Ana Paula Magalhães Rebelo

SESTRIN 2-MEDIATED MITOPHAGY: POTENTIAL REGULATION OF MITOHORMESIS AND PROMOTION OF METABOLIC HEALTH

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Anabela Rolo do Departamento de Ciências da Vida da Universidade de Coimbra e do Centro de Neurociências e Biologia Celular da Universidade de Coimbra e coorientação do Professor Doutor Carlos Palmeira do Departamento de Ciências da Vida da Universidade de Coimbra e do Centro de Neurociências e Biologia Celular da Universidade de Coimbra.

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Dissertation presented to the Faculty of Sciences and Technology of the University of Coimbra in partial fulfillment of the requirements for the degree of Master of Cellular and Molecular Biology, under supervision of Professor Anabela Rolo from Department of Life Sciences of the University of Coimbra and Center for Neurosciences and Cell Biology of University of Coimbra, and co-supervision of Professor Carlos Palmeira Department of Life Sciences of the University of Coimbra and Center for Neurosciences and Cell Biology of University of Coimbra.

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Resumo

As mitocôndrias desempenham um papel fundamental na homeostase celular. Para além do seu papel essencial na função energética, estes organelos produzem e eliminam espécies reativas de oxigénio. Historicamente, as espécies reativas de oxigénio estão associadas a stresse oxidativo. Contudo, cada vez mais é reconhecido o seu papel sinalizador, particularmente da mitocôndria para o resto da célula. A sestrina 2 é uma proteína indutível por espécies reativas de oxigénio, com propriedades antioxidantes e inibitórias da atividade do mTORC1. Adicionalmente, a sestrina 2 foi recentemente identificada como um elemento ativo na indução de mitofagia e consequentemente, reguladora da qualidade da população mitocondrial. Estas observações sugerem que a sestrina 2 pode desempenhar um importante papel na "mitohormesis". Este conceito estabelece que níveis moderados de produção de espécies reativas de oxigénio na mitocôndria, induzem uma resposta adaptativa aumentando a capacidade de resposta a stresses posteriores. Nesta perspetiva, este trabalho teve como objetivo a avaliação da indução de mitofagia por sestrina 2 como estratégia protetora contra o dano causado por hiperglicemia.

Células C2C12, que representam mioblastos de rato imortalizados, foram cultivadas durante 18 h em meio sem glucose, como um possível estímulo protetor. De seguida foi adicionada glucose ao meio de cultura, por forma a simular exposição a hiperglicemia, durante 24 h. Verificámos que 18 h de restrição de glucose diminuem a produção total de espécies reativas de oxigénio. Além disso, apesar de não ter sido observado um aumento significativo na formação mitocondrial de anião superóxido às 3 h, observou-se uma diminuição em células cultivadas durante 6 h em meio sem glicose. Observou-se também um aumento do conteúdo em sestrina 2 associado a fragmentação mitocondrial e estimulação de mitofagia. Não foram observadas alterações no conteúdo em caspase-3. Adicionalmente, células previamente sujeitas a deprivação de glicose e posteriormente cultivadas em hiperglicemia, durante 24 h, não apresentaram um aumento na produção de espécies reativas de oxigénio ou indução de mitofagia, contrariamente a células expostas apenas à condição de hiperglicemia. Estes

resultados sugerem que 18 h de deprivação de glicose podem ativar “mitohormesis” em C2C12, com a melhoria da função mitocondrial decorrente da estimulação de mitofagia por sestrina 2.

Este trabalho demonstrou que a diminuição da produção de espécies reativas de oxigénio em C2C12, após 18 h de deprivação de glicose, é acompanhada por uma diminuição do potencial de membrana, fragmentação mitocondrial e estimulação de mitofagia. Um aumento do conteúdo da sestrina 2 foi verificado após 6 e 18 h, mas não após 24 h em hiperglicemia. Em conclusão, este trabalho sugere que a deprivação de glicose, por um curto período de tempo, protege C2C12 do dano induzido por hiperglicemia mediante o aumento da eficiência mitocondrial.

Palavras-chave: Espécies reativas de oxigénio, “Mitohormesis”, Sestrina 2, Mitofagia e Hiperglicemia

Abstract

Mitochondria are major controllers of the overall cellular homeostasis. They represent the energy supply of the cell and have the capacity to produce and detoxify reactive oxygen species (ROS). Although historically associated with oxidative stress, ROS are ubiquitous signaling species from mitochondria to the rest of the cell. Sestrin 2 (Sesn2) is an ROS-inducible protein that exhibits antioxidant properties and suppression of mTORC1 activity. Moreover, in a recent study Sesn2 was identified as an active mitochondrial homeostasis mediator due to its regulation of mitophagy. Considering these features, Sesn2 is a potential regulator of mitohormesis. This concept defines that low-level of mitochondrial ROS generation ensures a rapid adaptation to a mild stress and enhances long-term resistance to subsequent stresses. In view of this, this work had the goal to evaluate if activation of mitophagy by Sesn2 could increase the resistance to hyperglycemia-induced cell dysfunction.

C2C12 cells, which are immortalized mouse myoblasts, were first cultured for 18 h in media without glucose, as a possible protective stimulus. Then glucose was added to the culture medium to simulate exposure to hyperglycemia for 24 h. Here we demonstrated that 18 h of glucose deprivation decreased total cellular ROS generation. Moreover, even though we did not observe significantly increased production of mitochondrial superoxide anion at 3 h, a subsequent decrease was observed at 6 h in glucose-deprived cells. Also, Sesn2 upregulation was observed after 6 and 18 h, which was accompanied by mitochondrial fragmentation and induction of mitophagy. These changes were not associated with alterations in caspase-3 content. Moreover, in cells previously cultured in media without glucose and then hyperglycemia for 24 h, there was no increase in ROS generation or induction of mitophagy, as opposed to cells cultured only in hyperglycemic conditions. Thus, we propose that short-term glucose deprivation resembles a mitohormetic induction condition where Sesn2 upregulation leads to enhanced mitochondrial function through the promotion of mitophagy.

In conclusion, 18 h of glucose deprivation significantly decreased total cellular ROS production in C2C12, which was accompanied by mitochondrial membrane

potential decrease, mitochondrial fragmentation, and mitophagy. Sesn2 upregulation was observed after 6 and 18 h of glucose deprivation, but not after the subsequent 24 h exposure to hyperglycemia. Our findings suggest that a previous exposure to glucose deprivation protects against hyperglycemic insult and might increase mitochondrial resistance.

Keywords: Reactive oxygen species, Mitohormesis, Sestrin 2, Mitophagy and Hyperglycemia

List of Figures

Figure 1. TCA cycle and oxidative phosphorylation bioenergetics 7
Figure 2. Mammalian macroautophagy, microautophagy, and chaperone-mediated autophagy pathways. 13
Figure 3. Pink1-Parkin-dependent mitophagy in mammalian cells. 14
Figure 4. LC3-interacting molecules-dependent mitophagy. 16
Figure 5. Hyperglycemia possible temporal impact on mitochondrial function. 21
Figure 6. Effects of different levels of stress in cellular and mitochondrial function. 23
Figure 7. Promotion of mitochondrial ROS generation by energetic deficits induces an overall adaptative response. 26
Figure 8. Representation of Saccharomyces cerevisiae TOR Complex 1 (TORC1) and TOR Complex 2 (TORC2). 30
Figure 9. Regulation of Nrf2 by Sestrin 2. 33
Figure 10. Sestrin 2 controls mTORC1 through GATOR complexes. 35
Figure 11. Sestrin 2 possible role during hormesis. 36
Figure 12. Evaluation of cellular ROS in C2C12 cells after 6 h (A) and 18 h (B) exposure to 0 and 35 mM of glucose (Glu). 45
Figure 13. Evaluation of mitochondrial superoxide anion in C2C12 cells after 3 and 6 h exposure to 0 and 35 mM glucose (Glu). 46
Figure 14. Mitochondrial membrane potential after 3 h exposure to 0 and 35 mM of glucose (Glu). 47
Figure 15. Mitochondrial membrane potential after 18 h exposure to 0 and 35 mM of glucose (Glu). 47
Figure 16. Tom20 immunofluorescence in C2C12 cells exposed to 18 h of 5.5, 35 and 0 mM of glucose. 49
Figure 17. PGC1-α content in C2C12 cells exposed to 18 h of 0 and 35 mM glucose (Glu). 50
Figure 18. Tfam content in C2C12 cells exposed to 18 h of 0 and 35 mM glucose (Glu). 50
Figure 19. Mtphagy dye live image in C2C12 cells exposed to 18 h of 5.5, 35 and 0 mM glucose. 51
Figure 20. Cleaved caspase-3 content in C2C12 cells exposed to 6 hours of 0 and 35 mM glucose (Glu). 52
Figure 21. Cleaved caspase-3 content in C2C12 cells exposed to 18 h of 0 and 35 mM glucose (Glu). 52
Figure 22. Sestrin 2 content in C2C12 cells exposed to 6 hours of 0 and 35 mM glucose (Glu). 54
Figure 23. Sestrin 2 content in C2C12 cells exposed to 18 h of 0 and 35 mM glucose (Glu). 54
Figure 24. Sestrin 2 immunofluorescence in C2C12 cells exposed to 18 h of 5.5, 35 and 0 mM of glucose (Glu). 55
Figure 25. LC3-I, LC3-II content and autophagic flux in C2C12 cells exposed to 18 h of 0 and 35 mM of glucose (Glu). 56
Figure 26. Evaluation of cellular ROS in C2C12 cells exposed to 24 h of hyperglycemia after 18 h in 0, 35 and 5.5 mM of glucose (Glu). 57
Figure 27. LC3-I and LC3-II mitochondrial content in C2C12 cells exposed cells exposed to 24 h of hyperglycemia after 18 h in 0 and 35 mM of glucose (Glu). 59
Figure 28. Sestrin 2 content in C2C12 cells exposed to 24 h of hyperglycemia after 18 h in 0, 35 and 5.5 mM of glucose (Glu). 60

Figure 29.Autophagic flux in C2C12 cells exposed to 24 h of hyperglycemia after 18 h in 0, 35 and 5.5 mM of glucose (Glu). 60
Figure 30. Sestrin 2, Cleaved Caspase-3 and LC3-I, LC3-II content in C2C12 cells after 24 h and 48h of electroporation knockdown Sestrin 2 induction. 62
Figure 31. Illustration of possible Nrf2 induction of Sestrin 2 by mitochondrial superoxide. 66
Figure 32. Illustration of possible Sestrin 2-involved protective pathways during short-term hypoglycemia. 69
Figure 33. Illustration of possible mitohormesis promotion of mitochondrial function from hyperglycemic. 72

List of Tables

Table 1. List of Antibodies used for Western Blot.. 37
Table 2. List of antibodies used for immunocytochemistry.. 37

List of Abbreviations

2,7- dichlorofluorescein (DCF)

2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA)

Acetyl-coenzyme A (Acetyl-CoA)

Activating transcription factor 4 (ATF4)

Adenosine diphosphate (ADP)

Adenosine monophosphate (AMP)

Adenosine Triphosphate (ATP)

AMP-activated protein kinase (AMPK)

Analysis of Variance (ANOVA)

Antioxidant response element (ARE)

Autophagy-related genes (ATG)

Bcl2/E1B 19 kDa-interacting protein 3-like protein (BNIP3)

Caloric restriction (CR)

Coenzyme Q / ubiquinol (CoQ/CoQH2)

cSesn (*Caenorhabditis Elegans* Sestrin)

Cytochrome C oxidase (COX)

Cytosolic E3 ubiquitin ligase (Parkin)

Deoxyribonucleic acid (DNA)

Dimethyl sulfoxide (DMSO)

Double-stranded DNA (dsDNA)

dSesn (*Drosophila melanogaster* Sestrin)

Dulbecco’s Modified Eagle Medium (DMEM)

Dynamin-related protein 1 (DRP1)

Electron transport chain (ETC)

Endoplasmic Reticulum (ER)

Fetal bovine serum (FBS)

Flavin adenine dinucleotide (FADH)

Glutathione peroxidase (GPX)

Heat shock protein 70 kDa protein (HSP70)

High-fat diet (HFD)

Hydrogen peroxide (H_2O_2)

Hydroxyl radical (OH^-)

Hypoxia-inducible factor 1 (HIF-1)

Inner mitochondrial membrane (IMM)

Inorganic phosphate (Pi)

Kelch-like ECH-associated protein 1 (KEAP1)

Kilodalton (kDa)

Knock-out (KO)

LC3-interacting region (LIR)

Lipid peroxides (ROOH)

Long- OPA 1 (L-OPA1)

Lysosomal-associated membrane protein 2A (LAMP-2A)

Mammalian target of rapamycin (mTOR)

Mammalian target of rapamycin complex 1, 2 (mTORC 1, 2)

Manganese-dependent superoxide dismutase (MnSOD)

Messenger RNA (mRNA)

Microtubule-associated protein 1A/1B-light chain 3 - PE phosphatidylethanolamine (LC3)

Mitochondrial apoptosis-induced channel (MAC)

Mitochondrial DNA (mtDNA)

Mitochondrial fission 1 (Fis1)

Mitochondrial inner/outer membrane transporter (TIM/TOM)

Mitochondrial membrane potential (MMP)

Mitochondrial Rho GTPase 1 (Miro1)

Mitochondrial transcription factor A (Tfam)

Mitochondrial unfolded protein response (UPR^{mt})

Mitofusin 1 (Mfn1)

Mitofusin 2 (Mfn2)

N-acetyl cysteine (NAC)

Nip3-like Protein X (Nix)

Nitric oxide (NO^-)

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)

Nuclear respiratory factors (NRF-1)

Optic Atrophy protein (OPA1)

Outer mitochondrial membrane (OMM)

Oxidative phosphorylation (OXPHOS)

Permeability transition pore (PTP)

Peroxiredoxin (Prx)

Phagophore assembly site (PAS)

Phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1)

Phosphate-buffered saline (PBS)

PPAR (peroxisome proliferator-activated receptor) coactivator-1α (PGC-1α)

Reactive Oxygen Species (ROS)

Ribonucleic acid (RNA)

Ribosomal RNA (rRNA)

Sequestosome 1 (p62/ SQSTM1)

Sestrin 1-3 Human (SESN1-3)

Sestrin 1-3 mouse (Sesn1-3)

Short-OPA1 (S-OPA1)

Singlet oxygen ($^1O2$)

Small interfering Ribonucleic acid (SIRNA)

Standard error of the mean (SEM)

Sulfiredoxin (Srx)

Sulforhodamine B (SRB)

Superoxide anion ($O2^-$)

Superoxide dismutase (SOD)

Target of rapamycin (TOR)

Tetramethylrhodamine methyl ester (TMRM)

Tricarboxylic acid (TCA)

Triple Knockout (TKO)

Tumor suppressor (p53)

Ubiquinone (Q)

Unc-51 like kinase (ULK)

Voltage-dependent anion-selective channel (VDAC)

β-guanadinopropionic acid (β-GPA)

Index

Resumo ---------- v
Abstract ---------- vii
List of Figures ---------- ix
List of Tables ---------- x
List of Abbreviations ---------- xi
Index ---------- 1
1. Objectives ---------- 3
2. Introduction ---------- 4
2.1. Mitochondria outline ---------- 4
2.1.1. ETC and OXPHOS ---------- 6
2.1.2. Mitochondrial biogenesis ---------- 7
2.1.3. Mitochondrial Dynamics ---------- 9
2.2. Autophagy ---------- 11
2.2.1. Mitophagy ---------- 13
2.3. Mitochondrial reactive oxygen species and oxidative stress ---------- 16
2.3.1. Hyperglycemia, oxidative stress, and mitochondrial dysfunction ---------- 18
2.3.2. Reactive oxygen species generation: A physiological role ---------- 22
2.3.3. Mitohormesis ---------- 24
2.4. Sestrins: A conserved stress-inducible family ---------- 28
2.5. The individual role of Sestrin 2 ---------- 31
2.5.1. Sestrin 2 and cellular response ---------- 32
3. Materials and Methods ---------- 37
3.1. Materials ---------- 37
3.1.1. Table 1. List of primary antibodies used for Western Blot ---------- 37
3.1.2. Table 2. List of primary antibodies used for Immunocytochemistry ---------- 37
3.1.3. Cell line ---------- 37
3.1.4. Cell culture maintenance ---------- 38
3.2. Methods ---------- 38
3.2.1. Mitohormesis induction ---------- 38
3.2.2. Measurement of ROS production ---------- 38
3.2.3. Measurement of mitochondrial membrane potential in C2C12 cells ---------- 39

3.2.4. Sulforhodamine B colorimetric assay ---------- 39

3.2.5. MitoSOX ---------- 40

3.2.6. Western blotting analysis ---------- 40

3.2.7. Subcellular fractionation of mitochondrial protein ---------- 41

3.2.8. Immunofluorescence of Sestrin2 and TOM20 ---------- 41

3.2.9. Mitophagy live cell imaging with Mtphagy dye ---------- 42

3.2.10. Small interfering RNA Sestrin 2 Silencing ---------- 42

3.2.11. Statistical analysis ---------- 43

4. Results ---------- 44

4.1. Short-term glucose deprivation resembles mitohormesis induction ---------- 44

4.1.1. Cellular ROS and mitochondrial superoxide decrease after short-term glucose deprivation but not in hyperglycemia ---------- 44

4.1.2. Mitochondrial membrane potential decreases in short-term glucose deprivation but not in hyperglycemia ---------- 46

4.1.3. Mitochondrial fragmentation after short-term glucose deprivation ---------- 48

4.1.4. Mitophagy and Tfam increase after short-term glucose deprivation ---------- 49

4.1.5. Short-term glucose deprivation does not induce caspase-3 activation ---------- 51

4.1.6. Sestrin 2 upregulation under glucose deprivation and hyperglycemic exposure ---------- 52

4.1.7. Glucose deprivation does not induce non-selective autophagy ---------- 55

4.2. Prior glucose deprivation exposure protects C2C12 from hyperglycemic-induced oxidative stress 56

4.2.1. Glucose deprivation does protect against hyperglycemic-induced ROS production ---------- 56

4.2.2. Glucose deprivation might increase mitochondrial resistance to hyperglycemic-induced mitochondrial damage ---------- 58

4.3. Sestrin 2 transient silencing protocol optimization ---------- 60

5. Discussion ---------- 63

5.1. Modulatory mitochondrial superoxide production during glucose deprivation is accompanied with Sestrin 2 upregulation ---------- 63

5.2. Glucose deprivation-dependent Sestrin 2 upregulation follows mitophagy induction without non-selective autophagic increase ---------- 65

5.3. Glucose deprivation pretreatment protects cells against hyperglycemic-induced oxidative stress ---------- 70

6. Bibliography ---------- 74

1. Objectives

The first purpose of the present work was the optimization of an exposure time with glucose deprivation that could resemble a mild oxidative stress. Follow optimization of this protocol, the goal was to evaluate if glucose deprivation might induce a mitohormetic response involving alterations in ROS generation. Next, we proposed to determine alterations in Sesn2 content and mitophagy status, to assess its association with a mitohormetic response. Finally, we aimed to determine if glucose deprivation, as a mild-stress, activates a protective response involving Sesn2 upregulation, thus preventing hyperglycemic damage.

In summary, this work was focused on establishing a mitohormetic-inducing protocol that might unravel the role of Sesn2 in mitohormesis, possibly involving mitophagic flux.

Introduction

1.1. Mitochondria outline

Mitochondria are double-membrane organelles that function as the heart of the eukaryotic cell metabolism. Maternally inherited, these cytoplasmic organelles were originated from symbiotic bacteria (Wallace, 2012). Mitochondria have central functions in the cell, which includes the majority of the cellular energy through the production of adenosine triphosphate (ATP), production and regulation of reactive oxygen species (ROS), buffering of cytosolic calcium (Ca^{2+}), and regulation of programmed cellular death (Galluzzi et al., 2012; Wallace, 2005). Mitochondria are particularly prominent in energy demanding tissues, namely in cardiac and skeletal muscle, liver, kidney, and neuronal, due to significant energy requirements from these type of cells (Ham & Raju, 2016). In the last years, scientists have learned how these organelles control cell migration, synapse formation, aging, epigenetics and their involvement in several pathologies ranging from ischemic disease to neurodegeneration, diabetes, and cancer (Scorrano, 2013). Thus, mitochondria have been intensively studied and suggested as important players in developing future therapies.

Reflecting the prokaryotic origin, mitochondria have their own genome and protein synthesis system. However, during its evolution, extensive transfer of mitochondrial genes to the nuclear chromosomes has resulted in only a minimal set of genes retained in the mitochondrial deoxyribonucleic acid (mtDNA) (Taanman, 1999). Nonetheless, mammalian mtDNA is a double-stranded DNA (dsDNA) molecule of 16.6 kb, which encodes 11 mRNAs (messenger ribonucleic acid (RNA)) (translated to 13 proteins), 2 rRNAs (ribosomal RNA) (12S and 16S rRNA), and 22 tRNAs (transfer RNA) (Gustafsson et al., 2016). Moreover, the two strands of mtDNA can be separated by buoyant density ultracentrifugation, and due to historical reasons are called heavy and light strands (Battey et al., 1978). Foremost in the remaining genetic material, 13 mtDNA-encoded genes constitute essential subunits of respiratory complexes I, III, IV, and V (Scarpulla, 2008). These complexes, in turn, are part of the oxidative phosphorylation (OXPHOS) system, which comprises approximately 90 proteins with dual genetic origin, either encoded by nuclear genes, translated on cytosolic ribosomes and imported into

mitochondria, or encoded by mtDNA and translated on mitochondrial ribosomes (Gustafsson et al., 2016; Scarpulla, 2008). Lastly, this mammalian mtDNA is packaged into compacted nucleoid structures, and the main packaging factor is the high-mobility group box domain protein called mitochondrial transcription Factor A (Tfam) (Brown et al., 2011; Kukat et al., 2011). The nucleus-encoded mitochondrial protein Tfam can bind, unwind and bend DNA without sequence specificity and, in addition, shows important functions in mtDNA transcription, mtDNA maintenance, replication and also mtDNA repair (Canugovi et al., 2010; Ekstrand et al., 2004; Fisher & Clayton, 1988; Fisher et al., 1992).

The referred genetic mitochondrial system is present in the organelle matrix, which is surrounded by two distinct functional membranes (Nunnari et al., 2012). The composition of the outer mitochondrial membrane (OMM) is similar to other eukaryotic membranes, whereas the inner mitochondrial membrane (IMM) resembles prokaryotic membranes in its high protein/lipid ratio and cardiolipin presence (Enríquez, 2016). Cardiolipin is responsible for the generation of positive curvature areas in the IMM, which in turn is characterized as a complicated network of tubules and lamellae called cristae (Enríquez, 2016). The cristae allow the presence of multiple electron transport chain (ETC) complexes, and the ATP synthase complexes, being the physical location of OXPHOS. Moreover, several selective and nonselective channels are present in the IMM and OMM, facilitating the communication between the cytosol and the metabolic center of the cell, about decisions regarding cell survival and death (Peixoto et al., 2012). These channels include the MAC (mitochondrial apoptosis-induced channel), mitochondrial porin or VDAC (voltage-dependent anion selective channel), the PTP (permeability transition pore), and the protein import translocases of the outer and inner membranes called TOM and TIM, respectively (Peixoto et al., 2012). In summary, mitochondrial structure composition comprises all the machinery required to assert their valuable role in the cell and, also, to ensure an intimate communication with the surroundings.

1.1.1. ETC and OXPHOS

Mitochondria are central to maintaining the delicate balance between life and death, especially given its principal role in energy production of ATP. Most of the ATP produced during aerobic glucose metabolism is from OXPHOS, one of the best-understood cellular processes at the genetic, molecular, structural, and biochemical level (Enríquez, 2016). Composed of respiratory complexes, electron carriers, and the F1FO-ATP synthase, the OXPHOS begins with the entry of electrons from catabolic processes such as glycolysis, fatty acid oxidation and the tricarboxylic acid (TCA) cycle to produce ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi) (Fig 1). In this process the ETC, which comprises four oxidoreductases, coupled electron transport with translocation of protons across the inner mitochondrial membrane (Dudkina et al., 2010). With the exception of complex II (succinate- quinone oxidoreductase), all the other are proton-pumping enzymes i.e. complex I (nicotinamide adenine dinucleotide (NADH)– ubiquinone oxidoreductase), complex III (cytochrome bc1) and complex IV (cytochrome *c* oxidase). These complexes are supported by membrane-embedded redox cofactors, ubiquinone (Q) (known as coenzyme Q (CoQ)) and soluble cytochrome *c* during the intra-protein electron transfer (Dudkina et al., 2010; Enríquez, 2016; Sazanov, 2015).

Complex I catalyze the transfer of electrons from NADH to reduce Q to ubiquinol (QH2). QH2 is subsequently used by complex III to reduce cytochrome *c* in the intermembrane space (IMS), and complex IV through cytochrome *c* reduces molecular oxygen, which is the ultimate electron acceptor (Enríquez, 2016; Sazanov, 2015). Ubiquinone also carries electrons from (flavin adenine dinucleotide reduced form) $FADH_2$, generated in succinate dehydrogenase in the citric acid cycle, to complex II (succinate–quinone oxidoreductase). This coupling of electrons flow to ATP synthesis was described by Peter Mitchell hypothesis in 1961, named as Chemiosmotic Theory (Mitchell, 1961). This theory infers that a coupled electrons transfer of energy with a proton circuit across the IMM drives OXPHOS, resulting in ADP phosphorylation (Jastroch et al., 2010). That is, the oxidation of molecules such as NADH and $FADH_2$ leads to the release of electrons that are passed through a series of carriers in respiratory chain complexes with increased oxidation potentials (Jastroch et al., 2010). Throughout

this exergonic process, protons from the mitochondrial matrix are pumped across the inner mitochondrial membrane, creating an electrochemical gradient known as the protonmotive force. The movement of protons back to the matrix through the ATP synthase drives the conversion of ADP and Pi to ATP.

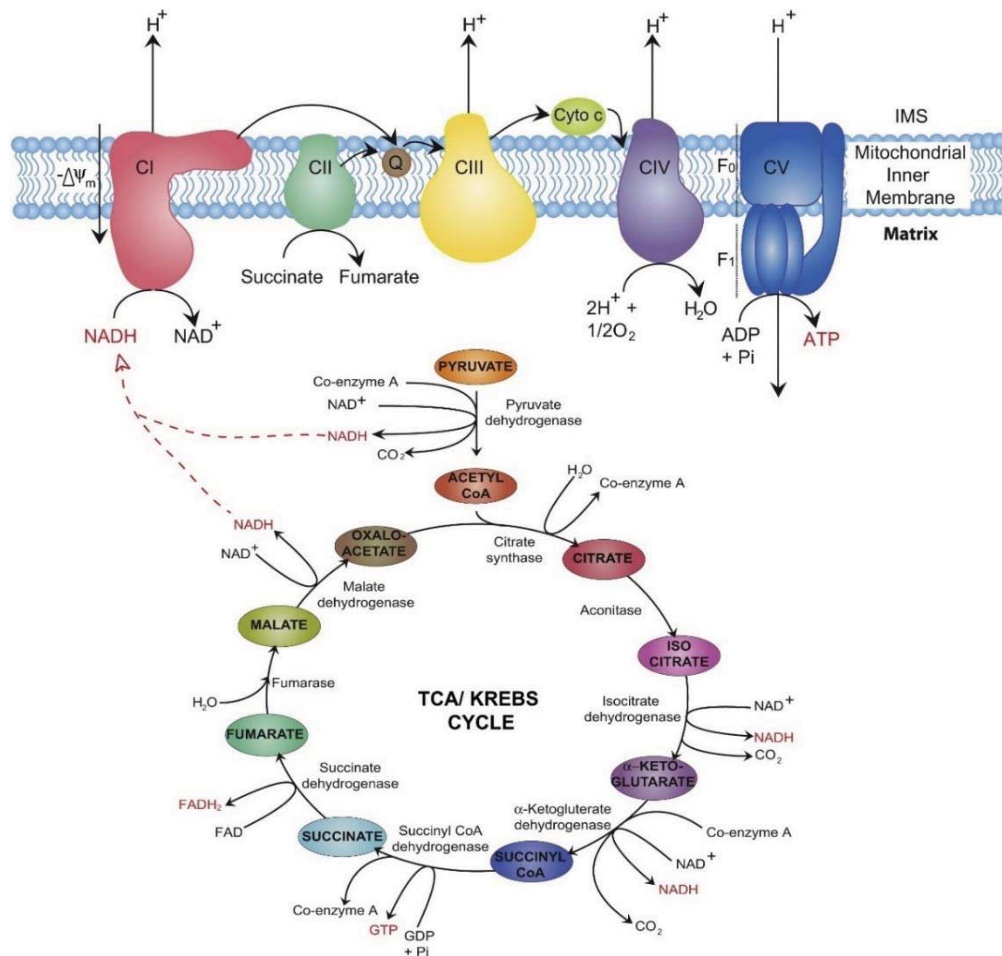


Figure 1. ***TCA cycle and oxidative phosphorylation bioenergetics***
The tricarboxylic acid (TCA), also known as Krebs cycle occurs after complete oxidation of sugars, fatty acid, and proteins. These substrates are catabolized to acetyl-CoA and enter the first stage of these eight subsequent enzymatic steps. This process begins with the entry of electrons from NADH and $FADH_2$ into the mitochondrial electron transport chain, driving oxidative phosphorylation (from Osellame, Blacker, & Duchen, 2012).

1.1.2. Mitochondrial biogenesis

Mitochondrial biogenesis can be simply defined as a cellular and molecular process that cause growth and division of pre-existing mitochondria (Jornayvaz & Shulman,

2010). The dynamics of this process and function involves a complex interplay that ultimately shapes bioenergetics capacity (Dominy & Puigserver, 2013). Moreover, concerning the limited number of proteins encoded by mitochondrial DNA, a correct mitochondrial biogenesis requires coordinated processes of synthesis and import of approximately 1000- 1500 proteins from cytosolic ribosomes (Baker et al., 2007). Also, mitochondrial fusion and fission must be coordinated along with mitochondrial variations in number, size, and mass, characterizing as well mitochondrial biogenesis progression (Baker et al., 2007). Environmental stresses, such as exercise, caloric restriction, low temperature, oxidative stress, cell division, and differentiation, can influence mitochondrial biogenesis through the activation of several signaling events, that lead to the activation of a particular transcription cascade (Jornayvaz & Shulman, 2010).

The master regulator of this process is known as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and was initially identified in mice exposed to cold temperatures, especially in thermogenic tissues, such as brown fat (Puigserver et al., 1998). PGC1-α regulates the process by being a co-transcription factor of the main mitochondrial enzymes, such as ATP synthase (β-subunit) and COX (cytochrome c oxidase) subunits (COX II and COX IV) (Wu et al., 1999). Indeed, PGC-1α induces mitochondrial biogenesis by activating different transcription factors, including nuclear respiratory factor 1 and 2 (NRF-1 and NRF-2), which link the mitochondrial transcription by targeting Tfam (Jornayvaz & Shulman, 2010). Nuclear respiratory factors (NRFs) govern the expression of nuclear genes that encode mitochondrial proteins, among which are subunits of the ETC, mitochondrial ribosomal proteins and key mitochondrial enzymes (Scarpulla, 2008). Through these transcription factors, PGC-1α coordinately increases mitochondrial biogenesis and respiration rates, in addition to the rise of uptake and utilization of substrates for energy production (Jornayvaz & Shulman, 2010).

Another major regulator of mitochondrial biogenesis is AMP-activated protein kinase (AMPK), the enzyme chief of the whole-body energy balance (Reznick et al., 2007). AMPK was identified as important in mitochondrial biogenesis through its chronic pharmacological activation with β-guanadinopropionic acid (β-GPA), a compound that increased NRF-1 binding activity, cytochrome *c* protein expression, and mitochondrial

content in skeletal muscle (Bergeron et al., 2001). Moreover, transgenic mice expressing a dominant-negative mutant form of AMPK in skeletal muscle fed with β-GPA confirmed the role of AMPK in mitochondrial biogenesis (Zong et al., 2002). No changes were detected in PGC-1α expression, mitochondrial DNA content and mitochondrial density, which were increased in wild-type mice fed with β-GPA (Zong et al., 2002). These findings and others studies with AMPK agonists have established the strong link between AMPK activation and mitochondrial biogenesis (Jayanthy et al., 2017; Winder et al., 2000). Also, CaMKIV, nitric oxide (NO^-) and sirtuins have been linked with mitochondrial biogenesis (Brenmoehl & Hoeflich, 2013; Lira et al., 2010; Wu et al., 2002). All together give rise to a complex transcription program that permits replacement of mitochondria or synthesis of newer components and their insertion in more efficient mitochondria.

1.1.3. Mitochondrial Dynamics

Mitochondria undergo plastic transformations that renowned their status as a highly dynamic organelle. The length, shape, size, and number of mitochondria are controlled by fusion (the joining of two organelles into one) and fission (the division of a single organelle into two) events, which make them essential in maintaining the overall morphology of the mitochondrial population. Hence, when unbalanced, these events can lead to dramatic transitions in mitochondrial shape, which therefore can influence crucial cellular functions, which go from Ca^{2+} signaling to generation of reactive oxygen species, neuronal plasticity, muscle atrophy, lymphocyte migration and even to lifespan (Campello & Scorrano, 2010).

Besides these dynamic processes, mitochondria adopt different morphologies depending on the cell type and, even in the same cell, it’s possible to see a range of heterogeneous mitochondrial morphologies. This engagement of several mitochondrial forms is supported by controlled processes of fusion and fission which actively silhouettes mitochondrial shape and size. Beyond that, mitochondrial internal structure, mainly at the inner membrane, can suffer organizational changes in response to their physiological state, and more remarkably, they can move to specific subcellular locations (Detmer & Chan, 2007).

Mitochondrial fusion results in a more interconnected mitochondrial network, and allows the exchange of DNA and metabolites between neighboring mitochondria, including damaged or senescent mitochondria (Nakada et al., 2001; Yoneda et al., 1994). Thus, mitochondrial fusion permits efficient mixing of mitochondrial content and promotes their endurance (Westermann, 2012). On the contrary, fission events produce smaller mitochondria that can operate individually elsewhere in the cell or are degraded by mitophagy (Elgass et al., 2013).

The first description of fusion events was described in yeast by Thomas & Wilkie in 1968 and later analyzed in others organism models, such as *Drosophila melanogaster* and mammalians (Bereiter-Hahn & Vöth, 1994; Cortese, Voglino, & Hackenbrock, 1998; Hales et al., 1997). Dynamin-like GTPases mediate the fusion of both mitochondrial OMs and IMs. In mammals, this process is mediated by the fusion proteins Mfn1 (Mitofusin 1) and Mfn2 (Mitofusin 2) and OPA1 (Optic Atrophy 1). Outer mitochondrial membrane fusion depends on both Mfn1 and Mfn2, which are highly homologous to dynamin-related GTPases, and have an N-terminal GTPase domain, two hydrophobic heptad repeats (HRs) and two transmembrane domains that insert them in the OMM (Scorrano, 2013). OM fusions mediated by mitofusins can be by homo- and heterotypic interactions that depend on Guanosine-5'-triphosphate (GTP) hydrolysis (Wai et al., 2016). Tissue-specific deletion of mitofusins in the central nervous system, heart, muscle, or liver compromise metabolic and organ function, highlighting their importance at both the cellular and organismal level (Dorn, 2015; Zorzano & Claret, 2015). Long-OPA1 (L-OPA1) form is also a dynamin-like GTPase, which is responsible for fusion of inner mitochondrial membranes. OPA1 is anchored to the IM by an N-terminal transmembrane domain, being the majority of the protein exposed in the IM space (Wai et al., 2016). It is possible to found multiple forms of OPA1 in mammalian cells and tissues due to alternative splicing and proteolytic cleavage (Wai et al., 2016). Newly synthesized OPA1 precursor molecules are targeted to mitochondria by an N-terminal mitochondrial targeting sequence that is cleaved off upon import into mitochondria generating L-OPA1. In addition, proteolytic cleavage of L-OPA1 into short-OPA1 (S-OPA1) can occur due to different cellular insults, which consequently causes mitochondrial fragmentation (Anand, Langer, & Baker, 2013). Thus, under stress conditions, mitochondrial fusion is inhibited due to the lack of L-OPA1, and thereby

ongoing fission events happen with the resulting fragmentation of the mitochondrial network (Anand et al., 2013; Ishihara et al., 2006; Wai et al., 2016).

Division of mitochondria is mediated by Dynamin-related Protein 1 (DRP1), a large GTPase that is recruited to the mitochondrial outer membrane via several receptor proteins (Mff, Fis1, MiD49, and MiD50) (Mishra & Chan, 2016). In response to several cellular signals, this protein is translocated from the cytosol to the OM, where its oligomerizes into multimeric spiral structures at future sites of division (Wai et al., 2016). While in OMM, DRP1 conformation changes due to GTP binding and hydrolase, and thereby leads to membrane constriction and scission (Mears et al., 2011). As an aftermath, cells lacking DRP1 adopt a hypertubular mitochondrial network configuration (Waterham et al., 2009). Thus, mitochondria can undergo morphologic transitions during metabolic flux and in response to cellular and environmental stresses (Galloway & Yoon, 2012). In addition, mitochondrial dynamics is more than a fusion and fission modulation of mitochondrial shape; it represents a central regulation of mitochondrial and cellular overall function by altering cellular susceptibility to death, respiratory properties and ROS generation (Picard et al., 2013).

1.2. Autophagy

Autophagy has been classified as a consolidated self-degradative process that remains from yeast to humans (Rabinowitz & White, 2010). In the majority of the circumstances, autophagy supports the cellular life by allowing the maintenance of cell homeostasis, either by removing potentially dangerous constituents such as protein aggregates and dysfunctional mitochondria or because it provides breakdown products to balance sources of energy at critical times (Glick, Barth, & Macleod, 2010; Rabinowitz & White, 2010). Three types of autophagy have been defined: macroautophagy, microautophagy, and a mechanistically separate process, chaperone-mediated-autophagy, all of them promote proteolytic degradation of cytosolic components at the lysosome (Glick et al., 2010). Lipids, proteins, and organelles are some of the cellular contents that are straightly taken by invaginations, protrusions, or septations of the lysosome membrane in the process of microautophagy (Glick et al., 2010; Zhang, 2015). In contrast, macroautophagy involves the sequestering of substrates within cytosolic double-membrane vesicles (named as autophagosome), that fuses later with the

lysosome to form an autolysosome (Feng et al., 2014). The substrates of this process can include either damaged organelles and cytosolic proteins, with the addition of invasive microbes (Feng et al., 2014). In the chaperone-mediated autophagy a protein named HSP70 (heat shock protein 70 kDa (kilodalton) protein), recognizes and translocates across the lysosomal membrane proteins with consensus KFERQ sequence, which are identified by the membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A), resulting in their unfolding and subsequent degradation (Glick et al., 2010; Zhang, 2015). However, autophagy can be relatively nonselective, targeting to lysosomal degradation virtually any portion of the cytoplasm (Mizushima & Komatsu, 2011).

The molecular machinery of autophagy, hereafter referred to as macroautophagy, shares most of the same with the selective micro-autophagy (Feng et al., 2014). Macroautophagy involves a complex molecular organization that starts with the autophagosome formation, and later nucleation, elongation, maturation, and degradation of autophagosomes (Wong & Cheung, 2011). In mammalian cells, phagophore membranes appear to initiate primarily from the endoplasmic reticulum (ER) (Hayashi-Nishino et al., 2009; Ylä-Anttila et al., 2009), though other cytosolic membrane sources have been suggested, such as the trans-Golgi and late endosomes (Axe et al., 2008; Mizushima & Klionsky, 2007). This process requires the coordinated nucleation and expansion of the isolation membrane, which starts with the assembly of proteins of the UNC51-like kinase (ULK) complex (Fig 2) (Lamb, Yoshimori, & Tooze, 2013). During the nucleation stage the ULK complex targets class III PI3K complex (consisting of beclin 1 (Atg6 in yeast), vacuolar protein sorting 15 (VPS15), VPS34 and ATG14), promoting a local generation of phosphatidylinositol 3-phosphate (Kaur & Debnath, 2015). In the expansion stage, the ATG12–ATG5–ATG16 complex is recruited to the autophagosome membrane where it facilitates the lipidation of microtubule-associated protein 1 light chain 3 (MAP1LC3; also known as LC3) with phosphatidylethanolamine (Lamb et al., 2013). The lipidated form of LC3, known as LC3-II, is later attached to the autophagosome inner membrane and has been proposed to function as a receptor for a particular substrate, p62/SQSTM1 (sequestosome 1) (Bjørkøy et al., 2005). Once the vesicle expansion is complete, the mature autophagosomes merge with the lysosomes, becoming self-degradation structures often called “autolysosomes” or “autophagolysosomes” (Mizushima, 2007). The inner

membrane of the autophagosome and the cytoplasm-derived materials contained in the autophagosomes are then degraded by lysosomal hydrolases (Mizushima, 2007). After degradation, monomeric units (e.g., amino acids) are exported to the cytosol for reuse as building molecules. Therefore, concerning the numerous stages of autophagy development, this process requires the efficient and consolidated regulation by upstream modulators to ensure the ending degradation.

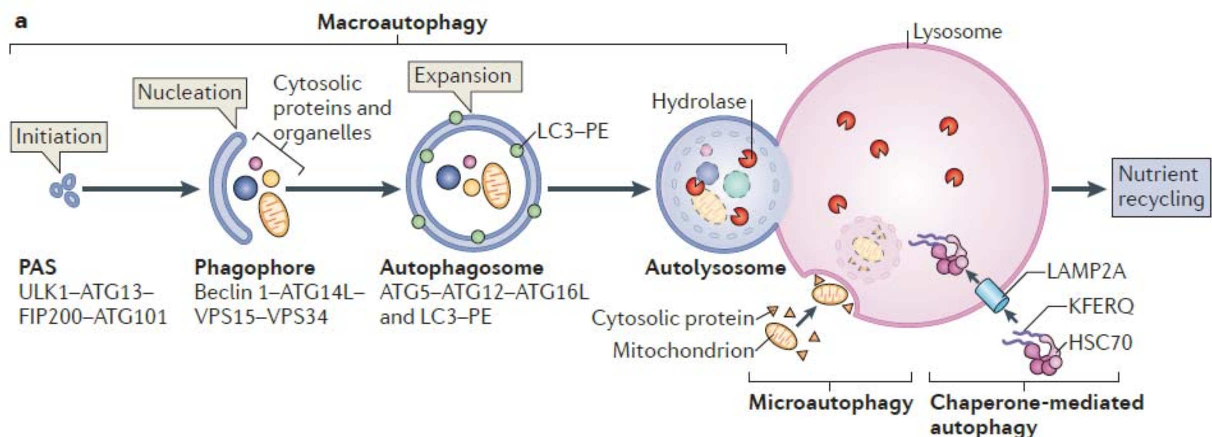


Figure 2. ***Mammalian macroautophagy, microautophagy, and chaperone-mediated autophagy pathways.***
The ULK complex mediates the formation of the phagophore assembly site (PAS), initiating the macroautophagy. Then, nucleation step occurs during simultaneous phagophore membrane elongation and autophagosome completion. The expansion of autophagic membranes requires conjugation of phosphatidylethanolamine (PE) to LC3 (the microtubule-associated protein 1 light chain 3, a principal mammalian homolog of yeast Atg8). Lately, autophagosome fusion with the lysosome leads to the formation of autolysosome. In addition, microautophagy comprises the direct incorporation of cellular elements by the lysosomal membrane. In chaperone-mediated autophagy, substrates are recognized and translocated to lysosomes in an LAMP2A-dependent manner. In all three processes, the autophagic cargo is degraded via lysosomal hydrolases (from Kaur & Debnath, 2015).

1.2.1. Mitophagy

Organellophagy (selective elimination of organelles via autophagy) sustains cellular homeostasis and adaptation to variable environments, by removing dysfunctional and surplus organelles (Okamoto, 2014). Mitophagy is the selective autophagy of damaged or dysfunctional mitochondria, which was firstly observed in mammalian cells by early electron microscopy studies, during stimulation of hepatocyte catabolism with glucagon (Duve & Wattiaux, 1966). Due to the prominent roles of mitochondria in processes such as energy conversion, calcium homeostasis, and programmed cell death (Nunnari & Suomalainen, 2012), their correct removal has been deeply associated with physiological deviations. Indeed, an interplay between

mitochondrial fission and decreased mitochondrial membrane potential (MMP), OPA1 levels and size are described as a process that might facilitate mitophagy (Twig et al., 2008).

Mitophagy can occur through many mechanisms. One well-established control mechanism is regulated by the MMP in collaboration with mitochondrial PINK1 (phosphatase and tensin homolog (PTEN)-induced kinase 1) and the cytosolic E3 ubiquitin ligase Parkin. When the mitochondrial population is healthy, PINK1 is continuously imported across the mitochondrial outer and inner membranes, and consequently cleaved by several mitochondrial processing peptidases (Kawajiri et al., 2010; Matsuda et al., 2010). While this situation is maintained, Parkin is found throughout the cytosol in an inactive form, without stable association with mitochondria (Matsuda et al., 2010). Upon mitochondrial damage and depolarization, PINK1 import system is impaired and the levels of this protein increase on their surface (Kawajiri et al., 2010; Matsuda et al., 2010). Subsequently, PINK1 forms a supermolecular complex together with the TOM components and undergoes intermolecular phosphorylation (Fig 3) (Okatsu et al., 2013).

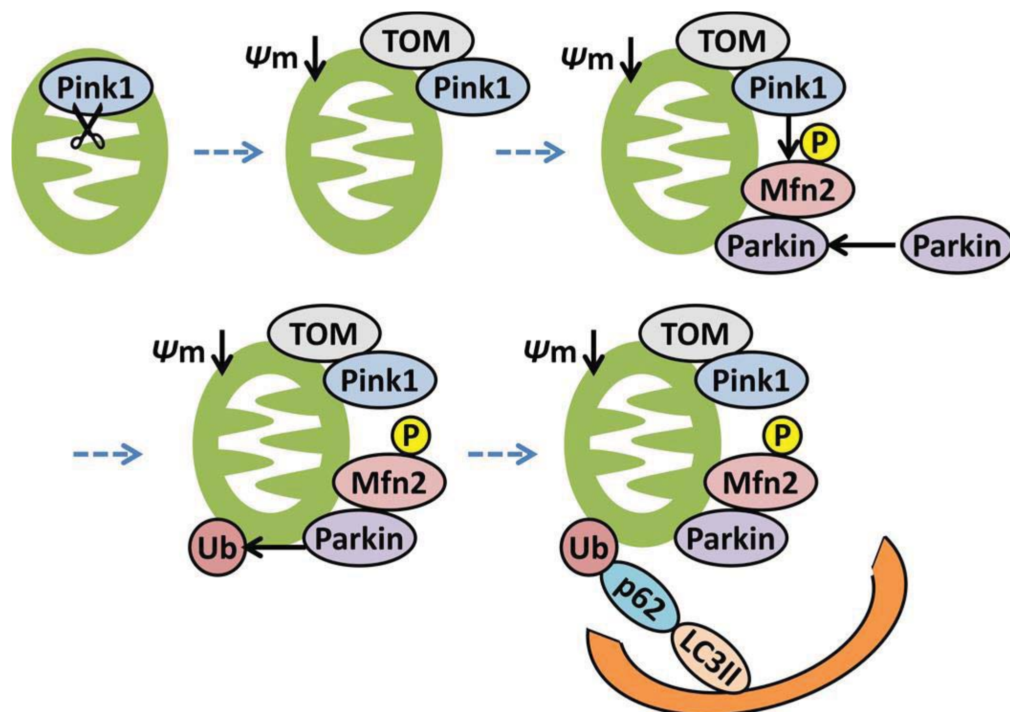


Figure 3. ***Pink1-Parkin-dependent mitophagy in mammalian cells.***
In functional mitochondria, Pink1 is successively degraded by mitochondrial proteases and peptidases. Upon mitochondrial depolarization, Pink1 accumulates at the outer membrane and recruits Parkin through phosphorylation of Mfn2, resulting in Parkin recruitment to the mitochondrial surface. Consequently, Parkin ubiquitinates surrounding proteins, which are detected by a ubiquitin- and LC3-

binding adaptor protein described as p62. With this process, abnormal mitochondria are identified and mediated to degradation by the cell (from Saito & Sadoshima, 2015).

These two last events are the prerequisite for Parkin recruitment to damaged mitochondria, along with PINK1 later phosphorylation of the Parkin ubiquitin-like (Ubl) domain (Okatsu et al., 2013). After activation, Parkin ubiquitinates several mitochondrial proteins, including VDAC, Mfn1/2, TOM, Fis1 (mitochondrial fission 1), Miro1 (Mitochondrial Rho GTPase 1) and mitochondrial hexokinase (Chan et al., 2011; Gegg et al., 2010; Kazlauskaite et al., 2014; Okatsu et al., 2012). The polyubiquitination catalyzed by Parkin is associated with the recruitment of 62/SQSTM1, a ubiquitin- and LC3-binding adaptor protein (Chan et al., 2011; Geisler et al., 2010; Okatsu et al., 2010). In resume, PINK1 accumulation in mitochondria is linked to Parkin recruitment and subsequent successive ubiquitination of mitochondrial proteins, which identifies the organelle for degradation.

In addition, mitophagy can happen independently of the PINK1/Parkin system, through three mitochondria-anchored receptors, NIX (Nip3-like Protein X), BNIP3 (Bcl2/E1B 19 kDa-interacting protein 3-like protein), and FUNDC1 (Fig 4)(FUN14 domain-containing protein 1) (Liu et al., 2012; Sandoval et al., 2008; Zhang et al., 2008). These proteins share an LC3-interacting region (LIR) that are essential for their mitophagy activities (Novak et al., 2010; Zhu et al., 2013). Bnip3 and Nix (also known as BNIP3L) are BH3-only proteins that activate mitochondria-mediated cell death as well as autophagy and mitophagy (Zhang & Ney, 2009). Thus, within a population of mitochondria, heterogeneity dueling of behaviors may decide the overall cellular response (Zhu et al., 2013). FUNDC1 is an outer mitochondrial protein that induces selective mitophagy in response to hypoxia (Liu et al., 2012; Zhu et al., 2013). FUNDC1-meadiated mitophagy is controlled by phosphorylation in the LIR motif, which affects its interaction with LC3 and leads to the incorporation of the mitochondrion as a specific cargo into LC3-bound isolation membrane for subsequent removal (Liu et al., 2012). These proteins, in addition to PINK1 and Parkin, reflect a cellular system to ensure mitochondrial degradation when necessary.

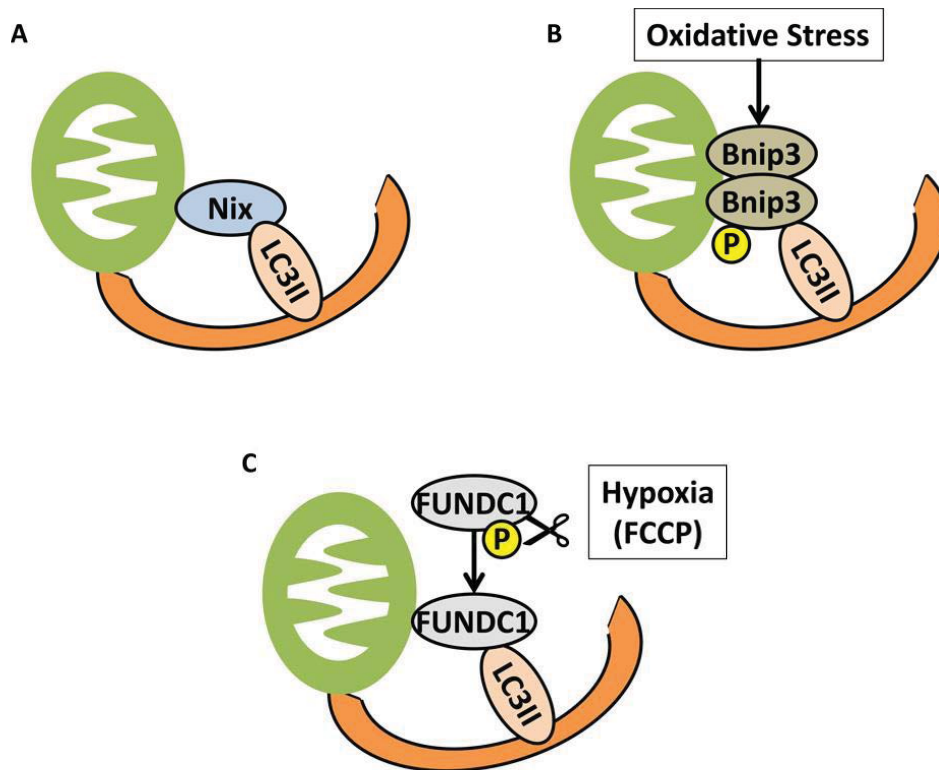


Figure 4. ***LC3-interacting molecules-dependent mitophagy.***
(A-B) BH3-only proteins with an LC3-interacting region (LIR), such as Nix and Bnip3 can regulate mitochondrial autophagy. Bnip3 suffers homodimerization-dependent activation under oxidative stress conditions. Subsequently, Bnip3 specific phosphorylation of the LIR causes LC3 binding and mitochondrial triggering to autophagy. (C) FUNDC1 dephosphorization under hypoxia or in response to FCCP-induced mitochondrial depolarization promotes mitophagy through interaction with LC3 (from Saito & Sadoshima, 2015).

1.3. Mitochondrial reactive oxygen species and oxidative stress

Mitochondria are the bulk ROS producer within cells due to the leak of single electrons to oxygen, while mitochondrial respiration and substrate oxidation occur (Andreyev, Kushnareva, & Starkov, 2005). ROS consist of oxygen free radicals, such as superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$), and nonradical oxidants, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O2$) (Dickinson & Chang, 2011; Murphy et al., 2011). Within mitochondria, $O_2^{\bullet-}$ is produced by the one-electron reduction of oxygen and represents the primary and most abundant ROS. Under physiological conditions, the levels of endogenous ROS are controlled by the primary antioxidant enzymes that include superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPX). For instance, the inactivation of $O_2^{\bullet-}$ into the

intermediate element H_2O_2 occurs through spontaneous or superoxide dismutase (SOD)-catalyzed dismutation (Fukai & Ushio-Fukai, 2011).

Three SOD isoforms were found in mammals with distinct subcellular localization: the copper/zinc-dependent isoform (Cu, Zn SOD, SOD1) (Beyer, Imlay, & Fridovich, 1991) is located in the mitochondrial intermembrane space and cytosol; the manganese-dependent isoform (Mn-SOD, SOD2) (Robinson, 1998) is found in the mitochondrial matrix; and Cu, Zn SOD is found in the extracellular space (ecSOD, SOD3) (Wiener & Mowen, 1978). In turn, H_2O_2 can diffuse across membranes and converted to water and molecular oxygen by CAT, GPX, and thioredoxin (Antunes & Cadenas, 2000; Roberts & Sindhu, 2009). When not metabolized, H_2O_2 in the presence of metal ions can be transformed to hydroxyl radical by Fenton reaction (Halliwell & Gutteridge, 1990). This molecule is highly reactive and has a severe detrimental impact on cellular constituents. Mitochondria are also equipped with nonenzymatic antioxidants, such as vitamin C or ascorbic acid, vitamin E, β-carotene, reduced glutathione and phenolic compounds (Roberts & Sindhu, 2009). Normally, mitochondria are able to scavenge ROS. However, ROS excessive release can surpass the whole scavenger response leading to their accumulation and consequent cell injury.

In terms of simplicity, the mitochondrial ROS amount translates the stressor gravity to the cellular response: while moderate stressor induces smaller quantities to promote cellular adaptation and cell survival, a detrimental stressor causes larger amounts of mitochondrial ROS that induce cellular damage and subsequent cell death (Ristow & Schmeisser, 2011; Roberts & Sindhu, 2009). In fact, excessive ROS formation from mitochondrial dysfunction has been associated with a variety of diseases such as diabetes, neurodegenerative disorders, and cancer (Ristow & Schmeisser, 2014). This last defines a cellular condition named as oxidative stress, in which ROS overproduction overwhelms the endogenous antioxidant capacity and consequently rise ROS beyond the normal/physiological threshold level (Birben et al., 2012). The triggering factors for oxidative stress may be diverse, oscillating from hereditary or acquired genetic defects (mutations) or environmental factors (radiation or toxins) to stochastic events such as metabolic fluctuations (Andreyev et al., 2005). These circumstances cause abrupt ROS production that favors a vicious cycle, since oxidative stress reciprocally aggravates ROS

production due to their capacity to cause unregulated oxidative damage of DNA, proteins, and lipids (Bentley et al., 2015).

Once mitochondria represent a major source of ROS, the free radical attack directly cumulates in oxidative injuries and progressive functional loss of these organelles (Guo et al., 2013). Unrepaired damage of mitochondrial DNA and mitochondrial respiratory chain, peroxidation (autoxidation) of mitochondrial phospholipids and perturbed mitochondrial calcium homeostasis are some of the events that promote mitochondrial dysfunction during this process (Guo et al., 2013). Subsequently, ROS also react with the nuclear DNA, structural and enzymatic cytosolic proteins and with non-mitochondrial polyunsaturated fatty acids of lipid membranes, thereby contributing to an extensive cellular injury. This severe damage results in cell death, and consequently whole organ and organism failure (Kozlov et al., 2011; Zorov et al., 2012).

1.3.1. Hyperglycemia, oxidative stress, and mitochondrial dysfunction

As mentioned before, under certain circumstances the leak of electrons from the ETC is amplified, leading to chronic oxidative stress and adverse effects. Hyperglycemia represents a glucotoxicity status, where cells or an organism are exposed to supraphysiologic concentrations of glucose. This condition incites the accumulation of ROS within mitochondria, which lately cause oxidative stress progress (Giugliano, Ceriello, & Paolisso, 1996). Given the glucose metabolism process, it is clear that an intracellular increase of glucose leads to a higher input of electron donors by the TCA cycle (NADH and $FADH_2$). Excessive NADH is reported to directly exacerbate production of $O_2^{\bullet-}$ by full reduction of flavin on complex I (Kussmaul & Hirst, 2006). However, complex I significant contribution of superoxide arises from reverse electron flow, which occurs when electrons are transferred back from ubiquinol through the ubiquinone binding site (Murphy, 2009). Moreover, in this condition, the proton gradient goes above threshold values resulting in hyperpolarization of MMP (Brownlee et al., 2001; Du et al., 2001). This, in turn, promotes complex III electron transport inhibition, and ubisemiquinone radical-generating Q cycle (Dröse & Brandt, 2008; Marchi et al., 2012). This occurs because respiratory chain complexes proton pumps are intimately

dependent on the transmembrane proton gradient and MMP. Normally, superoxide production is higher in complex I when compared with complex III. However, under hyperglycemic conditions, it seems that mitochondrial superoxide generation is more significant from complex III (Murphy, 2009). Thus, excessive electron slippage from non-injurious metabolism of glucose can, in conditions like hyperglycemia, lead to overproduction and/or mismanagement of ROS from complex I and III.

Being the main intracellular source of ROS, mitochondria are also the immediate target of ROS. Therefore, hyperglycemia-induced ROS generation can affect mitochondria dramatically. Mitochondrial damage/dysfunction by hyperglycemia can be characterized by mtDNA mutation, low respiratory contents and deteriorated antioxidant defense (Su, Kuo, & Liu, 2013). In addition, morphological changes can also occur in response to hyperglycemia. For instance, mitochondrial fragmentation has been described as a mediator of apoptosis and prerequisite for ROS increase (Yu at al., 2006). Although hyperglycemic-induced mitochondrial fission leads to ROS generation, this event was suggested as a physiological adaptation since its suppression promoted premature cell death (Yu et al., 2008). Thus, fission may allow a better distribution of metabolites and can contribute to dysfunctional mitochondria elimination by mitophagy. However, autophagy has also been shown to be affected by hyperglycemia. This, in addition to an increase fission, might explain the observed accumulation of small mitochondria, with disarrayed cristae and lower oxygen consumption rates during extended exposure to high glucose (Yu et al., 2006; Bonnard et al., 2008). Moreover, mitochondrial fragmentation accompanied by autophagy impairment can explain the increased mitochondrial ROS associated with hyperglycemia (Yu et al., 2006). In contrary, glucose-induced mitochondrial fragmentation has been described also to be triggered by ROS (Makino et al., 2010). These findings suggested that treatment with ROS scavengers would block the extreme fission events in response to elevated glucose concentrations leading to mitochondrial function protection (Makino et al., 2010). Concerning all these findings, dramatic mitochondrial network fission was shown to contribute to hyperglycemic-induced apoptosis in several studies (Jheng et al., 2012; Makino et al., 2010). Therefore, hyperglycemia temporal induction of either mitochondrial fragmentation or ROS generation elucidates the complex adaptation of cells in response to increased energetic flux. In interpreting the above-reviewed

literature, it is likely that mitochondrial fragmentation is a primary adaptive response to increased flux of energy fuel with long-term adverse effects on mitochondrial function.

Mitochondrial biogenesis control of mitochondrial function is also affected by hyperglycemia. In fact, hyperglycemia- induced ROS production in muscle from mice was associated with a decrease in the expression of PGC1-α mRNA and some of its target genes (Bonnard et al., 2008). Moreover, treatment with N-acetyl cysteine (NAC) restored mitochondrial density and structure (Bonnard et al., 2008). In vitro studies also supported mitochondrial biogenesis inhibition by hyperglycemia (Palmeira et al., 2007). In conclusion, these studies suggest that one of the explanations for hyperglycemic-mediated mitochondrial dysfunction by reduced expression of proteins involved in the OXPHOS system might due to a decrease in mitochondrial biogenesis machinery.

Autophagy can be stimulated to maintain cellular homeostasis by increasing energy demands or removing protein aggregates and damaged organelles (Rabinowitz & White, 2010). The removal of dysfunctional mitochondria from cells ensures a healthy pool of mitochondria and may be regulated by ATP and mitochondrial ROS production (Laar et al., 2011; Wei et al., 2014). Hyperglycemia provides an excess of the substrate to the respiratory chain, and consequently mitochondrial changes such as fragmentation and leak of electrons to form superoxide are increased. From these alterations or others, the ETC subunits become impaired, which promotes more superoxide production by hyperglycemia and ATP depletion. Although damaged mitochondria recognition to autophagy is mostly stimulated by the loss of MMP, deficient or decrease mitophagy is described in hyperglycemia-induced severe stress (Higgins & Coughlan, 2014; Zhan et al., 2015). A reasonable explanation for this is at the beginning of exposure to high levels of glucose; dysfunction mitochondria are eliminated by mitophagy, which prevents their accumulation and protects against oxidative stress (Lee et al., 2016; Xiao et al., 2017). However, the clearance of damaged mitochondria is blocked when the number of damaged mitochondria and the effects of stress overwhelms the mitophagic flux progression (Fig 5) (Kubli & Gustafsson, 2012; Matsui et al., 2013). In addition, impairment of mitophagic machinery by hyperglycemia may also occur as a counteract adaptation to mitochondrial biogenesis decrease to avoid loss of mitochondrial mass (Picard et al., 2013). The complexity to understand the hyperglycemic impact in

mitophagy results from the difficult temporal identification of cause and effect of lasting changes by excess glucose on a given cell.

The end-stage of mitochondrial dysfunction by hyperglycemia is the promotion of apoptosis (Smart & Li, 2007). Apoptosis is the process by which unwanted or damaged cells are eliminated during several physiological processes (Smart & Li, 2007). Appropriate modulation of apoptosis is critical for normal cellular homeostasis, whereas excessiveness promotes disease progression (Smart & Li, 2007). Mitochondria retain several apoptosis-inducing factors such as cytochrome *c*, being an integral part of apoptosis (Mignotte & Vayssiere, 1998; Wang, 2001). In hyperglycemia-induced apoptosis, the release of these proteins into the cytosol by mitochondrial outer membrane permeabilization (MOMP) or mitochondrial permeability transition pore (MPTP) has been intensively described (Cai et al., 2002; Szabadkai & Duchen, 2009).

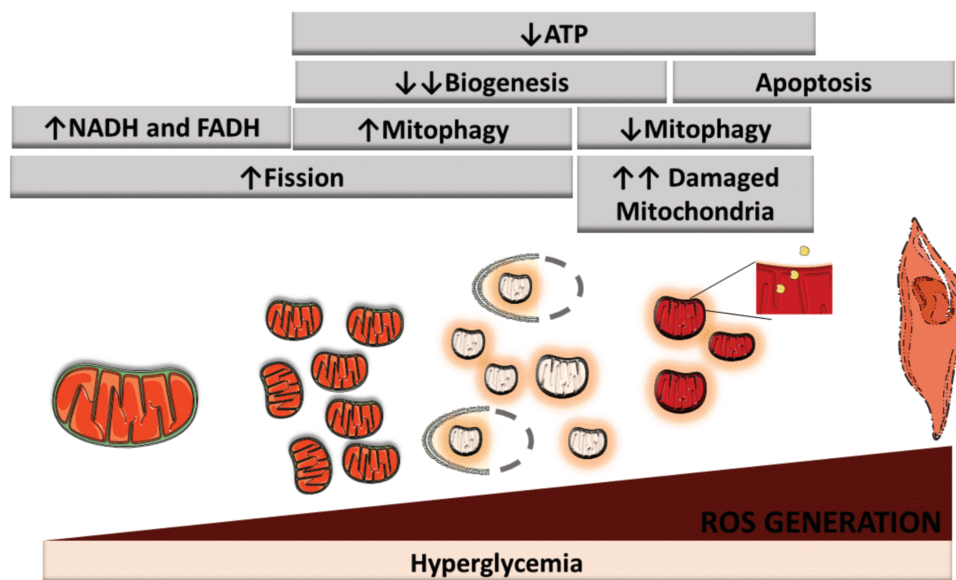


Figure 5. ***Hyperglycemia possible temporal impact on mitochondrial function.***
Increased intracellular glucose leads to a higher input of NADH and $FADH_2$ to OXPHOS. This stimulation promotes mitochondrial fragmentation, and all together support ROS generation increase. While ROS augment might induce mtDNA and nuclear DNA damage, and consequent mitochondrial biogenesis decreased, mitophagy is induced to eliminate non-functional mitochondria with limited OXPHOS and deteriorated antioxidant activity. When the number of damaged mitochondria and ROS production exceed the cell homeostasis machinery, the mitochondrial dysfunction and oxidative stress decide the fate of the cell. Therefore, apoptotic-inducing factors (yellow circles) release into the cytosol from an overall loss of mitochondrial integrity by hyperglycemia leads to cellular death (image created with **Servier Medical Art templates**).

1.3.2. Reactive oxygen species generation: A physiological role

Despite the harmful side of ROS accumulation and their broad association with disease and aging, ROS are also known as vital sensors and switches in physiological processes (D'Autréaux & Toledano, 2007). When the balance between their generation and scavenging is controlled, they can initiate cellular responses involved either in cell protection and adaptation or regulation of processes such as differentiation and autophagy (Sena & Chandel, 2012). For example, H_2O_2 rise and reversible induction were shown to regulate autophagosome formation, a critical step in autophagy development (Scherz-Shouval et al., 2007). Moreover, mild stress induction through transient low concentrations of H_2O_2 and rotenone (complex I inhibitor) were shown to trigger fission-dependent mitophagy (Frank et al., 2012). In these conditions, a moderate increase of mitochondrial ROS neither promoted cell death nor detectable mitochondrial dysfunction, but rather induced ROS signal cascades that culminate either in selective or overall autophagic cell protection. In contrast, ROS-mediated autophagy can also be induced in response to oxidative stress, from conditions such as starvation (Kroemer et al., 2010). From this condition, ROS-mediated autophagy may ensure cell survival from the current energetic deficit by maintaining energy levels through recycling of intracellular constituents (Kroemer et al., 2010; Li et al., 2013). Although ROS-mediated autophagy has been shown to support cellular survival, under severe circumstances, it can also promote cellular death (Kroemer et al., 2010).

In addition, numerous examples have clearly suggested a direct or indirect ROS regulation of cellular antioxidant responses (Adler et al., 1999). Moderate production of ROS during nonexhaustive exercise is one of the strong examples were mild stress leads to increased antioxidant capacity and cytosolic adaptive responses (Ji, 2002). By regulating stress kinases and transcriptions factors, ROS are feedback inducing signals that increase the cellular responsiveness upon alterations in ROS generation (Adler et al., 1999; Stone & Yang, 2006). Thus, inappropriate supplementation of antioxidant compounds can abolish the radicals involved in these beneficial cellular adaptations and consequently promote cellular sensitization to death (Poljsak et al., 2013). Therefore, ROS represent an alarm system that activates signaling intermediates to improve cellular reworking to non-optimal extracellular environmental changes. Moreover, their

total ablation does not enable the cell to adopt proper function and may have deleterious consequences in cell survival (Bentley et al., 2015). Curiously, an ROS role in the maintenance of homeostasis in the absence of a cellular stress has been hypothesized (Sena & Chandel, 2012). This hypothesis suggests that a moderate level of ROS promotes a wide range of tolerant signaling pathways that maintain cellular homeostasis, and are different from the ones activated following stress (Sena & Chandel, 2012). However, remains to be determined the veracity of this theory with future work. In summary, oxidative stress represents the switch of ROS as signaling and pro-survival molecules for harmful and death-promoting ones. Thereafter, it is crucial for the cell to sustain redox homeostasis with the control of ROS below a threshold level, keeping their function as signaling molecules, while reducing their toxic effects (Barbieri & Sestili, 2011) (Fig 6).

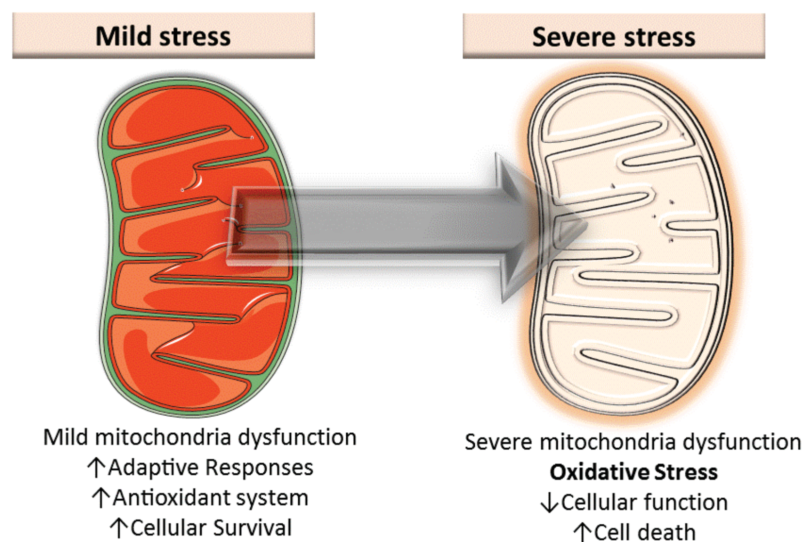


Figure 6. ***Effects of different levels of stress in cellular and mitochondrial function.***
Mild stresses can have a beneficial impact on cellular function by increasing antioxidant and adaptative responses. In contrary, prolonged or severe stresses can lead to ROS overproduction which causes oxidative stress. Consequently, mitochondria suffer an overall of detrimental changes which can equally affect others cytosolic constituents and promote cell death (image created with **Servier Medical Art templates**).

1.3.3. Mitohormesis

In the middle of the 20th century, the formation of oxygen free radicals in situ was discovered together with their association with mutations, cancer and aging (Gerschman et al., 1954). Indeed, Harman in 1956 stated that free radicals-induced accumulation of detrimental damages in cells would explain the aging process of organisms, referring this premises as the Free Radical Theory of Aging (Harman, 1956). Curiously, the interest in the free radical theory was limited because of the persistent doubt regarding the existence of oxygen free radicals in biological systems, despite some early reports (Commoner, Townsend, & Pake, 1954; Gerschman et al., 1954). After becoming conceptual their existence, mitochondria gained a central role in the free-radical theory, giving their generation of the disproportionately large amount of ROS in cells (Harman, 1972). In subsequent years, ROS have been considered exclusively unwanted by-products of oxidative phosphorylation and associated with cellular damage which accelerates aging and limits life span (Sanz, 2016).

Over the last decade, several studies have challenged the previous concept of ROS as detrimental and propagating molecules. Indeed, insights about the unexplored ROS function as signaling molecules with important regulatory roles in the longevity of higher organisms emerged, reflecting a different point of view concerning the role of mitochondrial ROS formation. Since then, it has been assumed that they serve as critical signaling molecules that are part of the communication between the mitochondria and other cellular compartments in response to physiological or pathophysiological variances (Chandel & Budinger, 2007; Finley & Haigis, 2009.; Kaelin, 2005; Pan, Schroeder, Ocampo, Barrientos, & Shadel, 2011; Schulz et al., 2007). Furthermore, studies started to directly link caloric restriction (CR) to the induction of low-level stress as the explanation of the consequent increased stress resistance and longevity, through an adaptative response defined as hormesis (Calabrese et al., 2007; Masoro, 1998). In general, hormesis theory defends that exposure to low continuous or higher intermittent sub-lethal doses of a stressor, that would otherwise be harmful at larger or chronic doses, promotes favorable biological adaptations which protect against greater subsequent stress (Merry & Ristow, 2016).

One particular form of hormesis has been associated specifically with the mitochondria, termed mitohormesis. In this paradigm, mild perturbations in mitochondrial homeostasis can activate nuclear and cytosolic responses that lead to a whole cell amplified protection from future perturbations (Yun & Finkel, 2014). Remarkably, the responses can be either transient cytoprotective mechanisms or long-lasting metabolic and biochemical changes and stress resistance (Merry & Ristow, 2016; Yun & Finkel, 2014). Indeed, many lifespan-extending interventions in model organisms, such as reduced signaling through the target of rapamycin (TOR) pathway, CR, and exercise, rely at least partially on mitohormesis (Pan et al., 2011; Schulz et al., 2007; Yang and Hekimi, 2010). The additional point is that mitochormesis implies the intriguing generation of signaling molecules from mitochondria, that result in benefits ranging from single acute protective adaptations to organismal-level longevity. Thus, some potential mitochondrial retrograde emitters have been described, such as ROS, MMP and calcium, mitochondrial unfolded protein response (UPR^{mt}), mitochondrial metabolites and mitokines (Merry & Ristow, 2016; Yun & Finkel, 2014).

The accumulation of short-term low levels of ROS either through intense physical activity or caloric restriction sustains the reduction of glucose metabolism, induction of mitochondrial metabolism and increment of lifespan (Cerletti et al., 2012; Merry & Ristow, 2016). Concerning these examples, it is referred that mitochondrial activity increase in response to energy depletion is the starting point of a downstream ROS-mediated adaptation (Ristow & Schmeisser, 2011). A possible explanation for this phenomenon is the activation of stress sensors such as AMPK (Ristow & Schmeisser, 2011) (Fig 7). AMPK is a heterotrimeric enzyme involved in energy homeostasis, to control the intracellular adenosine nucleotide levels (Hou et al., 2015; Lee et al., 2010; Liu et al., 2015; Seo et al., 2015). Its activation under a modest decrease of ATP and increase of either AMP (adenosine monophosphate) or ADP, leads to activation of catabolic pathways that promote the generation of more ATP (Mihaylova & Shaw, 2011). In addition, AMPK enhances mitochondrial function, increases mitochondrial content and oxidative capacity, through PGC1-α activation (Seo et al., 2015). In this manner, an energy deficit activates AMPK-mediated induction of mitochondrial metabolism and consequent ROS formation. The explanation for this is the ATP efficiency production from OXPHOS in comparison with aerobic glycolysis, whereas the first one produces 30

mol of ATP from 1 mol of glucose, its nonoxidative metabolism generates 2 mol of ATP (Michael Ristow & Schmeisser, 2011). Hence, enhanced mitochondrial respiration and biogenesis by CR activation of AMPK has been described from yeast to rodents, being a conserved pathway (Barros et al., 2004; López-Lluch et al., 2006). The association of CR with increased responsiveness to stress and longevity may involve ROS-mediated signals from mitochondria to the nucleus, which is referred as retrograde ROS signaling (Fig 7) (Ristow & Schmeisser, 2011).

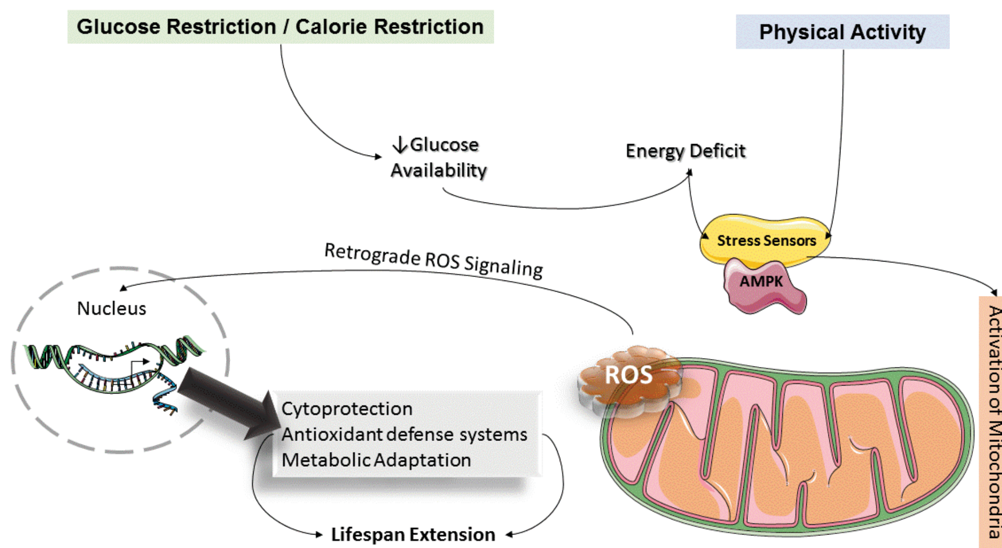


*Figure 7. **Promotion of mitochondrial ROS generation by energetic deficits induces an overall adaptative response.***

Lifespan-extending interventions cause an energy deficit that promotes stress sensors activation. These effectors lead to increased mitochondrial metabolism and promote mitochondrial ROS formation. Consequently, transfer of information from the mitochondria to the cytoplasm and nucleus occurs, resulting in mitochondrial retrograde signaling. Retrograde signaling pathways trigger adaptative cellular responses by coordinated changes in nuclear genetic expression, which promote longevity (Image based on Ristow & Schmeisser, 2011) (image created with **Servier Medical Art templates**).

The most described genetic alteration from this signaling is the upregulation of genes associated with the antioxidant response (Barros et al., 2004; Ristow & Zarse, 2010; Schulz et al., 2007). Since reduced or malfunction of the intracellular antioxidant response is associated with diseases such as diabetes and increasing age (Afanas'ev, 2010), an improvement of the same factor can explain the documented adaptations and lifespan extension by CR. Indeed, an enhanced antioxidant system has been found in CR-mediated mitohormesis in mitochondria from lifespan extending *Drosophila*

melanogaster and *Caenorhabditis Elegans* organisms (Schulz et al., 2007; Sharma et al., 2011). In addition, mimics of CR including AMPK activation like metformin, an anti-glycemic drug, were also shown to promote longevity through mitohormesis-induced augment of antioxidant response (De Haes et al., 2014). In humans, a CR regimen significantly changed markers of oxidative stress, as well (Meydani et al., 2011). This evidence was suggested to result from an increase in NF-E2-related factor-2 (NRF2), a transcription factor that promotes antioxidant response elements (ARE) signaling pathway, that induces antioxidant proteins expression (Nguyen et al., 2009). Notably, Nrf2/ARE decline response against oxidative stress has been described in aging by several evidences (Zhang, et al., 2015). Also, a study in humans suggested the mitohormetic role of exercise, by showing the physical activity induction of increased levels of PGC1- α, SOD and GPX on skeletal muscle, only in the absence of antioxidants (Ristow et al., 2009). For instance, one of the explored explanations for CR and exercise to reduce disease susceptivity and prolong longevity is by lowering oxidative damage through improvement of the endogenous antioxidative defense systems. A similar line of reasoning describes a possible increase of mitochondrial function in response to mitohormesis (Yun & Finkel, 2014). Although increased resistance towards ROS in response to life-extending conditions can by its own lead to a stronger protection of mitochondria from radicals, mitochondrial function improvement can also ensure an adequate response to circumstances such as nutrient overload and aging. Thus, regulation by mitohormesis of biogenesis, maintenance, and clearance of mitochondria can also confer the positive impacts of a mild induction of ROS signaling pathway. Exercise promotion of an increase in overall function of mitochondria is perhaps the literature most-described effective way to determine average life expectancy (Lanza & Nair, 2010). As mentioned before, physical endurance activity can stimulate PGC-1α expression, which augments tissue capacity for oxidative metabolism by increasing the expression and activities of mitochondrial proteins (Holloszy, 1967). Indeed, exercise acute activity leads to a long-term expression of PGC1-α, which is observed through increased mitochondrial content and volume in skeletal muscle (Chow et al., 2006). Given the association of malfunction and a decrease of AMPK and consequently biogenesis during aging, it seems clear that exercise can counteract these effects (López-Lluch et al., 2008). Moreover, disrupted biogenesis reduces mitochondrial turnover,

which may affect processes such as mitophagy, resulting in accumulation of damaged mitochondria in aged cells. Likewise, mitophagy plays a significant role in response to several stresses and is documented affected by aging (Diot et al., 2016). An induction of mitophagy during mitohormesis remains an unexplored topic. However, mitophagy-dependent removal of damaged mitochondria by forcing cells to rely on OXPHOS to produce energy was described, in addition to its induction by a mild stress (Diot et al., 2015; Frank et al., 2012). Thus, this may contribute to the increased mitochondrial function by exercise and CR, resulting in slower aging progression effects.

1.4. Sestrins: A conserved stress-inducible family

Sestrins are a distinct family of proteins that are expressed in mammals through three stress-responsive genes Sestrin 1-3 (Sesn1–3 in mouse, SESN1-3 in humans) (Budanov et al., 2010; Peeters et al., 2003). Invertebrates such as *Drosophila melanogaster* and *Caenorhabditis Elegans* have a single orthologue, while most known vertebrates have the three mentioned paralogs (Ho et al., 2016; Lee et al., 2013). They are respectively encoded by independent loci; p53-activated gene 26 (PA26) encodes for Sestrin 1 (Sesn1), hypoxia-inducible gene 95 (HI95) encodes to Sesn2, and Sestrin 3 (Sesn3) was identified as FoxO target (Budanov et al., 2002; Nogueira et al., 2008; Velasco-Miguel et al., 1999). The first member of the family, SESN1 gene is transcribed into three different mRNAs that encode three different protein products highly expressed in skeletal muscle, heart, brain, and liver (Budanov et al., 2002; Peeters et al., 2003; Velasco-Miguel et al., 1999). SESN2, instead, has only one transcript that encodes one protein product expressed mainly in kidney, liver, lung, and leucocytes (Budanov et al., 2002; Parmigiani & Budanov, 2016; Velasco-Miguel et al., 1999). The SESN3 gene is transcribed into two alternatively spliced mRNA isoforms and is found abundantly in skeletal muscle, kidney, brain, and small intestine (Budanov et al., 2002; Chen et al., 2010; Peeters et al., 2003). Thus, all the three Sestrin genes are expressed in most adult tissues, although at different levels (Parmigiani & Budanov, 2016).

The loss of the Sestrin gene in organisms such as *Drosophila melanogaster* and *Caenorhabditis Elegans* is not lethal and does not affect the healthy development. Nonetheless, inactivation of *Caenorhabditis Elegans* Sestrin (cSesn) shortens the

lifespan of the organism, while its overexpression extends it (Lee, Budanov, et al., 2010; Yang et al., 2013). Despite having no effect the *Drosophila melanogaster* Sestrin (dSesn) inactivation on lifespan, dSesn-deficient flies were characterized with multiple signs of accelerated ageing and imbalanced metabolism (Lee et al., 2013; Lee, Budanov, et al., 2010; Yang et al., 2013). Therefore, Sestrins have been strongly associated as active modulators of metabolic homeostasis through suppression of oxidative stress and regulation of AMPK- mammalian target of rapamycin (mTOR) signaling. This last referred protein is a conserved Ser/Thr kinase that controls cell growth (accumulation of mass) and metabolism in response to environmental cues (Wullschleger, Loewith, & Hall, 2006). mTOR is known to form two distinct complexes (mTORC1 and mTORC2 (mammalian target of rapamycin complex 1 and 2)), with different cellular roles (Fig 8). For instance, mTORC1 is known to impede autophagy by phosphorylating and inhibiting autophagy-related gene 13 (Atg13) and ULK1 (Kim et al., 2011; Ma & Blenis, 2009; Mayer & Grummt, 2006). Moreover, this protein activates anabolic processes, such as cellular lipid synthesis, that are aberrantly activated in age and obesity-associated metabolic pathologies (Lee et al., 2013). In fact, 20-day-old dSesn-null had higher content in triglycerides and, consequently, dSesn-null fat bodies showed decreased AMPK and increased target of rapamycin (TOR) activities (Lee, Budanov, et al., 2010). Furthermore, young (5-day-old) and 20-day-old mutants dSesn-null muscles showed mitochondrial abnormalities, including a rounded shape, occasional enlargement, and disorganization of cristae (Lee, Budanov et al., 2010). Also, dSesn-null muscles had increased accumulation of oxidative damage, which was associated with the dependent regulation of AMPK-TOR by dSesn (Lee et al., 2012; Lee & Budanov, et al., 2010). Thus, these findings suggest the regulatory function of Sesn2 on mitochondrial homeostasis, possible due to AMPK-TOR regulation and subsequently in metabolism.

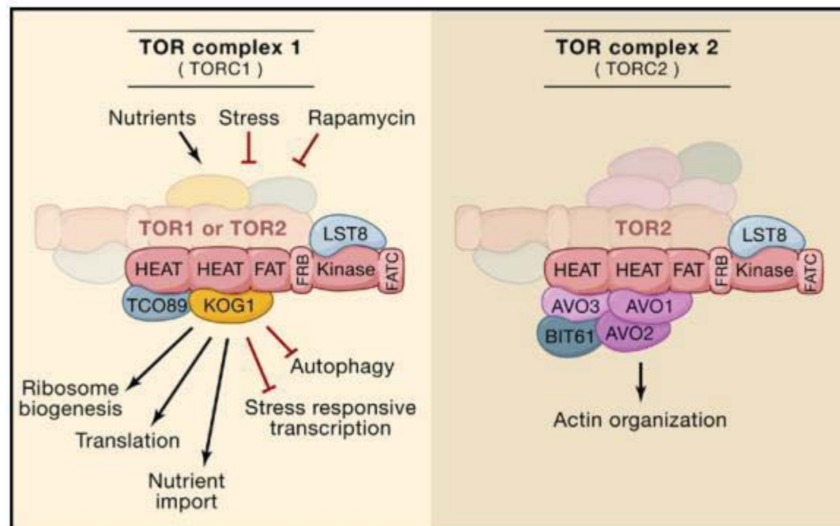


Figure 8. ***Representation of Saccharomyces cerevisiae TOR Complex 1 (TORC1) and TOR Complex 2 (TORC2).***
TORC1 is associated with pathways that induce accumulation of mass through the outputs described with black arrows. Stress and rapamycin can negatively affect TORC1 activation to avoid its inhibitory impact in autophagy and stress responsive transcription. In contrast, TORC2 is associated with the organization of the actin cytoskeleton (from Wullschleger et al. 2006).

In order to investigate the total contribution of Sestrins in mammals, Peng and colleagues generated null alleles for Sesn1, Sesn2, and Sesn3. This triple knockout (TKO) mice revealed Sestrins importance for postnatal survival (Peng et al., 2014). Indeed, they found that mTORC1 signaling was constitutively active in the liver, heart, and skeletal muscle (limbs) of TKO mice during neonatal fasting, leading to the incapacity of tolerating the fasting window that occurs in neonatal mammals between birth and suckling (Peng et al., 2014). Since this fasting period does not take place in invertebrates, Sestrin inactivation in *Drosophila melanogaster* or *Caenorhabditis Elegans* does not influence the postnatal survival of these organisms (Parmigiani & Budanov, 2016). Also, studies with the silencing of individual Sestrin elements in mammals does not lead to any obvious developmental abnormalities, potentially explained by the redundancy of the Sestrin proteins in most tissues (Lee et al., 2012; Parmigiani & Budanov, 2016; Peeters et al., 2003). However, with the characterization of single and double KO animals, starts to be revealed the impact of each Sestrin on stress response (Lee et al., 2012, 2013). In fact, some specific Sestrins are already recognized as upregulated by multiple signaling pathways in response to genotoxic, hypoxic, oxidative, and nutritional stress (Ho et al., 2016; Rhee & Bae, 2015). As an example, obese mice had Sesn2 accumulated in the liver and skeletal muscle, whilst Sesn1 and Sesn3 seemed unaltered

(Lee et al., 2012). In agreement, adult Sesn2 KO mice fed with high-fat diet (HFD) develop rapid blood sugar elevation, insulin resistance, hepatosteatosis, increased activation of the mTORC1, and accumulation of ROS when compared with the wild-type (Bae et al., 2013; Lee et al., 2013). Also, simultaneous loss of Sesn2 and Sesn3 promotes a similar metabolic pattern of glucose intolerance and insulin resistance, even in mice under a control diet (Lee et al., 2012). In light of these discoveries, Sesn2 has been suggested to play a promising role in the prevention of a broad range of diseases and in delaying metabolic syndrome-associated defects, by controlling metabolic derangements during hyper nutrition and normal aging. However, a lot of work is still needed to decipher the significance of each Sestrin, and to clarify its activities under different environmental stress conditions.

1.5. The individual role of Sestrin 2

Sesn2 has been mesmerizing the scientific field due to its stable stress-inducible metabolic regulatory activity, which protects and averts the accumulation of stress-induced damages, preventing an extensive range of age- and obesity-associated pathologies such as insulin resistance, muscle degeneration, cardiac dysfunction, mitochondrial pathologies, and tumorigenesis (Parmigiani & Budanov, 2016). The rousing interest in this member of Sestrins family stems from it's two seemingly unrelated physiological functions: reduction of ROS and inhibition of mTORC1. Recently, Kim and colleagues solidified these two dual roles of Sesn2 by resolving and showing that the crystal structure of Sesn2 is divided into two major subdomains: a N-terminal domain capable of reduce alkyl hydroperoxide radicals through its helix–turn–helix oxidoreductase motif and a C-terminal domain responsible for interacting this with GAP activity towards Rags 2 (GATOR2) and subsequent inhibition of mTORC1 (Kim et al., 2015). This protein is one of the two subcomplexes that constitutes the multiprotein GATOR (GATOR1 and GATOR2) (Bar-Peled et al., 2013; Chantranupong et al., 2014; Kim et al., 2015; Parmigiani et al., 2014). Its loss is associated with mTORC1 signaling insensitivity to amino acid starvation in several human cancer cell lines, and consequent hypersensitive to rapamycin (Bar-Peled et al., 2013). By targeting the GATOR2, which is upstream of mTORC1, Sesn2 ends up inhibiting the complex. As a result, Sestrin 2 multifaceted role allows Sestrins to fine tune the cell metabolism through regulation of

mTOR and ROS, and more recently due to the control of mitochondrial homeostasis (Ding et al., 2016; Parmigiani & Budanov, 2016).

1.5.1. Sestrin 2 and cellular response

All members of the Sestrin family are induced by oxidative stress, albeit due to different induction mechanisms (Budanov et al., 2004; Lee et al., 2013; Nogueira et al., 2008). In the case of Sesn2, several transcription factors have been described as Sesn2 inductors upon cellular stresses (Rhee & Bae, 2015). For instance, oxidative stress upregulates Sesn2 levels through Hypoxia-inducible Factor-1 (HIF-1), p53, activating transcription factor 4 (ATF4), Nrf2, PGC-1α (Garaeva et al., 2016; Ishihara et al., 2013; Park et al., 2014; Rhee & Bae, 2015; Shin at al., 2012). They are transcription factors that regulated the redox state of the cell and are involved in hypoxia, oxidative stress, and metabolic responses. As mentioned, oxidative stress loosely defines the interrelated phenomena of overproduction and/or mismanagement of ROS and consequently oxidative damage of cell constituents (Andreyev et al., 2005). Mitochondria are the most affected organelles by this cellular status, and their damage exacerbates the ROS levels. Initially, was supposed that Sesn2 function as a redox enzyme by catalyzing the regeneration of peroxiredoxins (Prxs), a family of thiol-containing peroxidases conserved from bacteria to mammals (Budanov et al., 2004; Wood et al., 2003). Prxs exert their protective antioxidant role in cells through their peroxidase activity, whereby hydrogen peroxide, and an extensive range of organic hydroperoxides (ROOH), are reduced and detoxified (Wood et al., 2003). Overoxidized Prxs have then to be recovered by reversibly hyper oxidization of the active-site cysteine of 2-Cys peroxiredoxins to cysteine sulfinic acid (Woo et al., 2009). This was precisely what Sesn2 was proposed to, in addition to sulfiredoxins (Srx) (Woo et al., 2009). However, Sesn2 direct sulfinyl reductase activity was later denied, whereas the reductase function of Srx has been confirmed by several studies (Woo et al., 2009). Indeed, it is now established that Sesn2 target Srx to instead restore the antioxidant function of Prx (Bae et al., 2013). Kim and colleagues also described that Sesn2 prevent ROS accumulation through the direct ROS scavenging, and via regulation of Nrf2 (Fig 9). Nrf2 regulates the activation of ARES, which includes Sesn2 gene (Shin et al., 2012), whereas Sesn2 induces Kelch-like ECH-associated protein 1 (Keap1)-p62 dependent autophagic degradation, increasing Nrf2

activity (Fig 9) (Itoh et al., 2010; Kensler et al., 2007). Keap1 is an adaptor of the ubiquitin ligase complex and functions as a Nrf2 repressor by targeting it for proteasomal degradation (Itoh et al., 2010; Kensler et al., 2007). However, upon conditions of increased ROS generation, the Keap1 interaction with phosphorylated p62 arises (Bae et al., 2013; Ichimura et al., 2013; Itoh et al., 2010). By physically interacting with ULK1 and p62, Sesn2 promotes ULK1-mediated p62 phosphorylation, which consequently prevents oxidative stress due to Nrf2 signaling activation (Ro et al., 2014).

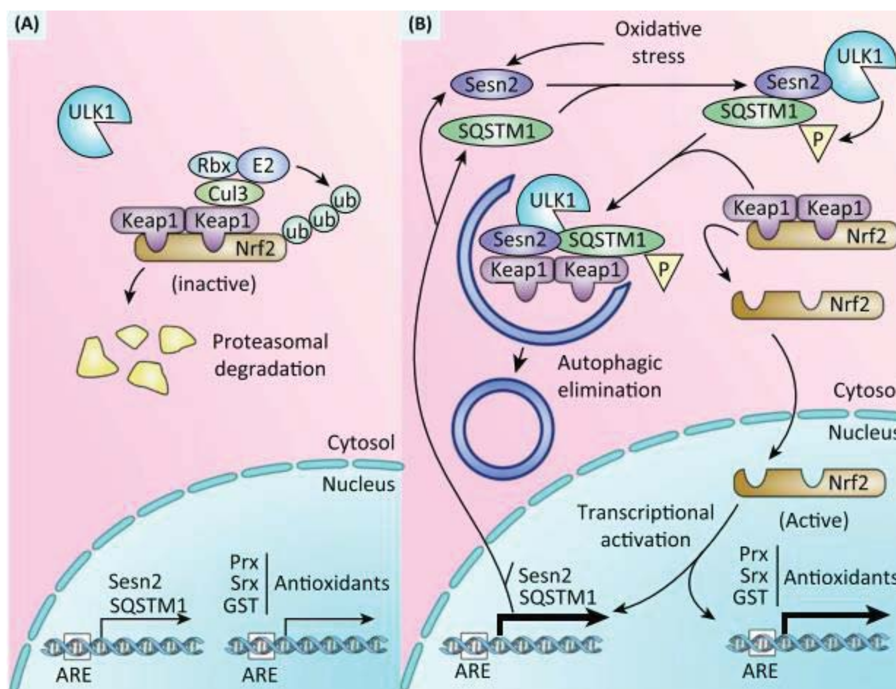


Figure 9. ***Regulation of Nrf2 by Sestrin 2.***
Under normal conditions, Nrf2 degradation by Keap1-mediated proteasomal degradation is always active (A). Conditions of increased ROS generation lead to upregulation of Sesn2 and p62 (SQSTM1) (B). The interaction between Sesn2 with p62 and ULK1 specifically targets Keap1 for autophagic elimination. This, in turn, favors Nrf2 translocation to the nucleus and induces the transcription increase of antioxidant response element (ARE), including Sesn2, and antioxidant enzymes such as Prx, Srx, and glutathione. (from Ho et al., 2016)

Parallel, Sesn2 avoids the hyperactivation of mTORC1, which alleviates ROS production in a different manner (Dirat et al., 2012; Lee, Budanov, et al., 2010; Rhee & Bae, 2015; Satapati et al., 2016; Seo et al., 2015). By effective elimination of ROS-producing damaged mitochondria, Sesn2-mediated autophagy through mTORC1 inhibition might also reduce ROS generation (Lee et al., 2010; Yen & Klionsky, 2008). Sestrin2 negatively regulates mTORC1 signaling through dependent induction of AMPK, a heterotrimeric enzyme involved in energy homeostasis. It is yet not clear how Sesn2

enhances the AMPK activity. However, indirect processes by which Sesn2 serves as an LKB1-AMPK scaffold protein to initiate AMPK activation by phosphorylation have been proposed (Budanov & Karin, 2008; Morrison et al., 2015). Moreover, a study with MCF7 breast cancer cells under genotoxic stress showed that SESN2 could lead to a direct increase of LKB1/ AMPKαβγ mRNA and protein expression (Sanli et al., 2012). Regardless of the exact mechanism by which Sesn2 can induce the phosphorylation or act as a transcription factor of these kinases, AMPK is involved in Sesn2-mTORC1 inactivation through the direct phosphorylation of TSC2 (Tuberous sclerosis complex) and the mTORC1 component raptor (Corradetti et al., 2004; Inoki et al., 2003; Shaw et al., 2004). Importantly, Sesn2 may also promote mitophagy through activation of AMPK-mediated ULK1 phosphorylation, which regulates ULK1 translocation to mitochondria and consequently starts their autophagic elimination (Tian et al., 2015).

Sesn2-mediated inhibition of mTORC1 may also occur due to AMPK-independent mechanisms. For example, proteomic studies have identified Sesn2 as GATOR2-interacting protein (Bar-Peled et al., 2013; Chantranupong et al., 2014; Kim et al., 2015; Parmigiani et al., 2014). Under normal conditions, GATOR2 showed to inhibit the GAP activity of GATOR1, thereby leading to the RagB activation by its guanylate exchange factor (GEF) Ragulator and translocation of mTORC1 to the lysosomal periphery (Bar-Peled et al., 2012). Sesn2 can inhibit the suppressive effects of GATOR2 toward GATOR1 by directly interacting with the WDR24 and Seh1L subunits of GATOR2 (Bar-Peled et al., 2012).Thus, freeing GATOR1 becomes an active GAP that binds to RagB, and consequently, releases mTORC1 from the lysosomal surface and causes mTORC1 inactivation (Fig 10) (Bar-Peled et al., 2012). Moreover, Sestrin2 evolutionarily leucine-binding pocket is in proximity with a highly conserved GATOR2 binding site, which reveals a possible mechanism by which leucine is sensing by the Sesn2-mTORC1 pathway couples cell growth to leucine availability (Saxton et al., 2015). Sesn2 control of GATOR2 activity also represses mTORC1 inhibition of autophagy, which might constitute another way of controlling mitochondrial elimination upon ROS damage.

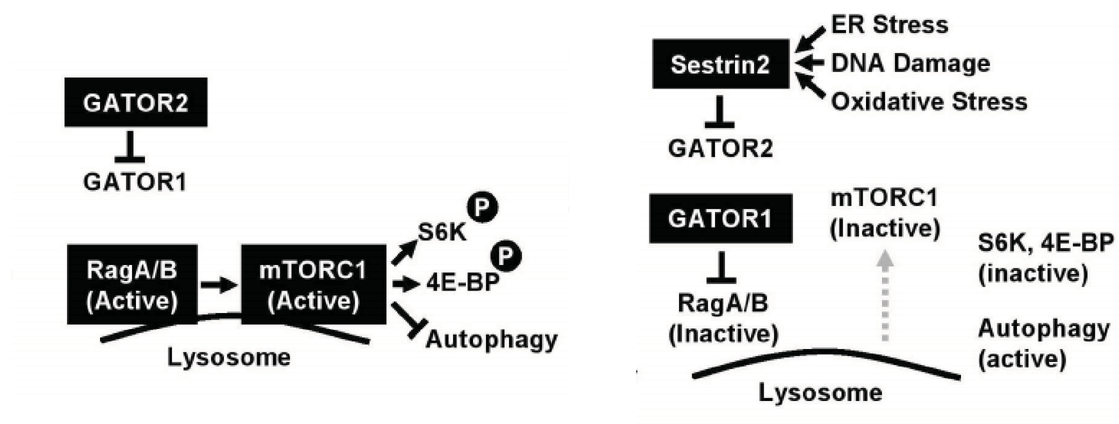


Figure 10. ***Sestrin 2 controls mTORC1 through GATOR complexes.***
Under non-stressed conditions, GATOR1 is constitutively inhibited by GATOR2, resulting in RagB recruitment of mTORC1 to lysosomal surface and activation of its downstream targets S6K and 4E-BP (left figure). Sesn2 expression following a cellular stress causes GATOR2 inhibition, consequently inactivating RagB. This, in turn, favors mTORC1 release from the lysosomal surface and mTORC1 signaling is subsequently inactivated (from Kim et al., 2015).

Recently, a direct activation of mitophagy by Sesn2 was revealed by increased nitric oxide in macrophages (Kim et al., 2016). By facilitating p62 aggregation and its binding to the mitochondrial surface, Sesn2 induces mitochondrial priming for mitophagy machinery recognition (Kim et al., 2016). Moreover, Sesn2 activation of this mechanism through the increase of ULK1 protein levels was also detected (Kim et al., 2016). In addition, Sesn2 activation of AMPK can also affect mitochondrial function (Lee et al., 2010). Sesn2-mediated AMPK activation may promote mitochondrial biogenesis, which compensates mitochondrial defects by producing new and functional mitochondria. Mitochondria, in turn, can fuse with damaged ones or ensure ATP cellular levels by restoring mitochondrial pool in a given cell. Moreover, consequently increased expression of genes involved in mitochondrial respiration can enhance the efficiency of mitochondrial respiration through downstream activation by AMPK of PGC1-α (Fernandez-Marcos & Auwerx, 2011).

Given the Sesn2 upregulation in response to numerous stresses (Budanov et al., 2002), it will be interesting to investigate whether some of these stresses can also induce Sesn2 and promote hormetic effects. Sesn2 multi-tasking role in mitochondrial function and as an antioxidant can be a molecular explanation for lifespan increase and prevention of age-associated degenerative disorders found in organisms exposed to life span extending conditions (Fig 11).

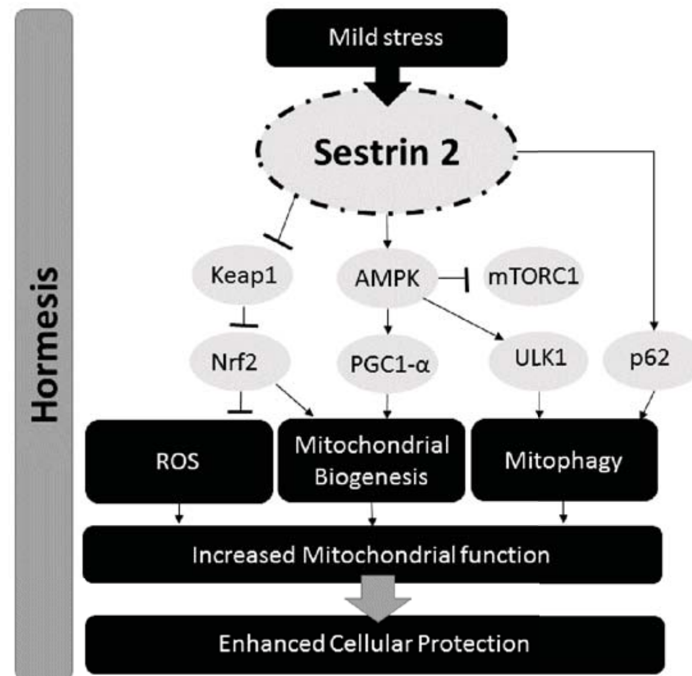


Figure 11. ***Sestrin 2 possible role during hormesis.***
Mild stress induction of Sesn2 expression can induce a hormetic adaptation by controlling molecular machinery involved in the antioxidant response, mitophagy and mitochondrial biogenesis. By managing damaged mitochondrial elimination and renovating the mitochondrial population, Sesn2 might increase mitochondrial adaptation for following cellular stresses, thereby ensuring cellular survival (based on Lee et al., 2013) (image created with **Servier Medical Art templates**).

2. Materials and Methods

2.1. Materials

Except when noted, all compounds were purchased from Sigma-Aldrich (St. Louis, MO). All reagents and chemicals used were of the highest grade of purity commercially available.

2.1.1. Table 1. List of primary antibodies used for Western Blot

Antibody	*Supplier*	*Dilution*	*Species*
Sestrin2	Santa Cruz	1:100	Mouse
α-Actin	Sigma	1:1000	Mouse
Tfam	Aviva Systems Biology	1:1000	Rabbit
PGC1-α	Cell Signaling	1:1000	Rabbit
LC3-I; LC3-II	Sigma	1:1000	Rabbit
VDAC	Abcam	1:1000	Rabbit
Cleaved Caspase-3	Cell signaling	1:500	Rabbit

Either anti-rabbit immunoglobulin G (IgG)-AP conjugated (1:2000), anti-mouse IgG conjugated antibody (1:2000), or substrate Qdot 625 streptavidin conjugate (1:2000) were purchased from Thermo Fisher Scientific.

2.1.2. Table 2. List of primary antibodies used for Immunocytochemistry

Antibody	*Supplier*	*Dilution*	*Species*
Sestrin 2	Santa Cruz	1:100	Mouse
Tom20	Santa Cruz	1:1000	Rabbit

Secondary Alexa Fluor 594 conjugated to anti-rabbit antibody (1:1000) was purchased from Invitrogen and Alexa fluor 594 donkey conjugated with anti-mouse (1:500) was purchased from Thermo Fisher Scientific.

2.1.3. Cell line

C2C12 immortalized mouse myoblast cell line was purchased American Type Culture Collection (ATCC). All cell care and maintenance was done according to the protocol recommended by ATCC.

2.1.4. Cell culture maintenance

Cells were grown in 75 cm^2 culture flasks at 37 °C in a humidified atmosphere with 5% CO_2 and maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO 31600-083), supplemented with 3,7 g/L $NaHCO_3$, antibiotic/antimycotic 1% (Thermo Fisher Scientific) and 10% of fetal bovine serum (FBS, Thermo Fisher Scientific). Cells were passaged or harvested for experiments upon reaching 80% of confluence, by detachment with 3 ml of 0,05% trypsin and 0,5 mM EDTA (TrypLE Express, Thermo Fisher Scientific) during 3 min in the incubator. DMEM with 10% FBS was then added to block the action of trypsin. Cells were collected in a 15 ml falcon tube and centrifuged at 200 x *g* for 3 min. Cellular pellet was resuspended in 10 ml of DMEM with 10% FBS, and 1 ml of this dilution was subcultured in a new 75 cm^2 flask.

2.2. Methods

2.2.1. Mitohormesis induction

C2C12 myoblasts were counted in a cell counter (TC10 Automated Cell Counter, Bio-Rad Laboratories, Hercules, CA) and plated at a density of 15,000 cells/cm^2 in 5.5 mM glucose DMEM with 10% fetal bovine serum, 3,7 g/L $NaHCO_3$, antibiotic/antimycotic 1% (Thermo Fisher Scientific). After 4 hours, glucose deprivation was performed by rinsing the cells twice with glucose-free medium (Gibco/Invitrogen) and incubating them in glucose-free medium with antibiotic/antimycotic 1% (Thermo Fisher Scientific), plus 10% FBS. After 18 h, cells were subcultured into two flasks for 24 h, either with 35 mM of glucose or normal glucose medium (5.5 mM) both with 10% FBS.

2.2.2. Measurement of ROS production

ROS production was determined fluorometrically, as described before (Palmeira et al., 2007). 2',7'-Dichlorodihydrofluorescin diacetate (H_2DCF-DA) was purchased from Sigma. Briefly, cells were collected by trypsinization and centrifugation and resuspended at 1×10^6 cells per 1 ml in culture medium without FBS or phenol red. Cells were loaded with 50 µM H_2DCF-DA prepared in Dimethyl sulfoxide (DMSO) for 30 min at 37 °C, washed and 200 µl containing 2×10^5 cells were loaded into a 96-well plate. The resulted fluorescence from the formation of oxidized derivatives was monitored at an excitation wavelength 485 nm and an emission wavelength 538 nm, to calculate the rate of ROS.

2.2.3. Measurement of mitochondrial membrane potential in C2C12 cells

MMP in C2C12 cells was measured with a fluorescent probe, tetramethylrhodamine methyl ester (TMRM). TMRM is a cell-permeant, cationic fluorescent dye that is readily sequestered by active mitochondria, accumulating electrophoretically in proportion to their MMP (Ehrenberg et al., 1988). TMRM was purchased from Thermo Fisher. Briefly, to monitor mitochondrial MMP, cells were plated and treated as described in 2.2.1. in 12-well plates. After exposure, the culture medium was aspirated, and the cells were loaded with 6.6 µM TMRM in DMSO in 1 ml of DMEM without FBS and phenol red for 15 min at 37 °C in the dark. After incubation, and replacement of the culture medium, fluorescence was measured using Victor3 plate reader (Perkin–Elmer). Fluorescence was measured using excitation and emission wavelengths of 485 and 590 nm respectively, at 37 °C. After recording basal fluorescence; mitochondrial MMP was estimated taking into account the complete depolarization by adding 2 mM of carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP). FCCP is an ionophore that disrupts ATP synthesis by permeabilizing the mitochondrial membrane to proton transport. Data was normalized taking into account protein count as determined in each well by the Sulforhodamine B method.

2.2.4. Sulforhodamine B colorimetric assay

Sulforhodamine B (SRB) was developed by Skehan and colleagues in 1990 (Papazisis et al., 1997; Skehan et al., 1990). This dye binds to basic amino acids of cellular proteins and from colorimetric evaluation permits an estimation of total protein mass which is proportional to cell number. Cells were seeded and treated as described in 12–well plates in a total medium volume of 1 ml per well. After MMP evaluation, cells were gently fixed in 12-well plates with ¼ volume of cold 10% (wt/vol) acid trichloroacetic acid (TCA) and incubated for 1 h at 4 °C. Subsequently, the cells were washed 4 times with water and air dried. Afterward, 500 µl SRB solution (0,5% in 1% acetic acid) was added to each well followed by 30 min incubation at room temperature. Cells were washed 4 times with 500 µl of 1% acetic acid to remove unbound excess dye and dried at room temperature. Finally, 1 ml of 10 mM Tris (pH10) was added to solubilize protein-bound dye for 5 min at room temperature in an orbital shaker. Absorbance was determined at 540 nm in a Victor3 plate reader (Perkin–Elmer).

2.2.5. MitoSOX

The mitochondrial superoxide formation was estimated using a mitochondrial superoxide anion specific indicator, named as MitoSOX (purchased from Molecular Probes). Cells were seeded and treated as described in a 24-well plate, after treatment cells were rinsed twice with 500 µl of pre-warmed DMEM without phenol red and FBS, and incubated with 5 µM of MitoSox and 5 µg/ml Hoechst 33342 both prepared in DMSO, for 10 minutes at 37°C in DMEM without FBS and phenol red. Cells were then washed, and fresh culture medium without FBS or phenol red was added. Live cell microscopy was used to capture images of MitoSOX stained cells with fluorescence microscope Niko Eclipse TS100 and software NIS-Elements Imaging software (Nikon). MitoSOX evaluation was done by individual cell fluorescence area emission quantified with NIS-Elements Imaging software from 40x images, and for each condition, at least 40 cells were counted.

2.2.6. Western blotting analysis

Myoblasts were washed with 1xPBS (pH 7,2) and collected from 100 mm dish using a plastic cell scraper, by suspension in 1 ml of PBS. The cellular suspensions were centrifuged at 10000g, for 3 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 30 µl of RIPA buffer containing 10 mM Tris–HCl, pH 7.5, 10 mM NaH2PO4, 130 mM NaCl, 1% Triton X-100. This last suspension was sonicated to complete cell lysis. Protein samples were long-term storage at -20°C and thawed to BCA quantification and western blot running. After protein extraction and quantification, samples were prepared with equal parts of Laemmli buffer 2x supplemented with β-Mercaptoethanol 5% (Bio-Rad Laboratories) and RIPA plus protein to load 50 µg of protein from each sample. Proteins (50 µg protein per lane) were separated using SDS-PAGE with an 8 or 15% gel and subsequently transferred to a polyvinylidene difluoride membrane (PVDF) (Bio-Rad Laboratories). Then blots were blocked in blocking buffer (BioRad) for 2 h at room temperature, and incubated with primary antibodies (Table 1) overnight at 4 °C. After incubation, the membranes were washed 3 times for 15 min with Tris-buffered saline (TBS)- 0,5% Tween solution and incubated with secondary antibodies for 1 h at room temperature. Membranes were then washed 3 times for 10 min with TBS - 0,5% Tween and incubated with substrate Qdot 625 streptavidin

conjugate (Thermo Fisher Scientific) for 15 min. Membranes were imaged using Bio-Rad Gel DocTM EZ Imager equipment and with the aid of Image Lab 4.1 Bio-Rad software.

2.2.7. Subcellular fractionation of mitochondrial protein

For subcellular fractionation to separate mitochondrial and cytoplasmic fractions, cells were seeded and treated as described in a 150 cm^2 dish. After exposures, cells were scraped in 4 ml of mitochondrial isolation buffer containing 250 mM of sucrose, 0,5 mM of EDTA and 10 mM of HEPES, pH adjusted to 7,4. Collected cells were centrifuged for 3 minutes at 3000 rpm and then resuspended in 2 ml of freshly mitochondrial isolation buffer. Further resuspension, cells were mechanically disrupted by passing through 15 strokes using a glass-Teflon homogenizer (reference A194). Cell slurries were centrifuged at 3000 rpm for 5 minutes to pellet. Supernatant fractions were collected and centrifuged at 10000 for 15 min to pellet mitochondria. Supernatant fractions represent the cytosolic fraction. The mitochondrial pellet was washed by resuspending in mitochondrial isolation buffer, spinning again at 10000 rpm for 15 minutes and lysed in RIPA with protease inhibitors.

2.2.8. Immunofluorescence of Sestrin2 and TOM20

Cells were seeded on glass coverslips in 12-well plates as previously described. After exposures, plates were rinsed twice with 1 x PBS. Coverslips were fixed in solution (400 µl) of paraformaldehyde 4% for 15 min at room temperature. Cells were then washed two times with PBS. Permeabilization buffer (2% BSA and 0,1% Triton) was added and incubated for 1 hour at room temperature, with shaking. Then, coverslips were washed 3 times with PBS and incubated 1 hour at 4°C with blocking buffer, while shaking. After incubation, cells were washed as indicated, and incubated with primary antibody for two hours at room temperature (Table 2), with shaking. Rewashed, and incubated with the secondary at room temperature for 1 hour. The cells were stained with 5 µg/ml Hoechst 33342 in PBS for 10 minutes to evaluate nuclear morphology and washed 3 times more with PBS. Coverslips were mounted in a fluorescent mounting medium (Dako) and images were recorded using a fluorescence microscope Niko Eclipse TS100 with software NIS-Elements Imaging software (Nikon). Sesn2 fluorescence was

evaluated by individual cell fluorescence area emission quantified with NIS-Elements Imaging software from 40x images. For each condition was at least counted 40 cells.

2.2.9. Mitophagy live cell imaging with Mtphagy dye

The mitophagy process was evaluated using a mitophagy detection kit (Dojindo) composed of Mtphagy Dye, a reagent for detection of mitophagy, and Lyso Dye, for staining of the lysosome. Mtphagy dye accumulated in intact mitochondria and is immobilized on it with a chemical bond, without fluorescence. When Mitophagy is induced, the damaged mitochondria fuse to the lysosome and then Mtphagy Dye emits a high fluorescence (excitation and emission wavelength respectively, 538 nm and 650 nm) due to the acidic pH of the lysosome. C2C12 were seeded in 24 well plates as described, and cultured at 37°C in a 5% CO2 incubator. After 3 h and confirmation of total adhesion, cells were washed with DMEM medium without phenol red and FBS twice and then incubated at 37°C for 30 minutes with 2 umol/L Mtphagy Dye diluted solution in DMSO. After incubation, the cells were washed twice with DMEM without phenol red and FBS and treated as indicated. After 18 h, mitophagy was observed by a fluorescence microscopy using a fluorescence microscope Niko Eclipse TS100 with software NIS-Elements Imaging software (Nikon). The images were taken with a 40x objective.

2.2.10. Small interfering RNA Sestrin 2 Silencing

Electroporation of C2C12 cells was performed using a Neon transfection system (MPK5000, Invitrogen), which utilizes gold-plated electrodes, in a thin plastic pipette tip for the electroporation chamber, resulting in a more uniform electric field. Two days before each electroporation, the cells were harvested into one T75 flask with a fresh medium such that the cells were 70% confluent on the day of the electroporation. In the day of electroporation, cells were harvested and counted as described in 3.1.4. For each electroporation, 500000 cells were resuspended in harm PBS and pellet by centrifugation 200x g for 3 minutes at room temperature. After PBS removal, cells were resuspended in 90 µl of Resuspension Buffer R (Invitrogen), and 10 µl of a 20 µM stock solution of Sestrin 2 small interfering RNA (siRNA) (Invitrogen) was added. This suspension was transiently transfected with 3 pulses of 1650 V and a pulse width of 10,

according to manufacturer's instructions. After transfection, myoblasts were seeded into culture dishes with free antibiotics medium, and at 24 and 48 h the efficiency of transfection was evaluated by western blot.

2.2.11. Statistical analysis

Data were presented as Means ± SEM. Statistical significance was assessed by using one-way ANOVA (for multiple groups comparison). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Short-term glucose deprivation resembles mitohormesis induction

3.1.1. Cellular ROS and mitochondrial superoxide decrease after short-term glucose deprivation but not in hyperglycemia

For a long time, increased formation of ROS was described as a harmful by-product of enhanced oxidative phosphorylation, and associated with aging progression and decreased life span (Harman, 1956). Nowadays, ROS function as physiological signaling molecules is starting to become recognized, namely their role as major mitohormetic emitters (Sena & Chandel, 2012; Yun & Finkel, 2014).

Since glucose restriction has been shown to promote mitochondrial metabolism, by causing increased ROS formation that cumulates in a hormetic extension of life span (Rovenko et al., 2015), we evaluated C2C12 ROS generation after 6 and 18 h in the glucose-free medium. As shown in Fig 12, the culture of cells in the glucose-free medium for 18 h induced a statistically significant decrease in ROS, when compared to the control (5.5 mM) and hyperglycemic (35 mM) conditions. This suggests that C2C12 response to glucose deprivation should involve an earlier adaptation that explains ROS values below the control at 18 h. Indeed, previous work revealed a short-term hyperglycemic reduction of oxygen consumption rates by C2C12 cells, whereas the opposite was verified in low glucose concentration (0,6 mM) (Li et al., 2013). These data, together with our results, suggest that myoblasts undergo an early metabolic shift that favors oxidative phosphorylation metabolism, and consequently lowering of ROS production by mitochondria during glucose restriction. This conclusion seems contradictory regarding glucose metabolism, where increased oxidative phosphorylation is described to augment ROS production (Barja, 2013). However, a mild stress from increased oxidative phosphorylation in response to short-term glucose deprivation can lead to ROS-mediated hormetic signaling that enhances antioxidant and mitochondrial function (Ristow & Schmeisser, 2011). This, in turn, ensures increased cellular overall adaptation to glucose deprivation and future stresses. To confirm this possibility, we evaluated mitochondrial superoxide generation with live-cell imaging of MitoSOX probe to detect a possible transient stress in the early period of exposure to glucose deprivation, that

could suggest mitohormesis induction. Even though we did not observe increased production of superoxide at 3 h, we did verify a subsequent decrease of this reactive species at 6 h when compared with the last time exposure (Fig 13). Therefore, the increase of mitochondrial superoxide might happen in the first 3 h in response to glucose deprivation. However, its detection can be complicated by a possible non-synchronized mild stress production within the cell culture population. Therefore, we suggest that mitochondrial superoxide increase might represent the mitochondrial signal that promotes cytosolic and nuclear changes that reduce total cellular ROS production at 18 h.

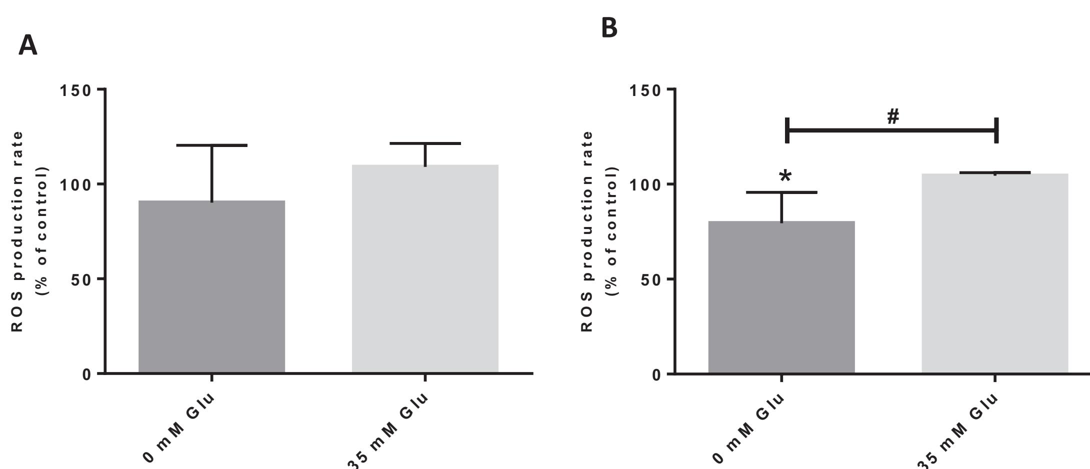


Figure 12. ***Evaluation of cellular ROS in C2C12 cells after 6 h (A) and 18 h (B) exposure to 0 and 35 mM of glucose (Glu).***
ROS generation was fluorometrically assayed using the probe H_2DCF-DA, as described in 2.2.2. of Methods section. Data are means ± SEM of different experiments (N=3), normalized against 5.5 mM of glucose (control). * indicates statistically significant difference in 0 mM versus control ($P < 0.05$), and # indicates statistically significant difference in 0 mM versus 35 mM ($P < 0.05$).

Despite not having observed an upregulated cellular ROS levels in hyperglycemic conditions, we did verify an increased production of mitochondrial superoxide at 6 hours in comparison with control and glucose-deprived cells (Fig 12 and 13). Previous work with this cell line exposed the dependence in anaerobic metabolism in medium (5.6 mM) and high glucose (23.3 mM) conditions (Li et al., 2013). This is a well-documented behavior from rapidly proliferating cells, in which myoblasts are an example (Li et al., 2013; Ryall, 2013). Nevertheless, these cells have functional oxidative phosphorylation machinery and activity, which is expected to be affected by hyperglycemia (Elkalaf et al., 2013). While myoblasts preference for anaerobic glycolysis may explain the non-

significant impact in overall ROS production at such short period as 18 h, we believe that either increased oxidative and anaerobic respiration happen during hyperglycemia, and from a longer period of exposure, we would observe the detrimental cellular effects described in the literature.

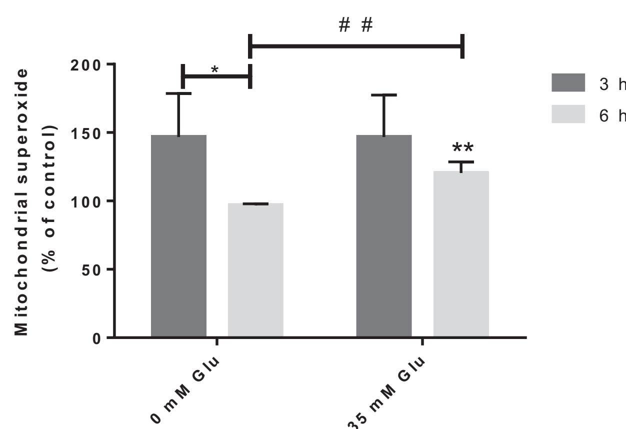


Figure 13. ***Evaluation of mitochondrial superoxide anion in C2C12 cells after 3 and 6 h exposure to 0 and 35 mM glucose (Glu).***
Mitochondrial superoxide generation was assayed using MitoSOX in fluorescence microscopy, as described in 2.2.5. of Methods section. Data are means ± SEM of different experiments (N=3), normalized against 5.5 mM of glucose (control). * indicates statistically significant difference between 3 h and 6 h in 0 mM, ** indicates statistically significant difference in 35 mM versus control at 6 hours ($P < 0.001$), and ## indicates statistically significant difference in 0 mM versus 35 mM at 6 hours ($P < 0.001$).

3.1.2. Mitochondrial membrane potential decreases in short-term glucose deprivation but not in hyperglycemia

MMP is critical for maintaining the physiological function of the respiratory chain to generate ATP. The causal relation from glucose deprivation increased respiration and decreased mitochondrial ROS production has been the focus of several studies (Barros et al., 2004). Moreover, mild mitochondrial uncoupling compounds have been described to mimic the beneficial effects of glucose deprivation (Barros et al., 2004). MMP reduction has been proposed either as a possible mitohormetic signal or mitohormetic response (Brand, 2000; Speakman et al., 2004; Yun & Finkel, 2014). By evaluating MMP with TMRM fluorescent dye, we observed glucose deprivation induction of mitochondrial membrane decreased at 18 h (Fig 15). One the other hand, non-significant change was found at 3 hours from the same condition (Fig 14). This indicates that the observed mitochondrial membrane decrease is not the mitohormetic signal, but a

possible mitohormetic response. We believe that mitochondrial depolarization works as a stimulus to recruit mitophagy machinery system PINK1/Parkin, which maintains a proper mitochondrial pool within the cell and, consequently, enhanced resistance to stress. Despite mild oxidative stress promotion of mitophagy has been established (Frank et al., 2012), its mitohormetic induction is still poorly explored.

The hyperglycemic-induced mitochondrial potential increase was verified at 18h (Fig 15), sustaining our conclusions described in 4.1.1. section.

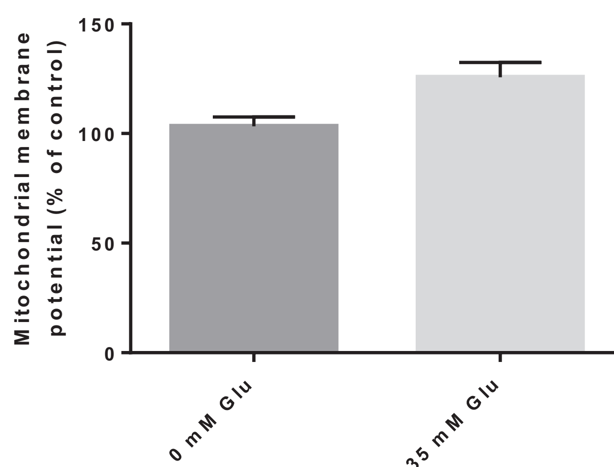


Figure 14. ***Mitochondrial membrane potential after 3 h exposure to 0 and 35 mM of glucose (Glu).*** Mitochondrial membrane potential was fluorometrically assayed using TMRM, as described 2.2.3. of Methods section. Data are means ± SEM of different experiments (N=3), normalized against 5.5 mM of glucose (control). No statistically significant differences were found (P < 0.05).

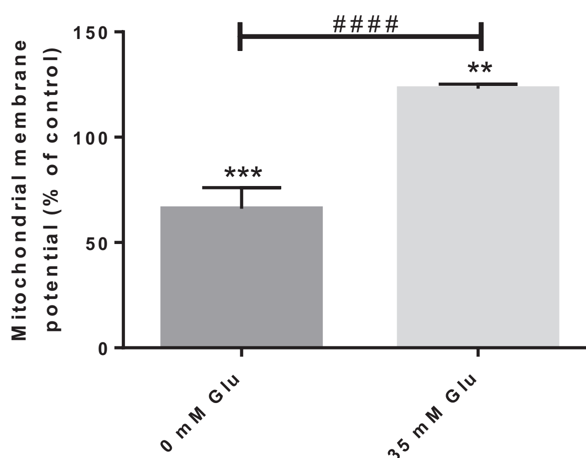


Figure 15. **Mitochondrial membrane potential after 18 h exposure to 0 and 35 mM of glucose (Glu).** Mitochondrial membrane potential was fluorometrically assayed using TMRM, as described 2.2.3. of Methods section. Data are means ± SEM of different experiments (N=3), normalized against 5.5 mM of glucose (control). ** indicates statistically significant difference in 35 mM versus control (P < 0.01), *** indicates statistically significant difference in 0 mM versus control (P < 0.001), and #### indicates statistically significant difference in 0 mM versus 35 mM (P<0.0001).

3.1.3. Mitochondrial fragmentation after short-term glucose deprivation

The dynamic nature of mitochondrial morphology is linked to the process by which dysfunctional mitochondria are initially distinguished from the functional ones. The spatial separation of dysfunctional mitochondria from the entire mitochondrial network can act as a mechanism to prevent further damage and is a prerequisite for impaired mitochondrial elimination.

Tom20 immunofluorescence allowed the assessment of mitochondrial network after 18 h in control, hyperglycemia and glucose-free conditions (Fig 16). In accordance with our previous data and suppositions, glucose-deprived myoblasts showed a fragmented mitochondrial network appearance. In addition, mitochondrial fragmentation is one morphologic change that has been described by elevated glucose concentrations (Trudeau, Molina, & Roy, 2011; Yu et al., 2008). We observed different mitochondrial distribution and less level of fragmentation from hyperglycemic conditions in comparison with glucose deprivation (Fig 16). We suggest that mitochondrial fragmentation in glucose-free medium ensures a durable induction of mitophagy and recycling of mitochondrial population due to a possible mitohormetic stress. A less mitophagic capacity is stimulated by hyperglycemia, which causes damaged mitochondrial accumulation and posterior ROS increased production. Moreover, mitochondrial cellular localization and distance from the nucleus were shown to alter transcriptional response (Al-Mehdi et al., 2012). The spread mitochondrial network observed in glucose-deprived cells may have a complex transcriptional mitohormetic meaning.

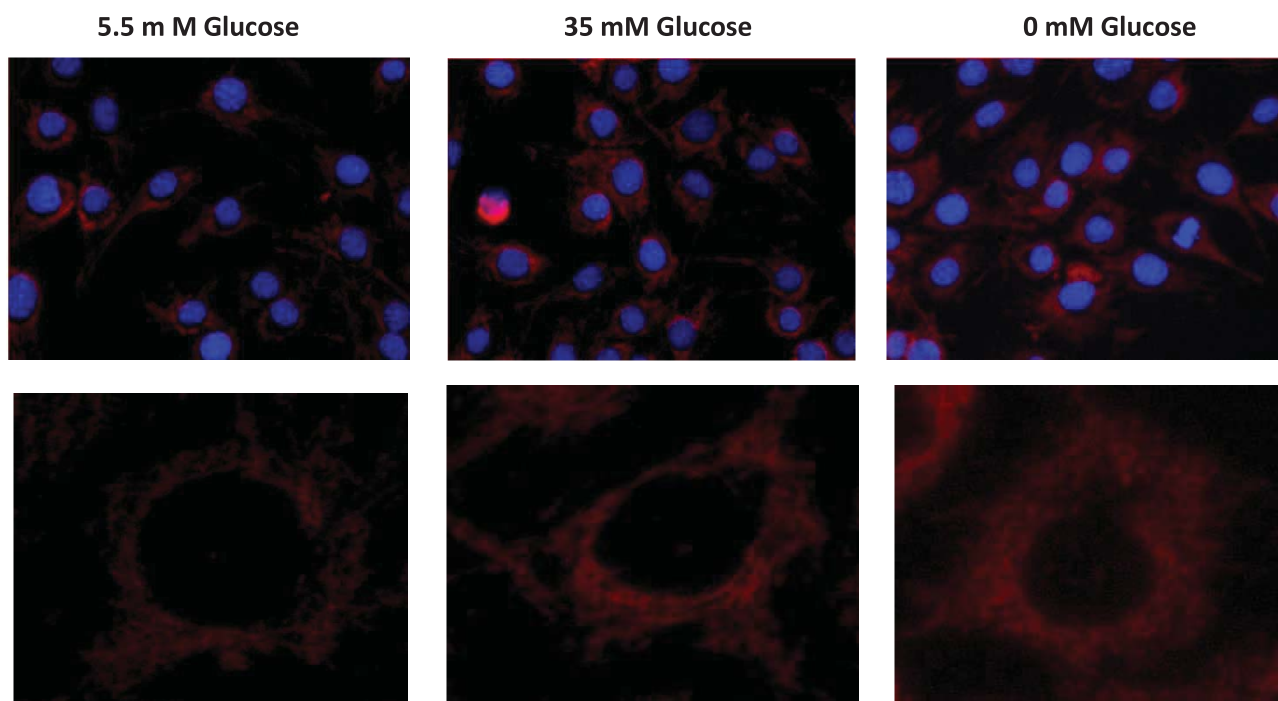


Figure 16. ***Tom20 immunofluorescence in C2C12 cells exposed to 18 h of 5.5, 35 and 0 mM of glucose.*** Mitochondrial network was visualized with Tom20 immunofluorescence as described in 2.2.8. of Methods section.

3.1.4. Mitophagy and Tfam increase after short-term glucose deprivation

Mitochondrial biogenesis and mitophagy represent two opposing cellular pathways, which coordinately regulate mitochondrial content to support energy metabolism, in response to cellular metabolic state, stress and other intracellular or environmental signals (Barbieri et al., 2013). Therefore, maintenance of mitochondrial homeostasis requires both clearances of damaged or unnecessary mitochondria, to generate fresh and functional mitochondria, in a coordinated way. Mild stress activates mitochondrial biogenesis (through the action of the key regulators such as PGC1-α or Tfam) and mitophagy (Frank et al., 2012; Liu & Brooks, 2012). However, it remains challenging to define if ROS production can induce a mitohormetic and coordinated induction of both. Despite no significant alterations in PGC1-α content between the different conditions, we verify an increase in Tfam protein content from glucose-deprived cells (Fig 17 and 18). Moreover, with the application of a specific mitophagy dye named Mtphagy, mitophagy was also significantly augmented (Fig 19). We suggest that short-term glucose induction of a possible mitohormetic response may increase mitochondrial biogenesis to sustain mitochondrial mass and function during intense mitophagic flux.

One the other hand, short-term elevated glucose concentrations did not affect mitochondrial biogenesis machinery and mitophagy, which may explain the expected detrimental effects of this condition to mitochondrial function during longer periods.

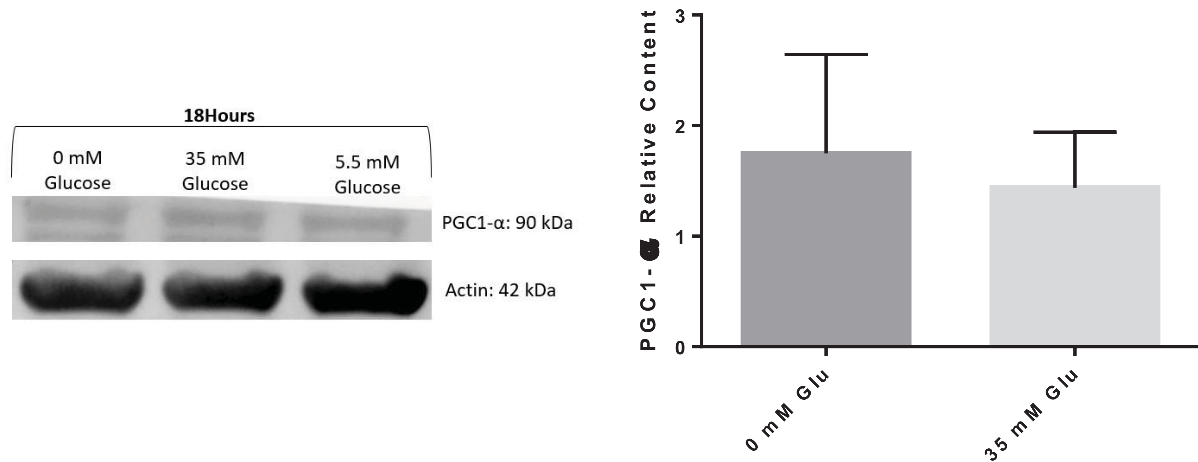


Figure 17. **PGC1-α content in C2C12 cells exposed to 18 h of 0 and 35 mM glucose (Glu).**
The PGC1-α content was evaluated by Western Blot as described in 2.2.6. of Methods section, in cells treated with 5.5, 35 and 0 mM of glucose. Data are means ± SEM of different experiments (N=3), normalized against 5.5 mM of glucose (control). No statistically significant differences were found ($P < 0.05$).

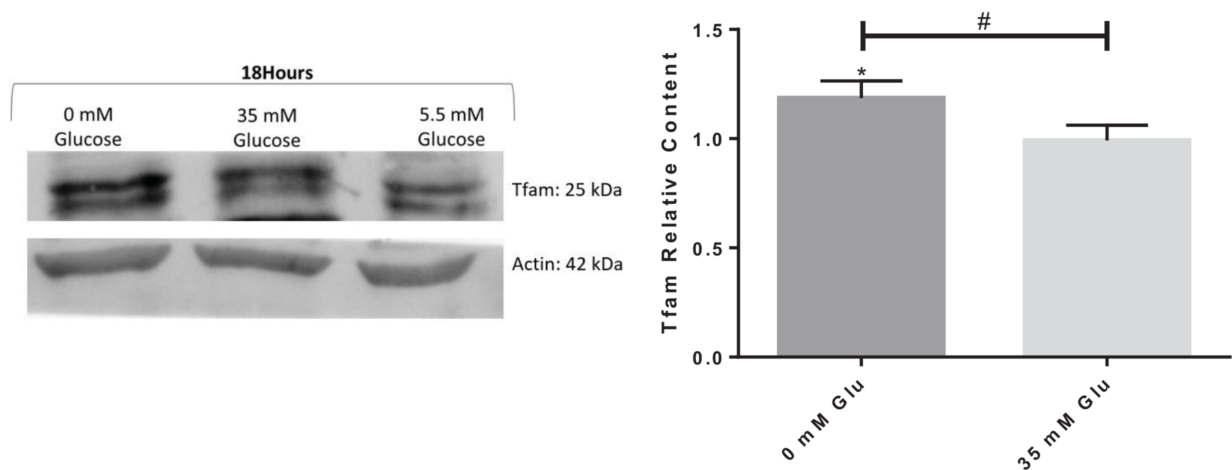


Figure 18. ***Tfam content in C2C12 cells exposed to 18 h of 0 and 35 mM glucose (Glu).***
Tfam content was evaluated by Western Blot as described in 2.2.6. of Methods section, in cells treated with 5.5, 35 and 0 mM of glucose. Data are means ± SEM of different experiments (N=3), normalized against 5.5 mM of glucose (control). * indicates statistically significant difference in 0 mM versus control ($P < 0.05$) and # indicates statistically significant difference in 0 mM versus 35 mM ($P < 0.05$).

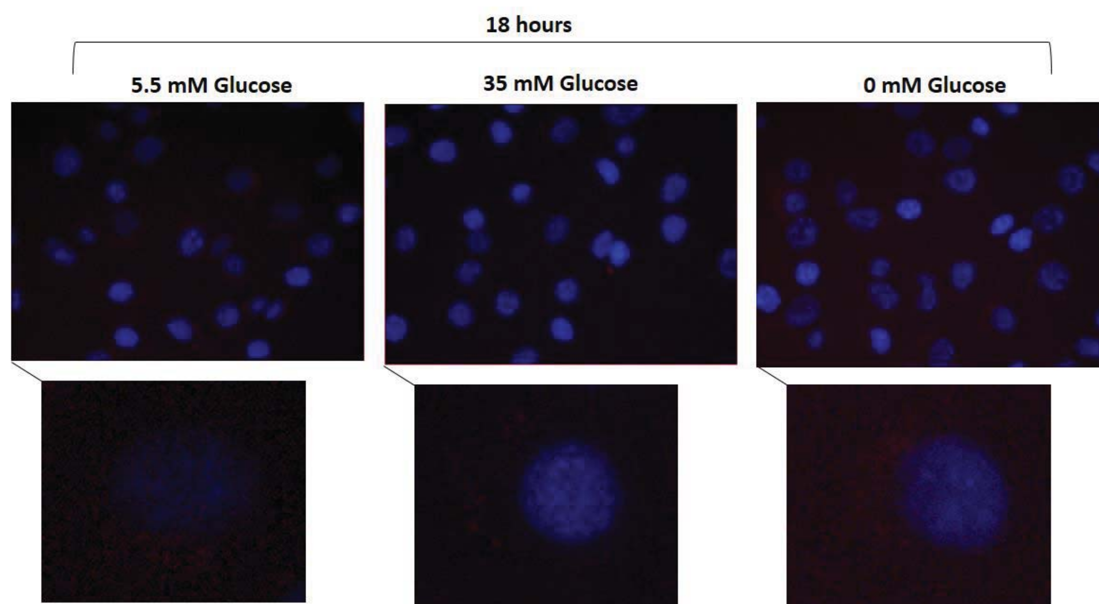


Figure 19. ***Mtphagy dye live image in C2C12 cells exposed to 18 h of 5.5, 35 and 0 mM glucose.*** Mitophagy increase was evaluated by microscopy fluorescence as described in 2.2.9. of Methods section, in cells treated with 5.5, 35 and 0 mM of glucose.

3.1.5.Short-term glucose deprivation does not induce caspase-3 activation

Mitochondria have multifaceted roles in cell death modulation (Parsons & Green, 2010). By working as a physical reservatory of pro-death factors within the cell, mitochondria are the master element of intrinsic apoptosis. In addition, mitochondrial bioenergetics, dynamics, and autophagic elimination are critical aspects that under certain circumstances can induce mitochondrial-mediated cell death (Ding & Yin, 2012; Ni, Williams, & Ding, 2015).

Once our cells showed mitochondrial fragmentation, decreased mitochondrial potential membrane and increased mitophagy during short-term glucose deprivation, we evaluated caspase-3 activation to clarify if these changes were persevering cell survival. Our data showed the absence of significant changes in caspase-3 cleaved form content at 6 h and 18 h in glucose-deprived myoblasts (Fig 20 and 21). This indicates that mitochondrial function alterations during short-term glucose deprivation seem not to compromise cell survival.

In concordance with our previous conclusions, where was suggested that 18 h is a limited time to observe the detrimental effects of hyperglycemia, such as ROS overall increased, we also did not verify caspase-3 activation (Fig 20 and 21).

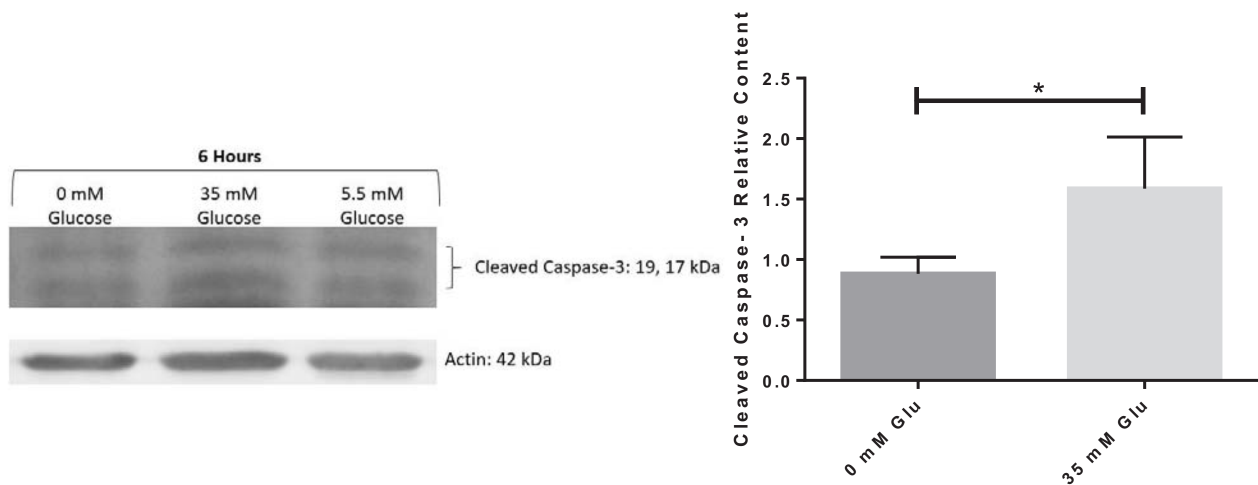


Figure 20. **Cleaved caspase-3 content in C2C12 cells exposed to 6 hours of 0 and 35 mM glucose (Glu).** Cleaved Caspase-3 content was evaluated by Western Blot as described in 2.2.6. of Methods section, in cells treated with 5.5, 35 and 0 mM of glucose. Data are means ± SEM of different experiments (N=3), normalized against 5.5 mM of glucose (control). * indicates a statistically significant difference in 0 mM versus 35 mM (P<0.05).

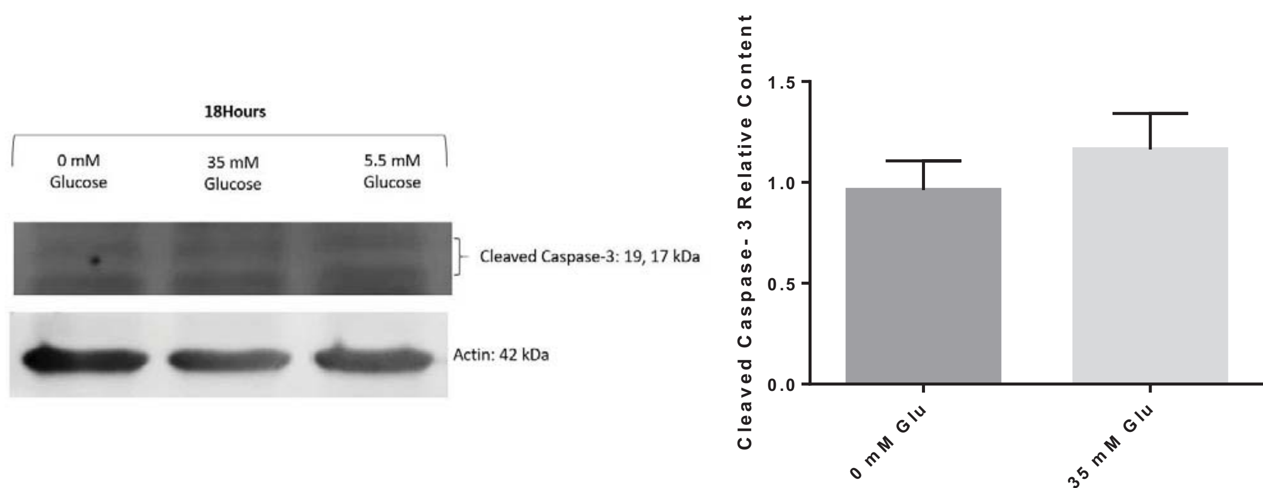


Figure 21. **Cleaved caspase-3 content in C2C12 cells exposed to 18 h of 0 and 35 mM glucose (Glu).** Cleaved Caspase-3 content was evaluated by Western Blot as described in 2.2.6. of Methods section, in cells treated with 5.5, 35 and 0 mM of glucose. Data are means ± SEM of different experiments (N=3), normalized against 5.5 mM of glucose (control). No statistically significant differences were found (P < 0.05).

3.1.6.Sestrin 2 upregulation under glucose deprivation and hyperglycemic exposure

The missing link between increased ROS production and followed upregulation of cellular antioxidant machinery represents a crucial point to better understand mitohormesis pathway. Concerning Sesn2 induction in response to several stresses and

its percussion in overall cellular adaptation, we believe that Sesn2 can represent an active element in mitohormesis by coordinating ROS generation, mitochondrial metabolism, and mitophagy.

To elucidate the pathways underlying a possible glucose-free mitohormetic induction, Sesn2 content was evaluated 6 and 18 h upon exposure of C2C12 cells to control, hyperglycemia, and glucose restriction conditions (Fig 22 and 23). Sesn2 content increased was noticeable after 6 and 18 h in glucose-deprived myoblasts, suggesting an early induction of this protein and sustained upregulation during this condition (Fig 22 and 23). Sesn2 has been described as important in glucose restriction by protecting cells from energetic stress-induced death (Dirat et al., 2012; Lee et al., 2012; Seo et al., 2015). We suggest that Sesn2 upregulation was mediated by a mild oxidative stress during the first 3 h of exposure to glucose-free medium, as an active element in cellular mitohormesis modulation. Indeed, it is highly likely that Sesn2 overexpression following a mild stress is connected to improved cellular resistance against current stress, whereas endogenous Sesn2 upregulation in response to a severe and prolonged stress plays a critical role in the prevention of cell death. Also, high glucose concentration increased of Sesn2 was only verified at 18 h of exposure (Fig 23). Our previous results showed mitochondrial superoxide increase but not total cellular ROS production at this time point (Fig 12 and 13). Therefore, we believe that mitochondrial superoxide causes the upregulation of Sesn2 even before hyperglycemia-induced cellular oxidative stress. Moreover, these results may also support the retrograde signaling between mitochondrial superoxide generation and Sesn2 expression, in which the durability and amount of mitochondrial superoxide production may target different cellular responses.

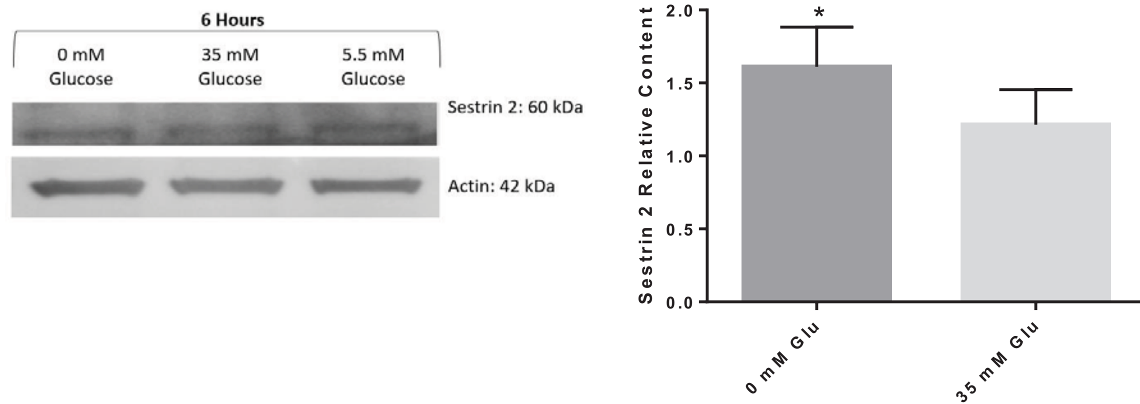


Figure 22. ***Sestrin 2 content in C2C12 cells exposed to 6 hours of 0 and 35 mM glucose (Glu).***
The Sesn2 content was evaluated by Western Blot as described in 2.2.6. of Methods section, in cells exposure 5.5, 35 and 0 mM of glucose. Data are means ± SEM of different experiments (N=3), normalized against 5.5 mM of glucose (control). * indicates statistically significant difference in 0 mM versus control ($P < 0.05$).

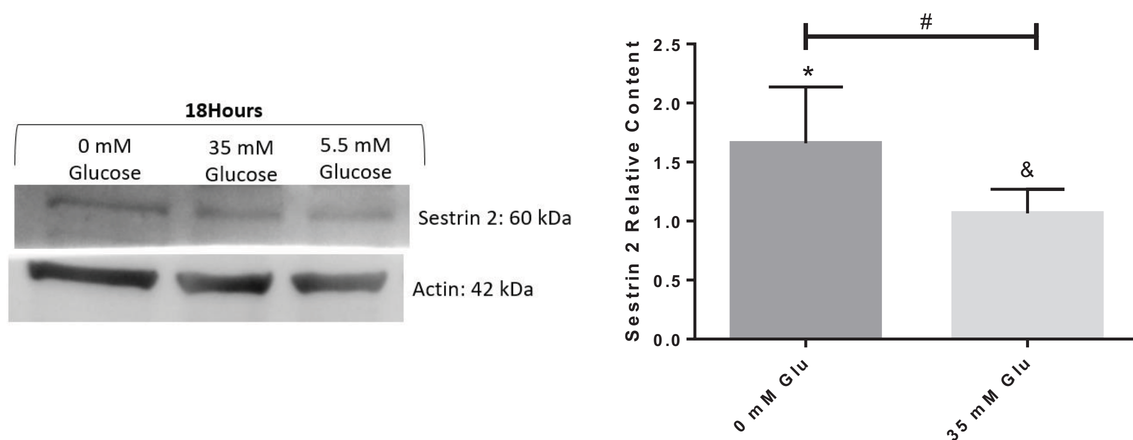


Figure 23. ***Sestrin 2 content in C2C12 cells exposed to 18 h of 0 and 35 mM glucose (Glu).***
The Sesn2 content was evaluated by Western Blot as described in 2.2.6. of Methods section, in cells exposure 5.5, 35 and 0 mM of glucose. Data are means ± SEM of different experiments (N=4), normalized against 5.5 mM of glucose (control). * indicates statistically significant difference in 0 mM versus control ($P < 0.05$), & indicates statistically significant difference in 35 mM versus control and # indicates statistically significant difference in 0 mM versus 35 mM ($P<0.05$).

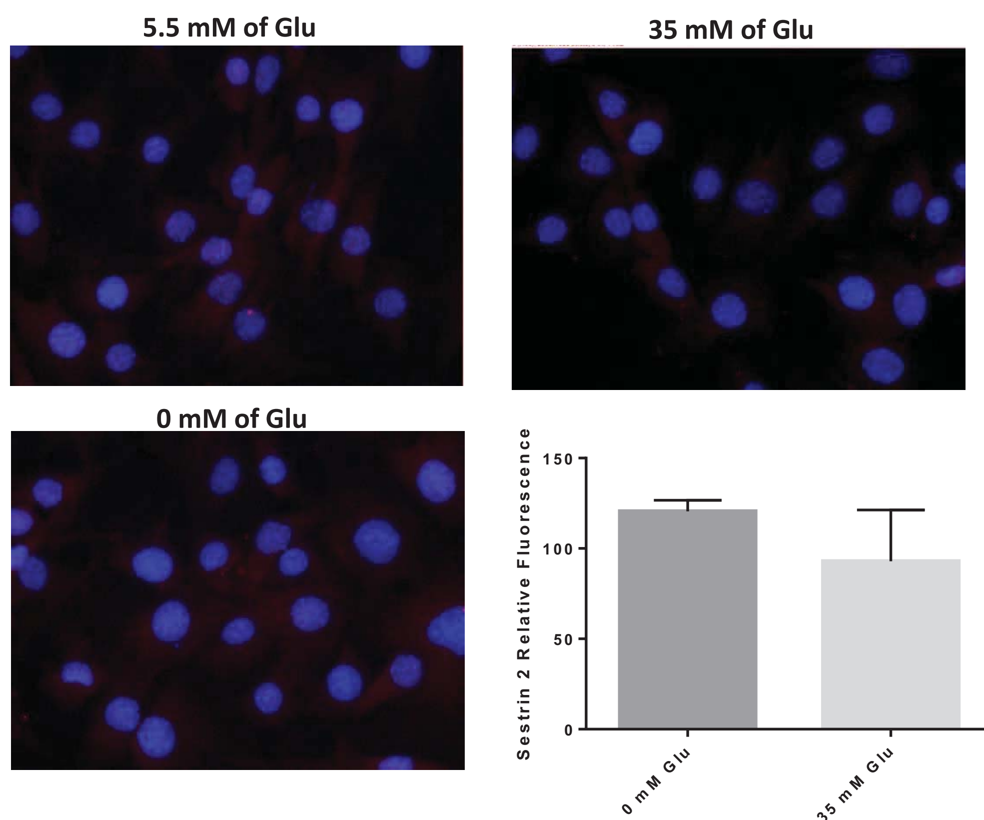


Figure 24. **Sestrin 2 immunofluorescence in C2C12 cells exposed to 18 h of 5.5, 35 and 0 mM of glucose (Glu).**
Sesn2 immunofluorescence was done as described in 2.2.8. of Methods section. Data are means ± SEM of different experiments (N=2), normalized against 5.5 mM of glucose (control). No statistically significant differences were found ($P < 0.05$).

3.1.7. Glucose deprivation does not induce non-selective autophagy

After 18 h in glucose-free medium, C2C12 cells showed in addition to Sesn2 content increase, a decrease in autophagic flux (Fig 25). This indicates that glucose deprivation does not induce autophagy, and moreover, it seems to reduce it. Despite the absence of control cells with and without lysosomal inhibitors at the different conditions in this work, we believe that as showed in a variety of cell lines, glucose deprivation does not induce non-selective autophagy, and can actually decrease basal autophagy and autophagic flux induced by a drug such as chloroquine (Ramírez-Peinado et al., 2013). Although it seems controversial this decline with the increase of Sesn2 that is usually associated with autophagy increase in several stressful conditions, we think that in a mitohormetic scenario Sesn2 upregulation can be more related to its antioxidant function or with selective autophagy modulation, such as mitophagy.

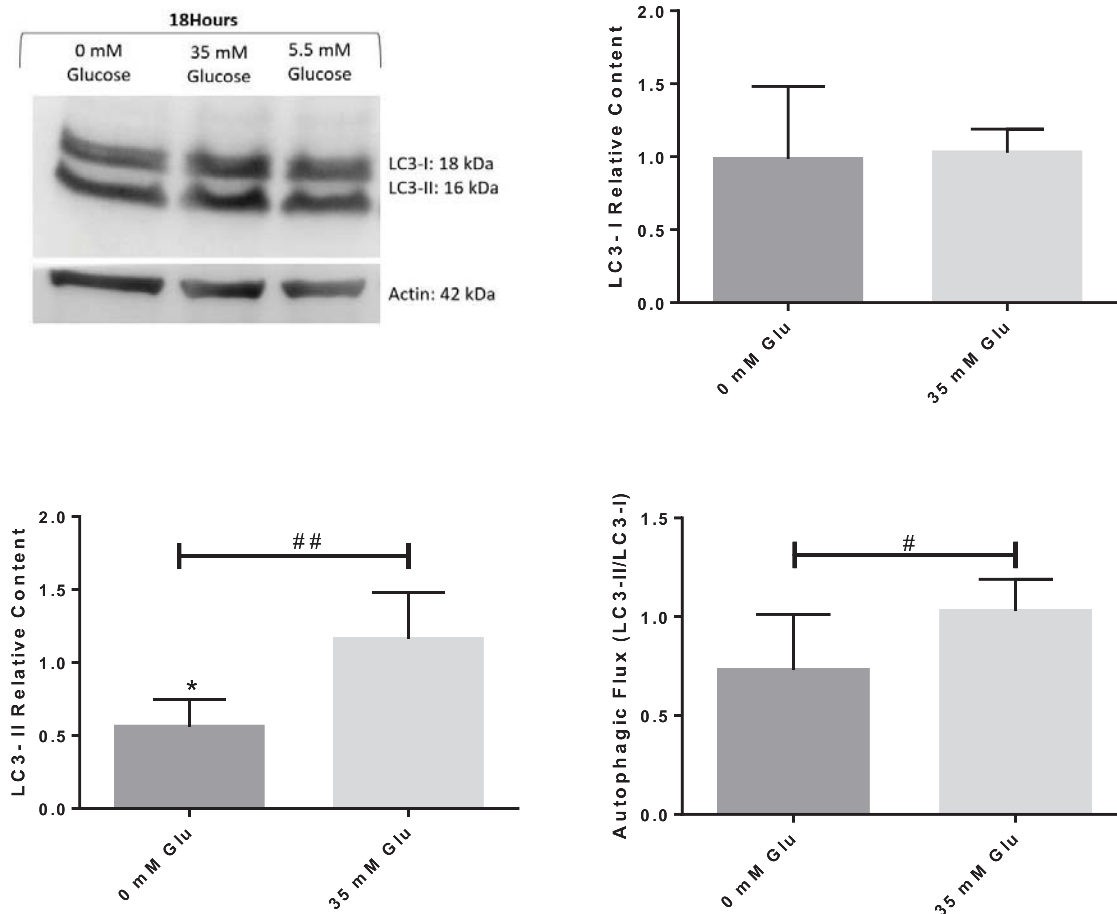


Figure 25. ***LC3-I, LC3-II content and autophagic flux in C2C12 cells exposed to 18 h of 0 and 35 mM of glucose (Glu).***

The LC3-I and LC3-II content were evaluated by Western Blot as described in 2.2.6. of Methods section, in cells exposure 5.5, 35 and 0 mM of glucose. Data are means ± SEM of different experiments (N=4), normalized against 5.5 mM of glucose (control). * indicates statistically significant difference in 0 mM versus control ($P < 0.05$), ## indicates statistically significant difference in 0 mM versus 35 mM ($P<0.01$) and # indicates statistically significant difference in 0 mM versus 35 mM ($P<0.05$).

3.2. Prior glucose deprivation exposure protects C2C12 from hyperglycemic-induced oxidative stress

3.2.1. Glucose deprivation does protect against hyperglycemic-induced ROS production

The mitohormetic pathway as seen during glucose restriction is described to be induced when the low availability of glucose translates in reduced energy levels, which consequently activates AMPK (Schulz et al., 2007). As a result, AMPK increases cellular

catabolism and respiration, which results in the increased ROS production and as result, activation of hormetic protective mechanisms to future stresses (Schulz et al., 2007).

Therefore, we attempted to identify if 18 h of glucose-free medium impact in C2C12 mitochondrial ROS production and mitochondrial changes could be beneficial for the following exposure to hyperglycemia. From literature, we expected that a previous glucose restriction period elicits adaptive changes that should enable a better tolerance to different stresses, such as hyperglycemia. Therefore, C2C12 cells were subjected to 18 h of glucose deprivation before hyperglycemic condition exposure for 24 h (35 mM). We observed non-significant impact of ROS production after 24 h of hyperglycemia from cells exposed to 18 h of glucose restriction, whereas control cells not pre-exposed to glucose deprivation had higher levels of ROS being produced (Fig 26). It seems that glucose deprivation protects C2C12 cells from the metabolic stress induced by 24 h of hyperglycemia.

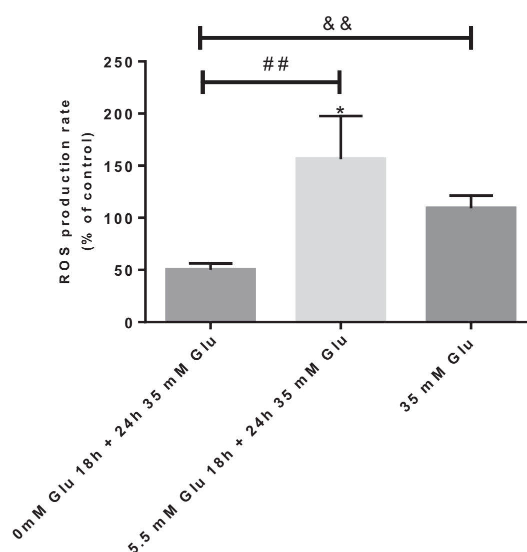
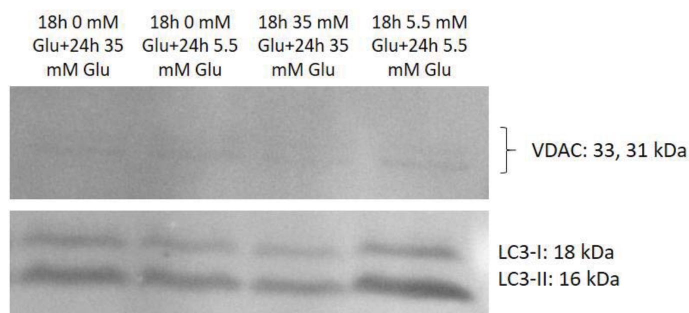
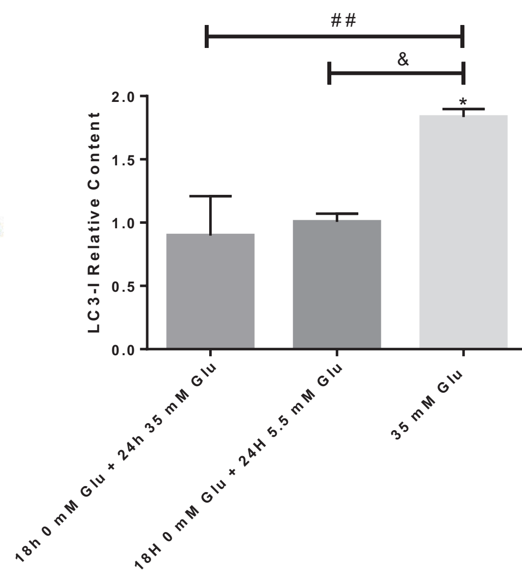


Figure 26. ***Evaluation of cellular ROS in C2C12 cells exposed to 24 h of hyperglycemia after 18 h in 0, 35 and 5.5 mM of glucose (Glu).***
ROS generation was fluorometrically assayed using the probe H_2DCF-DA, as described in 2.2.2. of Methods section. Data are means ± SEM of different experiments (N=3), normalized against 18 h of 5.5 mM of glucose + 24 h of 5.5 mM of glucose (control). * indicates statistically significant difference in 5.5 mM 18h + 24 h 35 mM versus control (P < 0.05), ## indicates statistically significant difference in 0 mM 18h + 24 h 35 mM versus 5.5 mM 18h + 24 h 35 mM (P<0.01), and && indicates statistically significant difference in 0 mM 18h + 24 h 35 mM versus 35 mM (P<0.01).

3.2.2. Glucose deprivation might increase mitochondrial resistance to hyperglycemic-induced mitochondrial damage

Mitochondrial degradation by mitophagy is fundamental when mitochondrial function is impaired, leading to the specific recognition and removal of damaged mitochondria. Conversely, induction of mitophagy has been observed in response to high glucose toxicity to attenuate cellular injury (Devi et al., 2017). Therefore, we evaluated the LC3-I and LC3-II content in mitochondrial fractions in cells exposed to 24 h of hyperglycemia after 18h of glucose deprivation, to verify if mitochondrial targeting to mitophagy was changed when compared with non-pre-exposed cells. Our data revealed that cells primarily exposed to glucose deprivation do not have variation in LC3-I and LC3-II content in mitochondria after 24 h in hyperglycemia, whereas an increase in these two proteins occurs without this pre-exposure (Fig 27). Moreover, we observed no change in these proteins in cells exposed to control conditions after 18h of glucose deprivation (Fig 27). Together, these findings suggest that high glucose induces mitochondrial autophagy, which is prevented by previous exposure to glucose deprivation. This result in conjunction with no changes in total cellular ROS production suggests that glucose deprivation increases mitochondrial resistance to hyperglycemic induced mitochondrial damage, consequently leading to a minor reliance in mitophagy to ensure cellular survival.





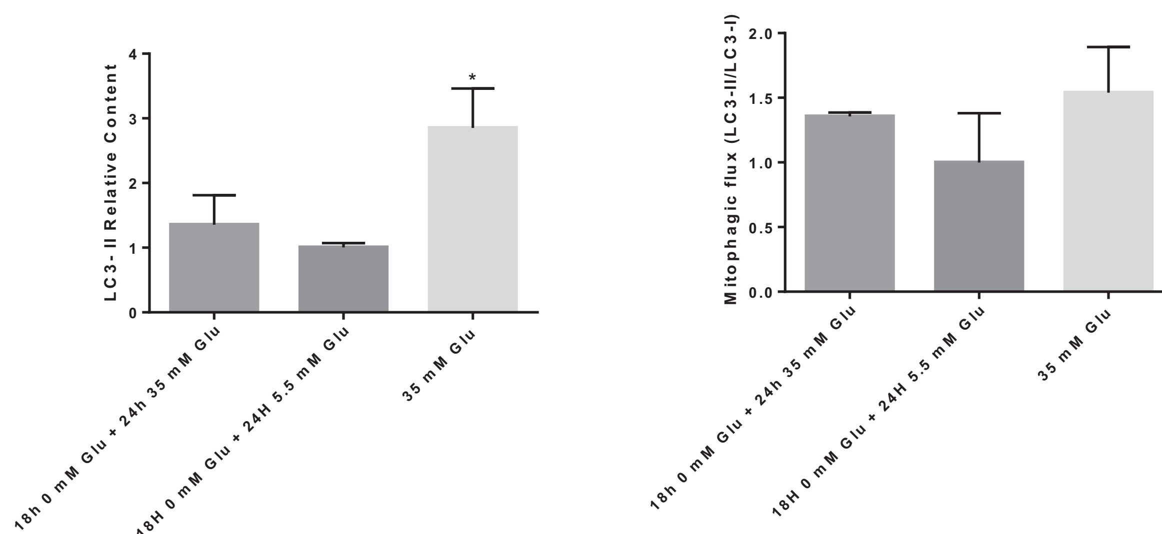


Figure 27. ***LC3-I and LC3-II mitochondrial content in C2C12 cells exposed cells exposed to 24 h of hyperglycemia after 18 h in 0 and 35 mM of glucose (Glu).***
The LC3-I and LC3-II content in mitochondrial fraction were evaluated by Western Blot as described in 2.2.6. and 2.2.7 of Methods sections, Data are means ± SEM of different experiments (N=3), normalized against 18 h of 5.5 mM of glucose + 24 h of 5.5 mM of glucose (control). * indicates statistically significant difference in 35 mM versus control (P < 0.05), ## indicates statistically significant difference in 0 mM 18h + 24 h 35 mM versus 35 mM (P<0.01) and & indicates statistically significant difference in 0 mM 18h + 24 h 5.5 mM versus 35 mM (P<0.01).

3.2.3. Previous glucose deprivation exposure does not change Sestrin 2 content and non-selective autophagy after hyperglycemic exposure

As glucose deprivation might induce cellular resistance against hyperglycemia through mitohormesis induction, we also evaluated Sesn2 content after 24 h of hyperglycemia in cells previously exposed to 18h of glucose deprivation. Despite not statistically significant, we suggest that glucose deprivation promotes an increase in antioxidant machinery in cells, which leads to a lower production of ROS when under high glucose concentrations (Fig 28). Therefore, Sesn2 can be a mitohormetic mediator during mild stress that in response to a new stress it is no longer necessary. This can mean, that Sesn2 upregulation can be required to the mitohormesis phenomenon induction, characterizing a fast antioxidant response promotion, which in further stresses does not need to be sustained. Results showed that autophagy seems also not to be affected (Fig 29).

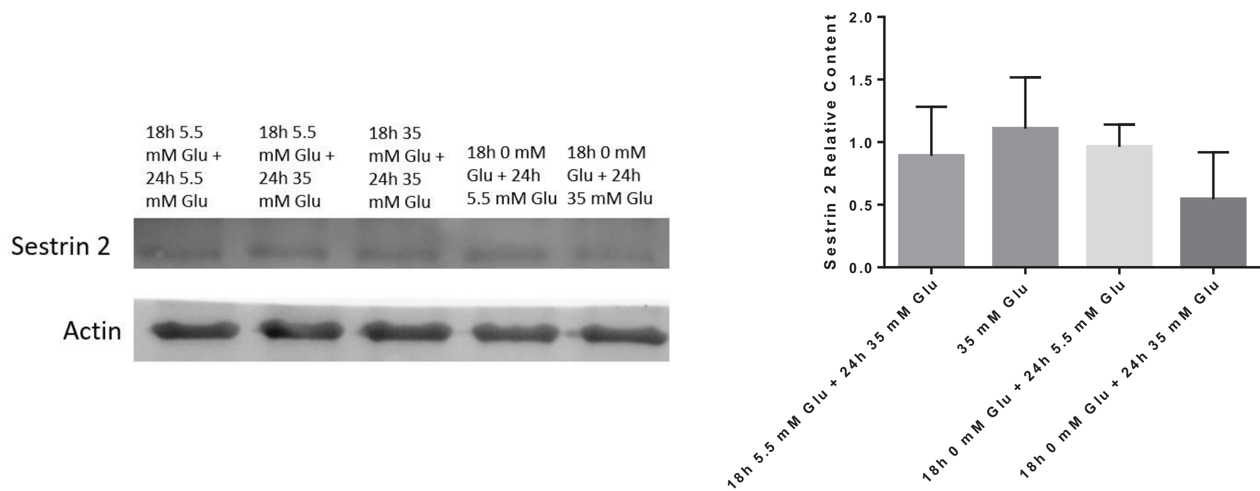


Figure 28. ***Sestrin 2 content in C2C12 cells exposed to 24 h of hyperglycemia after 18 h in 0, 35 and 5.5 mM of glucose (Glu).***

The Sesn2 content was evaluated by Western Blot as described in 2.2.6. of Methods section, Data are means ± SEM of different experiments (N=3), normalized against 18 h of 5.5 mM of glucose + 24 h of 5.5 mM of glucose (control). No statistically significant differences were found (P < 0.05).

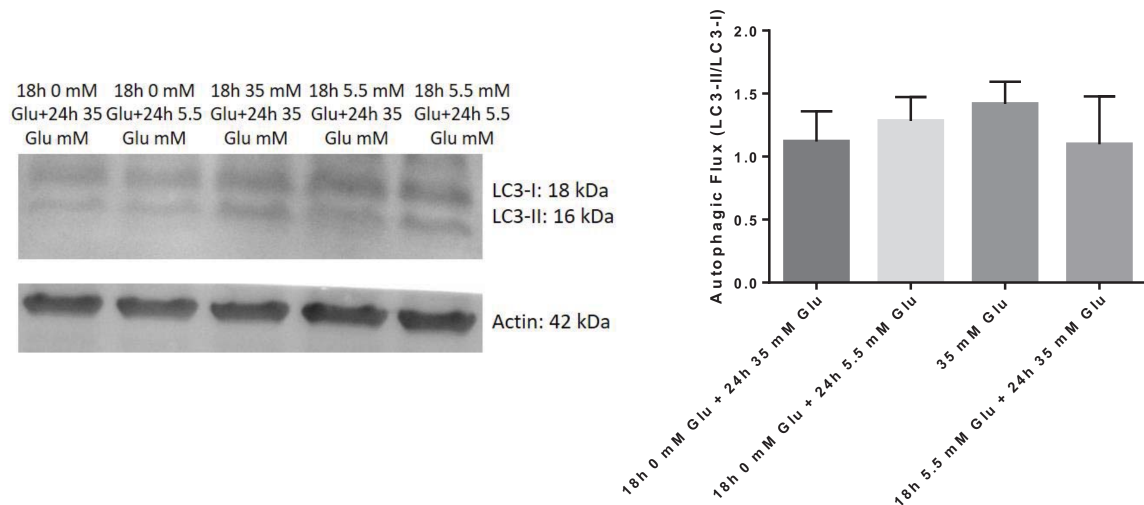


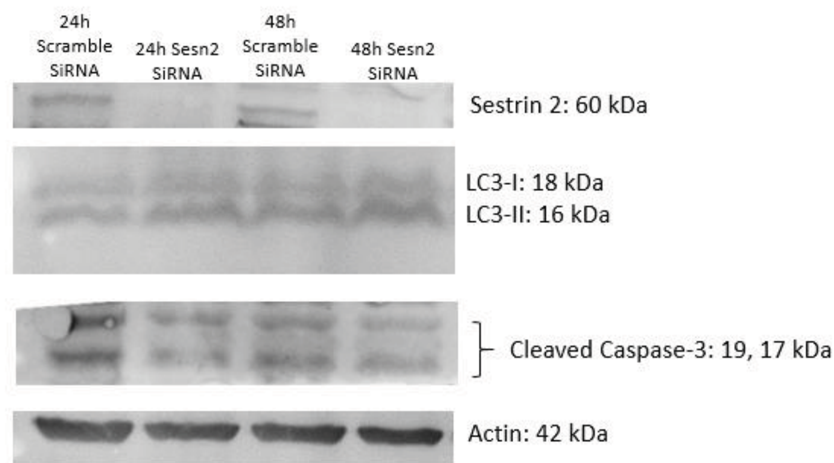
Figure 29. ***Autophagic flux in C2C12 cells exposed to 24 h of hyperglycemia after 18 h in 0, 35 and 5.5 mM of glucose (Glu).***

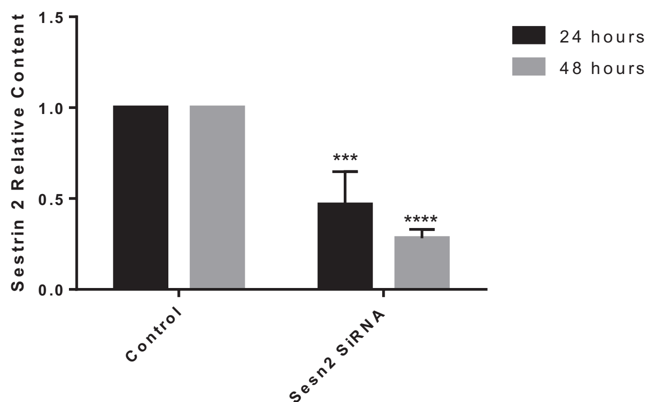
The LC3-I and LC3-II content were evaluated by Western Blot as described in 2.2.6. of Methods section, Data are means ± SEM of different experiments (N=3), normalized against 18 h of 5.5 mM of glucose + 24 h of 5.5 mM of glucose (control). No statistically significant differences were found (P<0.05).

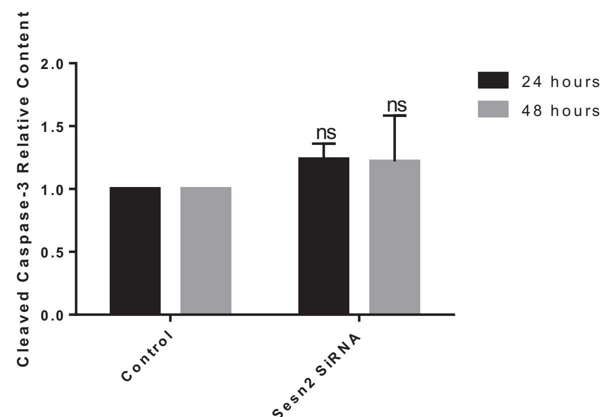
3.3. Sestrin 2 transient silencing protocol optimization

Despite Sesn2 upregulation along a possible mitohormetic phenomenon described in this work, its meaning and importance will need further studies. During the realization of this work, it was optimized a transient knockdown of Sesn2 in C2C12 cells, even if this

one was not applied in the described mitohormetic protocol induction. A reduction in the Sesn2 content expression above 50% was observed after 24 and 48 h of C2C12 electroporation with siRNA (Fig 30), without changes in caspase-3 activation (Fig 30). Despite not exposed to our results, a dramatical decrease of cellular growth was observed when compared with control cells. We believe that Sesn2 knockdown may not affect cellular survival under control conditions, but does dramatically influence cellular division and growth.







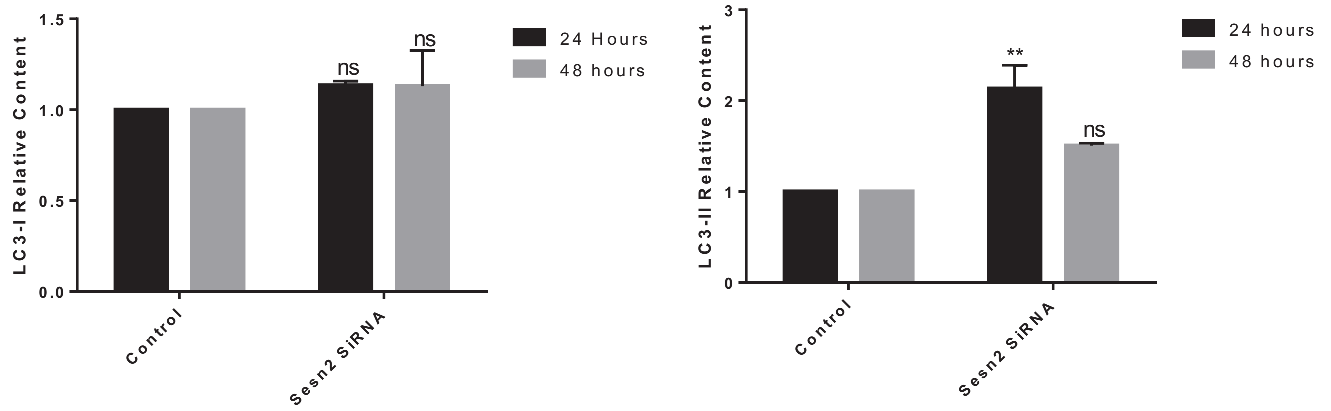


Figure 30. ***Sestrin 2, Cleaved Caspase-3 and LC3-I, LC3-II content in C2C12 cells after 24 h and 48h of electroporation knockdown Sestrin 2 induction.***

The protein content was evaluated by Western Blot as described in 2.2.6. of Methods section. Data are means ± SEM of different experiments (N=3). No statistically significant differences were found (P < 0.05).

4. Discussion

The first objective of this work was to establish a mitohormetic protocol in C2C12 cells, which could be used as an in vitro model of enhanced resistance against oxidative stress by prior exposure to a metabolic stressor. Herein, we found that glucose deprivation pretreatment protects C2C12 cells against hyperglycemic-induced oxidative stress in vitro. The results obtained in this work suggest the involvement of mitochondrial superoxide production in a retrograde pathway that promotes substantial mitochondrial respiration reduction accompanied with selective mitochondrial autophagy and biogenesis modulation. Given the ambivalent roles played by Sesn2 in cellular antioxidant adaptation, we appeal its possible importance as a downstream mitohormetic regulator. Moreover, with the recent association of this protein with mitophagy progression (Kim et al., 2016), we propose that mitophagy hormetic induction, and consequent stress-resistance adaptation, can be Sesn2-mediated.

4.1. Modulatory mitochondrial superoxide production during glucose deprivation is accompanied with Sestrin 2 upregulation

Limited glucose sources cause cellular metabolism to favor OXPHOS, aiming to maintain intracellular ATP supply (Rea & Johnson, 2003; Zhao et al., 2017). This, in turn, might result in an increased production of superoxide and hydrogen, which can cause ROS overload. Thus, several studies point to an increase in ROS production and consequent amplification of cell death during glucose withdrawal in different cell lines (Graham et al., 2012; Marambio et al., 2010). In contrast, increased mitochondrial respiration conditions are also described as possible enhancers of antioxidant and cryoprotective systems, being lifespan-extending interventions such as caloric restriction and specifically reduced glucose metabolism an example of it (Cerletti et al., 2012; Finley & Haigis, 2009; Masoro, 1998; Rea & Johnson, 2003; Yang et al., 2010). Within this scenario, the hypothesis that ROS activate a signal antioxidant response, which enhances defense capacity against oxidative stress, has been proposed in glucose-restricted *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis Elegans* studies (Kharade et al., 2005; Rea & Johnson, 2003; Rovenko et al., 2015; Schulz et al., 2007). In mammalian models, studies with reduction of monosaccharides, glucose,

and fructose showed to have different effects on mitochondrial function and antioxidant defense (Rizkalla, 2010; Shah et al., 2013). Nevertheless, the connection between antioxidant defense modulation with mitochondrial activity and/or ROS levels change still very scarce, even contradictory.

The current work shows non-statistically significant differences of mitochondrial superoxide after 3 h of exposure to glucose-free medium comparable to hyperglycemic-exposed myoblasts (Fig 13). Notably, a decrease in mitochondrial superoxide levels was verified in glucose-deprived cells after 6 h when compared with the 3 h production at this condition. Moreover, total cellular ROS production was described to be decreased after 18 h of glucose deprivation (Fig 12). From these results, it seems that C2C12 cells sensing of glucose deprivation lead to a transient mild production of mitochondrial superoxide, which quickly augments a cellular response that causes its own decay. This decrease was accompanied by Sesn2 content upregulation (Fig 23), which suggests a retrograde signal pathway induction from mitochondrial superoxide to the cytoplasm and nucleus. Usually, mitochondrial superoxide anion is rapidly converted to hydrogen peroxide by MnSOD, a more stable reactive species (Osburn et al., 2006). However, it has been hypothesized that in the presence of excessive superoxide, MnSOD becomes oxidized leading to superoxide accumulation (MacMillan-Crow et al., 1998). By being highly reactive, superoxide anion can consequently modify mitochondrial proteins, and through voltage-dependent anion channels transport, can affect cytosolic proteins as well (Budzińska et al., 2009.; Piskernik et al., 2008). As a matter of fact, enhanced longevity on *Caenorhabditis Elegans* has been described to possibly depend on mitochondrial superoxide transduction pathway that changes nuclear gene expression (Briedé et al., 2010; Cristina et al., 2009; Huang et al., 2002). Therefore, this early mitochondrial superoxide burst possibly implies dramatic extended cellular changes.

The Sesn2 expression is critically dependent on the Nrf2-ARE system, being an essential element of Nrf2-dependent antioxidant response (Rhee & Bae, 2015; Shin et al., 2012). Moreover, previous studies reported an increase in Nrf2 and Nrf2-ARE DNA binding by glucose deprivation in HepG2 cells (Lee et al., 2010; Seo et al., 2015). So, a simple explanation for the increase of Sesn2 in glucose-deprived cells can be explained by a Nrf-2 superoxide-dependent activation. Accordingly, diquat-treated mouse embryonic fibroblasts were described to have increased activation of Nrf-2 due to

superoxide anion production (Osburn et al., 2006). This result was only witnessed under diquat concentrations in which minimal toxicity was observed, illustrating the significant involvement of Nrf-2 in a mild oxidative stress condition (Osburn et al., 2006). Recently, Sesn2 upregulation by glucose starvation was shown to be mediated by cooperative activation of ATF4 and Nrf2 following their direct binding to the Sesn2 promoter (Ding et al., 2016). This cooperation has been described as a mechanism by which Sesn2 maximal expression can be induced (Budanov et al., 2004). Therefore, our results suggest that an early amplification of mitochondrial superoxide from a possible oxidative metabolic shift due to glucose deprivation may enhance Nrf2 activation. This can occur due to the direct reaction of superoxide with Keap1 or kinase signaling pathways (Huang et al., 2002; Velayutham et al., 2005). Hence, Nrf2 causes enhanced transcription of antioxidant-related genes as Sesn2, leading to ROS overall decrease. Moreover, Sesn2 can induce autophagic degradation of Keap1, reinforcing Nrf2 activation (Bae et al., 2013). Consistently, we detected Sesn2 content upregulation after 6 and 18 h of glucose deprivation, along with mitochondrial superoxide and total cellular ROS decrease (Fig 12, 13, 22 and 23). However, further work needs to confirm this explanation by Nrf-2 and Sesn2 expression, after concurrent treatment with an antioxidant such as N-acetyl-cysteine.

4.2. Glucose deprivation-dependent Sestrin 2 upregulation follows mitophagy induction without non-selective autophagic increase

Sesn2 protective induction under hypoglycemia has been intensively described (Ding et al., 2016; Dirat et al., 2012; Lee et al., 2012; Seo et al., 2015). Here, we show that expression of Sesn2 is also induced by glucose deprivation in undifferentiated C2C12 cells (Fig 22 and 23). After 3 h of glucose deprivation, mitochondrial superoxide production may reach a mild production, which follows decline to normal levels in glucose-deprived myoblasts (Fig 13). Also, we reported mitochondrial superoxide and total cellular ROS normalization production along with Sesn2 upregulation after 6 and 18 h (Fig 12 and 23). This might be due to two reasons. First, the superoxide boost would stimulate the Nrf2 activation of ARE-mediated antioxidant genes expression such as Sesn2, to decrease the intracellular superoxide/hydrogen peroxide levels (Nguyen et al., 2009). Although Sesn2 apparent absence of intrinsic catalytic antioxidant activity (Woo

et al., 2009), it suppresses ROS production through three pathways: upregulation of Nrf2 (Rhee & Bae, 2015), blocking of mTOR activation (Dirat et al., 2012; Kim et al., 2015;) and mitophagy induction (Ishihara et al., 2013; Kim et al., 2016) (Fig 31).

In addition, we also reported MMP decrease after 18 h exposure to glucose deprivation (Fig 15).

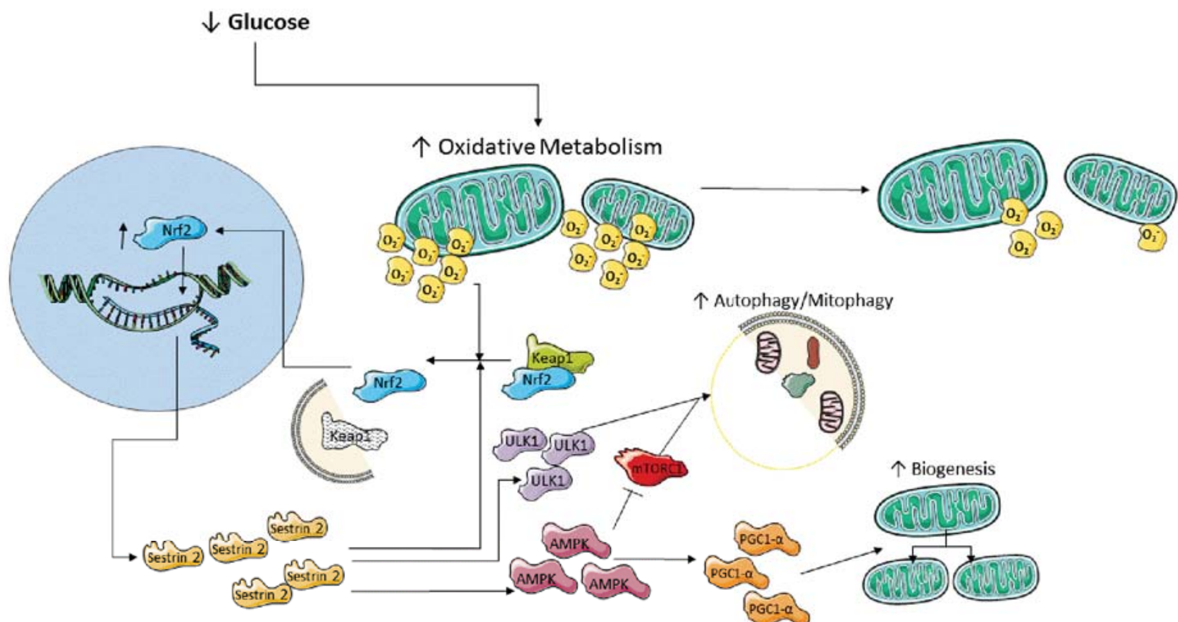


Figure 31. ***Illustration of possible Nrf2 induction of Sestrin 2 by mitochondrial superoxide.***
Mitochondrial superoxide moderate increased by glucose deprivation can directly or by relative derived non-radicals, such as hydrogen peroxide, affect Keap1 signaling. Consequently, Nrf2 is allowed to induce nuclear expression of antioxidant-related genes as Sestrin 2. With the induction of Sestrin 2 expression, several downstream pathways can be altered by its control of AMPK and ULK1. Indeed, these kinases might change overall cellular processes like autophagy, mitophagy and mitochondrial biogenesis (image created with **Servier Medical Art templates**).

MMP represents cell metabolic state and plays a major role in cell protection through adaptation to stress (Barbour & Turner, 2014; Palmeira & Rolo, 2012). As reported previously, withdrawal of growth factors or loss of the extracellular glucose supply leads to a decline in MMP (Howlin, 2014). Previous studies with longer hypoglycemic treatments have associated mitochondrial depolarization with cellular death promotion (Gottlieb et al., 2003; Isaev et al., 2008; Liu et al., 2003). In contrast, our results showed that there is no change in caspase-3 activation in glucose-deprived myoblasts (Fig 20 and 21). Caspase-3 has been identified as a hallmark of apoptosis, which can be either

dependent on or independent of mitochondrial cytochrome *c* release and caspase-9 (Porter & Jänicke, 1999). Nevertheless, evaluation of others apoptotic pathways and even necrotic systems needs to be done to understand the glucose deprivation influence in myoblasts cell survival.

Pursuing the described regulatory activity of Sesn2 in cell viability, we presumed that its upregulation plays a major role in hypoglycemic myoblasts adaptation. Accordingly, Sesn2 inactivation has been shown to strongly sensitize cells to cell death induced by glucose withdrawal (Ding et al., 2016; Dirat et al., 2012). One of the explanations for this sensitization is the Sesn2-mediated mTOR down-regulation (Dirat et al., 2012). As previously reported, Sesn2 potentiates several downstream effectors that promote mTORC1 activation attenuation and consequent increase in autophagy (Hou et al., 2015; Lee et al., 2010; Liu et al., 2015; Seo et al., 2015; Kim et al., 2015). Additionally, Sesn2 inhibition of mTOR was proposed to participate in the protective response against 2-deoxyglucose (2-DG)-induced apoptosis (Dirat et al., 2012). In contrast, several studies have pointed Sesn2 protection during hypoglycemic stress to pathways independent of autophagy induction (Ding et al., 2016; Seo et al., 2015). Intriguingly, our data reported induction of mitochondrial network fission accompanied with increased mitophagy, without a detectable increase in non-selective autophagy (Fig 16,19 and 25). Unchanged autophagic flux was verified in glucose-deprived myoblasts as described in a variety of cells lines (Ramírez-Peinado et al., 2013). It seems that autophagy can contribute to glucose and ATP homeostasis in particular circumstances. In this regard, some forms of starvation or drugs such as 2-DG may regulate signaling molecules that are involved in autophagy promotion besides mTOR, while glucose deprivation does not. However, we certainly cannot fully exclude that non-selective autophagy is induced to a not detectable extension. The decrease of lipidated LC3 form upon hypoglycemic treatment in myoblasts can be either interpreted as inhibition of autophagy or induction of autophagy because of LC3-containing autophagosomes faster degradation. For this reason, an additional comparison of treated cells with and without lysosomal inhibitors needs to be done in the future. We propose that under glucose withdrawal, mitophagy occurs in a truly selective manner and can proceed without a detectable increase in non-selective autophagy. Curiously, the inherent capability to degrade specifically mitochondria without inducing macroautophagy at the same time was described after

mild and transient oxidative stress induction with rotenone and hydrogen peroxide (Frank et al., 2012; Jendrach et al., 2008). These studies proposed that short incubations of low hydrogen peroxide or rotenone concentrations do not massively damage mitochondria, but rather initiate an ROS signaling cascade, leading to selective mitophagy promotion (Frank et al., 2012). Thus, mitophagy is indeed induced by mild oxidative stress in a very sensitive and accurate manner. To our knowledge, the data presented here show for the first time the reproduction of this phenomenon under a metabolic stress such as glucose deprivation. It seems that myoblasts short-term glucose restriction implies a low ROS production that it's translated in a selective increase of mitophagy. Moreover, mitophagy-dependent clearance of mitochondrial ROS can also explain the already exposed results (Scherz-Shouval & Elazar, 2011). Still, the mechanistic details inducing selective mitophagy of non-selective autophagy remain largely unclear. We hypothesized that Sesn2 could be involved in this mechanism, due to its recently discovered role in mitophagy (Ishihara et al., 2013; Kim et al., 2016). Nitric oxide-mediated Sesn2 suppression of inflammasome activation was explained by Sesn2 clearance of damaged mitochondria in macrophages (Kim et al., 2016). In this study, increased Sesn2 induced mitophagy activation through regulation of 2 synchronized procedures. First, Sesn2 causes “mitochondrial priming” by facilitating aggregation of p62 (SQSTM1) and its binding to ubiquitins on the mitochondrial surface. Secondly, Sesn2 activates specific autophagic machinery for the previously primed mitochondria via an increase of ULK1 protein levels. To complement our hypothesis, glucose deprivation was reported to promote the accumulation of p62 even without nonselective autophagy increase (Ramírez-Peinado et al., 2013). Based on these data and our results, we speculate that under short hypoglycemic periods Sesn2 may serve as an adapter protein that mediates p62 binding to ubiquitinated mitochondria, resulting in mitophagy. In addition, Sesn2 upregulation can control ULK1 levels through different ways (Kim et al., 2011; Kim et al., 2016; Ro et al., 2014), therefore supporting autophagosome formation and mitophagy. Moreover, mitophagy needs to be coordinated with mitochondrial biogenesis within the cell to preserve mitochondrial mass and mitochondrial homeostasis (Palikaras & Tavernarakis, 2014). Our results showed increased Tfam content during 18 h of glucose deprivation (Fig 18), which can also be supported by Sesn2 upregulation. Sesn2 can bind to AMPK, and either through

direct physical association or indirect transcriptional regulation, stimulate the formation of the AMPK holoenzyme and its phosphorylation and activation by upstream kinases (Budanov & Karin, 2008; Chen et al., 2010). Given AMPK activation of mitochondrial biogenesis through PGC-1α and the NRFs activation, Sesn2-induced AMPK activation can lead to increased mitochondrial biogenesis (Palikaras & Tavernarakis, 2014). Moreover, phosphorylation of ULK1 by AMPK leads to mitophagy in response to nutrients deprivation and, as such, this can be another pathway by which Sesn2 induces elimination of dysfunctional mitochondria (Egan et al., 2011). Thus, Sesn2 can be essential for autophagic removal of damaged mitochondria that leak electrons and produce pathogenic amounts of ROS.

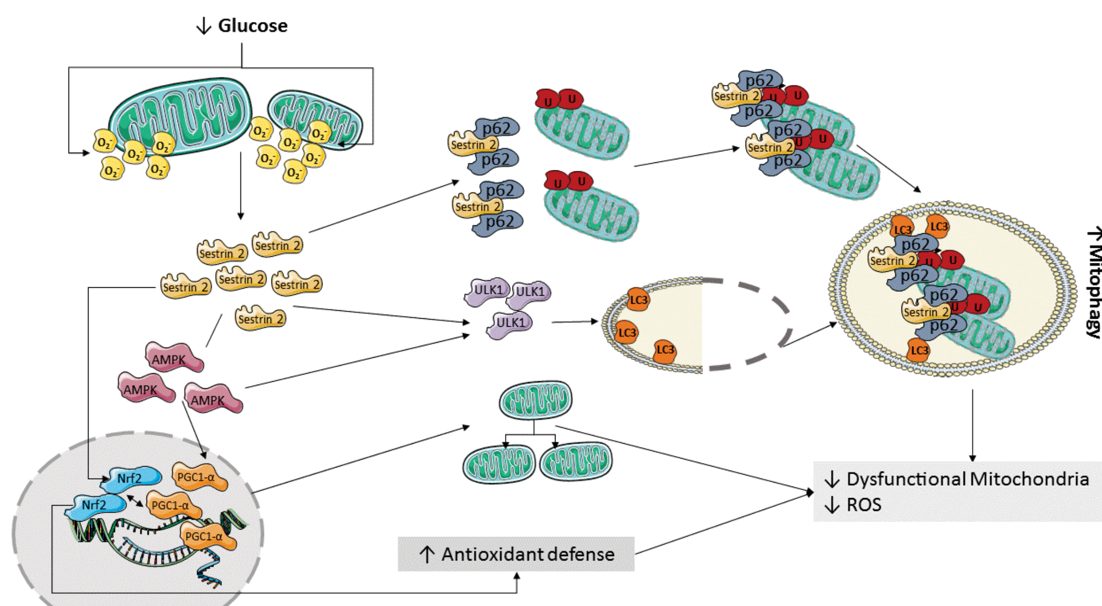


Figure 32. ***Illustration of possible Sestrin 2-involved protective pathways during short-term hypoglycemia.***
Glucose deprivation-induced Sestrin 2 content might increase antioxidant defense and mitophagy, resulting in an enhanced mitochondrial population quality. Sestrin 2 can trigger dysfunctional mitochondrial priming to removal by its direct effect on p62 aggregation and association with the mitochondrial surface. Moreover, Sestrin 2 also activates ULK1-mediated autophagosome formation, which reinforces mitophagy overall induction. This duality in the control of mitophagy by Sestrin 2 might explain the enhanced response to hyperglycemia in cells pre-exposed to glucose deprivation (image created with **Servier Medical Art templates**).

Our data do not contradict the view that prolonged hypoglycemic treatments can trigger autophagy and excessive ROS formation. Moreover, at higher ROS concentrations this and other quality control systems might just be overloaded,

resulting in permanent damage and reduced cell viability as described previously (Jendrach et al., 2008).

4.3. Glucose deprivation pretreatment protects cells against hyperglycemic-induced oxidative stress

The effects of continuous oxidative stress exposition on cells in vitro are well studied (Birben et al. , 2012). However, much less is known about the consequences of a single short ROS burst on the cellular and in particular on the mitochondrial level. In recent years, mitochondrial ROS association with an adaptive response called mitochondrial hormesis (mitohormesis), changed their scientific popularity (Schulz et al., 2007; Yun & Finkel, 2014). Mitohormesis defines that low levels of oxidative stress produced by mitochondria result in a long-term protective response, that promotes cell survival and stress resistance (Yun & Finkel, 2014). Accordingly, the concept of mitohormesis suggests a common interconnected explanation for the positive physiological effects of physical exercise, reduced calorie uptake and glucose restriction (Merry & Ristow, 2016). In contrast, increased production of mitochondrial ROS by hyperglycemia is known as a leading cause of the clinical complications associated with diabetes and obesity (Brownlee et al., 2001). To address the question whether the changes during glucose deprivation were reflected by alterations in the adaptive response to posterior metabolic stresses, the ROS production of non-treated cells and cells pretreated with a single period of 18 h of glucose deprivation were analyzed after 24 h under hyperglycemic condition (Fig 26). At the indicated time points the relative ROS content in cells pre-cultured in glucose deprivation was significantly decreased in comparison to non-exposed cells (Fig 26). Our findings suggest that glucose deprivation can activate mitochondrial perturbations that converge on similar downstream processes to enhance mitochondrial function and extend survival by controlling ROS production. Moreover, our data suggests that a mild production of mitochondrial superoxide acutely induced this response, though further investigation is necessary. It has been hypothesized in glucose-restricted *Saccharomyces cerevisiae* that ROS may induce antioxidant enzymes increased expression, ultimately leading to augmented defense capacity against ROS (Kharade et al., 2005). However, in eukaryotes, the existence of this process in a similar manner has not been fully elucidated (Calabrese et al., 2007;

Johnson et al., 2002). Also, mitohormesis has been suggested to play a significant role in mediating exercise-induced adaptations (Merry & Ristow, 2016). It seems that exercise-induced oxidative stress leads to cellular resistance against successive further perturbations, improving overall health and preventing chronic diseases (Yun & Finkel, 2014). The energetic demands of exercise closely resembled the glucose deprivation ones, thereby eliciting the same improvements in overall mitochondrial quantity and quality through mitochondrial biogenesis, dynamics (fusion and fission) and mitophagy (Drake, Wilson, & Yan, 2016). Thus, we proposed that a similar mitochondrial upgrade explains the enhanced resistance against hyperglycemic detrimental effects. As referred, glucose deprived myoblast showed slight activation of mitophagy and mitochondrial biogenesis, which theoretically results in the maintenance of a less vulnerable mitochondrial population and possibly in an increase in mitochondrial content. Additionally, mitochondrial morphology and internal arrangement of cristae number and shape, as well OXPHOS complexes content, defines respiratory capacity and cell viability (Cogliati, Enriquez, & Scorrano, 2016). Recently, mitochondrial dysfunction during aging was described in sedentary humans to be associated with OPA1 decline, and exercise was found to prevent this decline (Tezze et al., 2017). OPA1 function is beyond mitochondrial fusion in cooperation with Mfn 1 and 2, being its oligomerization capable of controlling cristae remodeling and mitochondrial bioenergetics (Frezza et al., 2006). With these evidences, we propose that increase in OPA1 complexes or other molecules that regulate oxidative efficiency are also involved in mitochondrial adaptation to secondary stresses after a mild oxidative stress. In addition, our data suggests no changes in mitophagy from pretreated cells after 24 h in hyperglycemia (Fig 27), which might happen due to the ablation of hyperglycemia-associated oxidative stress induction of mitophagy. This result in conjunction with no changes in total cellular ROS production might suggest that glucose deprivation increases mitochondrial resistance to hyperglycemic-induced mitochondrial damage, consequently leading to a minor reliance in mitophagy to cellular survival.

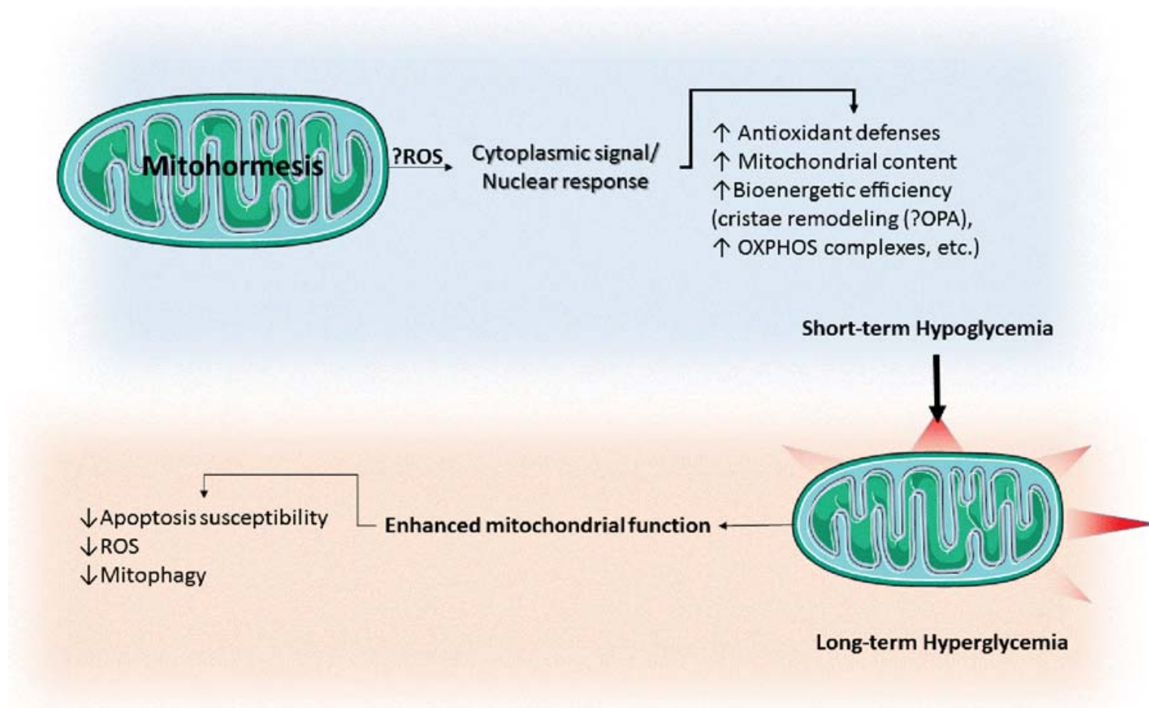


Figure 33. ***Illustration of possible mitohormesis promotion of mitochondrial function from hyperglycemic.***
Short-term hypoglycemia has been suggested as a mediator of mitohormesis through induction of a mild ROS generation with beneficial cytosolic and nuclear repercussions. Increased antioxidant defense, mitochondrial content, bioenergetic efficiency are some of the possible changes that might promote mitochondrial response to subsequent stresses. This, in turn, can influence apoptotic susceptibility, ROS formation and mitophagy induction from stressful conditions like hyperglycemia (image created with **Servier Medical Art templates**).

Overall, our study establishes a model that could support and allow for further investigation of mitohormesis: exposure to glucose deprivation promotes greater stress resistance against hyperglycemia, which was verify by prevention of hyperglycemic-induced mitophagy and ROS production. Considering the reported Sesn2 stimulation, and its capacity to ROS detoxification as well as other metabolism-controlling functions, we raise its potential role as regulator of mitohormesis. However, we disclose a strong limitation in terms of conclusion, since it was not shown the effects of Sesn2 default in hypoglycemia and pretreated myoblasts during hyperglycemia. However, the optimization of a transient silencing of this protein in the C2C12 undifferentiated cells during this study was achieved (Fig 33). In summary, this model has the potential to elucidate the effects of transient ROS on cell function and adaptation, which can bring valuable insights into the mechanism that improve cellular resistance to stress. In future,

this knowledge can potentiate therapies against premature aging and chronic metabolic disorders.

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