



Rapamycin requires AMPK activity and p27 expression for promoting autophagy-dependent *Tsc2*-null cell survival



Tania Campos^a, Javiera Ziehe^{a,1}, Francisco Fuentes-Villalobos^a, Orlando Riquelme^a, Daniela Peña^a, Rodrigo Troncoso^{b,c}, Sergio Lavandero^{b,d}, Violeta Morin^a, Roxana Pincheira^a, Ariel F. Castro^{a,*}

^a Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile

^b Advanced Center for Chronic Disease (ACCDiS), Facultad de Ciencias Químicas y Farmacéuticas & Facultad de Medicina, Universidad de Chile, Santiago, Chile

^c Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, Santiago, Chile

^d Department of Internal Medicine (Cardiology Division), University of Texas Southwestern Medical Center, Dallas, TX, USA

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ABSTRACT

Tuberous sclerosis complex (TSC) disease results from inactivation of the *TSC1* or *TSC2* gene, and is characterized by benign tumors in several organs. Because TSC tumorigenesis correlates with hyperactivation of mTORC1, current therapies focus on mTORC1 inhibition with rapamycin or its analogs. Rapamycin-induced tumor shrinkage has been reported, but tumor recurrence occurs on withdrawal from rapamycin. Autophagy has been associated with development of TSC tumors and with tumor cell survival during rapamycin treatment. mTORC1 and AMPK directly inhibit and activate autophagy, respectively. AMPK is hyperactivated in TSC cells and tumors, and drives cytoplasmic sequestration of the cell-cycle inhibitor p27KIP1 (p27). Whether AMPK and p27 are involved in rapamycin-induced autophagy and survival of TSC cells remain unexplored. Here, we show that inhibition of AMPK by compound C or by shRNA-mediated depletion of LKB1 reduces activation of autophagy by rapamycin in *Tsc2*-null cells. Similarly, shRNA-mediated depletion of p27 inhibited rapamycin-induced autophagy. In support of p27 lying downstream of AMPK on the activation of autophagy in *Tsc2*-null cells, a p27 mutant that preferentially localizes in the cytosol recovered the effect of rapamycin on autophagy in both p27- and LKB1-depleted cells, but a nuclear p27 mutant was inactive. Finally, we show that p27-dependent activation of autophagy is involved in *Tsc2*-null cell survival under rapamycin treatment. These results indicate that an AMPK/p27 axis is promoting a survival mechanism that could explain in part the relapse of TSC tumors treated with rapamycin, exposing new avenues for designing more efficient treatments for TSC patients.

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1. Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder that results from inactivating mutations in the *TSC1* or *TSC2* tumor suppressor gene. TSC disease is characterized by the development of benign tumors, most typically found in brain, kidney, heart, lung, skin and eye

Abbreviations: AMPK, 5' AMP-activated protein kinase; Atg13, autophagy-related protein 13; CDK, cyclin-dependent kinase; DAPI, 4',6'-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle's medium; DMSO, dimethyl sulfoxide; FDA, US Food and Drug Administration; GFP, green fluorescent protein; LAM, lymphangioleiomyomatosis; LC3, microtubule-associated protein light chain 3; LKB1, liver kinase B1; MEK1/2, mitogen-activated protein kinase kinase 1/2; mTORC1, mammalian/mechanistic target of rapamycin complex 1; NADPH, nicotinamide adenine dinucleotide phosphate; p27, cell-cycle inhibitor p27Kip1; p27CT, carboxy-terminus of p27; p27NT, amino-terminus of p27; PPP, pentose phosphate pathway; S6, ribosomal protein S6; TSC, tuberous sclerosis complex.

* Corresponding author at: Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universidad de Concepción, Barrio Universitario s/n, Concepción 4030000, región Bio Bio, Chile.

E-mail address: arcastro@udec.cl (A.F. Castro).

¹ Present address: Faculty of Life Sciences, University of Manchester, Manchester, UK.

[1,2]. A manifestation of TSC in the lung is lymphangioleiomyomatosis (LAM), a disease that also occurs by sporadic mutations in the *TSC* genes and is known as sporadic LAM [3]. The inactivation of the *TSC* genes leads to hyperactivation of the mechanistic target of rapamycin complex 1 (mTORC1) signaling, which is mainly responsible for the abnormal cellular behavior resulting in tumor development [4–6]. Thus, therapies for TSC and sporadic LAM have focused on inhibition of the mTORC1 signaling, being rapamycin and its analogs the most commonly used drugs. However, success of these therapies has been modest, and relapse occurs once treatment is discontinued [7,8].

Macroautophagy (hereafter autophagy) is a catabolic cellular process that has been recently associated with the development of TSC tumors and with survival of tumor cells to rapamycin treatment [9]. Autophagy is manifested by formation of a double membrane vesicle containing cytoplasmic components (autophagosome) that fuses with lysosomes (autolysosome) to degrade its content [10]. This process can suppress tumor initiation and progression by removing aggregated proteins and damaged organelles, and by limiting chromosomal instability [10,11]. Accordingly, mouse models with lost or lower expression of autophagy-related genes showed an increased incidence of cancer or

a higher susceptibility to chemical carcinogenesis. However, like TSC, acute activation of autophagy can support tumorigenesis [11,12]. Tumor cells activate autophagy to tolerate metabolic stress that arises in areas of reduced or intermittent blood supply [13]. This stress tolerance supports cell survival by maintaining sources of energy for tumor growth [12,14]. In addition, higher levels of autophagy are observed in response to chemotherapy, indicating that autophagy is also an adaptive response that enables tumor cells resistance to therapies [15].

Considering the pro-survival role of autophagy in response to metabolic and chemical stress, it is expected that drug-inhibition of autophagy in conjunction with other chemotherapies will be more effective to eliminate tumor cells. This notion has been tested in several cancer models, supporting the use of combined therapeutic approaches with autophagy drug inhibitors [16]. In TSC, inhibition of autophagy using chloroquine synergized with rapamycin to inhibit the survival of TSC cells, and the growth of tumors in a xenograft and a TSC mouse models [9]. Thus, a combined therapeutic approach with rapamycin and chloroquine could be more beneficial for TSC and sporadic LAM patients than rapamycin alone. Since chloroquine is already a FDA approved drug used to treat malaria, its clinical use in TSC and LAM patients could be expeditious. However, considering the homeostatic role of autophagy that prevents tumor initiation, it is still questionable a therapeutic window for safe use of autophagy drug inhibitors. Thus, it is important to identify the mechanism that drives the activation of autophagy in the context of the TSC genes inactivation, because it could provide more specific therapeutic approaches.

The mTORC1 signaling negatively regulates autophagy through phosphorylation of Ulk1/2 [17]. Accordingly, the hyperactivation of mTORC1 in TSC cells is correlated with low basal levels of autophagy, but sufficient to support tumorigenesis [9]. Opposite to mTORC1, AMP-activated kinase (AMPK) directly activates autophagy by phosphorylation of other residues of Ulk1 [18]. AMPK could also indirectly release mTORC1-dependent inhibition of autophagy through phosphorylation and activation of TSC2 [19], but this mechanism is absent in TSC cells. It is interesting that AMPK is also hyperactivated in TSC cells and tumors, and is driving cytoplasmic sequestration of the cell-cycle inhibitor p27KIP (p27) [20]. In other cells, AMPK-dependent stabilization of p27 is necessary for the activation of autophagy under metabolic stress conditions [21]. Considering these findings, we sought to investigate whether AMPK and p27 are involved in rapamycin-induced autophagy and survival of TSC cells under serum deprivation. Here, our results showed that AMPK activation and p27 expression are necessary for activation of autophagy during rapamycin treatment of *Tsc2*-null cells. Our results suggest that p27 lies downstream of AMPK in the rapamycin-dependent activation of autophagy, and this activity of p27 depends on the N-terminal portion of the protein where the CDK-inhibitory domain is located. Finally, we showed that p27-dependent activation of autophagy promotes *Tsc2*-null cell survival under rapamycin treatment. Thus, our work highlights a novel AMPK/p27 axis involved in rapamycin-dependent activation of autophagy and *Tsc2*-null cell survival, which could explain the relapse of TSC tumors treated with this drug.

2. Material and methods

2.1. Reagents

Rapamycin and bafilomycin A1 were obtained from Calbiochem (San Diego, CA), chloroquine was from Sigma-Aldrich (St Louis, MO). All these reagents were dissolved in dimethyl sulfoxide (DMSO). Complete protease and phosphatase inhibitors were purchased from Roche (Basel, Switzerland). For cDNA and shRNA transfection, Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) was used according to manufacturer's protocols. Lentiviral shRNA p27 plasmid (NM009875) and lentiviral shRNA LKB1 plasmid (NM011492) were obtained from Sigma-Aldrich (St Louis, MO). Lentiviral shRNA control (scramble) plasmid was donated by David Sabatini (Whitehead Institute, USA) and

lentiviral shRNA *Atg13* plasmid was donated by Do-Hyung Kim (University of Minnesota, USA), both through the Addgene organization. The pGFP-p27wt, pGFP-p27NT and pGFP-p27CT design and cloning were previously described [22]. Antibodies used in this study were LC3 (NBP1–19,167) from Novus (Vancouver, BC, Canada), p27 (M197), β -actin (69,879), MEK1/2 (81,504) and GFP (9996) from Santa Cruz Biotechnology (Dallas, TX), pS6 (Ser 235/236) (2211), S6 (2212), pAMPK (Thr 172) (2535), AMPK (2532) and LKB1 (3050) from Cell Signaling Technology (Danvers, MA), *Atg13* (AAS84643C) from Antibody Verify (Las Vegas, NV) and PARP (556,362) from BD Biosciences Pharmingen (San Jose, CA). Secondary antibodies anti-mouse and anti-rabbit conjugated to horseradish were purchased from Bio-Rad (Hercules, CA). Secondary antibodies used for immunofluorescence staining were anti-rabbit Alexa fluor 546 and anti-mouse Alexa 555 (Invitrogen, Carlsbad, CA).

2.2. Cell culture

Tsc2^{-/-} MEF cells (kindly provided by David Kwiatkowski, Brigham and Women's Hospital, Harvard University, USA), *Tsc2*^{-/-} MEF shCtrl and *Tsc2*^{-/-} MEF shp27 (generation previously described in [22]) were grown in DMEM medium with 10% FBS. For metabolic stress conditions, cells were deprived of serum for different time points (see figure legends).

2.3. Analysis of autophagy

Autophagy was detected by Western blot and immunofluorescence assays through measurement of LC3II accumulation [23]. Cells were treated with the lysosomal inhibitor bafilomycin A1 to allow autophagic vesicles to accumulate and be detected (autophagy flux) [23].

2.4. Cell survival and apoptosis assay

Cell survival was measured by using the colorimetric XTT Cell Proliferation Kit (Biological Industries, Kibbutz Beit Haemek, Israel), according to the manufacturer's instructions. Apoptosis was measured by using the luminescence Caspase3/7-Glo kit (Promega, Madison, WI), according to the manufacturer's instructions. Statistical analyses were performed using the non-parametric *t*-test. A *p*-value < 0.05 was considered significant.

2.5. Immunofluorescence microscopy

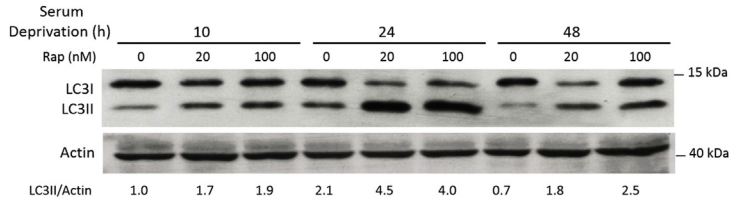
Cells plated on coverslips were fixed (4% paraformaldehyde), permeabilized (0.1% Triton X-100) and incubated with primary antibody (in blocking buffer, 1% BSA in PBS) overnight. After a washing step, fixed cells were incubated with the corresponding Alexa Fluor-coupled secondary antibody for 2 h. Mounting medium with DAPI (Vectashield, Burlingame, CA) was used. Images were obtained with an LMS 780 spectral confocal system (Zeiss, Jena, Germany). Identical exposure times and zoom (60 \times) were used for comparisons.

3. Results

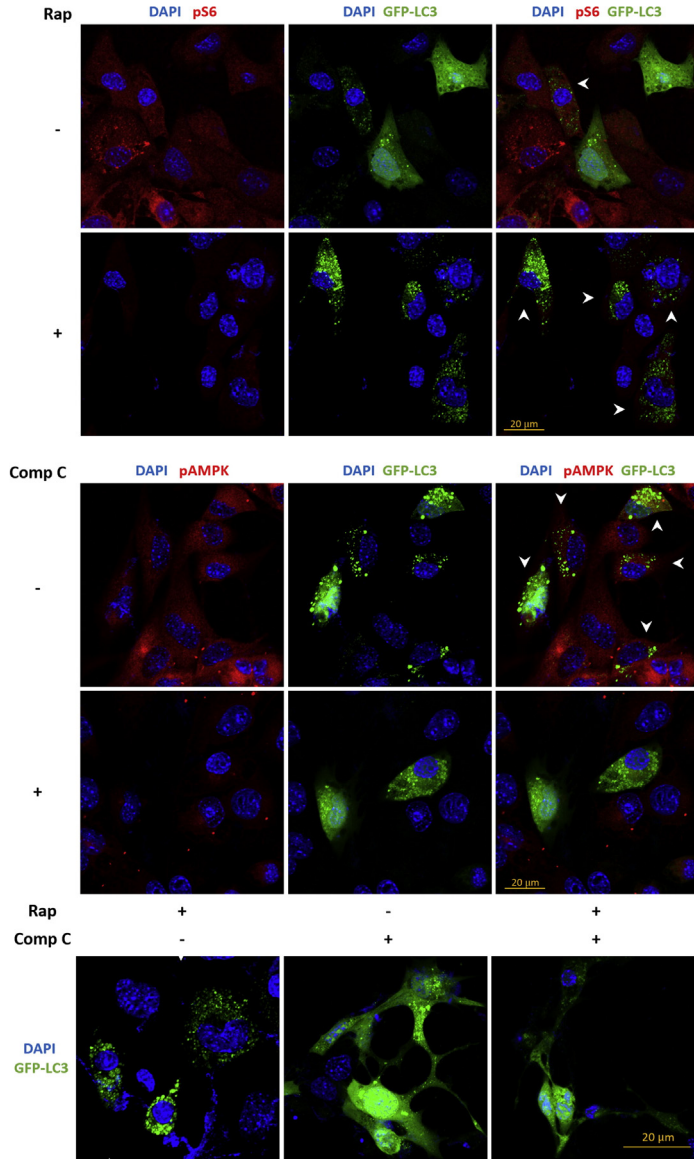
3.1. Rapamycin-induced autophagy is dependent on AMPK and p27

Inhibition of the mTORC1 pathway by rapamycin induces autophagy, leading to its activation in *Tsc2*-null cells under metabolic stress [9, 24,25]. However, the underlying mechanisms involved in the activation of autophagy in these cells are unknown. Since AMPK can activate autophagy [18] and is upregulated in TSC cells [20,26], we sought to investigate whether AMPK is involved in the rapamycin-dependent activation of the autophagic process in *Tsc2*-null cells. To monitor activation of autophagy, we measured the autophagic flux using the lysosomal inhibitor bafilomycin A1 and evaluating the protein levels of

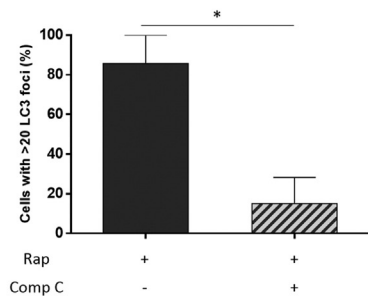
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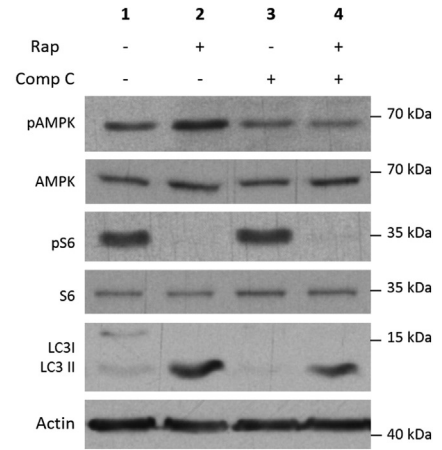
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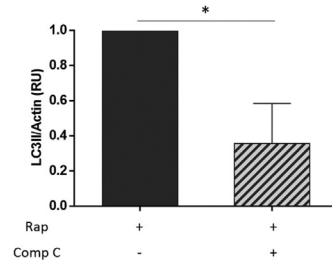
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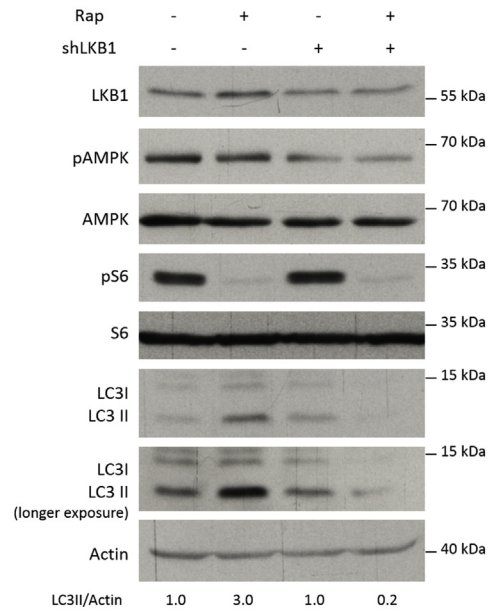
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E



F



LC3I and LC3II by Western blot. The results shown in Fig. 1 revealed an increase of LC3II levels in response to rapamycin treatment. Rapamycin, at a 20 nM concentration, induced the highest increase of LC3II levels after 24 h of serum deprivation (Fig. 1A). Thus, we used these conditions for the following experiments. Autophagy activation was also revealed by the appearance of LC3 punctate structures in immunofluorescence analysis. In these experiments, we confirmed that rapamycin reduced the levels of phosphorylation of S6 as expected by inhibition of the mTORC1 pathway. This effect correlated with the appearance of GFP-LC3 punctate structures (Fig. 1B, top panels). Inhibition of AMPK with compound C reduced the levels of phosphorylation of AMPK, and inhibited basal levels of autophagy as most of the cells showed diffuse GFP-LC3 fluorescence in the cytoplasm (Fig. 1B, middle panels). Quantification of number of GFP-LC3 foci per *Tsc2*-null cell showed that compound C also inhibited autophagy in rapamycin-treated cells (Fig. 1B, bottom panels and Fig. 1C). These results were confirmed by Western blot analysis, showing that compound C reduced rapamycin-dependent increase of LC3II levels, an effect correlated with reduction of AMPK phosphorylation (Fig. 1D and E). To confirm that rapamycin depends on AMPK activity for induction of autophagy in *Tsc2*-null cells, we inhibited AMPK activation in *Tsc2*^{-/-} and *Tsc2*^{+/+} MEFs by shRNA-mediated depletion of LKB1, the major kinase involved in AMPK activation [26]. Rapamycin inhibited the phosphorylation of S6 in control and LKB1-depleted *Tsc2*^{-/-} MEFs (Fig. 1F). However, it failed to increase LC3II levels in LKB1-depleted cells, consistent with inhibition of autophagy (Fig. 1F). In agreement with previous reports [9,24] basal levels of autophagy were high in *Tsc2*^{+/+} MEFs, and rapamycin was unable to further increase them. Even though depletion of LKB1 reduced the levels of AMPK phosphorylation in these cells, it was unable to affect autophagy under basal or rapamycin-treated conditions (Suppl. Fig. 1).

As a consequence of AMPK activation in *Tsc2*-null cells, p27 is stabilized in the cytosol [20]. We stably depleted p27 to analyze whether it is involved in rapamycin-induced autophagy in *Tsc2*-null cells. By immunofluorescence analysis, we confirmed that the levels of p27 are reduced in shRNAp27-treated cells. As expected, control *Tsc2*-null cells showed basal GFP-LC3 punctate staining that increases under rapamycin treatment, but p27-depleted cells showed diffuse GFP-LC3 fluorescence under basal and rapamycin-treated conditions (Fig. 2A). Quantification of number of GFP-LC3 foci per *Tsc2*-null cell confirmed that p27 depletion inhibited autophagy in rapamycin-treated cells (Fig. 2B). We also show by Western blot analysis that p27-depleted cells have reduced rapamycin-dependent increase of LC3II levels, even though the phosphorylation of S6 was blocked as in control cells (Fig. 2C and D). Altogether, our results indicated that AMPK activity and p27 expression are involved in activation of autophagy in *Tsc2*-null cells treated with rapamycin.

3.2. Rapamycin-induced autophagy depends on AMPK regulation of p27

To investigate whether the effect of AMPK on autophagy was dependent on the stabilization of p27 in the cytoplasm, we used an N-terminal p27 mutant (p27NT) characterized by its preferential cytosolic

localization and a C-terminal p27 mutant (p27CT) that mainly localizes in the nucleus ([22] and Fig. 3A). Consistent with the results in Fig. 2, rapamycin barely increased LC3II levels in p27-depleted cells (Fig. 3B, lane 1 vs. 2). Overexpression of p27NT significantly recovered the increase of LC3II levels in response to rapamycin (Fig. 3B, lane 3 vs. 4), while p27CT failed to do so (Fig. 3B, lane 5 vs. 6). We also analyzed the effect of these two p27 mutants in cells where AMPK was inhibited by depletion of LKB1. According to the role of AMPK in p27 stabilization, LKB1 depletion reduced the levels of p27 in the cytosol (Suppl. Fig. 2). In these cells, overexpression of p27NT resulted in a higher increase of LC3II levels in response to rapamycin than overexpression of p27CT (2.5 vs. 1.6 fold, Fig. 3C). Thus, these results are consistent with cytosolic p27 being downstream of AMPK for the activation of autophagy in rapamycin-treated cells.

3.3. Rapamycin promotes p27-dependent *Tsc2*-null cell survival

Rapamycin releases activation of autophagy under metabolic stress, protecting *Tsc2*-null cells from apoptotic cell death [9,24]. Accordingly, we found that rapamycin decreases activation of the apoptotic markers caspases 3 and 7 in *Tsc2*-null cells under serum deprivation (Fig. 4A). Consistent with the role of p27 in the effect of rapamycin on autophagy, rapamycin was unable to decrease the activation of caspases 3 and 7 in p27-depleted cells (Fig. 4B). To confirm that activation of caspases 3 and 7 was correlated with cell death, we analyzed the effect of rapamycin on cell survival. Rapamycin slightly, but significantly, increased *Tsc2*-null cell survival under serum deprivation (Fig. 5A–C, bars 1–2), which was consistent with the rapamycin inhibition of caspases 3 and 7 activities. *Tsc2*-null cell survival was decreased by inhibition of autophagy with bafilomycin A1 (Fig. 5A, bar 1 vs. 3), and rapamycin was unable to increase cell survival under this condition (Fig. 5A, bar 3 vs. 4). Cell survival was reduced in p27-depleted cells (Fig. 5A, bar 1 vs. 5). Consistent with the role of p27 in the regulation of autophagy, neither rapamycin nor bafilomycin A1 or combination of the two drugs could further affect cell survival of p27-depleted cells (Fig. 5A, bars 5 to 8), as they did it in control *Tsc2*-null cells (Fig. 5A, bars 1 to 3). We obtained similar results by inhibition of autophagy with chloroquine (Fig. 5B) or by shRNA-mediated depletion of Atg13 (Fig. 5C). Results shown in Fig. 5D confirmed shRNA-mediated depletion of Atg 13 expression in control and p27-depleted *Tsc2*-null cells. Altogether, our results suggest that p27-dependent activation of autophagy protects rapamycin-treated *Tsc2*-null cells.

4. Discussion

The association of LAM and TSC with aberrant mTORC1 activation has lead testing the clinical benefit of rapamycin treatment. This targeted therapy partially reduced the volume of angiomyolipomas and subependymal giant-cell astrocytomas, and improved clinical parameters of pulmonary LAM, but some of these benefits were lost when treatment was discontinued [8,27,28]. These results suggest that tumor cells engage survival mechanisms likely exacerbated during

Fig. 1. Rapamycin-induced autophagy is dependent on AMPK activity. A) MEF *Tsc2*^{-/-} cells were deprived of serum and treated with bafilomycin A1 (10 nM) and DMSO or Rap (20 and 100 nM) for 10, 24, 48 h. Lysates were characterized by immunoblotting of the indicated proteins. Numbers on the bottom show LC3II protein level determined by densitometric analysis and normalized to the corresponding actin level. B) MEF *Tsc2*^{-/-} cells were transiently transfected with pGFP-LC3 vector. Cells were deprived of serum, treated with bafilomycin A1 (10 nM) and DMSO, Rapamycin (Rap) (20 nM), Compound C (Comp C) (25 μM) or both drugs for 24 h as indicated. pS6 (red, upper panel) or pAMPK (red, middle panel) levels were analyzed by immunofluorescence and confocal microscopy. DAPI stained the nuclei (blue). A representative field is shown. Arrows indicate cells with GFP-LC3 foci. C) Quantification of GFP-LC3 foci from immunofluorescence experiments in A (bottom panel). Graph shows the percentage of cells with more than 20 GFP-LC3 punctate structures relative to the total GFP-positive cells in each indicated condition. D) MEF *Tsc2*^{-/-} cells were deprived of serum and treated with bafilomycin A1 (10 nM) and DMSO, Rap (20 nM), Comp C (25 μM) or both drugs for 24 h. Lysates were characterized by immunoblotting of the indicated proteins. E) Densitometry was used to analyze the data from experiments conducted as described in B. LC3II/Actin levels for Rap (lane 2) relative to LC3II/Actin levels in control condition (lane 1) were arbitrary considered as 1.0, and compared to LC3II/Actin levels for Rap + Comp C (lane 4) relative to LC3II/Actin levels in Comp C condition (lane 3). The result illustrates the effect of Comp C on Rap-induced increase of LC3II levels. F) MEF *Tsc2*^{-/-} cells were transiently transfected with shCtrl or shLKB1 vector. Cells were deprived of serum, treated with bafilomycin A1 (10 nM) and DMSO or Rap (20 nM) for 24 h. Lysates were characterized by immunoblotting of the indicated proteins. LC3II protein level was determined by densitometric analysis and normalized to the corresponding actin level. Numbers on the bottom show LC3II/actin levels for each Rap-treated condition (lanes 2 and 4) relative to the corresponding control condition arbitrary considered as 1.0 (lanes 1 and 3). Results are representative of at least three independent experiments, except that immunoblotting data from E are representative of two independent experiments. Data are expressed as the mean ± SD. *Statistical analysis: non-parametric *t*-test (*P* < 0.05).

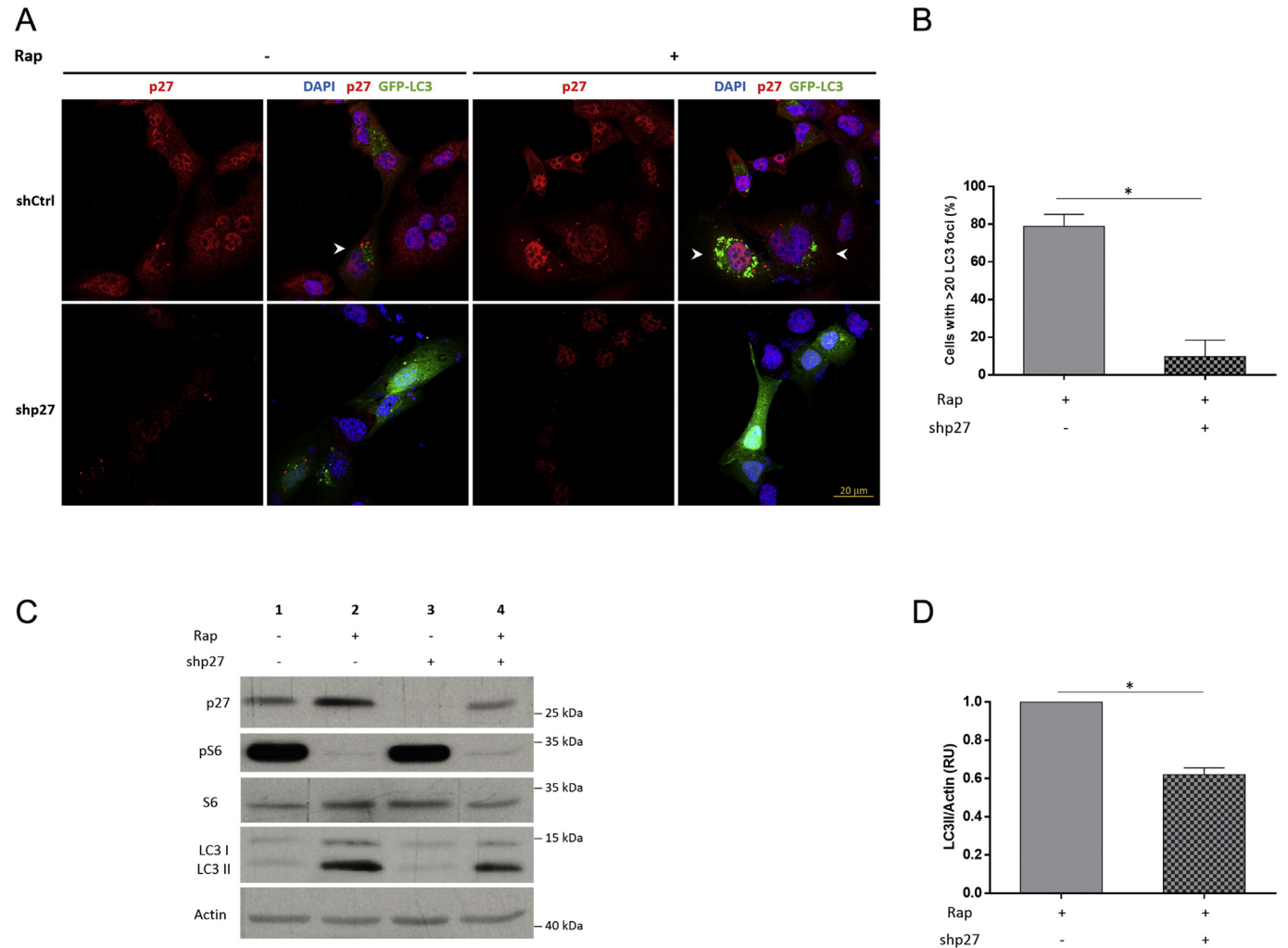


Fig. 2. Rapamycin-induced autophagy is dependent on p27kip1. A) MEF *Tsc2*^{-/-} shCtrl or MEF *Tsc2*^{-/-} shp27 cells were transiently transfected with pGFP-LC3 vector. Cells were deprived of serum, treated with bafilomycin A1 (10 nM) and DMSO or Rap (20 nM) for 24 h. p27 (red) levels were analyzed by immunofluorescence and confocal microscopy. DAPI stained the nuclei (blue). A representative field is shown. Arrows indicate cells with GFP-LC3 foci. B) Quantification of GFP-LC3 foci from immunofluorescence experiments in A. Graph shows the percentage of cells with more than 20 GFP-LC3 punctate structures relative to the total GFP-positive cells in each indicated condition. C) MEF *Tsc2*^{-/-} shCtrl or MEF *Tsc2*^{-/-} shp27 cells were deprived of serum, treated with bafilomycin A1 (10 nM) and DMSO or Rap (20 nM) for 24 h. Lysates were characterized by immunoblotting of the indicated proteins. D) Densitometry was used to analyze the data from experiments conducted as described in B. LC3II/Actin levels for Rap (lane 2) relative to LC3II/Actin levels in control condition (lane 1) were arbitrary considered as 1.0, and compared to LC3II/Actin levels for Rap + shp27 (lane 4) relative to LC3II/Actin levels in shp27 condition (lane 3). The result illustrates the effect of shp27 on Rap-induced increase of LC3II levels. All the results are representative of at least three independent experiments. Data are expressed as the mean \pm SD. *Statistical analysis: non-parametric t-test ($P < 0.05$).

rapamycin treatment. Our current study indicates that an AMPK/p27 axis underlies the rapamycin-dependent survival of *Tsc2*-null cells, revealing a molecular mechanism responsible for the cellular adaptation to this treatment.

Previous studies have indicated that autophagy is pro-tumorigenic in TSC and serves as a survival mechanism in response to rapamycin [9,24]. We now showed that AMPK activity is necessary for rapamycin-induced autophagy in *Tsc2*-null cells under serum deprivation. These results are consistent with a role of AMPK counteracting mTORC1 regulation of autophagy. In fact, AMPK is known to activate autophagy [18] and is found activated in TSC tumors and *Tsc2*-null cells [20,26]. Since mTORC1 is hyperactivated in *Tsc2*-null cells and negatively regulates autophagy, it is expectable that a balance between the AMPK and mTORC1 signaling determines the levels of autophagy in these cells. Compared to *Tsc2*-proficient cells, levels of autophagy were shown to be lower in *Tsc2*-null cells [9], suggesting that the hyperactivation of mTORC1 in these cells is prominent on the restriction of autophagy. Accordingly, blocking of mTORC1 activity by rapamycin

releases the inhibition of autophagy. How AMPK is inducing the autophagic process in rapamycin-treated *Tsc2*-null cells is unknown, but our results showed that AMPK depends on the expression of p27. In agreement with these results, Liang et al. found that AMPK is unable to activate autophagy in *p27*^{-/-} MEFs under metabolic stress [21]. A role of p27 in autophagy has also been reported in cardiomyocytes and glioma cells [29,30]. We have recently shown that p27 is also essential for Rheb-dependent activation of autophagy in colorectal cancer cells [22]. The effect of p27 seems to depend on its phosphorylation by AMPK on Thr198 and its consequent stabilization in the cytoplasm [21]. We previously found that the expression of the p27NT mutant that preferentially localizes in the cytoplasm recovers basal activation of autophagy in response to metabolic stress in p27-depleted *Tsc2*-null cells, while the nuclear p27CT mutant was inactive [22]. We now showed that p27NT can also recover rapamycin-induced autophagy. Together with previous reports, our data suggest an essential role of cytoplasmic p27 in the regulation of autophagy, and likely a direct effect of p27 downstream of AMPK on the process. Consistent with the latter,

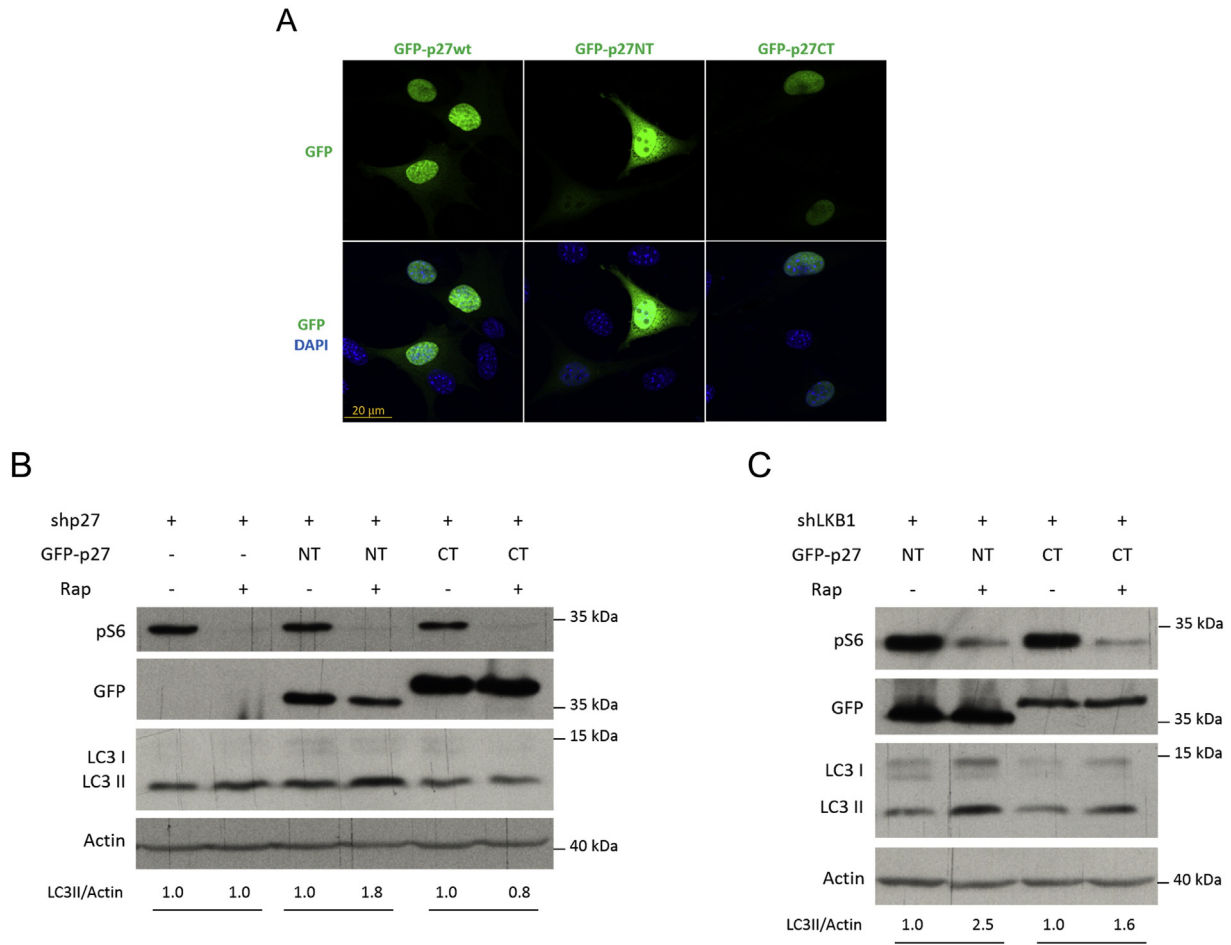


Fig. 3. Rapamycin-induced autophagy depends on AMPK regulation of p27kip1. A) MEF *Tsc2*^{-/-} cells were transiently transfected with pGFP-p27wt, pGFP-p27NT or pGFP-p27CT vector. The cells were analyzed by immunofluorescence and confocal microscopy. DAPI stained the nuclei (blue). A representative field is shown. B) MEF *Tsc2*^{-/-} shp27 cells were transiently transfected with pGFP-control, pGFP-p27NT or pGFP-p27CT. Cells were deprived of serum, treated with bafilomycin (10 nM) and DMSO or Rap (20 nM) for 24 h. Lysates were characterized by immunoblotting of the indicated proteins. LC3II protein level was determined by densitometric analysis and normalized to the corresponding actin level. Numbers on the bottom show LC3II/actin levels for each Rap-treated condition (lanes 2, 4 and 6) relative to the corresponding control condition arbitrary considered as 1.0 (lanes 1, 3 and 5). C) MEF *Tsc2*^{-/-} cells were transiently transfected with shLKB1 vector and pGFP-p27NT or pGFP-p27CT. Cells were deprived of serum, treated with bafilomycin A1 (10 nM) and DMSO or Rap (20 nM) for 24 h. Lysates were characterized by immunoblotting of the indicated proteins. LC3II protein level was determined by densitometric analysis and normalized to the corresponding actin level. Numbers on the bottom show LC3II/actin levels for each Rap-treated condition (lanes 2 and 4) relative to the corresponding control condition arbitrary considered as 1.0 (lanes 1 and 3). All the results are representative of two independent experiments.

p27NT was able to induce autophagy in cells where AMPK activity was inhibited by LKB1 depletion. It is unknown the mechanism by which p27 affects autophagy. Since p27NT contains the CDK-inhibitory

domain and retains the effect on autophagy, p27 interaction with CDKs may be involved in the regulation of the autophagic process. In this sense, CDKs have been shown to inhibit this cellular process [31].

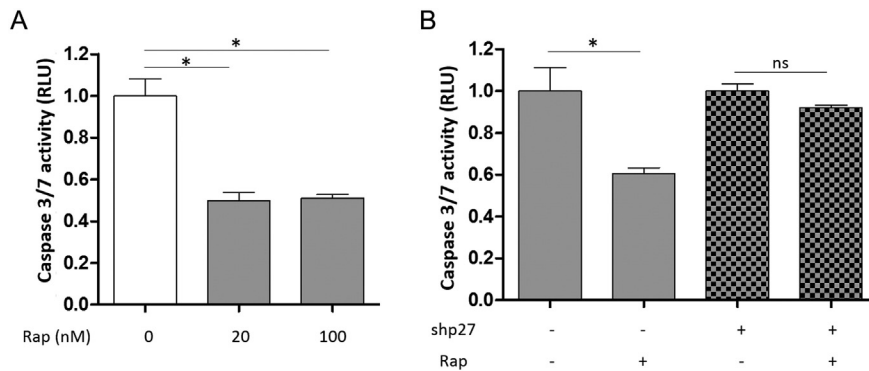


Fig. 4. Rapamycin prevents *Tsc2*-null cell apoptosis in a p27 kip1-dependent manner. A) MEF *Tsc2*^{-/-} cells were deprived of serum and treated with DMSO or Rap (20 and 100 nM) for 24 h. Cell death by apoptosis was evaluated by bioluminescent measurement of caspase 3 and 7 activity. Results are expressed as relative luminescent units (RLU). B) MEF *Tsc2*^{-/-} shCtrl or MEF *Tsc2*^{-/-} shp27 cells were deprived of serum and treated with DMSO or Rap (20 nM) for 24 h. Apoptosis was assessed by bioluminescent measurement of caspase 3 and 7 activity. Results are expressed as relative luminescent units (RLU). All the results are representative of at least three independent experiments performed in quintuplicate. Data are shown as mean ± SD. **P* < 0.05, non-parametric *t*-test.

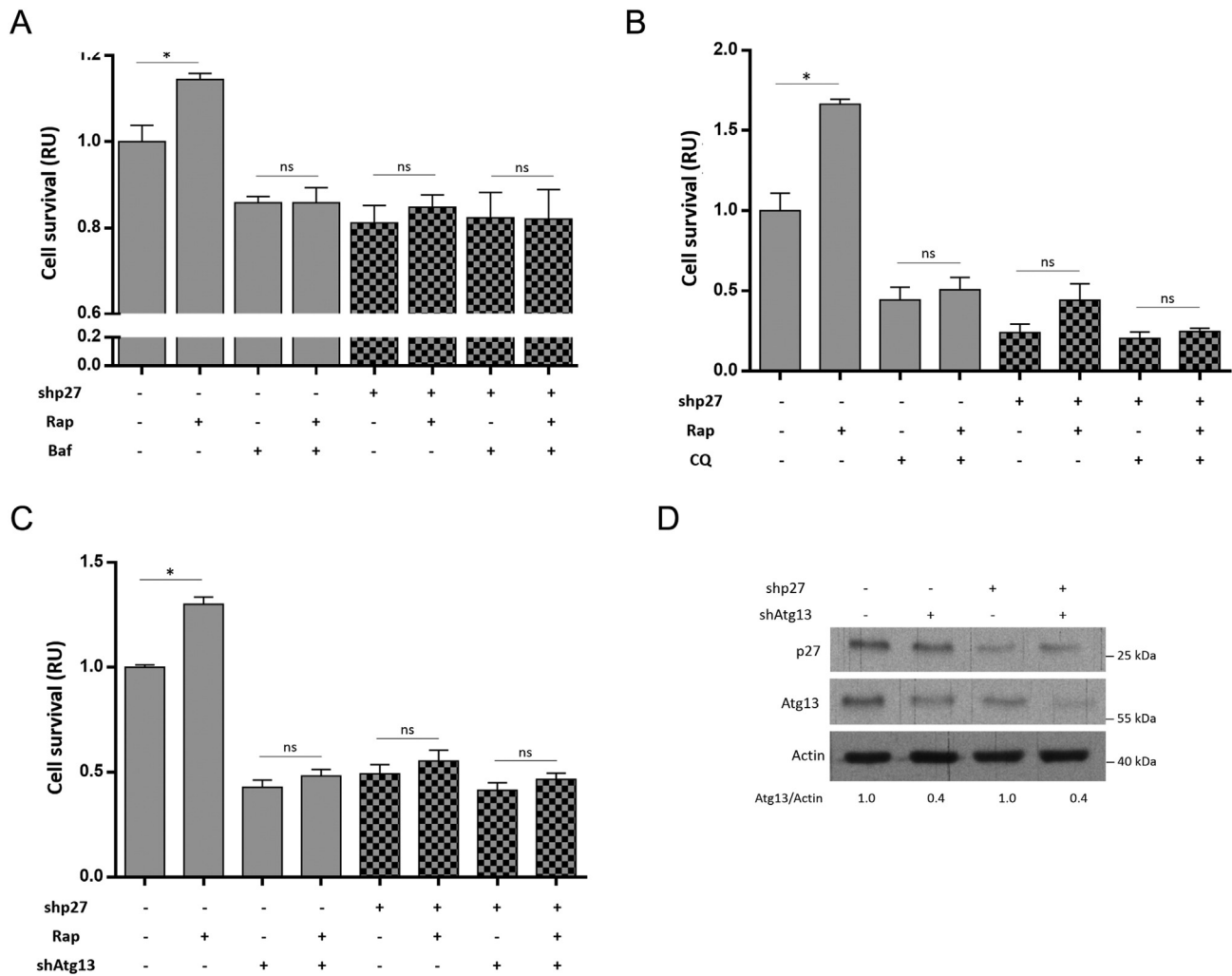


Fig. 5. Rapamycin promotes p27 kip1-dependent Tsc2-null cell survival. A) MEF *Tsc2*^{-/-} shCtrl or MEF *Tsc2*^{-/-} shp27 cells were deprived of serum and treated with DMSO, bafilomycin A1 (Baf) (10 nM), Rap (20 nM) or both drugs for 24 h. Cell survival was estimated by colorimetric XTT measurement and the results are expressed as relative units (RU). B) Same as in A), but using chloroquine (10 μ M) instead of bafilomycin A1. C and D) MEF *Tsc2*^{-/-} shCtrl or MEF *Tsc2*^{-/-} shp27 cells were transiently transfected with shCtrl or shAtg13 vector, deprived of serum and treated with DMSO or Rap (20 nM) for 24 h. (C) Cell survival was estimated by colorimetric XTT measurement and the results are expressed as relative units (RU) (D). Lysates were characterized by immunoblotting of the indicated proteins. Atg13 protein level was determined by densitometric analysis and normalized to the corresponding actin level. Numbers on the bottom show Atg13/actin levels relative to the corresponding control condition arbitrary considered as 1.0. Results are representative of at least three independent experiments performed in quintuplicate. Data are expressed as mean \pm SD. * $P < 0.05$, non-parametric *t*-test.

Thus, p27 may counteract CDKs' regulation of autophagy under metabolic stress, but this mechanism awaits investigation.

AMPK and p27 have been previously shown to increase survival of *Tsc2*-null cells in the absence of serum [20], but whether their effect is related to activation of autophagy was unknown. Our studies demonstrated that p27 regulation of autophagy promotes basal and rapamycin-dependent increase of cell survival in the absence of serum. Because p27 stabilization in the cytosol is characteristic of cells with absent *Tsc2* expression [20] and cytosolic p27 seems to be necessary for autophagy activation [21], we could expect that targeting this pathway will be deleterious in a *Tsc2*-null cellular context. Although AMPK is also involved in the cytosolic localization of p27 in metabolically stressed *Tsc2*-proficient cells [20], we were unable to detect any effect on autophagy by inhibition of AMPK activity in *Tsc2*^{+/+} MEFs. Furthermore, in the context of a potential therapy, *Tsc2*-null cells are more likely to be affected because they are frequently confronted to metabolic stress caused by the outgrowth of the tumor mass and, consequently, to activation of autophagy.

Our findings may have broader implications because deregulation of autophagy has also been involved in several neurodegenerative disorders and cancer. Neurodegenerative disorders have been associated

with defective autophagy [32]. On the other hand, like in TSC, acute activation of autophagy is pro-tumorigenic in cancer because it increases the survival of cancer cells [33]. Accordingly, studies in models of these diseases indicate that autophagy-modulating drugs should be considered for their treatment [32,33]. Thus, understanding the mechanisms involved in the regulation of autophagy is essential for finding efficient therapies. Whether the regulation of autophagy by the AMPK/p27 axis extends to other pathophysiological conditions awaits further investigations.

In favor of potential therapy targeting autophagy activation in TSC, it was shown that inhibition of autophagy with chloroquine in combination with rapamycin effectively reduced the growth of *TSC2*-null xenograft tumors and development of renal tumors in *Tsc2*^{-/+} mice [9]. Similarly, resveratrol was shown to induce apoptosis and prevent induction of autophagy in response to rapamycin treatment in *TSC2*-null and LAM cells, which was correlated with inhibition of *TSC2*-null cell lung metastases in mice [24]. However, *Tsc2*-deficient cells seem to metabolically adapt to the inhibition of autophagy, which could eventually result in tumor cells resistance to these treatments. In fact, chloroquine induces metabolic adaptation of *Tsc2*-null cells through the pentose phosphate pathway (PPP), which generates NADPH and

sustains the proliferation of *Tsc2*-null cells [25]. It is interesting that LKB1 and AMPK have been shown to maintain intracellular levels of NADPH through PPP in cancer cells, which promotes cancer cell survival and tumorigenesis [34]. Since AMPK is activated in *Tsc2*-null cells [20, 26], it is important to determine whether AMPK is also controlling PPP in these cells. Together with our current results, these studies would place AMPK as a central promoter of *Tsc2*-null tumor cell survival in response to metabolic stress and therapies, consequently, as a central target for efficient therapies for TSC and LAM patients.

5. Conclusion

AMPK/p27 axis is involved in rapamycin-dependent activation of autophagy and *Tsc2*-null cell survival. These results could explain the relapse of TSC tumors treated with rapamycin.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

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