

Review

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Review

Targeting the Hippo Pathway in Melanoma

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Abstract: Melanoma is the most aggressive form of skin cancer. In the advanced stage of development it is resistant to currently available therapeutic modalities. Increased invasiveness and metastatic potential depend on a number of proteins involved in various signal transduction pathways. Hippo signaling plays an important role in malignant transformation. Dysfunctions of the Hippo pathway initiate the expression of tumor growth factors and is associated with tumor growth and metastasis formation. This review summarizes the recent achievements in studying the role of the Hippo pathway in melanoma pathogenesis and points to the potential specific targets for anti-melanoma therapy.

Keywords: hippo signaling; melanoma; therapy

Introduction

Melanoma belongs to the most aggressive human cancers. In a metastatic phase it is resistant to the conventional treatment. Clinically it is a highly heterogeneous disease, which may translate into the systemic therapy failures. Despite a recent progress in the identification of a number of therapeutic targets or inhibitors of immune check-points, metastatic melanoma is still incurable. So far little is known about its pathogenesis, also the specific biomarkers which would allow for a better diagnosis and prediction of disease course are missing. Such a high invasiveness and metastatic potential of melanoma result from mutations and activation of different signal transduction pathways. The Hippo signaling is responsible for a growth control and differentiation of tissues and organs. It is also largely involved in tumor formation and metastasis. However, the specific mechanisms how the Hippo pathway controls melanoma are not known. Characterization of the key proteins involved in major signaling pathways and the analysis of their interactions will allow for a better understanding of the mechanisms of melanoma pathogenesis and identification of its specific clinical subtypes. This in turn will allow in the future for identification of the specific molecular targets for a more effective treatment.

The Hippo pathway

The Hippo signaling was initially discovered in *Drosophila melanogaster*. It is an evolutionary conserved pathway which controls organ growth during the development of all metazoans [1,2]. The main role of the Hippo pathway is a thorough supervision and regulation of organ size, cell proliferation, apoptosis, and stemness in response to intrinsic and extrinsic signals that act through G-protein-coupled receptors [3–5]. Dysregulation of the pathway results in organ and tissue hypertrophy and is associated broadly with neoplastic phenotype and cancer metastasis [6].

Main Components

Human Hippo pathway is composed of three interlinked parts, including upstream regulatory components, the core kinase cascade, and the downstream transcriptional machinery. The core of the Hippo pathway acts rather as a signalling network than a definite transduction cascade [7]. It contains a conserved kinase cassette consisting of Mst1/2, Lats1/2, and MOBKL1A/B and MOBKL1B (Mob1) kinases [8,9]. MST1/2 (Mammalian Ste20-like kinases 1/2) forms the complex with SAV1 (*Salvador* Family WW Domain Containing Protein 1) which phosphorylates and activates the kinase complex composed of LATS1/2 (Large tumour suppressor 1/2) and MOB1 (*MOB* Kinase Activator) [10–14]. While activated LATS1/2 phosphorylates and inactivates YAP1 (Yes association protein 1) and its paralog TAZ (also known as WWTR1) [11,15]. In their unphosphorylated states, YAP1 and TAZ easily enter the nucleus and function as transcriptional activators. However, while phosphorylated, YAP1 and TAZ are bound by 14-3-3 proteins, and consequently sequestered in the cytosol and degraded by proteasome complex [14,16].

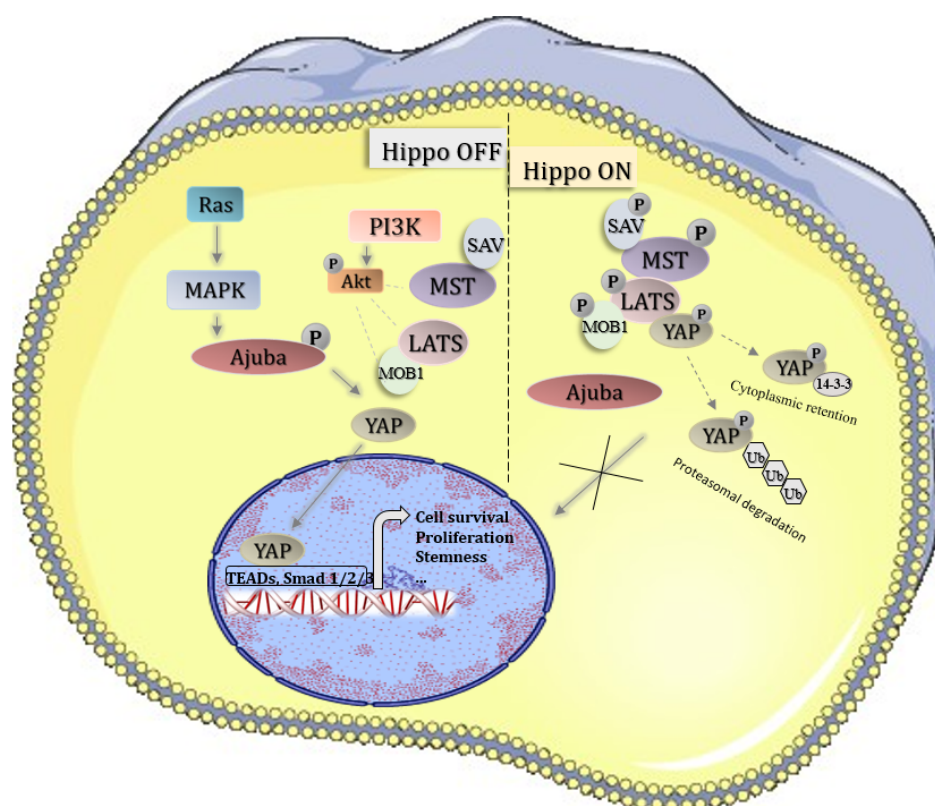


Figure 1. Hippo pathway signaling; inactive Hippo leads to YAP oncogene transport to the nucleus and activation of pro-survival genes; active Hippo leads to YAP phosphorylation and its degradation in cytoplasm .

Upstream Regulators of the Core Kinase Cascade

The first identified upstream components of the Hippo pathway in *Drosophila* were two FERM-domain containing proteins: Merlin (NF2 in mammals) and Expanded (FRMD6 in mammals) [17]. NF2 (Merlin) is the product of the Neurofibromatosis type 2 (NF2) tumour suppressor gene and a member of ezrin-radixin-moesin (ERM) protein family [18,19]. In its closed inactive conformation, the CTD is joined with the N-terminal FERM domain. Phosphorylation of C-terminal residues disrupts the connection and liberates FERM to bind cell adhesion molecules. Mer/NF2 functions in complex with WWC1 (Kibra) and FRMD6 as an upstream effector of Hippo pathway in order to regulate cell and growth and suppress tumour development. WWC1-NF2-FMRD6 protein complex activates MST1 protein through autophosphorylation [18,20]. WWC1 is an apical protein containing two amino-terminal WW domains, an internal C2-like domain and carboxy-terminal glutamic acid-

rich region. To promote Hippo signalling, NF2 binds to Kibra (WWC1 in mammals) [17]. The last upstream regulator of the Hippo pathway is a transmembrane protein Crumbs (Crb) which functions in a complex with FRMD6 [17].

The Core of the Hippo Signaling

The Mammalian Ste20-Like Kinases (MST) 1/2

Mammalian Ste20-like kinases (MST1/2) are classified among STE20 kinases. Primarily MST1/2 were described as elemental proapoptotic kinases, which are activated through signals linked to stress-induced apoptosis [21,22]. Mst1/2 activity is modulated both by autophosphorylation and caspase-cleavage [23]. Following autophosphorylation of their threonine residues (Tre183 for MST1 and Tre180 for MST2), MST1/2 phosphorylates protein SAV1 (also referred to as WW45), thus initiating the Hippo pathway [24]. Conversely, following caspase-cleavage, modified MST protein with liberated N-terminal kinase domain enters the nucleus and induces chromatin condensation by phosphorylating histone H2B, therefore causing DNA fragmentation and apoptosis [11,25]. Mst1/2 activity is also regulated by heterodimerization with RASSF family proteins, which are tumour suppressors. Frequently, in human cancers expression of RASSF proteins is restricted through epigenetic silencing [24]. The experiments performed on mice proved that MST1/2 proteins are essential for proper development. Single knock-out of one MST1 or MST2 allele does not lead to any disorders. However, mice having both alleles knocked-out die in embryonal stadium due to developmental defects [26].

Large Tumor Suppressors (LATS) 1/2

LATS1 and LATS2 belong to the serine-threonine kinases family and are involved in cell proliferation, apoptosis and regulation of the cell cycle. They are mainly active in the early prophase as LATS1 cooperates with CDC2/cyclin A and LATS2 binds to the proteins in centrosome region enabling the formation of kinetic spindle. Additionally, LATS1 negatively regulates G2/M transition by down-regulating CDK1 kinase activity [27]. Correspondingly, LATS2 inhibits cell growth at the G1/S control point via downregulating cyclin E/CDK2 kinase activity [28]. In regard of the Hippo pathway, LATS1 and LATS2 are tumour suppressors that inhibit the oncogenic nuclear function of YAP/TAZ and TEAD. Following phosphorylation by MST1/2, LATS1/2 connect with their coactivator MOB1 and undergo autophosphorylation. Simultaneously, SAV plays an important role of a scaffold protein for assembling MST1/2 and LATS1/2 [29,30]. Consequently, this active LATS1/2-MOB1 complex phosphorylates transcriptional coactivators YAP and TAZ on their multiple HxRxxS motifs, thereby leading to their inactivation [31].

Mps One Binder Kinase Activator (MOB) 1

MOB 1 is an essential coactivator of LATS kinases. Overexpression of MOB1 triggers LATS1/2 activation and consequently leads to proliferation inhibition or even to apoptosis. Contrarily, decrease of MOB1 expression increases the rate of cell division. MOB1 deficiency or reduction is frequently associated with tumorigenesis, especially in human skin melanoma, colorectal cancer and non-small cell lung cancer [32].

Hippo Pathway Effectors

Yes-Associated Protein (YAP)

YAP is an effector protein in the Hippo pathway. As it does not have a DNA-binding domain, it is classified as a transcriptional coactivator, which interacts with transcriptional factors in order to regulate gene expression [33]. There were two isoforms of YAP identified: YAP1 with single WW domain (domain consisting of 40 amino acids, rich in proline and with two conservative tryptophan residues – WW) and YAP2 with double WW domain [34]. WW domain proteins have an ability to bind other proteins that exhibit PY motifs, being proline-rich modules [35]. Besides the WW domain,

YAP1 and YAP2, contain an SH3 domain-binding motif and PDZ-binding motif in their C-terminus [36]. YAP activity depends on its cellular localisation, since only while being in cell nucleus it may exert its functions. Contrarily, following the phosphorylation of serine residue in position 127, YAP binds with 14-3-3 protein and therefore it is inactivated and restrained outside nucleus. Apart from serine 127, there are 4 other consensus HXRXXS motifs, that may be phosphorylated (S61, S109, S164, and S381). Nonetheless, S127 and S381 are regarded as the critical sites related to oncogenic phenotype. Phosphorylation of solely S127 suffices to impede YAP activity [37]. Phosphorylated YAP is either sequestered in the cytoplasm via interaction with 14-3-3 protein or subjected to proteasomal or autophagy-induced degradation [38]. As YAP chromosomal locus 11q22 is commonly amplified in cancer cells, its expression and nuclear localization is frequently elevated in human cancers [39]. YAP performs a role of an oncogene in medulloblastoma, glioblastoma multiforme, squamous cell carcinoma, small cell lung cancer, liver cancer, colorectal cancer, pancreatic cancer and ovarian cancer [40]. Noteworthy YAP overexpression leads to neoplastic transformation as cells gain ability to proliferate infinitely and uncontrollably, to undergo epithelial–mesenchymal transition (EMT) and to evade apoptosis [41]. Additionally, YAP and its paralog TAZ make cancer cells evade immune surveillance [42–44]. Adversely, while in most solid tumours, YAP is an oncogene, in haematological cancers it plays a tumour-suppressor role [45].

TEA Domain (TEAD) Factors

TEAD belongs to a family of transcriptional factors, which mediate the main transcriptional output of the Hippo pathway in response to YAP and TAZ activity [46,47]. All members of TEAD family contain identical DNA-binding domain, which connects with M-CAT motif (5'-CATTCCCT-3'). TEAD transcription factors are expressed in every kind of tissue. However, depending on tissue type, the levels of particular TEAD factors vary and their physiological response may differ. For example YAP protein is indispensable for the full transcriptional activity of TEAD2. Interaction between TEAD1–4 and their major binding partners, YAP and TAZ, causes VGLL4 dissociation from TEAD1–4 and anchorage of YAP/TAZ to chromatin. Consequently TEAD-mediated gene transcription induces cell proliferation and inhibits apoptosis [4,48].

Response to Cell Density

As it is known that organ growth is controlled by the intrinsic network of pathways of which the Hippo pathway plays the pivotal role. The organ development depends on cell number and density, cell size as well as extracellular factors [49]. The increased adherens and tight junctions in confluent cells contribute to activation of LATS and inactivation of YAP/TAZ [50]. Detachment of the cells inactivates YAP/TAZ and triggers anoikis [51]. At low cell density LATS kinase remains inactive, which leads to YAP/TAZ activation and evasion from cell-contact inhibition [16].

Hippo Pathway in Cancer

The Hippo pathway signaling is dysregulated in a variety of cancers leading to YAP/TAZ activation which in turn leads to hyperproliferation of cancer cells and lack of apoptosis, stem cell maintenance and epithelial-to–mesenchymal transition, increased invasion and metastatic potential, oxidative stress and finally resistance to therapies [52].

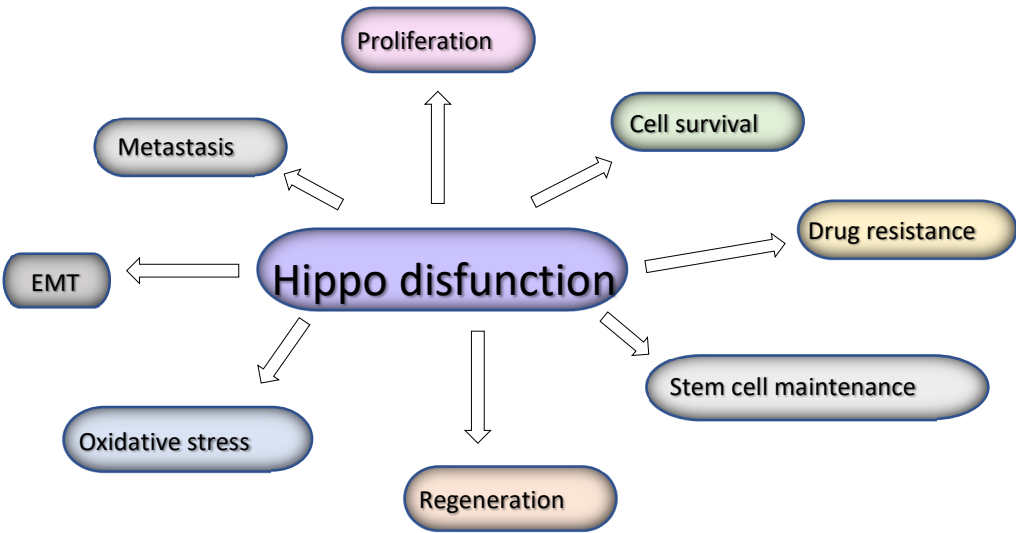


Figure 2. Hippo pathway dysfunction leads to several events linked with cancer progressio.

However, those event result from the mutual cooperation with other prominent signaling pathways such as the Wnt, G protein-coupled receptor (GPCR), epidermal growth factor (EGF), BMP/transforming growth factor beta (TGFβ), JAK/STAT and Notch pathways [52,53]. In melanoma, the interaction between the Hippo with PI3K/Akt (Akt) and ERK/Raf/Ras (MAPK) pathways is essential for tumor growth control [54].

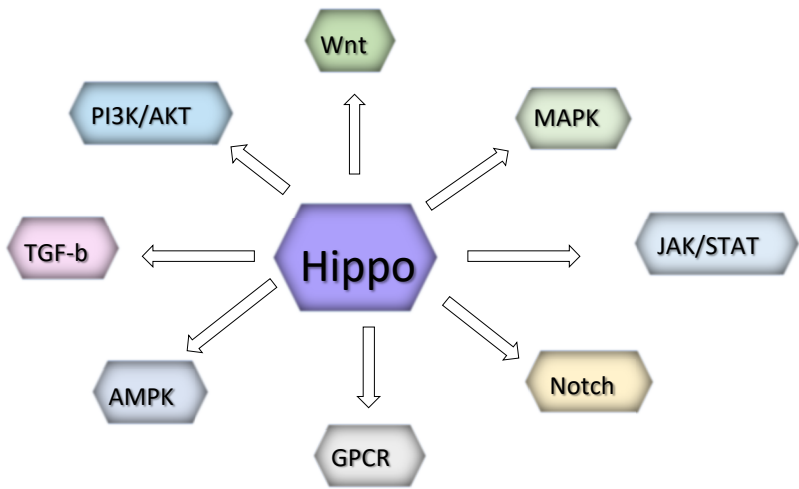


Figure 3. Hippo signaling highly impacts several pathways involved in melanoma development.

Hippo Pathway in Melanoma Biology and Treatment

Hippo pathway components are involved in determination of the overall number of cutaneous melanocytes. Mechanical signals mediated by contacts between melanocytes and the basement membrane, other melanocytes and keratinocytes are integrated in space and time to activate the Hippo signaling as it takes place in other tissues. These signals are complemented by biochemical markers send from keratinocytes, and transferred from surface receptors to cellular components through protein phosphorylation [55]. The clinical data confirmed that the deregulation of the Hippo pathway often correlated with poor prognosis for melanoma patients [56].

Hippo Signaling and BRAF/MEK Inhibitor Resistance

Targeted therapy with BRAF and MEK inhibitors significantly improved the prognosis of melanoma patients. However, it still demonstrates some limitations [57,58]. The vast majority of patients treated with BRAF inhibitors acquires the resistance within 1 or 2 years. Defining the mechanisms of that phenomenon is an emerging issue to improve the outcomes. It has been shown previously shown that YAP promote resistance to anti-cancer therapies. In the past decade, the YAP mechanotransduction pathway has been implicated in the resistance to BRAF and MEK inhibitors. Understanding the mechanisms leading to YAP-related resistance to RAF and MEK inhibitors is essential to develop more effective treatment. Lin et al. confirmed the unanticipated functional cross-talk between YAP and RAF-MEK signaling. The authors investigated whether YAP regulates the response to targeted inhibition of BRAF signaling in several tumor cell lines harboring BRAF, KRAS or NRAS activating mutations [59]. They found that YAP1 suppression enhanced the response to both vemurafenib and trametinib in A2058 and WM793 melanoma cell lines, and A2058 melanoma xenografts. Most likely YAP enhances the expression of antiapoptotic factors such as BCL2 family member protein BCL-xL in resistant cells and YAP knockdown prevent that process. Moreover, YAP upregulation in BRAF- or RAS-mutant tumor cells might promote resistance to RAF and MEK inhibitors in patients. Accordingly, they observed high levels of YAP in the majority of melanoma samples with mutant BRAF V600E. Interestingly, patients harboring BRAF V600E mutation who responded to RAF and MEK inhibitors (CR) exhibited lower YAP expression in the pretreatment tumor samples, and those with incomplete response had higher YAP expression before treatment [59]. The above study clearly shows that YAP acts a survival factor in melanoma cells and its downregulation may be an effective way to enhance the therapeutic effects of RAF and MEK inhibitor based treatment.

Searching deeper into the mechanisms of BRAFi resistance, Kim et al. looked at the connection between Hippo pathway and anti-melanoma immune response. They genetically modified three melanoma cell lines (SKMEL28, WM3248, and A375) to activate YAP, and performed a set of in vitro experiments to test whether YAP activation changes the cytokine release by those cells, and how it influences the cytotoxic abilities of lymphocytes. They developed that YAP activation in BRAFi-resistant melanoma cells directly inhibited cytotoxic T-cell responses, and the mechanism they proposed was the induction of PD-L1 expression on tumor cells [60,61].

Kim et. al. studied the mechanism of BRAF inhibitor PLX4032 resistance in melanoma cell lines and linked it with actin cytoskeleton remodeling. They demonstrated that melanoma cells resistant to PLX4032 exhibit an increased content of stress fibers in actin cytoskeleton, which appears to promote the nuclear accumulation of YAP/TAZ [62–64]. After knocking down of YAP in PLX4032-resistant SK-MEL28 and WM3248 cell lines, they observed the reduced viability of the cells, whereas overexpression of constitutively active YAP induced resistance of the parental melanoma cell lines. YAP/TAZ knockdown influenced also EGFR and phosphorylated AKT levels, and resulted in significant enrichment of downregulated genes related with cell cycle and mitosis in PLX4032-resistant cells. Activated YAP overexpression suppressed the expression of SOX10 and MITF, and upregulating EGFR. The authors also showed that both c-MYC protein and mRNA levels are dependent on YAP/TAZ. Conversely, inhibition of actin polymerization and actomyosin tension suppresses both YAP/TAZ activation and PLX4032 resistance. The results of the above study suggest that inhibition of actin remodeling may be a potential strategy to suppress the resistance to BRAF-inhibitor based therapies [62]. Also Monaghan-Benson found the connection between Hippo pathway and BRAF-related modification of cell cytoskeleton. They found that mutated BRAF was associated with a Rac-dependent cadherin switch in melanoma cells. Rac1 acts to modify the cytoskeleton. This gene is highly mutated in melanoma cells and loss of its function can change Hippo pathway regulation [55,65].

Recently Dieter et al. have described in Nature a novel approach for breaking YAP/TAZ-driven resistance to MAPK pathway inhibitor treatment. First, they transiently activated YAP1 in BRAFV600E mutant melanoma cell lines and established that it is YAP1 who induces the resistance to BRAF and MEK inhibitors. Moreover, YAP1 showed to drive the MITFlow/AXLhigh phenotype which seems to be a potential biomarker predicting response to MAPKi therapeutic approaches.

Next, using a genome-wide CRISPR/Cas9 functional screen the authors identified the essential genes in MAPKi-resistant cells with activated YAP1. They distinguished *SLC35B2* gene which appeared to play a crucial role in that process. *SLC35B2* is responsible for heparan sulfation expression and positively correlates with tumor progression. After suppressing the gene they noticed that such modification sensitizes the cells to BRAF inhibition [66].

BRAF inhibitor resistance can be also triggered by the ubiquitin ligase- dependent degradation of LATS1 and MST2 in BRAFi resistant cell lines. Romano et. al. demonstrated that mutant BRAFV600E binds to and inhibits MST2 preventing the MST2-dependent apoptosis. Treatment of BRAFi resistant cells with proteasome inhibitors resulted in the rescue of proapoptotic MST2 and LATS1 signalling. The results from a small cohort of patients with resistance to BRAFi confirm that MST2 downregulation might be associated with the acquisition of resistance in human melanoma [67].

YAP/TAZ in Melanoma Invasion and Metastasis

A connection between the Hippo signaling targets and melanoma pathogenesis has been subject of several studies. Nallet-Staub et al. provided an evidence that endogenous YAP/TAZ contribute to the invasiveness and metastatic behavior of melanoma cells. They performed profiling of human melanocytic lesions and melanoma cell lines for endogenous YAP and TAZ expression, followed by specific YAP/TAZ knockdown and overexpression experiments in melanoma cell lines to analyze the anchorage-independent growth, invasion into Matrigel, human skin reconstructions, and in vivo tumor growth and metastasis formation. They found that knockdown of either of these Hippo effectors lead to reduced clonogenic and invasive capacity in vitro, and decreased their ability to form lung metastases after intravenous injection in mice, while YAP/TAZ overexpression had the opposite effects. [68,69]. Interestingly, TAZ levels were higher than YAP in melanoma cells, and its knockdown gave better anti-melanoma response than YAP knockdown. The authors of the above study noticed that overexpression of both YAP and TAZ leads to enhancement of their nuclear activity despite the presence of their phosphorylated forms in the cytoplasm [68,70] YAP/TAZ overexpression increased anchorage-independent growth and invasion into Matrigel, hereby confirming a direct role for endogenous YAP and TAZ in controlling melanoma cell metastatic potential. This study corroborates the findings of Lamar *et al.* from 2012 who showed that overexpression of YAP enhanced the frequency and size of melanoma tumors in lungs after intra-vein injection, in a TEAD binding dependent manner [71]. They used A375 melanoma cell line for a Luminex-based in vivo assays and found that the domain of YAP that interacts with the TEAD/TEF family of transcription factors is essential for tumor cell proliferation, transformation, migration, and invasion. They also observed that the ability of melanoma cells to form metastases is significantly correlated with increased TEAD transcriptional activity [71]. They generated an activated form of YAP by introducing the S127A mutation into YAP and found that it dramatically enhanced tumor growth and metastasis in mice. Mutation of the TEAD-interaction domain prevented tumor formation. Concluding, it seems that YAP-mediated melanoma growth and metastasis requires a functional TEAD-interaction domain. In addition, YAP exerts its prometastatic effects at both the primary and metastatic site, and enhances several processes which contribute to metastasis.

YAP/TAZ and CSC and EMT Phenotype

Kim et al. studied whether upstream elements of the Hippo pathway and the markers of epithelial-to-mesenchymal transition E-cadherin and N-cadherin (Kim et al., submitted) are correlated with YAP1 and TAZ expression. They found that the majority of melanoma cell lines tested had lost E-cadherin expression which was replaced with the expression of N-cadherin, which in turn was associated with a more invasive phenotype. Regarding YAP/TAZ, all cell lines strongly expressed TAZ and many additionally expressed YAP [72].

A group of scientists from the University of Maryland School of Medicine searched for the role of YAP/TAZ in cancer stem cells (CSC). They studied BRAFi resistant melanoma stem cell spheroids

(MCS) and noticed their enhanced growth and matrigel invasion, together with elevated YAP1, TAZ and TEAD levels. That effect was reversed after knockdown of TEAD proteins. They also performed knockdown and overexpression experiments to confirm a BRAFi resistance role of YAP1 and TAZ, since the inhibition of YAP1 function with verteporfin reduced YAP1/TAZ level and restored sensitivity to BRAFi [73]. YAP1 expression can be downregulated after treatment with the combination of a histone deacetylase inhibitor LBH589 and a bromodomain inhibitor I-BET151 as it was shown in melanoma xenografts. Both drugs induced a caspase-dependent apoptosis which except of YAP1 decrease involved the downregulation of AKT pathway [74].

YAP/TAZ as Prognostic Factors

Menzel et. al. used the comparative genomic hybridization (CGH) data of 48 human primary melanomas and 10 melanoma metastases to analyze the involvement of YAP1 gene in melanoma development. They found that in twenty tumors YAP1 gene or YAP1-activating PAK1 gene were amplified or there was a copy number loss of LATS2 or NF2 suppressor genes. Also, patients with high YAP1 expression showed decreased survival [75].

YAP1 activity has been shown to be elevated especially in constitutively invasive melanoma cells. Zhang et. al. demonstrated that YAP1 hyperactivity can switch melanoma cells from proliferative to invasive phenotypes and promote spontaneous melanoma metastasis *in vivo* while compromising the growth of primary tumors. Moreover, that switch cannot be reversed and in consequence such invasive cells either die or downregulate YAP1 overexpression to increase the chance of survival [76].

Zhang et. al. depleted YAP and/or TAZ in several melanoma cell lines, and used patient-derived xenografts (PDX), to test the therapeutic effects of YAP targeting. They discovered that the sensitivity of YAP1 or TAZ targeting was independent of classical BRAF/NRAS driver mutations; however, it was variable between different cell lines or patients. They also showed that YAP1 is elevated in most melanomas, and may be considered as a survival factor for melanoma cells. From the other side, YAP1 level was also increased in benign nevi, the vast majority of which do not transform to invasive state. From that view, YAP1 does not seem to be a good prognostic biomarker for YAP1-targeted therapy and other marker proteins need to be elucidated [77].

Yuan et al. analyzed different genetic variants (single nucleotide polymorphisms) of the Hippo pathway genes in melanoma patients to check whether they were associated with survival rates. The authors demonstrated that *YAP1* rs11225163 C>T, *TEAD1* rs7944031 A>G and *TEAD4* rs1990330 C>A significantly modulated survival rates of melanoma patients. Moreover, based on genotyping data they could distinguish different prognostic groups. This study confirmed the importance of combining genetic features with clinicopathological characteristics while assessing the patient prognosis [30].

YAP/TAZ Signaling Regulation

The evidence that YAP overexpression is an essential factor for tumor progression and targeted drug resistance to targeted drugs is obvious. However, little is known about the regulatory mechanisms of YAP/TAZ signaling. Studies has shown that it can be directly or indirectly regulated by non-coding RNAa (ncRNAs). Choe et al. demonstrated that miR-550a-3-5p directly suppressed oncogenic YAP and exerted tumor-suppressive activity. Treatment of vemurafenib-resistant melanoma cells with miR-550a-3-5p reduced phosphorylation of AKT, and marginally modulated phosphorylated ERK, and in consequence improved the sensitivity of the drug. The authors also observed that the resistant WM3248 melanoma cell line showed a higher level of EGFR and maintained ERK/AKT activity in response to vemurafenib comparing with control cells. miR-550a-3-5p overexpression inhibited various oncogenic properties, including cell proliferation, anti-apoptosis, migration, invasion, and cancer stemness, which was consistent with the effects of YAP inhibition [78].

Hippo signaling regulation is clearly essential for melanoma development. Kim et. al. indicated that in melanoma cells the transcription of TAZ/TEAD target genes may be regulated by PIN1-STK3 axis. PIN1 is one of the factors which inhibit the Hippo pathway. It associates with STK3 (MST2) and promotes its ubiquitin-mediated degradation, to induce the nuclear translocation of TAZ oncogene and subsequent transcriptional activation of the TAZ/TEAD complex. TAZ/TEAD enhances the transcription of specific genes such as *CTGF* which promotes cell proliferation, epithelial-mesenchymal transition, invasion, and cellular transformation [79,80]. Conversely, depletion of PIN1 in melanoma cells leads to retention of TAZ in the cytoplasm followed by CTGF decrease and final inhibition of melanoma development. The authors demonstrated that PIN1 increases the mRNA and protein expression of TAZ/TEAD target genes in A375 cells promoting tumorigenicity, and STK3 overexpression suppressed the tumorigenicity of those cells. These results were further confirmed by *in vivo* experiments with syngeneic mouse model. Moreover, TCGA analysis of SKCM patients revealed a significant negative correlation between PIN1 and STK3 mRNA levels [81].

While studying the mechanisms of melanoma pathogenesis the researchers from China focused on the role of the latent transforming growth factor binding protein (LTBP). They have previously demonstrated that LTBP4 may serve as a diagnostic marker for melanoma in the GSE46517 dataset [82]. Patients with low expression of that gene had poorer prognosis. It has been shown that LTBP4 stabilizes the TGF- β receptor complex and regulates TGF- β 1 activity, while TGF- β 1 regulates the nuclear translocation of YAP1 protein [83]. Thus, LTBP4 regulates the Hippo pathway by affecting TGF- β 1 activity. Wang with his group silenced LTBP4 in melanoma cells and observed the enrichment of YAP1 in the nucleus and inhibited phosphorylation of YAP1 protein in the cytoplasm. Thus, they indicated that LTBP4 promotes the phosphorylation of YAP1 and reduces its nuclear translocation, leading to the activation of the Hippo signaling [84].

The Core of the Hippo Pathway in Melanoma Suppression

LATS1/2 in Tumor Growth Regulation

Although most studies on the Hippo signaling engagement in melanoma is focused on YAP1 oncotarget, several evidences point to the high importance of its core kinases in the regulation of that cancer. Hippo pathway consists of four tumor suppressors: SAV1, MST1/2, LATS1/2, and MOB kinase activators. The role of the upstream kinases is to degrade their oncotargets (YAP/TAZ), and inhibit melanoma growth and metastasis. SAV1 is involved early in the Hippo pathway and is required for activation of LATS1/2. Moreover, SAV1 directly interacts with AKT kinase and suppresses AKT-mediated tumorigenesis; also, it promotes apoptosis. It is clear that SAV1 and LATS1/2 play essential roles in tumor suppression.

Romano et al. demonstrated that SAV1 and LATS1/2 expression can be induced by triptonide. Triptonide is a minor component in the Chinese herb *Tripterygium wilfordii* and exerts a strong anti-cancer effect with low toxicity. Enhanced expression of SAV1 and LATS1/2 leads to degradation of oncogenic YAP, and suppression of MITF and AKT. These events strongly inhibit melanoma cell tumorigenicity, migration, invasion, and lung metastasis [85].

Our previous study showed that LATS1 silencing reduces the expression of the melanogenesis markers and melanin synthesis in primary melanocytes and melanoma cells. That phenomenon was associated with increased growth of LATS1 knocked down human xenografts in nude mice. Moreover, silencing LATS1 resulted in enhanced oxidative stress [86].

Vittoria et. al. studied the controlled BRAFV600E expression in a melanocyte cell line and discovered that the expression of oncogenic BRAFV600E leads to the significant increase in LATS phosphorylation and in a consequence the nuclear exclusion of YAP and decrease in the expression of the YAP target genes CYR61 and AMOTL2. Also, it restrains oncogenic melanocyte proliferation *in vitro* due to the Hippo pathway activation. Next, the authors searched for the mechanism of that activation and found that since inhibition of MEK1/2 or ERK1/2 kinases resulted in YAP/TAZ phosphorylation, Hippo pathway activation is entirely mediated by the general hyperactivation of MAPK signaling. *In vivo* experiments showed that the functional impairment of the Hippo pathway

in melanocytes promoted cutaneous melanomagenesis. They showed that *Lats1/2*^{-/-} mice formed tumors with strong p-ERK staining suggesting that *LATS1/2* depleted melanocytes evolved to hyperactivate the MAPK pathway. In addition to highlighting the role of *LATS1/2* kinases in melanoma development, that study also pointed to YAP/TAZ as promising therapeutic targets as constitutively active YAP caused extended tumor formation in zebrafish.

In 2016 Moroishi et. al. published a prominent paper which describes a suppressing role of *LATS1/2* in melanoma immunity. They discovered that inactivation of *LATS1/2* in tumor cells strongly suppresses tumor growth in immune-competent, but not immune-compromised mice, which is caused by the induction of anti-tumor immune responses. *LATS1/2*-deficient melanomas secreted nucleic-acid-rich extracellular vesicles (EVs) that stimulated the host TLRs-MYD88/TRIF-IFN nucleic-acid-sensing pathways, inducing anti-tumor inflammatory responses. Using three different melanoma cell lines in different syngeneic mouse models they showed that *LATS1/2* deletion abolished the growth of SCC7 tumors and highly reduced tumor growth and the metastasis of B16 and 4T1 cells [87].

It has been recently demonstrated that *LATS1* kinase directly links Hippo pathway with apoptosis. Garcia-Gutierrez et al. performed the set of experiments to identify new interactions of *LATS1* with apoptotic markers. They found that *LATS1* forms complexes with SMAC which is dependent on RASSF1A expression. SMAC is a mitochondrial protein that promotes cytochrome c-dependent caspase activation. The *LATS1*-SMAC complex requires the presence of the IAP family member XIAP. Although XIAP inhibits SMAC alone, it seems that *LATS1* bound to SMAC counteracts that effect resulting in an increase of SMAC levels due to protein stabilization. SMAC then promotes the ubiquitination and subsequent degradation of XIAP leading to apoptosis. In a parallel study, the authors observed the loss of *MST2* and *LATS1* expression in BRAFⁱ resistant melanoma cell lines. They showed that oncogenic BRAF can inhibit *MST1/2* in these cell lines, prevents *MST2*-*LATS1* interactions and *LATS1*-dependent apoptosis [88].

LATS and microRNA in Melanoma Tumorigenesis

Hu et al. using an online tool for target prediction analysis of microRNA (miRDB) linked *LATS2* kinase with miR-135b expression and showed its role in melanoma tumorigenesis. The authors compared miR-135b levels in 20 melanoma tissues and analyzed the impact of miR-135b on cell proliferation, migration, or apoptosis in either primary melanocytes or the A-375 melanoma cell line. MiR-135b expression was significantly upregulated in melanoma tissue. Overexpression of miR-135b in primary melanocytes promoted cell proliferation and migration, and miR-135b inhibition reversed that effect. The proposed mechanism of such process was that miR135b through targeting *LATS2* prevents inhibitory effects of that kinase [89].

Surprisingly, *LATS1* phosphorylation of YAP1 may also indirectly promote the growth of a small population of cancer cells named Tumor Repopulating Cells (TRCs). TRCs were discovered by Jiu et. al. who mechanically selected a small sub-population of cancer cells with high tumorigenic and proliferative capacity and named it Tumor Repopulating Cells (TRCs). Those cells showed to efficiently repopulate tumors at distant organs in mice, and were independent of surface CSC markers. Using microarrays the authors screened for potential factors contributing to enhanced proliferation of TRCs and identified Nuclear protein 1 (Nupr1) which was markedly reduced in that population. YAP1 phosphorylation by *LATS1* leads to the downregulation of Nupr1, leading to the downregulation of p53 and upregulation of Nestin and Tert. All those events help to promote the proliferation and self-renewal of TRCs [90].

MST1/2 Activation and Suppression of Tumor Growth

The efficacy of the Hippo pathway is significantly dependent on Merlin which is an upstream regulator of *MST1/2* Ser/Thr kinases and responds for inhibition of cell proliferation, and invasiveness [91]. Murray et al. assessed the correlation between Merlin expression and response to oxidative stress. They found that higher merlin levels in human melanoma cells promote the H₂O₂-

induced activation of MST1/2 and suppression of tumor growth. Merlin knockdown promoted post-confluence cell proliferation and subcutaneous growth of WM1552C human melanoma cells.

Feng et. al. analyzed the interactions between the MAPK and Hippo pathways in four melanoma cell lines. They found that RAF-1 forms a complex with MST-2. The suppression of RAF-1 prevents RAF-1/MST-2 complex formation leading to MST-2 activation, and subsequent inhibition of cell proliferation, migration and invasion, and apoptosis promotion. [92] These results stay in agreement with another study on interactions between different signaling pathways. Notably, Romano et al. reported that RAF-1 mutation stimulates both the Hippo and MAPK pathways, simultaneously driving apoptosis and proliferation, whereas concomitant MST-2 downregulation switches signaling to cell proliferation, transformation and survival [93].

Yu et al. linked Hippo pathway with curcumin-induced melanoma cell apoptosis. They found that curcumin activated MST1-dependent cell death in cultured melanoma cells. Curcumin enhances ROS production which activates MST1. Activated MST1 then promotes cell apoptosis probably through inducing JNK activation, Foxo3a nuclear accumulation, and Bim-1 expression. MST1 knockdown prevented curcumin-induced activation of caspase-3 and caspase-9 and Bim expression, which contributed to cancer cell apoptosis [94].

Kang et. al. studied a link between the Hippo pathway and fascin in melanoma. Fascin is upregulated in various cancers including melanoma. It increases melanoma tumorigenesis and stemness via interaction with MST2 and inhibition of MST2 homodimer formation and kinase activity, and thus reduction of LATS activity and enhancement of TAZ (but not YAP1) stability. The results point to fascin/Hippo axis as a potential therapeutic target for melanoma. Knockout of fascin significantly reduces tumorigenicity and stemness of melanoma whereas ectopic expression of fascin exerts opposite effects. Thus, targeting fascin could also become an effective strategy for advanced melanoma [95].

Summary

Hippo pathway has been investigated in tumorigenesis due to its particular role in cell growth and metastasis. It affects several biological processes such as cell proliferation, migration and invasion, epithelial-to-mesenchymal transition, cancer stem cell formation, apoptosis, cell cycle and polarity. Hippo pathway effector components are often found mutated or downregulated in a variety of cancers including melanoma, and the target genes are upregulated. Therapies targeted to the Hippo pathway components may largely improve the survival rates of melanoma patients. The thorough analysis of the Hippo pathway in melanoma and investigation of its relationship with other key signaling pathways involved in the tumor formation will provide a better understanding of the mechanisms of melanoma pathogenesis and will help to develop more effective treatments.

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