Drosophila*, is considered the major partner of both YAP and TAZ in executing their activities within the Hippo pathway. The 4 mammalian TEF/TAED transcription factors are widely expressed and regulate transcription in specific tissues during certain development stages [69]. It was shown that TAED1/TEF2 and YAP share a large number of target genes [51, 70, 71]. In support of this evidence, TEAD1 and TEAD2 double-knockout mice display similar phenotypes to YAP knockouts [69]. Furthermore, ablation of TAED/TEF expression decreases the ability of YAP/TAZ in promoting anchorage independent growth and EMT (epithelial to mesenchymal transition) [51, 71, 72]. Recently Dupont and collaborators have identified YAP and TAZ as the nuclear principal complex of mechanical signals exerted by extracellular matrix (ECM) rigidity and cell shape. This regulation requires Rho GTPase activity and tension of the actomyosin cytoskeleton but is independent from the Hippo/LATS cascade. YAP/TAZ is required for differentiation of mesenchymal stem cells induced by ECM stiffness and for survival of endothelial cells regulated by cell geometry [73].

The exact role of YAP has yet to be defined since it appears to be able to act as an oncogene or as a tumor suppressor depending on the cellular context. YAP1 was shown to bind long forms of p73 and p63, while not to wt p53, thereby potentiating p73- and p63-induced apoptosis [74, 75]. In particular, p73 recapitulates the most well-characterized p53 antitumoral effects, from growth arrest and apoptosis to senescence. YAP imparts transcriptional target specificity to p73 in promoting either growth arrest or apoptosis in response to different stimuli [76–78].

3.2. The Complexity of Upstream Regulators: FRMD6, Mer, and Kibra. As mentioned above, the complexity of molecular links between the upstream regulators and the core kinases in mammals has not been clarified either for *Drosophila*. The mammalian genome contains homologs for all the reported upstream regulators of the Hippo pathway. Notably, it encodes more than one paralogue for each *Drosophila* component, thus increasing complexity and the need for further investigation. Two homologs for Kibra, KIBRA/WWC1 and WWC2 and for Expanded, FRMD6 and FRMD1, while only one for Merlin, NF2, were identified. Interestingly, they often differ in protein structure compared to *Drosophila* counterparts. One Ex homolog for FRMD6 does not possess the extended C-terminal portion that is required for growth inhibition activity of Ex and binding to Kibra [20, 79]. No interaction between FRM6 and MST1/2 has been confirmed, in contrast to the described interaction between Ex and Hippo [21]. Also Mer/NF2 is a FERM domain-containing protein and the most investigated. It is a tumor suppressor, whose mutations trigger neurofibromatosis 2, mainly characterized by tumor insurgence in the nervous system [80, 81]. It has a prominent role in growth inhibition triggered by C-adherin-based cell contact. Growth inhibitory action of Mer/NF2 appears to stem from controlling the distribution and signaling of membrane receptors. In fact, in Merlin K/D cells the activation and internalization of the EGF receptor are also maintained in high-cell-density conditions [82].

Furthermore, contrasting data for Mer/NF2 involvement in developing hepatocellular carcinoma (HCC) and tumors of the bile duct were reported. It is worthy to note that in specific *Merlin* $^{-/-}$ liver an increased proliferation of hepatocytes and of bile ducts was reported, coupled with minor LATS and YAP phopshorylation and increased YAP nuclear export [83]. Conversely, in this context, other authors did not observe any alterations in YAP phosphorylation and localization [84].

3.3. The Core Kinases: MST, LATS, and MOB. The ortholog human counterparts of core kinases Hpo and Warts are represented by the proapoptotic MST1/2 and LATS1/2 kinases [58, 59]. MST1/2 are serine-threonine kinases, better known for their ability to initiate apoptosis when overexpressed through a combination of p53- as well as JNK-mediated pathways [85, 86]. Generally, apoptosis induced by different stimuli is coupled with the activation of kinases MST1/2, which result themselves as substrates for caspases 3, 6, and 7 cleavage. This produces highly active catalytic fragments, which are mainly localized in the nucleus, where they exert their proapoptotic function [85–87]. As mentioned above, loss of function of the MST1/2 ortholog Hpo shows a phenotype characterized by a marked overgrowth due to accelerated cell-cycle progression and deregulated apoptosis. Exogenous MST2 expression can successfully rescue this phenotype. MSTs become activated by autophosphorylation in the threonine residues within their activation loop domain. Inhibition of dimerization and autophosphorylation of MST2 exerted by RAF1 was reported [88]. In this latter context, expression of *rassf1a* is able to release MST2 from RAF1 inhibition, thus inducing apoptosis [77]. Moreover, PP2A phosphatase dephosphorylates MST1/2 kinases as shown by two different groups [42, 89]. How autophosphorylation and activation of MST kinases are triggered by unknown extracellular stimuli remain to be elucidated, and okadaic acid treatment or siRNA-mediated knockdown of PP2A promote MST1/2 phopshorylation and activation. Interestingly, Guo and collaborators very recently showed that *rassf1a* activates MST1 and MST2 by preventing their dephosphorylation. Specifically, they observed that *rassf1a* knockdown, which is a frequent phenomenon in human tumors, leads to a dramatic decreased in MST1/2 levels exerted by phosphates. They also observed that restoring *rassf1a* expression and function promotes the formation of active MST1/2 by counteracting the role of phosphates. This is one of the first examples of a tumor suppressor acting as an inhibitor of a specific dephosphorylation pathway.

In the Hippo pathway context, MST substrates include LATS and MOB1. LATS1/2 kinases control cellular homeostasis, negatively regulating cell division cycle 2 (CDC2) and favoring G2/M arrest [90–92]. LATS2 was also reported to induce G1/S arrest [93]. In fact, both overexpressions of LATS1 and 2 dramatically inhibit both cell proliferation and anchorage-independent growth [47, 94] in various cell lines. It is also true that loss of LATS1/2 leads to a broad variety of tumors, such as soft tissue sarcoma and leukemia [95]. In light of these data, these proteins are believed to be strong tumor suppressors. Recent data addressed LATS involvement in tumor suppressive as well as oncogenic pathways, such as p53, RAS, and Akt signaling pathways. Interestingly, LATS2 can bind to MDM2 protein, thus inhibiting its E3 ubiquitin ligase activity to stabilize p53, which in turn favors the transcription of LATS2 [96]. Up until now, YAP and TAZ are the main LATS substrates identified in its kinase activity, but yet they only mediate some of the effects of LATS, thus indicating the existence of other substrates, such as Snail [97], DYRK1A [98], and LATS1 and LATS2 [99].

In the Hippo pathway context, LATS activity is supported by MOB1. This protein, which corresponds to the human ortholog of the Mats adaptor protein, binds to and phosphorylates LATS kinases, favoring YAP and TAZ proto-oncogenes phosphorylation and inhibiting their nuclear activity. MOB1 binding to LATS kinases is strongly enhanced upon phosphorylation of MOB1 by MST1/2 kinases [46]. Loss of MOB1 function results in increased cell proliferation and decreased cell death, suggesting that MOB1 functions, as well as the other Hippo pathway components, as a tumor suppressor protein.

4. *rassf1a* Signaling into Hippo Pathway

Due to the absence of enzyme activity, Ras-Association Domain Family (*rassf*) are noncatalytic-proteins. They are often referred to as "scaffold proteins," which are ubiquitously expressed in normal tissue and described in literature as a strong tumor suppressor family of proteins (reviewed in [100]). The *rassf*s family comprise ten members from *rassf1* to *rassf10*. Among them only *rassf1a* shares the closest homology to *Drosophila rassf* (*drassf*) (reviewed in [101]). *rassf1a* exhibits strong tumor suppressor function [102]. Loss of *rassf1a* allele is a frequent occurrence in primary human cancers [103, 104]. Furthermore, hypermethylation of *rassf1a* promoter is very often correlated with oncogenic phenotypes. Concomitantly, the identification of specific point mutations of *rassf1a* impinges on the ability of this protein to inhibit tumor cell growth [105, 106]. About 15% of primary tumors show point mutations of *rassf1a* [107]. Two independent research groups generated *rassf1a* knockout mice [108, 109]. Both these mice showed a phenotype with greatly increased susceptibility to tumor formation. Pursuing the hypothesis that the protein-protein interaction of YAP pattern changes as a consequence of different stimuli, Matallanas and colleagues followed the behavior of *rassf1a* after triggering apoptosis [77]. They showed that *rassf1a* disrupts the inhibitory complex between RAF1 and MST2 and favors the physical association between MST2 and LATS1 concomitantly, therefore, leading to YAP1 phosphorylation and nuclear relocalization where it binds to p73 and potentiates its apoptotic activity (Figure 2). It was also shown that the FAS active receptor induces *rassf1a* to compete with RAF1 in binding to MST2, thus promoting the formation of a LATS1 complex. This results in the translocation of YAP from the cytoplasm to the nucleus. These findings may suggest that the activation of the *rassf1a* complex indirectly diverts LATS1 from phosphorylating YAP, thus making it available for different phosphorylation events.

In addition, it is also able to enter into the nucleus where it can activate the transcription of p73 target genes involved in apoptosis.

It is worthy to note that in 2009, Hamilton and collaborators identified a novel DNA damage pathway that is activated by ATM kinase, involving *rassf1a* and Hippo pathway members [110]. They showed that, upon DNA damage, *rassf1a* becomes phopshorylated by ATM on Ser131. This event seems to be necessary in promoting MST2 binding to *rassf1a*, potentiating MST2 and LATS1 proapoptotic activity leading to p73 stabilization. Thus, this confirms findings observed in previous *in vitro* experiments showing that the *rassf1a* peptide containing an ATM putative domain is a substrate for ATM phosphorylation [111, 112].

More recently, the interaction, between *rassf1a* and SAV Hippo pathway member [113], was shown to potentiate p73-dependent apoptosis [114]. While this effect does not seem to require direct interaction between *rassf1a* and MST kinases, it was shown to trigger apoptosis via the MST/LATS pathway [77]. It is also true that SAV acts as a scaffold protein connecting MST kinases with LATS kinases [115] and that the expression of exogenous SAV can greatly enhance this proapoptotic signal [113]. Consequently, it is reasonable for authors to speculate the existence of a functional axis involving *rassf1a*-MST-SAV-LATS-YAP in promoting p73-induced apoptosis. Altogether, these findings show a close functional interconnection between *rassf1a*, Hippo, and p53 family tumor suppressor effects.

RASFF1A functions as a negative regulator of cardiomyocyte hypertrophy [116]. The latter displays an enlargement in size of cardiomyocytes, which is very often associated with heart failure [117]. It was proposed that a large number of protooncogenes, which are expressed in the heart, could possibly mediate this aberrant process [118]. *rassf1a* exon1α knockout mice exhibit normal cardiac morphology at 12 weeks of age. Notably, the application of a pressure overloaded the transverse aortic constriction causing massive cardiac hypertrophy, among the severest reactions ever to be reported [116]. This may suggest that *rassf1a* plays a role in contrasting overproliferation of cardiomyocytes. Interestingly, the authors observed that *rassf1a* in this cellular system greatly opposes the RAS-RAF1-ERK1/2 signal pathway. Not long ago, it was proposed that the activation of RAF by RAS requires a complex regulation of many adaptor molecules including the involvement of CNK1 (connector enhancer of kinase suppressor of RAS). This protein is able to form a complex with *rassf1a*, increasing *rassf1a*-induced cell death [119]. In light of these data authors speculated about a possible imbalance in the ratio of the components of the scaffold complex required for RAS signal transmission. CNK1 was also found to interact with MST1 and MST2, requiring MST kinases to induce apoptosis. Deleting the MST1 segment that mediates binding to *rassf1a* also eliminates the physical association between MST1 and CNK1. To sum up, CNK1 binds to *rassf1a* and promotes apoptosis through a pathway that requires *rassf1a* and MST kinases [119]. This mechanism may be the underlying factor behind *rassf1a*'s action in preventing cardiomyocytes hypertrophy. Supporting this, Del Re and collaborators showed that *rassf1a* is an endogenous activator of MST1 in the heart. They also found that in cardiac fibroblasts the *rassf1a*/MST1 pathway negatively regulates TNF-α that is believed to be a key mediator of hypertrophy and consecutive cardiac dysfunction [120]. Altogether, these findings highlight the importance of a crosstalk between *rassf1a* and components of the human Hippo pathway in preventing cardiac dysfunction due to aberrant overproliferation of cardiomyocytes. Of note, other Hippo pathway members were shown to be involved in heart development and size, such as YAP [121], Dch1-FAT [122], LATS2 [123], and SAV [124].

5. *rassf5* and *rassf6*

Other *rassf* family members were involved in modulating the activity of Hippo pathway components. The first RAS interactor discovered within this family was *rassf5* [125], often called Novel Ras Effector 1 (NORE1). This isoform that shares up to 60% homology with *rassf1*, is the most common isoform. As for many *rassf*s, it was demonstrated to be a centrosomal protein that can bind to the microtubule scaffold structure. This event appears to be required for growth inhibition and consequently tumor suppression activity, which is achieved through the inhibition of ERK signaling [126]. Furthermore, it has been reported that active RAS binds to *rassf5*-MST1 complex thereby conferring the role of the RAS effector complex in mediating the proapoptotic function of KiRASG12V [127]. RASFF5 and the MST1 pro-apoptotic kinase are involved in a physical interaction, thus forming an active complex where RAS interacts upon serum stimulation consequently leading to its pro-apoptotic function. Furthermore, the interaction of *rassf1a* and NORE1 with MST1 appears to be controversial. In fact, an inhibition of MST kinases activity by coexpression with the complex NORE1-*rassf1a* in excess was reported [128]. At the same time, by *in vivo* experiments, overexpression of *rassf1a* together with MST2 was shown to increase kinase activity of MST2 consequently potentiating its pro-apoptotic effect [77, 113, 129].

In 2009, Ikeda and collaborators showed that another *rassf* member, *rassf*6, can bind to MST2 kinase. This protein is known to induce apoptosis [130, 131]. When *rassf*6 is bound to MST2, *rassf*6 inhibits MST2 activity, thus, inhibiting its role in the Hippo pathway. Conversely, the release of MST2 from *rassf*6 causes apoptosis in a WW45-dependent manner (*Drosophila* SAV). Therefore, *rassf*6 impinges the Hippo proapoptotic pathway by inhibiting MST2, but it is *per se* able to induce apoptosis through a parallel Hippo mechanism. In fact, MST2 is responsible for apoptosis induced through Hippo signaling and through a *rassf6*-WW45-mediated pathway [131].

6. Concluding Remarks and Future Perspectives

In conclusion, the Hippo pathway is a signaling pathway that regulates cell proliferation and cell death. It is a kinase cascade that phosphorylates and negatively regulates transcription by transcriptional coactivators. As summarized

above, the loss of function of the Hippo pathway triggers tumorigenesis. Accordingly, the downregulation of the Hippo pathway is frequently observed in human cancers. Aberrant activation of Hippo downstream executors, YAP1 and TAZ, induce epithelial-mesenchymal transition and the expression of stem-cell markers in cancer cells. Quite recently, the Hippo and the *rassf* pathways have emerged to be closely linked. The tumor suppressor *rassf* proteins were shown to induce cell-cycle arrest and apoptosis. Stimuli activating the Hippo pathway simultaneously induce *rassf*-dependent biological events. Thereby, the Hippo and *rassf* pathways cooperate in preventing tumorigenesis. Reintegration of the Hippo pathway and *rassf* functions should be implemented in cancer therapy. However, it is also true that if this cross-talk results disproportionate, the consequence will be excessive apoptosis and consecutive organ dysfunction. In such cases, the involvement of the Hippo/*rassf* inhibitors will be useful. The relationship between the Hippo and *rassf* pathways is probably not restricted to cancer biology since many of the Hippo components also regulate adipogenesis, osteogenesis, and myogenesis. As discussed above, a growing body of evidence shows that this relationship between *rassf* and the Hippo pathways also occurs in cardiac tissue inhibiting cardiac hypertrophy and playing a critical role in preventing heart failure. Based on what has been described and in light of the synergistic effects observed on the interaction within *rassf* and components of Hippo signaling in preventing defects of proper biological development such as insurgence of many human diseases, much more work is needed to further investigate the importance of this physiological relationship.

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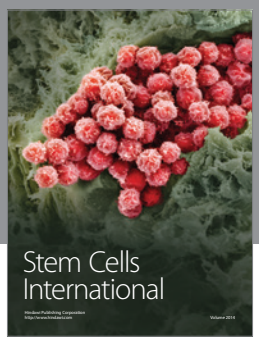

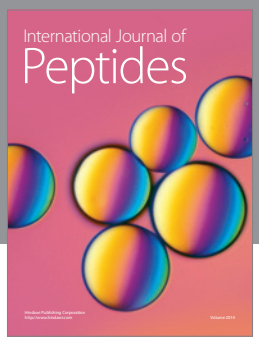

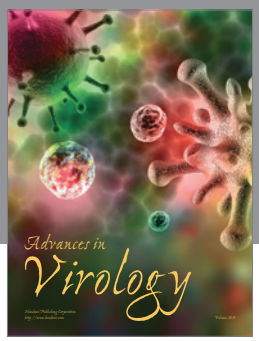

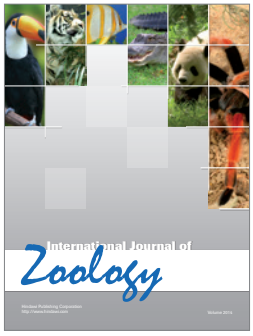

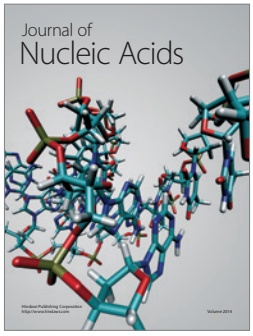

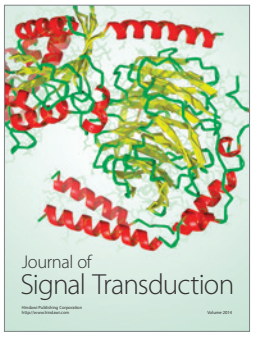

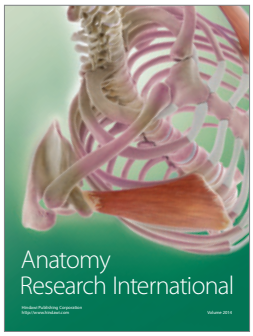

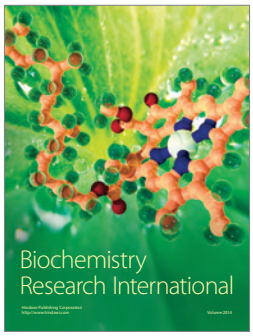

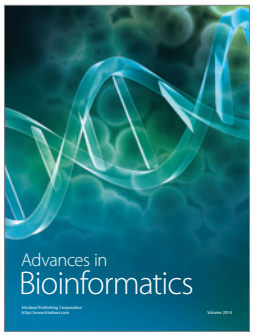

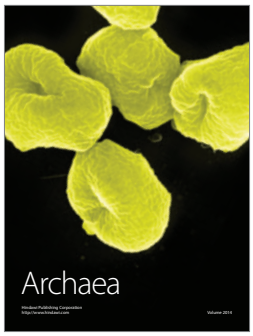

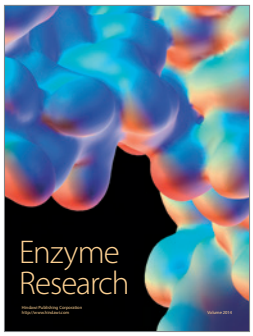

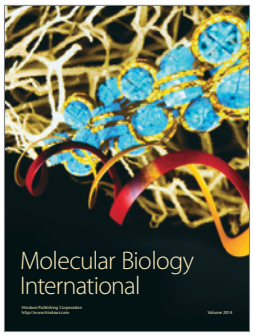

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