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Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes

Robert B. Hamanaka and **Navdeep S. Chandel**[1,2,*]

[1] Department of Medicine, Division of Pulmonary and Critical Care Medicine, Northwestern University Medical School, Chicago, IL 60611, USA

[2] Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL 60611, USA

Reactive oxygen species (ROS) have historically been viewed as toxic metabolic byproducts and causal agents in a myriad of human pathologies. More recent work, however, indicates that ROS are critical intermediates of cellular signaling pathways. Although it is clear that dedicated cellular ROS producers such as NADPH oxidases participate in signaling, evidence suggests that mitochondrial production of ROS is also a tightly controlled process, and plays a role in the maintenance of cellular oxidative homeostasis and propagation of cellular signaling pathways. Production of ROS at mitochondria thus integrates cellular energy state, metabolite concentrations, and other upstream signaling events and has important implications in cellular stress signaling, maintenance of stem cell populations, cellular survival, and oncogenic transformation.

Production of ROS by the Mitochondrial Electron Transport Chain

Mitochondria are a principal source of cellular reactive oxygen species (ROS). Whereas mitochondrial ROS production has commonly been thought of solely as the result of inefficiencies in the electron transport chain, a role for mitochondrial ROS in the propagation of cellular signaling pathways has emerged, leaving the questions of if and how ROS production at mitochondria is specifically regulated by these pathways in order to dictate biological outcomes. This review discusses pathways which impinge on and depend on mitochondrial ROS production and their important implications for biology both at the cellular and organismal level.

ROS are produced by mitochondria during oxidative metabolism through the one-electron reduction of molecular oxygen (O_2), forming superoxide anion ($O_2{\bullet}^-$). Superoxide is the proximal ROS produced by mitochondria and is converted to hydrogen peroxide (H_2O_2) through the action of superoxide dismutases (SODs) both within the mitochondria and in the cytosol. Complexes I, II, and III of the electron transport chain contain sites wherein electrons can prematurely reduce oxygen, resulting in the formation of superoxide [1, 2]. Although complexes I and II produce ROS only into the matrix, complex III can produce ROS on both sides of the mitochondrial inner membrane [1, 3]. This is of interest in the field of signaling, as ROS produced into the intermembrane space theoretically have an easier route to the cytosol to act as signaling molecules than do ROS produced into the matrix [4]. There are other non-

*Corresponding author. Tel: +1-312-503-2549; Fax: +1-312-503-0411, nav@northwestern.edu.

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respiratory chain enzymes that produce superoxide in mitochondria, including glycerol-3-phosphate dehydrogenase; however the contribution of these enzymes to total mitochondrial ROS production remains unclear [2, 5].

For a given cell, the rate of ROS production from the electron transport chain varies with the amount of electron carriers present in a state capable of reacting with O_2. This is affected by the concentration of a given electron carrier in a cell as well as the rate of electron supply and release from each carrier [2]. These factors can change depending on the biological state of a given cell, respiratory rate, mitochondrial inner membrane potential, and posttranslational modifications or damage to the respiratory chain. Several groups have measured the rate of mitochondrial superoxide production *in vitro*, leading to the estimation that between 0.15% and 2% of cellular oxygen consumption results in superoxide [2, 5]. Unfortunately, little is known about the regulation of mitochondrial function *in vivo*; thus it is unknown how much superoxide is produced by mitochondria *in vivo* [2].

There are many excellent reviews that discuss in detail the production of ROS by each mitochondrial complex, as well as the biochemical mechanisms of oxidative signaling modifications [1, 2, 5–7]. The purpose of this review is to discuss specific cellular signaling pathways that impact or require mitochondrial ROS production. We attempt to explain the current evidence for ROS involvement in these pathways, although in many cases, direct biochemical evidence has yet to be provided. Future work must focus on providing a greater understanding of how upstream signals impinge on mitochondrial ROS production, as well as identifying the proximal targets of ROS in each pathway.

Hypoxia-induced production of mitochondrial ROS is required for the cellular response to hypoxia

Exposure of cells to low oxygen (hypoxia) leads to the activation of signaling pathways that promote adaptive transcriptional programs, reduce cellular oxygen usage, and decrease cellular energy consumption. Paradoxically, hypoxia leads to an increase in mitochondrial production of ROS. Current evidence suggests that this H_2O_2 emission from mitochondria during hypoxia is a central upstream regulator of many of the cellular responses to hypoxia [8–14].

The cellular response to hypoxia requires the induction of the hypoxia inducible transcription factors (HIFs) that consist of a stable β-subunit and one of three labile α-subunits (HIF-1α, HIF-2α, and HIF-3α) [15]. During hypoxia, the normally degraded HIF-α subunits become stabilized, allowing for transcriptional transactivation and expression of genes regulating erythropoiesis, glycolysis, angiogenesis, cell cycle, and survival [15]. The normoxic turnover of HIF-α subunits requires proline-directed hydroxylation of HIF-α subunits that targets them for recognition by the von Hippel-Lindau tumor suppressor protein and subsequent proteasomal degradation [16]. This hydroxylation reaction is carried out by a family of 2-oxoglutarate-dependent dioxygenases termed prolyl hydroxylases 1, 2, and 3 (PHD1-3). During hypoxia, PHD activity is inhibited, allowing for stabilization of HIF-α subunits.

The requirement of mitochondrial ROS for hypoxia-mediated HIF stabilization was first suggested by studies in ρ^0 Hep3B cells. These cells do not contain mitochondrial DNA and thus demonstrate no electron transport or ROS production; moreover, they are incapable of HIF-mediated transcription during hypoxia due to a lack of HIF-α subunit stabilization [8]. Further experiments using mitochondrial inhibitors, antioxidants, and genetic targeting of mitochondrial proteins suggested that hypoxia induces ROS production from mitochondrial complex III, creating a cytosolic signal that stabilizes HIF [11, 17–19]. Later work demonstrated that the ability of complex III to pump protons or to participate in oxidative phosphorylation is not required for ROS generation at this complex. Thus, cells that express a mutant

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cytochrome *b* subunit are unable to consume oxygen for oxidative phosphorylation, but are able to produce complex III-derived ROS and stabilize HIF during hypoxia [20]. HIF stabilization afforded by mitochondrial ROS is a result of PHD inhibition; however, the mechanism of this inhibition is not yet known [20].

In addition to the transcriptional regulation mediated by HIFs, hypoxia also requires that cells expend less energy to minimize oxygen usage. A major consumer of cellular ATP is the Na/K-ATPase, which can account for 20–80% of oxygen expenditure in mammalian cells [21]. Exposure of cells to hypoxia reversibly suppresses Na/K-ATPase activity via a mitochondrial ROS-mediated process that promotes the rapid endocytosis of this protein complex [12]. The cellular function of the Na/K-ATPase is to transport sodium and potassium ions across the plasma membrane to maintain cellular ionic gradients. Thus, hypoxic downregulation of Na/K-ATPase activity has important implications for airway fluid absorption during hypoxia [22].

Hypoxia-induced Na/K-ATPase endocytosis requires the activity of AMP-activated protein kinase (AMPK) [23]. AMPK is ubiquitously expressed and facilitates ATP production while suppressing ATP usage in energy-stressed cells. During hypoxia, AMPK phosphorylates and activates protein kinase C zeta (PKCζ) [23], which then phosphorylates Na/K-ATPase α-subunits on serine-18, leading to endocytosis of the complex [12]. Mitochondrial ROS production triggers AMPK activation during hypoxia, providing a link between ROS and Na/K-ATPase inhibition [13, 23]. In addition to regulating the Na/K-ATPase, AMPK phosphorylates and activates the tuberous sclerosis complex during hypoxia. This leads to inhibition of mTOR activity and further conservation of cellular energy by inhibiting the energetically-costly process of protein translation [24].

Another important organismal adaptation to hypoxia is constriction of pulmonary arteries which allows for the diversion of blood from poorly oxygenated regions of the lung. Hypoxia triggers contraction of pulmonary artery smooth muscle cells through an increase in cytosolic calcium from both intracellular and extracellular stores [25]. This increase in calcium and subsequent pulmonary artery vasoconstriction are both dependent on hypoxic mitochondrial ROS production [9, 14]. Thus, the cellular response to hypoxia requires the mitochondrial generation of ROS to propagate signaling events that regulate transcription, calcium stores, and energy stores at the cellular level. At the organismal level, mitochondrial ROS regulate airway fluid absorption and oxygen exchange in the pulmonary vasculature.

Hypoxic generation of ROS at mitochondrial complex III occurs through a process termed the Q-cycle. Complex III accepts electrons donated to coenzyme Q by mitochondrial complexes I and II and transfers them to cytochrome *c*. Whereas coenzyme Q accepts two electrons from either complex I or II, cytochrome *c* is only capable of reduction with one electron. Thus complex III must sequentially remove each electron from reduced coenzyme Q (ubiquinol, QH_2). Removal of the first electron of ubiquiol results in the formation of the radical ubisemiquinone (QH•). Normally, the unpaired electron of ubisemiquinone is transferred to the cytochrome *b* center of complex III; however, this electron can also react with molecular oxygen to form superoxide [2, 26]. Thus, genetic targeting of the complex III subunit Rieske iron-sulfur protein, the protein required for electron transfer from ubiquiol, abolishes ROS formation during hypoxia, preventing the activation of downstream signaling effectors [17, 19, 20].

Although much work has gone into demonstrating the role of mitochondrial ROS in hypoxic signaling, it remains unclear whether ROS induction during hypoxia is an intrinsic property of mitochondria, or if other cellular factors are required. Indeed, it is counterintuitive that a decrease in the concentration of a reactant would lead to an increase in product formation; thus upstream pathways might be required. If hypoxic ROS emission is intrinsic to the electron

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transport chain, possible explanations include increased half-life of ubisemiquinone during hypoxia or increased access of ubisemiquinone to oxygen in the inner membrane during hypoxia. It is also possible that complex III produces relatively more superoxide on the outer side of the mitochondrial inner membrane during hypoxia than during normoxia [27]. This could result from a conformational change of complex III within the inner membrane during hypoxia leading to more ROS emitted into the intermembrane space without affecting total ROS levels produced by complex III. The mechanism of hypoxic induction of mitochondrial ROS production will be the subject of intense study in the years to come.

The PI3-Kinase pathway induces mitochondrial ROS emission

Activation of phosphoinositide-3-kinase (PI3K) by ligation of growth factors to their cellular receptors promotes recruitment of Akt to the plasma membrane and its subsequent activation [28]. Akt positively regulates the mammalian target of rapamycin (mTOR), which is a key regulator of mitochondrial oxygen consumption and oxidative capacity [29]. Akt activation is associated with increased oxygen consumption as well as an increase in total cellular ATP derived from both glycolytic and oxidative sources [30, 31]. Conversely, rapamycin-mediated inhibition of mTOR activity results in reduced oxygen consumption and oxidative capacity [29].

Akt activation is also associated with the accumulation of mitochondrial ROS [31]. This ROS induction might stem from an increase in mitochondrial metabolism, but other factors might also play a role [32, 33]. Regardless of the effect of Akt activation on the creation of mitochondrial ROS, Akt activity is a key inhibitor of mitochondrial ROS scavenging. Akt phosphorylates forkhead box O (FOXO) transcription factors on three conserved residues, resulting in cytosolic sequestration through interaction with 14-3-3 adaptor proteins [34]. FOXOs comprise a family of transcription factors that promote the expression of genes associated with cell cycle arrest, stress resistance, apoptosis, and tumor suppression. Of note, FOXOs robustly regulate the expression of mitochondrial manganese superoxide dismutase (*SOD2*) and catalase [35, 36]. Thus, activation of the PI3K-Akt pathway not only results in increased production of ROS from mitochondria through metabolic pathways, but also the inability to scavenge mitochondrial ROS through inhibition of FOXOs.

FOXOs regulate mitochondrial ROS, proliferation, survival, and differentiation

FOXO-mediated transcription is activated upon cellular stress, including oxidative stress, via phosphorylation events mediated by JNK (c-Jun N-terminal kinase), MST1 (mammalian Sterile20-like 1), or AMPK [34].

The physiological significance of FOXO-dependent regulation of antioxidants has been demonstrated in the ability of FOXOs to block the cardiac hypertrophic response. As mammalian cardiomyocytes do not proliferate, increased workloads are met with cell growth. Prolonged hypertrophy, however, leads to congestive heart failure and death due to arrhythmias [37]. The hypertrophic response is associated with increased production of ROS from mitochondrial and non-mitochondrial sources, which leads to aberrant signaling via downstream pro-growth pathways [38, 39]. Induction of FOXO3a activity during the hypertrophic response thus increases mitochondrial ROS scavenging, attenuating pro-growth signaling and protecting cardiomyocytes from prolonged hypertrophic growth [40].

Recently, FOXO-mediated antioxidant transcription has been implicated in the maintenance of stem cell populations. Deletion of *Foxo1*, *3*, and *4* in hematopoietic stem cells (HSCs) results in cell cycle entry and terminal differentiation [41]. Remarkably, administration of antioxidants

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to FOXO-deficient mice rescued this defect in the HSC compartment, suggesting the necessity of ROS regulation in stem cell maintenance. This observation corresponds with the HSC defects observed in mice deficient for the DNA damage activated kinase *Atm* (ataxia telangiectasia mutated) [42]. *Atm* deficiency leads to increased cellular ROS levels in HSCs, resulting in activation of p38 mitogen-activated protein kinase (MAPK) and mitotic entry; treatment of mice with antioxidants or p38 inhibitors rescued this stem cell hyperproliferation [42].

The regulation of ROS in stem cell populations is not restricted to the hematopoietic compartment. Deletion of the polycomb gene *Bmi1* leads to mitochondrial dysfunction and increased ROS production in thymocytes [43]. The thymocyte maturation defect characteristic of *Bmi1*−/− mice is largely rescued by treatment with antioxidants. Cellular ROS levels also increase upon the differentiation of both neural and epithelial stem cells [44–46]. Thus, a paradigm appears to be set in which ROS levels increase as a population of stem cells differentiates. Low levels of ROS are required for quiescence and stem cell maintenance, whereas ROS induction leads to proliferation and differentiation programs. Indeed, work in *Drosophila melanogaster* has corroborated the essential role of ROS regulation in stem cell populations. ROS levels increase as *Drosophila* multipotent hematopoietic progenitors differentiate [47]; overproduction of mitochondrial ROS leads to precocious differentiation into mature blood cells whereas increased scavenging ability inhibits differentiation.

ROS regulate the activity of phosphatases

In the cytosol, oxidation of cysteines remains the best-studied oxidative signaling modification [6]. Oxidation of the cysteine sulfhydryl group can alter protein–protein interactions, the DNA binding activity of transcription factors, and the catalytic activity of enzymes. Additionally, oxidation of two intra- or intermolecular cysteines forms disulfide bridges allowing for conformational changes or oligomerization of proteins [6, 7]. Mitochondrial ROS play a large role in maintaining the oxidative homeostasis of the proteome as inhibition of mitochondrial ROS production drastically decreases the total number of cellular protein disulfide bonds [48].

The best-described class of ROS targets is phosphatases. These enzymes oppose the activity of protein kinases and possess a reactive cysteine in their catalytic domain that is required for enzymatic activity. The low pK_a of this cysteine allows it to act as a nucleophile for catalysis of the dephosphorylation reaction, but also makes it a target for oxidation by ROS [6, 7]. Protein tyrosine phosphatase 1B was the first phosphatase demonstrated to be inhibited by ROS under physiological conditions [49, 50]. Since then, ROS have been shown to inhibit other classes of phosphatases, including phosphatase and tensin homolog (PTEN, lipid phosphatase) and MAPK phosphatases [51–53].

Mitochondrial ROS regulate NF-κB and TNFα-mediated cell death

Tumor necrosis factor alpha (TNFα) is a pleiotropic cytokine that acts on its receptors (TNFRs) to promote either cell survival or cell death [54]. The outcome of TNFα exposure on cell fate depends on the activities of two separate TNFR complexes, one of which mediates survival through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [55]. The c-Jun N-terminal kinase (JNK) is an important mediator of pro-apoptotic signaling downstream of TNFα. Upon binding of TNFα to its receptors, JNK activity is rapidly, but transiently, stimulated [56]. However, in the absence of NF-κB, this JNK activation is greatly extended, allowing for increased cell death [57, 58].

Although NF-κB regulates the expression of several anti-apoptotic genes, the key regulator of NF-κB-mediated inhibition of the JNK pathway is the mitochondrial antioxidant protein SOD2 [53]. Upon treatment with TNFα, mitochondrial production of ROS is increased [59]. Cells without

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intact NF-κB lack SOD2 induction and accumulate ROS at much higher levels than their wild-type counterparts when treated with TNFα, corresponding with increased JNK activation [53, 60]. SOD2 is thus essential for cellular survival following TNFα treatment [61–63], and enforced expression of SOD2 in the absence of NF-κB can counteract TNFα-induced cell death [53]. Furthermore, treatment of cells with antioxidants inhibits TNFα-induced killing of NF-κB-deficient cells [60, 64].

The mechanism by which SOD2 confers survival to cells treated with TNFα lies in the ability of ROS to inactivate JNK phosphatases [53]. Treatment of cells with TNFα thus leads to mitochondrial ROS production, oxidation of JNK phosphatase catalytic cysteines, and activation of JNK signaling and cell death. SOD2 provides a protective effect by lowering cellular ROS levels and decreasing JNK activation after TNFα treatment [53]. Thus, NF-κB promotes survival through regulation of mitochondrial antioxidant protein expression during the response to TNFα.

Mitochondrial ROS contribute to cellular transformation

Cellular transformation is a multistep process requiring acquisition of activated oncogenes and loss of tumor-suppressor genes, leading to usurpation of cellular signaling pathways controlling proliferation, survival, and metabolism [65, 66]. Transformed cells acquire capabilities such as anchorage-independent growth, limitless proliferation, and evasion of apoptotic signals. Another common trait of transformed cells is oxidative stress [67, 68]. Activation of oncogenes, aberrant metabolism, mitochondrial dysfunction, and loss of the tumor suppressor p53 can all promote increased ROS accumulation in cancer cells [69].

Traditionally, the high ROS levels observed in tumors were viewed as triggers for high mutation rates and genomic instability which promote tumor progression. Although DNA damage undoubtedly contributes to cellular transformation, the role of ROS as signaling intermediates is also crucial. In a mouse model of myc-driven tumorigenesis, tumor growth is inhibited by treatment with antioxidants [70]. However, the effect of antioxidant treatment was a result of the inhibition of HIF accumulation: expression of a stable HIF mutant alone could provide resistance to antioxidant treatment. Similarly, antioxidants inhibited tumor growth in a mouse model of Kaposi's sarcoma. In this case, treatment was associated with reduced Akt and HIF activities in tumors[71].

Recent work demonstrates that mitochondrial ROS directly contribute to cellular transformation induced by either Ras or Myc overexpression. The NAD-dependent deacetylase sirtuin 3 (SIRT3) acts as a tumor suppressor through its regulation of FOXO activity and ROS maintenance [72]. SIRT3 deacetylates FOXO3a, promoting its nuclear localization and transcriptional activation [40, 72]. Moreover, *Sirt3*−/− murine fibroblasts display increased levels of mitochondrial ROS accumulation (compared to wild-type cells) when either Myc or Ras oncogenes are expressed. This ROS accumulation is associated with decreased flux through mitochondrial complexes I and III (possibly allowing for increased escape of electrons to O_2), and reduced cellular levels of SOD2 [72]. *Sirt3*−/− cells become transformed after expression of either Myc or Ras, whereas wild-type cells require expression of both oncogenes. Expression of SOD2 inhibited this oncogene-mediated transformation of *Sirt3*−/− cells, suggesting that mitochondrial ROS play an important role in the S*irt3*−/− phenotype. These finding are consistent with observations in *Sod2*−/− fibroblasts, which like their S*irt3*−/− counterparts, are capable of transformation with single oncogenes [72]. Interestingly, SIRT3 is present in mitochondria, where it regulates the acetylation and activity of a variety of metabolic enzymes, suggesting additional ways that it might affect mitochondrial ROS production [73].

The available data suggests that elevated ROS levels are required for tumor growth and can promote transformation, even acting as the "second hit" in the Knudson two-hit model of

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carcinogenesis. One can then envision a model in which oncogene expression leads to increased levels of mitochondrial ROS production. In normal cells, high ROS levels activate tumor suppressors, leading to senescence or apoptosis. However, loss of tumor suppressor function allows cells to sustain high levels of ROS, which further activates proliferative, angiogenic, and survival pathways, which allow increased ROS accumulation and the continuation of a 'vicious cycle'. The equilibrium of ROS concentration will thus be reached at the point where maximal signaling is permitted without causing irreversible damage to cellular components. Indeed, this seems to be the case as compounds that raise ROS levels can effectively and selectively kill a variety of tumor cell lines [31, 74, 75].

ROS-mediated signaling is not only involved in tumor initiation and maintenance; it also promotes metastasis. Cells harboring the G13997A mutation in the gene encoding the ND6 subunit of mitochondrial complex I are characterized by respiratory deficiency and high ROS production. This mutation is also associated with high HIF activity and increased metastatic potential [76, 77]. Transfer of mutant mitochondria to a poorly metastatic tumor cell line was sufficient to increase the metastatic potential of the recipient cell line. Notably, this metastasis was inhibited by treatment with antioxidants [76].

Concluding remarks: Mitochondrial ROS regulate the fitness of organisms-A heretical model

The common notion of ROS solely as damaging agents likely stems from two factors. First, the discovery of the first antioxidant enzymes (superoxide dismutase) came decades before the discovery that non-phagocytic cells also possess enzymes (NADPH oxidases) whose sole biological function is to produce ROS [78, 79]. Second, high cellular ROS levels are associated with cancer, diabetes, inflammatory diseases, ischemia-related diseases, and neurodegeneration, and are thus commonly thought to contribute to human pathologies and aging [80–83]. This line of thinking has led to multiple clinical trials using antioxidants to scavenge ROS, yet these trials have consistently failed, and in some cases, actually increased mortality [84–87].

In addition to these clinical trials, genetic experiments in mouse models have cast doubt on the idea that less is better when it comes to ROS. *Sod2*−/− mice die before birth, and their heterozygous littermates exhibit mitochondrial damage and oxidative modifications to both nuclear and mitochondrial DNA. However, these heterozygous mice have a normal lifespan [88, 89]. Furthermore, *Sod2* transgenic mice do not have an increased lifespan compared to wild-type, and depending on the level of transgene expression, these mice display growth retardation and decreased fertility [90, 91]. Various combinations of *Sod2*, cytosolic *Sod1*, or catalase transgenes also fail to extend the lifespan of mice [92].

To make matters more complicated, recent publications draw a correlation between high oxidative stress and lifespan extension. Mice heterozygous for the mitochondrial glutathione peroxidase 4 or the ubiquinone biosynthesis protein CLK1 display high levels of mitochondrial oxidative stress, yet live longer than their wild-type counterparts [93, 94]. These results correlate with those from *Caenorhabditis elegans*, in which inactivation of *clk-1* or *sod-2* is associated with increased lifespan [95, 96]. Furthermore, in *C. elegans*, lifespan extension mediated by glucose restriction is associated with increased mitochondrial metabolism and ROS production. Treatment of worms with antioxidants abolished this life extension [97].

Thus, there is no unified model of mitochondrial ROS as a detrimental or beneficial agent in biology. The term hormesis is used to describe nonlethal stress doses that induce stress responses and an adaptive, beneficial effect on organismal fitness. This theory can be applied to mitochondrial ROS [97], and indeed, part of the fitness of *Clk1*+/− mice is attributed to the

ability of mitochondrial ROS to modulate HIF function and inflammation [98]. Based on the entirety of published observations, we propose a new view of mitochondrial ROS in which low levels of ROS are required for cellular processes such as proliferation and differentiation. Cellular stress can cause increased ROS levels, which can promote adaptation and possibly organismal fitness. Even higher levels of ROS will trigger senescence or cell death. Levels of ROS that cause irreversible damage to cellular proteins, DNA, or lipids might only be seen under super-physiological conditions such as UV irradiation or direct treatment of cells in culture with oxidants. Thus, future work might center on the modulation (as opposed to focusing on reduction) of mitochondrial ROS production to achieve a beneficial therapeutic outcome.

Box 1

Mitochondrial complex III produces superoxide through the Q-cycle

Mitochondrial complexes I and II transfer two electrons to coenzyme Q (ubiquinone, Q), forming reduced coenzyme Q (ubiquinol, QH_2). These electrons are transferred from ubiquinol to cytochrome *c* at mitochondrial complex III. As cytochrome *c* is only capable of reduction by a single electron, complex III function occurs through a bifurcated process termed the Q-cycle. Complex III binds coenzyme Q at two sites, the intermembrane space-proximal Q_o site, and the matrix-proximal Q_i site. Complex III receives ubiquinol at the Q_o site. The Rieske iron-sulfur protein (RISP) then removes one electron from ubiquinol and transfers it to cytochrome c_1, from which it is then transferred to cytochrome *c*, and finally to complex IV (cytochrome oxidase). This one electron oxidation of ubiquinone results in the transient formation of ubisemiquinone (QH•).

The remaining electron of ubisemiquinone is then transferred by RISP to cytochrome *b* where it passes through two heme groups (b_L and b_H), before it is subsequently used to reduce another molecule of ubiquinone at the Q_i site, forming ubisemiquinone. After a second round of this cycle, coenzyme Q at the Q_i site is fully reduced and can then be oxidized at the Q_o site. By oxidizing coenzyme Q on the intermembrane space side of the inner membrane, and reducing it on the matrix side, complex III produces a net flux of protons from the matrix to the intermembrane space.

Ubisemiquinone formed at the Q_o site of complex III is capable of donating its free electron directly to oxygen, forming superoxide. Genetic targeting of RISP expression prevents formation of ubisemiquinone at the Q_o site, rendering cells both respiratory incompetent and incapable of superoxide formation at complex III. Mutation of cytochrome *b* severely decreases electron flow through complex III, rendering cells respiratory incompetent. However, the presence of RISP allows for ubisemiquinone formation and superoxide is formed in cells lacking cytochrome *b*activity.

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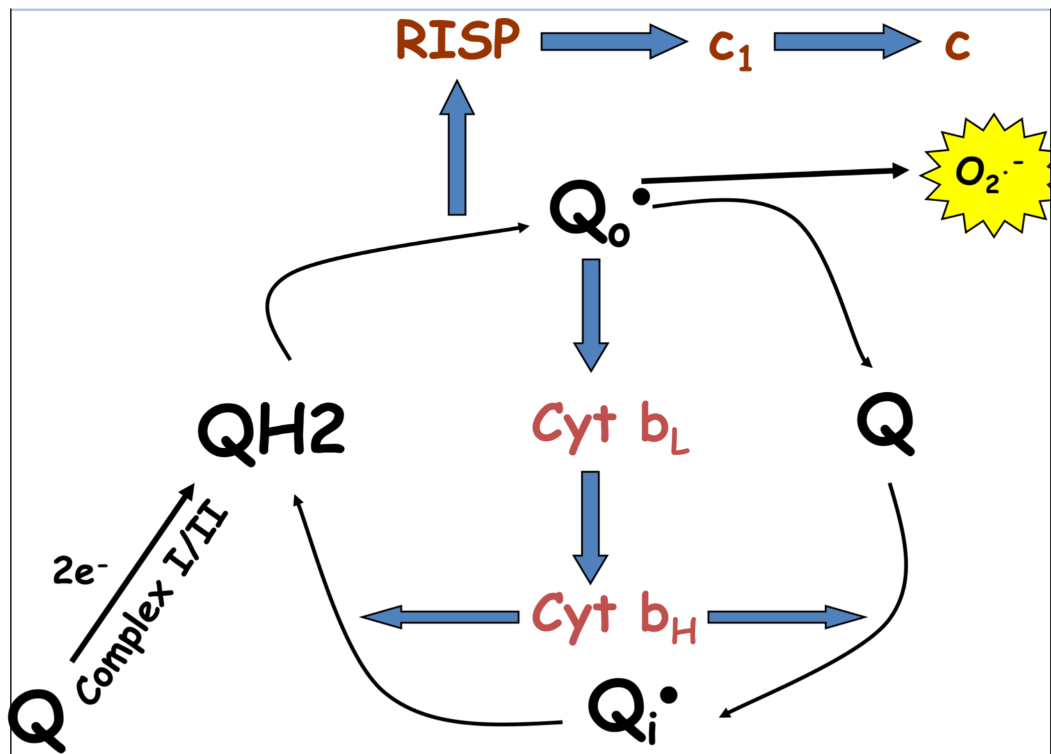

Figure I.
Mitochondrial complex III produces superoxide through the Q-cycle.

Box 2

Regulation of phosphatases by hydrogen peroxide-mediated oxidation of cysteine residues

The catalytic cysteine of phosphatases has a low pK_a, allowing the cysteine thiol group (SH) to exist as a thiolate anion (Figure Ia, S−). Thiolate anions are better nucleophiles than thiols, allowing these catalytic cysteines to participate in dephosphorylation reactions, but also making them more susceptible to oxidation than thiols. Hydrogen peroxide (H_2O_2) readily oxidizes thiolate, yielding sulfenic acid (Figure Ib, SO−), inhibiting enzyme activity. Under high concentrations of H_2O_2, SO− can undergo further oxidation to generate sulfinic (Figure Ic, SO_2−) and sulfonic (Figure Id, SO_3−) acids. Sulfinic and sulfonic acids represent irreversible oxidative modifications, preventing reactivation of the phosphatase. A common mechanism to prevent irreversible oxidation of catalytic cysteines is to encorporate the SO − intermediate into a disulfide (S-S) bond or into a sulfenic-amide (S-N) bond. Disulfides are formed by reaction of SO− with either an inter- or intra- molecular cysteine (Figure Ie, f), or with glutathione (Figure Ig). Sulfenic-amide bonds are formed by nucleophilic attack of the backbone nitrogen atom of the adjacent residue on SO− (Figure Ih). The actions of glutathione reductase (GRX) or thioredoxin (TRX) restores the oxidized enzymes back to their reduced state.

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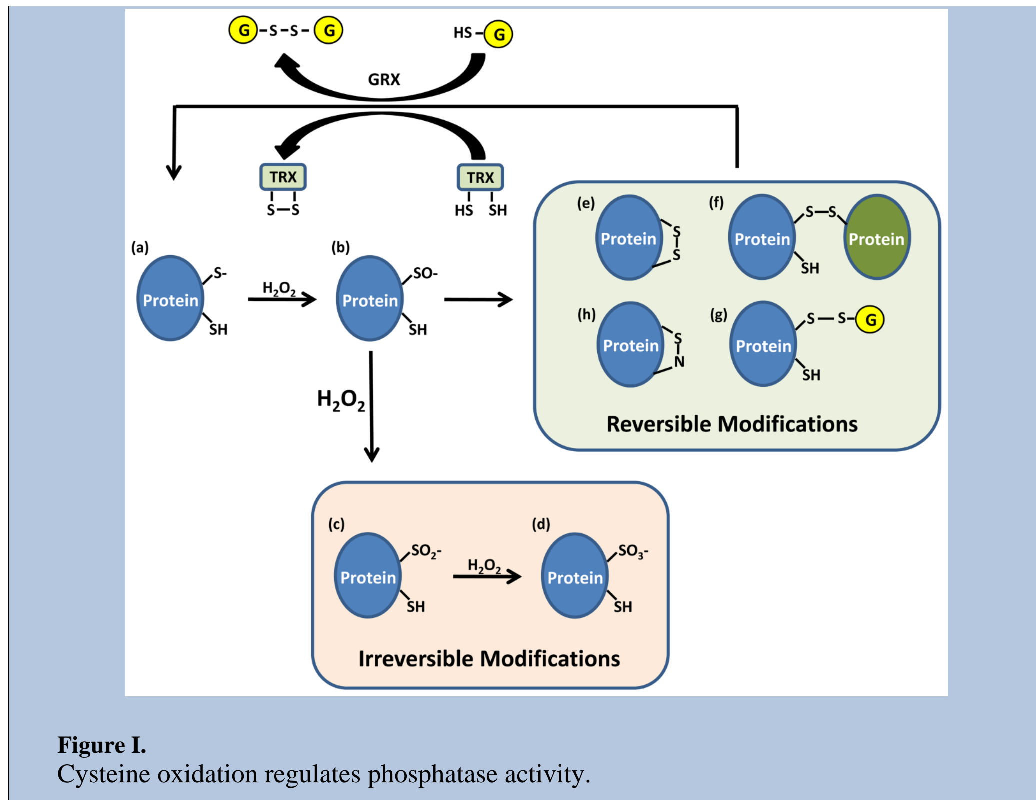

Figure I.
Cysteine oxidation regulates phosphatase activity.

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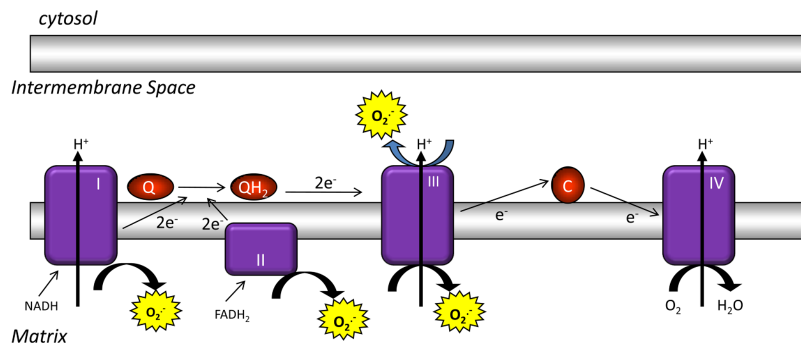

Figure 1. The mitochondrial electron transport chain produces ROS

Mitochondrial complexes I and II use electrons donated from NADH and $FADH_2$ to reduce coenzyme Q. Coenzyme Q shuttles these electrons to complex III, where they are transferred to cytochrome *c*. Complex IV uses electrons from cytochrome *c* to reduce molecular oxygen to water. The action of complexes I, III, and IV produce a proton electrochemical potential gradient, the free energy of which is used to phosphorylate ADP at ATP synthase. Complexes I, II, and III produce superoxide through the incomplete reduction of oxygen to superoxide. Whereas complexes I and II produce superoxide only into the mitochondrial matrix, complex III produces superoxide into both the matrix and the intermembrane space.

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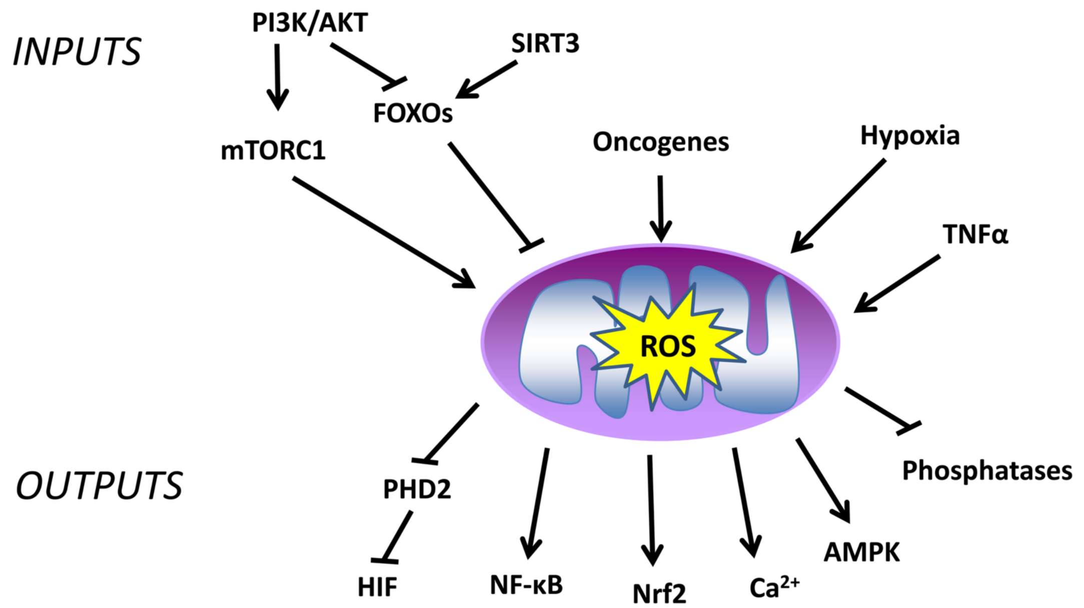

Figure 2. Signaling inputs and outputs of mitochondrial ROS signaling

There are multiple inputs that regulate the generation of mitochondrial ROS (e.g., hypoxia, PI3K, TNFα, and oncogenes). These ROS activate multiple outputs including phosphatases, transcription factors, and kinases.

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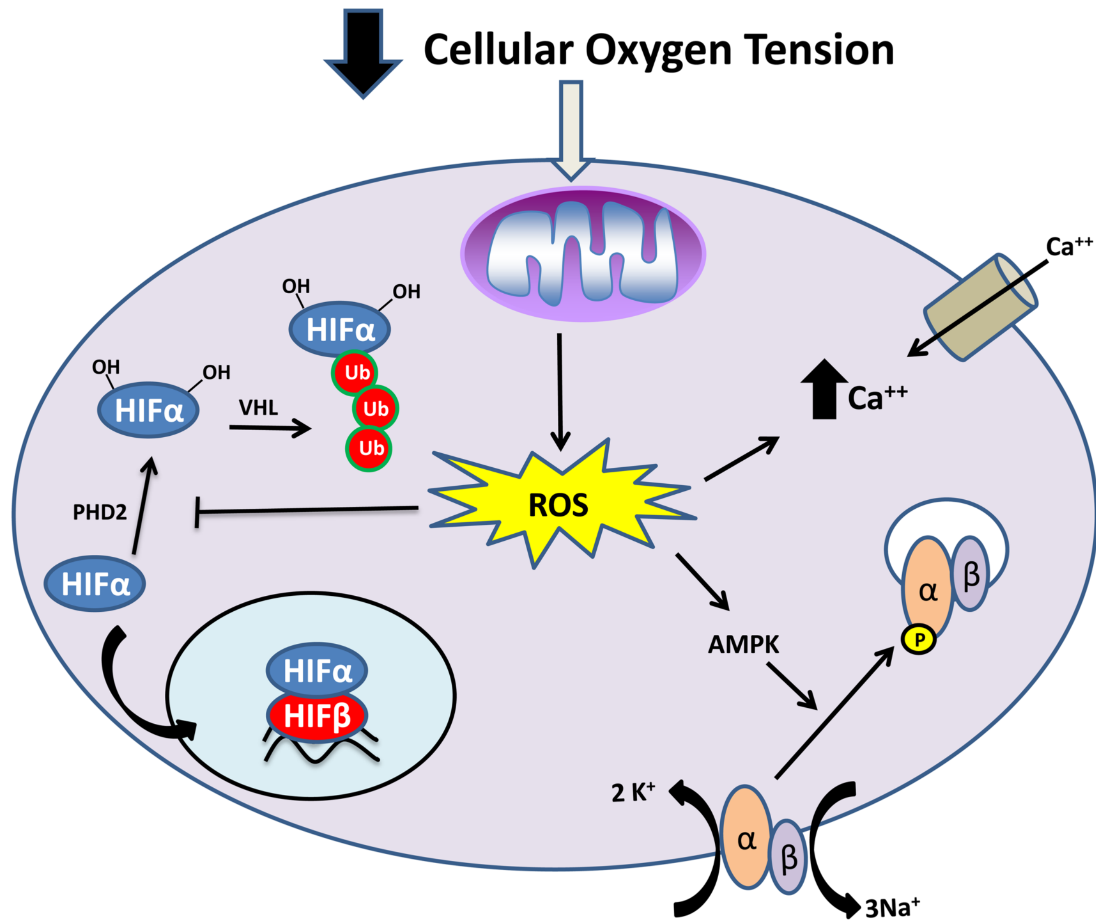

Figure 3. Mitochondrial ROS regulate the cellular response to hypoxia

Hypoxia leads to an induction in the production of mitochondrial ROS. These ROS inhibit the activity of PHD2, leading to stabilization of HIFα subunits (blue) and transcriptional activation. Mitochondrial ROS generated during hypoxia regulate increases in cellular calcium uptake and contraction of pulmonary arteries. Mitochondrial ROS also lead to activation of AMPK, allowing increased cellular energy conservation. AMPK phosphorylates the α-subunit (peach) of the Na/K ATPase leading to endocytosis.

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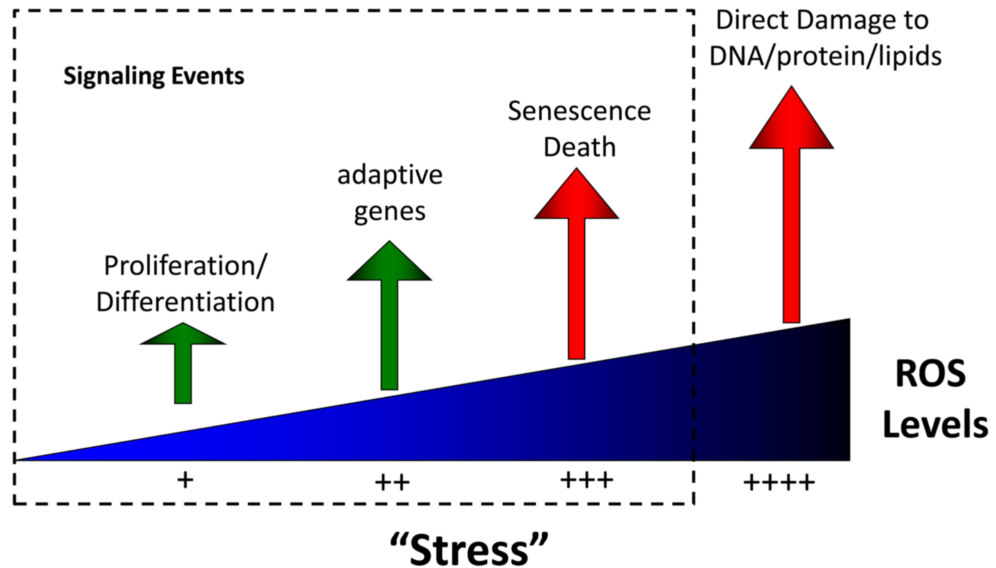

Figure 4. Mitochondrial ROS levels are crucial for biological outcomes
Low levels of mitochondrial ROS production are required for cellular processes such as proliferation and differentiation. An induction in ROS production will lead to adaptive programs including the transcriptional upregulation of antioxidant genes. Even higher levels of ROS will signal the initiation of senescence and apoptosis. Non-signaling, irreversible damage to cellular components is only observed under the highest levels of cellular ROS.