Nrf2 as a regulator of mitochondrial function: energy metabolism and beyond

Noemí Esteras[1] & Andrey Y. Abramov[1]

[1]Department of Clinical and Movement Neurosciences. UCL Queen Square Institute of Neurology. Queen Square, London UK.

Co-corresponding authors: Dr Noemí Esteras n.gallego@ucl.ac.uk & Prof Andrey Y. Abramov a.abramov@ucl.ac.uk

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Abstract

Mitochondria are unique and essential organelles that mediate many vital cellular processes including energy metabolism and cell death. The transcription factor Nrf2 (NF-E2 p45-related factor 2) has emerged in the last few years as an important modulator of multiple aspects of mitochondrial function. Well-known for controlling cellular redox homeostasis, the cytoprotective effects of Nrf2 extend beyond its ability to regulate a diverse network of antioxidant and detoxification enzymes. Here, we review the role of Nrf2 in the regulation of mitochondrial function and structure. We focus on Nrf2 involvement in promoting mitochondrial quality control and regulation of basic aspects of mitochondrial function, including energy production, reactive oxygen species generation, calcium signalling, and cell death induction. Given the importance of mitochondria in the development of multiple diseases, these findings reinforce the pharmacological activation of Nrf2 as an attractive strategy to counteract mitochondrial dysfunction.

Abbreviations

$\Delta\psi_m$ – Mitochondrial Membrane Potential

ARE – Antioxidant Response Element

CREB - cAMP response element–binding protein

DMF – Dimethyl fumarate

ER – Endoplasmic reticulum

ETC – Electron transport chain

FTD – Frontotemporal dementia

GSK3β - Glycogen synthase kinase-3 β

IMM – Inner mitochondrial membrane

IMS – Intermembrane Space

Keap1 - Kelch-like ECH-associated protein 1

MCU – Mitochondrial Calcium Uniporter

Mfn1 & Mfn2 – Mitofusin 1 & Mitofusin 2

mPTP – mitochondrial Permeability Transition Pore

mROS – Mitochondrial Reactive Oxygen Species

mtDNA – Mitochondrial DNA

nDNA – Nuclear DNA

NRF-1 – Nuclear Respiratory Factor 1

NRF-2 – Nuclear Respiratory Factor 2

Nrf2 - Nuclear factor erythroid-derived 2 (NF-E2)-Related factor 2

OMM – Outer mitochondrial membrane

OxPhos – Oxidative Phosphorylation

PC – Pyruvate carboxylase

PDH – Pyruvate Dehydrogenase

PGC-1α - Peroxisome proliferator-activated receptor (PPAR) Gamma Coactivator 1-alpha)

PINK1 - PTEN-induced putative kinase 1

PPARs - Peroxisome Proliferator-Activated Receptors

ROS – Reactive Oxygen Species

SOD – Superoxide Dismutase

TCA – Tricarboxylic Acid

TFAM – Mitochondrial Transcription Factor A

TIM – Translocase of the Inner Membrane

TOM – Translocase of the Outer Membrane

TRX2 – Thioredoxin-2

UCPs – Uncoupling proteins

VDAC – Voltage-dependent anion channel

VGCCs – Voltage-dependent calcium channels

Introduction

Mitochondrial organization and structure: importance for energy production through oxidative phosphorylation

Mitochondria are essential organelles for multiple aspects of cellular function, the most-well known energy production. Their structure consists of a matrix surrounded by two major membranes, the inner (IMM) and outer (OMM) mitochondrial membranes, with an intermembrane space (IMS) between them. This structure determines the organization of the mitochondria in different compartments, and most of its cellular functions depend on their unique characteristics and protein composition. Both membranes are very different in terms of permeability. The outer OMM, in contact with the cytosol, is a highly permeable membrane that allows the free diffusion of ions and small molecules through porins as the voltage-dependent anion channels (VDACs). Bigger molecules and proteins are transported through translocases such as the TOM (Translocase of the Outer Mitochondrial Membrane) complex. In contrast, the IMM is a highly impermeable membrane with a very large protein content, which includes multiple specific carrier proteins that mediate the transport of ions and molecules. The IMM is invaginated, and the internal compartments defined by the invaginations are called cristae, which are separated to the rest of the IMM by cristae junctions (Figure 1). Cristae extend deeply into the matrix and increase largely the surface of the IMM compared to the OMM, and further subdivide the IMM into the inner boundary membrane (IBM) and the cristae membrane (CM). The cristae membrane is essential for the bioenergetic function of the mitochondria, as it contains the protein complexes that make up the electron transport chain (ETC), and the mitochondrial ATP synthase, whose coupled function is essential for ATP generation through oxidative phosphorylation (OxPhos), the most efficient cellular pathway for ATP synthesis. Due to its importance, cristae take up most of the mitochondrial volume in tissues with high energy demand such as the cardiac or skeletal muscle, while in tissues with lower energy requirements such as the liver, the mitochondrial matrix, where most of the biosynthetic enzymes are located, is more expanded [1]. The regulation of the structure of the different individual respiratory complexes ~~and~~, their assembly into supercomplexes in the cristae membrane and its role in mitochondrial function has gained a lot of attention in the recent years thanks to the application of cryo-electron microscopy, for a recent detailed review see [2].

Generation of ATP by OxPhos in the mitochondria is achieved by the driving force generated by mitochondrial respiration, a sequential chain of redox reactions through the ETC (Figure 1). Briefly, the ETC is formed by four protein complexes (I-IV) in the cristae membrane and two mobile electron carriers: the membrane-embedded ubiquinone (or coenzyme Q) and the soluble cytochrome c. During mitochondrial respiration, the ETC transfers electrons in a series of redox reactions from the reduced equivalents NADH and in less extent $FADH_2$ originated in different metabolic pathways (such as Krebs cycle), to its final acceptor O_2 to generate H_2O. Complex I or NADH:ubiquinone oxidoreductase is the largest complex in the ETC and transfers electrons from matrix NADH to ubiquinone (CoQ), leading to its reduction to ubiquinol. Complex II or succinate dehydrogenase links ETC with the Krebs cycle, as it is also a component of it, and also transfers electrons to reduce CoQ to ubiquinol, through $FADH_2$. Complex III or cytochrome c reductase, accepts the electrons from Complex I and II carried by ubiquinol (which is re-oxised to ubiquinone) and in turn reduces cytochrome c, which shuttles to complex IV (cytochrome c oxidase) that finally donates 4 electrons to its final acceptor O_2 to generate H_2O (Figure 1). The transport of electrons through complexes I, III and IV is coupled with the translocation of protons (H^+) from the mitochondrial matrix to the IMS, creating a proton gradient with a chemical (ΔpH) and electrical component (membrane potential, $\Delta\psi m$, which is the major contributor) across the IMM. The protonmotive force of this electrochemical proton gradient is used by the F_1F_o-ATP synthase or complex V, which acts as a rotational motor system that allows the controlled re-entry of protons into the mitochondrial matrix. As H^+ flow down the gradient from the IMS to the matrix, the rotation catalyses the phosphorylation of ADP to generate ATP. Importantly, the F_1F_o-ATP synthase can also work in reverse, hydrolysing ATP as an ATPase and using the released energy to pump protons to the IMS to sustain the $\Delta\psi$ [3], as in addition to ATP generation, $\Delta\psi m$ drives other essential mitochondrial functions such as calcium uptake [4]. Mitochondrial respiration and phosphorylation of ADP by complex V are coupled mechanisms, and proton leak across the membrane (rather than through complex V) lowers the coupling efficiency, reducing ATP generation. Mitochondrial uncoupling can occur through different mechanisms with physiological and pathological roles [5]. The uncoupling proteins (UCPs) family are mitochondrial anion carrier proteins that mediate most of the induced proton leak into the matrix. Among them, UCP1 plays an essential role in non-shivering thermogenesis, by uncoupling respiration from ATP synthesis in brown adipose tissue mitochondria, inducing energy dissipation in the form of heat.

Mitochondrial ROS

Mitochondria represents the main source of reactive oxygen species (ROS) production in the cell. This is greatly due to the large presence of O_2 in this organelle and its interaction with electrons that prematurely leak out of the ETC, which is the major contributor (although not the only one) to mitochondrial ROS (mROS) production. Complex I (principally) and III are shown to be the major molecular sites of mROS production in the ETC, predominantly in the primary form of superoxide anion (O_2^{*-}) but also through hydrogen peroxide (H_2O_2), and depending on the site of production, mROS are generated to the matrix and/or the IMM [6] (Figure 1). Interconversion of the different ROS species might also lead to the production of the highly reactive hydroxyl radical (OH*) in the Fenton reaction, one of the major contributors to the oxidation of lipid membranes, leading to cellular damage.

Production of mROS is influenced by the $\Delta\psi_m$ [7] and the activity of the ETC, although with different consequences depending on the specific context and site of action. For example, an increase in mitochondrial respiration can increase mROS production if it is driven by an augmented substrate supply (the ETC becomes more reduced) or decrease mROS if it is driven by proton leak or higher ATP demand (the ETC becomes more oxidised) [6]. Similarly, both an increase [3] or a decrease [8] in $\Delta\psi$m when mitochondrial respiration is inhibited might lead to higher mROS production.

Apart from the ETC, other sources of mROS comprise different mitochondrial enzymes, including matrix enzymes from the Krebs cycle such as the α-ketoglutarate dehydrogenase [9] or the OMM flavoenzyme monoamine oxidase (MAO), which catalyses the oxidative deamination of monoamines with the concomitant production of H_2O_2.

mROS are not merely by-products of mitochondrial activity, but also exert key signalling and physiological roles within cells [10]. This is particularly important in the brain, where, as an example, mROS serve as oxygen sensors in astrocytes [11] and modulate glutamatergic signalling in neurons [12]. However, excessive mROS production leads to the oxidation of macromolecules such as DNA, lipids and proteins, causing cellular damage which has deleterious consequences for cells [13]. To counteract excessive mROS production, mitochondria employ different endogenous antioxidant mechanisms. Highly instable O_2^{*-} is rapidly converted to the more stable and membrane-permeable H_2O_2 spontaneously or enzymatically by two different superoxide dismutase

(SOD) systems: Mn-SOD in the matrix and Cu,Zn-SOD in the IMS. In addition, mitochondria also count with the NADPH-dependent glutathione (GSH) and thioredoxin-2 (TRX2) antioxidant systems, and catalase in specific tissues, to clear excess of H_2O_2 (Figure 1).. The disturbance in the balance between mROS production and antioxidant defence either by an excessive mROS production or a depletion of the antioxidant defences, leads to oxidative stress, which is involved in the pathogenesis of various chronic conditions, including neurodegenerative, metabolic or cardiovascular ~~disease~~diseases. Modulation of excessive mROS has been shown to be protective in different neurodegenerative (and other) disorders, pointing at a potential pharmacological target for disease-modifying therapies [3, 12, 14].

Mitochondrial metabolic pathways

Mitochondria is a central metabolic hub for energy production that integrates many metabolic pathways derived from the oxidation of the major nutrients (carbohydrates, lipids and proteins). Although a small fraction of the total cellular ATP generated occurs in the cytosol, mitochondria accounts for the majority of the ATP produced, due to the high efficiency of OxPhos. In addition, the mitochondrial matrix hosts key metabolic reaction pathways such as the tricarboxylic acid (TCA) cycle, the beta-oxidation of fatty acids or several essential amino-acid catabolic pathways. The TCA or Krebs cycle, named after its discoverer Sir Hans Kreb, consists of a series of enzymatic reactions leading to the generation of different intermediate metabolites that are transported to the cytosol for biosynthetic and signalling purposes [15], and the provision of the reduced equivalents NADH and $FADH_2$, that will later serve as substrates for the mitochondrial ETC eventually leading to ATP production, as explained before (Figure 1). Different substrates with varied origins are able to feed the TCA cycle to maintain it running. The cycle begins with the condensation of oxaloacetate (OAA) with acetyl-CoA to generate citrate, followed by 7 more enzymatic reactions that will ultimately regenerate OAA. Acetyl-CoA might derive from the catabolism of glucose, fatty acids and amino-acids, so serves as an important metabolic regulator. Regarding glucose, the first steps of the glucose metabolism occur in the cytosol, leading to the generation of its end-product pyruvate, which is a key metabolic molecule for the brain and other tissues [16], and has additional sources such as amino-acid metabolism or lactate conversion through lactate dehydrogenase. In addition to pyruvate, glycolysis generates 2 ATP and 2 NADH per each glucose molecule. For complete glucose aerobic oxidation to maximize the ATP yield

(up to 25 more ATP molecules per starting glucose molecule), pyruvate is then imported into the mitochondria, through VDAC in the OMM and by the mitochondrial pyruvate carrier (MPC) in the IMM. Once in the mitochondria, the pyruvate dehydrogenase complex (PDH) leads to the decarboxylation of pyruvate to Acetyl-CoA and its further oxidation in the TCA cycle. Alternatively, pyruvate might be carboxylated by pyruvate carboxylase (PC) to replenish the TCA cycle with oxaloacetate (Fig. 1). Amino-acid catabolism occurs on the other hand through different transamination/deamination reactions leading to the removal of the amino group and the conversion of different amino-acids to their respective carbon skeletons (alpha-ketoacids), leading to the production of intermediates of the TCA cycle, pyruvate or acetyl-CoA. As an example, in glutaminolysis, mitochondrial glutaminase (GLS) converts glutamine into glutamate, and glutamate dehydrogenase (GDH) catalyses the conversion of glutamate into α-ketoglutarate, that is incorporated into the TCA to replenish TCA metabolites, representing a critical metabolic pathway in cancer cells [17] (Figure 1). Finally, mitochondrial beta oxidation of fatty acids consists of four cyclic steps occurring in the mitochondrial matrix which lead to the sequential break-down of a long-chain acyl-CoA molecule into several acetyl-CoA. Each cycle of beta-oxidation leads to the production of 1 acetyl-CoA, NADH and $FADH_2$, which will be used in the TCA and ETC. To be incorporated into the mitochondria, depending on their chain length, fatty acids are first activated by conjugation with coenzyme A (CoA) by acyl-CoA synthetases (some of which are located in the OMM). Mitochondrial CPT1 (carnitine:palmitoyltransferase I) in the OMM, CPT2 (carnitine:palmitoyltransferase II) in the IMM and CAT (carnitine:acylcarnitine translocase) coordinate their transport into the matrix in exchange for carnitine (Figure 1).

Importantly, different cell types have preferred substrate sources for ATP generation, i.e. brain relies mostly on glucose oxidation while cardiac muscle cells in the heart obtain most of their energy from fatty acids oxidation [18].

Mitochondrial Calcium Modulation

Highly linked to its bioenergetics function, mitochondria play an essential role in the buffering and shaping of the cytosolic calcium transients occurring in the cytosol in response to different stimulus such as neuronal transmission or cardiac contraction. Mitochondria use their $\Delta\Psi m$ to uptake calcium into their matrix through the high capacity, low affinity mitochondrial calcium uniporter (MCU) located in the IMM. Mitochondrial calcium content needs to be tightly regulated,

so calcium influx is followed by calcium efflux, that in excitable cells such as neurons or cardiomyocytes, occurs in exchange of Na^+ through the Na^+/Ca^{2+} exchanger NCLX [19] (Figure 1). Importantly, several matrix dehydrogenases and metabolite carriers in the mitochondria are activated by calcium, which enhances mitochondrial respiration and ATP production [20], suggesting a physiological role for mitochondrial calcium in the adaptation of the cells (in particular those with high calcium activity such as skeletal muscle, cardiac cells or neurons) to their high energy requirements. However, excessive calcium accumulation, specially under conditions of oxidative stress, triggers the opening of the mitochondrial permeability transition pore (mPTP), a high conductance mitochondrial channel whose composition and structure are a major area of debate. Opening of the mPTP leads to an unselective permeability of the IMM to small solutes leading to the collapse of the $\Delta\Psi m$, mitochondrial swelling, and necrotic and apoptotic cell death due to the release of cytochrome c to the cytosol (Figure 1). ~~Due to~~As a result of its broad effects in cellular function, mitochondrial calcium homeostasis impairment is involved in many neurodegenerative and other disorders [4].

Mitochondria homeostasis

Mitochondria organize within the cell through a highly dynamic and complex tubular network regulated by different highly interconnected processes: they are able to divide and fuse through fission and fusion, they are transported within the cell to deliver their functions, new mitochondria are generated from other in the process of biogenesis, and damaged mitochondria are degraded in the mitochondria-specific autophagic pathway of mitophagy (Figure 2).

- ### Mitochondria biogenesis

Mitochondria are not generated *de novo,* but through the replication and growth of pre-existing mitochondria. They are unique organelles thought to originate from an endosymbiotic bacteria ancestor that was eventually integrated and established in a host cell. As a result, current mitochondria still contain their own DNA (mtDNA) located in the mitochondrial matrix, with bacteria-like features such as a circular structure. Mitochondrial genome consists of 37 genes, of which 13 encode core subunits of the ETC respiratory complexes I, III and IV, and ATP synthase; and the rest tRNAs and rRNAs required for their translation within the mitochondria- [21]. The remaining mitochondrial proteins – the vast majority with ~1500 in humans - are encoded by nuclear DNA (nDNA), therefore it is necessary a synchronized coordination between both

mechanisms for correct mitochondrial replication. In addition, mitochondrial proteins encoded by the nuclear genome are synthesized as precursors in the cytosol and need to be targeted, translocated to the mitochondria, and assembled to be able to deliver their functions. Translocases in the outer (TOM) and inner (TIM) membrane, the import motor PAM (presequence translocase-associated motor), or the ATP-driven chaperone mitochondrial heat-shock protein 70 (mtHsp70) among others are key components of this machinery [22].

Mitochondrial biogenesis is induced by different stimulus such as high energy demand, exercise, caloric restriction, low temperature, inflammation or oxidative stress. It requires the coordinated expression of gene products from both nuclear and mitochondrial DNA, in a process modulated by different transcription factors. Importantly, the transcription and translation of the mtDNA relies on factors encoded in the nucleus. Key effectors in this process include the nuclear respiration factors NRF-1 and NRF-2 (not to be mistaken with Nrf2) which provide an important functional link between both organelles (Figure 2A). NRF-1 and NRF-2 activate the transcription of nuclear genes encoding mitochondrial proteins essential for respiration and import, such as subunits of the ETC or TOMM20, as well as the expression of transcription factors essential for mtDNA processing, such as Tfam (mitochondrial transcription factor A) or TFB1M and TFB2M (mitochondrial transcription specificity factors) [23, 24]. Other nuclear transcription factors also play a role in mitochondrial biogenesis, such as the estrogen-related receptors (ERRs) and peroxisome proliferator-activated receptors (PPARs), which regulate genes involved in mitochondrial fatty acid oxidation essential for heart and muscle, for an extended review see Scarpulla *et al*. [25]. Upstream, different transcriptional coactivators play an essential role in controlling gene expression without binding DNA, but by orchestrating the function of DNA-binding transcription factors. The transcriptional coactivator PGC-1α (Peroxisome proliferator-activated receptor (PPAR) γ coactivator 1-alpha) is considered the master regulator of mitochondrial biogenesis and cellular energy metabolism, as it activates not only key transcription factors for mitochondria such as PPARs, ERRs, NRF-1 and NRF-2; but also orchestrates a wider variety of genes involved in metabolism [26]. PGC-1α is a coactivator of most nuclear receptors and other transcription factors and is highly expressed in tissues with high metabolic capacity, such as the skeletal muscle, heart, brain, liver, or brown fat. Importantly, PGC-1α is highly inducible and its levels can be upregulated under various stimulus including cold exposure, exercise, or caloric restriction, thus integrating physiological signalling into molecular pathways

targeting mitochondria and metabolism. Regulation of PGC-1α occurs at the mRNA level and by protein post-translational modifications in response to a variety of cellular pathways. For example, AMPK (AMP-activated protein kinase) and SIRT1 (sirtuin 1) act as metabolic sensors and activate PGC-1α via phosphorylation and deacetylation respectively, upon energy deprivation [27, 28]. In particular, SIRT1 is a NAD^+ -dependent deacetylase often described as a NAD^+ sensor, while AMPK act as a sensor of low ATP levels that modify the ATP:AMP ratio. Besides, AMPK and SIRT1 are interconnected and can regulate each other [29]. Thus, metabolic changes can be sensed and integrated by these pathways leading to the activation of the master regulator of mitochondrial biogenesis and metabolism PGC-1α (Figure 2A).

Regulation of PGC-1α also occurs at the level of transcription. PGC-1α promoter contains binding sites for transcription factors such as myocyte enhancer factor 2 (MEF2), forkhead box class-O (FoxO1), activating transcription factor 2 (ATF2), and cAMP response element–binding protein (CREB), where many pathways converge [30]. Transcriptional regulation of PGC-1α through these mechanisms responds to a variety of stimuli that also depend on the tissue. Just to give some examples of the myriad of regulatory pathways, PGC-1α was originally discovered as a cold-inducible coactivator of the nuclear receptor PPAR γ in brown adipose tissue and skeletal muscle from mice [31]. Puigserver *et al* showed that PGC-1α activates the transcription of the mitochondrial uncoupling protein 1 (UCP-1), pointing at its role in uncoupling mitochondrial respiration and activating thermogenesis. β-adrenergic/cAMP pathway appeared to be upstream of the PGC-1α-mediated regulation of thermogenesis. In the liver, fasting-induced glucagon and glucocorticoids activate PGC-1α through cAMP/CREB. CREB-dependent activation of PGC-1α leads to the expression of genes involved in hepatic gluconeogenesis, such as phosphoenolpyruvate carboxykinase (PEPCK), essential to maintain glucose homeostasis under fasting conditions [32]. In the skeletal muscle, the role of the calcium-dependent pathways calcineurin-A and calmodulin kinase (CaMK) stand out in the regulation of mitochondrial biogenesis through PGC-1α expression [33]. Calcium-mediated stimulation of MEF2/HDAC pathways were also shown to be key regulators of PGC-1α in promoting mitochondrial biogenesis in cardiomyocytes [34]. In the brain, the brain-derived neurotrophic factor (BDNF) was shown to stimulate PGC-1α through ERKs and CREB, promoting mitochondrial biogenesis and dendrite spines formation in the hippocampus [35].

Given the role of mitochondrial biogenesis in many essential cellular pathways and tissues, its defects have been implicated in a number of pathologies, from mitochondrial and metabolic disorders to pathologies in tissues with a high energy demand such as the heart, the skeletal muscle or the brain. For this reason, activation of mitochondrial biogenesis is currently explored as a potential therapeutic target [36].

- **Mitochondrial turnover**

Besides mitochondrial fission and fusion, and in a process opposed to biogenesis, clearance of damaged mitochondria is also an essential mechanism to control mitochondrial quantity and quality and maintain a healthy cellular pool. The mechanism of mitophagy consists of a selective form of autophagy in which damaged mitochondria are targeted, recruited into autophagosomes and degraded in the lysosomes. Mitophagy plays an essential role in physiological pathways, such as the critical removal of mitochondria during erythroid maturation [37], or the selective degradation of paternal mitochondria during embryogenesis [38]; and is triggered by different cellular stressors to maintain mitochondria quality control. Thus, hypoxia, mtDNA damage, oxidative stress and mitochondrial depolarization are able to activate mitophagy through different molecular pathways, for a recent review see [39].

One of the most studied mitophagy routes is the PINK1/Parkin-mediated pathway (Figure 2B). The mitochondrial kinase PINK1 (PTEN-induced putative kinase 1) is under basal conditions imported into the mitochondria and degraded in the matrix. However, depolarization of the mitochondria under stress conditions prevents PINK1 import, leading to its selective accumulation in the surface of the OMM. This leads to the recruitment of the E3 ubiquitin ligase Parkin to the OMM starting a positive amplification loop, where Parkin ubiquitinates and targets for degradation different OMM proteins. Phospho-ubiquitin in the mitochondrial surface is then recognized by the autophagy regulator p62, triggering their LC3-mediated engulfment in the autophagosome and posterior degradation in the lysosome [40]. Defects in this pathway are closely related to neurodegeneration, since loss-of-function mutations in PINK1 and Parkin genes are the most common causes of autosomal recessive and early onset Parkinson's disease. However, at present, observation of PINK1/Parkin-mediated mitophagy *in vivo* in cultured neurons and mouse brain is still very limited compared to non-neuronal models, suggesting alternative mitophagy pathways occurring in the brain [41] and the potential relevance of additional roles for PINK1/Parkin besides

mitophagy. In this regard, PINK1 itself has been shown to modulate mitochondrial complex I activity, among other mitophagy-independent mechanisms [42]. Likewise, mitophagy also takes place through PINK1/Parkin-independent mechanisms which are thought to contribute largely to basal mitophagy necessary for mitochondrial housekeeping observed in energy-demanding tissues such as the brain [43]. Several proteins in the OMM act as mitophagy receptors, able to interact with and recruit key components of the autophagosome membrane such as LC3 and trigger mitophagy independently of ubiquitin. These include among others BNIP3 and its homolog NIX, well-known for its role in programmed mitophagy during erythrocyte differentiation [37] and hypoxia, or the IMM phospholipid cardiolipin, whose externalization to the OMM upon mitochondrial damage triggers the recruitment of mitochondria into autophagosomes [44] (Figure 2).

Due to its essential roles in mitochondrial quality control, a fine regulation of mitophagy is essential, with either excessive or inefficient mechanisms leading to cellular death. Impairment of mitophagy has been involved in the pathogenesis of many disorders, fundamentally, but not restricted~~,~~ to, those affecting tissues with high energy demand such as neurons and cardiac cells [45]. Targeting of mitophagy is currently being explored as a disease-modifying ~~therapies~~therapy for some of these disorders, with a variety of pharmacological tools being tested at the moment [46].

- **Mitochondrial dynamics**

Mitochondrial dynamics comprise the coordinated opposite processes of fission and fusion constantly taking place in the mitochondrial network. Fusion and fission lead to a continuous ~~remodelation~~remodelling of the mitochondrial organisation reflected by changes in their shape, length and morphology. Mitochondrial dynamics are essential for their function and adaptation to the cellular needs and also serve as a quality control mechanism for preserving mitochondria under different circumstances: fusion allows the exchange of metabolites and components between healthy and unhealthy mitochondria to dilute the effects of stress; while fission induces the segregation of damaged mitochondria for later degradation [47]. Both processes are mediated by the action of a number of dynamin-related GTPases and their regulators, necessary to maintain an adequate balance between them.

Mitochondrial fusion involves the sequential fusion of both the OMMs and the IMMs of different mitochondria. Fusion of the OMM occurs through the GTPases mitofusins 1 and 2 (Mfn1 and Mfn2) present in the OMM. Mitofusins assemble as homo/hetero-dimers to tether adjacent mitochondria and induce the fusion process [48]. Subsequent fusion of the IMM is mediated by the IMM GTPase OPA1 (optic atrophy protein 1), with a vital role of the IMM phospholipid cardiolipin in the process [49] (Figure 2C).

Fission is primarily mediated by Drp1, a cytosolic dynamin-related GTPase recruited to the OMM by different mitochondrial receptor proteins: mitochondria fission factor (Mff), mitochondrial dynamics protein of 49 kDa and 51 kDa (MiD49 and MiD51), and fission 1 (Fis1) [50]. Importantly, mitochondrial excision occurs at marked sites of mitochondria-ER contacts. Pre-constriction of the mitochondria at those sites appears to be necessary prior to Drp1 recruitment to facilitate its function, and the endoplasmic reticulum plays an essential role in the process [51] (Figure 2C).

An adequate equilibrium between fission and fusion processes is essential for mitochondrial function. Imbalances in mitochondrial dynamics have been correlated with the pathogenesis of different diseases, and mutations in genes encoding components of the fission/fusion machinery have been identified in a subset of neurological disorders [52].

- **Mitochondrial motility**

Distribution of mitochondria within the cell is essential for the correct development of their functions, and they can be transported long or short distances and anchored to specific sites to respond to specific cellular demands, which also depend on the cell type. We will extend this information for neurons, where mitochondrial motility plays an essential role given their specific metabolic needs and unique structure.

Neurons – mitochondrial motility and the essential role of mitochondria in synaptic transmission

Mitochondrial motility is critical in highly specialized, polarized and potentially long cells such as neurons, in which high energy-consuming processes occur, specially at the synapses, the junctions between neurons where they communicate to each other using chemical and electrical signals. Neurons are in general structured in three differentiated areas: the soma or cell body and two

different types of processes extending out of it: the dendrites and the axon, which most commonly play specific and differentiated roles in synaptic transmission, with axons typically acting as the pre-synaptic site and dendrites as the post-synaptic, although this is not exclusive [53], with many neuronal types behaving in diverse ways [54].

The axon is structurally a long projection (up to a meter in some motor neurons) commonly branched at their terminal end. Axons typically fire and transmit action potentials, and axon terminals classically act as pre-synaptic sites, containing clusters of neurotransmitters-filled synaptic vesicles. The firing of an action potential induces a calcium influx through voltage-dependent channels (VGCCs) that triggers the release of the neurotransmitters to the synaptic cleft which will bind to their specific receptors in the post-synaptic neuron. On the other hand, dendrites are generally thick, short and branched processes forming a large-surface arborized structure around the neuron which typically acts as the post-synaptic membrane, containing a number of neurotransmitter receptors which receive the afferent information from the pre-synaptic neuron and effect the synaptic transmission.

The different processes involved in synaptic transmission account for most of the ATP consumption in the brain [55]. These include the pumping of ions to restore the electrochemical gradients after the generation of action potentials and synaptic transmission, and vesicle release and recycling, which are frequently fuelled by different ATPases (plasma membrane Na^+/K^+ -ATPase and Ca^{2+}- ATPase, or vacuolar H^+-ATPase) [56]. ATP is mainly provided by the mitochondria, which also contributes to the synaptic homeostasis by acting as a highly localized calcium buffering system shaping local calcium signals in pre and post-synaptic events and modulating neurotransmitter release [19, 57]. Importantly, mitochondrial calcium homeostasis is highly linked to bioenergetics: increased mitochondrial calcium uptake enhances ATP production suggesting a role for mitochondrial calcium in the cellular adaptation to the energy demands. Indeed, motility and recruitment of mitochondria to specific regions of the neuron is modulated by calcium [58-60]. Due to its essential roles for cellular function, mitochondrial calcium impairment is involved in many neurodegenerative disorders [4].

In addition to its roles in calcium buffering and bioenergetics, mitochondria also participate in the modulation of synaptic transmission via reactive oxygen species (mROS) signalling [12, 61]. mROS are able to regulate glutamatergic excitatory neurotransmission in cortical neurons, playing

an important role both in physiology and pathology [12]. mROS modulate the trafficking of specific glutamate receptor subunits (GluA1 and NR2B) via redox regulation [12]. In frontotemporal dementia (FTD) neurons, where mROS production is enhanced as a result of impaired mitochondrial bioenergetics [3], this leads to an upregulation of the surface expression of these receptors, increased calcium influx and excitotoxicity (neuronal death induced by excessive glutamatergic activation) that could be prevented with mitochondrial antioxidants. These data points at the modulation of mitochondrial ROS as a strategy to prevent calcium-induced neuronal death and modulate glutamatergic neurotransmission [12]. mROS were also shown to modulate inhibitory synapses in the cerebellum. Accardi *et al* showed that reactive oxygen species produced in the mitochondria were able to regulate the strength of post-synaptic $GABA_A$ receptors at inhibitory synapses of stellar cells [61].

It is therefore clear that mitochondria ~~is~~are essential for different aspects of neuronal transmission. Given the distinct specific cellular roles occurring in the different neuronal areas, mitochondria need to be adequately distributed between the soma and the higher energy-demanding sites axon and dendrites. Indeed, the density of mitochondria differs between neurons and within areas or a neuron, with a higher percentage of them localizing in dendrites [62].

In mature neurons, around one third of mitochondria are mobile, while the rest is docked to a specific area, and both processes are essential for neuronal function [63]. Mitochondria are transported by opposing motor proteins over neuronal microtubule tracks in the direction determined by the microtubule polarity. In the uniformly polarized axons, kinesin (KIF5 family) mediates the anterograde transport (plus-end) of mitochondria to distal areas, while dynein is used for retrograde transport (minus-end) towards the soma [64]. KIF5 and dynein motors couple to mitochondria through motor adaptor proteins such as Trak1 and Trak2, and Miro1 and 2 proteins, calcium-sensitive GTPases in the mitochondrial outer membrane that serve as motility regulators [65, 66]. Ca^{2+} binding to Miro as a result of elevated cytosolic Ca^{2+} levels (such as during synaptic activity) ~~diminish~~diminishes mitochondrial motility, thus allowing mitochondria recruitment and immobilization to areas of high demand. Docking of mitochondria in axons is mediated by syntaphilin, which anchors mitochondria to microtubules playing an essential role in pre-synaptic function [63].

- **Interaction of the different pathways of mitochondrial homeostasis**

Importantly, the different mitochondrial functions and homeostatic mechanisms ~~and mitochondrial functions~~ are highly interconnected. Mitochondrial oxidative stress induces an imbalance in fission-fusion events leading to mitochondrial fragmentation [67]. Defects in fission-fusion might impair mitochondrial transport, since mitochondrial size influences its motility; while impaired mitochondrial transport also influences mitochondrial dynamics, as stationary mitochondria are less likely to fuse. PINK1/Parkin route, for example, in addition to mitophagy, influences mitochondrial motility, dynamics and biogenesis in an orchestrated pathway for mitochondrial quality control. Promotion of fission by the PINK-1 mediated recruitment of Drp1 to the mitochondria and block of fusion by PINK1/Parkin-mediated degradation of mitofusins, lead to the segregation of mitochondria destined for mitophagy from the mitochondrial network [68], while PINK1/Parkin-mediated degradation of Miro induces mitochondria arrest contributing to the isolation of the damaged mitochondria for selective and adequate clearance [69]. PINK1/Parkin activation also promotes mitochondrial biogenesis by phosphorylation and targeted-degradation of the PGC-1α repressor PARIS [70], therefore contributing to the restoration of the mitochondrial mass.

Nrf2 pathway and its relation to mitochondrial function

Impairments or disbalances in the multiple aspects of mitochondrial function or quality control are closely related to different disorders, specially, but not restricted to, those affecting tissues with high energy requirements such as the heart, the skeletal muscle or the brain. Multiple pathways have been shown to regulate these processes, and among them, the role of Nrf2 (nuclear factor erythroid-derived 2 (NF-E2)-related factor 2 - not to be mistaken with the nuclear respiratory factor 2 NRF-2) has gained a lot of attraction in the recent years. Nrf2 is a transcription factor with a well-known cytoprotective function by inducing the expression of multiple genes encoding detoxifying and antioxidant enzymes. As part of its cytoprotective activity, growing evidence has shown its role in modulating different aspects of mitochondrial function [71].

Nrf2 levels are tightly regulated and under basal conditions, it is sequestered in the cytoplasm and targeted for continuous proteasomal degradation by different ubiquitin-ligase systems (Figure 3). The most well-known is the Cullin 3 (Cul3) RING-box 1 (RBX1) E3 ubiquitin ligase complex, which needs the substrate adaptor protein Kelch-like ECH-associated protein 1 (Keap1) to ubiquitinate Nrf2. Keap1 is a cysteine-rich protein located in the cytoplasm which also serves as a

crucial sensor for oxidative stress [72]. Keap1's highly reactive Cys residues are modified by electrophilic or oxidative signals, thus preventing Nrf2 ubiquitination and degradation and allowing its translocation to the nucleus to exert its function as a transcription factor. Any other pathways that impair Nrf2-Keap1 interaction or promote Keap1 degradation are also able to activate Nrf2, such as the p62-dependent inactivation of Keap1 [73]. Nrf2 levels are also controlled by other ubiquitin-ligase systems independent of Keap1. Glycogen synthase kinase-3 (GSK3)-mediated phosphorylation of Nrf2 targets the protein for degradation by the proteasome through β-TrCP/Cul 1 [74], involving pathways that participate in GSK-3 regulation such as PI3K/Akt or Wnt signalling in the mechanism of Nrf2 activation (Figure ~~2~~3).

By either mechanism, disruption of Nrf2 continuous degradation allows the translocation of the transcription factor to the nucleus promoting the transcription of its target genes. Nrf2, prior heterodimerization with sMaf (small musculoaponeurotic fibrosarcoma) proteins, binds specific DNA sequences, known as ARE (antioxidant response element) present in the promoter of its target genes. These include genes involved in xenobiotics detoxification; in NADPH regeneration, ROS detoxification including the thioredoxin, sulfiredoxin and glutathione antioxidant systems, or proteins involved in mitochondrial and intermediary metabolism, among others [71].

Due to its cytoprotective role, Nrf2 activation could represent an important ~~pharmacological~~ therapeutic strategy in disorders where mitochondrial dysfunction and oxidative stress are hallmarks of the pathology [75, 76]. Nrf2 can be pharmacologically induced by different molecules, recently reviewed in [77]. The majority of the most studied pharmacological activators are electrophilic compounds that covalently modify the Cys residues in Keap1, inducing conformational changes that prevent Nrf2 ubiquitination and degradation. These include sulforaphane, TBE-31, dimethyl fumarate (DMF), omaveloxolone and a variety of natural compounds. In an effort to improve the selectivity of Nrf2 activators, non-electrophilic compounds that aim to reversibly inhibit the protein-protein interaction of Nrf2-Keap1 are being developed [77]. Importantly, a number of pharmacological activators of the pathway are in various stages of clinical development, with some of them currently being tested in a number of clinical trials and some already approved as disease- modifying therapies for different disorders [77]. These include the recently approved fumaric acid ester derivates: dimethyl fumarate (DMF) currently in use for the treatment of relapsing-remitting multiple sclerosis and psoriasis [78], and diroximel fumarate

and monomethyl fumarate also approved for multiple sclerosis [79, 80]. Ursodiol (ursodeoxycholic acid) is used for the treatment of primary biliary cirrhosis, and although its cytoprotective mechanisms are still under debate, upregulation of Nrf2 appears to play an important role [81].

Work in cellular and animal models has confirmed that the improvement of different aspects of mitochondrial function is an essential component of the benefits of Nrf2 activation for different disorders such as epilepsy [82], amyotrophic lateral sclerosis/frontotemporal dementia [83], Parkinson's disease [84] or Friedrich's ataxia [85].

Next, we will review the more specific roles of Nrf2 in regulating the different aspects of mitochondrial function.

Nrf2 in bioenergetics and metabolism

Multiple evidence points at the important role of Nrf2 in enhancing different pathways involved in energy production by upregulating mitochondrial bioenergetics and metabolism [71] (Figure 1). Many enzymes involved in those pathways have ARE sequences and have been shown to be transcriptionally activated by Nrf2. Chromatin immunoprecipitation-sequencing (ChIP-Seq) identified novel target genes of Nrf2 involved in mitochondrial metabolism in mouse embryonic fibroblasts from mice with constitutive accumulation or deficiency of Nrf2 [86] and in lymphoid cells treated with the Nrf2 activator sulforaphane [87]. Further studies of the Nrf2 transcriptome and proteome in combination with bioinformatic analyses showed the Nrf2-dependent upregulation of genes encoding different subunits of the respiratory complexes such as ubiquinone and complexes I-IV, the alpha and beta subunits of the ATP synthase, the mitochondrial pyruvate carrier, and various enzymes of the TCA cycle, glucose and fatty acid metabolism in lung cells in response to sulforaphane [88], in the skeletal muscle of inducible Nrf2 activation mouse models [89], in resting and stimulated Keap1-KD macrophages [90]; and/or in human iPSC-derived cardiomyocytes with silenced Keap1 [91]. Conversely, knock-out of Nrf2 downregulated different proteins associated with mitochondrial respiratory function, including a subunit of complex I encoded by *NDUFS6* gene and enzymes involved in the Krebs cycle, glucose and fatty acid metabolism, such as the isocitrate dehydrogenase, a regulatory component of the pyruvate dehydrogenase phosphatase or the mitochondrial long-chain specific acyl-CoA dehydrogenase [89, 90]. Additionally, Nrf2 might indirectly regulate these pathways by preventing the oxidative

thiol modifications that have been found to affect the activity of different enzymes involved in glucose metabolism, TCA cycle and ETC [75, 92].

Functionally, studies in isolated mitochondria, neurons and embryonic fibroblasts from transgenic mouse models showed that Nrf2 regulates mitochondrial respiration. Holmstrom *et al.* demonstrated that in cells from Nrf2-KO mice, respiration was impaired leading to a lower mitochondrial membrane potential and ATP production [93]. Impaired respiration was not caused by differences in the expression of the different respiratory complexes. Instead, the underlying cause was a lower substrate availability for the ETC complexes I and II, proved by the smaller NADH and $FADH_2$ pools induced by Nrf2 deficiency. The slower rates of regeneration of NADH and $FADH_2$ when respiration was inhibited, point at a less efficient activity of the TCA in the production of these substrates [84]. Importantly, the lower efficiency of OxPhos in Nrf2-KO neurons led to reduced ATP levels and their reliance on glycolysis for ATP production. Conversely, Nrf2 constitutive activation by Keap1-KD led to the opposite scenario. Genetic Nrf2 activation increased the rate of respiration and the availability of substrates, leading to an enhanced ATP production by OxPhos and a higher mitochondrial membrane potential in these cells [93]. Given the high energy requirements of neurons~~,~~ and their dependence on an adequate mitochondrial function to achieve it, as detailed in the previous section, it is expected that Nrf2-KO neurons might present an increased vulnerability and impaired function. Indeed, aged Nrf2-KO mice show cognitive deficits relative to WT animals, linked to impaired synaptic activity and mitochondrial bioenergetics [94, 95]. Likewise, a genetic background of Nrf2-deficiency aggravates the neuropathological effects of tau and amyloid pathology in the brain [96]. Conversely, pharmacological activation of Nrf2 has emerged as a very attractive strategy to counteract the mitochondrial bioenergetic deficits that are often a hallmark in many neurodegenerative disorders, as previously reviewed [75]. ~~Similarly~~In a similar way to Nrf2-deficiency, Parkinson's disease (PD) pathophysiology is associated with impaired mitochondrial respiration due to a lack of substrates for the ETC [97]. Nrf2 activation with sulforaphane or omaveloxolone (RTA-408) was able to revert the bioenergetic alterations in neurons of the PINK1-deficiency model of PD and prevent neuronal death, proving the role of Nrf2 activation in mitochondrial bioenergetics [84]. Likewise, the pharmacological activation of Nrf2 with sulforaphane, TBE-31 or RTA-408, increased the NADH pool available for mitochondrial respiration and restored mitochondrial membrane potential in fibroblasts from patients with p62-

deficiency associated to frontotemporal dementia and amyotrophic lateral sclerosis [83]. The neuroprotective activity of omaveloxolone was also demonstrated *in vivo* and *in vitro* in the prevention of the development of seizures [82]. Prolonged seizure-like activity leads to synchronous Ca^{2+} oscillations that are associated with mitochondrial depolarization, ATP and GSH depletion and neuronal damage [98], and ROS overproduction in NADPH oxidase and xanthine oxidase [99]. Provision of omaveloxolone after the start of status epilepticus led to an increase in GSH and ATP levels that prevented the associated neuronal damage and the spontaneous seizures that typically develop for several weeks after the induction of status epilepticus [82]. Importantly, omaveloxolone exerted part of its neuroprotective effects by enhancing the mitochondrial bioenergetic function and providing ATP, thus counteracting the increased energy demands imposed by the high-frequency calcium oscillations occurring during seizures. Taken together, this data show that the modulation of mitochondrial bioenergetics through pharmacological activation of Nrf2 has proven to be a promising target in neurodegenerative disorders, due to the high energetic demand of neurons and their reliance on mitochondria [75].

Data suggests Nrf2 increases the availability of mitochondrial substrates by enhancing the activity of the TCA cycle. In agreement with this, Singh *et al.* demonstrated that Nrf2 regulates the flow of carbon through the TCA cycle [100] (Figure 1). An increase in glucose utilization in the TCA cycle was shown in tumorigenic and not tumorigenic tissues from Keap1-KD mice, while Nrf2-KO tissues displayed a dramatic reduction of glucose utilized in the TCA cycle compared to WT [100]. Among its target genes, several enzymes of the TCA cycle and components of the mitochondrial pyruvate carrier are upregulated by Nrf2 [88, 89].

Upstream of the TCA cycle, Nrf2 also influences glucose metabolism. Higher glucose uptake upon Nrf2 activation has been shown in fibroblasts [101] and myocytes [102]. Upon cellular uptake, glucose might be used in two main pathways: glycolysis and pentose phosphate pathway, and Nrf2 has been shown to upregulate both. Nrf2 activation is known to increase NADPH production, a cofactor necessary for the function and regeneration of many antioxidant pathways modulated by Nrf2, such as the thioredoxin, peroxiredoxin or glutathione systems [103]. Some studies point at a larger utilization of glucose by the pentose phosphate pathway to generate NADPH upon Nrf2 activation [101]. Nrf2 also generates NADPH by inducing the expression of the cytosolic malic

enzyme 1 (ME1), which catalyses the reversible conversion of malate to pyruvate and links the Krebs cycle and the glycolytic pathway [87]. In addition to increasing the expression of many key glycolytic enzymes [104], Nrf2 has also been shown to functionally stimulate glycolysis in different cellular models [90, 105, 106]. Recently, a direct link coupling glucose metabolism and the Nrf2-Keap1 pathway was described through the discovery of the role of the endogenous glycolytic metabolite methylglyoxal in the modulation of Nrf2 activity through Keap1 [107].

There is a well-documented effect of Nrf2 in modulating the levels of different components of the fatty acid metabolism machinery [90], in which the Nrf2-dependent activation of PPAR plays an important role [108]. Fatty-acid oxidation is essential for energy production in different tissues such as cardiac cells, and Nrf2 has been shown to enhance its efficiency, contributing to an increased availability of substrates for mitochondrial respiration and ATP production [109] (Figure 1).

Finally, Nrf2 also plays a role in modulating amino-acid catabolism, which is particularly essential for cancer progression. Nrf2 overactivation in cancer cells leads to metabolic reprogramming and increased glutaminolysis [110].

Of note, duration and intensity of the Nrf2 activation influence the metabolic response of cells. Tsakiri *et al.* have shown that while mild or short-lived activation of Nrf2 increased the lifespan of *Drosophila,* overactivation of the pathway led to metabolic deregulation and aging acceleration [111]. Importantly, in many human cancers Nrf2 regulation is lost, and Nrf2 is persistently activated, which is associated with a poor prognosis. In this context, Nrf2 appears to play differential metabolic roles to sustain the rapid proliferation and therapeutic resistance of these cells. Together with other oncogenic pathways, aberrant Nrf2 activation in cancer cells promotes metabolic reprogramming leading to reduced mitochondrial respiration and increased glucose utilization for glutathione synthesis [112].

Nrf2 and mitochondrial ROS production

Nrf2 and mitochondrial ROS production are interconnected processes, in close relation with other mitochondrial functions modulated by Nrf2, such as mitochondrial bioenergetics. As such, mROS production is upregulated in the brain of Nrf2 deficient mice as a result of their impaired mitochondrial respiration [113]. Increased production of mROS in the absence of Nrf2 was closely

related with the bioenergetics impairments previously observed in these cells, where Nrf2 deficiency impaired mitochondrial respiration due to diminishment of substrates availability [93]. Treatment of Nrf2-KO neuronal-astrocytic co-cultures with pyruvate to feed the TCA cycle and increase the availability of NADH for respiration, significantly diminished mROS production, confirming that the limitation of respiration substrates leading to impaired activity of complex I are the underlying causes for the increased mROS production in Nrf2-KO brain cells [113]. Interestingly, constitutive Nrf2 activation in Keap1-KD mice brain also led to an increase in mROS production compared to WT cells, probably linked to the slight hyperpolarization of the mitochondria in these cells [113].

It is widely accepted that mitochondrial ROS are able to activate Nrf2, although it is still not well-known how Nrf2 senses mitochondrial redox homeostasis. Limited reports exist of a direct mechanism of mROS leading to Nrf2 activation [114, 115]. Given the short half-life of superoxide, the major specie of ROS produced in the mitochondria, this effect would likely be mediated by its rapid conversion to the membrane-permeable hydrogen peroxide, which would translocate to the cytosol and then activate Nrf2. Other reports show that it is the disruption of the mitochondrial GSH pool and the inhibition of its thioredoxin system which leads to Nrf2 activation and not the mitochondrial production of superoxide or hydrogen peroxide alone [116]. Interestingly, complex I activity has also been shown to induce Nrf2 activation through fumarate accumulation and independently of mROS [117]. In addition, Nrf2 is recruited to the mitochondrial OMM [118] through an interaction with PGAM5 protein, which tethers a ternary complex containing Nrf2 and Keap1 [119]. Disruption of this complex ~~increased~~was shown to increase Nrf2 activity, and it has been proposed that Nrf2 mitochondrial location to the OMM might serve as a sensor of mitochondrial function and ROS production to induce Nrf2 activation [119] (Figure 1).

Accordingly, Nrf2 activation in different tissues has been shown to be protective against oxidative-induced mitochondrial damage by different mechanisms [85, 114, 118]. Strom *et al* showed that cardiomyocytes overexpressing Nrf2 exposed to hydrogen peroxide were resistant to changes in mitochondrial morphology and function [118]. Likewise, Nrf2 activation was also required for Down Syndrome fibroblasts survival, which presented oxidative stress and mitochondrial dysfunction as a result of increased mitochondrial ROS production [114]. Omaveloxolone, a novel pharmacological Nrf2 activator, prevented neuronal death induced by hydrogen peroxide in

fibroblasts from patients of Friedrich Ataxia, a neurodegenerative condition characterized by atrophy in the cerebellum [85]. As part of the protective mechanism, Nrf2 activation improved different aspects of mitochondrial function, including counteracting the excessive endogenous mitochondrial ROS production and lipid peroxidation that these fibroblasts present.

Nrf2 in mitochondrial calcium

Mitochondrial stress-induced cellular death is frequently triggered by the opening of the mitochondrial permeability transition pore (mPTP), which occurs under an environment of mitochondrial calcium and ROS overload. Mitochondria isolated from hearts of Nrf2-deficient mice showed a higher sensitivity to mPTP opening after being exposed to calcium than WT ones [118]. In agreement, different studies have shown a protective effect of Nrf2 activation in preventing mPTP opening induced by different insults. In LPS-treated microglia, Nrf2 activation diminished mPTP induction and protected the cells from apoptosis [120]. Studies in isolated liver mitochondria of rats treated with sulforaphane, also showed that Nrf2 activation prevented oxidative stress-induced mPTP opening [121]. The effects appeared to be mediated by the Nrf2-dependent increase in mitochondrial antioxidant defence. Sulforaphane induced an increase in the levels of mitochondrial glutathione, glutathione peroxidase 1, thioredoxin 2, malic enzyme 3 as found in the mitochondrial fractions isolated from the animals [121]. Taken together, these results suggest that the Nrf2-induced prevention of mPTP opening might also offer a neuroprotective mechanism in neurons with inhibited mitochondrial calcium efflux leading to mitochondrial calcium overload, such as Parkinson's disease and tauopathies [4, 97, 122] (Figure 1).

A relationship between Nrf2 and mitochondrial calcium uptake through MCU and its regulatory proteins has been recently linked to cancer progression in various tumour types. Jin *et al* reported an elevated expression of MCUR1, one of the regulators of the mitochondrial calcium uniporter MCU, in metastatic hepatocellular carcinoma cells [123]. Increased mitochondrial calcium uptake through the uniporter elevated ROS production and activated Nrf2, whose nuclear translocation induced Notch signalling pathway leading to tumour progression. Inhibiting mitochondrial calcium uptake, mROS, Nrf2 or Notch prevented the metastatic phenotype of the cells [123]. Activation of Nrf2 mediated by an increase in mitochondrial calcium uptake in pancreatic ductal adenocarcinoma also promoted metastasis via cystine transporter SLC7A11 [124]. It has also been suggested that MCU could be regulated by Nrf2 in oral squamous cell carcinoma [125]. In these

tumour cells, which also present an increased expression of MCU and Nrf2, silencing of MCU didn´t affect Nrf2 levels. Instead, Nrf2 silencing led to reduced MCU expression and MCU-dependent expression of its regulatory proteins MICU1 and MICU2. This might suggest a role for Nrf2 in modulation of mitochondrial calcium signalling in cancer by mediating the transcriptional regulation of MCU, although more research would be necessary to prove this finding [125]. Interestingly, a recent proteomic study also pointed at the upregulation of *MCU* among a battery of genes induced by Nrf2 activation in skeletal muscle [89].

Nrf2 in mitochondrial biogenesis

One of the first roles of Nrf2 in modulating mitochondrial function came from the observation that Nrf2 induces mitochondrial biogenesis in different tissues. As part of its mechanism of action, Nrf2 is able to promote the expression of different genes implicated in mitochondrial biogenesis. Pantadosi *et al*. showed for the first time that Nrf2 mediated the role of heme-oxygenase-1 (HO-1) induced by low doses of carbon monoxide (CO), in promoting mitochondrial biogenesis in ~~cardiomyocites~~cardiomyocytes [126]. Authors demonstrated that Nrf2 was able to bind 4 ARE sequences present in the promoter of *NRF-1* transcription factor that mediates mitochondrial biogenesis (Figure 4). Mechanistically, CO induced the expression of heme-oxygenase 1 (HO-1) leading to Akt/PKB activation. Akt phosphorylation of GSK3β led to its inhibition, promoting Nrf2 translocation to the nucleus to bind its target sequences in the *NRF-1* promoter [126]. In addition to NRF-1, Nrf2 activation has also been linked to an upregulation of the mitochondrial transcription factor *TFAM* during mitochondrial biogenesis in the skeletal muscle induced by exercise [127].

Evidence has also shown the close relationship between Nrf2 and the main regulator of mitochondrial biogenesis, the transcriptional coactivator PGC-1α. ~~Similarly~~In a similar fashion to Nrf2, PGC-1α also coordinates the expression of a large proportion of molecules involved in antioxidant defence and both are induced by oxidative stress [128]. The complementary and overlapping actions of both factors in modulating cytoprotective pathways suggests they might form a regulatory loop, although a direct molecular interaction between both hasn´t been established yet [129] (Figure 4A). It has been suggested that Nrf2 might regulate PGC-1α transcriptionally, and indeed PGC-1α contains two ARE sequences, although it hasn´t been ~~shown yet~~fully established if Nrf2 is able to bind them. Likewise, it hasn´t been proven yet if PGC-1α

acts as a direct transcriptional co-activator of Nrf2 gene. However, multiple reports point at the interaction between both pathways. Aquilano *et al*. showed that in a model of aging, PGC-1α controlled Nrf2 expression, leading to Nrf2 binding to its target sequences in the promoter of *GCLC* gene encoding γ-glutamylcysteine ligase protein, which participates in GSH biosynthesis [128]. PGC-1α silencing abrogated Nrf2 activation and mediated increase in γ-glutamylcysteine ligase expression, indicating PGC-1α modulates Nrf2 in this cell model. Likewise, in experiments done in microglia, induction of the HO-1/Nrf2 pathway increased mitochondrial biogenesis and metabolism in a mechanism controlled by PGC-1α [130]. Authors show that Nrf2 transfection or pharmacological activation with sulforaphane led to an increase in the activity of PGC-1α promoter, as shown by a luciferase-dependent increase, suggesting Nrf2 might control the expression of PGC-1α in this model, by a mechanism that needs to be further elucidated [130]. Possible routes linking both pathways have been suggested. In a model of ischemia/reperfusion, Choi *et al*. proposed that GSK3β mediated the functional interaction between PGC-1α and Nrf2 [131]. Authors show that PGC-1α activation upregulated Nrf2 via inactivation of GSK3β by its p38-dependent phosphorylation, leading to Nrf2 stabilization and translocation to the nucleus. Importantly, GSK3β itself has been also found to modulate PGC-1α [132]. Likewise, different pathways mediate the synergistic activity of Nrf2 and PGC-1α, including PPARγ, ERK1/2 and AMPK. Nrf2 has been shown to regulate the expression of PPARγ (the canonical target of PGC-1α) by stimulating its promoter activity, pointing at a role of Nrf2 in modulating adipocyte differentiation [133]. The kinase AMPK, which is an important modulator of PGC-1α as explained before, is also able to activate the Nr2 pathway [134], while ERK1/2 was shown to upregulate both PGC-1α and Nrf2 leading to increased mitochondrial biosynthesis and neuroprotection during hypoxia [135] (Figure 4).

In agreement with the role of Nrf2 in promoting mitochondrial biogenesis, the liver of Nrf2 deficient mice present a lower mitochondrial content in comparison with WT mice [136] and multiple pharmacological and physiological Nrf2 activators have been shown to promote mitochondrial biogenesis in various tissues, recently reviewed in [137].

Mitochondrial biogenesis has been highly studied in the skeletal muscle, as it represents one the main adaptations of this organ to exercise, and it is considered to be responsible for the benefits of exercise to health. Different studies show that Nrf2 is induced by exercise and mediates

mitochondrial changes in the skeletal muscle response to ~~exercise~~it [138, 139]. Merry and Ristow demonstrated that Nrf2 is induced during exercise by ROS and NO, and increases mitochondrial biogenesis in the skeletal muscle by upregulating NRF-1 and TFAM transcription factors [127]. Likewise, pharmacological activation of Nrf2 with DMF upregulated mitochondrial biogenesis and mitochondrial function *in vitro* and *in vivo* [140]. Importantly, this effect was shown for the first time in patients, where an increase in mtDNA was observed in lymphocytes from multiple sclerosis patients under treatment with DMF, the first Nrf2 activator approved in clinics [140]. This data points at an additional therapeutic target of Nrf2 activation for mitochondrial or muscular diseases. DMF and its bioactive metabolite MMF have also demonstrated to mediate a neuroprotective action in a mouse model of Parkinson's disease via Nrf2-dependent mitochondrial biogenesis in brain [141]. Several other studies have focused on the role of the pharmacological activation of this pathway in physiological aging and age-related disorders pointing at a beneficial effect in muscle and cognitive function [137, 142].

Nrf2 in mitophagy

There is a direct connection between Nrf2 and different autophagic mechanisms, including mitophagy, demonstrated by the transcriptional regulation of different autophagic mediators induced by Nrf2 (Figure 4B). Murata *et al.* showed that Nrf2 transcriptionally regulates PINK1 expression via binding to the four ARE sequences present in *PINK1* promoter [143]. The study further showed that Nrf2 induction with *tert*-butylhydroquinone (tBHQ) greatly increased PINK1 expression in different cell lines, including mature iPSC-derived neurons, while Nrf2 silencing abrogated this effect. Interestingly, oxidative stress had a critical role in mediating Nrf2-induced PINK1 expression, which was prevented in the presence of antioxidants [143]. The positive feedback loop between Nrf2 and the autophagy substrate p62 ~~in~~ by which they are able to activate each other has also been well documented in the literature. It is known that p62 is able to activate Nrf2, by different mechanisms such as interacting with the Nrf2-binding site of Keap1 [73] or sequestering Keap1 upon p62 accumulation [144], therefore halting Nrf2 degradation and facilitating its translocation to the nucleus to bind its target promoters. Importantly, Jain *et al.* showed that p62 is one of the target genes of Nrf2, and induces its expression by binding into an ARE sequence in its promoter in response to oxidative stress. Conversely, p62 impairs Keap1-Nrf2 interaction, and therefore promotes Nrf2 translocation to the nucleus which further stimulates

p62 production to sustain a loop of Nrf2 activation [145]. Although this loop has a physiological role in modulating the response to oxidative stress, a chronic activation as a result i.e. of impaired autophagy leading to the accumulation of p62, might result in persistent Nrf2 activation that ~~might~~could contribute to tumour progression [146]. On the other hand, pharmacological activation of Nrf2 with omaveloxolone (RTA408) was shown to counteract the mitochondrial dysfunction induced by p62 deficiency in ALS/FTD patient cells [83]. Interestingly, functional experiments showed that genetic Nrf2 overexpression induced mitophagy in a *Drosophila* model of Pink1/Parkin deficiency by a mechanism that was also independent of p62, suggesting that Nrf2 modulates additional mitophagy pathways, although the exact mechanism was not further elucidated in the study [147].

Parkinson's disease is often a candidate for disease-modifying therapies aimed at promoting mitochondrial function and specifically mitophagy, given the close genetic and pathophysiological relationship of these processes with the pathogenesis of PD. In addition, an impairment of the Nrf2 response has also been associated with the progression of PD [148], therefore, the pharmacological activation of Nrf2 appears as a promising strategy. Multiple studies have shown the neuroprotective role of Nrf2 activators in Parkinson's disease, with some of them focusing on its role in modulating mitochondrial function [149]. As part of it, Lastres-Becker *et al.* showed that the Nrf2 activator DMF was neuroprotective in a mouse model of synucleopathy by modulating p62-dependent autophagy [148]. However, despite the direct connections between Nrf2 and the components of the mitophagy pathway, functional evidence of increased mitochondrial degradation via mitophagy upon pharmacological activation of Nrf2 is still scarce in PD and other cellular models. East *et al.* showed that the small molecule PMI (p62-mediated-mitophagy inducer) was able to induce mitophagy by upregulating p62 in a Nrf2-dependent mechanism [150, 151]. This effect was independent of the mitochondrial bioenergetic state, and although less efficiently, it also triggered mitophagy in the presence of an impaired Pink1 or Parkin pathway, pointing at its potential beneficial use in PD [150]. Intriguingly, induction of mitophagy appeared to depend on the specific molecular mechanism of Keap1 inhibition- and therefore Nrf2 activation induced by this molecule. As opposed to traditional electrophilic Nrf2 activators that covalently bind and modify Cys residues in Keap1, PMI is protein-protein interaction inhibitor, a non-electrophilic compound that activates Nrf2 by impairing Nrf2 and Keap1 interaction. While PMI and other structurally unrelated molecules with the same mechanism of action showed a similar

induction of mitophagy, traditional electrophilic inducers of Nrf2 such as sulforaphane failed to show the same effects [151]. In agreement, Zeb *et al*. have recently reported that Keap1-Nrf2 protein-protein interaction inhibitors stimulate Parkin-mediated mitophagy without affecting the mitochondrial membrane potential [152]. However, in this case results pointed at a preferential role for the inhibition of Keap1 interaction with the OMM protein PGAM5 in the process, rather than being mediated by Nrf2. Taken together, these results support the therapeutic potential of non-electrophilic Nrf2 inducers for mitophagy induction. It shouldn't be disregarded either the different mechanisms independent of mitophagy and explained in previous sections by which Nrf2-dependent induction of PINK1 pathway might play a role, such as complex I activation or other mitochondria quality controls mechanisms such as mitochondrial dynamics or biogenesis.

Nrf2 in mitochondrial dynamics

Recent papers in the literature point at a general pro-fusion effect of Nrf2 activation in mitochondrial dynamics. Constitutive activation of Nrf2 by Keap1 inhibition or treatment with two well-known Nrf2 pharmacological activators -sulforaphane and dimethyl fumarate – led to Drp1 degradation and a hyperfused mitochondrial network in fibroblast and neuronal cultures, pointing at its potential use in clinic to target mitochondrial dynamics imbalance [153]. Excessive mitochondrial fragmentation as a result of reduced fusion or augmented fission is often a common event induced by pathological insults such as oxidative stress and has been associated with different disorders [67]. In this context, a protective role for Nrf2 by inhibiting the pro-fission effect of Drp1 has been described in various cellular models exposed to different pharmacological activators of Nrf2. Hoe *et al*. have recently shown that Nrf2 activation induced by mitoquinone (better known to be a mitochondrial antioxidant but which also exerts protective roles via Nrf2 activation) prevented Drp1-mediated mitochondrial fission induced by LPS in alveolar epithelial cells during acute lung injury [154]. Mitoquinone prevented mitochondrial recruitment of Drp-1, inhibited Mff and Fis1 expression and up-regulated the pro-fusion protein Opa1. Importantly, this effect was loss when Nrf2 was pharmacologically inhibited. Mitochondrial fragmentation also appears to be an important pathogenic mechanism in atherosclerosis. The triterpenoid ilexgenin A improved endothelial function by promoting Drp1 degradation, and therefore preventing mitochondrial fission, in a Nrf2-dependent mechanism [155]. Of note, sulforaphane, a well-known pharmacological activator of the Nrf2 pathway, was shown to inhibit mitochondrial fission by preventing Drp1 recruitment in the mitochondria in retinal pigment epithelial cells, but,

surprisingly, this effect appeared to be independent of Nrf2 or Keap1 [156]. Nrf2 activation also promoted a pro-fusion phenotype in inflammatory macrophages [90]. Ryan et *al*. have recently shown that the LPS-induced activation of Keap1-KD macrophages led to an increase in the mitochondrial fusion proteins Opa1, Mfn1 and Mfn2, and conversely, Nrf2-KO macrophages presented an increase in the mitochondrial fission factors Mff and Mief2, as revealed by proteomics [90]. The structural analysis of the mitochondria by imaging proved that prolonged stimulation of the macrophages with LPS led to a switch in mitochondrial morphology, leading to a fused/elongated network in Nrf2 activation which was suppressed in Nrf2 deficiency [90].

In other tissues, experiments have shown a pro-fission role for Nrf2 activation. Nrf2 plays an important role in skeletal muscle function, and some of these effects have been shown to be mediated by the modulation of the mitochondrial function, including dynamics. Aged Nrf2-KO mice show exacerbated sarcopenia, a progressive and generalised skeletal muscle disorder leading to loss of muscle mass and function closely related to aging. Nrf2 deficiency aggravated the disease by reducing skeletal muscle biogenesis and dynamics, leading to a reduced mitochondrial fission phenotype [157]. Interestingly, exercise, which is a recommended intervention for sarcopenia, also mediates part of its beneficial roles through Nrf2-induced improvement of mitochondrial dynamics [158]. In this context and tissue, Nrf2 activation either pharmacologically with ~~sulphoraphane~~sulforaphane or by exercise, promoted Drp1-induced mitochondrial fission by stabilizing Drp1, and improved skeletal muscle function.

Nrf2 in mitochondrial transport

Effects of Nrf2 in modulating mitochondrial transport haven't been explored in deep yet. O'Mealey et al described a role for Nrf2-Keap1-PGAM5 (a mitochondrial phosphatase) complex in mitochondrial motility [159]. Authors showed that Nrf2 depletion inhibits mitochondrial retrograde trafficking induced by proteasome inhibition [159], in a mechanism involving the Keap1-metiated aberrant degradation of Miro2 protein rather than Nrf2 activity *per se*. Miro2 is involved in the anchoring the mitochondria to microtubule motor proteins and authors show its levels were consistently lower in Nrf2-KO brains [159].

Concluding remarks

In the recent years, there has been an increasing interest in understanding the role of Nrf2 in regulating different aspects of mitochondrial homeostasis. Nrf2 has emerged not only as a regulator of mitochondrial function, recent studies also point at its role in controlling mitochondrial quality control, biogenesis and dynamics, with a clear interplay between them. As part of these functions, experimental data support the enhancement of energy production via increased efficiency of oxidative phosphorylation and regulation of different metabolic pathways induced by Nrf2. Data from *in vitro* and *in vivo* models confirm the protective effects of modulating mitochondria bioenergetics via Nrf2 activation in neurodegenerative diseases, characterized by an increased vulnerability to energy deprivation. Promotion of mitochondrial biogenesis and adequate dynamics and clearance further contribute to maintaining and adequate mitochondrial homeostasis.

Pharmacological activation of Nrf2 has shown promising results as a disease-modifying therapy in preclinical and clinical studies for chronic disorders. The role of Nrf2 in enhancing mitochondrial function points at an additional cytoprotective mechanism that needs to be taken in account in further studies. A comprehensive understanding of the mechanisms of Nrf2-induced mitochondrial regulation, its specificity in different tissues, and the importance of a controlled Nrf2 activation will be essential for the development of future therapeutic strategies.

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DECLARATION OF INTEREST

The authors declare no competing interests

FIGURE LEGENDS

Figure 1 – Mitochondrial function and metabolism: the role of Nrf2.

Multiple metabolic pathways converge in the mitochondria. Glucose is up-taken into cells by GLUT and is then metabolized through glycolysis, generating pyruvate, ATP and NADH; or through the pentose phosphate pathway, generating NAPDH which is essential for GSH regeneration and antioxidant defence. In addition to glycolysis, pyruvate can be produced from lactate and malate, and is transported into the mitochondria through VDAC in the OMM and the MPC in the IMM. Once in the mitochondria, PDH converts pyruvate into Acetyl-CoA, which enters the Krebs cycle, a cyclic series of enzymatic reactions leading to the interconversion of different metabolites and the production of NADH and $FADH_2$, which serve as substrates for respiration and generation of ATP through OxPhos. Other metabolic pathways can also feed the Krebs cycle and generate respiration substrates. Fatty acids are incorporated into the mitochondria thorough CPT1/CAT/CPT2 after their activation by ACS. Once in the mitochondria, the several steps of the β-oxidation of fatty acids lead to the sequential generation of Acetyl-CoA molecules that will enter the Krebs cycle, NADH and $FADH_2$. In the process of glutaminolysis, glutamine is uptaken into the mitochondria and incorporated into the Krebs cycle after its conversion by GLS and GDH into the Krebs cycle metabolite α-ketoglutarate. Some dehydrogenases in the Krebs cycle are activated by calcium, which enters the mitochondria through MCU and leaves the mitochondria through NCLX. An excessive accumulation of calcium in the mitochondria in the context of high ROS levels triggers apoptosis by inducing mPTP opening, a high conductance mitochondrial channel leading to unselective permeability of the IMM.

Mitochondria cristae contain the components of the ETC (CI-CIV) and the ATP synthase (C-V) necessary for ATP generation through OxPhos. During mitochondrial respiration, the ETC transfers electrons through a series of redox reactions from the respiratory substrates NADH and $FADH_2$ to its final acceptor O_2. Sequential transfer of electrons between the different complexes is coupled with the translocation of H^+ to the IMS through CI, CIII and CIV, generating an electrochemical gradient, whose major component is the $\Delta\psi m$. The protonmotive force of this gradient is used by the F_1F_o-ATP synthase or complex V to generate ATP as the H^+ flow down the gradient back into the matrix.

Leak of electrons from the ETC might interact with O_2, leading to the production of ROS mostly in the form of superoxide O_2^{*-}, which is rapidly converted to H_2O_2 spontaneously or by SOD. H_2O_2 is detoxified by different mitochondrial antioxidant mechanisms such as catalase or GSH, which depends on NADPH to regenerate. Interconversion and excessive ROS production might lead to the generation of the highly reactive hydroxyl radical (OH*), causing cellular damage.

Nrf2 has been shown to regulate different metabolic pathways and aspects of mitochondrial function, as indicated by the red stars. Nrf2 enhances mitochondrial metabolism by increasing efficiency of ATP production by OxPhos; enhancing glucose uptake and pyruvate flux through the Krebs cycle and upregulating fatty acid oxidation and glutaminolysis. Nrf2 is also able to increase the antioxidant defence by inducing some of its enzymes, upregulating the pentose phosphate pathway and increasing NADPH availability necessary for GSH regeneration. Furthermore, Nrf2 activation prevents mPTP opening and apoptosis. Importantly, Nrf2 can be recruited to the OMM by a ternary complex with PGAM5/Keap1.

Abbreviations: ACS, acyl-CoA synthetase; CAT, carnitine:acylcarnitine translocase; CI-CV, Complex I to V; CJ, cristae junctions; CM, cristae membrane; CoQ, coenzyme Q; CPT1-CPT2, Carnitine:PalmitoylTransferase I and II; Cyt C, cytochrome C; ETC, Electron Transport Chain; GLS, mitochondrial glutaminase; GDH, Glutamate dehydrogenase; GLUT, glucose transporter; GR, Glutathione Reductase; GSH, Glutathione; IMM, inner mitochondrial membrane; IMS, intermembrane space; LDH, lactate dehydrogenase; MCU, Mitochondrial Calcium Uniporter; ME-1, malic enzyme 1; MPC, mitochondrial pyruvate carrier; mPTP, mitochondrial Permeability Transition Pore; NCLX, Sodium/Calcium Exchanger; OMM, outer membrane space; OxPhos, Oxidative Phosphorylation; PC, Pyruvate Carboxylase; PDH, Pyruvate dehydrogenase; ROS, reactive oxygen species; SDH, Succinate Dehydrogenase; SOD, Superoxide Dismutase; VDAC, Voltage-Dependent Anion Channel.

Figure 2 – Mitochondrial homeostasis

A) Mitochondria biogenesis consists of the generation of new mitochondria through the replication and growth of pre-existing ones and requires the coordinated expression of proteins encoded in the nuclear and mitochondrial DNA. PGC-1α is the master regulator of the process and can be activated by different stimulus. Metabolic changes induce AMPK (AMP-activated protein kinase) which senses the ATP:AMP ratio; and SIRT1 (sirtuin 1) which acts as a NAD+

sensor. In turn, both activate PGC-1α via post-translational modifications. Cold exposure, fasting and exercise are also able to transcriptionally up-regulate PGC-1α expression. PGC-1α is a transcriptional coactivator of key transcription factors which mediate the expression of essential mitochondrial proteins necessary for mitochondrial biogenesis. Those transcription factors include PPAR (Peroxisome Proliferator-Activated Receptors), ERR (Estrogen-Related Receptors) and the nuclear respiratory factors NRF-1 and NRF-2, which importantly also induce the expression of the Mitochondrial Transcription Factor A (tFAM). tFAM is essential to produce core subunits of the electron transport chain encoded in the mitochondrial DNA (mtDNA).

B) Mitophagy involves the selective degradation of mitochondria via autophagy in the lysosomes. PINK1/Parkin-dependent mitophagy starts with the accumulation of PINK1 in the OMM (outer mitochondrial membrane) upon mitochondrial damage, as opposed to the continuous import and degradation of PINK1 occurring in basal conditions. PINK1 then recruits the E3 ubiquitin ligase Parkin to the OMM, starting a positive amplification loop of ubiquitination and phosphorylation of different OMM proteins. Phospho-ubiquitin is recognized by the autophagy regulator p62, triggering their LC3-mediated engulfment in the autophagosome for degradation in the lysosomes. Mitophagy receptors present in the OMM including BNIP3, NIX or cardiolipin are also able to recruit mitochondria into autophagosomes independently of PINK1/Parkin.

C) Mitochondrial dynamics consist of the continuous fission and fusion of the mitochondrial network. Mitochondrial fusion results in the merge of metabolites and components from two different mitochondria and involves the sequential fusion of the OMM through the mitofusins (Mfn-1 and Mfn-2) present in adjacent mitochondria; and the IMM (inner mitochondrial membrane) through Opa1 (optic atrophy protein 1). Mitochondrial fission is primary mediated by Drp1 (dynamin related protein) which excises the membrane at sites previously marked and pre-constricted by mitochondria-ER (endoplasmic reticulum) contacts.

Figure 3 – Nrf2 regulation

Nrf2 is a transcription factor that promotes the expression of its target genes by binding specific ARE (Antioxidant Response Elements) sequences present in their promoter. Nrf2 levels are tightly

regulated by its continuous degradation in the proteasome mediated by different ubiquitin-ligase systems. 1) Keap1 (Kelch-like ECH-associated protein 1) is a cysteine-rich adaptor protein located in the cytoplasm that binds Nrf2 so it can be ubiquitinated and targeted for degradation by the Cullin 3 (Cul3) RING-box 1 (RBX1) E3 ubiquitin ligase complex upon basal conditions (A). (B) Keap1 highly reactive Cys residues are modified by electrophiles or oxidative stress, leading to a conformational change that prevents Nrf2 ubiquitination and degradation, allowing its translocation to the nucleus. (C) Molecules that prevent Nrf2-Keap1 interaction such as p62 or different pharmacological protein-protein interaction inhibitors (PPI) also prevent Keap1-mediated Nrf2 degradation leading to its translocation to the nucleus. 2) GSK3β regulates Nrf2 degradation by the β-TrCP/Cul 1 ubiquitin ligase complex. Under basal conditions (d), GSK3β phosphorylation of Nrf2 targets the protein for degradation through this system. When GSK3β is inhibited, such as after its phosphorylation by p38 or PI3K/Akt signalling pathways, Nrf2 degradation via this pathway is inhibited, allowing its translocation to the nucleus to exert its function as a transcription factor.

Figure 4 – Role of Nrf2 in mitochondrial biogenesis and turnover

A) Nrf2 promotes mitochondrial biogenesis through various mechanisms. Nrf2 and the master regulator of mitochondrial biogenesis, the transcriptional coactivator PGC-1α, are closely linked, interconnected, and have synergistic actions in promoting mitochondrial biogenesis. Both have been shown to upregulate each other by different mechanisms including the inhibition of Nrf2 degradation by GS3Kβ-inactivation, which also stimulates PGC-1α. In addition, both are induced by different cellular pathways such as AMPK and ERK1/2. PGC-1α is a transcriptional coactivator of key transcription factors that mediate the expression of essential mitochondrial proteins necessary for mitochondrial biogenesis. Those transcription factors include PPAR (Peroxisome Proliferator-Activated Receptors), ERR (Estrogen-Related Receptors) and the nuclear respiratory factors NRF-1 and NRF-2, which induce the expression of the Mitochondrial Transcription Factor A (tFAM). tFAM is essential to produce core subunits of the electron transport chain encoded in the mitochondrial DNA (mtDNA). Nrf2 further contributes in the process by upregulating NRF-1, PPAR and tFAM via binding to ARE (Antioxidant Response Elements) sequences in their promoters.

B) Nrf2 is connected with different autophagic mechanisms via the upregulation of PINK1 and the autophagy substrate p62. In addition, p62 also stimulates Nrf2 expression by inhibiting its degradation, either by impairing Keap1-Nrf2 interaction or promoting Keap1 sequestration. This establishes a positive feedback loop of Nrf2 and p62 activation. Nrf2 activation has been shown to stimulate autophagy and mitophagy via PINK1 and p62 dependent and independent mechanisms.

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