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TSC2 N-terminal lysine acetylation status affects to its stability modulating mTORC1 signaling and autophagy

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ABSTRACT

There is a growing evidence of the role of protein acetylation in different processes controlling metabolism. Sirtuins (histone deacetylases nicotinamide adenine dinucleotide-dependent) activate autophagy playing a protective role in cell homeostasis. This study analyzes tuberous sclerosis complex (TSC2) lysine acetylation, in the regulation of mTORC1 signaling activation, autophagy and cell proliferation. Nicotinamide 5 mM (a concentration commonly used to inhibit SIRT1), increased TSC2 acetylation in its N-terminal domain, and concomitantly with an augment in its ubiquitination protein status, leading to mTORC1 activation and cell proliferation. In contrast, resveratrol (RESV), an activator of sirtuins deacetylation activity, avoided TSC2 acetylation, inhibiting mTORC1 signaling and promoting autophagy. Moreover, TSC2 in its deacetylated state was prevented from ubiquitination. Using MEF *Sirt1* +/+ and *Sirt1* −/− cells or a SIRT1 inhibitor (EX527) in MIN6 cells, TSC2 was hyperacetylated and neither NAM nor RESV were capable to modulate mTORC1 signaling. Then, silencing *Tsc2* in MIN6 or in MEF *Tsc2* −/− cells, the effects of SIRT1 modulation by NAM or RESV on mTORC1 signaling were abolished. We also observed that two TSC2 lysine mutants in its N-terminal domain, derived from TSC patients, differentially modulate mTORC1 signaling. TSC2 K599M variant presented a lower mTORC1 activity. However, with K106Q mutant, there was an activation of mTORC1 signaling at the basal state as well as in response to NAM. This study provides, for the first time, a relationship between TSC2 lysine acetylation status and its stability, representing a novel mechanism for regulating mTORC1 pathway.

1. Introduction

Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance in different tissues [1]. Two major phases can be distinguished in progression to T2DM. During the first one, an increase in pancreatic β cell mass by hyperplasia and hypertrophy is important to compensate for systemic insulin resistance. In the second one, β cells start to fail and die by apoptosis, leading to overt diabetes [2]. Under insulin resistance, mammalian/mechanistic target of rapamycin complex 1 (mTORC1) is hyperactive and essential for the compensatory mechanisms that lead to augmented beta cell mass, but might act as a double-edged sword in a later phase and contribute to beta cell apoptosis, involved in the pathogenesis of T2DM [3]. Autophagy is a physiologic process that eliminates damaged organelle and aggregated proteins. Our group and others have determined that autophagy, is a protective mechanism under different stress situations in several cell lines and tissues, promoting cell survival [4–7].

Insulin signaling modulates longevity in a great variety of organisms such as worms, flies, and mice [8], and rapamycin or knockdown of *MTOR* or *S6K1* can extend life span in different species [9–13]. In addition, tuberous sclerosis complex 1 (TSC1) and 2 (TSC2) activation, which negatively controls mTORC1 signaling through its GAP (GTPase activating protein) towards *Rheb* (Ras homolog enriched in brain), prolongs longevity in *Drosophila* [14]. Aging or a hypercaloric diet is associated with mTORC1 hyperactivity, leading to a defective autophagy and an increase in ER stress, contributing to insulin resistance [15]. In this regard, calorie restriction modulates changes in both nicotinamide adenine dinucleotide (NAD^+) and sirtuins levels, activating autophagy, and has been related with mammalian longevity [16]. Mammalian

Abbreviations: ARD1, arrest defective type 1; Atg, autophagy related; CHX, cycloheximide; GAP, GTPase activating protein; LC3B, microtubule-associated protein 1 light chain 3 beta; mTORC1, mammalian/mechanistic target of rapamycin complex 1; NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; NAT, N-terminal acetyl transferase; OAADPr, *O*-acetyl ADP-ribose; P70S6K, ribosomal protein S6 kinase polypeptide; RAPA, rapamycin; RESV, resveratrol; Rheb, Ras homologue enriched in brain; Sir2, silent information regulator 2 protein; SIRT1, sirtuin 1; shRNA, short-hairpin RNA; TSC1, tuberous sclerosis complex 1; TSC2, tuberous sclerosis complex 2; T2DM, type 2 diabetes mellitus.

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sirtuins are composed of seven members (SIRT1–7) stimulated by energy deprivation, calorie restriction and resveratrol [17,18]. Sirtuins are histone deacetylases NAD^+-dependent homologous to the yeast silent information regulator 2 (Sir2) protein. SIRT1–3 catalyzed deacetylation reaction yielding nicotinamide and *O*-acetyl ADP-ribose (OAADPr) along with the deacetylated lysine [19]. Then, the nicotinamide product is a noncompetitive inhibitor of sirtuins [20].

The different localizations of SIRT proteins in the cell have an important implication in physiologic or pathologic metabolism processes. SIRT1 and SIRT2 shuttle between the nucleus and cytoplasm [21–23]. Protein acetylation modulates autophagy and can increase life span in yeasts as well as in nematodes [24]. SIRT1 interacts with TSC2 in HeLa cells, without affecting TSC2 acetylation [25]. The N-terminal acetyl-transferase (NAT), arrest-defective protein 1 (ARD1) interacts with TSC2, acetylates the first residue methionine, stabilizing it and inhibiting mTORC1 activity [26]. Furthermore, it has recently been published the interaction between SIRT1 and p70S6K. This interaction diminishes p70S6K acetylation degree, favoring the phosphorylation by mTORC1 [27].

Many reports indicate a differential regulation of TSC2 by both activating and inactivating phosphorylation sites [28]. However, the consequences of TSC2 lysine acetylation remain to be explored. Here, in this report we describe TSC2 lysine acetylation as a novel mechanism in the control of mTORC1 signaling and its consequences affecting essential processes, such as autophagy and proliferation.

2. Material and methods

2.1. Antibodies and reagents

The following antibodies were obtained from Cell Signaling Technology (Beverly, MA): anti pan-acetylated Lysine, #9441, anti-LC3B #4108, anti-p70S6K #9202, anti-phospho-p70S6K (Thr389), #9205, anti-TSC2, #9442, anti-phospho-AMPK (T172), #2531. From Sigma-Aldrich: anti-HA H6908, anti-Flag M2F1804, anti-β-actin A5316. From Santa Cruz Biotechnology: anti-GFP sc-9996, anti-SIRT1 sc-15404, anti-TSC2 sc-893. Other antibodies were used as follows: anti-phospho-S6 Ribosomal Protein (Ser235/236) from Thermo Scientific, MA5-15140, and anti-mono- and polyubiquitinylated proteins FK2 conjugated with peroxidase (HRP) or FK2H from Enzo Life Sciences, BML-PW0150. Chloroquine C6628, cycloheximide C7698, 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide (EX-527) E7034, nicotinamide N3376 and propidium iodide P4170, were from Sigma-Aldrich; rapamycin 553210 and resveratrol R5010 were from Merck; and geneticin was from Santa Cruz (G418).

2.2. Cell culture

Mouse insulinoma 6 (MIN6) cell line originally described in [29] were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose supplemented with 15% FBS (fetal bovine serum), 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol. MIN6 Scr and *Tsc2* shRNA cell lines were generated as described in [7]. Cells were maintained at 37 °C in an atmosphere of 5% CO_2. Mouse embryonic fibroblasts (MEFs) and human embryonic kidney 293 (HEK 293T) cells were grown in DMEM containing 25 mM glucose supplemented with 10% FBS. MEF *Tsc1* −/− *and Tsc2* −/− were generous gifts of Dr. Kwiatkowski (Harvard Medical School, Boston). Primary cultures of MEF *Sirt1* +/+ and *Sirt1* −/− MEFs were generous gifts of the Maria Monsalve Lab (IIB, CSIC), and were immortalized by retrovirus-mediated transfection of attenuated SV40 T-antigen. After 5 h, the medium was refreshed and 72 h later, cells were selected with puromycin (1 μg/ml) for three weeks. Alternatively we used immortalized *Sirt1* +/+ and *Sirt1* −/− MEFs generously provided by Leonard Guarente (MIT, Boston).

2.3. Flow cytometry

For cell cycle analysis, trypsinised adherent and non-adherent cells were collected by centrifugation and fixed with cold ethanol (70% v/v). The cells were then washed, resuspended in PBS, and incubated with RNase for 30 min at 37 °C. After addition of 0.05% propidium iodide (w/v), cellular DNA content was quantified by flow cytometry.

2.4. Immunoprecipitation and Western blot

After treatment, cells were washed twice with PBS and lysed for protein extraction according to standard procedures. Protein determination was performed by the Bradford dye method, using the Bio-Rad (Hercules, CA) reagent and BSA as the standard. For immunoprecipitation, equal amounts of protein (200–600 μg) were immunoprecipitated at 4 °C o/n with the corresponding antibodies. The immune complexes were collected on protein A-agarose beads (Roche Applied Sciences) and protein samples were submitted to Western blot analysis. After SDS–PAGE, gels were transferred to Immobilon P PVDF membranes (Merck–Millipore). Then, membranes were blocked with 5% non-fat dried milk and incubated overnight with antibodies at 4 °C. Immunoreactive bands were visualized using the ECL Western blotting protocol (GE Healthcare, Little Chalfont, UK).

2.5. Lentivirus production and cell infection

To generate MIN6 *Tsc2 shRNA* cells, HEK 293T cells were co-transfected using Lipofectamine2000 with lentiviral packaging plasmid pMD2.G (Addgene, 12259) and psPAX2 (Addgene, 12260), along with lentiviral vector pLKO.1 neo or hygro for shRNA production (Addgene, 13425 and 24150 respectively). Different sequences were cloned between EcoRI (Roche, 10-200-310-001) and AgeI (New England Biolabs, R-0552S) sites of pLKO.1 lentiviral vector, following the recommendations from Addgene. Sequences of oligonucleotides used for knocking-down Tsc2 were as follows:

Tsc2-sense: 5′-CCGGcccgatatgtgttctccaaCTCGAGttggagaacacatatcgggTTTTTG-3′,

Tsc2-antisense: 5′ AATTCAAAAAcccgatatgtgttctccaaCTCGAGttggagaacacatatcggg-3′.

Pairs were annealed and cloned into pLKO.1 using restriction enzymes from Roche and New England Biolabs and a T4 ligation kit (Roche Applied Sciences, 11635379001).

Supernatants containing lentiviral particles from 24 and 48 h after HEK293T transfection were collected and passed through 0.45 μm filters. MIN6 cells were infected with lentiviral particles in polybrene (8 μg/ml) supplemented-media. After 24 h cells were used for experiments.

2.6. Measurement of TSC2 protein stability

HEK293T cells were seeded in 60 mm plates with fresh medium (DMEM 10% FBS). The following day, cells were stimulated with cycloheximide (100 μg/ml) for 30 min, and then, cells were treated with NAM (5 mM) or RESV (50 μM) for 4 h. After treatment, cells were washed twice with PBS and lysed for protein extraction according to standard procedures.

2.7. Retroviral production and cell infection

To generate stably transfected *Tsc2* +/+ MEF cells with EGFP-LC3B, the Phoenix packaging cells were transfected with retroviral vector p-EGFP-LC3B (Addgene, 11546) [30] using Lipofectamine2000 (Invitrogen, 11668-019). Supernatants containing retroviral particles were collected 48 h after transfection and passed through 0.45 μm filters. Cells were

infected with retroviral particles in polybrene (8 μg/ml) supplemented-media and selected with geneticin (250 μg/ml) for three weeks. Cells were subcloned by limiting dilution.

2.8. Transient transfection and co-transfection experiments

HEK 293T cells were transiently co-transfected with HA-TSC2 and Flag-SIRT1-WT or GFP-SIRT1-NLS2 with Lipofectamine2000 (Invitrogen, 11668-019). Alternatively, HEK 293T cell were co-transfected with the different constructs of tagged-V5 TSC2, K106Q, K599M or WT TSC2 protein and Flag-SIRT1-WT. After 24 h, the medium was replaced by fresh medium and the following day, cells were stimulated with RESV or NAM for 2 h.

2.9. Violet crystal assay

MIN6 cells were seeded in 12-well plates at a density of 5000 cells/cm^2 in DMEM supplemented with FBS 2%. After the addition of the agonist, cells were washed twice with cold PBS and stained with 0.2% violet crystal (w/v) in 2% ethanol (v/v) for 10 min. Plates were rinsed with ddH2O, dried, and after addition of 1% sodium dodecyl sulfate (w/v), absorbance at 560 nm was determined for each time point.

2.10. Statistics

Statistically significant differences between mean values were determined using the paired Student *t*-test in the Graphpad statistical analysis software package. Differences were considered statistically significant at $P < 0.05$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and n.s indicates no statistical significance.

3. Results

3.1. SIRT1 deacetylates TSC2 modulating mTORC1 signaling and autophagy in HEK293T

TSC2 protein is important in the control of mTORC1 signaling in pancreatic β cells *in vitro* [31]. It is described that TSC2 is interacting with SIRT1 in HeLa cells [25]. However, it is not known whether TSC2 can be modulated by lysine acetylation. To elucidate the role of this post-translational modification on TSC2 and on the control of mTORC1

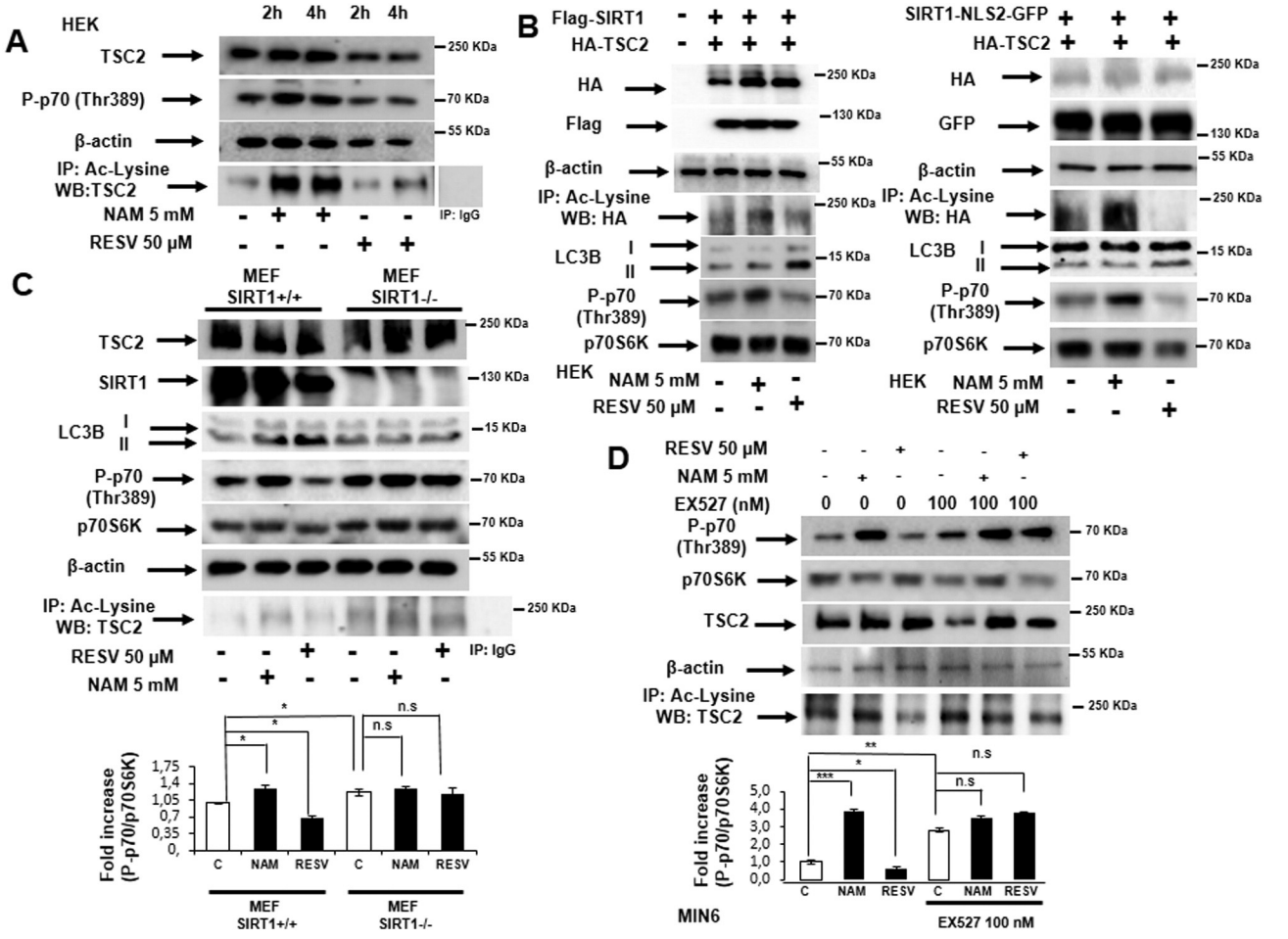

Fig. 1. TSC2 lysine acetylation status modulates mTORC1 signaling and autophagy by SIRT1 activity. (A) Endogenous anti-pan acetylated lysine was immunoprecipitated from HEK 293T cells cultured in complete medium and stimulated or not with nicotinamide (NAM, 5 mM) or resveratrol (RESV, 50 μM) for 2 or 4 h. Western blot was probed with anti-TSC2 antibody. (**B**) HEK 293T cells were transiently co-transfected with HA-TSC2 and either Flag-SIRT1-WT (left) or SIRT1-NLS2-GFP (right). 1 d after transfection, the medium was replaced, and 24 h later, cells were subjected to 2 h NAM or RESV treatment or not (control). Cell lysates were immunoprecipitated with anti-pan-acetylated-lysine proteins and Western blotting of anti-HA antibody. (**C**) *Sirt1* +/+ and *Sirt1* −/− MEFs were stimulated with NAM or RESV for 2 h and cell extracts submitted to immunoprecipitation and Western blot for analysis of TSC2 acetylation and mTORC1 signaling. (**D**) MIN6 cells were pre-treated or not with a SIRT1 inhibitor (EX527) during 30 min, then were stimulated with NAM or RESV for 2 h. Cell extracts were tested for mTORC1 activity and TSC2 acetylation levels by Western blotting. All the blots are representative from at least 3 independent experiments, and the corresponding fold-increase in the ratio P-p70 (Thr389)/p70S6K is shown in the graphs below expressed as mean ± standard deviation (s.d.) (**C**,**D**). $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$; n.s indicates no statistical significance.

signaling, we stimulated HEK293T cells with different sirtuins modulators (NAM or RESV) for 2 and 4 h. NAM increased TSC2 lysine acetylation and RESV prevented TSC2 acetylation (Fig. 1A). The effect of NAM and RESV on TSC2 lysine acetylation was quantified (Supplemental Fig. 1). To corroborate these data obtained with the endogenous proteins, we co-transfected HEK 293T cells with expression construct encoding HA-TSC2 and Flag-SIRT1. After immunoprecipitation of acetylated-lysine using specific antibodies, we detected an increase in TSC2 acetylation in response to NAM, and a reduction in response to RESV (Fig. 1B). TSC2 acetylation status correlated with an increase in the phosphorylation of p70 S6 Kinase (p70S6K) (Thr389), which indicates an increase in mTORC1 activation. The induction of autophagy was assessed by the conversion of cytosolic LC3B-I into lipidated LC3B-II, and was inversely related to mTORC1 activity (Fig. 1B).

SIRT1 is a histone deacetylase mainly found in the nucleus. However, SIRT1 contains both nuclear localization sequences (NLS) and nuclear export signals (NES), facilitating nuclear-cytoplasmic shuttling [22]. We co-transfected HEK 293T cells with HA-TSC2 and with Flag-SIRT1, SIRT1-WT-GFP or a variant of SIRT1, which has both NLS sequences mutated (SIRT1-NLS2-GFP), which is exclusively located in the cytoplasm. The localization of both SIRT1-GFP and SIRT1-NLS2-GFP variants were confirmed by immunofluorescence. Furthermore, we detected both endogenous and exogenous SIRT1 by Western blot (Supplemental Fig. 2). HEK 293T cells were transiently co-transfected with HA-TSC2 and Flag-SIRT1-WT or with a cytoplasm-specific SIRT1 (GFP-SIRT1-NLS2), and after NAM 2 h-treatment we observed an increase in TSC2 acetylation, and concomitantly, activation of mTORC1 activity and inhibition of autophagy. In contrast, RESV reduced TSC2 acetylation, leading to reduced mTORC1 activity and increased autophagy (Fig. 1B).

To confirm the role of SIRT1 in the control of TSC2 acetylation, we used *Sirt1* +/+ and *Sirt1* −/− MEFs, as well as MIN6. In *Sirt1* +/+ MEFs, NAM stimulated mTORC1 signaling and increased TSC2 acetylation, while RESV diminished mTORC1 and abolished TSC2 acetylation. On the other hand, the acetylation state of TSC2 and mTORC1 activity was greatly upregulated in *Sirt1* −/− cells. In contrast, the effects of NAM or RESV on TSC2 acetylation state and mTORC1 signaling were abolished in *Sirt1* −/− cells (Fig. 1C). Furthermore, we observed an increased TSC2 ubiquitination as well as a decreased TSC2 protein content in *Sirt1* −/− MEFs (Supplemental Fig. 3). These data were confirmed in MIN6 cell line treated with a potent chemical inhibitor of SIRT1 enzymatic activity (EX527). We observed that MIN6 cell line have a higher

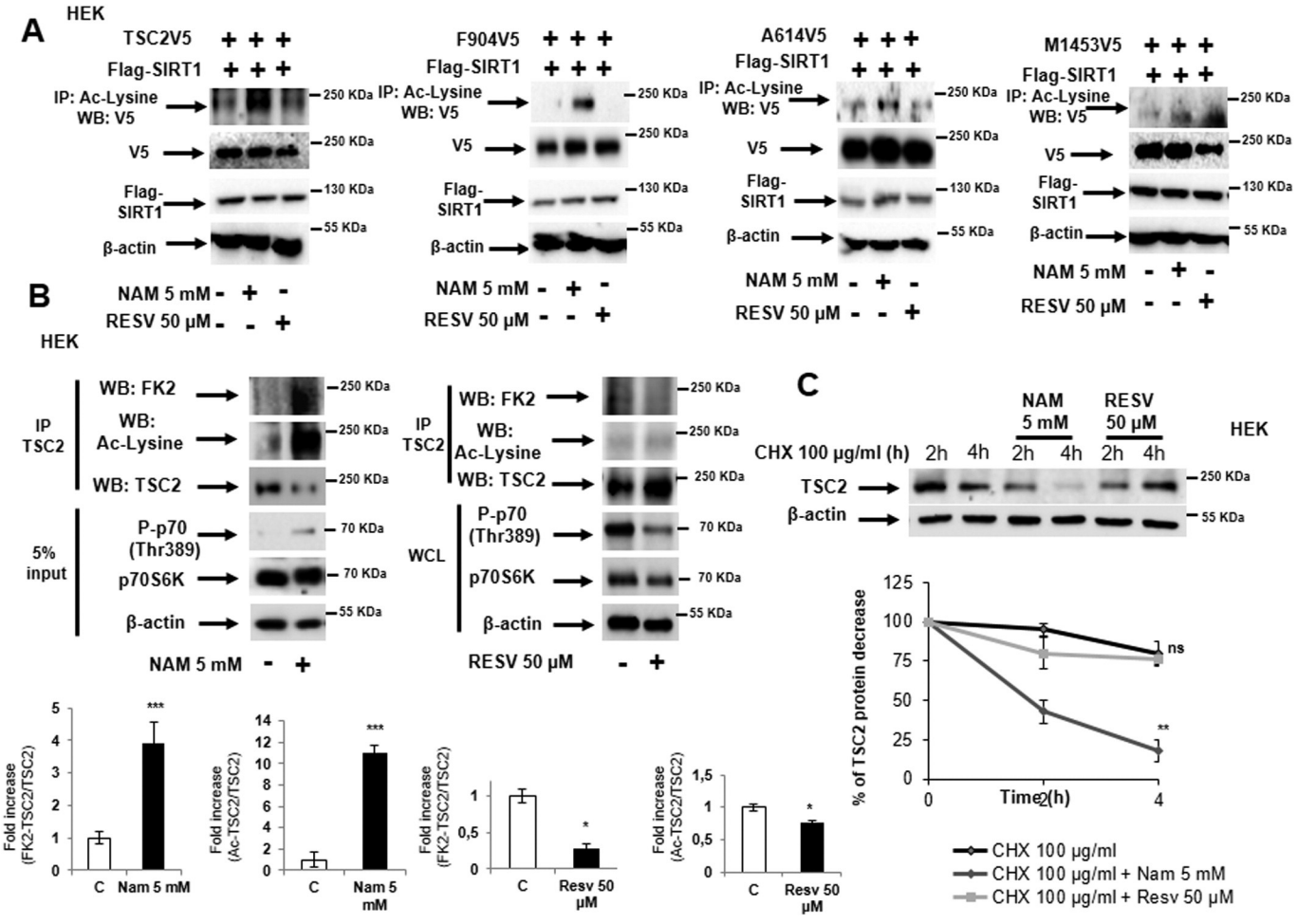

Fig. 2. SIRT1 deacetylates the N-terminal region of TSC2 and regulates ubiquitin binding. (**A**) HEK 293T cells were transiently co-transfected with different V5-tagged truncated constructs of TSC2 and Flag-SIRT1. After 24 h of transfection, the medium was replaced and 24 h later, cells were treated with NAM or RESV for 2 h and subjected to IP using antibodies against pan-acetylated lysine, followed by Western analysis. (**B**) HEK 293T cells were stimulated with NAM or RESV for 2 h, followed by IP of TSC2 and Western analysis for the detection of mono and poly-ubiquitinated proteins or acetyl-lysine with FK2 antibody. To corroborate that the agonists worked properly, Western blot analysis from the supernatant after the addition of prot-A-agarose, in the case of NAM, or whole cell extracts, in the case of RESV, were analyzed for testing mTORC1 signaling. All the blots are representative from at least 3 independent experiments, and the corresponding fold-increase in the ratio FK2-TSC2 and Ac-Lys-TSC2/TSC2 are shown in the graphs below expressed as mean ± standard deviation (s.d.) (**C**,**D**). $^{*}P < 0.05$; $^{***}P < 0.001$. (**C**) HEK293T cells were stimulated with cycloheximide (CHX, 100 μg/ml) for 30 min, and then, cells were treated with NAM (5 mM) or RESV (50 μM) for 4 h. After treatment, cells were washed twice with PBS and lysed for protein extraction for TSC2 Western blotting analysis. The graph represents the % of decrease in the ratio TSC2/β-actin. Values were obtained from 3 independent experiments and are expressed as means ± s.d. $^{**}P < 0.01$.

susceptibility to changes in NAD^+ levels in comparison with MEF cell line (Fig. 1D).

3.2. TSC2 is regulated by lysine acetylation in its amino terminal region affecting to its stability

To assess the region of TSC2 that is required for modulation by SIRT1, we co-transfected HEK 293T cells with expression constructs encoding Flag-SIRT1 and different V5-epitope tagged N-terminal or C-terminal regions of TSC2 (Supplemental Fig. 4). NAM increased, and RESV inhibited acetylation of full length TSC2 as well as two partial TSC2 proteins containing the N-terminal region. However, using a partial C-terminal region of TSC2 containing GAP module, we did not observe any change in acetylation in response to neither NAM nor RESV (Fig. 2A).

To study if TSC2 lysine acetylation status plays a role in its stability controlling mTORC1 signaling, we submitted HEK 293T cells to NAM or RESV, and we analyze TSC2 ubiquitination status with FK2 antibody, which recognizes mono- and poly-ubiquitinated proteins but no free ubiquitin. In response to NAM, there was an increase in TSC2 lysine acetylation and FK2-TSC2 concomitantly, favoring its degradation by ubiquitin system. In contrast, RESV diminished TSC2 acetylation as well as ubiquitination, facilitating its stability (Fig. 2B). In this regard, we detected a lower amount of TSC2 in NAM treatment when we corrected the levels of immunoprecipitated TSC2. In contrast, after RESV stimulation, an increased in TSC2 protein levels was observed after the immunoprecipitation analysis (Fig. 2B). To corroborate this differential observation, we submitted HEK 293T to cycloheximide, an inhibitor of protein biosynthesis, for 30 min, and then, cells were stimulated with NAM or RESV for 2 and 4 h. The level of TSC2 protein in response to RESV (80% at 4 h after treatment) was significantly higher when compared to NAM (40% at 4 h after treatment) (Fig. 2C), indicating a direct effect of acetylation in diminishing TSC2 stability and degradation.

3.3. An acetyl-mimetic TSC2 mutant enhances mTORC1 signaling

Tuberous sclerosis complex (TSC) is a genetic multisystem disorder associated with constitutive overactivation of the mammalian target of rapamycin (mTOR) pathway and characterized by development of benign tumors in several organs. TSC disorder is caused by mutations within the *Tsc1* or *Tsc2* genes that inactivate the genes' tumor-suppressive function and drive hamartomatous cell growth. To determine the possible residue(s) of TSC2 lysine acetylation, we used 3 different programs for prediction of acetylation in internal residues (PAIL) software (http://bdmpail.bocuckoo.org) [32], Lysacet 1.1 (http://www.biosino.org/LysAcet/) [33] and Musite (musite.net) [34]. Then, we decided to explore the effects of two TSC2 mutants in lysine residues K106 and K599 (K106Q and K599M) that were predicted as possibly

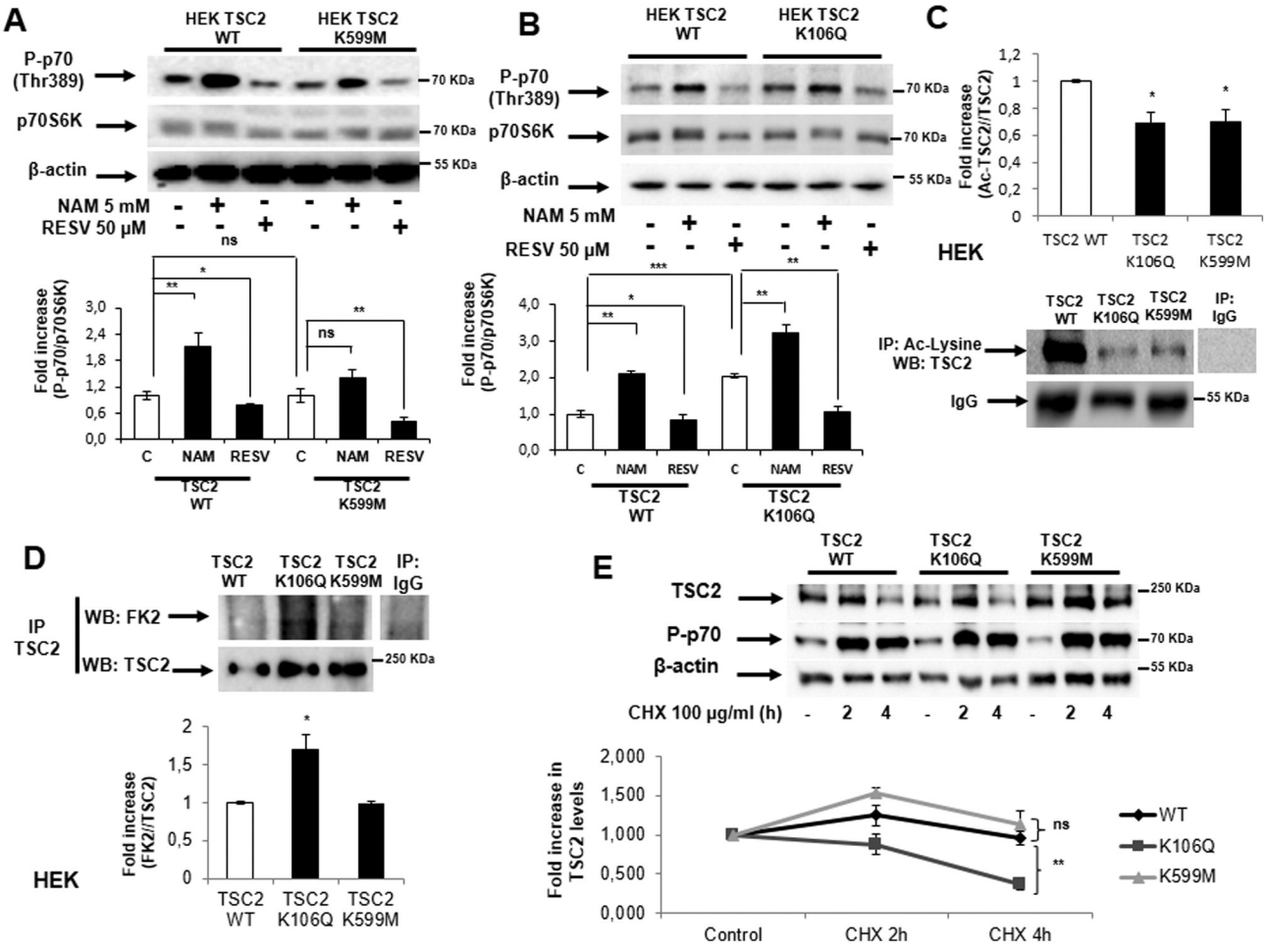

Fig. 3. TSC2 K599M mutant presents lower mTORC1 activation in response to NAM and TSC2, whereas K106Q variant shows a highly increased basal mTORC1 activity. HEK 293T cells were transiently co-transfected with Flag-SIRT1 and TSC2 WT or the TSC2 K599M or K106Q mutant form, 1 d after transfection, the medium was replaced, and 24 h later, cells were stimulated for 2 h- with NAM or RESV, and cell extracts were tested to check mTORC1 activity (**A**–**B**). All the blots are representative from at least 3 independent experiments and the corresponding fold-increase in the ratio P-p70 (Thr389)/p70S6K is shown in the graphs below and are expressed as mean ± s.d., * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. n.s indicates no statistical significance. Cell extracts were then submitted to immunoprecipitation of TSC2 protein to determinate its acetylation and ubiquitination status. The corresponding fold-increase in the ratio Ac-Lys-TSC2/TSC2 (**C**) and FK2-TSC2/TSC2 (**D**) of the blots are representative from 3 independent experiments and are expressed as mean ± s.d., * $P < 0.05$.

acetylated by the different programs, located in the N-terminal region of the protein (1–904) [35,36]. Multiple putative deacetylation sites by SIRT1 protein were identified using a web server for lysine acetyl transferase-specific acetylation site prediction program using a new method based on sequences and functions based prediction (ASEB: acetylation set enrichment-based) [37,38]. Then, K106 and K599 are two *bona fide* candidate residues affected by acetylation probably regulated by SIRT1 activity modulation (Supplemental Table 1). In order to validate that both residues were indeed affected by acetylation/deacetylation status we co-transfected HEK 293T with Flag-SIRT1 and TSC2 wild-type (TSC2-WT) or TSC2 K599M. NAM stimulated mTORC1 signaling at a lower extent in K599 M variant compared to the WT. RESV was also capable of diminishing mTORC1 (Fig. 3A). Interestingly, another mutant form of TSC2 from a patient with TSC (K106Q), which represents an acetyl-mimetic variant, revealed a higher mTORC1 basal activity when compared with TSC2WT. Then, we analyzed if these TSC2 mutants presented a differential autophagy activation in response to NAM or RESV. We observed that the ratio LC3B-II/β-actin increased in response to RESV in TSC2 WT and in K599M mutant. However, in K106Q there was a reduction in LC3B-II/β-actin ratio. For analyzing autophagic flux, we submitted the cells to RESV with or without CQ. Our data indicate that in K599M mutants there is a positive autophagic flux in response to RESV. In contrast, in K106Q there is a blockade in the autophagic flux (Supplemental Fig. 5). Furthermore, NAM and RESV still were able to modulate mTORC1 signaling (Fig. 3B). The acetylation status diminished in both TSC2 mutants, indicating that K106 and K599 are really acetylated (Fig. 3C). In addition, a higher ubiquitination was observed in the acetyl-mimetic mutant compared with the WT protein. In contrast, the K599 TSC2 mutant presented a lower ubiquitin ligation (Fig. 3D). Then, we analyzed TSC2 protein stability using the different mutant forms of TSC2. When we compared TSC2 protein levels, we clearly observed a clear reduction in TSC2 stability in K106Q mutant compared with TSC2 WT (TSC2 WT) or TSC2 K599M (Fig. 3E).

3.4. Regulation of mTORC1 signaling and autophagy by SIRT1 modulators in a dose-dependent manner

To explore the role of mTORC1 modulation in the control of autophagy activation, we subjected MIN6 cells to different NAM and RESV treatments. In response to RESV, we observed dose-dependent reduction in mTORC1 signaling and concomitantly, an increase in LC3B-II form (Fig. 4A and C). In contrast, after NAM stimulation, mTORC1 activity increased in a dose-dependent manner and we did not detect an increase in LC3B-II (Fig. 4B and D). Similar effects were obtained in the time-course using both treatments in MIN6 cells (Supplemental Fig. 6A and 6B). Accordingly, we explored the role of NAM and RESV in HEK 293T cells, and similar results were also observed (Supplemental Fig. 6C). In addition, we analyzed the effects of NAM and RESV on the regulation of autophagic flux. MEFs WT stably transfected with GFP-LC3B were pre-treated with chloroquine (CQ), a lysosomotropic agent

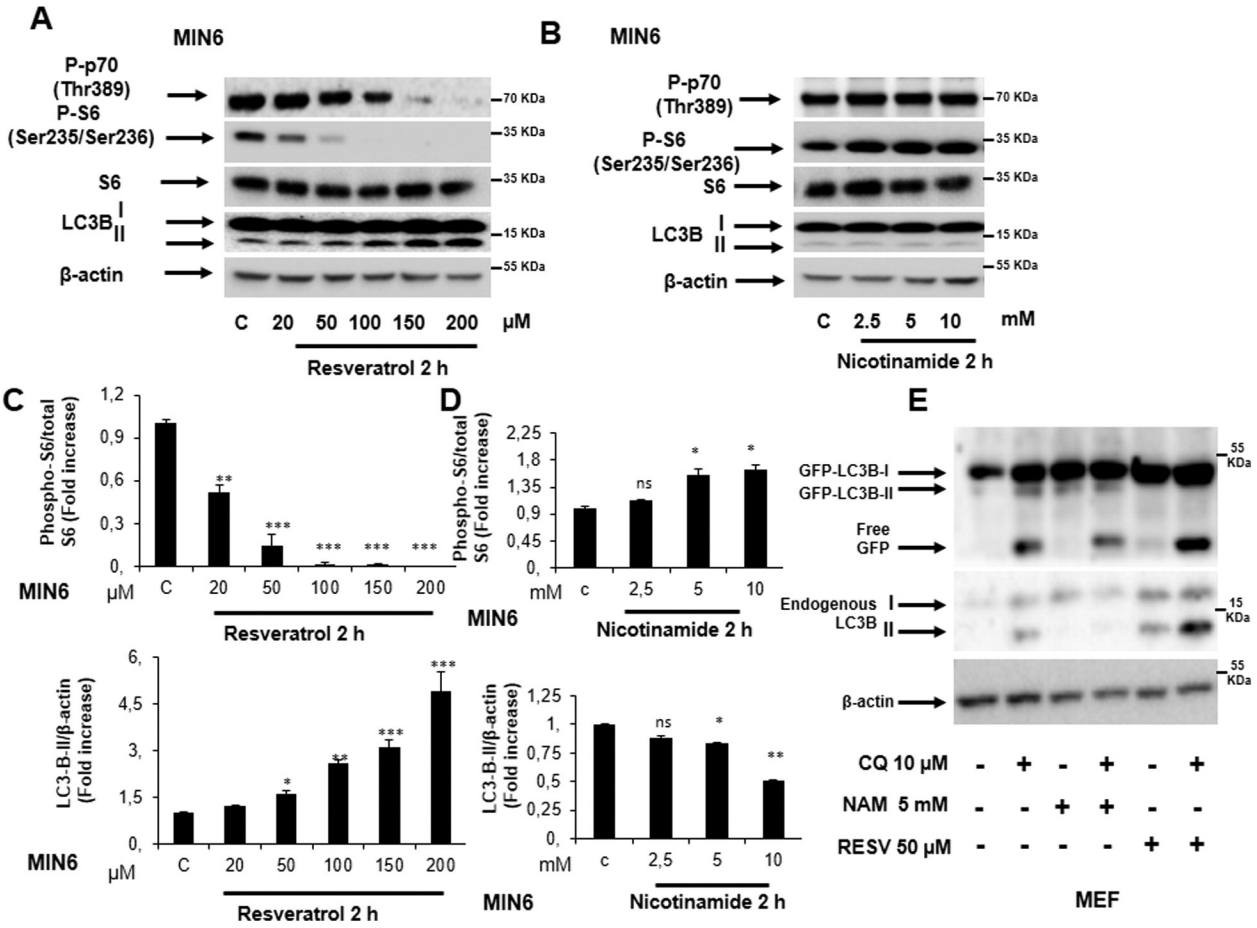

Fig. 4. NAM stimulates and RESV inhibits mTORC1 signaling modulating autophagy in a time and dose-dependent manner. MIN6 cells were cultured in complete medium and untreated or treated with the indicated concentrations of RESV (**A**) or NAM (**B**) for 2 h. (**C–D**) Plots representing the quantification of three different experiments and statistical analysis of phospho-S6/total S6 or LC-3B/β-actin stimulated with RESV (**C**) or NAM (**D**) for 2 h. Representative blots from three independent experiments are shown. (**E**) *Tsc2* +/+ MEFs stably transfected with EGFP-LC3B construct were stimulated with NAM (5 mM) or RESV (50 μM) in the presence or absence of CQ (10 μM). LC3B protein levels as well as free GFP levels were analyzed by Western blotting. A representative blot from three independent experiments is shown. Fold-increase of phospho-S6 (Ser235/Ser236)/total S6 and LC3B-II/β-actin are shown and are expressed as mean ± s.d., * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

which blocks autophagolysosome formation and protein degradation by raising lysosomal pH, in order to inhibit a proper autophagy response. Then, cells were stimulated with NAM or RESV, and autophagic flux was determined by the generation of free-GFP. CQ increased the autophagic marker LC3B-II as well as free-GFP, NAM had no effect on free-GFP neither on LC3B-II, while RESV further increased free-GFP in the presence and in the absence of chloroquine (CQ). These data clearly indicate that RESV is a potent inductor of autophagic flux (Fig. 4E). To assess that RESV activates AMPK signaling pathway properly, we submitted MEF TSC2 +/+ and TSC2 −/− cells to RESV at 50 μM. As it can be observed, RESV stimulated AMPK signaling in both cell types (Supplemental Fig. 7).

3.5. The effects of nicotinamide and resveratrol on mTORC1 signaling and autophagy modulation are TSC2-dependent

To further study the role of TSC2 in the control of mTORC1 by NAM and RESV, we used *Tsc2*-knockdown MIN6 cells and *Tsc2* +/+ and −/− MEFs. As expected, *Tsc2* knockdown induced sustained mTORC1 activation in MIN6 as well as in MEF *Tsc2* −/− (Fig. 5A and B). NAM stimulated mTORC1 signaling, and inhibited autophagy in control MIN6 and *Tsc2* +/+ MEFs, while RESV diminished mTORC1 signaling and increased the autophagic marker LC3B-II in the same cell lines (Fig. 5A and B). Neither NAM nor RESV affected mTORC1 signaling or autophagy in *Tsc2* −/− MEFs or MIN6 *Tsc2* knockdown cells (Fig. 5A and B). These data suggest that TSC2 is necessary for mediating the effects of both NAM and RESV, on mTORC1 signaling and autophagy.

3.6. Nicotinamide stimulates and resveratrol blocks cell proliferation

Others and we have demonstrated that mTORC1 is a key molecule that affects pancreatic β cell proliferation *in vitro* and *in vivo* [31,39], as well as pancreatic β cell mass *in vivo* [40–43]. We determined that changes in mTORC1 signaling affect cell proliferation in response to NAM or RESV. Under growing conditions, NAM treatment increased pancreatic β cell numbers from 24 to 72 h (Fig. 6A). In contrast, RESV as well as rapamycin, a specific inhibitor of mTORC1 and a classic inductor of autophagy, reduced cell numbers (Fig. 6A). To further confirm the effect of both NAM and RESV on proliferation, we performed cell cycle analysis by flow cytometry in MEFs WT. In addition, NAM increased the S/G2/M population in cell cycle analysis by flow cytometry (Fig. 6B). When we treated MEFs with RESV, an accumulation of cells in S/G2/M phase was also observed (Fig. 6B), but the total cell number was reduced, indicating that RESV blocks cell cycle progression (Fig. 6C). To corroborate that TSC2 in its acetylated state promotes cell proliferation, we transiently transfected HEK or MIN6 cells with the different TSC2 constructs. TSC2 K106Q increased cell proliferation in both cell types. In contrast, TSC2 K599M mutant diminished cell progression (Supplemental Fig. 8).

4. Discussion

The TSC2-TSC1 complex controls mTORC1 activation by acting as a GAP towards *Rheb*. TSC2 is phosphorylated by multiple kinases that either activate or inactivate the complex [28]. However, nothing is known about TSC2 lysine acetylation and its role in the modulation of mTORC1 activity. Interaction of TSC2 with SIRT1 in HeLa cells has been previously reported [25]. In that paper, the authors described for the first time that SIRT1 and TSC2 interacts *in vitro*, not affecting to TSC2 acetylation and modulating mTOR signaling. Although the authors did not detect acetylation of TSC2 by the use of immunoblotting nor by mass spectrometry, it cannot be ruled out that possibility. Here, in this paper, we demonstrate that TSC2 is lysine acetylated and this modification directly affects mTORC1 activity. Our data indicates that NAM, a specific sirtuin inhibitor, increases TSC2 acetylation favoring mTORC1 activation. In contrast, RESV, a sirtuin activator, avoids TSC2 acetylation and blocks activation of mTORC1. Arrest-defective protein 1 (ARD1), an atypical acetyl transferase, which presents both N-terminal acetyl

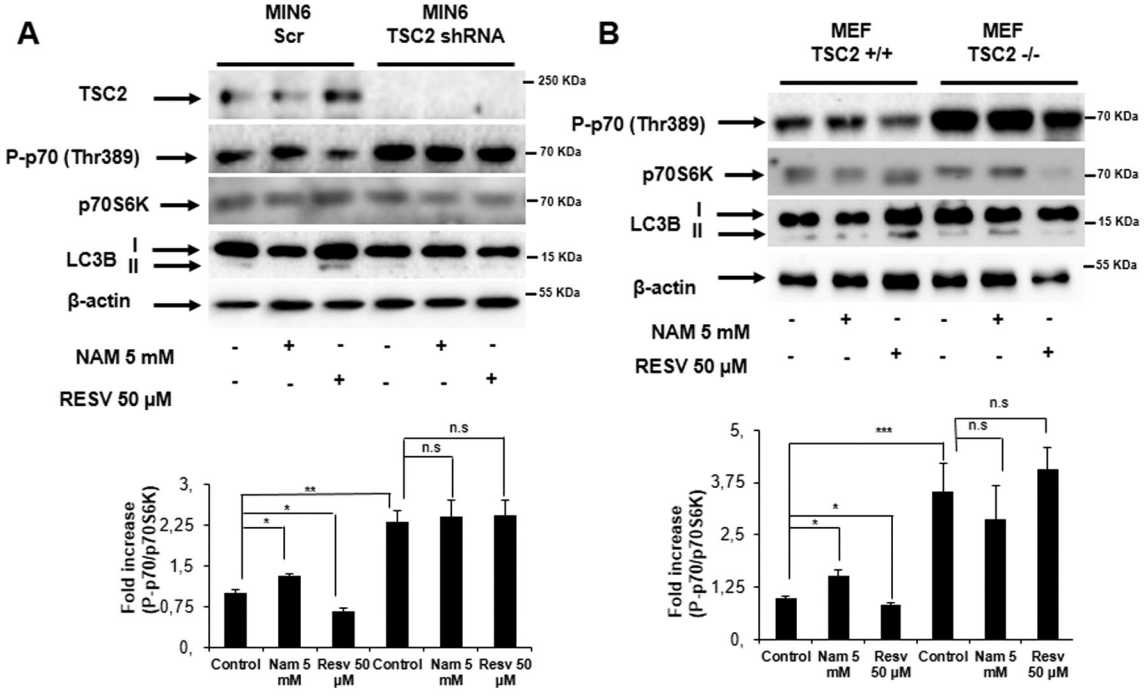

Fig. 5. The effects of NAM or RESV on mTORC1 signaling and autophagy modulation are TSC2-dependent. (**A**) MIN6 Scr and *Tsc2* shRNA or (**B**) *Tsc2* +/+ and *Tsc2* −/− MEFs were stimulated or not with 5 mM of NAM or 50 μM of RESV for 2 h in complete medium. Representative immunoblots indicating mTORC1 activity and LC3B levels from 3 independent experiments are shown. Fold-increase in the ratio P-p70 (Thr389)/p70S6K is shown in the graphs below and are expressed as mean ± s.d., * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. n.s indicates no statistical significance.

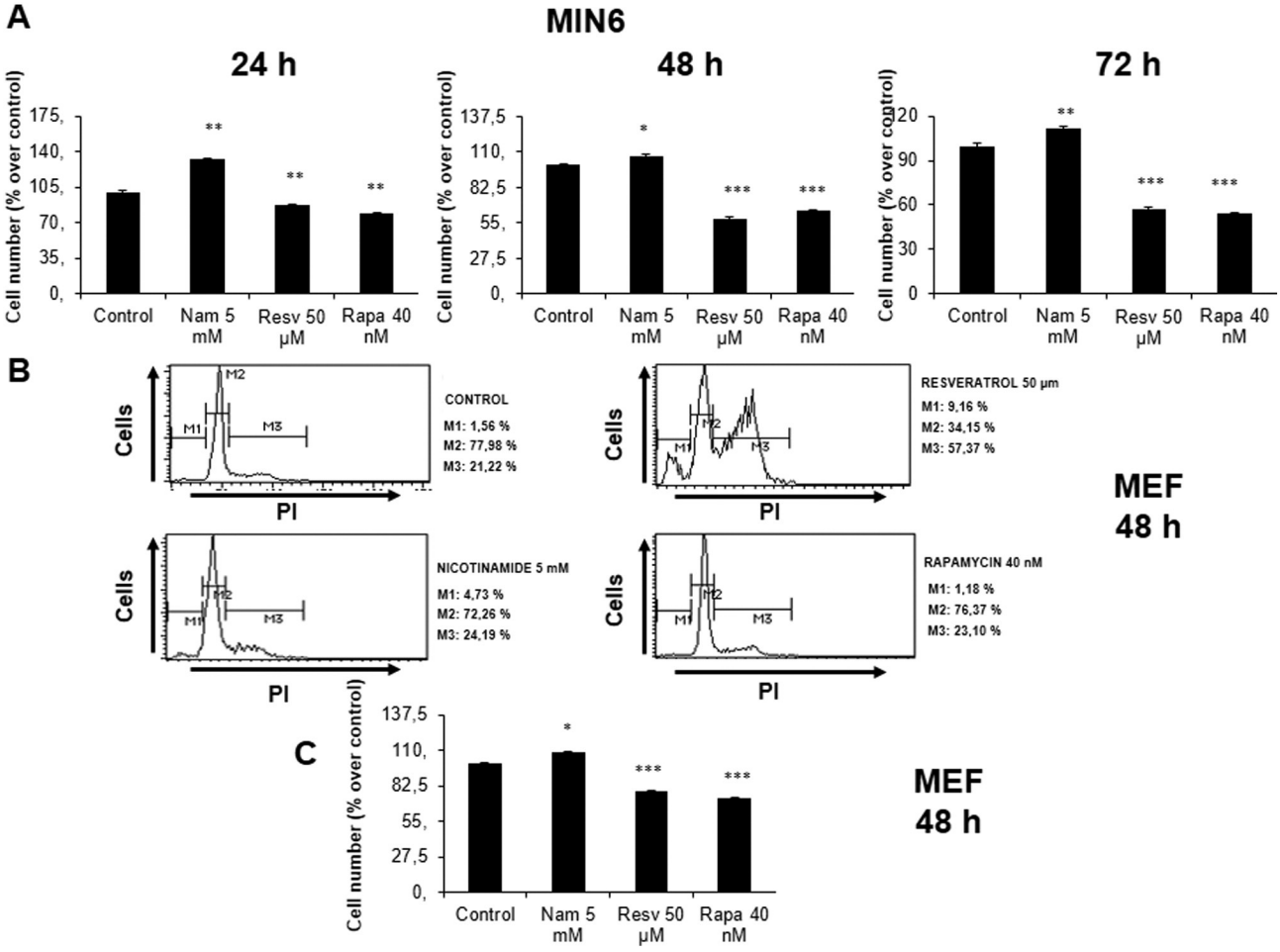

Fig. 6. NAM stimulates and RESV impairs cell proliferation. (**A–C**) MIN6 cells and MEFs cells were incubated in DMEM medium containing 25 mM glucose, 2% FBS and treated with NAM, RESV or rapamycin (RAPA) at the indicated doses for 2 h. Then, the medium was then replaced for the indicated times. The next day this protocol was repeated. (**A**) MIN6 cells were submitted to violet crystal assay. Percentages of cell number over control from 24 h to 72 h are shown. (**B–C**) MEFs cells from 48 h were fixed and DNA content was measured by PI staining and flow cytometry (**B**). These cells were also submitted to violet crystal assay. Percentages of cell numbers from 48 h are shown, expressed as mean ± s.d., * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

transferase as well as lysine acetylation activities interacts with TSC2, increasing its N-terminal acetylation. This modification stabilizes TSC2 and represses mTORC1 activation. This observation is in contrast to our data presented in this report, probably explained by the different type of modification examined. In Kuo's paper, ARD1 did not affect TSC2 lysine acetylation levels [26]. In contrast, ARD1 present a lysine acetyl transferase activity in other proteins such as beta catenin, modulating proliferation [44].

It is known that protein acetylation affects to different enzymes that control metabolism [45]. Furthermore, this modification is dependent on cellular energetic status [46]. For instance, acetylation of human phosphoenolpyruvate carboxykinase 1 (PEPCK1), was associated with a decreased in protein stability due to its interaction with UBR5, an ubiquitin ligase that promotes its proteasomal degradation. Conversely, the deacetylase SIRT2 stabilized PEPCK1, promoting its gluconeogenic action [47]. In contrast, acetylation of ATP-citrate lyase, a protein involved in the cytosolic synthesis of acetyl-CoA, stabilizes the enzyme, promoting lipid biosynthesis [48]. Our results indicate that TSC2 lysine acetylation (stimulation with NAM) elicits an inhibition of TSC2 protein by promoting its ubiquitination state and its degradation by ubiquitin system, thereby stimulating mTORC1 signaling. In fact, our results clearly show that NAM affects to the half-life of TSC2 protein increasing cell proliferation. The same results were observed when we used the acetyl-mimetic mutant form in K106 (K106Q), which it is susceptible to be regulated by acetylation using different prediction programs. In this regard, two TSC2 disease mutants (R611Q and R905Q), consisting in a change between an arginine, that is not modified by acetylation, with glutamine, considered as an acetyl-mimetic, which present an increased in ubiquitination, with the outcome of total level of TSC2 protein decreased in both mutants [49]. In contrast, the stimulation with RESV was associated with a lower level of TSC2 acetylation status as well as a deubiquitination, concomitantly with an increased in half-life of TSC2. Using musite software, we could detect K599 as a possible lysine modified by acetylation. In this regard, using a specific mutant form of K599M we observed the same results as RESV stimulation, increasing half-life of the protein as well as a reduction in cell proliferation. It is important to take into account that the mTORC1/p70S6K/S6 signaling pathway is involved in protein synthesis and, under normal conditions, should be activated. However, after RESV treatment, which mimics a calorie restriction, TSC2 acetylation is blocked, inhibiting mTORC1 signaling.

Using a SIRT1 mutant that localized exclusively in the cytoplasm (SIRT1-NLS2-GFP), NAM further stimulated, and RESV stronger diminished TSC2 acetylation compared to the effects observed in SIRT1-WT. In this regard, enucleated cells expressing a cytoplasmic-restricted SIRT1 mutant were able to induce autophagy after RESV or spermidine stimulation [50]. We have observed that mTORC1 can be modulated by TSC2 lysine acetylation, which is controlled by the cytosolic action of SIRT1. In another study it was shown that under starvation conditions, SIRT1 can directly control autophagy by deacetylation of

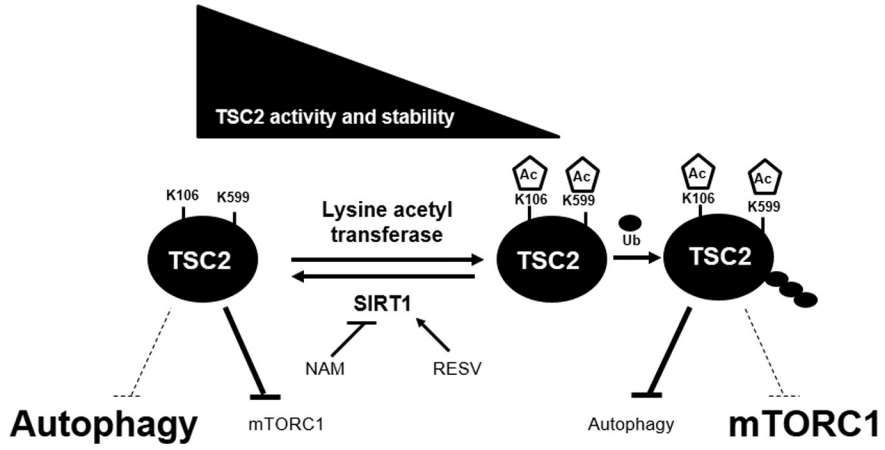

Fig. 7. Scheme depicting the differential proposed effect of NAM and RESV on mTORC1 signaling and modulation of proliferation and autophagy.

autophagy related (Atg) proteins [51]. Accordingly, RESV was not able to induce autophagy in MEF *Sirt1* −/− MEFs. However, it was still able to inhibit mTORC1 signaling. RESV stimulated AMPK and could inhibit mTORC1 by a TSC2-independent pathway through Raptor Ser722 and Ser792 phosphorylation [52]. Here, we demonstrate that SIRT1 is sufficient for the control of mTORC1. Recently, an additional mechanism that could control mTORC1 activation independently from SIRT1 was described. RESV inhibited leucine-induced mTOR activation promoting mTOR and DEP domain-containing mTOR-interacting protein (DEPTOR) association [53]. DEPTOR is a component of both mTORC1 and mTORC2 complexes and negatively modulates mTOR activation [54]. Our results indicate that in MEF *Sirt1* −/− there was an increase in TSC2 acetylation and mTORC1 signaling, which indicates that SIRT1 is a negative regulator of mTORC1 and accordingly, an increase in the TSC2 acetylation basal levels. The data obtained in MIN6 cells with the SIRT1 inhibitor (EX-527), corroborated the data obtained in *Sirt1* −/− cells. Basal lysine acetylation levels are increased for around 1800 proteins in *Sirt1* −/− MEFs, affecting essential processes such as chromatin regulation, metabolism and differentiation [55].

It is well established that mTORC1 activity is involved in cell cycle progression under normal conditions and is dysregulated in cancer, T2DM and in other pathologies [56]. Then, using different mTORC1 inhibitors could be a promising option for therapy of these diseases. In fact, TSC2 is considered as a tumor suppressor since its elimination provokes an increase in protein synthesis and, indirectly, in cell growth [39]. NAM stimulates and RESV inhibits mTORC1 signaling in a time and dose-dependent manner. Interestingly, K106Q mutant, which increased mTORC1 signaling, enhanced cell proliferation at the basal state. In contrast, K599M, which decreased mTORC1 signaling, prevented proliferation. After RESV stimulation, we observed a decrease in the number of cells. Paradoxically, in the flow cytometry we detected an accumulation in the S and G2/M phase. This effect has been observed in other cell types and it could indicate that RESV presents a role in DNA repair and checking replication before mitosis transition [57,58]. In contrast, NAM inhibits and RESV stimulates autophagy. Concomitantly, K106Q mutant, apart from increasing cell proliferation and mTORC1 signaling, was able to inhibit autophagic flux. In contrast, K599 M, inhibited cell proliferation, decreased mTORC1 signaling and promoted autophagic flux. These effects are observed in control MIN6 cells and *Tsc2* +/+ MEFs. *Tsc2* knockdown activates mTORC1 but, interestingly, mTORC1 and autophagy modulation by both NAM and RESV were impaired in MIN6 *Tsc2* knockdown cells and in *Tsc2* −/− MEFs. These data indicate that the modulation of mTORC1 is TSC2-dependent, as previously suggested [25]. Previously we showed that *Tsc2* knockdown in pancreatic β cells, or overexpression of a constitutive active form of Rheb (Rheb Q64L), inhibit autophagy, and these cells are more susceptible to endoplasmic reticulum (ER) stress [4]. Upon TSC2 depletion, neither NAM nor RESV were able to mediate their respective effects on mTORC1 signaling and autophagy. To summarize, Fig. 7 depicts a proposed mechanism for the modulation of autophagy and proliferation in response to NAM or RESV through the control of K106 and K599 acetylation status by SIRT1 activity. However, further studies will be necessary for a better understanding of the role of differential TSC2 acetylation in the control of cell homeostasis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2016.08.006.

Transparency document

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

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Author contributions: AGA and CG designed and performed all of the experiments and wrote the paper, MN provided constructs and contributed to the discussion. MB contributed to the assessment and discussion of the manuscript.

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