The tuberous sclerosis complex (TSC) is a genetic disease characterized by growth of hamartomas in different organs including brain, kidney, lung, skin, and heart (Crino et al., 2006). These lesions are a source of morbidity and mortality in patients with TSC, as they may cause intractable epilepsy, autism, developmental delay, renal, and pulmonary failure. Known causes of TSC are loss of function mutations in TSC1 and TSC2 genes. The TSC1 and TSC2 gene products, together with a third subunit TBC1D7, regulate of YAP by mTOR and autophagy reveals a therapeutic target of tuberous sclerosis complex

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Genetic studies have shown that the tuberous sclerosis complex (TSC) 1–TSC2–mammalian target of Rapamycin (mTOR) and the Hippo–Yes-associated protein 1 (YAP) pathways are master regulators of organ size, which are often involved in tumorigenesis. The crosstalk between these signal transduction pathways in coordinating environmental cues, such as nutritional status and mechanical constraints, is crucial for tissue growth. Whether and how mTOR regulates YAP remains elusive. Here we describe a novel mouse model of TSC which develops renal mesenchymal lesions recapitulating human perivascular epithelioid cell tumors (PEComas) from patients with TSC. We identify that YAP is up-regulated by mTOR in mouse and human PEComas. YAP inhibition blunts abnormal proliferation and induces apoptosis of TSC1–TSC2-deficient cells, both in culture and in mosaic Tsc1 mutant mice. We further delineate that YAP accumulation in TSC1/TSC2-deficient cells is due to impaired degradation of the protein by the autophagosome/lysosome system. Thus, the regulation of YAP by mTOR and autophagy is a novel mechanism of growth control, matching YAP activity with nutrient availability under growth-permissive conditions. YAP may serve as a potential therapeutic target for TSC and other diseases with dysregulated mTOR activity.

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form a ternary complex having GTPase activating protein (GAP) activity for the GTPase Rheb and therefore inhibiting mTOR complex 1 (mTORC1) kinase activity (Manning and Cantley, 2003; Dibble et al., 2012). The majority of TSC lesions contain multiple cell types of the mesenchymal lineage, as in the case of angiomyolipomas, lymphangioleiomyomatosis, and angiofibromas. A unique cell type named perivascular epithelioid cell (PEC) is constantly present in mesenchymal TSC lesions, such as angiomyolipomas and lymphangioleiomyomatosis, based on morphological features and the common expression of melanocytic and myogenic markers (Martignoni et al., 2008). Hence, these lesions are now officially classified, along with other tumors, as PEComas. Their cell of origin and the molecular mechanisms underlying their pathogenesis remain poorly defined.

TSC1/2–mTORC1 pathway senses and integrates a diversity of upstream signals, including growth factors, amino acids, oxygen, energy status, and stress, to control growth (Laplante and Sabatini, 2012). By phosphorylating specific effector proteins, mTORC1 up-regulates protein, RNA, DNA, glycogen, and lipid synthesis, as well as energy metabolism, including glycolysis and mitochondrial respiration. In addition, mTORC1 inhibits the initiation of autophagy, which is a eukaryotic catabolic pathway that sequesters cellular organelles and proteins in double-membrane autophagosomes, and delivers and degrades the cargos in lysosomes (He and Klionsky, 2009; Mizushima and Komatsu, 2011; Choi et al., 2013). Allosteric mTORC1 inhibitors, such as rapamycin derivatives, have been shown to be effective to the treatment of TSC lesions, including subependymal giant cell astrocytomas, angiomyolipomas, and lymphangioleiomyomatosis (McCormack et al., 2011; Bisler et al., 2013; Franz et al., 2013). However, the tumors started regrowth when the treatment was discontinued. Moreover, the molecular nature underlying the regulation of abnormal proliferation and survival of TSC1/2-null cells downstream of mTORC1 remains to be fully understood.

In this study, we generated a mosaic Tsc1 mutant mouse model that developed renal mesenchymal lesions showing similarities with human PEComas of TSC patients. To gain insights into the pathogenesis of these lesions, we screened the transcriptional outputs of several signaling pathways that govern proliferation, differentiation and maintenance of multipotency during development, including Notch, Wnt, Hedgehog, and Hippo pathways. Strikingly, we revealed a transcriptional signature of the Hippo pathway in Tsc1 mutant kidneys. Hippo signaling pathway is a critical regulator of organ size during development, first identified in Drosophila and highly conserved in mammals (Pan, 2010; Halder and Johnson, 2011; Tapon and Harvey, 2012; Yu and Guan, 2013). Central to Hippo pathway is a kinase cascade composed of the Ste20-like protein kinase Hippo (MST1/2 in mammals) and the NDR family protein kinase Warts (Lats1/2 in mammals), disruption of which lead to tissue overgrowth and tumorigenesis in Drosophila and mice. Warts (Lats1/2 in mammals) kinase in turn phosphorylates and inactivates transcriptional co-activator Yorkie (YAP/TAZ in mammals), the downstream effector of Hippo pathway. Yorkie and the mammalian orthologues YAP/TAZ have been reported to control proliferation, survival, epithelial to mesenchymal transition and multipotency, which may have oncogenic properties when their activity is not kept in balance (Huang et al., 2005; Camargo et al., 2007; Dong et al., 2007; Zhao et al., 2007, 2008; Brittle et al., 2010; Cordenonsi et al., 2011; Halder et al., 2012; Tumaneng et al., 2012; Calvo et al., 2013). We identified that YAP was up-regulated by mTOR in mouse and human TSC1/2-null cells. Inhibition of YAP, either genetically or pharmacologically, greatly attenuates the abnormal proliferation and induces apoptosis of TSC1/2-null cells, both in vitro and in PEComas of mosaic Tsc1 mutant mice. Finally, we demonstrated that YAP accumulation in TSC1/2-deficient cells was due to impaired degradation of the protein by autophagy in a mTOR–dependent manner. These results suggested YAP as a potential therapeutic target for TSC and other diseases with dysregulated mTOR activity.

RESULTS

Generation of mosaic–Tsc1KO mice that develop multisystem lesions showing similarities with human TSC

Because TSC is a multiorgan mosaic disease with early manifestations in infancy and childhood, we generated mice that were homozygous for a floxed Tsc1 allele (Tsc1<sup>fl/fl</sup>), and carried a ubiquitously expressed and temporally regulated Cre transgenic allele (CAGGCre-ER<sup>TM</sup>; Hayashi and McMahon, 2002; Meikle et al., 2007). Depending on the dose of Tamoxifen (TM), Cre-ER<sup>TM</sup> stochastically causes recombination of floxed alleles in a variable percentage of cells in every tissue (Hayashi and McMahon, 2002), thus potentially targeting cells that give rise to the heterogeneous lesions. To circumvent the embryonic lethality of whole-body Tsc1 deletion (Kobayashi et al., 2001), we injected a relatively low dose of TM (10 mg/kg) i.p. into pregnant dams at embryonic day 15.5 (E15.5). Cre-ER<sup>TM</sup>-positive pups were viable at birth and reached young adulthood at the same Mendelian frequency as the control littermates. However, the majority of Cre-ER<sup>TM</sup>-positive mice did not survive for longer than 10 wk. We therefore focused our analysis on 6-wk-old mice before the onset of lethality. At this age, recombination of the Tsc1 allele was seen in every organ analyzed at 30–80% frequency, depending on the organ (Fig. 1 a). These data indicate that generalized mosaicism for complete Tsc1 loss was achieved in these mice. Henceforth, these mutant mice shall be referred to as mosaic–Tsc1KO.

Histological analysis of mosaic–Tsc1KO mice was performed in multiple tissues, with special attention to anatomical locations that are commonly affected in TSC. The cerebral cortex revealed a disorganization of the cortical layers and the presence of enlarged dysplastic neurons immunoreactive with antibodies against phosphorylated ribosomal protein S6 (pS6), a marker of mTORC1 activity (Fig. 1 b), as previously observed upon brain specific deletion of Tsc1 (Meikle et al., 2007; Zhou et al., 2011). Furthermore, the dermal layer of the skin was thickened whereas the hypodermal fat
and muscular layers of the skin of mosaic-Tsc1 KO mice. Macroscopically, the fur of the mutant mice was fairer than in their littermates (unpublished data). Their kidneys were enlarged and highly susceptible to hemorrhage (Fig. 2 a). Kidney failure was underscored by a threefold increase of the plasma urea levels (Fig. 1 d). In conclusion, the mosaic-Tsc1 KO mice develop a multisystem and early onset disease resembling different aspects of human TSC.

Mosaic-Tsc1 KO mice develop renal mesenchymal lesions recapitulating PEComas associated with human TSC

We investigated whether the stochastic Tsc1 deletion at E15.5 of embryogenesis target cells gives rise to mesenchymal lesions, such as PEComas. We examined the mutant kidneys in detail, as the observed hemorrhages might be caused by vascular lesions, and hemorrhage is an important complication of TSC renal angiomyolipoma (Bissler et al., 2013). Consistent with the phenotype in other Tsc1 mutant animal models (Onda et al., 1999; Zhou et al., 2009), the mosaic-Tsc1 KO kidneys were polycystic, a sign of abnormal proliferation of tubular epithelial cells (Fig. 2 b). However, immunostaining with Vimentin antibody, a marker of mesenchymal cells, revealed several lesions (Fig. 2 b), appearing as small, well defined, fasciculated nodules (Fig. 2 c). Cells within the lesions expressed additional mesenchymal markers, such as H-Caldesmon and α-smooth muscle actin (αSMA; Fig. 3, a and b). These nodules were proliferative, as demonstrated by immunostaining with Ki67 antibodies, and displayed high mTORC1 signaling, as shown by phosphorylation of rpS6 (Fig. 2 c). They were frequently observed near vessels (Fig. 2 c), and/or contained disorganized vascular components highlighted by CD31+ endothelial cells and PDGF receptor β– and αSMA-double positive pericytes (Fig. 3, a and b). Importantly, these mesenchymal lesions also showed immunoreactivity with the HMB45 antibody recognizing the melanocytic marker Pmel (also named gp100), which is a pathognomonic feature of PEComas (Fig. 2 d). Moreover, the mRNA levels of several melanocytic markers, including Mart1, Cathepsin K, Pmel, and Tyrp1, were sharply increased in mutant kidneys (Fig. 2 e). Finally, immunoblot analysis of protein extracts confirmed the induction of both melanocytic (Mart1) and myogenic (αSMA) markers, along with mTORC1 activation in mosaic-Tsc1 KO kidneys (Fig. 2 f). To monitor Cre recombinase activity within the lesions, the mosaic-Tsc1 KO mice were crossed with transgenic mice carrying a flox-stop-flox-lacZ reporter at the Rosa26 locus. As shown in Fig. 3 c, the epithelial cells lining the cysts and the majority of the cells in the mesenchymal lesions underwent Cre-mediated recombination and were therefore Tsc1 deficient.

To directly compare the mesenchymal lesions found in mosaic-Tsc1 KO kidneys with human specimens, we screened samples of renal angiomyolipomas occurring in TSC patients. As shown in Fig. 2 g, the majority of human angiomyolipomas were surrounded by several smaller lesions, so called microhamartomas (or microscopic angiomyolipomas), which shared the common expression of cathepsin K, α-SMA, and...
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Figure 2. Mosaic-Tsc1KO mice develop renal mesenchymal lesions recapitulating PEComas associated with human TSC. Kidneys from ctrl (n = 3) and mosaic-Tsc1KO (n = 3) mice were analyzed at 6 wk of age. (a) Macroscopic photos and representative H&E staining. Arrows indicate hemorrhages. (b–d) Expression of vimentin (b and c), phospho-rpS6, and Ki67 (c) and Hmb45/Pmel (d) was assessed by immunohistochemistry. H&E staining was shown on left in c. Arrows in b indicate vimentin+ microhamartomas in mosaic-Tsc1KO kidneys. For immunohistochemistry analysis, similar stainings were found in three different ctrl/mosaic-Tsc1KO mice (three sections per mouse). (e) Expression of Mart1, Cathepsin K, Pmel, and Tyrp1 mRNA was assessed by qRT-PCR. Mean ± SEM; n = 3 mice/group; *, P < 0.05. (f) Expression of α-SMA, Mart1, p-rpS6, and total rpS6 protein was assessed by Immunoblot. (g) Expression of the indicated proteins in three distinct human angiomyolipoma (AML) samples was assessed by immunohistochemistry. Arrows indicate the microhamartoma. Bar, 100 µm unless otherwise indicated. Insets show higher magnification of the lesions indicated with squares.

Pmel. It is likely that the small lesions represent early developmental stage of angiomyolipomas. Importantly, these microhamartomas exhibited a striking similarity with their murine counterparts in terms of morphology and immunophenotype. In conclusion, mosaic-Tsc1KO mouse kidneys develop renal mesenchymal lesions similar to the PEComas observed in human TSC patients. However, we did not observe any kidney angiomyolipomas in these mice. This may be because of the premature mortality seen in these mice, precluding further lesion development into full blown angiomyolipomas.
transcriptional targets of the Hippo pathway (Tumaneng et al., 2012). This stimulation was blunted by treating the mice every other day for 2 wk with the rapamycin derivative temsirolimus (5 mg/kg), suggesting a tight control of the Hippo pathway by mTOR in Tsc1 mutant kidneys. Next, we examined the levels of YAP, whose function is required for the transcriptional regulation of CTGF, Areg, and Cyr61 downstream of the Hippo pathway. Although YAP mRNA levels were not affected by Tsc1 deletion and mTOR inhibition (Fig. 4a), the protein amount was tightly correlated with mTORC1 activity as assessed by phosphorylated rpS6 levels in kidneys (Fig. 4b). The increase in YAP is up-regulated by mTOR in renal lesions of mosaic-Tsc1KO mice and human angiomyolipoma

The phenotype of mosaic-Tsc1KO mouse kidneys consisted of enlarged organ size, polycystosis, and microscopic PEComas. To gain mechanistic insights, we screened transcriptional outputs of developmental pathways involved in tissue growth, planar cell polarity, and multipotency. These included target genes of Notch, Wnt, Hedgehog, and Hippo pathways (Fig. 4a and not depicted). The mosaic-Tsc1KO kidneys had a two- to eightfold increase in mRNA levels of connective tissue growth factor (CTGF), amphiregulin (Areg), and Cyr61, all known transcriptional targets of the Hippo pathway (Tumaneng et al., 2012). This stimulation was blunted by treating the mice every other day for 2 wk with the rapamycin derivative temsirolimus (5 mg/kg), suggesting a tight control of the Hippo pathway by mTOR in Tsc1 mutant kidneys. Next, we examined the levels of YAP, whose function is required for the transcriptional regulation of CTGF, Areg, and Cyr61 downstream of the Hippo pathway. Whereas YAP mRNA levels were not affected by Tsc1 deletion and mTOR inhibition (Fig. 4a), the protein amount was tightly correlated with mTORC1 activity as assessed by phosphorylated rpS6 levels in kidneys (Fig. 4b). The increase in
Figure 4. YAP is up-regulated by mTOR in renal lesions of mosaic-Tsc1KO mice and human angiomyolipoma. (a and b) Expression of indicated genes was determined by qRT-PCR (a) and immunoblot analysis (b) in kidney samples from Ctrl mice treated with placebo, mosaic-Tsc1KO mice treated with placebo, or temsirolimus. Mice were injected i.p. with vehicle or temsirolimus (5 mg/kg) every other day for 2 wk before sacrifice. Mice were sacrificed at 6 wk of age. Mean ± SEM; n = 3 mice/group; *, P < 0.05, **, P < 0.01. (c) Expression of indicated proteins was analyzed by immunoblot in nuclear and cytoplasmic protein extractions from ctrl and mosaic-Tsc1KO mouse kidney samples. Similar results were found in three different mice/genotype. (d) YAP expression was assessed on kidney sections from ctrl and mosaic-Tsc1KO mice by immunohistochemistry. Arrows indicate cells with YAP nuclear localization. Bars, 100 µm. Similar stainings were found in three different mice/genotype (three sections per each mouse). (e) Expression of indicated proteins was analyzed by immunoblot in control human kidney and angiomyolipoma samples. (f and g) YAP expression was analyzed by immunohistochemistry on human angiomyolipoma samples (N, normal kidney; T, Tumor). Arrows indicate cells with YAP nuclear localization. Bars, 100 µm. Insets show higher magnification of the lesions indicated with squares. Similar results were found in 7/7 different human angiomyolipoma samples.
YAP levels was detected both in cytosol and nuclei (Fig. 4 c). TAZ, the homologue of YAP, was also up-regulated at the protein level in mosaic-Tsc1/2-null cells (Fig. 4 b).

To localize the kidney cell compartment expressing YAP, immunostaining was performed (Fig. 4 d). In adult floxed control littermates, YAP was found in scattered tubular epithelial cells of the kidney cortex. Strikingly, Tsc1 deletion caused a widespread expression of YAP in both tubular epithelial cells lining the cysts and in spindle-shaped mesenchymal cells of the kidney cortex. Notably, in the mosaic-Tsc1/2 KO mouse kidneys, a large fraction of YAP was localized in the nuclei, consistent with an active function as a transcriptional regulator.

To determine whether the activation of the YAP pathway also occurred in human TSC, first we examined YAP, CTGF, and Areg protein levels in TSC angiomyolipomas by immunoblotting (Fig. 4 e). The activation of mTORC1, as assessed by 4EBP1 and rpS6 phosphorylation, and the increased YAP protein were seen in all samples examined. Importantly, the transcriptional targets CTGF and Areg were also expressed at higher levels in angiomyolipomas. Because the levels of TAZ were more variable among the human samples analyzed, we decided to focus our analysis on YAP in this study. Next, we analyzed YAP localization in the different cell types of human angiomyolipomas and in the adjacent kidney structures. As shown in Fig. 4 f, high levels of YAP immunoreactivity were detected in all cell types that could be found in angiomyolipomas, i.e., adipocytes, smooth muscle cells, and blood vessel components. In the adjacent kidney regions, YAP protein was almost undetectable, except for small YAP-positive cysts of epithelial origin lining the lesion. The YAP antibody also stained small human renal micro-hamartomas that closely resembled the mesenchymal lesions observed in mosaic-Tsc1/2 KO mouse kidneys (Fig. 4 g). Finally, we examined other human TSC-related PEComas from different organs. In pulmonary lymphangioleiomyomatosis, hepatic angiomyolipomas, and in renal angiomyolipomas, YAP expression invariably correlated with mTORC1 activity in tumor cells (unpublished data). After screening a total of 10 PEComas, YAP was found to be consistently highly expressed in all the specimens and can be considered a novel marker. Interestingly, we detect YAP expression already at early stages of human PEComa development, suggesting a putative role in the origin of the lesion.

**YAP is required for the abnormal proliferation and survival of Tsc1/2-null cells in vitro**

We then examined whether YAP contributed to the growth phenotype of TSC1/TSC2-deficient cells. We cultured mouse embryonic fibroblasts (MEFs) that were Tsc2−/− and Tsc2+/+ MEFs, which were in p53−/− background (Zhang et al., 2003). YAP protein levels were increased by threefold in the Tsc2-null cells (Fig. 5 a). This effect was also observed in nuclear fractions (not depicted), and was paralleled by increased expression of the YAP target CTGF (Fig. 5, a and g). Thus, the up-regulation of the YAP pathway can also be observed in a cell-autonomous manner upon TSC complex deficiency. This effect could not be ascribed to a relief of Hippo/Lats inhibition of YAP in Tsc2-null cells, because the amount of Ser112-phosphorylated YAP, a site of the Lats1/2 kinases (Zhao et al., 2010), paralleled the increase in total YAP protein in the mutant cells (Fig. 5 a). Tsc2 deletion is known to provide a proliferative advantage over wild-type controls in serum starvation conditions (Zhang et al., 2003). After 24 h of serum starvation, the phosphorylation of the mTORC1 substrate rpS6 and the YAP protein levels were shut off in controls, while remaining high in Tsc2−/− cells (Fig. 5 b). As expected, cell counting over a 3-d period after serum starvation indicated that the number of Tsc2−/− cells steadily increased as opposed to wild type control (Fig. 5 c). Importantly, the proliferation of Tsc2−/− cells was blunted by YAP knockdown (Fig. 5, c and d). Cell viability was also sensitive to YAP levels, as indicated by the drop in cell number at the 72-h time point (Fig. 5 c), and the increase in TUNEL+ apoptotic cells (Fig. 5 e). The effect of YAP knockdown was mimicked by treatment with verteporfin, a compound that was reported to interfere with YAP binding to the TEAD1−4 transcription factors, and accordingly inhibited CTGF mRNA expression in Tsc2−/− cells (Fig. 5, f and g; Liu-Chittenden et al., 2012). YAP levels were also increased after TSC1 and TSC2 knockdown by RNAi in human embryonic kidney 293 (HEK293) cells (Fig. 5 h). The concomitant knockdown of YAP impaired the proliferative advantage of TSC1-depleted HEK293 cells in serum-starved conditions (Fig. 5 i). Collectively, these data demonstrate that YAP activity is, at least partly, required to promote proliferation of TSC complex-deficient cells in vitro.

**Inhibition of YAP pharmacologically or genetically attenuates abnormal proliferation and induces apoptosis of Tsc1-null cells in vivo**

To assess the effects of YAP inhibition in vivo, mosaic-Tsc1/2 KO mice and control mice were treated i.p. with verteporfin (100 mg/kg) every other day for 10 d (Liu-Chittenden et al., 2012). The YAP inhibitor reduced the kidney/body weight ratio by 50% (Fig. 6 a), and attenuated overall kidney size and polycystosis in mutant mice without affecting control kidneys (Fig. 6 b). These effects were concomitant to a reduction in CTGF expression (Fig. 6 c). The proliferation of the mesenchymal lesions was severely affected by verteporfin treatment, as indicated by Ki67 staining of mosaic-Tsc1/2 KO kidneys 2255 (mosaic-Tsc1/2/KO;Yap−/−) mice. The offspring mice were injected with TM at postnatal day 2 (P2), to generate mosaic-Tsc1/2 KO carrying a mutant allele of Yap (mosaic-Tsc1/2/KO;Yap−/−). The heterozygous deletion of Yap was sufficient to reduce YAP protein levels in mosaic-Tsc1/2 KO mice (Fig. 7 c). This was accompanied by a sharp attenuation of the kidney phenotype as compared with mosaic-Tsc1/2 KO mice, as assessed by the kidney to body weight ratio (Fig. 7 a), kidney size and polycystosis (Fig. 7 b), and mesenchymal cell proliferation (Fig. 7, d and e). The
Figure 5. YAP is required for the abnormal proliferation and survival of TSC1/2-null cells in vitro. (a and b) Expression of indicated proteins was analyzed by immunoblot in Tsc2+/+ and Tsc2−/− MEFs (these MEFs were generated in p53−/− background) cultured with or without serum for 24 h. Similar results were observed in three independent experiments. (c–e) Tsc2+/+ and Tsc2−/− MEFs transfected with siCtrl or siYAP were cultured in serum starvation conditions for 72 h. (c) Cell number was counted in different groups. Three independent experiments were performed and analyzed (Mean ± SEM; **, P < 0.01). (d) Expression of YAP protein was analyzed by immunoblot at the 24-h time point. Similar results were observed in three independent experiments. (e) Apoptotic cells were identified by TUNEL assay at the 48-h time point. The percentage of TUNEL+ cells was determined by counting TUNEL+ nuclei among at least 500 cells in distinct fields of each experiment. Three independent experiments were performed and analyzed. Mean ± SEM; *, P < 0.05, **, P < 0.01. (f and g) Tsc2+/+ and Tsc2−/− MEFs were cultured under serum starvation conditions and treated with DMSO or Verteporfin for 72 h. (f) Cell number was counted. Three independent experiments were performed and analyzed. Mean ± SEM; *, P < 0.05, **, P < 0.01. (g) Expression of Ctgf mRNA was assessed by qRT-PCR in different treatments. Three independent experiments were performed and analyzed. Mean ± SEM; *, P < 0.05, **, P < 0.01. (h and i) Percentage of BrdU+ HEK293 cells with indicated treatments under serum deprivation conditions for 24 h was assessed by 3-h BrdU pulse-labeling before harvest. Three independent experiments were performed and analyzed. Mean ± SEM; **, P < 0.01, ***, P < 0.001.
Figure 6. Verteporfin treatment inhibits proliferation and induces apoptosis of Tsc1-null cells in vivo. Mice were administered i.p. with vehicle or verteporfin at a dose of 100 mg/kg every other day for 10 d before sacrifice. Mice were sacrificed at 6 wk of age. Three independent experiments were performed and mice in different treatments were pooled for analysis. (a) Kidney/body weight ratio of Ctrl and mosaic-Tsc1 KO mice treated with placebo or Verteporfin. Mean ± SEM; *, P < 0.05, ***, P < 0.001. (b) Representative pictures of hematoxylin and eosin staining of kidney sections from Ctrl and mosaic-Tsc1 KO mice treated with placebo or Verteporfin. Bar, 1 mm. (c) Expression of indicated mRNAs was analyzed by qRT-PCR in kidney samples from Ctrl and mosaic-Tsc1 KO mice treated with placebo or Verteporfin. Mean ± SEM; *, P < 0.05, **, P < 0.01. (d) Percentage of Ki67 and αSMA double-positive cells in α-SMA+ mesenchymal lesions in the indicated kidneys. Immunofluorescence staining and counting were performed on three sagittal sections from different kidney regions for each mouse. Representative images are shown. Mean ± SEM; n = 3 mice/group; **, P < 0.01; Bar, 100 µm. Insets show the lesions indicated with arrows. (e) In situ apoptotic cells were assessed by TUNEL assay on the kidney sections from Ctrl and mosaic-Tsc1 KO mice treated with placebo or verteporfin. The number of TUNEL+ cells were counted in ten distinct fields of each section. Three sections were analyzed per each mouse. Representative images are shown. Arrows indicate TUNEL+ epithelial cells and arrowheads show TUNEL+ mesenchymal cells. Insets show the lesions indicated with arrows. Mean ± SEM; n = 3 mice/group; *, P < 0.05; Bar 100 µm.
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residual lesions observed in mosaic-Tsc1KO;Yap+/− kidneys displayed rpS6 phosphorylation, suggesting efficient recombination (unpublished data). Whole-body heterozygous deletion of Yap (Yap+/−) did not inhibit kidney growth and proliferation (Fig. 7, f–h), indicating that the reduction of YAP gene dosage was particularly effective in a background of Tsc1 loss of function. Thus, strategies targeting YAP activity might be beneficial in treating TSC-related kidney overgrowth and PEComa formation.

YAP is accumulated in TSC-deficient cells due to blockage of autophagy in a mTOR-dependent manner
YAP is under the tight control of a variety of upstream regulators (Tumaneng et al., 2012). To gain mechanistic insights

Figure 7. Heterozygous deletion of Yap compromises the proliferation of renal mesenchymal lesions in mosaic-Tsc1KO mice. Three independent tamoxifen injection experiments were performed and female mice of indicated genotypes were pooled for analysis. (a) Kidney/body weight ratio of the indicated mice sacrificed at 6 wk of age (Ctrl, n = 4; mosaic-Tsc1KO, n = 5; mosaic-Tsc1KO;Yap+/−, n = 3). (b) Representative pictures of hematoxylin and eosin staining of kidney sections from the indicated mice. (c) Expression of indicated proteins was analyzed by immunoblot in mouse kidney samples from the indicated genotypes. (d) Percentage of Ki67 and α-SMA double-positive cells in α-SMA+ kidney lesions from mice of the indicated genotypes. Immunofluorescence staining and counting were performed on three sagittal sections from different kidney regions for each mouse. Bars, 100 µm. Mean ± SEM, **, P < 0.01. (e) Percentage of BrdU and Vimentin double-positive cells in Vimentin+ mesenchymal lesions in the indicated kidneys. BrdU (50 mg/kg) was injected i.p. 24 h before sacrifice. Immunofluorescence staining and counting were performed on three sagittal sections from different kidney regions for each mouse. Representative images are shown (Ctrl, n = 4; mosaic-Tsc1KO, n = 5; mosaic-Tsc1KO;Yap+/−, n = 3). Insets show the lesions indicated with arrows. Bars, 100 µm. Mean ± SEM, **, P < 0.01; Bar, 100 µm. (f) Kidney/body weight ratio of Yap+/+ (n = 3) and Yap+/− (n = 3) mice. (g) Hematoxylin and eosin staining of kidney sections from Yap+/+ and Yap+/− mice. Bar, 1 mm. (h) Percentage of Ki67+ cells per mm2 in kidney sections from Yap+/+ and Yap+/− mice (n = 3). The number of Ki67+ cells were counted in 10 distinct fields per each section. Two sections were analyzed per each mouse.
residues by the Lats1/2 kinases that causes cytosolic retention and promotes protein degradation by the proteasome (Huang et al., 2005; Zhao et al., 2010). However, no major alteration in Lats1/2 kinase activity was detected in Tsc2−/− cells and kidney tissues, as indicated by the Ser-112 YAP phosphorylation (Fig. 5 a and not depicted). Accordingly, 8-h treatment with the proteasome inhibitor MG132, while leading to a significant accumulation of polyubiquitinated proteins, did not affect YAP levels in either Tsc2−/− or control cells in growing conditions (unpublished data). Intriguingly, the YAP...

Figure 8. YAP is accumulated in TSC deficient cells due to blockage of autophagy. (a) Expression of indicated proteins was analyzed by immunoblot in Tsc2+/+ and Tsc2−/− MEFs treated with or without chloroquine (CQ) for 8 h. Similar results were observed in three independent experiments. (b, c) Localization of YAP and Lamp2 was analyzed by immunofluorescence in Tsc2+/+ and Tsc2−/− MEFs treated with or without CQ (20 µM) for 24 h. Insets show part of the cells indicated with squares. 50 cells were counted for yellow punctas in each group. Three independent experiments were performed and analyzed (mean ± SEM; *, P < 0.05, **, P < 0.01; Bar 10 µm). (d and e) Expression of indicated proteins was analyzed by immunoblot in Tsc2+/+, Tsc2−/− and Atg7+/+, Atg7−/− MEFs culturing with or without Torin1 for 24 h. Similar results were observed in three independent experiments. (f) Immunoblot analysis of YAP and LC3 in HeLa cells with indicated treatments. YAP levels were normalized with β-actin. Similar results were observed in three independent experiments. (g) Immunoprecipitation analysis of interacting proteins of flag-tagged YAP in the indicated culturing conditions for 0.5 h. Similar results were observed in three independent experiments. (h) Expression of indicated proteins was analyzed by immunoblot in ctrl (n = 3) and proximal tubule–specific Atg7 knockout mouse kidney samples (n = 3).
phosphorylation by Lats1/2 kinases has been reported to induce a rapid degradation of YAP in high- but not low-density culture conditions (Zhao et al., 2010), the latter pertaining to the experimental setup used in this study. Thus, in growing cells with no contact inhibition and with high mTOR activity, YAP levels are controlled by an alternative mechanism.

**TSC1/2** mutant cells have impaired autophagic flux (Parkhutik et al., 2011; Dibble et al., 2012), as indicated by the reduced conversion of LC3-I to the cleaved and lipidated form LC3-II (Fig. 8 c). We wondered whether an alteration in the lysosomal route of degradation might explain the YAP accumulation in Tsc2−/− MEFs. The lysosomotropic drug and autophagy inhibitor chloroquine (CQ) led to the accumulation of LC3-II that normally undergoes lysosomal turnover. This effect was paralleled by the accumulation of YAP protein in control MEFs, whereas the amount of YAP was not further increased by chloroquine in Tsc2−/− cells (Fig. 8 a). In MEFs, YAP protein was localized in cytosol, nucleus, and cytolytic vesicles, a fraction of which was also positive for the lysosomal marker Lamp2 (Fig. 8 b). Impairment of lysosomal degradation by chloroquine treatment increased the amount of YAP detectable in the lysosomes, indicating that YAP is a cargo for lysosomal degradation. In Tsc2−/− cells, the amount of YAP localized in the lysosomes was reduced as compared with control cells in both untreated and chloroquine-treated conditions (Fig. 8, b and c). Notably, interactome studies in *Drosophila* cells revealed a strong association of the Hippo pathway with endocytosis and vesicle-trafficking complexes (Kwon et al., 2013).

To analyze the crosstalk between mTOR kinase activity and YAP, we used torin1, a potent ATP competitive inhibitor of mTOR (Thoreen et al., 2009), as assessed by the dephosphorylation of the mTOR substrate 4EBP1 (Fig. 8, d and e). Torin1 treatment of Tsc2−/− MEFs promoted LC3 lipidation, indicating an induction of the autophagic flux, which was accompanied by a reduction of YAP levels (Fig. 8 d). The effect of Torin1 was also observed in autophagy–competent control MEFs (Fig. 8, d and e). However, in autophagy deficient Atg7−/− MEFs, mTOR inhibition was largely unable to induce YAP degradation (Fig. 8 e). Similar to Torin1 treatment, nutrient starvation also promoted YAP degradation concomitantly with the induction of autophagy in a chloroquine-sensitive manner (Fig. 8 f). Of note, ectopic Flag-YAP co-immunoprecipitated with the autophagy cargo receptor p62 and with the upstream kinase Lats1 in basal conditions (Fig. 8 g). Upon autophagy induction by nutrient starvation together with chloroquine treatment to induce a late autophagy block, the interaction with p62 and Lats1 was, respectively, up- and down-regulated. Finally, the in vivo deletion of Atg7 in epithelial cells of kidney proximal tubules was sufficient to cause the accumulation of YAP in lysates from the whole mouse kidneys (Fig. 8 h). In conclusion, mTOR regulates YAP degradation through autophagy, which is dependent on ATG7. Our data favor a model in which the control of YAP lysosomal degradation by mTOR matches YAP activity with nutrient availability in growth permissive conditions.

**DISCUSSION**

We delineate a crosstalk between two master regulators of growth, mTOR and YAP. Mechanistically, the impairment of autophagy, a result of mTOR hyperactivity in TSC1/2 mutant cells, leads to the accumulation of the oncogene YAP. The consequence is the establishment of the growth-promoting YAP transcriptional program, thus defining a novel protumoral target downstream of mTOR. By establishing a novel multisystem mouse model of TSC and by screening human TSC samples, we reveal that this molecular mechanism is required for the tumorigenesis of TSC-related kidney lesions and in PEComas.

Rapamycin derivatives (rapalogs) are approved for the treatment of TSC lesions, including subependymal giant cell astrocytomas, angiomyolipomas, and lymphangiomyomatosis (Bissler et al., 2013; Franz et al., 2013). In many cases, these allosteric inhibitors of mTOR constituted a major improvement in the management of the disease. However, long-term administration of rapalogs may have undesirable side effects, including dislipidemia, hyperglycemia, stomatitis, skin rash, lung fibrosis, leukopenia, and anemia. Our data suggest that preclinical trials targeting the YAP pathway, alone or in combination with rapalogs, should be considered for TSC, as they may limit the adverse effects. Importantly, a large variety of G protein–coupled receptors (GPCRs) have been recently identified as positive and negative up-stream regulators of the Hippo–YAP pathway (Yu et al., 2012). Future studies should define the GPCR present in the different TSC lesions and test their activity on YAP. Pharmacological agents acting on the angiotensin and adrenergic GPCRs are well tolerated and have interesting actions that should be considered in this scenario. Angiotensin antagonists ameliorate renal injury in conditions of hypertension and fibrosis (Remuzzi et al., 2002). Propranolol, a β adrenergic antagonist, has striking efficacy against infantile hemangioma, possibly due to inhibition of angiogenesis and pericyte functions (Léauté-Labrèze et al., 2008). Whether these agents act on YAP and are effective against TSC PEComas are remarkable possibilities to explore.

**MATERIALS AND METHODS**

**Mice.** *Tsc1fl/fl* and *CAGGCre-ER* mice were obtained from The Jackson Laboratory. *Yapfl/fl* mice were previously described (Xin et al., 2011). R26-floxstop-LacZ mice were obtained from The Jackson Laboratory. *Yapfl/+* and *Yapfl/fl* mice were shared by U. Apte. Kidney-proximal tubule-specific Atg7 knockout mouse samples, using a modified PEPCK-driven Cre, were provided by Z. Dong. Kidney-proximal tubule-specific Atg7 knockout mouse samples, using a modified PEPCK-driven Cre, were provided by Z. Dong. All animal studies were approved by the Direction Départementale des Services Vétérinaires (Prefecture de Police, Paris, France; authorization number 75–1313) and the ethical committee of Paris Descartes University. Mice were housed in a 12-h light/dark cycle and fed a standard chow. All animal used in this study were on mixed genetic background (C57BL/6J, BALB/cJ, or 129/SvJae), with control mice being Cre-negative littermates of mutant mice. Female mice were used unless otherwise indicated. Tamoxifen (Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich; 10 mg/ml) was injected i.p. into pregnant females with a dose of 10 mg/kg at E15.5 (the day of vaginal plug observed was considered as E0.5). 1 mg/ml tamoxifen prepared in corn oil was used to inject subcutaneously the pups (10 mg/kg) at postnatal day 2 to generate mosaic-*Tsc2/KO; Yapfl+/*.*

Blood was collected using tubes with EDTA (RABE LABORTECHNIK).
centrifuged at 500 g for 3 min. Plasma was isolated and analyzed for urea levels with an AU400 instrument (Olympus).

**Human TSC samples.** Human samples were retrieved from the files of the Department of Pathology and Diagnostics of the University of Verona and from Institut Bergonie of Bordeaux. The frozen samples included five angio-myolipoma samples and two resected kidney samples as consequences of TSC-unrelated diseases, pyelonephritis, or oncocytoma. Paraffin-embedded human PEComa samples included seven angiomylipomas, two lymphangio- leiomyomatosis, and one hepatic angiomylipoma. Human studies were ethically approved by the Comité de Protection des Personnes (CPP Ile de France III) with the reference file #AT144.

**Cell culture and reagents.** HEK293, Tlx2-/-p53-/- and Tlx2-/-p53-/- MEFs were cultured in DMEM with 10% FBS, 1% penicillin/streptomycin. Atg7+/+ and Atg7-/- MEFs were provided by M. Komatsu (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; Komatsu et al., 2005) and cultured in DMEM with 10% FBS, 1% nonessential amino acid (Invitrogen), 1% penicillin/streptomycin. All cells were cultured at 37°C and 5% CO2. 5 x 10⁵ cells were seeded per well in a 6-well plate and cultured over-night before treatment with MG132 (Tocris), Chloroquine (Sigma-Aldrich), or Torin1 (Tocris). For RNA experiments, 2 x 10⁶ HEK293 cells were seeded per well in a 6-well plate, cultured overnight, transfected with siRNAs targeting TSC1, TSC2, and ctrl siRNA (QIAGEN) using DharmaFECT1 Transfection Reagent. For nutrient starvation, cells were cultured in EBSS (Invitrogen) for half an hour or 1 h. Flag-YAP plasmid was obtained from Addgene (#19045).

**Immunohistochemistry and immunofluorescence.** Mouse tissues were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 4 µm. Sections were deparaffinized and hydrated before being incubated in citrate buffer (pH6) sub-boiling for 10 min. Sections were blocked in 3% Goat normal serum, 1% BSA, and 0.1% Triton X-100 (Millipore), fixed in 100% cold methanol, blocked in 3% goat normal serum, 1% BSA, and 0.1% Triton X-100/PBS, and incubated with the following anti bodies: anti-YAP (#sc-101199; Santa Cruz Biotechnology, Inc.) and anti-BSA, and 0.1% Triton X-100/PBS, and incubated with the following anti bodies overnight at 4°C: anti–phospho-rpS6 (#2217), anti-phospho- rpS6(Ser240/244; #2232), anti-p4EBP1(Thr37/46; #2855), anti-p-YAP (Ser127; #4911), anti-Lats1 (#3477), anti-TSC2 (#4308), and anti-Ub (#9396; Cell Signaling Technology). Nuclear and cytoplasmic protein extractions were performed with NE-PER kit (#78835; Thermo Fisher Scientific). Flag-tagged YAP protein complex was immunoprecipitated from cell lysates with anti-Flag antibody (#F1804; Sigma-Aldrich) and protein G-Sepharose (#17-0618-01; GE Healthcare). Immunocomplexes were washed in cell lysis buffer three times and analyzed by immunoblotting.

**BrdU labeling.** Cells were incubated with 10 µg/ml BrdU (Sigma-Aldrich) for 3 h before harvest. Cells were washed, fixed in 4% paraformaldehyde for 15 min, incubated in citrate buffer (pH 6.0) at sub-boiling temperature, and detected with an anti-BrdU antibody (#11710376001; Roche). The percentage of BrdU incorporation was determined by counting BrdU nuclei among at least 200 cells in distinct fields. For in vivo studies, mice were injected i.p. with BrdU (50 mg/kg) for 24 h before sacrifice.

**TUNEL assay.** TUNEL assays were performed using In Situ Cell Death Detection kit (Roche) according to the manufacturer’s instructions.

**Tensiriolimus and verteporfin treatments.** Tensiriolimus (LC Laboratories) was dissolved in 100% ethanol (25 mg/ml), aliquoted, and stored at −20°C. 1 µg/ml working solution was prepared in 5% Tween-80, 5% PEG400, and 86% PBS before injection. Mice were injected i.p. with vehicle or tensiriolimus (5 mg/kg) every other day for 2 wk. Verteporfin (Selleckchem) was administered in DMSO (100 mg/ml), aliquoted, and stored at −80°C. Working solution was prepared at 10 mg/ml in PBS freshly before use. Mice were administered i.p. with vehicle or verteporfin solution at a dose of 100 mg/kg every other day for 10 d. In vitro, cells were treated with DMSO or verteporfin with a dose of 4 µM.

**Quantitative RT-PCR.** Total RNA was extracted from tissue or cells using an RNAeasy Mini kit (QIAGEN), following the manufacturer’s instructions. Single-strand cDNA was synthesized from 1 µg of total RNA with SuperScript II (Invitrogen) and random hexamer primers. Real-time quantitative PCR was performed on MX3005P instrument (Agilent) using a Brilliant III SYBR Green QPCR Master Mix (Agilent). Relative amounts of the indicated mRNAs were determined by means of the 2^−ΔΔCT method, normalizing with β-actin levels. The primer sequences used were mouse β-actin, 5'-GTGACGTGGTACATCCGTAAGA-3' and 5'-GCCGGA-CTCAGTGACTACCT-3'; mouse Mart1, 5'-TCCCAAGAAAGGCCCCAGA-AGC-3' and 5'-ACGGTGGGAACCCAGGGCCT-3'; mouse Cathepsin K, 5'-AAAGGTTGTCAGAAGATGACGGGAC-3' and 5'-TCTTCAAGTATTCCGCCCCGGTC-3'; mouse P53, 5'-TCGAGTGACCTCCGATCTTC-3' and 5'-GGGTTTGCCCTTTTT-3'; mouse Gli2, 5'-CTCAGCTGGTTCTTGCTGAGAT-3' and 5'-GGGTTTGCCTCGTCTGC-3'; mouse Gli1, 5'-TGCTTTGTTGCTGCTTCGCT-3' and 5'-GGGTTTTGTTTCTGCTCTG-3'; mouse Lamin A/C, 5'-GGGGTTCCCTTCAACCTG-3' and 5'-GGGGTTTCTGCTCTGC-3'; mouse YAP, 5'-GTTTCTCTCTTTTGATCT-3' and 5'-GGGGTTTCTGCTCTGC-3'; mouse GHI, 5'-TGAGTGTCTTCCGGAGCCAGCA-3' and 5'-GGGGTTTCTGCTCTGC-3'; mouse TGFβ, 5'-CGGTTTTCTGCTTCGCT-3' and 5'-GGGGTTTCTGCTCTGC-3'; mouse G-CSF, 5'-GAGGTTGCTGCTTCGCT-3' and 5'-GGGGTTTCTGCTCTGC-3'; mouse BMP2, 5'-GGGGTTTCTGCTCTGC-3' and 5'-GGGGTTTCTGCTCTGC-3'; mouse Bmp7, 5'-GGGGTTTCTGCTCTGC-3' and 5'-GGGGTTTCTGCTCTGC-3'; mouse BMP1, 5'-GGGGTTTCTGCTCTGC-3' and 5'-GGGGTTTCTGCTCTGC-3'.

**Immunoblotting and immunoprecipitation.** Cells and tissues were lysed in cell lysis buffer (#8903; Cell Signaling Technology) with complete protease inhibitor cocktail and phosphoSTOP phosphate inhibitor cocktail (Roche). Protein extracts were resolved by SDS-PAGE, transferred to PVDF membranes and incubated with the following primary antibodies: anti-α SMA (#A2547; Sigma-Aldrich); anti-Mart1 (#sc-20032; Santa Cruz Biotechnology, Inc.); anti-GAPDH (#ab9485); anti-CTGF (#ab6992; Abcam); anti-AREG (#sc-7450; Invitrogen); anti-LC3 (#20231–100); anti-p62/SQSTM (#H00008878–M01; Abnova); anti-β-actin (#A5441); anti-α-tubulin (#F5168; Sigma-Aldrich); anti-YAP (#4912); anti-YAP/TAZ (#8418); anti-Lamin A/C (#2032); anti-p56 (#2217); anti-phospho-rpS6(Ser240/244; #3845); anti-p-EP1(Thr37/46; #2855); anti-p-YAP (Ser127; #4911); anti-Lats1 (#3477); anti-TSC2 (#4308); and anti-Ub (#9396; Cell Signaling Technology). Nuclear and cytoplasmic protein extractions were performed with NE-PER kit (#78835; Thermo Fisher Scientific). Flag-tagged YAP protein complex was immunoprecipitated from cell lysates with anti-Flag antibody (#F1804; Sigma-Aldrich) and protein G-Sepharose (#17-0618-01; GE Healthcare).

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Statistical analysis. Data are shown as means ± SEM. Analysis was performed by unpaired, two-tailed, Student’s t test unless otherwise indicated. P < 0.05 was considered statistically significant.

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REFERENCES


Bissler, J.J., J.C. Kingswood, E. Radzikowska, B.A. Zonenbe...


