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Sirtuin 5 protects mitochondria from fragmentation and degradation during starvation

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ABSTRACT

During starvation, intra-mitochondrial sirtuins, NAD^+ sensitive deacylating enzymes that modulate metabolic homeostasis and survival, directly adjust mitochondrial function to nutrient availability; concomitantly, mitochondria elongate to escape autophagic degradation. However, whether sirtuins also impinge on mitochondrial dynamics is still uncharacterized. Here we show that the mitochondrial Sirtuin 5 (Sirt5) is essential for starvation induced mitochondrial elongation. Deletion of Sirt5 in mouse embryonic fibroblasts increased levels of mitochondrial dynamics of 51 kDa protein and mitochondrial fission protein 1, leading to mitochondrial accumulation of the pro-fission dynamin related protein 1 and to mitochondrial fragmentation. During starvation, Sirt5 deletion blunted mitochondrial elongation, resulting in increased mitophagy. Our results indicate that starvation induced mitochondrial elongation and evasion from autophagic degradation requires the energy sensor Sirt5.

1. Introduction

Mitochondrial oxidative phosphorylation (OXPHOS) converts most of the energy found in nutrients into ATP, required for cellular endergonic reactions. These key organelles also regulate several other important physiological processes, such as calcium homeostasis, oxidative stress and apoptosis [1]. Mitochondria are highly dynamic organelles: they form a reticulum continuously remodeled by fission and fusion events. In mammalian cells, mitochondrial fragmentation is mainly regulated by DRP1 [2]. During mitochondrial fission, DRP1 is recruited to the mitochondrial outer membrane allowing membranes constriction. Fusion of mitochondria is primarily controlled by mitofusin (MFN) 1 and 2, located on the mitochondrial outer membrane, and OPA1, located in the inner membrane [3]. By controlling the shape, length and number of mitochondria, fission and fusion events modulate mitochondrial functions, including OXPHOS, reactive oxygen species (ROS) production and apoptosis [4,5]. Because ATP production must be maintained, mitochondrial structure and activity need to be finely regulated at different levels to allow survival of cells in challenging metabolic conditions.

Nutrient excess is associated with lower mitochondrial mass, lower mitochondrial DNA (mtDNA) amount and impaired OXPHOS, whereas low nutrient availability promotes OXPHOS [6–9]. In obese and diabetic patients, mitochondrial respiration and ATP production are impaired, activities of the respiratory chain and tricarboxylic cycle enzymes are lowered, and ROS production is increased [6–8]. The mitochondrial structure is also modified by nutrient availability. During starvation, mitochondria elongate *in vitro* and *in vivo* [9,10]. This elongation process allows maintaining ATP production *via* lower mitochondrial degradation and increased dimerization and activity of ATP synthase [9]. Nutrient excess is associated with fragmented mitochondria in different *in vitro* and *in vivo* models [11,12]. For instance, deletion of the protease OMA1 impairs OPA1 processing and causes obesity [13]. Biopsies of skeletal muscle from subjects with type II diabetes and obesity reveal mitochondria of smaller size [14,15]. Therefore, mitochondrial fusion and fission mechanisms represent a key step in the mitochondrial adjustment to challenging nutrient conditions.

Recently, sirtuins emerged as crucial molecular sensors of cellular energy balance [16,17]. Since sirtuin enzymatic activity requires NAD^+ as a cofactor, any energy stress that modifies NAD^+ levels impacts on their activity. For instance, several sirtuins target proteins are involved in the increased lipid and amino acid oxidation to derive energy during fasting [18,19]. There are seven sirtuin members in mammals, among which mitochondria-localized sirtuins (including Sirt3 and Sirt5) play a critical role in mitochondrial functions. During fasting, caloric restriction and exercise, the rise in NAD^+ level increases Sirt3 expression and activity [20]. Activated Sirt3 deacetylates several proteins to impact on mitochondrial processes, such as OXPHOS, fatty acid oxidation, ketone body production and oxidative stress management [20]. Disruption of Sirt3 either by genetic ablation or during high-fat diet results in hyperacetylation of mitochondrial proteins, mitochondrial dysfunctions and accelerated development of metabolic abnormalities [21,22]. Sirt5 was recently shown to possess a strong desuccinylase activity [23–25].

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Analyses of the lysine succinylation proteome in wild-type (*Sirt5*$^{+/+}$) and Sirt5 knock out cells (*Sirt5*$^{-/-}$) revealed several potential targets of this sirtuin within mitochondria, including enzymes involved in OXPHOS, tricarboxylic cycle, amino acid degradation and fatty acid metabolism [25,26]. Therefore, sirtuins represent key modulators of mitochondria, allowing this organelle to adjust according to nutrient availability, to maintain metabolic homeostasis and eventually, survival.

Despite the apparently convergent effect on the metabolic adaptation to challenging metabolic conditions, the interaction between sirtuins and mitochondrial dynamics is still uncharacterized. We set out to genetically investigate how Sirt5 impacts on mitochondrial activity and structure. We show that deletion of Sirt5 leads to mitochondrial DRP1 accumulation, mitochondrial fragmentation and accordingly to mitochondrial degradation during autophagy. These results suggest that Sirt5 modulate mitochondrial dynamics in order to maintain metabolic homeostasis during challenging metabolic conditions.

2. Materials and methods

2.1. Cell culture and transfection

Immortalized wild-type (*Sirt5*$^{+/+}$) and Sirt5 knock-out (*Sirt5*$^{-/-}$) mouse embryonic fibroblasts (MEFs) were kindly provided by Dr. Yingming Zhao (University of Chicago) and Dr. David Lombard (University of Michigan). MEFs were maintained in culture with high glucose (4.5 g l^{-1}) DMEM (Dulbecco's modified Eagle's medium) supplemented with 2 mM glutamine, 1 mM pyruvate and 10% (v/v) fetal bovine serum. All of the cells were kept in a 5% CO_2 atmosphere at 37 °C and the medium was renewed every 2 to 3 days. Analysis and cell harvesting were always performed when confluency was about 80–90%. Mitochondrially targeted dsRED (mtRFP) was a gift from M. Zaccolo (Venetian Institute of Molecular Medicine, Padua, Italy). Sirt5-FLAG was obtained from Addgene (plasmid 13816). Plasmids were transfected using polyethylenimine (PolySciences). Analyses were performed 48 h following transfection. For starvation assays, cells were incubated in Hank's Balanced Salt Solution (HBSS) for the indicated period of time, as indicated in [9].

2.2. Subcellular fractionation, chemical cross-linking and western blot

The cells were harvested, resuspended in isolation buffer (250 mM sucrose, 1 mM EDTA, 5 mM HEPES, pH 7.4) and disrupted with 25 strokes using a 25G needle. The total cell lysate was centrifuged at 500*g* (4 °C) to remove cells debris and nuclei. The supernatant was kept and centrifuged at 12,500*g* for 10 min (4 °C). Then, the supernatant was kept (cytosolic fraction) and the pellet was resuspended and the centrifugation cycle was repeated. Finally, the mitochondrial fractions were obtained from the last pellet. Purity of the different fractions was tested by immunoblotting of the cytosolic α-tubulin and the mitochondrial proteins SDHA.

For cross-linking experiments, *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs were treated with the non-cleavable crosslinker bismaleimidohexane (BMH; 1 mM) at 4 °C. After 30 min, the reaction was stopped by addition of 100 mM glycine and cells were harvested in lysis buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, supplemented with protease inhibitors), and incubated at 4 °C. After 30 min, the lysate was centrifuged at 12,500*g* for 10 min at 4 °C. The supernatant was then processed for western blotting.

For western blotting, the samples were boiled in SDS PAGE sample buffer and subjected to SDS-PAGE (12.5%). Alternatively, mitochondria from *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs were solubilized with *n*-dodecylmaltoside (2.5 g mg/mg protein) in 1 M 6-aminocaproic acid, 50 mM BisTris, pH 7.0 buffer, supplemented with glycerol and Coomassie Blue, and subjected to 5–13% gradient BN-PAGE. The separated proteins were then transferred onto a PVDF membrane.

Proteins were immunodetected using antibodies against Sirt5 (Cell Signaling; 8782), α-tubulin (Cell Signaling; 3873 RRID:AB_1904178), SDHA (Abcam; ab14715), succinyllysine (PTM Biolabs; PTM-401), OPA1 (BD Biosciences; 612607), DRP1 (BD Biosciences; 611113), pDRP1-S616 (Cell Signaling; 3455), pDRP1-S637 (Cell Signaling; 4867), MFN2 (Sigma; M6444), MFN1 (Abnova; H00055669-M04 RRID:AB_581724), Fis1 (ProteinTech; 10956-1-AP RRID:AB_2102532), MiD49 (ProteinTech; 16413-1-AP), MiD51 (ProteinTech; 20164-1-AP), MFF (Sigma; HPA010968), β-actin (Sigma; A1978 RRID:AB_476692), FLAG (Sigma; F1804), Hsp60 (Santa Cruz; sc-13966 RRID:AB_2121457), NDUFA9 (Abcam; ab14713 RRID:AB_301431), COX1 (Abcam; ab14705 RRID:AB_2084810) and MnSOD (Santa Cruz; sc-30080). Primary antibodies were revealed with a horseradish peroxidase-conjugated F(ab′)$_2$ fragment of anti-mouse or anti-rabbit fragment-specific (Jackson ImmunoResearch Laboratories, West Grove, PA). The blots were visualized using enhanced chemiluminescence (ECL) Plus from Amersham. Labels were quantified by densitometric analysis using the Image J (NIH) software.

2.3. Oxygraphic measurements

Mitochondrial oxygen consumption assays were performed in a glass chamber equipped with a Clark oxygen electrode (Hansatech). Cell respiration was measured using 2×10^6 cells ml^{-1} at 37 °C in a 2 ml chambers at a stirring rate of 750 rpm. Three different states of endogenous respiration with intact cells were measured: (i) basal respiration representing the endogenous physiological coupled state, (ii) respiration with oligomycin (2 μg ml^{-1}) representing the non-coupled resting respiration, and (iii) maximal uncoupled respiration induced by FCCP (0.5 μM steps with 2 μM final concentration) providing a measure of the maximal capacity of ETS under conditions of physiological substrate supply in the intact cell. Respiratory control ration (RCR) was calculated from the ratio between uncoupled and non-coupled respiration.

2.4. Imaging

The shape of the mitochondrial network was evaluated using confocal microscopy of live cells. MEFs seeded onto 24-mm round glass coverslips transfected and treated as indicated were placed on the stage of the IMIC Andromeda system (FEI Munich GmbH) equipped with a 60× oil objective (UPLAN 60× oil, 1.35NA, Olympus), 488 and 561 nm excitation lasers, FF01-446/523/600/677 (Semrock) as emission filter and an Orca 03G CCD camera (Hamamatsu).

Morphometric analysis of mitochondrial shape was carried out as described previously [3]. For each experiment, 50 cells were randomly selected. Stacks of 50 images separated by 0.2 μm along the z axis were acquired. Three-dimensional reconstruction and volume rendering of the stacks were carried out with the appropriate plug-in of ImageJ (NIH).

2.5. Statistical analyses

Data were analyzed with Student's *t*-test and two-way ANOVA as appropriate, using GraphPad Prism (7.00). Significance was assessed at the 0.05 (or lower) level for all tests.

3. Results

3.1. Sirt5 ablation reduces mitochondrial respiration and causes mitochondrial fragmentation

In order to address the relationship between Sirt5, mitochondrial function and shape, we first examined the subcellular localization of Sirt5. Immunoblotting for Sirt5 on subcellular fractions indicated that Sirt5 was retrieved in cytosol and mitochondria (Fig. 1A), consistent

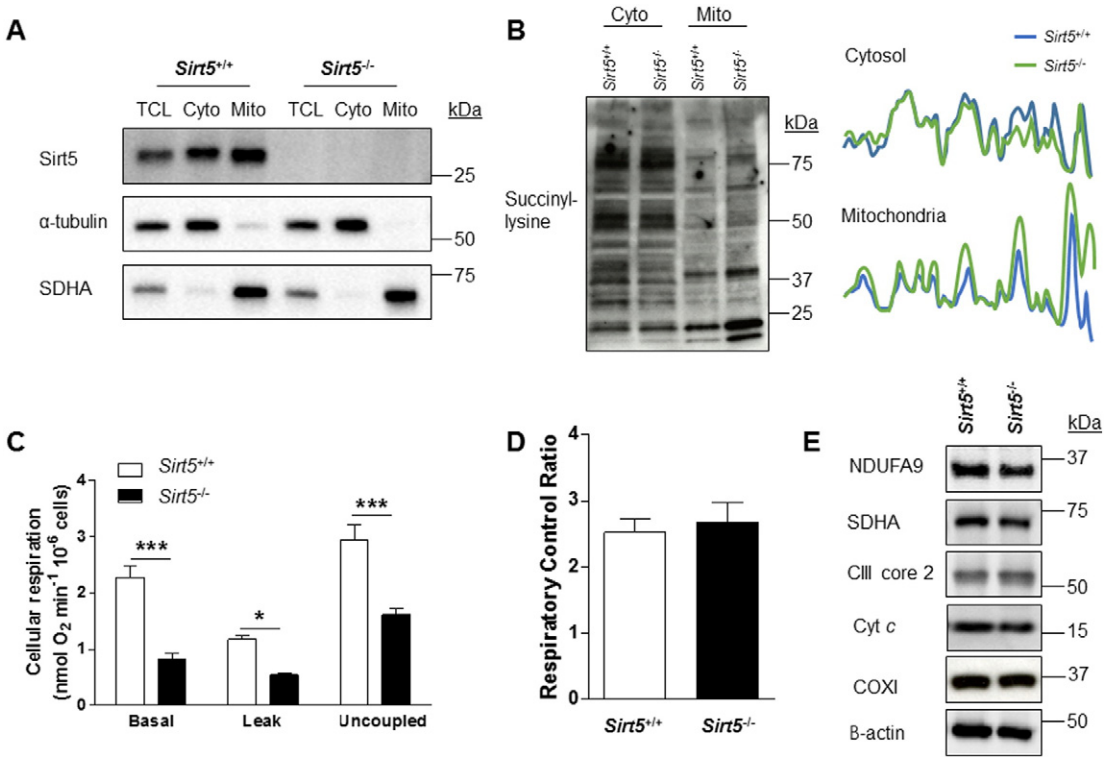

Fig. 1. Deletion of Sirt5 alters mitochondrial respiration. (A) Representative immunoblots (n = 3) of Sirt5, the cytosolic α-tubulin and the mitochondrial succinate dehydrogenase A (SDHA) in total cellular lysate (TCL), cytosolic fraction (Cyto) and mitochondrial fraction (Mito) of *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ mouse embryonic fibroblasts (MEFs) showing the presence of Sirt5 in both cytosolic and mitochondrial compartments. (B) Left, representative immunoblots (n = 3) of succinylated lysine in cytosolic fraction (Cyto) and mitochondrial fraction (Mito) of *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs. Right, band quantification of the immunoblot showing a specific increase of succinylation for several proteins in the mitochondrial fraction. (C) Basal, leak and uncoupled cellular respiration are lower in intact *Sirt5*$^{-/-}$ as compared to *Sirt5*$^{+/+}$ MEFs (n = 5–6). (D) The respiratory control ratio (as calculated by the ratio between uncoupled and leak respiration) is not different between *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs (n = 5–6). (E) Representative immunoblots (n = 3) of the mitochondrial complex I subunit NDUFA9, complex II subunit SDHA, complex III subunit core 2 (CIII core 2), cytochrome *c* (Cyt c), complex IV subunit I (COX I) and beta-actin in total cell lysate of *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs, showing no difference of mitochondrial content upon deletion of *Sirt5*. Data are presented as means ± SEM. $^{*}p < 0.05$; $^{***}p < 0.001$, according to two-way ANOVA followed by Bonferroni post-hoc test.

with previous observations [27,28]. Despite the observed cytoplasmic localization, lysine succinylation was however increased only in the mitochondrial fraction upon genetic deletion of Sirt5, whereas succinylation of cytosolic proteins seemed unaffected (Fig. 1B). These results suggest that mitochondrial Sirt5 is probably more enzymatically active than cytosolic Sirt5 and called for an analysis of the effect of Sirt5 deletion on mitochondrial function. We therefore measured cellular respiration in intact wild-type (*Sirt5*$^{+/+}$) and *Sirt5*$^{-/-}$ mouse embryonic fibroblasts (MEFs). Basal, oligomycin-insensitive (indicative of proton leak across mitochondrial inner membrane), as well as uncoupled respiration induced by the protonophore carbonyl cyanide 4-*p*-trifluoromethoxy phenylhydrazone (FCCP) were all lower in *Sirt5*$^{-/-}$ MEFs than in *Sirt5*$^{+/+}$ MEFs (Fig. 1C). Accordingly, the respiratory control ratio (*i.e.*, the ratio between the uncoupled respiration and the leak respiration) was not significantly different between *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs (Fig. 1D). Overall, these results indicate that deletion of *Sirt5* globally reduces mitochondrial respiration *in situ*, without altering electron transfer, passive proton leak or mitochondrial membrane potential across the inner mitochondrial membrane. The levels of several mitochondrial proteins involved in OXPHOS appeared similar between showed similar *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFS (Fig. 1E), suggesting that the decrease of mitochondrial activity upon *Sirt5* deletion is not due to lower mitochondrial content.

Since mitochondrial shape can also affect mitochondrial activity and respiration, we then compared mitochondrial shape in wild-type and *Sirt5*$^{-/-}$ MEFs expressing a red fluorescent protein targeted to mitochondria (mtRFP). Visual inspection of confocal mtRFP images (Fig. 2A) and morphometric analysis (Fig. 2B) revealed that mitochondria were fragmented in *Sirt5*$^{-/-}$ MEFs (cells with fragmented mitochondrial network: *Sirt5*$^{+/+}$: 19.48 ± 3.40%; *Sirt5*$^{-/-}$: 34.01 ± 3.06%). The observed fragmentation was a specific consequence of Sirt5 ablation, since reconstitution of *Sirt5*$^{-/-}$ MEFs with ectopically expressed Sirt5-FLAG completely corrected the phenotype (Fig. 2B and C). Along the same line, forced expression of Sirt5 in wild-type MEFs increased the proportion of cells with elongated mitochondria (Fig. 2B and C), indicating that it participates in the modulation of mitochondrial shape.

3.2. Levels of Sirt5 modulate DRP1 subcellular localization

In order to understand the molecular mechanism linking absence of Sirt5 to mitochondrial fragmentation, we monitored by immunoblotting levels of the core mitochondria-shaping machinery proteins OPA1, DRP1 and Mfns in wild-type and *Sirt5*$^{-/-}$ cells. While we could not detect differences in OPA1 and MFN1, we observed an important increase in MFN2 levels and a slight increase and/or change in isoform expression of DRP1 (Fig. 2E). However, re-expression of Sirt5 in *Sirt5*$^{-/-}$ MEFs did not reverse the change of MFN2 levels (Supplementary Fig. 1), suggesting that MFN2 is not involved in the fragmented mitochondrial phenotype observed upon deletion of Sirt5. Therefore, we hypothesized that DRP1 might be involved in the regulation of mitochondrial shape by Sirt5. To address this, we compared the localization of DRP1 in cytosolic and mitochondrial fractions of wild-type and *Sirt5*$^{-/-}$ MEFS. Levels of DRP1 were lower in cytosolic fractions and higher in mitochondrial fractions of *Sirt5*$^{-/-}$ MEFs (Fig. 3A). Moreover, ≈200 kDa DRP1-positive band appeared on *Sirt5*$^{-/-}$ mitochondria (Fig. 3A), suggesting its oligomerization to allow mitochondrial

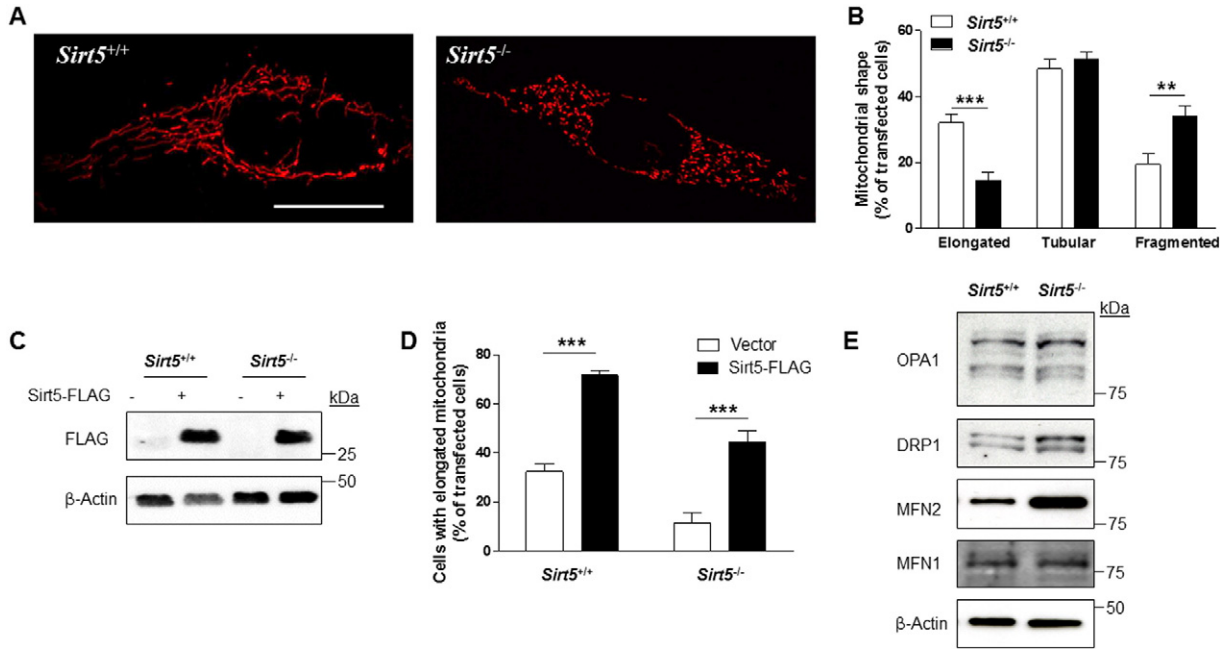

Fig. 2. Sirt5 modulates the structure of the mitochondrial network. (A) Representative micrographs showing the shape of the mitochondrial network of *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs grown on coverslips and transfected with a mitochondria-targeted (mt) RFP (n = 6–9). Scale bar: 20 μm. (B) Morphometric analysis of data presented in *A* (n = 6–9), showing that deletion of *Sirt5* decreases the elongation and increases fragmentation of the mitochondrial network. (C) Representative immunoblots of FLAG and β-actin (n = 3) showing the expression of ectopic Sirt5-FLAG construct in *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs. (D) Morphometric analysis of *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs grown on coverslips and co-transfected with mtRFP and Sirt5-FLAG (n = 3), showing that overexpression of Sirt5 induces elongation of mitochondria in both *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs. (E) Representative immunoblots of OPA1, DRP1, MFN2, MFN1 and β-actin in total cell lysate of *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs (n = 3; see also Supplementary Fig. 1). Data are presented as means ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$, according to two-way ANOVA followed by Bonferroni post-hoc test.

membrane constriction and ultimately, fragmentation of mitochondria [2]. To confirm the presence of high molecular weight DRP1 oligomers in *Sirt5*$^{-/-}$ MEFS, we performed two independent experiments. First, immunoblotting for DRP1 in lysates of wild-type and *Sirt5*$^{-/-}$ MEFs treated with the non-cleavable cross-linker bismaleimidohexane (BMH) revealed increased DRP1 multimers in *Sirt5*$^{-/-}$ MEFs (Fig. 3B and Supplementary Fig. 2A). Second, BN-PAGE also revealed higher levels of high molecular weight DRP1 (Fig. 3C and Supplementary Fig. 2B). Altogether, these results suggest that increased mitochondrial DRP1 localization and oligomerization participate in the mitochondrial fragmentation observed in *Sirt5*$^{-/-}$ MEFs. Different mechanisms can promote DRP1 recruitment to mitochondria, including phosphorylation status of DRP1 on S616 and S637 and outer mitochondrial membrane receptors for DRP1. Immunoblottings revealed similar levels of

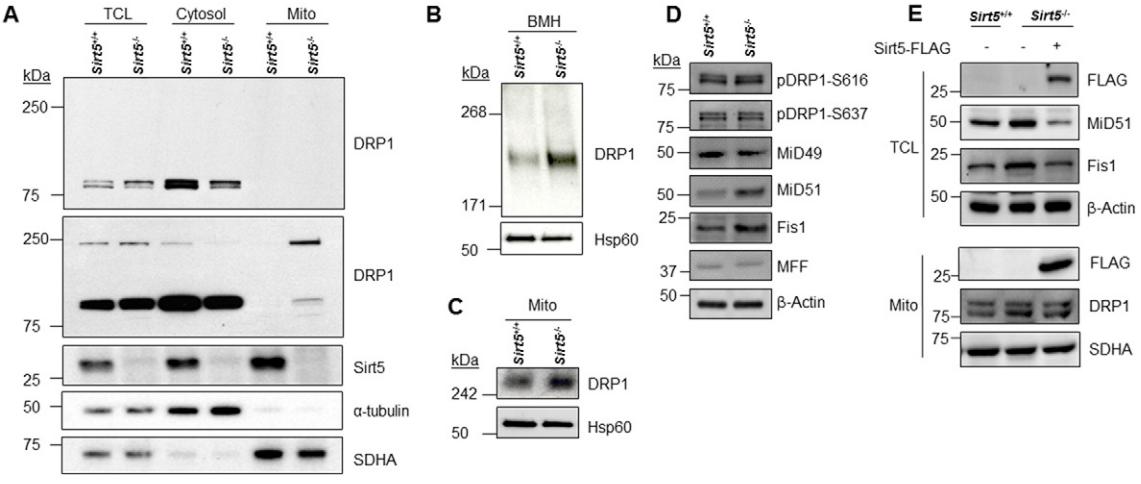

Fig. 3. Sirt5 impacts on the subcellular localization and oligomerization of DRP1. (A) Representative immunoblots of DRP1 (low and high exposition), Sirt5, α-tubulin and succinate dehydrogenase A (SDHA) in total cellular lysate (TCL), cytosolic fraction (Cyto) and mitochondrial fraction (Mito) of *SIRT5*$^{+/+}$ and *SIRT5*$^{-/-}$ MEFs (n = 3). The results obtained show decreased levels of DRP1 in cytosolic fraction and increased levels of DRP1 in mitochondrial fraction in *Sirt5*$^{-/-}$ MEFs. (B) Representative immunoblots of DRP1 and Hsp60 in total cell lysate obtained from *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs treated with the cross-linker bismaleimidohexane (BMH) (see also Supplementary Fig. 2A for quantifications). (C) Representative immunoblots of DRP1 and Hsp60 of *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ mitochondrial lysate separated by Blue-Native PAGE, showing higher levels of DRP1 multimers in *Sirt5*$^{-/-}$ MEFs (see also Supplementary Fig. 2B for quantifications). (D) Representative immunoblots (n = 3) of pDRP1-S616, pDRP1-S637, MiD49, MiD51, Fis1 and MFF in total cell lysate obtained from *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs, showing specific increase in the levels of MiD51 and Fis1 upon deletion of Sirt5 (see also Supplementary Fig. 2C for quantifications). (E) Representative immunoblots (n = 3) of FLAG, MiD51, Fis1 and β-actin in total cell lysate (TCL), and of FLAG, DRP1 and SDHA in mitochondrial fractions (n = 3–4), showing that reintroduction of Sirt5 in *Sirt5*$^{-/-}$ MEFs re-establishes the total levels of MiD51 and Fis1 as well as the levels of mitochondrial DRP1 observed in *Sirt5*$^{+/+}$ MEFs (see also Supplementary Fig. 2D for quantifications).

pDRP1-S616 and pDRP1-S637 in wild-type and *Sirt5*$^{-/-}$ MEFs (Fig. 3D), suggesting that deletion of Sirt5 does not promote DRP1 recruitment to mitochondria through phosphorylation-dependent regulation of DRP1. We next examined the levels of the mitochondrial outer membrane proteins MiD49, MiD51, Fis1 and MFF which were proposed to act as receptors of DRP1 and promote mitochondrial fission [29]. Immunoblottings revealed higher levels of MiD51 and Fis1 in *Sirt5*$^{-/-}$ MEFs whereas the levels of MiD49 and MFF were similar between wild-type and *Sirt5*$^{-/-}$ MEFs (Fig. 3E and Supplementary Fig. 2C). The re-introduction of Sirt5 in *Sirt5*$^{-/-}$ MEFs reversed the impact of Sirt5 deletion on the levels of MiD51 and Fis1, and the recruitment of DRP1 to mitochondria (Fig. 3E and Supplementary Fig. 2), suggesting that deletion of Sirt5 impacts on MiD51 and Fis1 to increase the recruitment of DRP1 to mitochondria and promote mitochondrial fission.

3.3. Sirt5 is required for mitochondrial elongation induced by starvation

The activity of sirtuins depends on the NAD^+/NADH ratio, being upregulated when NAD^+ levels are increased, like during starvation [30] during which DRP1 is retained in the cytoplasm and mitochondria elongate [9]. Therefore, we hypothesized that Sirt5 could modulate mitochondrial morphology during nutrient deprivation. When *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs transfected with mtRFP were starved [9], mitochondria elongated only in wild-type MEFs but not in *Sirt5*$^{-/-}$ MEFs (Fig. 4A and B). Immunoblottings of the different mitochondria-shaping proteins during these conditions revealed lower levels of pDRP1-S637 and increased levels of DRP1 in starved *Sirt5*$^{-/-}$ MEFs (Fig. 4C and Supplementary Fig. 3B) whereas no significant changes were observed for the other pro-fission proteins MiD49, MiD51, Fis1, MFF or the pro-fusion proteins OPA1, MFN1 and MFN2 (Fig. 4C and Supplementary Fig. 3). Globally, these results suggest that Sirt5 is a critical regulator of the adaptive response of mitochondrial shape during nutrient deprivation.

The elongation of mitochondria during starvation protects the organelles from autophagic degradation [9,10]. Thus, *Sirt5* deletion is expected to accelerate mitochondrial degradation during starvation, by inhibiting their elongation. When we measured mitophagy by following over time the relative abundance of a panel of mitochondrial proteins (NDUFA9, COXI and MnSOD) we found that mitochondrial degradation occurred at a faster pace in *Sirt5*$^{-/-}$ MEFs (Fig. 5A). Importantly, the mitochondrial degradation was blocked when starved *Sirt5*$^{-/-}$ MEFs were treated with the autophagy inhibitor bafilomycin (Fig. 5B), indicating that mitochondrial elongation to evade mitophagy during starvation requires Sirt5.

4. Discussion

Sirtuins are crucial regulators of metabolism, but their role in the control of mitochondrial morphology is unclear. Here we show that the mitochondrial Sirt5 regulates mitochondrial morphology by controlling the amount of DRP1 recruited to mitochondria. Notably, Sirt5 is required for starvation-induced mitochondrial elongation and therefore to protect mitochondria from autophagic degradation during limited nutrient availability.

Post-translational modifications of mitochondrial proteins emerged recently as important processes in the regulation of metabolism during challenging conditions. In this context, sirtuins are energy sensors able to directly modulate the enzymatic activity of several metabolic enzymes [30]. Sirt5 is thought to act mainly as a demalonylase and desuccinylase [23,31]. Hundreds of mitochondrial, but also cytosolic and nuclear proteins, can be succinylated, and become

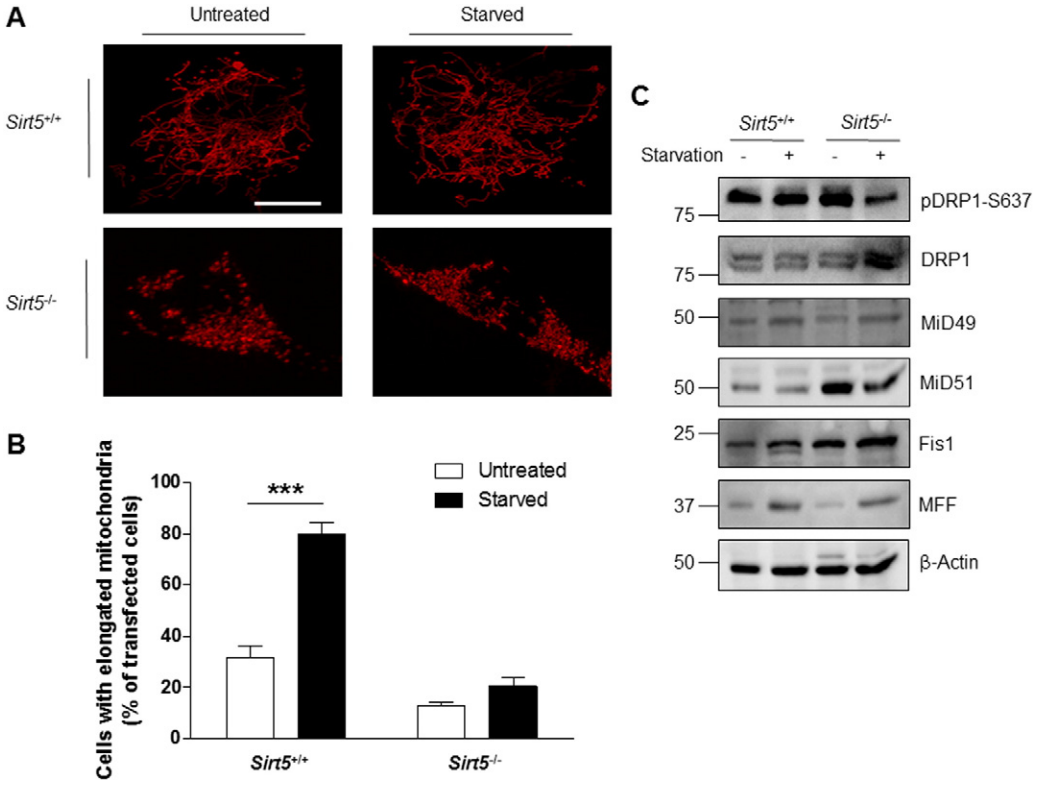

Fig. 4. Sirt5 is required for starvation-induced mitochondrial elongation. (A) Representative micrographs of untreated and starved *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs transfected with mtRFP. Where indicated, cells were starved (incubated in HBSS) for 2.5 h. Scale bar: 20 μm. (B) Morphometric analysis of data presented in *A* (n = 3–5), showing that elongation of mitochondria during starvation is abolished in *Sirt5*$^{-/-}$ MEFs. (C) Representative immunoblots (n = 3) of pDRP1-S637, DRP1, MiD49, MiD51, Fis1, MFF and β-actin in total cell lysate of untreated and starved *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs (treated as in A), showing that the abolition of mitochondrial elongation is associated with lower phosphorylation of DRP1-S637 and higher levels of DRP1 in starved *Sirt5*$^{-/-}$ MEFs. Data are presented as means ± SEM. ***$p < 0.001$, according to two-way ANOVA followed by Bonferroni post-hoc test.

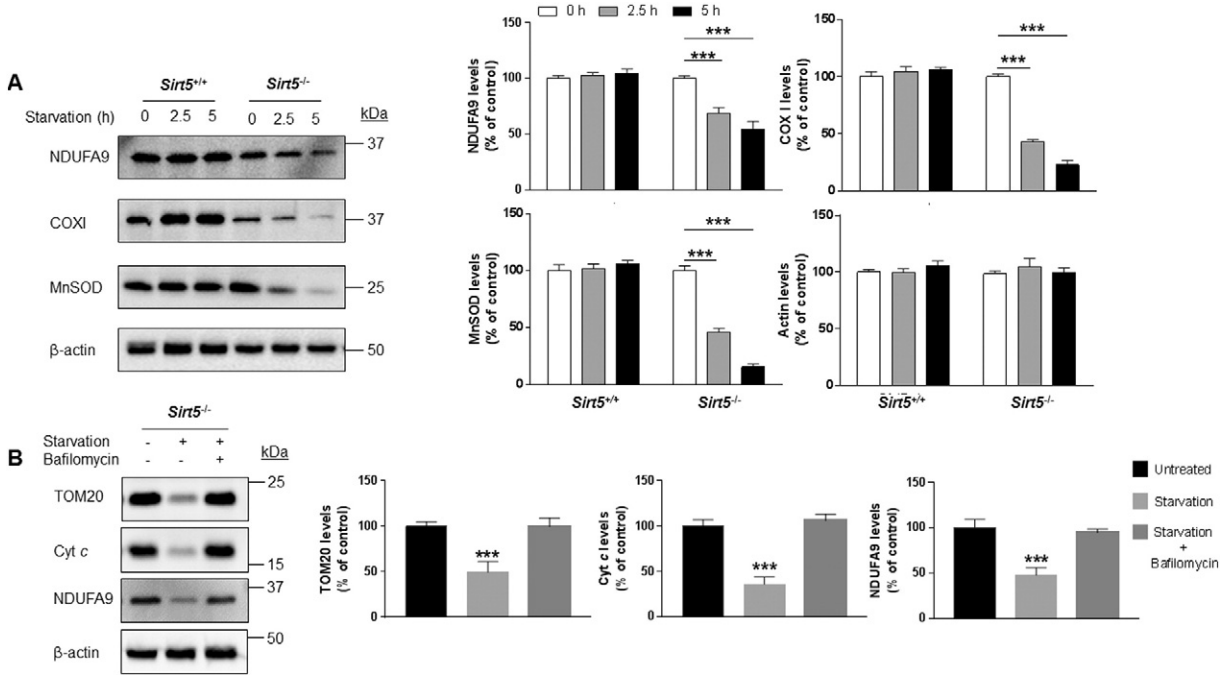

Fig. 5. Deletion of Sirt5 induces degradation of mitochondrial proteins during starvation. (A) *Left*, representative immunoblots of NDUFA9, COXI, MnSOD and β-actin in total cell lysate of *Sirt5*$^{+/+}$ and *Sirt5*$^{-/-}$ MEFs starved during 0, 2.5 and 5 h, showing that mitochondrial proteins are specifically degraded in starved *Sirt5*$^{-/-}$ MEFs. *Right*, quantification of data (n = 3). Protein levels are expressed as percentage of protein levels observed in untreated cells (0 h). These data were analyzed using a two-way ANOVA followed by Bonferroni post-hoc test. (B) *Left*, representative immunoblots of TOM20, Cyt c, NDUFA9 and β-actin in total cell lysate of *Sirt5*$^{-/-}$ MEFs, showing that starvation-induced degradation of mitochondrial proteins is blocked by the autophagy inhibitor bafilomycin. Cells were starved with or without bafilomycin (1 μM) during 5 h. *Right*, quantification of data (n = 3). Protein levels are expressed as percentage of protein levels observed in untreated cells. These data were analyzed with a one way ANOVA followed by Dunnett's multiple comparison test. Data are presented as means ± SEM. ***$p < 0.001$, according to two-way ANOVA followed by Bonferroni post-hoc test.

hypersuccinylated in *Sirt5*$^{-/-}$ models [24–26,32]. Succinyl-coA and NAD^+ (both essential for succinylation of proteins) are good indicators of the metabolic status of a cell [33], suggesting that desuccinylation pathways involving Sirt5 are important to adjust metabolism. Indeed, Sirt5 was shown to modulate succinylation of the ketogenic enzyme 3-hydroxy-3-methylglutaryl-CoA-synthase 2 [25] and to impact on the enzymatic activity of pyruvate dehydrogenase complex and of succinate dehydrogenase [26]. Therefore, Sirt5 modulates metabolism by targeting directly different metabolic enzymes to maintain cellular homeostasis during stress. The results presented here show higher levels of the DRP1 mitochondrial receptors MiD51 and Fis1, which increases association of DRP1 with mitochondria and mitochondrial fragmentation in *Sirt5*$^{-/-}$ MEFs. Therefore, our study demonstrates that other processes, including mitochondrial dynamics, are also modulated by Sirt5.

Mitochondrial shape has profound impact on mitochondrial physiology, including ATP production, apoptosis and mitophagy [3] and is modified according to the cellular energetic state. Inhibitors of the electron transport proteins can induce fragmentation whereas cells cultured in galactose-glutamine medium (which stimulate mitochondrial metabolism [34]) have elongated or tubular mitochondria [35]. Accordingly, several signaling pathways triggered by challenging metabolic conditions target mitochondrial-shaping proteins and mitochondria elongate during starvation [9,10]. In such conditions, the starvation-induced mitochondrial elongation depends on the decreased activity of the pro-fission DRP1 (due to lower phosphorylation of DRP1-S616) and on the increased phosphorylation of DRP1-S637 by PKA which favors the cytosolic retention of DRP1 [9,10]. Sirtuins-dependent signaling pathways also target mitochondrial-shaping proteins. Sirt1 activation by nicotinamide induced mitochondrial fragmentation in fibroblasts [36]. In contrast, overexpression of *Sirt3* rescued mitochondrial fragmentation in cortical neurons expressing the mutant superoxide dismutase $SOD1^{G93A}$ [37]. Sirt3 was shown to deacetylate OPA1 to increase mitochondrial fusion during stress [38]. Recently, decreased levels of MFN2 and OPA1 associated with lower mitochondrial fusion were reported in *Sirt5*-silenced cells [39]. In basal conditions, Sirt5 seems to impact on mitochondrial shape through levels of MiD51, Fis1 and subcellular localization of DRP1. However, the absence of mitochondrial elongation in starved *Sirt5*$^{-/-}$ MEFs was associated with higher levels of DRP1 and lower phosphorylation of DRP1-S637 whereas no change of MiD51 and Fis1 was observed. Globally, these results suggest that Sirt5 can target simultaneously different mitochondrial-shaping proteins to adapt mitochondrial functions according to nutrient availability. In future studies, it will be important to determine if Sirt5 can target directly these proteins through deacylation events and/or indirectly through different signaling pathways. For instance, Sirt5 could target the protein kinase A (PKA) and/or the phosphatase calcineurin to modulate the phosphorylation status of DRP1-S637 and consequently the translocation of DRP1 to mitochondria [3]. Sirt5 could also affect the activity of adenosine monophosphate-activated protein kinase (AMPK) which phosphorylates MFF to induce mitochondrial fission during energy stress [40].

Degradation of cytosolic components and organelles by autophagy is also considered as a protective mechanism during challenging metabolic conditions. Autophagy is activated by nutrient starvation to mobilize cellular energy and nutrient sources and sustain cellular homeostasis [41]. Different sirtuins are able to modulate autophagy. For instance, alteration of autophagy activation upon starvation is observed in *Sirt1*$^{-/-}$ MEFs [42]. Silencing of *Sirt1* also abolished induction of autophagy by nutrient deprivation in human cancer cells, and by caloric restriction in *C. elegans* [43]. Sirt1 can directly modulate the autophagic machinery through deacetylation of Atg5, 7 and 8 [42], whereas the expression of different components of the autophagic machinery is enhanced by Sirt1-dependent deacetylation and activation of FoxO transcription family members [44]. Activation of autophagy is increased

in *Sirt3*$^{-/-}$ MEFs upon starvation [45]. Sirt3 also deacetylates FoxO3 to promote autophagic elimination of damaged mitochondria [46]. A recent study showed that silencing of *Sirt5* increases the levels of the autophagy receptor BNIP3 in mitochondrial fractions whereas overexpression of *Sirt5* decreases the levels of this protein in mitochondria [39]. Consistent with these results, we observed higher degradation of mitochondrial proteins upon starvation in *Sirt5*$^{-/-}$ MEFs (Fig. 5). Altogether, these results suggest that Sirt5 modulates degradation of mitochondrial proteins through autophagy during challenging metabolic conditions.

Therefore, Sirt5 connects two different processes that are crucial for metabolic homeostasis and cellular survival during challenging metabolic conditions such as starvation. Indeed, we showed that Sirt5 is required for (i) mitochondrial elongation induced by starvation, and (ii) to protect mitochondria from degradation during such conditions. Sirt5 likely acts in parallel with other sirtuins at different levels of mitochondrial physiology, including enzymatic activities of mitochondrial proteins, mitochondrial shape and mitophagy, to maintain survival in challenging metabolic conditions. For instance, Sirt3 targets OPA1 to increase mitochondrial fusion [38,45], whereas our results show that Sirt5 target MiD51, Fis1 and DRP1 to decrease mitochondrial fragmentation, suggesting that sirtuins target different mitochondrial shaping proteins to lower degradation of mitochondria during starvation. Therefore, we can propose that the metabolic rearrangement occurring during low nutrient availability involves Sirt5-dependent direct regulation of (i) key metabolic pathways and (ii) mitochondrial structure.

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Transparency document

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

Conflict of interest

The authors declare no conflict of interest.

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