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New insights into the pathophysiology of the tuberous sclerosis complex: Crosstalk of mTOR- and hippo-YAP pathways in cell growth

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Tuberous Sclerosis Complex (TSC) is a genetic disease causing uncontrolled growth of hamartomas involving different organ systems. In the last decade, dysregulation of the mTORC1 pathway was shown to be a main driver of tumor growth in TSC. Recently, a new crosstalk was detected between the mTORC1 and the Hippo-YAP pathway, another major cell signaling cascade controlling cell growth and organ size. Elucidating this connection is an important step in understanding the complexity of TSC, enabling new pharmacological targets and therapeutical options.

Keywords: Tuberous Sclerosis Complex, signal transduction, mTOR, Hippo/YAP, pharmacology

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Introduction

Tuberous sclerosis complex

Tuberous sclerosis complex (TSC) is a rare autosomal dominant disorder with a prevalence of approximately 1:6000 live births.[1] The disease is characterized by the growth of hamartomas, which may involve multiple organs, including the brain, kidneys, lungs, skin, heart, and retina. Depending on the localization of the hamartomas, the manifestations of TSC can be potentially devastating. The involvement of the brain may lead to significant morbidity and mortality in childhood and adolescence, such as due to intractable severe epilepsy caused by cortical tubers or hydrocephalus with life-threatening symptoms due to a growing giant cell astrocytoma. In adulthood, hamartomas of the kidneys (angiomyolipomas - AML) may cause severe life-threatening hemorrhage, whereas hamartomas of the lungs (lymphangioleiomyomatosis - LAM), predominantly affecting females, can lead to respiratory failure.[2]

Both AML and LAM are classified as PEComas, because they contain a specific cell-type called perivascular epitheloid cells (PECs). Since there is no counterpart of PECs in healthy tissues, the origin of this cell-type remains obscure, as they reveal melanocytic as well as myogenic markers in histology.[3]

TSC and mTORC1

TSC is caused by mutations in either *TSC1* located on chromosome 9q34, or *TSC2* located on chromosome 16p13.3, which encode the proteins hamartin, and tuberin, respectively.[2] Both proteins form a heterodimeric TSC complex that integrates inputs of upstream signals, such as growth factors, cellular nutrients and energy levels, stress, oxygen, and cytokines, to regulate mammalian target of rapamycin complex 1 (mTORC1).[4] mTORC1 is a master regulator of cell growth.[5] Its activation leads to multifaceted downstream events, including the control of mRNA translation via phosphorylation of S6 kinase 1 (S6K1) and 4E binding protein 1 (4EBP1), glycolysis, lipid synthesis, the pentose phosphate pathway, and de novo pyrimidine synthesis, as well as the inhibition of autophagy through phosphorylation of the kinase complex consisting of unc-51-like kinase 1/mammalian autophagy related gene 13/focal adhesion kinase family-interacting protein of 200 kDa (ULK1/Atg13/FIP200).[4,6] mTORC1 is activated by direct binding to the small G-protein Ras homolog enriched in brain (Rheb).[4] TSC2 is a GTPase–activating

protein (GAP) and acts as an upstream inhibitor of mTORC1.[4]

The growth of hamartomas in TSC requires the inactivation of both alleles of *TSC1* or *TSC2*. This loss of heterozygosity is assumed to be caused by an independent somatic mutation, according to the Knudson's hypothesis.[7] This mosaicism in TSC contributes to the high variations in phenotype and organ manifestations, even in the same mutation in a family.[2]

In the past years, mTORC1 allosteric inhibitors, such as everolimus and rapamycin, have shown to inhibit the growth of hamartomas in TSC in Phase III studies/[8,9] These studies led to the approval of everolimus to treat TSC-associated giant cell astrocytomas and AML. Yet, in about 50% of treated individuals the tumor regression was not satisfactory, suggesting the involvement of other mTORC1-unrelated pathways or the need of more potent mTORC1 inhibitors.

Hippo-YAP signaling pathway

The Hippo signaling pathway is another master regulator of organ size often involved in tumorigenesis. It was first described in Drosophila and contains a set of 4 genes encoding kinases and adaptor proteins which are highly conserved throughout different species.[10-13] The core of the Hippo pathway in Drosophila and mammalian orthologs comprises of the nuclear dbf2-related (NDR) family protein kinase Warts (LATS1/2 in mammals), the WW domain-containing protein Salvador (hww45 in mammals), the Ste20-like protein kinase Hippo (MST1/2 in mammals), and the adaptor protein Mob-as-tumor-suppressor (Mob1 in mammals). A loss of function in either of these genes leads to a tissue overgrowth phenotype characterized by increased cell proliferation and decreased cell death. Merlin (NF2 in mammals) is an upstream tumor suppressor protein that promotes Warts phosphorylation by the Hippo-Salvador kinase complex at the plasma membrane.[14]

The downstream effector of the Hippo pathway is the transcriptional coactivator Yorkie (yes-associated protein – YAP and the transcriptional coactivator with PDZ-binding motif – TAZ in mammals.[10])

In mammals, non-phosphorylated activated YAP/TAZ transfer to the nucleus, bind to TEAD transcription factors to promote expression of several target genes, including the connective tissue growth factor (CTGF), the EGF family member amphiregulin (AREG), Cyr61, and the IAP family member BIRC5, subsequently inducing cell proliferation and suppressing apoptosis.[15] YAP/TAZ are inactivated by phosphorylation by the LATS1/2 kinases. They are sequestered in the cytoplasm via 14-3-3 and ubiquitin-dependent degradation.[16] YAP/TAZ serve as mechanosensors and mechanotransducers and receive inputs from changes in the physical cellular milieu such as cell-cell-contact, polarization of epithelial cells, or ligand-receptor association.[17,18] Subsequently, junctional complexes, polarity complexes, some G protein-coupled receptors (GPCR), or leukemia inhibitory factor (LIF) initiate the signaling cascade and activate the MST kinase, which phosphorylates and activates the LATS1/2 kinases.[17]

Crosstalks between mTORC1 and Hippo-YAP pathways in cell growth

Because activated YAP/TAZ induce the key features of cancer development, i.e., cell proliferation and inhibition of apoptosis, YAP/TAZ are defined as proto-oncogene proteins. There is increasing evidence of cancer related pathways coordinating with Hippo-YAP signaling, such as Wnt, TGFβ, Ras, or Sonic hedgehog.[19]

Crosstalks between mTOR and Hippo-YAP pathways have also been uncovered. One of the YAP target genes, miR-29, inhibits the upstream component PTEN, subsequently activating AKT and Mtor.[20]Recently MST1 was suggested to control the proliferation of glioma cells through binding to AKT, subsequently negatively regulating AKT and mTOR activities.[21]Also, the TSC protein complex has been shown to interact with several upstream components of Hippo pathway, such as Merlin and Crumbs homolog 3 (CRB3).[22] Recently, Liang et al. shed light into another crosstalk between these 2 master regulators of cell growth at the level of autophagy in the disease model of TSC.[23]

A New Mouse Model for PEComas

Mouse models addressing TSC manifestations, such as the Tsc1 and Tsc2 knockout mice, have been previously reported. Both models showed a similar phenotype revealing renal cystadenomas and hepatic hemangiomas.[24] Another kidney-specific mouse model targeting epithelial tubular cells could induce the formation of multiple cysts.[25] Until recently, none of these models could create organ pathologies in the kidney or the lungs resembling the PEComa characteristics of human AML or LAM.

Liang et al.[23] now generated a whole body mosaic *Tsc1* mutant mouse model that develops renal mesenchymal lesions with similarities to human PEComas. In this model, the mice are homozygous for a floxed *Tsc1* allele (Tsc1fl/fl) and also carry a ubiquitously expressed and temporally regulated Cre transgenic allele (CAGG-Cre-ERTM), which, depending on the dose of intraperitoneal tamoxifen injection into the pregnant dam at E15.5, induce the recombination of floxed alleles in a variable percentage (30–80%) of cells in every organ of the newborn pups. With focus on the renal lesions, mutant mice revealed enlarged kidneys with small hemorrhages and polycystic lesions as well as well-defined small nodules adjacent to vessels. These lesions were Tsc1-deficient, showed high levels ribosomal phospho S6 as a marker of mTORC1 activation along with disorganized vascular endothelial cells and pericytes. In addition, they also expressed several mesenchymal markers such as vimentin, H-Caldesmon, α-smooth muscle actin, the proliferation marker Ki67 and the melanocytic marker Pmel, which are pathognomonic features of PEComas.[3] Analysis of human TSC specimens revealed microhamartomas surrounding the AML lesions possibly representing an early stage of AML. Interestingly, these microhamartomas revealed a very similar morphology and immunophenotype to the murine lesions. However, the mouse model did not generate advanced AMLs.

In summary, this new mouse model combines several typical characteristics of human TSC including (1) whole body mosaicism; (2) evolution of disease very early in perinatal stage; and (3) presence

of PEComa-like lesions in kidneys, which makes it a promising model for PEComas.

Up-Regulation of YAP and mTORC1 in TSC

Liang et al.[23] further investigated possible links between different master regulators of cell growth and found evidence for connections between the mTORC1 and Hippo-YAP pathways. These studies revealed elevated mRNA levels for transcriptional targets of the Hippo-YAP pathway, such as CTGF, AREG, and Cyr61. The renal lesions of the mosaic *Tsc1* knockout mice revealed elevated levels of YAP in the tubular epithelial cells lining the cysts and in the spindle-shaped mesenchymal cells of the renal cortex. In these cells, the YAP activity is detected in the cytosol and in a large fraction in the nuclei, suggesting an active transcriptional function in these lesions. In all 5 analyzed human AML samples, YAP and mTORC1 were up-regulated in all cell types of the AML including adipocytes, smooth muscle cells, blood vessels, and microhamartomas. This observation supports the hypothesis that YAP upregulation happens early and seems to be crucial for AML development. In addition, overexpression and nuclear localization of YAP were also observed in TSC-associated pulmonary LAM cells and hepatic PEComa cells (**Fig. 1**).

Treatment with verteporfin, a YAP-TEAD binding inhibitor, markedly rescued the renal phenotype of mosaic *Tsc1* knockout mice. The kidney size virtually normalized with clearly less cystic reorganization. Also a decrease in CTGF expression and cell proliferation as well as an increase in apoptosis was observed. When the mosaic *Tsc1* knockout mouse was crossed with a floxed YAP allele, heterozygous deletion of YAP attenuated the kidney phenotype compared with the mutants. All findings in cell cultures using $Tsc2^{-/-}$ mouse embryonic fibroblasts (MEFs) demonstrated an up-regulation of YAP in terms of an increase of protein levels, transcriptional activity and CTGF expression in comparison to $Tsc2^{+/+}$ MEFs. Remarkably, this occurred in a cell-autonomous way since $Tsc2^{-/-}$ MEFs continue to proliferate in spite of serum starvation conditions. Upon either YAP knockdown or addition of verteporfin the proliferation decreased and apoptosis was induced.

Degradation of YAP is mTORC1 Dependent

Liang et al.[23] further investigated at which step(s) of the pathway the crosstalk between the Hippo-YAP and mTORC1 occurs. No differences were found at the level of YAP transcription or the YAP phosphorylation and degradation step through LATS1/2. Hence, it should be noted that the activity of the Hippo-YAP pathway depends on the density of the cells receiving input from the cellular environment. While in high cell density conditions YAP is inactivated by LATS1/2, in conditions of low cell density, however, an alternative inactivation path of YAP has been hypothesized. Based on the known impairment of lysosomal degradation in TSC1/2 mutant cells, $Tsc2^{-/-}$ MEFs were analyzed under low cell density conditions and in the presence of chloroquine, a pharmacological inhibitor of lysosomal degradation. YAP accumulated in the lysosomal fraction, providing evidence that YAP is a cargo for lysosomal degradation. Under mTORC1 inhibition with the ATP competitive inhibitor Torin, the autophagic degradation of YAP increased. Further, autophagy-deficient $Atg7^{-/-}$ MEFs as well as in vivo deletion of Atg7 in epithelial cells of renal proximal tubules and deletion of Atg7 in 2 liver mouse models (adenoviral-Cre Atg7 f/f and Albumin-Cre transgene Vps15 f/f) all showed elevated accumulation of YAP in lysates. In summary, the mTORC1 and Hippo-YAP pathway crosstalk occurs during autophagy, which is also ATG7 dependent.

Potential Pharmacological Strategies Targeting YAP

The Hippo-YAP pathway provides several potential pharmacological targets for TSC-associated lesions (**Fig. 2**).

Originally used in photodynamic therapy to treat subfoveal choroidal neovascularization of the eye, verteporfin is a substance that interferes with the binding of YAP to TEAD transcription factors.[26] The data of our study clearly demonstrates that verteporfin is able to suppress the abnormal proliferation and survival of TSC1/2 mutant cells both in vitro and in vivo. Yet, further studies are required to outline the efficacy and tolerability of verteporfin in treating TSC-associated lesions.

GPCRs have shown to be positive or negative upstream regulators of the Hippo-YAP pathway depending on the coupled G protein.[27] Substances interacting with GPCRs have therapeutic potential. For example, Angiotensin (AT) II antagonists, which are drugs used against hypertension, also reveal protective effects on renal parenchymal damage and fibrosis due to chronic kidney disease.[28] In a recent study, TSC patients with polycystic kidney disease receiving antihypertensive treatment with AT II antagonists were retrospectively evaluated to have less AML burden than untreated patients. Moreover, AML cells treated with AT II in vitro revealed increased proliferation that could be blocked by the AT II antagonist valsartan.[29] These data suggest preventative therapeutic potential of AT II antagonists to treat AML.

Propranolol, a β-adrenergic antagonist and antihypertensive drug, also interacts with GPCR. In addition, propranolol is also used to treat infantile hemangiomas due to its possible effects on angiogenesis and pericyte functions.[30] Since facial angiofibromas in TSC are characterized as highly vascular lesions as well, propranolol would certainly be an interesting therapeutical candidate to test.

Statins control YAP activity through the mevalonate pathway. Statins are inhibitors of the rate-limiting enzyme HMG CoA reductase, subsequently inhibiting Rho GTPase geranylgeranylation, and finally YAP/TAZ nuclear localization and transcriptional tasks. Simvastatin has been shown to inhibit proliferation and survival of TSC2-null cells in vitro.[31] In a mouse model for LAM,

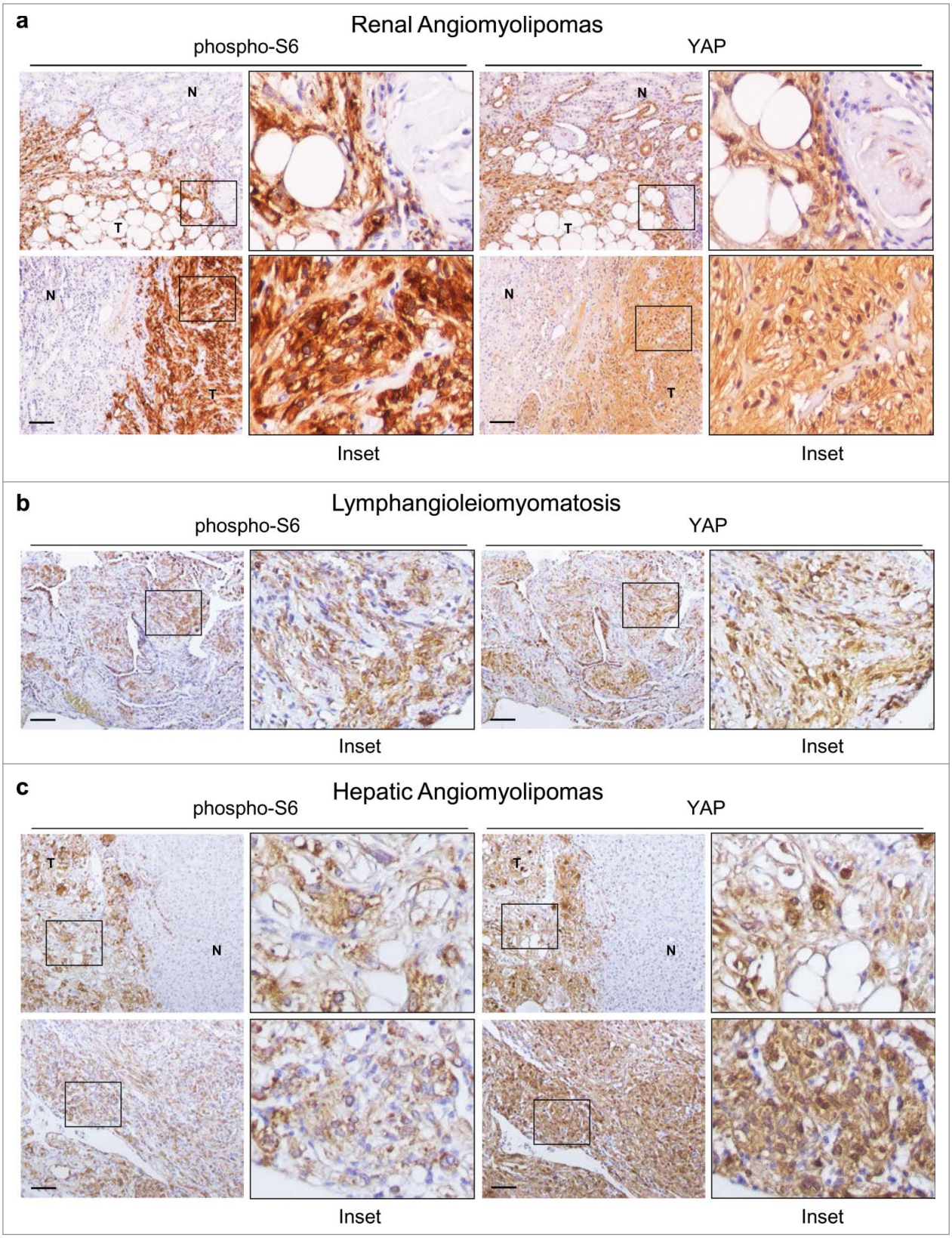

Figure 1. YAP levels correlate with rpS6 phosphorylation in human TSC-related PEComas. Immunohistochemical analysis of rpS6 phosphorylation and YAP expression in human PEComa samples associated with TSC **a**, renal angiomyolipomas; **b**, pulmonary lymphangioleiomyomatosis; **c**, hepatic angiomyolipoma) (N, normal tissue; T, tumor). Scale bar, 100 μm.

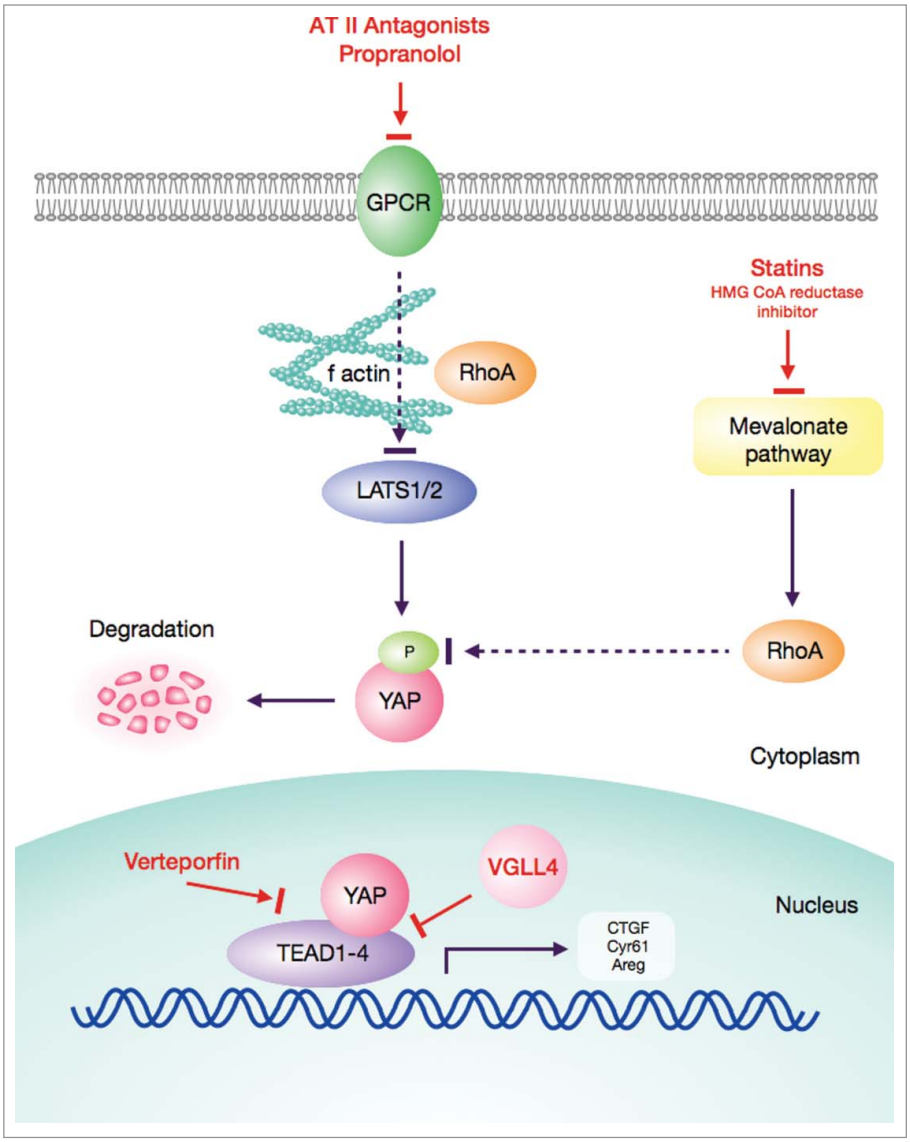

Figure 2. Schematic representation of potential pharmacological targets in the Hippo/YAP pathway for TSC lesions.[26,27,31,33,34] Propranolol and AT II antagonists interact with GPCR Gα and subsequently inhibit LATS1/2 in a RhoA and f actin dependent manner: YAP remain unphosphorylated and tranfer into the nucleus to initiate transcription. Statins inhibit HMG CoA reductase and subsequently the mevalonate pathway: the phosphorylation of YAP is not inhibited by RhoA, which leads to enhanced degradation of YAP. This process is independent from LATS1/2. In the nucleus, verteporfin interferes with the binding of YAP to TEAD1-4, whereas VGLL4 competitively inhibits the binding of YAP to TEAD1-4.

generated by injecting TSC2-null cells into nude mice, simvastatin in combination with rapamycin was more effective than either substance alone in preventing the growth of pulmonary TSC2-null lesions and destruction of lung parenchyma in mutants.[32]

Another interesting approach involves peptides that inhibit the interaction between YAP and TEAD transcription factors. The mammalian vestigial-like proteins (VGLL) bind competitively to TEAD1-4 through their Tondu domains.[33] Peptides mimicking VGLL's Tondu domains have already been generated and were able to inhibit YAP-induced cell proliferation in human primary gastric cancer cells and in a helicobacter pylori-infected mouse model for gastric cancer.[34] This option has not yet been explored in TSC-related models.

Finally, detecting the responsible cargo receptor that transports YAP to autophagosomes would be pathbreaking to the development of further therapeutical approaches.

Given the fact that both the mTORC1 and Hippo-YAP pathways comprise an extended signaling network that controls organ size, it is in fact no surprise to find crosstalks between these 2 pathways in a complex disease like TSC. Liang et al.[23] recently demonstrated the first crucial steps in this very promising field, providing new insights for new potential pharmacological targets. The success of mTOR inhibitors in the therapy of TSC is evident, but not all patients respond sufficiently to this therapeutical strategy. In complex diseases like TSC, with multi-system and multi-organ involvement and with a wide spectrum of clinical manifestations and individual phenotypes, it seems wise to further investigate and address different pharmacological targets for a more customized and multimodal therapy.

Key Messages

- Liang et al.[23] generated a new TSC1 mosaic mouse model that shows promising characteristics as a PEComa model.
- YAP upregulation seems to be crucial in early stages of AML development and perhaps in other PEComa-related lesions associated with TSC.
- The crosstalk between the mTORC1 and Hippo-YAP pathways occurs at the level of autophagy, resulting in accumulation of YAP in the lysosomes when mTORC1 is disinhibited. This process is Atg7 dependent.
- These results provide new perspectives on potential pharmacological strategies by inhibiting the functions of YAP through verteporfin, GPCR, statins and VGLL peptide mimics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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