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Bioenergetic and autophagic control by Sirt3 in response to nutrient deprivation in mouse embryonic fibroblasts

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Synopsis

Sirtuin 3 (Sirt3) is an NAD-dependent deacetylase localized to mitochondria. Sirt3 expression is increased in mouse muscle and liver by starvation, which could protect against the starvation-dependent increase in oxidative stress and protein damage. Damaged proteins and organelles depend on autophagy for removal and this is critical for cell survival but the role of Sirt3 is unclear. To examine this, we used Sirt3 knockout (KO) mouse embryonic fibroblast cells, and found that under basal conditions, Sirt3 KO cells exhibited increased autophagy flux compared to Wildtype (WT) cells. In response to nutrient deprivation, both WT and KO cells exhibited increased basal and ATP linked mitochondrial respiration, indicating an increased energy demand. Both cells exhibited lower levels of phosphorylated mTOR, and higher autophagy flux, with KO cells exhibiting lower maximal mitochondrial respiration and reserve capacity and higher levels of autophagy than WT cells. KO cells exhibit higher phospho-JNK and phospho-c-Jun than WT cells under starvation conditions. However, inhibition of JNK activity in Sirt3 KO cells did not affect LC3-I and LC3-II levels, indicating the Sirt3-regulated autophagy is independent of the JNK pathway. Caspase 3 activation and cell death are significantly higher in Sirt3 KO cells compared to WT cells in response to nutrient deprivation. Inhibition of autophagy by chloroquine, exacerbated cell death in both WT and Sirt3 KO cells, and by 3-methyladenine exacerbated cell death in Sirt3 KO cells. These data suggest that nutrient deprivation-induced autophagy plays a protective role in cell survival, and Sirt3 decreases the requirement for enhanced autophagy and improves cellular bioenergetics.

Keywords

Sirt3; autophagy; apoptosis; JNK; c-Jun

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Author Contributions: QL performed autophagy studies. GAB performed the cellular bioenergetics studies. AV and DG provided the MEFs, figure and methods related to the generation of the MEFs. QL, GAB, VDU and JZ designed and evaluated the experiments and wrote the paper.

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Introduction

Autophagy is a highly conserved process that occurs ubiquitously in all eukaryotic cells [1]. It is an intracellular bulk degradation pathway for delivery of proteins and organelles to lysosomes or vacuoles, where they are degraded and recycled. Autophagy participates in turnover of long-lived proteins and the removal of damaged proteins or organelles under normal conditions; and is activated in response to stresses such as deprivation of nutrients and oxidative stress [2, 3]. Autophagy is a tightly regulated multi-step process, involving the initiation by the surrounding of cytoplasmic constituents with isolation membrane known as phagophore, which forms a closed double membrane structures called autophagosomes. Later, the autophagosome fuses with lysosomes to form autophagolysosomes, where their contents are then degraded by hydrolytic enzymes [4]. Microtubule-associated light chain 3 (LC3), the mammalian homologue of yeast Atg8, is proteolytically processed and then conjugated to phosphatidyl-ethanolamine (PE) prior to its insertion into autophagosomes [5]. Lipidated LC3 remains associated with autophagosomes until fusion with lysosomes, at which point intra-autophagosomal LC3 is degraded [6, 7]. The level of LC3-II has been shown to be proportional to the abundance of autophagosomes [6]. Autophagy has been shown to be important for clearance of damaged proteins and organelles, such as the mitochondria, if in deficit, protein and bioenergetic function is compromised [8, 9]. How autophagy is regulated by intracellular signaling mechanisms is then of critical interest.

The Sirtuin family proteins are NAD⁺-dependent deacetylases with homology to the yeast silent information regulator 2 (Sir2) [10]. Increased expression of Sir2 can extend the life span of model organisms, and the activity of Sir2 is required for survival under caloric restriction for organisms such as yeast and flies [11]. The mammalian Sirtuin family has 7 Sirtuins (Sirt1–Sirt7), which are important in many physiological events, such as aging, cell metabolism, apoptosis, and cell cycle regulation [12–14]. Among the 7 Sirtuins, it has been shown that Sirt1 overexpression can stimulate autophagy while Sirt1 deficiency exhibited attenuated autophagy under starvation [15]. The mechanisms of how Sirt1 contributes to the regulation of autophagy are still unclear. Whether other Sirtuins have similar function in autophagy, and through regulation of autophagy play a protective role for cell and organismal survival, are also unknown. Sirt3 is of particular interest because of its localization to the mitochondrion [16, 17], a key organelle in cellular metabolism and bioenergetics. Other Sirtuins that are localized to the mitochondria also include Sirt4, and Sirt5 [18]. Proteomics analyses have revealed that at least 20% of mitochondrial proteins are acetylated and every major metabolic pathway contains acetylated proteins [19]. Sirt3 KO mice showed significant hyperacetylation of mitochondrial proteins, which is absent in Sirt4 or Sirt5 KO mice [20], suggesting that Sirt3 may be the main deacetylase in mitochondria. Sirt3 has been shown to regulate the function of mitochondria proteins, including MnSOD, through its deacetylation activity [21–23]. It has been demonstrated that Sirt3 expression is significantly increased in starved mouse muscle and liver [24, 25]. Similarly, caloric restriction also augments the level of Sirt3 and is required for caloric restriction-mediated reduction of oxidative damage [26].

In the present study, we investigated the role of Sirt3 in the regulation of autophagy using Sirt3^{−/−} mouse embryonic fibroblasts. We demonstrated that Sirt3 deficiency resulted in impaired cellular bioenergetics, greater autophagy flux both under basal and starvation conditions. The greater autophagy flux seemed to be unable to suppress cell death as we found that cell death is more pronounced in Sirt3 KO cells compared to WT cells in response to starvation, accompanied by higher activated caspase 3 levels. Inhibition of autophagy by 3-methyladenine (3-MA) and chloroquine exacerbated cell death in Sirt3 KO cells, supporting a protective role of autophagy against cell death in this context.

MATERIALS AND METHODS

Reagents

3-methyladenine (3-MA), chloroquine and rapamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). SP600125 (anthra[1,9] pyrazol-6(2H)-one) and N-methyl-substituted pyrazolanthrone (N1-methyl-1,9-pyrazolanthrone) were from EMD Millipore (Billerica, MA, USA).

Antibodies

Anti-cleaved caspase 3 antibody, anti-total cJun, anti-phospho-c-Jun ser63 and ser73 antibodies, anti-phospho-JNK and anti-JNK antibodies, anti-phospho-mTOR and anti-mTOR antibodies, anti-phospho-p70S6K and anti-p70S6K antibodies were purchased from Cell Signaling (Danvers, MA, USA). Anti-LC3 antibody was from Sigma-Aldrich (St. Louis, MO, USA). Anti- β -actin monoclonal antibody was from Sigma-Aldrich. Anti-p62 antibody was obtained from Novus Biologicals (Littleton CO, USA). Anti-Becn1, anti-Mfn1, anti-Mfn2, anti-cathepsin D, anti-cathepsin B and anti-GRP78 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Drp1 and anti-GabarpL1 antibodies were from Abcam (Cambridge, MA, USA). Anti-LAMP1 antibody was from Developmental Studies Hybridoma Bank. Horseradish peroxidase-labeled secondary antibodies for enhanced chemiluminescence system detection were from Pierce (Rockford, IL USA).

MEF cell culture

The animals were a gift from Dr. Chuxia Deng [27]. All animal studies were conducted at the IACUC-approved Animal Facility in Northwestern University Feinberg School of Medicine. Experiments were performed in accordance with animal protocols approved by Northwestern University Feinberg School of Medicine. For isolation of MEFs from Sirt3^{+/+} and Sirt3^{-/-} mice were bred and a pregnant female was used to harvest embryos between day 12.5–14.5 d of gestation [27, 28]. After dissection from the uterus, embryo head, liver and other inner tissues were carefully removed (embryo head can be used for isolating DNA and genotyping). All blood clots were removed by washing embryos with 1–2 ml of PBS and then tissue was minced with razor blades and placed into a 15 ml tube with 2 ml of trypsin. After incubation for 30 min at 37°C, the cellular suspension was transferred to a 10 cm dish with MEF medium. Cells were allowed to grow to confluency and frozen down for further used. These cells were subsequently used to isolate RNA for RT-PCR and whole cell lysates for western immunoblotting with primers and antibodies to confirm the loss of SIRT3 (Supplemental Figure 1). For experiments, MEF cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 50 U/ml penicillin/streptomycin (Invitrogen) and 0.1 mM β -mercaptoethanol (Fisher) at 37°C, and 5% CO₂. We used passage 5 for all our studies. Cell death was measured by trypan blue exclusion assay. Starvation/nutrient deprivation was performed by switching the medium to Hanks buffered Saline Solution (HBSS). As shown by prior studies, this condition has been used for studying starvation-induced autophagy in many cell lines including MEFs [15, 29–35].

Measurement of mitochondrial function

To measure mitochondrial function in mouse embryonic fibroblasts, we used a Seahorse Bioscience XF24 Extracellular Flux Analyzer. The Seahorse Bioscience XF24 Analyzer creates a transient, 7 μ l chamber that allows for the measurements of oxygen consumption in real time [36, 37]. We have seeded cells at 40,000 cells per well because oxygen consumption rate (OCR) at this density is within machine detection range and in linear

proportion to seeding density. Concentration of oligomycin, FCCP, and antimycin A were titrated to have maximal non-toxic effect to mitochondrial respiration to be 1 μ M, 1 μ M and 10 μ M respectively. After completion of the experiments, total protein in each well was determined by the DC protein assay (BioRad) and the OCR as measure in pmol/min was normalized to cell protein.

Western blot analysis

Cells were collected in lysis buffer containing 50mM Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA, 1% Triton X-100 and supplemented with protease inhibitor mixture (Roche). Homogenates were centrifuged at 15,000 $\times$ g for 15 min at 4°C. Protein concentrations were determined by detergent-compatible protein assay (Bio-Rad). Protein extracts were mixed with 5 $\times$ sample loading buffer and boiled for 5 min. Thirty to fifty micrograms of protein was resolved on 12% SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked in 5% non-fat dry milk or 5% horse serum (for cathepsin D antibodies detection) in TBST (50mM Tris-HCl, 150mM NaCl, pH7.4, 0.1% Tween 20) for 30 min at room temperature. The membranes were then incubated overnight at 4°C with primary antibodies: anti-cleaved caspase3 1:1000; anti-p62 1:6000; anti-beclin1 1:1000; anti-LC3 1:6000, anti-actin 1:8000, anti-phospho c-Jun ser63 and ser73 1:1000, anti-phospho mTOR and mTOR 1:1000, anti-phospho-p70S6K and p70S6K 1:1000, anti-Mfn1, and anti-Mfn2 1:1000, anti-Drp1 and anti-GabarapL1 1:1000, anti-LAMP1, anti-cathepsin D and anti-cathepsin B 1:1000. The membranes were then washed 4 times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. After washing for 40 min with TBST, the membranes were developed using enhanced chemiluminescence (ECL) substrate kit. We used Image J software to quantify the western blot band intensity.

Statistical analysis

Data are reported as means $\pm$ SEM. Comparisons between two groups were performed with unpaired Student's *t*-tests. Comparisons among multiple groups or between two groups at multiple time-points were performed by either one-way or two-way analysis of variance, as appropriate. A *p* value of less than 0.05 was considered statistically significant.

Results

SIRT3 effects on cellular bioenergetics

To examine the effect of Sirt3 KO on mitochondrial function under normal and starvation conditions, we cultured WT and Sirt3 KO mouse embryonic fibroblasts (MEFs) and measured oxygen consumption rate (OCR) both in XF medium (8.28 g/L DMEM lacking sodium bicarbonate, 1 g/L D-glucose, 0.11 g/L sodium pyruvate, and 4 mM L-glutamine) and under starvation conditions in Hanks buffered Saline Solution (HBSS), using the Seahorse XF24 analyzer [38–40]. In XF medium basal OCR for the WT and Sirt3 KO cells was not significantly different (Figure 1A). Both WT and Sirt3 KO cells exhibited a stimulation of basal OCR in HBSS (Figure 1B) which was due to a combination of an increased ATP linked respiration and proton leak. The addition of the proton ionophore, FCCP, allows an estimation of the maximal OCR and this was significantly decreased in the Sirt3 KO cells compared to the WT control under starvation conditions. The difference between the basal and maximal respiration represents the bioenergetic reserve capacity which the cells can use under conditions of stress and was decreased in the Sirt3 KO. These data can be used to calculate the State apparent which allows an estimation of the activity of the mitochondria in a cellular setting [38]. In complete media both the WT and Sirt3 KO cells had a similar State apparent which is close to State 3.72 which suggests the mitochondria are turning over at approximately 25% of their maximal capacity under basal

conditions. In contrast, under starvation conditions the state apparent fell to approximately 40% for the WT and is significantly lower at 50% of maximal for the Sirt3 KO (Figure 1C). Taken together these data indicate that under starvation conditions ATP demand increases and maximal capacity decreases consistent with increased stress on the cell and lower substrate availability for oxidative phosphorylation. This response is significantly exacerbated in the Sirt3 KO suggesting that deacetylation has an important contribution to modulation of mitochondrial metabolism in response to starvation.

Sirt3 KO MEFs showed increased autophagic activity in response to starvation

To determine whether Sirt3 plays a role in autophagy, we cultured Sirt3 WT and KO MEFs and compared autophagic flux in these cells. Under non-starvation conditions, steady-state LC3-I and LC3-II are both higher in Sirt3 KO cells compared to WT cells (Figure 2A–C). To measure autophagic flux, we measured LC3-I and LC3-II levels in the presence of chloroquine. Both WT and Sirt3 KO cells exhibited decreased LC3-I and increased LC3-II in response to chloroquine. At this condition, both LC3-I and LC3-II levels were still higher in KO cells compared to WT cells, consistent with the KO cells having higher autophagic flux (Figure 2A–C).

In response to starvation, p-mTOR and its substrate p-p70S6K were significantly decreased (Figure 3A–E). While LC3-II levels are unchanged, LC3-I levels were decreased in both WT and KO cells (Figure 3F–H). Autophagic flux assays demonstrated that, in the presence of chloroquine under starvation conditions, Sirt3 KO MEFs displayed higher LC3-II levels compared to WT MEFs (Figure 4A–C), again indicating an enhanced autophagic flux of the Sirt3 KO cells under starvation.

Further we tested whether the activation of autophagy in KO cells compared to WT cells, under both basal and starvation conditions, was associated with any changes in major autophagy proteins. No difference was observed between Sirt3 WT and KO MEFs in normal condition or in response to starvation for autophagy proteins, p62, Beclin1 or GABARAPL1 (Supplemental Figure 2), suggesting that alteration of autophagy in Sirt3 KO MEFs was independent of changes in p-mTOR-, p62-, Beclin 1- and GABARAPL1 levels. Levels of mitochondrial fusion and fission proteins Mfn1, Mfn2 or Drp1 were unchanged in normal condition and in response to starvation (Supplemental Figure 3). Importantly there was no difference in the levels of these proteins between WT and KO cells. The only changes we consistently observed were that under basal conditions, lysosomal protein, LAMP1 was modestly decreased (Supplemental Figure 3), mature cathepsin D was increased (Supplemental Figure 3), while mature cathepsin B is unchanged in either normal condition or in response to starvation (Supplemental Figure 3) in Sirt3 KO MEFs.

Phosphorylated-c-Jun and -JNK were higher in Sirt3 KO MEFs compared to WT cells in response to starvation

To further determine the mechanisms underlining autophagy alteration in Sirt3 KO cells, we studied stress kinase JNK and its substrate c-Jun. Under basal conditions, there was no significant difference in the levels of p-c-Jun and p-JNK in KO cells compared to the WT cells. However, p-c-Jun was significantly increased in response to starvation in KO cells but not in WT cells. P-JNK was significantly decreased in response to starvation in both WT and KO cells, with higher p-JNK in KO cells than WT cells (Figure 5A–D). We used different concentrations of the JNK inhibitor SP600125 to decrease the p-JNK activity in Sirt3 KO MEFs exposed to starvation and established that 2.5 μ M of SP600125 can decrease the p-JNK level in Sirt3 KO MEFs to similar level in WT cells (Figure 6A). While decreased the activation of JNK in Sirt3 KO cells (Figure 6B–D), JNK inhibitor SP600125

did not change LC3-I and LC3-II levels in Sirt3 KO MEFs (Figure 6B, E-F), suggesting that the Sirt3 regulated autophagy in response to starvation is independent of JNK activation.

Sirt3 KO MEFs exhibited increased cell death in response to starvation

To study the effect of Sirt3 deficiency on cell survival in response to nutrient deprivation, we cultured Sirt3 WT and KO MEFs in HBSS for starvation for 4 hr then determined cell viability. As shown in Figure 7A, both WT and Sirt3 KO cells showed decreased viability in response to starvation, with cell viability decreased to a greater extent under starvation conditions in Sirt3 KO cells compared to WT cells. In WT MEFs, the switch to nutrient-depleted conditions resulted in an increased caspase 3 activation, this response was further increased by about 50% in Sirt3 KO MEFs (Figure 7B–C), suggesting that Sirt3 is important for attenuating apoptosis activation in response to starvation.

To examine the role of autophagy in regulating cell death in Sirt3 KO MEFs, we treated Sirt3 WT and KO MEFs with inhibitors of autophagy. We used chloroquine and 3-MA to inhibit completion and initiation of autophagy respectively. We found that chloroquine or 3-MA alone did not induce cell death under basal conditions in either WT or Sirt3 KO cells. However, treatment with chloroquine and 3-MA both further decreased cell survival in Sirt3 KO MEFs in response to starvation. Chloroquine but not 3-MA also decreased WT cell survival in response to starvation (Figure 7A). An inducer of autophagy rapamycin treatment resulted in a low level (20%) of toxicity to the cells under basal conditions, perhaps due to its role in inhibition of protein synthesis. However, rapamycin had no further effect on the Sirt3 WT and KO MEFs cell viability in response to starvation (Figure 7A), suggesting the effect of Sirt3 on autophagy may be downstream of mTOR. Consistent with lack of an effect of the JNK inhibitor on autophagy, inhibition of JNK did not affect cell viability in Sirt3 KO MEFs in response to starvation (Figure 7D), suggesting the cell death is independent of autophagy and the JNK pathway in Sirt3 KO MEFs.

Discussion

Evidence is accumulating that protein acetylation is an evolutionarily conserved metabolic regulatory mechanism involved in coordinating different metabolic pathways, including autophagy in response to different conditions [41]. Even though Sirt3 is a mitochondrial protein, both nuclear and cytosolic targets of Sirt3 deacetylation have also been identified [42–44]. Previous studies have reported diverse roles for Sirt3 in mediating cell death. For example, Sirt3 is required for apoptosis induced by selective silencing of Bcl-2 in HCT116 human epithelial cancer cells under basal conditions [45]. Sirt3 is an essential mediator of JNK2-regulated apoptosis operating under basal conditions in HCT116 cells while it is dispensable for Sirt1-regulated apoptosis. In contrast, Sirt3 is not required in apoptosis pathway in HCT116 cells exposed to extrinsic inducers of apoptosis such as UV-irradiation and 5-fluorouracil, indicating that stress-induced apoptosis bypasses the need for the pro-apoptotic functioning of Sirt3. Sirt3 KO MEFs exhibited decreased stress-induced apoptosis in response to either IR or camptothecin [28]. In the present study, we found that Sirt3 KO MEFs exhibited complex changes including changes to cellular bioenergetics and increased autophagic flux. Nonetheless, the increased autophagy in Sirt3 KO seems to play a protective role against nutrient-deprivation induced cell death.

With regard to cellular bioenergetics, under starvation conditions Sirt3 KO MEFs exhibited decreased maximal OCR, reserve capacity, and State_{apparent}, consistent with a diminished capacity to respond to stress. These data are not consistent with previous studies using control cDNA transfected WT and Sirt3 KO mouse embryonic fibroblast which showed a significant decrease of basal OCR in the Sirt3 knock down [46]. However, these data were

not normalized to protein and bioenergetic analysis was not complete so changes due to differences in cell number cannot be ruled out.

So far, we were not able to determine the molecular events that led to increased autophagy in Sirt3 KO cells. Several potential mechanisms have been ruled out. TOR (target of rapamycin) is a key component that coordinately regulates the balance between growth and autophagy in response to cellular physiological conditions and environmental stress. We found, as reported in the literature, that nutrient starvation induces autophagy in mammalian cells through inhibition of mTOR [47]. However, mTOR was inhibited to the same extent in both WT and Sirt3 KO cells, suggesting the regulation of autophagy by Sirt3 under starvation is independent of the mTOR pathway. Starvation also had no effect on p62, Beclin1, GABARAPL1, nor on Mfn1, Mfn 2 or Drp1 suggesting no change in mitochondrial fission and fusion. Lysosomal cathepsin D plays an important role in decreasing both α -synuclein and mutant huntingtin toxicities and cathepsin B is important in decreasing mutant huntingtin toxicity in cultured cells [48, 49]. The decreased LAMP1 and increased mature cathepsin D may be a reflection of decreased lysosomal number and compensatorily increased lysosomal aspartic protease activity, but since these changes are relatively modest it is unlikely they are making a major contribution to the Sirt3-dependent response to starvation induced autophagy. JNK and c-Jun phosphorylation were found to be significantly higher in Sirt3 KO MEFs compared to WT MEFs in response to starvation. This is consistent with prior studies that Sirt3 KO MEFs exhibit higher level of p-JNK [50], as activated JNK can phosphorylate c-Jun. Inhibition of JNK activation did not change LC3-I and LC3-II levels in Sirt3 KO MEFs. These observations suggested JNK is not playing a major role in Sirt3 regulated autophagy in response to nutrient deprivation.

Autophagic flux is higher in Sirt3 KO cells compared to WT cells both in normal medium and in response to nutrient deprivation. The higher autophagic flux did not alter viability in normal medium in response to Sirt3 KO. However, significantly more cell death occurred in Sirt3 KO cells compared to WT cells in response to nutrient deprivation. Inhibition of autophagy completion by chloroquine significantly decreased cell survival both in WT and in Sirt3 KO cells, whereas inhibition of autophagy initiation by 3-MA significantly decreased survival of Sirt3 KO cells in response to nutrient deprivation, without affecting the survival of WT cells. These observations demonstrate that autophagy plays a protective role in response to starvation both in WT cells and in Sirt3 KO cells. However, the decreased mitochondrial reserve capacity in the Sirt3 KO cells in nutrient deprivation suggests that this high level of autophagy is not adequate to remove the damaged mitochondria sufficiently rapidly. This is also consistent with the observation that the Sirt3 KO is associated with increased mitochondrial reactive oxygen species generation [28, 51, 52]. Sirt3 then appears to be playing a modulatory role between cell survival, mitochondrial ROS formation and maintenance of mitochondrial function in response to nutrient deprivation. It is tempting to extrapolate these studies to the potential impact on dietary interventions such as caloric restriction. One implication would be that the response of subjects to such interventions will depend on a functional Sirt 3 pathway. Future *in vivo* studies will be needed to determine the role of Sirt3 modulated autophagy in diet, exercise, longevity and susceptibility to cancer and degenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Sirt3	Sirtuin3
3-MA	3-methyladenine
CQ	chloroquine;

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Figure 1. Sirt3 KO MEF cells exhibited decreased mitochondrial function in response to starvation compared to WT cells

(A) WT and Sirt3 KO MEFs were plated at 40,000 cells/well in XF24 plates and grown overnight. Using the XF24 Seahorse bioanalyzer, the mitochondrial oxygen consumption rate (OCR) was determined either in XF media or in HBSS for 2 hours. OCRs were between 8–12 pmol O₂/min/μg protein. Then OCRs were measured after injection of Oligomycin (O), FCCP (F) and Antimycin A (A). Data = mean ± SEM, n=5. (B) Using the OCR traces shown in A, Basal, ATP-linked, Proton Leak, Maximal, reserve capacity, and non-mitochondrial OCR were calculated. (C) Comparison of State_{apparent}. Data = mean ± SEM, n=5, *p* < 0.05; * vs wt XF media; # vs Sirt3 KO XF media; and † vs wt HBSS.

Figure 2. Sirt3 KO cells exhibited altered autophagy activity in complete media

(A). Sirt3 WT and KO MEF cells were cultured in complete media with or without 40 μ M CQ for 4 hr. Western blot analyses were performed with anti-LC3 antibody. β -actin western blots were used as loading controls. Quantification of relative levels of LC3-I (B), and LC3-II (C) from western blot band intensity and shown in the graphs. * p < 0.05 between WT and KO, # p < 0.05 compared to without CQ.

Figure 3. Sirt3 KO cells exhibited increased autophagy in response to starvation

(A). Sirt3 WT and KO MEF cells were starved in HBSS for 0 and 4 hr. Western blot analyses were performed with anti- p-mTOR, total mTOR, p-p70S6K, and total p70S6K antibodies. β-actin western blots were used as loading controls. Quantification of p-mTOR (B), total mTOR (C), p-p70S6K (D), and total p70S6K (E) levels from western blot band intensity were shown in the graphs. * $p < 0.05$ compared to 0 hr. (F). Sirt3 WT and KO MEF cells were starved in HBSS for 0 and 4 hr. Western blot analyses were performed with anti-LC3 antibody. β-actin western blots were used as loading controls. Quantification of relative

LC3-I (G) and LC3-II (H) levels from western blot band intensity and shown in the graphs.

* $p < 0.05$ between WT and KO, † $p < 0.05$ compared to 0 hr.

Figure 4. Sirt3 KO cells exhibited altered autophagic flux

(A). Sirt3 WT and KO MEF cells were treated with 40 μ M CQ for 4 hr in HBSS. Autophagy flux was shown by western blot performed with anti-LC3 antibody. β -actin western blots were used as loading controls. Quantification of relative LC3-I (B) and LC3-II (C) levels from western blot band intensity and shown in the graphs. * p < 0.05 between WT and KO; # p < 0.05 compared to without CQ.

Figure 5. p-JNK and phosphorylation of c-Jun at both Ser63 and Ser73 sites were increased in Sirt3 KO cells under starvation

(A) and (C) Sirt3 WT and KO MEF cells were starved in HBSS for 0 and 4 hr. Western blot analyses were performed with anti-p-JNK, total JNK, p-c-Jun Ser63, p-c-Jun Ser73, total c-Jun antibodies. β-actin western blots were used as loading controls. (B) and (D) Relative p-JNK, p-c-Jun Ser63 and p-c-Jun Ser73 levels were quantified by band intensity and shown in the graphs. * $p < 0.05$ compared to WT 4 hr, # $p < 0.05$ compared to 0 hr.

Figure 6. Effect of JNK inhibitor SP600125 on autophagy in Sirt3 WT and KO MEF cells under starvation

(A) Sirt3 WT and KO MEF cells were cultured in HBSS for 4 hr. Sirt3 KO MEFs were treated with different doses of JNK inhibitor SP600125, 0 μM, 1 μM, 2.5 μM, 5 μM and 10 μM. JNK activity inhibition was confirmed by western blot with anti-p-JNK and JNK antibodies. (B) Sirt3 KO MEF cells were cultured in HBSS for 4 hr with or without 2.5 μM of SP600125 and 40 μM of CQ. LC3 and p-JNK WB were performed with anti-LC3, p-JNK and JNK antibodies. (C) and (D) Relative p-JNK and JNK levels were quantified by band intensity and shown in the graphs. *p<0.05 compared to without SP600125. (E) and (F)

Relative LC3-I and LC3-II levels were quantified by band intensity and shown in the graphs.
* $p < 0.05$ compared to without CQ.

Figure 7. Sirt3 KO MEF cells exhibited increased cell death in response to starvation in HBSS and the cell death was exacerbated by 3-MA or CQ treatment

(A). Sirt3 WT and KO MEF cells were treated with or without 5 mM 3-MA, 40 μM CQ or 50 nM rapamycin in complete media or in HBSS for 4 hr. Cell viability was determined by trypan blue exclusion counting. * $p < 0.05$ compared to WT in HBSS, # $p < 0.05$ compared to WT or KO control in HBSS, † $p < 0.05$ compared to control WT or KO in complete media.

(B). More activated caspase 3 in Sirt3 KO MEF cells compared to WT cells in response to starvation. Sirt3 WT and KO MEF cells were starved in HBSS for 0 and 4 hr. Western blot analyses were performed with anti-cleaved caspase 3 antibody. β-actin western blots were used as loading controls. (C). Quantification of cleaved caspase 3 levels from western blot

band intensity. Student t-test was performed. * $p < 0.05$ compared to 0 hr, # $p < 0.05$ compared to WT 4 hr. (D). Sirt3 WT and KO MEF cells were cultured in complete medium or in HBSS for 4 hr with or without 2.5 μ M SP600125. Cell viability was determined by trypan blue exclusion counting. * $p < 0.05$ compared to complete medium, # $p < 0.05$ compared to WT in HBSS.

Figure 8. A schematic model for the regulation of autophagy and cell death in Sirt3 KO MEFs
 Loss of Sirt3 has been shown to lead to increased reactive oxygen species formation. We have found that loss of Sirt3 increases both basal and starvation induced autophagy, and sensitizes cell death in response to starvation. The increased autophagy in Sirt3 cells is important for cell survival, since inhibition of autophagy in Sirt3 KO cells by 3-MA or chloroquine exacerbates cell death in response to starvation.