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Review

The H⁺-ATP synthase: A gate to ROS-mediated cell death or cell survival[☆]

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ABSTRACT

Cellular oxidative stress results from the increased generation of reactive oxygen species and/or the dysfunction of the antioxidant systems. Most intracellular reactive oxygen species derive from superoxide radical although the majority of the biological effects of reactive oxygen species are mediated by hydrogen peroxide. In this contribution we overview the major cellular sites of reactive oxygen species production, with special emphasis in the mitochondrial pathways. Reactive oxygen species regulate signaling pathways involved in promoting survival and cell death, proliferation, metabolic regulation, the activation of the antioxidant response, the control of iron metabolism and Ca²⁺ signaling. The reversible oxidation of cysteines in transducers of reactive oxygen species is the primary mechanism of regulation of the activity of these proteins. Next, we present the mitochondrial H⁺-ATP synthase as a core hub in energy and cell death regulation, defining both the rate of energy metabolism and the reactive oxygen species-mediated cell death in response to chemotherapy. Two main mechanisms that affect the expression and activity of the H⁺-ATP synthase down-regulate oxidative phosphorylation in prevalent human carcinomas. In this context, we emphasize the prominent role played by the ATPase Inhibitory Factor 1 in human carcinogenesis as an inhibitor of the H⁺-ATP synthase activity and a mediator of cell survival. The ATPase Inhibitory Factor 1 promotes metabolic rewiring to an enhanced aerobic glycolysis and the subsequent production of mitochondrial reactive oxygen species. The generated reactive oxygen species are able to reprogram the nucleus to support tumor development by arresting cell death. Overall, we discuss the cross-talk between reactive oxygen species signaling and mitochondrial function that is crucial in determining the cellular fate. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

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Abbreviations: AIF, apoptosis inducing factor; Akt, v-Akt murine thymoma viral oncogene; AP-1, activator protein 1; ARE, antioxidant responsive element; ASK1, apoptosis signal-regulated kinase 1; ATM, ataxia telangiectasia mutated; Duox, Dual oxidase enzymes; DUSP3, dual-specific phosphatase 3; ETC, electron transport chain; GPXs, glutathione peroxidases; GSH, glutathione; GST, glutathione S-transferase; HIF1 α , Hypoxia Inducible Factor 1; HO1, heme oxygenase-1; IER3, immediate early response gene; InsP3R, InsP3 receptor; IF1, ATPase Inhibitory Factor 1; IRE, iron-responsible elements; IRP, iron regulatory protein; JNK1, c-Jun N-terminal kinase 1, monoamine oxidase (MAO); mROS, mitochondrial reactive oxygen species; NF κ B, nuclear factor kappa-light-chain-enhancer; NOX, NADPH oxidase; Nrf2, NFE2-like 2; O₂⁻, superoxide radical; [•]OH, hydroxyl radical; OONO, peroxynitrite; OXPHOS, oxidative phosphorylation; p66Shc, 66 kDa proto-oncogene; SERCA, sarco/endo-plasmic reticulum Ca²⁺-ATPase; Src, homologous-collagen homologue (Shc) adaptor; PI3K, phosphoinositide-3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PP2A, protein phosphatase 2A; PRXs, peroxiredoxins; PTEN, phosphatase and tensin homolog; PTP, permeability transition pore; PTP1b, phosphotyrosine protein phosphatase; Ref-1, redox factor-1; RNS, reactive nitrogen species; ROS, reactive oxygen species; RyR, ryanodine receptor; SODs, superoxide dismutases; UTR, untranslated region; VHL, von Hippel-Lindau; $\Delta\psi/m$, mitochondrial membrane potential

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1. Introduction

Oxidative stress is a phenotypic trait of many tumors. Main causes of this phenotype are the increased generation of reactive oxygen species (ROS) and the dysfunction of the antioxidant systems in cancer cells. ROS generation and scavenging are tightly connected to the metabolic state of the cell and especially to the activity of mitochondria. Nowadays, it is accepted that the roles played by cellular ROS are highly dependent on the level at which they are being produced. In this regard, it has been reported that high levels of ROS lead to increased cell death inhibiting tumorigenesis and metastasis [1], whereas low levels of ROS have an effect in promoting tumorigenesis by activating the signaling pathways that regulate proliferation, angiogenesis and metastasis [2,3], stressing the relevance of ROS as important signaling molecules that regulate cell fate. In this review we will briefly summarize: (i) the sites of production, mechanism of action and signaling pathways that are activated by ROS and (ii) the role of the mitochondrial H⁺-ATP synthase in ROS-signaling cell death or cell survival paying, in the latter case, special attention to the new physiological function unveiled for the

ATPase Inhibitory Factor 1 (IF1) as a main regulator of the oncogenic phenotype in some prevalent carcinomas.

2. ROS dynamics and signaling

2.1. Major cellular sites of ROS production

Most intracellular ROS are derived from the superoxide radical (O_2^-), which is the product of the one electron reduction of O_2 (Fig. 1). Superoxide is then converted to hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD1, SOD2 and SOD3) (Fig. 1). The enzymes peroxiredoxins (PRXs), glutathione peroxidases (GPXs) and catalase are responsible for removing cellular H_2O_2 (Fig. 1), a process that is tightly regulated [4]. H_2O_2 can also react with iron to generate hydroxyl radicals ($\cdot OH$) that are main drivers of the modifications in proteins, lipids and DNA that result in oxidative stress (Fig. 1).

Several enzymes produce superoxide radical in the cell. Among them, NADPH oxidase is the best described enzymatic source of superoxide that uses NADPH as an electron donor (Fig. 1) [5,6]. NADPH oxidases include the Nox family members (Nox1–5) and the Dual oxidase enzymes (Duox1–2) that are expressed in numerous tissues [6–8]. These enzymes play important roles in cell signaling, regulation of gene expression, cell death, differentiation and growth [9]. Nox enzymes have developed different regulatory mechanisms depending on their function [6,8,10–12]. ROS produced by Nox proteins can act both intra- and extra-cellularly. These enzymes generate superoxide at the plasma membrane, in endosomes and in the endoplasmic reticulum [13,14]. ROS produced by Nox2 have a main physiological role in the respiratory burst that occurs in phagocytes. Nox1 in the colon and Duox1 and 2 in the lung also play important roles in host defense [15]. However, ROS derived from Nox also participate in signaling as they can specifically and reversibly alter the activity, localization and half-life of proteins in response to various stimuli [9]. The phosphoinositide-3-kinase (PI3K) [16] and nuclear factor kappa-light-chain-enhancer of activated cell (NFkB) [13] pathways are two important signaling routes in which NADPH oxidases are involved. Fibroblasts over-expressing Nox1 displayed increased levels of superoxide and exhibited a transformed phenotype [17]. Moreover, it has been described that Nox1

signals angiogenic and tumorigenic effects through hydrogen peroxide [18]. Excess ROS produced by Nox5 have also been related to cancer [19,20].

A substantial portion of cellular ROS is generated in mitochondria. There are eight sites in mitochondria that have the ability to produce ROS [21]. The mitochondrial electron transport chain (ETC) is the major site of non-enzymatic formation of superoxide radical (Fig. 2). The ETC is composed of four multiprotein complexes (I–IV) located in the inner mitochondrial membrane. Complexes I, II and III have the ability to produce superoxide as a result of the flux of electrons through the ETC. Complexes I, II and III produce ROS within the mitochondrial matrix whereas complex III also generates ROS and releases it into the intermembrane space (Fig. 2) [22]. Importantly, ROS generated in the intermembrane space are supposed to access the cytosol in a faster way which may confer them signaling advantages [3,23]. ROS are released to the cytosol through voltage-dependent channels that are constituents of the permeability transition pore (PTP) and by the inner membrane anion channel (IMAC) [24,25]. The transition of ROS from mitochondria to the cytosol is crucial in the regulation of programmed cell death geared by mitochondria [25,26].

Other important sources of mitochondrial ROS (mROS) are p66Shc and monoamine oxidase (MAO) (Fig. 2). The protein p66Shc plays key roles in the oxidative stress response by inducing apoptosis under stressful conditions (Fig. 2) [27]. p66Shc acts as a redox protein due to its capability to interact and oxidize cytochrome c (Fig. 2) [28]. MAO is a flavoenzyme bound to the outer mitochondrial membrane that catalyzes the oxidative deamination of neurotransmitters and monoamines. MAO represents a significant source of ROS production in brain mitochondria where it has been shown to generate ROS in a much higher amount than the respiratory chain [29]. In fact, MAO is involved in multiple neuropathologies and myocardial diseases and its inhibition is likely to provide a promising target for the relief of the oxidative stress that is associated with these pathologies [30].

The overproduction of ROS in response to metabolic stress triggered by hypoxia or chemotherapy promotes an oxidative stress that has been invariably linked to multiple pathologies including neurodegenerative diseases, diabetes, cancer and premature aging [3]. Nowadays, it is indubitable that mROS are important signaling intermediates in the

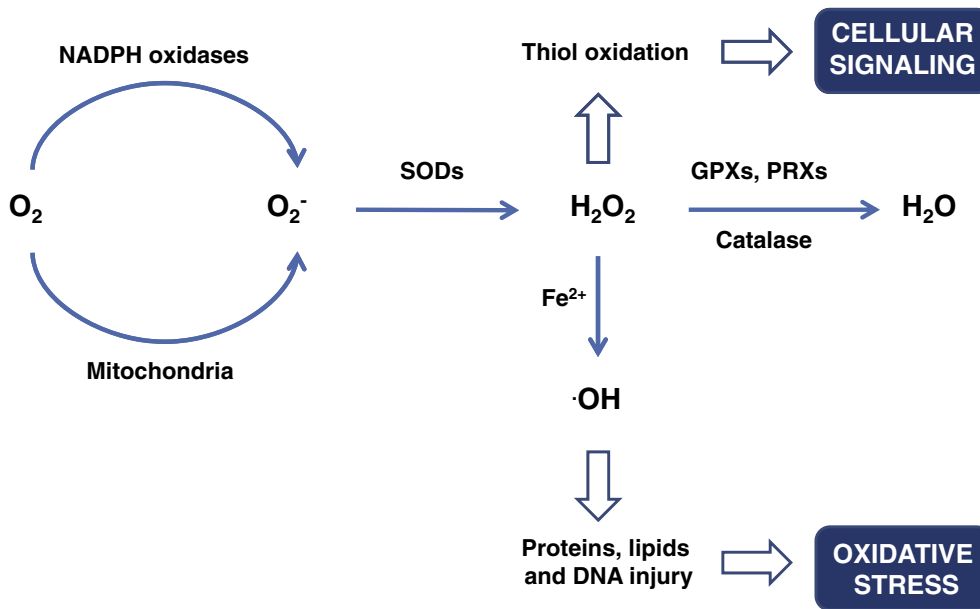


Fig. 1. The metabolism of oxygen. Superoxide is mainly produced by NADPH oxidases and by the mitochondrial respiratory chain. Superoxide is converted to hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs). H_2O_2 is converted to water (H_2O) by glutathione peroxidases (GPX), peroxiredoxins (PRX) or catalase. H_2O_2 is the main player in ROS cellular signaling because it can promote posttranslational modifications in proteins by thiol oxidation. The reaction of H_2O_2 with iron (Fe^{2+}) generates hydroxyl radicals ($\cdot OH$) that are responsible for lipid, protein and DNA damage, promoting oxidative stress.

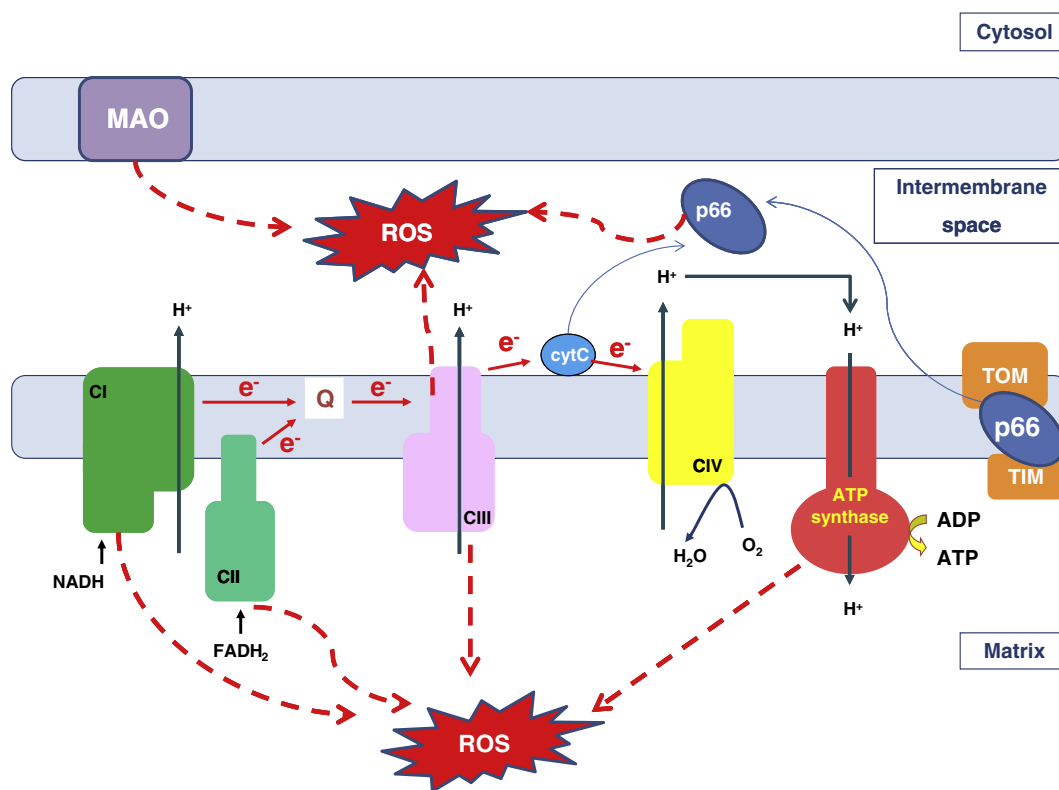


Fig. 2. Overview of mitochondrial ROS production. The scheme shows the relevant sites of ROS production by mitochondria. The transfer of electrons obtained by the oxidation of NADH and FADH₂ to molecular oxygen by respiratory complexes in the inner mitochondrial membrane is depicted by continuous red lines. The formation of the proton gradient generated by respiration and its utilization for the synthesis of ATP by the H⁺-ATP synthase in oxidative phosphorylation is indicated. Complexes I, II and III produce superoxide (discontinuous red lines, ROS) and release it in the matrix. Complex III also releases ROS into the intermembrane space. The activity of the H⁺-ATP synthase also modulates the levels of ROS produced by mitochondria. ROS are also generated by the redox protein p66Shc which interacts with TOM-TIM protein import complexes and with cytochrome c (cyt c). MAO, which is located in the outer mitochondrial membrane, represents an additional source of ROS in mitochondria.

communication of the organelle with other compartments and cellular processes for maintenance of homeostasis under different conditions and for adaptation to stress [3]. In fact, mROS can balance between survival and cell death in a process that is highly dependent on the levels at which they are being produced [31]. For instance, the mitochondrial release of H₂O₂ in hypoxia activates the transcription factor hypoxia inducible factor 1 (HIF1 α), which is required for metabolic adaptation under low oxygen tension [32,33]. In addition, the mitochondrial release of H₂O₂ has been reported to activate key signaling proteins such as c-Jun N-terminal kinase 1 (JNK1), p53 and NF κ B [34–36]. Numerous reports highlighting the importance of mROS-dependent signaling in a variety of systems and processes have emerged in the literature of the last decade [3,31,37–39].

It is well established that mROS production is highly dependent on the proton motive force as has been shown by titration of the mitochondrial membrane potential ($\Delta\psi/m$) with uncouplers [40–42]. Perhaps, the best characterized site of ROS production in mitochondria is at complex III of the ETC (Fig. 2) [43]. The production of superoxide during forward electron transfer at complex III is low but significantly increases by reverse electron flow under conditions of hypoxia [44] or cellular toxicity [40,42] from substrates that feed electrons to complex II. The fact that mitochondria produce more ROS under low oxygen levels is intriguing because it seems to contradict the dependence of mROS formation on the availability of oxygen that was observed in isolated mitochondria [43,45]. It is likely that mROS generated in cells in response to hypoxia depend on additional factors of the hypoxic cellular environment by mechanisms that remain to be elucidated (see [43] for details). In general, cellular conditions that slow-down the rate of electron transfer to molecular oxygen in the respiratory chain (Fig. 2) at high values of $\Delta\psi/m$ favor the generation of superoxide radical in mitochondria. One

such situation is provided by inhibition of the back-flow of H⁺ through the H⁺-ATP synthase (Fig. 2) that is mediated by the over-expression of IF1 in human carcinomas [38,46–48] (see following section). In this situation, $\Delta\psi/m$ provides a link between energy metabolism, ROS production and cell fate [47].

2.2. Cysteine oxidations regulate the activity of ROS transducers

The regulation of signaling pathways by ROS is exerted by their ability to promote reversible posttranslational modifications of proteins [42,49,50]. Hydrogen peroxide is more stable than superoxide radical and is also capable of crossing biological membranes so the protein modifications mediated by ROS are predominantly H₂O₂-dependent. However, superoxide itself has been shown to be involved in aging related processes [51]. H₂O₂ can reversibly oxidize thiol groups (–SH) of redox-reactive cysteine (Cys) residues on proteins to form disulfide bonds (–S–S–) or sulfenic acid (–SOH), the latter can be further oxidized to sulfinic (–SO₂H) and sulfonic (–SO₃H) acids (Fig. 3) [31,52]. Sulphinic acid (–SOH) can react with glutathione (GSH) to become glutathionylated (–SSG) (Fig. 3). These oxidative modifications result in changes in the structure of the targeted proteins thereby affecting its activity in the signaling pathways in which they are involved. In addition, the generation of intermolecular disulfide bonds can promote the homo- and/or heterodimerization of proteins that lead to the activation or repression of the signal transducers. Thiol groups can also react with reactive nitrogen species (RNS) forming S-nitrosothiol groups (–SNO) (Fig. 3). With the exception of sulfonic and sulfinic acids that are essentially irreversible reactions the modification of cysteines is reversible by the action of the reducing systems of the cell such as glutathione, thioredoxin and peroxiredoxin (Fig. 3) that are critical

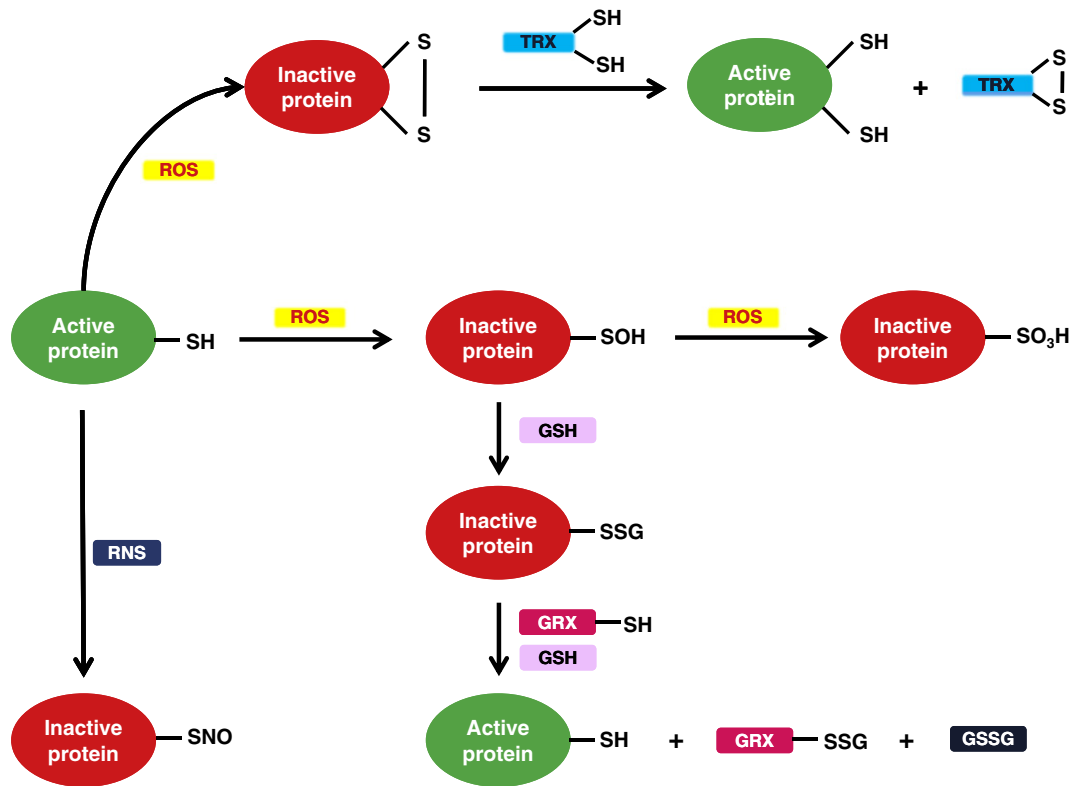


Fig. 3. Regulation of protein activity by ROS. Proteins are regulated by ROS through the oxidation of thiol groups ($-SH$) of redox-reactive cysteine residues. Oxidation of these residues by ROS can form reactive sulfenic acid ($-SOH$) that can undergo further oxidation to sulfonic ($-SO_3H$) acid or form disulfide bonds with nearby cysteines ($-SS-$). These modifications lead to the inactivation of the proteins. Disulfide bonds can be reduced by thioredoxin (TRX) reductase that recovers the activity of the protein. Sulphenic acid ($-SOH$) can also react with glutathione (GSH) becoming glutathionylated ($-SSG$). Protein activity can be recovered by the action of glutaredoxin (GRX) that recognizes glutathionylated substrates and utilizes glutathione (GSH) for the reduction of the $-SSG$ groups. Thiol groups can also react with reactive nitrogen species (RNS) to form S-nitrosothiol groups ($-SNO$) that also trigger protein inactivation.

elements in redox sensing and signaling (Fig. 3) [53–55]. Phosphatases such as phosphotyrosine protein phosphatase (PTP1b), phosphatase and tensin homolog deleted on chromosome ten (PTEN) and MAPK phosphatase are known examples of proteins involved in signaling pathways inactivated by H_2O_2 oxidation of cysteines [56,57]. Superoxide also reacts with nitric oxide (NO), forming highly reactive and potentially damaging peroxynitrite ($OONO^-$) [58]. The formation of peroxynitrite from O_2^- can lead to reversible glutathionylation of proteins on reactive cysteines, as has been reported for the Na^+-K^+ ATPase [59]. The inactivation of aconitase by reaction of superoxide with $(FeS)_4$ clusters provides an example of the inhibition of the activity of a metabolic enzyme mediated by the interaction of O_2^- and iron [60,61].

2.3. Overview of signaling pathways regulated by ROS

Low or transient ROS levels can activate kinases and/or inhibit phosphatases involved in a wide variety of cell signaling processes by oxidizing critical cysteine residues of the proteins (Fig. 4) [62,63]. Proteinases and matrix metalloproteins have also been described as ROS targets [64]. Thioredoxin that catalyzes the reversible reduction of disulfides to a dithiol in ROS targeted proteins (Fig. 3) interacts in its reduced state with apoptosis signal-regulated kinase 1 (ASK1) that is activated under oxidative stress (Fig. 4) [65]. Thioredoxin–ASK1 interaction blocks oligomerization of ASK1 and its subsequent activation [66,67]. When thioredoxin is oxidized by ROS, it disassociates from ASK1 allowing protein oligomerization and subsequent activation through auto-phosphorylation [68]. This kinase mediates apoptosis by regulating the JNK and p38 MAPK pathways [69]. The regulation of differentiation [70] and immune signaling [71] mediated by ASK1 through the p38 MAPK pathway are other important biological effects triggered by ROS. Importantly, thioredoxin is also involved in the regulation of AMPK

activity by preventing the oxidation of cysteine residues in the α subunit of the metabolic stress kinase [72]. This elegant study links oxidative stress and metabolism demonstrating that a reducing enzyme is a critical cofactor controlling the activation of AMPK, a key regulator of metabolism and cell survival in situations of energy stress [72] (Fig. 4).

The best-characterized example of ROS-mediated inactivation of phosphatases by the oxidation of the active site cysteine residue of the enzyme is that of protein tyrosine phosphatase 1b (PTP1b). The inactivation of this enzyme results in the promotion of MAPK and growth factor signaling pathways initiated from different stimulus (Fig. 4) [62,73–76]. Additionally, ROS can inactivate the dual-specific phosphatase 3 (DUSP3) that is another protein tyrosine phosphatase involved in the dephosphorylation of ERK1/2 causing the sustained activation of the ERK1/2 signaling pathway [77] that plays an essential role in cell proliferation, differentiation, invasion, and apoptosis [78–80].

The PI3K pathway which is important for cellular growth, survival and proliferation (Fig. 4) [81] can be affected by the redox state of the cell through different mechanisms [82]. For example, Akt that is a main protein kinase downstream in the pathway has been shown to be activated by H_2O_2 [83]. The target of ROS in the PI3K pathway is the tumor suppressor PTEN, a phospholipid phosphatase that converts PIP3 back to PIP2 thus acting as a negative regulator of the pathway [82,84]. PTEN is inhibited by hydrogen peroxide through disulfide bond formation between the active site cysteine (Cys124) and a vicinal cysteine residue [85–87]. Through PTEN, the PI3K pathway is subject to reversible redox regulation by ROS generated by growth factor stimulation [88]. PTEN oxidation is reversed by cytoplasmic peroxiredoxin II that eliminates the H_2O_2 generated in response to growth factors [85]. Mitochondrial-generated ROS can also inhibit PTEN affecting the angiogenesis process [89]. Protein phosphatase 2A (PP2A) which is involved in the dephosphorylation of Akt [90] and the inactivation of the PI3K/Akt pathway

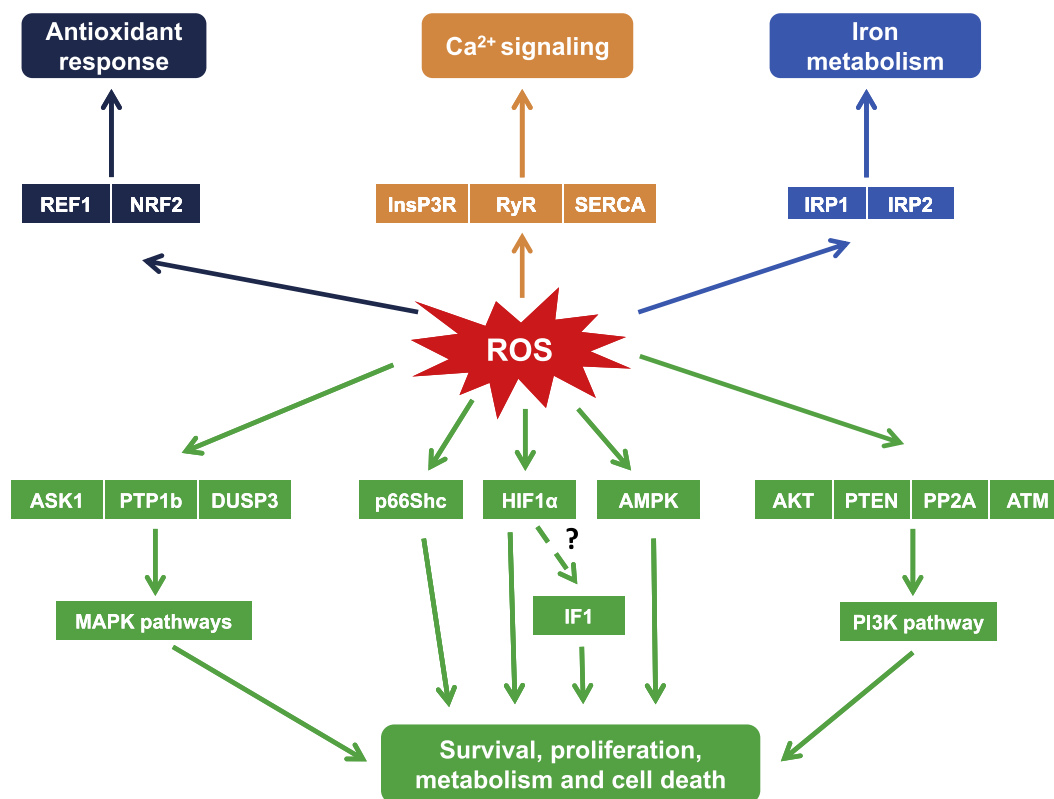


Fig. 4. Signaling pathways regulated by ROS. ROS regulate several signaling pathways with key roles in cellular processes by affecting the activity of critical molecules. ROS can mediate survival, proliferation, metabolism and cell death by regulating the activity of proteins involved in MAPK pathways such as, ASK1, PTP1b, and DUSP3. Proteins participating in the PI3K pathway such as AKT, PTEN, PP2A and ATM are also direct targets of ROS. HIF1 α , p66Shc and AMPK are also regulated by ROS. ROS modulate the antioxidant response through the action of REF1 and NRF2. The iron regulatory proteins IRP1 and IRP2 that are involved in iron homeostasis and InsP3R, RyR and SERCA that participate in the regulation of Ca²⁺ signaling are also targeted by ROS.

[91] is another redox sensitive phosphatase. By inhibiting these important phosphatases the AKT signaling pathway is deregulated promoting uncontrolled cellular proliferation and enhanced survival and growth. Ataxia-telangiectasia mutated (ATM) protein (Fig. 4) is a PI3K-like serine/threonine protein kinase that is activated under stressful conditions and phosphorylates various proteins involved in cellular proliferation, death, survival and DNA repair [92,93]. ATM is preferentially activated by DNA double strand breaks and also acts as a sensor of oxidative stress [94]. ATM protein is also regulated by a redox sensitive mechanism via the formation of active ATM dimers through intermolecular disulfide bond formation (Fig. 3) [95].

The hypoxia inducible transcription factor HIF1 α is also a target of ROS (Fig. 4) [23]. HIF1 α is responsible for the coordination of the cellular responses to decrease oxygen availability [96,97]. During normoxia prolyl hydroxylation of HIF1 α promotes its association with the von Hippel-Lindau (VHL) tumor suppressor that targets the protein for ubiquitination and degradation. However, during hypoxia the hydroxylation of proline in HIF1 α is inhibited due to the inactivation of prolyl hydroxylases. mROS are involved in the stabilization of HIF1 α to promote the transcriptional activity of the protein [32,98–101]. The stabilization of HIF1 α under low oxygen conditions requires the generation of ROS in complex III of the ETC [102]. In other words, the mitochondrial respiratory chain acts as an O₂ sensor that activates a signaling cascade to stabilize HIF1 α through the production of ROS. First evidences illustrating that mROS are involved in the regulation of HIF1 α aroused from studies in cells depleted of mitochondrial DNA (ρ^0 cells) that failed to stabilize HIF1 α under hypoxia [32]. HIF1 α stabilization is blunted by treating the cells with mitochondrial antioxidants emphasizing that mitochondria are the source of ROS under hypoxic conditions [32,33]. Moreover, inhibitors of the mitochondrial electron transport chain that block HIF1 α activation in hypoxia have also been described [103]. The

mROS mediated regulation of HIF1 α is implicated in regulating tumorigenesis by controlling genes involved in metabolism, angiogenesis, and metastasis [97,104].

As discussed previously mROS produced in p66Shc play relevant roles in the activation of the apoptotic pathway and in the regulation of life span [27]. The production of mROS by this protein leads to mitochondrial damage and apoptosis under oxidative or genotoxic stress conditions [105]. The mechanisms that mediate mitochondrial translocation of p66Shc and its proapoptotic activity seem to be controlled by protein phosphorylation [106], although it has also been suggested that ROS production and the initiation of apoptosis by p66Shc are also redox sensitive by formation of two disulfide bonds in the protein (Fig. 4) [107]. The interaction observed between p66Shc with the TOM-TIM protein import complexes [105,108] and with cytochrome c [28] is also a mechanism involved in the proapoptotic function of the protein (Fig. 2).

An important point in the cellular response to increased levels of ROS is the redox regulation of transcription factors that activate the antioxidant defense system (Fig. 4). The transcription factor redox factor-1 (Ref-1) is a multifunctional protein that translocates to the nucleus upon exposure to genotoxic agents and H₂O₂ and initiates a protective response of the cell from DNA and oxidative damage [109,110]. Ref-1 regulates the transcriptional activity of several key transcription factors involved in cellular defense such as activator protein 1 (AP-1), p53, NF κ B and HIF1 α by its redox sensitive cysteine residues [111–113]. The antioxidant genes glutathione S-transferase (GST) [114], NADPH quinone oxidoreductase-1 (NQO1) [115] and heme oxygenase-1 (HO1) [116,117] are regulated by an enhancer termed the antioxidant responsive element (ARE) [118]. H₂O₂ activates transcription of these genes via the ARE element and involves a complex set of redox regulated proteins [119]. The primary transcription factor involved in ARE

activation is the redox-sensitive transcription factor NFE2-like 2 (Nrf2) [120] (Fig. 4) that is translocated into the nucleus under oxidative stress [121]. In the nucleus Nrf2 dimerizes with the small Maf proteins and binds the ARE to activate ARE-dependent transcription of target genes in ROS homeostasis [118,122]. Interestingly, the transcriptional activity of Nrf2 is also regulated by Ref-1 [123] indicating the collaboration of these proteins in the detoxification of the cell under oxidative stress.

Extensive literature has related ROS and Ca^{2+} signaling and their effects on apoptosis, aging and cardiovascular diseases [124] (Fig. 4). ROS can modify the properties and activities of some of Ca^{2+} channels and transporters [125,126]. In fact, some of the proteins involved in Ca^{2+} signaling such as the InsP3 receptor (InsP3R) [127], the ryanodine receptor (RyR) channels [128] and the sarco/endo-plasmic reticulum Ca^{2+} -ATPase (SERCA) [129] have been shown to be sensitive to ROS.

In addition, ROS signaling is also important for the maintenance of iron homeostasis (Fig. 4). Iron is an essential element that plays crucial roles in cell proliferation and metabolism as it represents a functional constituent of various enzymes. Excessive levels of free iron can generate ROS via the Fenton reaction [130,131] promoting deleterious oxidative stress to the cells (Fig. 1). Iron regulatory proteins-1 and -2 (IRP1 and IRP2) regulate the expression of many genes involved in iron transport and storage at the posttranscriptional level by interacting with iron-responsive elements (IRE) in the 5'- or 3'-untranslated region (UTR) of the mRNAs. IRP1 and IRP2 have redox sensitive target sites that are subjected to redox regulation by H_2O_2 and nitric oxide [132–135]. Taken together, the IRE-IRP regulatory system is also regulated by ROS to elicit a defense mechanism against iron-catalyzed oxidative stress.

3. Old and new functions of the H^+ -ATP synthase

3.1. The H^+ -ATP synthase, a core hub in energy and cell death regulation

The oxidation of glucose in the cytoplasm and the subsequent oxidation of pyruvate in mitochondria provide the energy, reducing power and carbon skeletons required for the maintenance of cellular homeostasis and proliferation [47,136]. Normoxic cells oxidize most of the pyruvate to CO_2 in mitochondria and the electrons collected onto NADH and FADH_2 are transferred to the complexes of the respiratory chain to generate the proton electrochemical gradient that is used for the synthesis of ATP in oxidative phosphorylation (OXPHOS) (Fig. 2). ATP is synthesized by the mitochondrial H^+ -ATP synthase, a reversible engine of the inner mitochondrial membrane that provides most of the ATP that is required to maintain cellular activities in normal aerobic differentiated cells [47]. The mammalian H^+ -ATP synthase consists of two main domains: a membrane-bound hydrophobic FO portion, which contains the proton channel, and the soluble catalytic F1 portion that encloses the adenine nucleotide binding sites at the α/β subunit interface [137,138]. Both regions are linked together by a central and a peripheral stalk. In normal aerobic cells under phosphorylating conditions, the re-entrance of protons into the mitochondrial matrix (Fig. 2) triggers the rotation of the c-ring in FO and of the attached central stalk to induce the conformational changes in the β -F1-ATPase subunit that drive the synthesis of ATP (Fig. 2).

The cellular availability of ATP, NADH and some metabolic intermediates coordinates at short-term the flux of glucose consumption by regulating the activity of key enzymes of the glycolytic pathway and mitochondrial dehydrogenases, to limit the production of biological energy as it is being demanded [47]. In other words, the efficient production of biological energy by OXPHOS determines the rate of glucose consumption, which is nowadays formulation of the Pasteur Effect [139]. When the cells have a limited supply of oxygen or have a genetic or epigenetic impairment that restrains OXPHOS, glycolysis is enhanced [47,140]. When short-term regulation of enzyme activities of energy metabolism is not enough to cope with the energetic demand cells onset the gene expression programs required for adaptation. Examples that are

relevant in this regard are the induction of glycolysis during adaptation to hypoxia [141], the rewiring of metabolism in cancer [142] and in differentiation of somatic cells [143], the onset of the bioenergetic function of mitochondria during adaptation of mammals to the aerobic extrauterine environment [144,145] and the metabolic reprogramming that accompanies stem cell differentiation [146,147].

3.2. The H^+ -ATP synthase in signaling cell death

Down-regulation of oxidative phosphorylation (OXPHOS) and the concurrent activation of aerobic glycolysis are hallmarks of proliferating cancer cells [140,148]. Whereas the increase of glycolysis in the majority of carcinomas is nowadays out of question, the role of OXPHOS modifications in tumor development and progression is still debated [47]. Nevertheless, it has been shown that a dysfunctional OXPHOS promotes cellular proliferation and invasion [47,149] whereas an increase in oxidative metabolism halts cellular proliferation and tumor progression [47,140,150,151]. In this regard, the activity of OXPHOS has been demonstrated to be specifically required for the execution of cell death [47,152–154]. In particular, molecular components that participate in OXPHOS, such as cyt c, AIF and subunits of the H^+ -ATP synthase are needed for the execution of cell death [40,155–158]. Hence, bioenergetics and cell death are two master tasks of mitochondria that are molecularly and functionally integrated [47].

The impairment of mitochondrial energy production by metabolic stress and/or in response to chemotherapy leads to increased ROS generation through respiratory chain electron leakage. ROS can signal mitochondrial geared cell-death pathways or activate transcription programs aimed at cell survival, two opposite cellular fates that largely depend on the intensity of the ROS signal [1,31]. The induction of cell death by different stressful conditions promotes $\Delta\psi_m$ collapse that is preceded by transient mitochondrial hyperpolarization [40,156,159] and the subsequent production of mROS which is highly dependent on $\Delta\psi_m$ [40,42,160]. It has been suggested that mROS produced in response to cell death stimulation occurs by reverse electron flow from complex II-linked respiratory substrates into complex I of the ETC (Fig. 2) because its production can be inhibited with rotenone [40,42,161]. Consistent with a role for mROS in the execution of cell death [1,162], extensive protein carbonylation of cellular proteins as well as covalent modifications in mitochondrial proteins have been reported in response to staurosporine treatment [40]. The generation of mROS preceded the release of cyt c, the activation of caspase 3 and cell death [40]. Upon inhibition of mitochondrial respiration with staurosporine [163] it is suggested that the hydrolysis of glycolytic ATP by reverse functioning of the H^+ -ATP synthase maintains $\Delta\psi_m$ [40,164–166]. In this situation, the inhibition of the activity of the H^+ -ATP synthase with oligomycin blunted mitochondrial hyperpolarization and ROS production, prevented the oxidation and modification of mitochondrial proteins, and delayed the release of cyt c and the execution of cell death [40,164].

In contrast to these findings, 1,4-benzodiazepine-derivative Bz-423 signals apoptosis by the induction of ROS production from the mitochondrial respiratory chain as a result of the inhibition of the H^+ -ATP synthase [167]. The antagonistic effects on apoptosis of oligomycin and Bz-423 seem to be dependent on the different mechanism by which these compounds inhibit the enzyme [167]. Similarly, the drug 3,3'-diindolylmethane also promotes ROS-mediated cell death by inhibiting the H^+ -ATP synthase [168]. In any case, these findings support a role for the activity of the H^+ -ATP synthase in controlling the extent of oxidative damage to mitochondrial constituents that will effectively swamp the cells into death [40]. Consistently, the cell death response to different chemotherapeutic agents varies largely depending upon the relative activity of the pathways that sustain energy metabolism (Fig. 5) [40,169]. In fact, highly glycolytic cells with negligible contribution of OXPHOS for ATP provision have a cell-death resistant phenotype because mROS signaling after chemotherapeutic targeting is blunted (Fig. 5) [40,169]. Overall, the down-regulation of the H^+ -

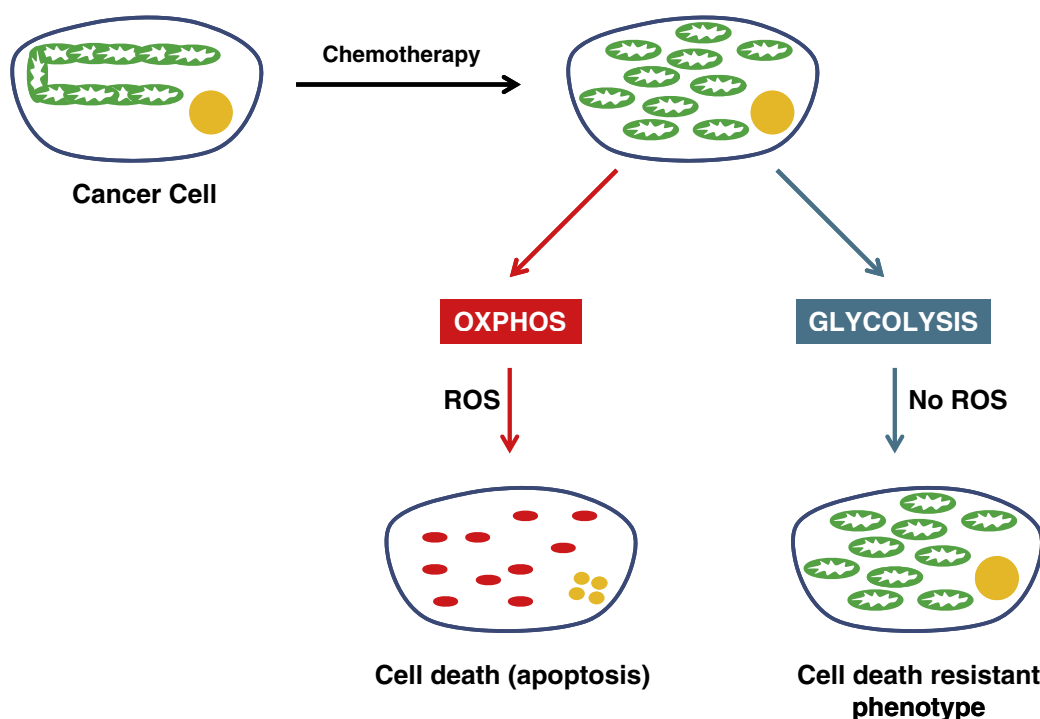


Fig. 5. Energy metabolism determines the cell-death pathway of cancer cells. Susceptibility of cancer cells to death stimulus is highly dependent on their metabolic phenotype. The rapid dismantling and fragmentation of the mitochondrial tubular network into small mitochondria (green) occur as a first response to a death stimulus to chemotherapeutic agents. Tumor cells with a significant activity of oxidative phosphorylation (OXPHOS) will produce high levels of ROS contributing to the oxidation of mitochondrial proteins (red dots) and the release of the mitochondrial arsenal involved in the execution of cell death (fragmented nucleus in yellow). Cells with a diminished activity of OXPHOS and thus an increased activity of the glycolytic pathway will not generate ROS under conventional chemotherapy which results in a resistant cell death phenotype.

ATP synthase, and thus of OXPHOS, is part of the molecular strategy adapted by cancer cells to avoid reactive oxygen species-mediated cell death.

Interestingly, it has been shown that the activity of the H^+ -ATP synthase per se is inhibited by ROS [170,171]. The impact of ROS on the activity of the complex is also observed in chloroplasts where the γ subunit seems to be a main target for ROS oxidation [172]. These findings further emphasize the tightly regulated connections that exist between the H^+ -ATP synthase, energy production, ROS generation and cell death.

The point of no return in cell death is the permeabilization of the inner mitochondrial membrane to low molecular weight solutes, the so-called PTP opening [173–175]. Although the molecular composition of the PTP remains unknown recent findings support that a critical component of the high-conductance channel is subunit c of the H^+ -ATP synthase [176]. Moreover, it has been shown that dimers of the H^+ -ATP synthase form a channel with electrophysiological properties identical to those of the PTP [177]. Non-specific ROS-mediated modifications of mitochondrial constituents could represent a critical point of regulation of the mitochondria-gear cell death pathway. Protein oxidation could define the threshold value of irreversible damage of the mitochondria and the set-point for the release of the mitochondrial arsenal that controls cell death (Fig. 5) [40]. Indeed, oxidative stress promotes cell death by increasing the susceptibility of the opening of the PTP [178]. PTP opening is linked to oxidative stress since it has been shown to be dependent on the NADPH redox state [179,180] and promoted by thiol oxidation [181,182]. Consistently, it has been reported that PTP opening can be stimulated by the addition of exogenous sources of ROS and prevented by antioxidants in pro-apoptotic conditions [180]. Interestingly, PTP opening also increases mitochondrial ROS production illustrating a retro-amplification cascade when the decision to execute cell-death has been taken [183].

Consistent with the tumor suppressor function of mitochondrial activity there is a large body of data supporting that OXPHOS, both under

basal conditions or in response to chemotherapeutic agents abolishes tumorigenicity (see [47] for updated review). A likely mechanism that explains the preferential death of cancer cells when forced to oxidize mitochondrial substrates is the overproduction of superoxide radical as a result of the stimulation of mitochondrial metabolism [40,47,184]. Both genetic [185] and pharmacological [186] studies have shown that the PGC1 α -mediated improvement of mitochondrial activity and metabolism restrains cancer progression by increasing ROS-mediated apoptosis in cancer cells [185].

A diverse set of genetic, epigenetic and environmental mediated mechanisms interfere with mitochondrial bioenergetics of the cancer cell [47,187]. One such mechanism, which is observed in many prevalent human carcinomas, is the down-regulation of the expression of the catalytic subunit of the mitochondrial H^+ -ATP synthase (β -F1-ATPase) relative to the enzyme of glycolysis glyceraldehyde-3-phosphate dehydrogenase [140,188]. This finding has been confirmed and extended to other carcinomas (see [140] for other studies), providing a “bioenergetic signature” of cancer [140,188] of clinical applicability. Indeed, as assessed in large cohorts of colon [188–190], lung [191, 192], breast [193] and ovarian carcinomas [194] and in cells of acute myeloid leukemia patients [195] the altered bioenergetic signature of the tumors predicts a worst overall and/or disease-free survival for the patients. Down-regulation of the bioenergetic signature is also functionally linked to the resistance to chemotherapy in many different cancer cells [47,196,197], in colon cancer patients [190] and in chronic [198] and acute [195] leukemia patients. In general, the bioenergetic signature represents a functional index of metabolic activity of the cells because it correlates, both in vitro [199] and in vivo [192], with the rate of glucose utilization. Overall, these findings emphasize that a diminished bioenergetic activity of mitochondria in the cancer cell predisposes to cancer onset and progression, highlighting the emerging role that the H^+ -ATP synthase plays as a master regulator of cell death [40,156,177,200, 201]. Consistently, cancer progression requires the silencing of the bioenergetic activity of mitochondria [199] not only by down-regulating

the content of the H⁺-ATP synthase as above-discussed but also by over-expressing the natural physiological inhibitor of the enzyme, the so-called ATPase Inhibitory Factor 1 (IF1) [38,46,47].

3.3. The ATPase Inhibitory Factor 1 (IF1)

In mitochondria, ATP hydrolysis by the H⁺-ATP synthase is inhibited by the ATPase Inhibitory Factor 1 (IF1) that reversibly binds to the enzyme (Fig. 6) [202,203]. The natural inhibitor of the H⁺-ATP synthase is a low molecular weight (~10 kDa) mitochondrial protein which inhibited the soluble ATPase, but did not interfere with its coupling activity [204]. IF1 is encoded in the nuclear ATP1F1 gene located on chromosomes 1 and 4 in human and mouse, respectively. IF1 has been described in mammals, birds, amphibious, nematodes, yeast and plants and shows considerable sequence identity among various eukaryotes [205]. It is absent in bacteria and chloroplasts. Three different isoforms of the human protein are produced by alternative splicing. Isoform 1 is the longest one, it codifies for a protein of 106 amino acids (12.2 kDa; pI = 10) which is, by far, the protein more abundantly expressed in most human tissues [46,47]. Isoforms 2 and 3 codify for proteins of 71 (7.9 kDa; pI = 8.5) and 60 (6.6 kDa; pI = 9.3) amino acids, respectively. The inhibitor protein has an N-terminal presequence of 25 residues for targeting the protein into the mitochondrial matrix which is cleaved off after import [206,207].

The interaction between IF1 and the H⁺-ATP synthase depends on the pH of the mitochondrial matrix and is affected by changes in the $\Delta\psi_m$ [202,207]. IF1 binds β , α and γ subunits of the H⁺-ATP synthase due to its activation under low pH conditions blocking ATP hydrolysis and preventing a useless waste of energy [207]. The substitution of histidine 49 in the IF1 sequence by a lysine renders a mutant form of IF1 (H49K) that is as active as IF1 in the inhibition of the ATP hydrolase activity but less sensitive to the regulation by pH [138,202,203,208,209]. The participation of E26 in the pH regulated inhibitory activity of bovine IF1 has also been suggested [210]. Bovine IF1 has been shown to have oligomeric states, tetramer and dimer, favored by pH values above and below 6.7, respectively [202]. Dimerization and activation of IF1 occur by formation of an antiparallel α -helical coiled-coil in its C-terminal region which places the N-terminus (inhibitory regions) of

the monomers at opposite ends of the dimer, allowing the dimeric IF1 to bind two domains of F1-ATPase simultaneously [202]. The structure of the inhibited F1-ATPase complex with bound IF1 in the presence of ATP has been solved and confirms that the N-terminal region of the dimeric inhibitor is bound to F1-ATPase [138]. The inhibitory 42L–58K segment of IF1 has been shown to interact with α/β pair of subunits of the F1-ATPase domain. It is suggested that this interaction inhibits the conformational inter-conversions of the catalytic sites involved in ATP hydrolysis and hence rotatory catalysis [138]. Hence, the mechanism of inhibition of the ATPase by IF1 arises from the disruption of the catalytic site.

The physiological function ascribed to IF1 in normal hypoxic cells is to inhibit the hydrolase activity of the H⁺-ATP synthase [202,203,211,212]. Until recently, the role of IF1 in preserving cellular ATP in myocardial ischemia [209,211] and in ischemic preconditioning [209] has been the most extensively studied functions of IF1. However, the transformation of mitochondria from ATP producers into ATP consumers under depolarizing conditions has been recently questioned [213]. Upon mitochondrial depolarization IF1 has been identified as an essential gene to promote PARK2 recruitment onto mitochondria to establish the selective autophagic program of mitophagy [214]. More recent findings support a relevant role for IF1 in controlling the ferrochelatase activity of mitochondria, and hence heme biosynthesis, in erythroid cells [215]. Surprisingly, a recently ill-defined knockout IF1 mouse model has revealed no alterations in phenotype [216]. The absence of IF1 is known to occur in Luft's disease, a mitochondrial myopathy of the striated muscle [209,217,218]. The disorder is characterized by a hypermetabolic state, mitochondria with densely packed cristae and a loosely coupled OXPHOS [209,217,219], suggesting additional functional roles for IF1 in the regulation of the H⁺-ATP synthase of muscle mitochondria. It has been shown that long rows of dimers of the H⁺-ATP synthase promote the high local curvature of the inner membrane at cristae ridges [220–223]. Interestingly, aging seems to melt down the inner-membrane cristae of mitochondria by age-dependent dissociation of ATP synthase dimers [224]. It has been suggested that IF1 regulates the oligomeric state of the H⁺-ATP synthase increasing the density of cristae and the formation of dimeric ATP synthase complexes [212,225–227]. However, this suggestion has been questioned [228–230].

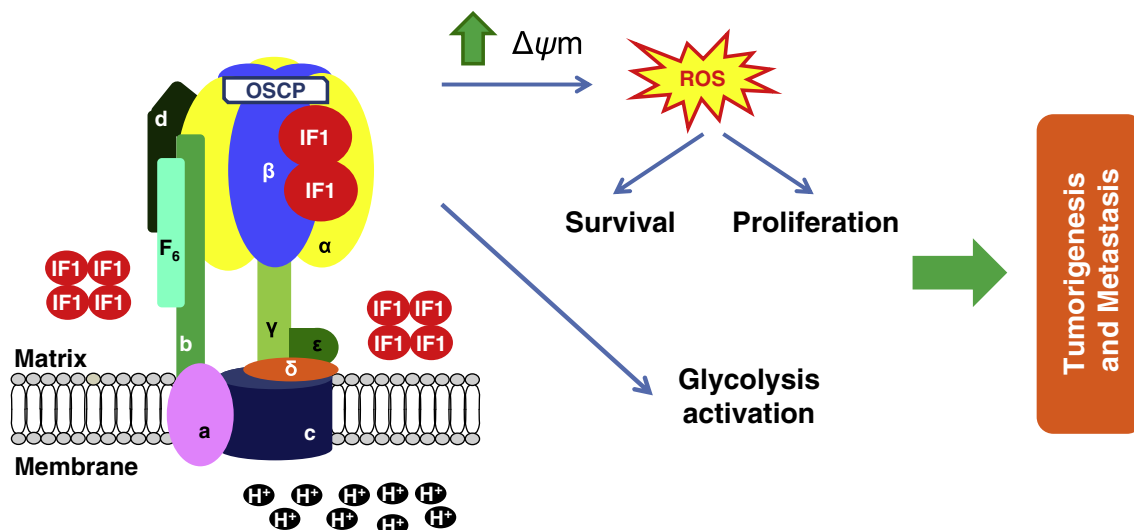


Fig. 6. IF1 mediates the oncogenic phenotype by ROS signaling. The illustration shows a schematic organization of the mitochondrial H⁺-ATP synthase. Several subunits are color-coded and labeled. In tumor cells, the ATPase Inhibitory Factor 1 (IF1) (in red) is highly over-expressed and binds the catalytic α/β interface. This interaction prevents synthesis of ATP triggering metabolic reprogramming towards an enhanced glycolytic phenotype. Inhibition of the H⁺-ATP synthase also promotes an increase in the mitochondrial membrane potential ($\Delta\psi_m$) and the subsequent increase in superoxide radical production (ROS). ROS signaling will activate proliferation and survival pathways in the nucleus of the cell. All these events represent hallmarks of cancer as they promote tumorigenesis and metastasis.

3.4. IF1 is a master regulator of energy metabolism in cancer and in stem cells

A differential expression level of IF1 has been reported between different mouse [231] and human tissues [46,48]. Moreover, cardiomyocytes of low heart rate species (human) have a higher expression level of IF1 than cardiomyocytes of fast heart rate species (rat, mouse) [209,211], which might explain the differential preservation of cellular ATP upon sublethal ischemic episodes. Interestingly, normal colon, lung and breast tissue have negligible expression of IF1 [38,46,48]. In contrast, mitochondria of almost all lung, colon, breast and ovarian carcinomas analyzed in large cohorts of cancer patients show an overwhelming increase in the expression of IF1 [48]. We have demonstrated that the over-expression of IF1 results in the inhibition of the ATP synthetic activity of the H^+ -ATP synthase and the switch of the cells to an enhanced aerobic glycolysis [38,46]. On the contrary, silencing of IF1 enhances the H^+ -ATP synthase activity and reduces aerobic glycolysis, strongly supporting a crucial role for IF1 in mediating the metabolic switch experienced by cancer cells [38,46]. Likewise, IF1 is present in human mesenchymal stem cells (hMSCs) as well as in stem cells of the prostate and in the Lieberkühn crypts of the colon [147]. Consistent with a master role for IF1 in the regulation of energy metabolism we have shown that the regulated degradation of IF1 triggers metabolic rewiring from aerobic glycolysis to a predominant OXPHOS in the differentiation process of hMSCs into osteocytes [147], once again stressing the biological relevance of this protein in regulating energy metabolism of proliferating cells.

3.5. The role of IF1 in ROS signaling cell survival

The IF1-mediated inhibition of the H^+ -ATP synthase results in mitochondrial hyperpolarization and the subsequent production of superoxide radical in colon [38] and other cancer [48] cells (Fig. 6). Therefore, besides the role of IF1 in rewiring energy metabolism [46,232], the over-expression of IF1 also triggers a retrograde ROS signal to the nucleus to establish the appropriate adaptive cellular program needed to support tumor development [38,48]. IF1 mimics the inhibitory effect of oligomycin in reprogramming energy metabolism and in inhibiting apoptosis [40,156]. Remarkably, it has been demonstrated that the ROS-mediated response in colon cancer cells in response to the over-expression of IF1 signals to the nucleus an NF- κ B-dependent adaptation that includes enhanced proliferation, invasion and cell survival [38]. An IF1 ROS-mediated resistance to cell death has also been demonstrated in other cancer cells [48]. The ROS-signaling pathways (Fig. 4) activated in response to IF1 over-expression that arrest cell death in many prevalent human cancer cells still remain to be investigated [48]. An alternative, not mutually exclusive mechanism of action of IF1 to prevent cell death is that it might contribute to stabilize the oligomerization state of the H^+ -ATP synthase to preserve mitochondrial *cristae* impeding in this way the release of cyt c [233]. In this regard, IF1 has been suggested to increase the density of mitochondrial *cristae* by the formation of dimeric ATP synthase complexes [212]. In this situation, IF1 would represent a negative regulator of PTP opening [177] contributing to the evasion of cell death. Consistently, recent findings in a conditional transgenic mouse expressing H49K (a gain-of-function mutant of human IF1) in neurons that also inhibits the H^+ -ATP synthase support a ROS-mediated metabolic and structural pathway to prevent neuronal cell death *in vivo* after an excitotoxic insult [234]. Overall, these findings strongly support that IF1 plays a master role in the regulation of energy metabolism and in retrograde communication to the nucleus other features of the oncogenic phenotype such as cell survival [47,187].

3.6. Regulation of IF1 activity and expression and other IF1 paradoxes

In silico analysis of the promoter region of the human ATP1F1 gene reveals the existence of potential *cis*-acting responsive elements for transcription factors involved in cancer. Data from high-throughput

ChIP-sequencing confirmed the binding of several transcription factors involved in the regulation of cell cycle (NF-YB, NF-YA, In1), proliferation (c-FOS, Sp1, c-MYC) inflammation and cell death (NF- κ B, TAF1) in the proximal promoter region of the ATP1F1 gene. However, the regulation of IF1 expression in human carcinomas is exerted at post-transcriptional levels [48]. In fact, IF1 has a very short half-life (~2 h) in colon cancer cells [48] being degraded by a mitochondrial protease [147]. However, the mitochondrial protease involved in IF1 degradation has not been identified despite our recent attempts using a large siRNA screen [147]. It has been suggested that the hypoxia regulated transcription factor HIF1 α participates in controlling IF1 expression (Fig. 4) [235]. Moreover, it has been shown that the immediate early response gene (IER3) binds the C-terminus of IF1 to render the protein prone to proteolytic digestion in HeLa cells [236]. However, recent findings in different cancer cell lines seem to exclude the participation of HIF1 α [48] and of IER3 [48] in controlling IF1 expression in colon, lung and breast carcinomas.

Paradoxically, tissues with very high activity of OXPHOS (liver, muscle, neurons) show a very high content of IF1 [38,46,48,234], which would imply the partial IF1-mediated inhibition of the H^+ -ATP synthase in these tissues, which is obviously not the case. As recently pointed out [48], these findings suggest that besides the well characterized pH controlled binding of IF1 to β -F1-ATPase [202], an additional mechanism should regulate the biological activity of IF1 specially in tissues that naturally over-express the protein. It has been described that IF1, in addition to binding subunits of the H^+ -ATP synthase, also binds other membrane proteins of mitochondria in a pH and $\Delta\psi$ independent manner [237]. Specifically, it has been shown that the binding to one of these membrane proteins (~5–6 kDa) hampers the activity of IF1 as an inhibitor of the ATPase [237]. Hence, it is conceivable that tissues that normally over-express IF1 could also express a putative receptor that might act as a negative regulator of IF1 in order to counterbalance its inhibitory activity, contributing in this way to the fine-tuning of OXPHOS. Alternatively, tissue-specific post-translational modifications of IF1 [238] could explain its activity on the H^+ -ATP synthase. The biological relevance of these modifications and the proteins and signals involved are unknown but they are likely to be relevant to understand IF1 expression and activity.

Interestingly, the study of IF1 expression in tumors of large cohorts of breast and colon cancer patients supports that it represents a marker of clinical outcome [48]. Surprisingly, colon and breast cancer patients with high tumor expression of IF1 have a better prognosis in terms of time of disease relapse [48] which suggests that cancer cells with a low expression of IF1 should be more likely to metastasize [48]. The molecular bases of this paradox are presently unknown.

4. Final remarks

We have outlined the main pathways that lead to superoxide production in mitochondria, the primary mechanism by which ROS modulate the activity of proteins involved in signaling pathways and their biological consequences in the cell response. The mitochondrial H^+ -ATP synthase is presented as a key transducer in controlling energy metabolism, ROS-mediated cell death or the retrograde ROS response that allows the acquisition of an enhanced proliferation and the resistance to cell death, key features of the phenotype observed in carcinomas. ROS signaling by mitochondria is important in cancer onset and progression and it might represent the remnant mechanism that allowed the successful symbiosis of the two organisms that gave rise to the aerobic eukaryotic cell. A master regulator of mitochondrial ROS production with clear effects in promoting the malignant phenotype of cancer cells is the ATPase Inhibitory Factor 1. Characterizing the regulation of its expression and/or activity is a critical issue to understand the regulation of OXPHOS in different mammalian tissues and of many of the hallmarks of the cancer phenotype. The development of tissue specific mouse models with regulated expression of IF1 will contribute to

portray its functional role in cellular physiology and pathophysiology. Overall, we can conclude that bioenergetics, ROS production and cell death are master tasks of mitochondria that are molecularly and functionally integrated. Unveiling the mechanisms that mediate these signaling networks will bring up new opportunities for cancer therapies.

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