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Chapter 1

REGULATION OF CYTOCHROME *C* IN RESPIRATION, APOPTOSIS, NEURODEGENERATION AND CANCER: THE GOOD, THE BAD AND THE UGLY

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ABSTRACT

Despite the fact that over 200 phosphorylation sites have been mapped on the mitochondrial oxidative phosphorylation (OxPhos) complexes, very little is known about the relevant cell signaling pathways and the terminal kinases and phosphatases that control these phosphorylations. Within OxPhos, cytochrome *c* (Cyt*c*) plays a special role because it is not only involved in electron transport but is also a key executer of apoptosis when it is released from the mitochondria. It is therefore not surprising that Cyt*c* is regulated by phosphorylation. Four phosphorylation sites have been mapped on mammalian Cyt*c*, two of which have been studied functionally, demonstrating that both respiration and apoptosis are under the control of signaling pathways that have yet to be identified. We here review the regulation and multiple functions of mammalian Cyt*c*, including respiration, reactive oxygen species (ROS) scavenging under healthy conditions, ROS production via $p66^{Shc}$, and cardiolipin oxidation during apoptosis. We propose targeting Cyt*c* by manipulation of signaling cascades as a therapeutic avenue in conditions including neurodegeneration and cancer.

ABBREVIATIONS

Apaf-1	apoptotic protease-activating factor 1
CPP	cell-penetrating peptide
COX	cytochrome *c* oxidase
Cyt*c*	cytochrome *c*
Cyt*c*-T	testes cytochrome *c*
$\Delta\Psi_m$	mitochondrial membrane potential

ETC	electron transport chain
OxPhos	oxidative phosphorylation
ROS	reactive oxygen species

1. INTRODUCTION

Mitochondria are increasingly recognized not only as essential for human health but also as active players and executers of cell destiny. They are not only the production sites of more than 90% of the cell's energy but also integrate numerous signals, allowing responses from within the cell via retrograde regulation that have consequences far beyond the cell's borders. They are a major site of redox signaling and reactive oxygen species (ROS) production [1]. Earlier studies proposed that 1-2% of consumed oxygen is converted into superoxide [2], whereas another study reported significantly lower values of 0.15% with palmitoyl carnitine as substrate and even lower levels with other substrates [3]. ROS are mainly generated at electron transport chain (ETC) complexes I and III, which release superoxide towards the matrix and the intermembrane space, respectively, at high membrane potentials [4-6]. Since superoxide generated by complex III can easily leave the mitochondria it can directly signal to other components of the cell. The earlier view that ROS are merely damaging byproducts of the ETC has more recently changed towards the emerging picture that ROS play an important physiological role in cell signaling [7].

A disturbance of the balance between efficient generation of ATP and basal levels of ROS production is seen not only in traditional mitochondrial diseases, but also in numerous common pathologies, which are increasingly associated with mitochondrial dysfunction.

Both decreased energy levels and/or increased ROS have been associated with diabetes, cancer, acute inflammation (as in sepsis), neurodegenerative diseases, and ischemia reperfusion injury (as seen in stroke and myocardial infarction) [8-13].

Mitochondria are also key players in aging and show decreased capacity to produce ATP over time in combination with increased mitochondrial damage including mutations in mitochondrial DNA [14, 15]. During conditions of stress, various signals can initiate the intrinsic programmed cell death process through mitochondrial type II apoptosis.

Interestingly, life-sustaining and life-threatening functions, via OxPhos and apoptosis, converge on the small electron carrier cytochrome *c* (Cyt*c*).

Cyt*c* is one out of an estimated 1000-1500 nuclear encoded mitochondrial proteins [16] that, in addition to the 13 OxPhos proteins that are encoded by the mitochondrial DNA, constitute the mitochondrial proteome. Of those nuclear encoded mitochondrial proteins about half are expressed across all tissues with the remainder being expressed in a tissue-specific manner [17].

The existence of Cyt*c* as a part of respiration was first suggested by McMunn in 1884 [18] and rediscovered 40 years later by Keilin [19], whereas it took another 70 years to demonstrate that it is required for the execution of type II apoptosis [20]. Recently there has been a flurry of discoveries related to the regulation of Cyt*c*, and new functions have been described. A molecule that is essential in energy production and in apoptosis, as well as other functions, is likely to be subject to various regulatory signals. In what follows, we describe the structure and function of mammalian Cyt*c* and its regulation, focusing on recent advances in the understanding of cell signaling. We then propose a model according to which phosphorylation of Cyt*c* provides an overarching means of regulation of Cyt*c* in its most important roles.

2. CYTOCHROME *C* STRUCTURE

Under healthy conditions Cyt*c* is located in the mitochondrial intermembrane space and transfers packages of single electrons from the bc_1 complex to cytochrome *c* oxidase (COX).

Import of Cyt*c* into the mitochondrial intermembrane space occurs through the heme-lacking apo-Cyt*c* form of the protein. Protein import does not require the mitochondrial membrane potential ($\Delta\Psi_m$), ATP, or the Mia40-dependent pathway [21].

Mature Cyt*c* is then formed by the covalent attachment of a heme group, a process catalyzed by the enzyme Cyt*c* heme lyase [22].

In all eukaryotes, Cyt*c* folds into a tight, nearly spherical ball with a diameter of ~32Å, studded with 13-18 lysine side chains that contribute to its high isoelectric point, and an additional 10-11 aspartate and glutamate residues. The position and surface distribution of these charged amino acids is highly conserved from fungi to vertebrates.

Mature mammalian Cyt*c* contains 104 amino acids (~12 kDa), lacking the start-methionine (Figure 1A). It is evolutionarily conserved and highly positively charged with a pI of about ~9.6.

Cyt*c* was one of the earliest mammalian proteins successfully analyzed by X-ray crystallography, with the first structure of oxidized horse heart Cyt*c*

published in 1967 [23]. Later higher resolution structures of Cyt*c* allowed a more detailed view of its structural properties.

The heme group is covalently attached to the protein through thioether bonds with cysteines 14 and 17 (numbering is based on the mature peptide lacking the N-terminal methionine). The heme iron is in a hexacoordinate configuration with amino acid ligands His18 and Met80. The Met80-heme iron bond causes a weak absorption band at 695 nm in the spectrum of oxidized Cyt*c*, which is an indicator of correct folding. The heme group is only 7.5% solvent-exposed on one corner (Figure 1C), where electrons enter and leave the protein, and it is otherwise surrounded by a hydrophobic environment composed of aliphatic and aromatic amino acid side chains. [24

This environment, together with the heme iron ligands His18 and Met80, was proposed to account for the high redox potential (about 260 mV) of mammalian Cyt*c*. [25.

3. PRO-LIFE FUNCTIONS OF CYTOCHROME *C*: ELECTRON TRANSPORT, ROS SCAVENGING, AND REDOX-COUPLED PROTEIN IMPORT

Cyt*c* is an essential component of aerobic energy metabolism, shuttling electrons from the bc_1 complex to COX. COX transfers these electrons to oxygen, and water is formed. Mice null for Cyt*c* die around midgestation [26], when energy metabolism switches from mainly glycolytic to mainly aerobic [27].

Electrostatic interactions between Cyt*c* and a binding pocket in its electron acceptor cytochrome *c* oxidase (COX) are the chief forces that drive its interaction with COX, including both the associative docking of reduced (Fe^{2+}) Cyt*c* to COX and its dissociative release as oxidized Fe^{3+} Cyt*c*. Heteronuclear single quantum coherence spectral analysis using ^{1}H-^{15}N Cyt*c*, revealed that the overall affinity of oxidized Cyt*c* for COX is only slightly, but still significantly, weaker than that of reduced Cyt*c* [28]. These studies suggest that these on and off interactions are driven chiefly by a relatively small number of charged lysines in the N-terminal (αA) and long C-terminal (αE) α-helices of Cyt*c*, as seen in Figure 1A and B. These two helices pack onto each other in the folded structure, situated 180° opposite from the 50s helix αB.

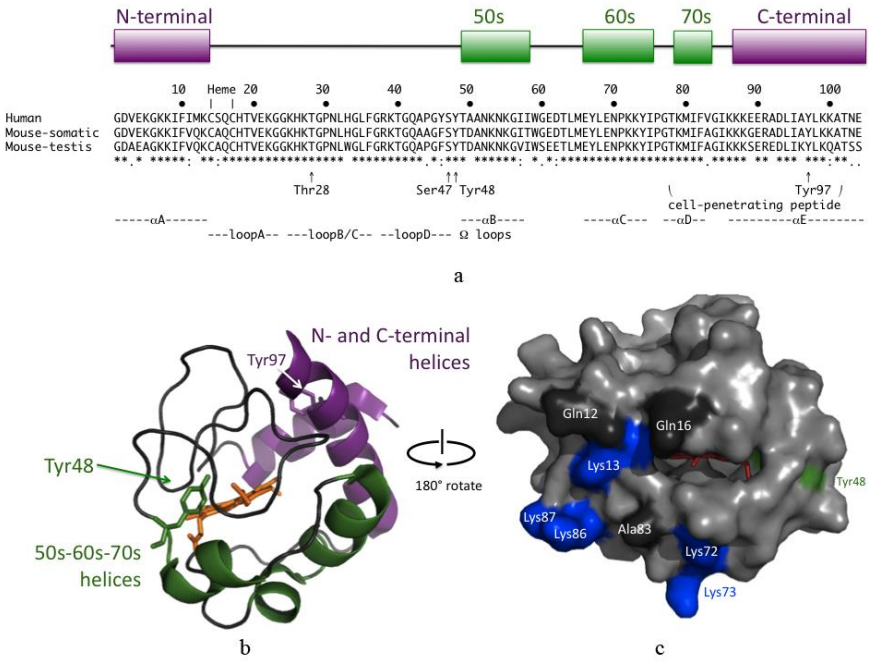


Figure 1. Cytochrome *c* sequence, domain structure, and phosphorylation sites. A, Human (NM_018947), mouse somatic (CAA25899) and testis (NP_034119) Cyt*c* show 91% (human-mouse somatic), 82% (human-mouse testis), and 86% (mouse somatic-mouse testis) conservation. Helical regions are depicted as boxes (top). Residues that have been identified as phosphorylation sites (Thr28, Ser47, Tyr48, and Tyr97) are designated with arrows, and the cell penetrating peptide region comprised of amino acids 77-101 is indicated below the alignment. B, Ribbon structure of horse heart Cyt*c* with highlighted helical regions (magenta) implicated in binding to COX by the Sakamoto model. [28 Crystal structure data of horse heart Cyt*c* (PDB ID: 1HRC; [24]) were processed with the program MacPyMOL 1.2. Tyr48 and 97, which can be phosphorylated and for which functional data are available, are highlighted. C, Space-fill model highlighting charged residues predicted to be crucial for Cytc-COX binding based on the Roberts model [29]. In this model Cyt*c* and COX are positioned for efficient electron transfer between the heme crevice of Cyt*c* and the Trp104 electron entry point in COX subunit II with a distance of <4Å. Cyt*c* residues involved in binding with polar interactions are Gln12, Lys13, Gln16, Lys72, Lys73, Ala83, Lys86, and Lys87 with residues on COX at distances <6Å. Interestingly, all but 2 (Lys16 and 72) of these Cyt*c* residues that bind with COX [29] have undergone evolutionary selection in the primate lineage to reduce the electrostatic nature of the interaction. [155]

Upon Cyt*c* binding to COX, NMR signals from residues in the three Ω loops and helices αB, αC, αD (50s, 60s, 70s) show little or no chemical shift. These studies suggest that structural changes on Cyt*c* during binding and dissociation are mainly confined to Cytc's αA and αE helices, colored purple in Figure 1A and B. One interpretation would be that this area of the protein is the face of Cyt*c* with which it interacts with COX most intimately. It should be noted, however, that other convincing models exist, based on computer predictions [29] that align Cytc on COX for efficient electron transfer between the heme crevice of Cyt*c* and the Trp104 electron entry point in COX subunit II with a distance of <4Å. This model involves Cyt*c* residues Gln12, Lys13, Gln16, Lys72, Lys73, Ala83, Lys86, and Lys87 for polar interactions with residues on COX at distances <6Å, thus extending the interaction area into the αD domain and beyond but questioning the importance of the αE region for binding (Figure 1C).

A second 'pro-life' function of Cyt*c* is redox-coupled protein import. Proteins that localize to the mitochondrial intermembrane space and the inner membrane and contain twin CX_3C or CX_9C motifs are imported by the Mia40/ Erv1 pathway [30-32]. Here, protein import is coupled to Mia40/Erv1-mediated disulfide bond formation, which locks the proteins in their mature tertiary structure and in the mitochondria. The electrons derived from the disulfide reaction are transferred from Mia40 to Erv1 and finally Cyt*c* [33], thus feeding additional electrons into the ETC.

In its third 'pro-life' function Cyt*c* acts as a ROS scavenger. Among the most common ROS are superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$); their buildup, if unregulated, will trigger apoptosis [34]. Several cellular lines of defense exist to protect the cell from ROS. These include enzymatic ROS scavengers such as superoxide dismutatases and catalase, as well as non-enzymatic scavengers such as glutathione. Cyt*c* is yet another enzyme that can detoxify superoxide [35, 36]. Since Cyt*c* accepts single electrons it can accept an electron from superoxide, generating oxygen. This reaction requires Cyt*c* to be in the oxidized form. Furthermore, it was demonstrated that Cyt*c* operates as an H_2O_2 scavenger [37]. In contrast to the detoxification of superoxide, conversion of H_2O_2 is catalyzed by both reduced and oxidized Cyt*c*. Cyt*c* constantly switches between reduced and oxidized states in a respiring cell. Therefore the detoxification reactions of both superoxide and H_2O_2 can take place. In conclusion, the redox capabilities of Cyt*c* make it an ideal antioxidant for the cell, especially given its location at a site within the mitochondria where the ROS load is very high. The pro-life functions of Cyt*c* are summarized on the left side of Figure 2.

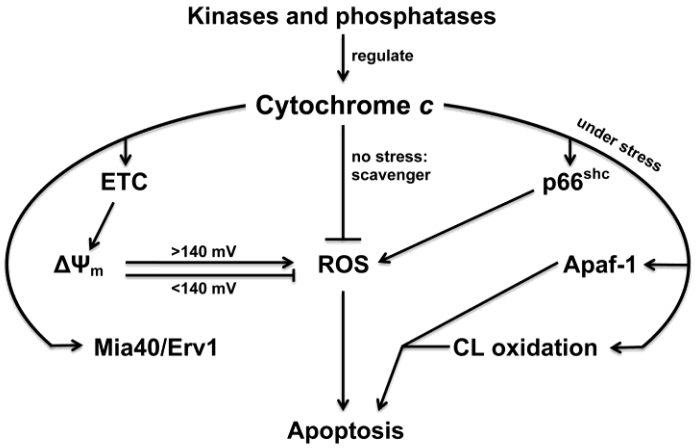


Figure 2. Model that the main functions of cytochrome *c* are regulated by Cyt*c* phosphorylation. Cyt*c* is essential for electron transfer as part of the electron transport chain (ETC), redox-mediated protein import, and as a ROS scavenger under healthy conditions. This is mediated by phosphorylation of Cyt*c*, which partially inhibits ETC flux and prevents mitochondrial membrane potential ($\Delta\Psi_m$) hyperpolarization, which would otherwise cause ROS production at ETC complexes I and III. Under conditions of stress, dephosphorylation of Cyt*c* increases ETC flux leading to $\Delta\Psi_m$ hyperpolarization and thus production of ROS, which can trigger apoptosis. Additional mechanisms involving Cyt*c* operate during conditions of stress including ROS production through the $p66^{shc}$ pathway and cardiolipin (CL) peroxidation catalyzed by Cyt*c*, which allows it to dissociate from CL and to be released into the cytosol, where the unphosphorylated form of Cyt*c* can bind to Apaf-1, triggering apoptosis.

4. THE APOPTOSOME: CYTOCHROME *C* RELEASE AND BINDING TO APAF-1 IS REQUIRED TO TRIGGER APOPTOSIS

The number of published studies of the role of Cyt*c* in apoptosis has increased exponentially since 1996 when Liu and colleagues first demonstrated in a cell free apoptotic system that Cyt*c*, in addition to other components including dATP, is required to execute the program [20]. It is now textbook knowledge that release of Cyt*c* from the mitochondria into the cytosol and its binding to apoptotic protease-activating factor 1 (Apaf-1) are key steps for triggering apoptosis. Cyt*c* binding to Apaf-1, the latter of which contains a dATP cofactor, induces hydrolysis of the dATP, which is subsequently replaced by exogenous dATP to form the apoptosome [38].

The apoptosome then recruits several pro-caspase-9 molecules and promotes cleavage to their active form.

Apoptosome-bound caspase-9 mediates cleavage and activation of caspase-3, a major committer and executor of apoptosis. [39, 40] Apoptosis involving the release of Cyt*c* is a process well established and conserved in mammals but more controversial in lower organisms. It was recently suggested that the intrinsic (type II) apoptotic pathway arose once, before the emergence of the deuterostomes, and that portions of the pathway have been lost in some lineages [41]. Several studies have indicated that it is also operates in organisms such as the protostome *Drosophila* [42], the very primitive metazoan *Nematostella* [43], and apparently even yeast [44], but seems to be absent in *C. elegans*.

4.1. Release of Cytochrome *C* Precedes Apoptosome Formation

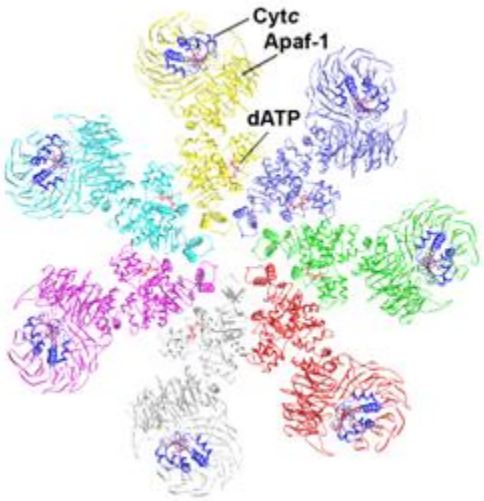
The formation of the apoptosome requires the release of Cyt*c* from the mitochondria, which is an interesting and controversial topic. Although the release process is often considered to be the consequence of mitochondrial membrane rupture or mitochondrial permeability transition pore (PTP) formation, there are studies suggesting a different modus operandi. First, there is evidence that release of Cyt*c* is independent of PTP formation and that it precedes it [45, 46]. Second, mitochondria have the capability to reversibly release and take up Cyt*c*, with the latter restoring ETC function [47]. This may involve a special feature of Cyt*c*; it contains cell-penetrating peptide (CPP) sequences [48], allowing it to cross membranes in a non-traditional fashion (see also section 6.2). The region of Cyt*c* facilitating membrane translocation most efficiently is composed of amino acids 77 through 101 (Figure 1A).

4.2. Regulation of Apoptosis at the Level of Cytochrome *C*

If CPP-mediated transition pore-independent release of Cyt*c* is the preferred mechanism it may be subject to regulation, including by phosphorylation. Preliminary support for this idea is provided by a study showing that neuroprotective insulin treatment leads to Tyr97 phosphorylation of brain Cyt*c* [49]. Furthermore, phosphorylation of Tyr97, which is located within the 77-101 CPP (Figure 1A), leads to a suppression of Cyt*c* release from the mitochondria, as discussed in section 5.3.

When Cyt*c* is released into the cytosol, it binds to Apaf-1, and Apaf-1 assembles into the heptameric apoptosome (Figure 3). Little is known about the possible regulation of Cyt*c*'s interaction with Apaf-1 and the formation of the apoptosome. The binding of Cyt*c* to Apaf-1 is known to be electrostatic in nature, and mutagenesis studies suggest that lysines 7, 8, 13, 25, 39, 72, 86, 87, and 88 participate in binding to negatively-charged aspartate residues in the WD-40 domain of Apaf-1 [50, 51]. Binding of nucleotides, especially ATP, to several of these lysine residues prevents the interaction between Cyt*c* and Apaf-1, thus presenting a means of regulating apoptosome formation [51]. The heptameric structure of the apoptosome (Figure 3A) suggests regulation by Cyt*c* concentration, and mathematical modeling suggests that the optimal Cyt*c*/Apaf-1 ratio for apoptosome formation is one-to-one. Higher relative amounts of Cyt*c* lead to preferential formation of tetrameric and larger oligomers, which by themselves are unable to form heptamers [52]. Another study also supports the concept that concentration of free Cyt*c* is a regulating factor. tRNAs are thought to inhibit the reaction of Cyt*c* with Apaf-1. Mei et al. found that Cyt*c* binds to mitochondrial and cytosolic tRNAs, inhibiting Cyt*c*-induced apoptosome formation [53]. The authors speculated that since tumor cells synthesize tRNA at high rates, the suppression of apoptosis as seen in cancer could be caused by tRNA binding to Cyt*c*. Such a mechanism could be implicated in tumorigenesis and be a target for therapy in the future. In addition, various studies have shown that oxidized Cyt*c* is more competent than reduced Cyt*c* to form the apoptosome and to activate caspases (reviewed in [54]). Therefore redox signaling may be an important regulatory component of apoptosome-formation.

Another possible mechanism to regulate apoptosis is through Cyt*c* phosphorylation, which may interfere with Cyt*c*-Apaf-1 binding. A recent model of the apoptosome based on electron density maps at 9.5Å shows the sevenfold symmetry (Figure 3A) [55]. In the model the four amino acids of Cyt*c* that can be phosphorylated show various distances to the nearest residues of Apaf-1: Cyt*c* Thr28 is within 6Å of Apaf-1 Trp884; Cyt*c* Ser47 is within 5Å of Apaf-1 Ala763 and within 6Å of Ile800; Cyt*c* Tyr48 is within 6Å of Apaf-1 Ile800; and Cyt*c* Tyr97 is the most distant residue of the four, with the closest amino acids of Apaf-1 (Asp1106 and Glu659) within 14Å (Figure 3B). Further structural analyses and refinements are necessary to strengthen the model. Type II apoptosis is a multi-faceted process that involves additional important reactions in which Cyt*c* is directly or indirectly involved, including ROS generation by the ETC and the Cyt*c*-p66^{shc} pathway, and oxidation of Cyt*c*-bound cardiolipin, which precedes Cyt*c* release from the mitochondria.



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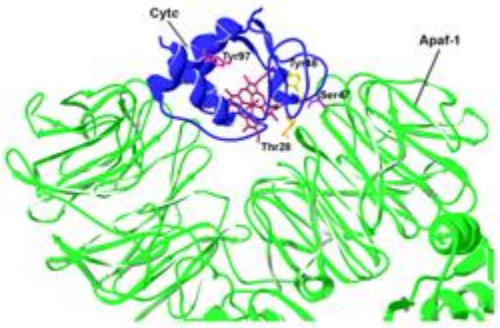

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Figure 3. Interaction of cytochrome *c* with Apaf-1 based on the Akey model of the apoptosome [55]. Crystal structure data of the model (PDB ID: 3J2T) were used and processed with the program Swiss-PDBViewer 3.7. A, Overview depicting the sevenfold symmetrical structure of the apoptosome, highlighting Cyt*c* and co-factor dATP. B, Center-side view of the Cyt*c*-Apaf-1 interaction. Cyt*c* residue Thr28 is located within 6Å of Apaf-1 Trp884; Cyt*c* Ser47 is within 5Å of Apaf-1 Ala763 and within 6Å of Ile800; Cyt*c* Tyr48 is within 6Å of Apaf-1 Ile800; and Cyt*c* Tyr97 is within 14Å of Apaf-1 Asp1106 and Glu659. In the model, Cyt*c* docks to the WD40 domain of Apaf-1 (green) where the largely β-sheet structure is evident.

These topics are discussed in section 6 below and summarized in Figure 2 (right side). All these mechanisms work hand in hand and all have to be considered simultaneously to get a comprehensive picture of the role of Cyt*c* in apoptosis.

5. REGULATION OF CYTOCHROME *C*

The last step of the ETC, the transfer of electrons from Cyt*c* via COX to oxygen, is the proposed rate-limiting step of the ETC in mammals in intact cells [56-61].

It is therefore not surprising that all major forms of regulation have been identified for both Cyt*c* and COX (for a review of COX regulation see [62]).

These include expression of tissue-specific isoforms only found in COX and Cyt*c* among OxPhos complexes, allosteric regulation, and regulation via posttranscriptional modification, in particular by phosphorylations, the latter of which have been mapped on all OxPhos complexes (reviewed in [63]).

5.1. Isoforms

In mammals, two Cyt*c* isoforms exist, the so-called somatic isoform found in almost all tissues and cell types, and a testes-specific isoform (Cyt*c*-T). Cyt*c*-T was first discovered in the testicular germinal epithelial cells of rodents [64]. It is the predominant isoform found in sperm. Mouse somatic and testes Cyt*c* share 86% sequence identity (Figure 1A).

Although their overall functions are similar there are a few interesting differences between the isoform pair. Cyt*c*-T has a threefold higher activity to reduce H_2O_2 but a fourfold higher apoptotic activity [65]. It was proposed that these Cyt*c*-T-specific differences reduce ROS damage in sperm cells but are at the same time a selection tool to eliminate damaged sperm to ensure efficient DNA transmission by intact sperm.

Interestingly, Cyt*c*-T can induce apoptosis through both the intrinsic and extrinsic pathways, suggesting that both death pathways converge at Cyt*c* [66].

Cyt*c*-T is absent in primates and the gene mutated to a non-transcribed pseudogene [67], implying that human Cyt*c* has to carry out testis- and non-testis-specific functions.

5.2. Allosteric Regulation

Due to its high positive charge it is not surprising that Cyt*c* binds anions that are known to affect the interaction and thus the kinetics of Cyt*c* and COX. One such anion, ATP, binds to both enzymes [68-70] and this binding inhibits the electron transfer kinetics [71, 72] by altering the high-affinity Cyt*c*-COX binding site to a low-affinity site. [68] Since ATP is the ultimate product of OxPhos, it leads to an inhibition under conditions when energy is plentiful, thus working as an energy sensor. This regulation is also important in light of ROS production. Under conditions when almost all cellular ADP has been converted into ATP, and ATP synthase does not utilize $\Delta\Psi_m$, further pumping of protons by the ETC would lead to a buildup or hyperpolarization of $\Delta\Psi_m$, a condition known to lead to excessive ROS production. [73] ATP-dependent allosteric regulation requires phosphorylations on COX because no inhibitory effect of ATP was observed after *in vitro* dephosphorylation of cow liver COX [62]. Several 'enabling' sites have been proposed, including on COX subunits I, IV, and Vb [74-76]. Therefore, regulation by adenine nucleotides should be viewed and interpreted in combination with phosphorylations of COX and Cyt*c*.

5.3. Phosphorylation of Cytochrome C

In the past few years over 200 phosphorylation sites have been reported for the entire OxPhos system [63]. This finding was made possible by increasingly sensitive mass spectrometry methodology. For almost all of these mapped sites, the corresponding signaling pathways including the kinases and phosphatases are unknown.

In addition, some authors questioned how biologically relevant some of those phosphorylations are due to low stoichiometry and the possibility of unspecific auto-phosphorylations at sites with a higher affinity to ATP [77]. However, there is now clear evidence for the biological significance of at least a small number of phosphorylations including those on Cyt*c* and COX, which have been studied in more detail (for a review of COX phosphorylations see [78]). Here we will focus on Cyt*c*.

It is perhaps not surprising that the first phosphorylation site on Cyt*c*, Tyr97 in cow heart, was only recently reported, in 2006 [79], likely because most traditional protocols to purify mitochondrial proteins do not consider and thus do not maintain such phosphorylations.

We have therefore modified these protocols and added unspecific phosphatase inhibitors, such as fluoride for Ser/Thr phosphatases and activated vanadate for Tyr phosphatases, which we use for the purification of Cyt*c* and COX [80]. For the purification of Cyt*c* we initially purified mitochondria [79]; we now omit this isolation step and directly extract Cyt*c* from cells or tissues under conditions preserving phosphorylations in all steps [81].

Mass spectrometry showed that Cyt*c* from cow heart is Tyr97 phosphorylated under normal conditions [79], i.e., without external stimulation or inhibition of signaling pathways.

Spectral analysis of oxidized Tyr97-phosphorylated Cyt*c* revealed a small shift of the 695 nm absorption band. Since this absorption is due to the Met80-heme iron bond, phosphorylation of Cyt*c* in the periphery (i.e., an area not involved in binding to COX based on the Roberts model [29]; see Figure 1C) apparently affects the heme environment. Tyr97-phosphorylation leads to an inhibition of the Cyt*c*-COX reaction as seen by enhanced sigmoidal kinetics with K_M values of COX for Cyt*c* of 5.5 μM Cyt*c* substrate concentration compared to 2.5 μM for unphosphorylated Cyt*c*.

Analysis of liver Cyt*c* also revealed tyrosine phosphorylation but, surprisingly, on a different site, Tyr48, located in the frontal area of the protein (Figure 1C), closer to the site at which Cyt*c* binds to COX [81]. Interestingly, the spectrum of Tyr48 phosphorylated Cyt*c* was not altered but the kinetics were distinct compared to both unphosphorylated and Tyr97-phosphorylated Cyt*c*. In the reaction with COX, phospho-Tyr48 Cyt*c* showed hyperbolic kinetics similar to unphosphorylated Cyt*c*. Importantly, maximal turnover was more than 50% reduced for the phosphorylated form.

Both heart and liver-specific phosphorylations of Cyt*c* occur on residues that are highly conserved in eukaryotes (Figure 1A).

Interesting, both Tyr48 and 97 phosphorylations lead to an inhibition in the reaction with COX via a reduction of maximal turnover and a shift from hyperbolic to sigmoidal kinetics, respectively, suggesting tissue-specific adaptations of Cyt*c* regulation.

Our findings of partial inhibition of respiration as a consequence of Cyt*c* Tyr48 and 97 phosphorylation fits our working model (Figure 2). We propose that under healthy conditions mammalian OxPhos does not operate at maximal capacity, thus avoiding high mitochondrial membrane potentials, which lead to an exponential increase of ROS production at $\Delta\Psi_m$>140 mV [73].

In addition to changes in mitochondrial respiration, phosphorylation of Cyt*c* may also impact its other functions including apoptosis, a life and death decision process in which Cyt*c* plays a crucial role.

As discussed above, the role of Cyt*c* in apoptosis is several-fold and includes multiple stages during the process, including cardiolipin peroxidation, its release from the mitochondria, and its interaction with Apaf-1 to induce the cell's execution. *In vitro* work with phosphomimetic Cyt*c* indeed suggests such a role. Replacement of Tyr48 with phosphomimetic Glu, which mimics the negative charge of the phosphate group, produced kinetics similar to those observed using *in vivo* phosphorylated Cyt*c* with purified COX, suggesting that the replacement is a good model system [82].

Strikingly, Tyr48Glu Cyt*c* showed no detectable downstream caspase activation, suggesting that this site can function as a switch for the regulation of apoptosis. Only two mutations have been reported in human patients. One of them is Tyr48His in a family from Italy [83] in which the patients presented with low platelet counts in their blood (thrombocytopenia). This mutation adds a positive charge to a site that is crucial for energy production and apoptosis. Interestingly, this mutation results in decreased respiration similar to the phosphomimetic substitution, but has the opposite effect on apoptosis which is further activated compared to wild-type. The second mutation reported in humans, Gly41Ser, also causes a relatively mild form of thrombocytopenia [84]. Similar to the Tyr48His mutation, Gly41Ser increases apoptotic activity. The mutation leads to protein folding changes in the Cyt*c* heme environment [85]. It is unclear why no other phenotype is observed in these patients and why no other tissues are affected. One explanation might be that there are several signaling pathways to fine-tune Cyt*c* activity in apoptosis, a concept supported by the presence of multiple tissue-specific phosphorylation sites. Together with the substitutions at Tyr48, the Gly41Ser substitution argues for a localization of an epitope of Cyt*c* on the 40s Ω loop D controlling the mitochondrial pathway of apoptosis, which we suggest is the Apaf-1-interacting face of Cyt*c* (Figure 1A and B).

For Tyr97 no data with phosphomimetic mutants is currently available and it is thus unclear how involved this site is in the regulation of apoptosis. However, binding of Cyt*c* to Apaf-1 involves several amino acids located on the surface of Cyt*c* required for enveloping the molecule as suggested by earlier mutational analysis of Cyt*c*, revealing several key residues required for formation of the apoptosome.

Lys7, which is located near Tyr97, is one of them, without which binding affinity is greatly diminished [50]. Phosphorylation of Tyr97 may lead to salt bridge formation with Lys7, potentially capturing an important residue for the interaction with Apaf-1. It will be interesting to see if the Cyt*c*-Apaf-1 interaction is affected by such a posttranslational modification.

For the step upstream, the release of Cyt*c* from the mitochondria, we have recently shown that treatment with a single bolus of insulin is neuroprotective in a rat model of global brain ischemia/reperfusion injury, shielding CA1 hippocampal neurons from cell death [49]. Insulin treatment results in robust phosphorylation of Cyt*c* Tyr97 in ischemic pig brain *in vitro* as well as in normal and ischemic rat brain *in vivo*. Importantly, it also coincides with inhibition of Cyt*c* release from the mitochondria (see section 6.2 for a possible mechanism). Taken together with the observation that Tyr97 phosphorylation reduces ETC flux, thereby preventing $\Delta\Psi_m$ hyperpolarization and thus ROS production, these findings suggest that Tyr97 phosphorylation reduces oxidative stress and acts as an anti-apoptotic signal. The existence of tyrosine phosphorylations, which can suppress Cyt*c* release from the mitochondria (Tyr97), and limit cardiolipin oxidation and abolish the execution of the caspase cascade (Tyr48), suggests a fourfold control of apoptosis, by suppression of: 1) $\Delta\Psi_m$ hyperpolarization, 2) cardiolipin peroxidation, 3) Cyt*c* release, and 4) the formation of a functional apoptosome (Figure 2). Such multi-level regulation might be expected for a function as crucial as the life-or-death decision for the cell. Once the corresponding kinases and phosphatases involved in Cyt*c* phosphorylation are known they may be specifically targeted to prevent or induce cell death in conditions such as neurodegeneration and cancer, respectively.

Two more phosphorylation sites, Thr28 and Ser47 (Figures 1A and 3B), have recently been reported on human skeletal muscle Cyt*c* by high throughput phospho-proteomic mass-spectroscopy analysis [86]. This indicates that Cyt*c* in this tissue is controlled by a signaling pathway distinct from liver and heart. No information exists about the effects of these phosphorylations or about the ratio of phosphorylated versus unphosphorylated Cyt*c*.

Other posttranslational modifications have been reported for Cyt*c*. In particular, Cyt*c* was proposed to be an important target of tyrosine nitrations as reaction products of peroxynitrite, which forms in the presence of nitric oxide and superoxide [87]. Although the amount of tyrosine nitrated Cyt*c* is rather low *in vivo* it has been studied quite intensively. In wild-type Cyt*c*, Tyr67 is preferentially nitrated, leading to structural changes and the loss of the heme iron-Met80 bond [88]. As expected, this modification also causes changes in the redox characteristics of Cyt*c*, a strong inhibition of the reaction with purified COX [89], and inhibition of downstream caspase activation [90]. The authors also generated single tyrosine mutants of Cyt*c*, in which the other three tyrosines were replaced with phenylalanines, and reported various effects on function for each individual site after nitration.

They concluded that nitration of Tyr74 resulted in the second largest effects after Tyr67. In addition, Tyr74 nitration abolishes downstream caspase activation but still allows Cyt*c* to bind to Apaf-1 [91].

Interestingly, one study reported that Tyr48 nitration completely blocks downstream caspase activation [87], similar to phosphomimetic Tyr48Glu Cyt*c* [82]. Tyrosine nitration introduces a relatively bulky group with a negative charge similar to tyrosine phosphorylation. Tyrosine nitrations might thus be considered a phosphotyrosine-mimetic substitution, perhaps better than glutamate substitutions for resembling tyrosine phosphorylations.

6. REDOX SIGNALING AND CYTOCHROME *C*

Redox signaling occurs when a change in the level of a reactive oxygen or nitrogen species or a change in the redox state of a molecule causes a response in a biological system [92]. Even the change in the redox state of Cyt*c* as part of its role as an electron carrier in the ETC can constitute a redox signal, as in the example of feedback control described below (see section 6.1). Recent work has shown that Cyt*c* in its role as an executer of apoptosis can respond to redox signaling. Such signals arise from the ETC as a generator of ROS, from $p66^{shc}$, which oxidizes Cyt*c* and catalyzes the production of hydrogen peroxide, and from the oxidation of cardiolipin. Cyt*c* is bound to cardiolipin at the inner mitochondrial membrane, which causes Cyt*c* to partially unfold, giving rise to peroxidase capability, oxidation of cardiolipin, and its own release into the cytosol, where it binds to Apaf-1 to form the apoptosome. The reader should bear in mind that these processes, since they deal with the life and death of the cell, are necessarily tightly regulated, likely with multiple checkpoints (Figure 2).

6.1. $p66^{shc}$ and Cytochrome *C*

$p66^{shc}$ is an adapter protein that, if rendered inoperative, increases resistance to oxidative stress and increases lifespan [93-95]. $p66^{shc}$ is found throughout the cell, including the mitochondria. Under stress conditions, it oxidizes Cyt*c*, transferring an electron to oxygen thus catalyzing the production of hydrogen peroxide [96], which may act as an apoptotic signal [97]. In fact, release of Cyt*c* correlates with the H_2O_2 production activity of $p66^{shc}$ [98].

Superoxide and subsequently peroxide production by $p66^{shc}$ could contribute to the ability of Cyt*c*, as described below, to oxidize cardiolipin, enabling the release of Cyt*c* into the cytosol.

A principal role of shc family members is assembly of signaling complexes. Consistent with this role it has recently been shown that $p66^{shc}$ acts to assemble a PKCδ/Cyt*c*/retinol complex in the intermembrane space of the mitochondria, thereby regulating mitochondrial respiration [99, 100]. In that case, the redox state of Cyt*c* acts as a feedback sensor of the activity level of the ETC, and the signaling complex accordingly adjusts the amount of fuel entering the tricarboxylic acid cycle.

Phosphorylation of $p66^{shc}$ Ser36 appears to be an essential regulator of function and increases with age in several organs including lung, liver, and skin, causing increased ROS production and oxidative damage as was shown in aged mice [101]. However, the possibility that phosphorylation of Cyt*c* can regulate the interaction between Cyt*c* and $p66^{shc}$ remains to be explored. Such a regulation would not be surprising given its effect on apoptosome formation and downstream caspase activation (see also section 5.3).

6.2. Cytochrome C, Cardiolipin, and Apoptosis

About 15% of Cyt*c* is bound to cardiolipin, a mitochondria-specific phospholipid that is primarily found in the inner mitochondrial membrane [102]. During apoptosis, trans-membrane migration of cardiolipin enhances its association with Cyt*c* in the intermembrane space, resulting in complexes formed of cardiolipin and Cyt*c* [102]. Before Cyt*c* can be released from the mitochondria during apoptosis, a redistribution of cardiolipin occurs [103]. Almost 80% of the cardiolipin is located in the inner mitochondrial membrane where it constitutes about 20% of the total membrane lipids. During apoptosis a lipid exchange occurs that results in almost 40% of the cardiolipin of the inner mitochondrial membrane translocating to the outer membrane [104].

The binding of cardiolipin to Cyt*c* may proceed in various ways (reviewed in [105]); nevertheless, under appropriate conditions, Cyt*c* is bound by cardiolipin in such a way that it partially unfolds, acquires peroxidase capability, oxidizes and dissociates from cardiolipin, and initiates apoptosis [102]. It is likely that tight binding requires deep penetration of one (or of two) acyl chains into the protein, reaching the heme pocket region. Such binding requires lysines 72, 73, and 79, as shown by Lys72/73/79Asn mutants in horse heart Cyt*c* [106].

The Spiro group has elucidated a possible sequence of partial unfolding as a result of cardiolipin binding, using horse heart Cyt*c*. At pH 3, thermal unfolding causes loss of the hydrogen bond between His26 and Pro44, which initiates a rapid transformation of the 40s Ω loop to a β-sheet, and extension of the β-sheet into the 60s and 70s helices. These changes cause disruption of the Met80-heme covalent bond, opening of the heme crevice, which allows access of peroxide to the heme and induction of peroxidase activity [107].

Activation of peroxidases that function via a heme group, such as Cyt*c*, usually involves a protein-bound radical. An intermediate of the cardiolipin peroxidation process appears to be a tyrosyl radical, or rather various tyrosyl radicals. Initially, Tyr97 was identified as the site of a tyrosyl radical [102, 108, 109]. The Kagan group later found in studies of tyrosine mutated Cyt*c* that of the four tyrosines that exist in horse heart Cyt*c*, the Tyr67Phe mutation caused the greatest decrease of peroxidase activity of the mutants, and was a likely involved tyrosyl radical [110]. Rajagopal et al., [111] found through electronic paramagnetic resonance (EPR) spectroscopy that Tyr46 and 48 were the putative radical sites, and Tyr/46/48/Phe mutants showed similar EPR signals in all variants, indicating that the radicals are formed on more than one site. Of the two tyrosines, Tyr46 is present in humans but not conserved in mammals and it is therefore unlikely to be a key residue for cardiolipin peroxidation. The conclusion that there are various sites of radical formation seems most reasonable at this time.

Cell signaling, specifically phosphorylation of Cyt*c* tyrosine residues, may also affect cardiolipin peroxidation, as we propose (Figure 2). Pecina et al. [82] used rodent phosphomimetic Tyr48Glu substituted Cyt*c* and reported significantly decreased Cyt*c*-cardiolipin binding and decreased but not abolished peroxidase activity compared to the wild type. It is possible that the bulky phosphate group identified on this position in liver *in vivo* may show an even more pronounced effect on the peroxidase activity of Cyt*c*. Since cardiolipin oxidation precedes Cyt*c* release from the mitochondria, the phosphorylation state of Cyt*c* likely controls this rather early step in the apoptotic cascade.

The Cyt*c*-cardiolipin interaction not only enhances the peroxidase capacity of Cyt*c*, it also leads to the formation of discrete pores in membranes through which Cyt*c* can escape into the cytosol. Groves and colleagues observed Cyt*c* and dextran leakage across cardiolipin-containing membranes into the vesicle interior, which occurred in the absence of other apoptotic machinery [112]. “Bursts” of leakage were interpreted as the opening of stable pores.

The pores may be the result of the ability of Cyt*c* to cause curvature of the cardiolipin-containing membranes. Pletneva and colleagues [113] found, with time-resolved FRET measurements of dansyl variants of horse heart Cyt*c*, that late in the process of cardiolipin binding by Cyt*c*, when the greatest unfolding of the native structure occurs, the C-terminal helix of the protein is unfolded and extended. Interestingly, the C-terminus of Cyt*c* has been identified as containing cell-penetrating peptide (CPP) sequences (Figure 1A, amino acids 77-101), which allow proteins to cross membranes, and which induced apoptosis in an astrocytoma cell line [48]. These CPPs, when in an open conformation, may allow Cyt*c* to translocate into the cytosol independently of the permeability transition pore.

7. CYTOCHROME *C* IN THE CONTEXT OF NEURODEGENERATION AND CANCER

Cyt*c*, as a protein with functions crucial to life and death, is clearly relevant in the context of therapeutics. This is perhaps most clear in the cases of neurodegenerative diseases and cancer. In neurodegenerative conditions a significant cause of damage is excessive and uncontrolled apoptotic activity.

Mitochondrial dysfunction leading to release of Cyt*c* and induction of apoptosis has been demonstrated in a broad range of neurodegenerative conditions, including acute trauma such as stroke [114-117] and traumatic spinal cord injury [118, 119], and chronic conditions such as multiple sclerosis (MS) [120, 121], Parkinson's Disease [122-124], Huntington Disease [125-127], and amyotrophic lateral sclerosis (ALS) [128-130]. Excessive apoptotic cell death could be prevented if Cyt*c* release into the cytosol or Cyt*c* binding to Apaf-1 could be inhibited.

The mechanisms discussed in the course of this review have led to a number of proposed therapies for neurodegenerative diseases. For example, the Szeto group has investigated the synthetic mitochondrial peptide SS-31, finding that it selectively targets cardiolipin and inhibits Cyt*c* peroxidase activity by protecting the heme, mitigating ischemia-reperfusion injury [131, 132]. The anti-inflammatory and neuroprotective tetracycline-derivative minocycline was investigated by Patriarca et al. who found that it binds to Cyt*c*, decreasing its peroxidase activity and caspase-3 activation [133]. Its anti-apoptotic effect may therefore result from the inhibition of cardiolipin peroxidation by Cyt*c*, or from the inhibition of binding of Cyt*c* to Apaf-1.

Ji and colleagues employed a brain-permeable mitochondria-targeted nitroxide electron scavenger to prevent cardiolipin oxidation in the brain, and were able to inhibit apoptosis that otherwise would occur in rat models of traumatic brain injury *in vitro* and *in vivo* [134].

Since $p66^{shc}$ has been found to be involved in MS, Savino and coworkers [135] investigated the role of $p66^{shc}$ in that disease (see also [136]). They found that deletion of $p66^{shc}$ protects from neurodegenerative stress in experimental autoimmune encephalomyelitis (EAE), a murine model of MS, and inhibits $p66^{shc}$-mediated opening of the mitochondrial permeability transition pore. It therefore could serve as a marker for early detection of MS and other neurodegenerative diseases, and its inhibition could constitute a treatment for such diseases.

We and others have shown that insulin protects hippocampal cells from cell death [137, 138].

As mentioned above, we were able to demonstrate that such treatment leads to Tyr97 phosphorylation, which suppresses its release from the mitochondria [49]. It may be this phosphorylation, predicted to maintain controlled and healthy cell respiration, that is at least in part responsible for the neuroprotective effect of insulin.

In cancer, two typical characteristics related to the life and death functions of Cyt*c* are resistance to apoptosis [139-141] and the preferential switch from aerobic energy metabolism to glycolytic energy metabolism, even in the presence of oxygen, known as the Warburg effect [10, 142].

Glycolytic metabolism is able to produce the ATP that rapidly proliferating cells need, along with precursors of cellular biosynthesis [143], and targeting the glycolytic nature of rapidly proliferating cancer cells has emerged as a promising therapeutic strategy [144-147]. However, cancers constantly evolve against attempts to limit their survival, whether the mechanism of death is apoptosis or other death pathways [148].

With respect to apoptosis, Jones et al. identified a cell penetrating peptide sequence in the C-terminus of Cyt*c*, and added to it an N-terminal sequence that increased the apoptotic potency attributable to the C-terminal section of Cyt*c* alone [48]. The authors foresee the ability to synthesize a class of synthetic proteins that would enhance apoptosis in tumor cells.

Johnson and colleagues have shown that in brain tumors, traditionally refractory to chemotherapy, cytosolic Cyt*c* is sufficient to activate apoptosis in brain tumor cell lines, whereas normal brain cell lines are resistant to apoptosis from cytosolic Cyt*c* [149]. The authors attributed the difference to levels of Apaf-1, which were high in the cancer cells compared to normal tissues.

Thus Cyt*c* may prove to be a therapeutic agent that can selectively kill cancer cells, sparing normal cells in the brain. Another promising strategy, because of development of resistance to chemotherapeutic agents, would be to combine chemotherapy with enhanced apoptosis. For example, since Cyt*c* in its reduced state is less capable of triggering apoptosis, Barros et al. have shown that depleting cells of glutathione or glutathione-S-transferases (by such agents as DNA hairpins or the neurotoxin veratridine) increased the cytotoxic activity of methotrexate in breast cancer cells [150].

Intriguingly, there appears to be a mechanistic connection between the two characteristic features of cancer cells discussed in this section; that is, resistance to apoptosis and a preference in cancer for glycolysis. This connection was suggested by Plas and Thompson [151], and followed up by Bonnet et al. [152], who showed that the small molecule dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase, decreases cancer growth *in vitro* and *in vivo*, activates aerobic respiration, and promotes apoptosis through release of Cyt*c*. Vaughn and Deshmukh continued this line of research by investigating the similarities between cancer cells and postmitotic neurons, which last for the lifetime of the organism [153]. Both types of cells inhibit the apoptotic pathway, and they both extensively metabolize glucose. The authors suggested that the regulatory feature shared by postmitotic neurons and cancer cells is the redox effect of glycolytic metabolism on Cyt*c*. They further observed that oxidized but not reduced Cyt*c* promotes apoptosis and showed that, both in postmitotic neurons and in cancer cells, glycolytic metabolism, chiefly through the action of the pentose phosphate pathway, reduces Cyt*c* and therefore decreases apoptosis. Similar conclusions where obtained in a study using a yeast model for metabolic switching [154]. Suppression of aerobic respiration inhibited apoptosis, while enhancement of respiration increased it.

CONCLUSION

Cyt*c* plays a key role in pro-life and pro-death processes. Those include electron transfer, radical scavenging, and redox-coupled protein import on the one side, and cardiolipin oxidation, ROS generation through $p66^{shc}$ or as part of the ETC, and apoptosome formation on the other side (Figure 2).

Regulation of these processes is of utmost importance for the fate of the cell. Reversible phosphorylations are arguably the most important regulators and four such modifications have been identified on Cyt*c*.

Identification of the involved kinases, phosphatases, and signaling pathways that modulate them would allow a deeper understanding of the crosstalk between pathways that leads to a final resolution of the cell's destiny. It would thereby also allow targeted control of Cyt*c*, which could lead to the development of therapeutic interventions that, for example, increase OxPhos relative to glycolysis in tumors to upset the normal metabolic preference of most cancers, and increase sensitivity to apoptosis. In other conditions, such as neurodegenerative diseases, phosphorylations that inhibit apoptosis could be enhanced and utilized as neuroprotective therapy.

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REFERENCES

[1] Jezek, P., Hlavata, L. (2005) Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *Int. J. Biochem. Cell Biol.* 37: 2478-2503.

[2] Richter, C., Park, J. W., Ames, B. N. (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl. Acad. Sci. US* 85: 6465-6467.

[3] St-Pierre, J., Buckingham, J. A., Roebuck, S. J., Brand, M. D. (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J. Biol. Chem.* 277: 44784-44790.

[4] Han, D., Canali, R., Rettori, D., Kaplowitz, N. (2003) Effect of glutathione depletion on sites and topology of superoxide and hydrogen peroxide production in mitochondria. *Mol. Pharmacol.* 64: 1136-1144.

[5] Rottenberg, H., Covian, R., Trumpower, B. L. (2009) Membrane potential greatly enhances superoxide generation by the cytochrome bc1 complex reconstituted into phospholipid vesicles. *J. Biol. Chem.* 284: 19203-19210.

[6] Murphy, M. P. (2009) How mitochondria produce reactive oxygen species. *Biochem. J.* 417: 1-13.

[7] Finkel, T. (2012) Signal transduction by mitochondrial oxidants. *J. Biol. Chem.* 287: 4434-4440.

[8] Blake, R., Trounce, I. A. (2013) Mitochondrial dysfunction and complications associated with diabetes. *Biochim. Biophys. Acta*.

[9] Kadenbach, B., Arnold, S., Lee, I., Hüttemann, M. (2004) The possible role of cytochrome *c* oxidase in stress-induced apoptosis and degenerative diseases. *Biochim. Biophys. Acta* 1655: 400-408.

[10] Modica-Napolitano, J. S., Kulawiec, M., Singh, K. K. (2007) Mitochondria and human cancer. *Curr. Mol. Med.* 7: 121-131.

[11] Samavati, L., Lee, I., Mathes, I., Lottspeich, F., Hüttemann, M. (2008) Tumor necrosis factor α inhibits oxidative phosphorylation through tyrosine phosphorylation at subunit I of cytochrome *c* oxidase. *J. Biol. Chem.* 283: 21134-21144.

[12] Duvigneau, J. C., Piskernik, C., Haindl, S., Kloesch, B., Hartl, R. T., Hüttemann, M., Lee, I., Ebel, T., Moldzio, R., Gemeiner, M., Redl, H., Kozlov, A. V. (2008) A novel endotoxin-induced pathway: upregulation of heme oxygenase 1, accumulation of free iron, and free iron-mediated mitochondrial dysfunction. *Lab. Invest.* 88: 70-77.

[13] Sanderson, T. H., Reynolds, C. A., Kumar, R., Przyklenk, K., Hüttemann, M. (2013) Molecular mechanisms of ischemia-reperfusion injury in brain: pivotal role of the mitochondrial membrane potential in reactive oxygen species generation. *Mol. Neurobiol.* 47: 9-23.

[14] Bratic, A., Larsson, N. G. (2013) The role of mitochondria in aging. *J. Clin. Invest.* 123: 951-957.

[15] Hwang, A. B., Jeong, D. E., Lee, S. J. (2012) Mitochondria and organismal longevity. *Curr. Genomics* 13: 519-532.

[16] Calvo, S. E., Mootha, V. K. (2010) The mitochondrial proteome and human disease. *Annu. Rev. Genomics Hum. Genet.* 11: 25-44.

[17] Pagliarini, D. J., Calvo, S. E., Chang, B., Sheth, S. A., Vafai, S. B., Ong, S. E., Walford, G. A., Sugiana, C., Boneh, A., Chen, W. K., Hill, D. E., Vidal, M., Evans, J. G., Thorburn, D. R., Carr, S. A., Mootha, V. K. (2008) A mitochondrial protein compendium elucidates complex I disease biology. *Cell* 134: 112-123.

[18] McMunn, C. A. (1884) On myohaematin, an intrinsic muscle-pigment of vertebrates and invertebrates, on histohaematin, and on the spectrum of the suprarenal bodies. *Proceedings of the Physiological Society* 5: 24-26.

[19] Keilin, D. (1925) On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants. *Proc. R. Soc. London Ser. B* 98: 312–339.

[20] Liu, X., Kim, C. N., Yang, J., Jemmerson, R., Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86: 147-157.

[21] Diekert, K., de Kroon, A. I., Ahting, U., Niggemeyer, B., Neupert, W., de Kruijff, B., Lill, R. (2001) Apocytochrome c requires the TOM complex for translocation across the mitochondrial outer membrane. *Embo J.* 20: 5626-5635.

[22] Dumont, M. E., Cardillo, T. S., Hayes, M. K., Sherman, F. (1991) Role of cytochrome c heme lyase in mitochondrial import and accumulation of cytochrome c in Saccharomyces cerevisiae. *Mol. Cell. Biol.* 11: 5487-5496.

[23] Dickerson, R. E., Kopka, M. L., Borders, C. L., Jr., Varnum, J., Weinzier, J. E. (1967) A centrosymmetric projection at 4A of horse heart oxidized cytochrome c. *J. Mol. Biol.* 29: 77-95.

[24] Bushnell, G. W., Louie, G. V., Brayer, G. D. (1990) High-resolution three-dimensional structure of horse heart cytochrome c. *J. Mol. Biol.* 214: 585-595.

[25] Salemme, F. R. (1977) Structure and function of cytochromes c. *Annu. Rev. Biochem.* 46: 299-329.

[26] Li, K., Li, Y., Shelton, J. M., Richardson, J. A., Spencer, E., Chen, Z. J., Wang, X., Williams, R. S. (2000) Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell* 101: 389-399.

[27] Morriss, G. M., New, D. A. (1979) Effect of oxygen concentration on morphogenesis of cranial neural folds and neural crest in cultured rat embryos. *J. Embryol. Exp. Morphol.* 54: 17-35.

[28] Sakamoto, K., Kamiya, M., Imai, M., Shinzawa-Itoh, K., Uchida, T., Kawano, K., Yoshikawa, S., Ishimori, K. (2011) NMR basis for interprotein electron transfer gating between cytochrome c and cytochrome c oxidase. *Proc. Natl. Acad. Sci. US* 108: 12271-12276.

[29] Roberts, V. A., Pique, M. E. (1999) Definition of the interaction domain for cytochrome c on cytochrome c oxidase. III. Prediction of the docked complex by a complete, systematic search. *J. Biol. Chem.* 274: 38051-38060.

[30] Chacinska, A., Pfannschmidt, S., Wiedemann, N., Kozjak, V., Sanjuan Szklarz, L. K., Schulze-Specking, A., Truscott, K. N., Guiard, B., Meisinger, C., Pfanner, N. (2004) Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. *Embo J.* 23: 3735-3746.

[31] Stojanovski, D., Bragoszewski, P., Chacinska, A. (2012) The MIA pathway: a tight bond between protein transport and oxidative folding in mitochondria. *Biochimi. Biophys. Acta* 1823: 1142-1150.

[32] Wrobel, L., Trojanowska, A., Sztolsztener, M. E., Chacinska, A. (2013) Mitochondrial protein import: Mia40 facilitates Tim22 translocation into the inner membrane of mitochondria. *Mol. Biol. Cell* 24: 543-554.

[33] Allen, S., Balabanidou, V., Sideris, D. P., Lisowsky, T., Tokatlidis, K. (2005) Erv1 mediates the Mia40-dependent protein import pathway and provides a functional link to the respiratory chain by shuttling electrons to cytochrome c. *J. Mol. Biol.* 353: 937-944.

[34] Aoki, H., Kang, P. M., Hampe, J., Yoshimura, K., Noma, T., Matsuzaki, M., Izumo, S. (2002) Direct activation of mitochondrial apoptosis machinery by c-Jun N-terminal kinase in adult cardiac myocytes. *J. Biol. Chem.* 277: 10244-10250.

[35] Korshunov, S. S., Krasnikov, B. F., Pereverzev, M. O., Skulachev, V. P. (1999) The antioxidant functions of cytochrome c. *FEBS Lett.* 462: 192-198.

[36] Pereverzev, M. O., Vygodina, T. V., Konstantinov, A. A., Skulachev, V. P. (2003) Cytochrome c, an ideal antioxidant. *Biochem. Soc. Trans.* 31: 1312-1315.

[37] Wang, Z. B., Li, M., Zhao, Y., Xu, J. X. (2003) Cytochrome c is a hydrogen peroxide scavenger in mitochondria. *Protein Pept. Lett.* 10: 247-253.

[38] Kim, H. E., Du, F., Fang, M., Wang, X. (2005) Formation of apoptosome is initiated by cytochrome c-induced dATP hydrolysis and

subsequent nucleotide exchange on Apaf-1. *Proc. Natl. Acad. Sci. US* 102: 17545-17550.

[39] Green, D. R. (2000) Apoptotic pathways: paper wraps stone blunts scissors. *Cell* 102: 1-4.

[40] Wang, X. (2001) The expanding role of mitochondria in apoptosis. *Genes Dev.* 15: 2922-2933.

[41] Bender, C. E., Fitzgerald, P., Tait, S. W., Llambi, F., McStay, G. P., Tupper, D. O., Pellettieri, J., Sanchez Alvarado, A., Salvesen, G. S., Green, D. R. (2012) Mitochondrial pathway of apoptosis is ancestral in metazoans. *Proc. Natl. Acad. Sci. US* 109: 4904-4909.

[42] Arama, E., Bader, M., Srivastava, M., Bergmann, A., Steller, H. (2006) The two Drosophila cytochrome C proteins can function in both respiration and caspase activation. *EMBO J.* 25: 232-243.

[43] Zmasek, C. M., Zhang, Q., Ye, Y., Godzik, A. (2007) Surprising complexity of the ancestral apoptosis network. *Genome Biol.* 8: R226.

[44] Madeo, F., Herker, E., Wissing, S., Jungwirth, H., Eisenberg, T., Frohlich, K. U. (2004) Apoptosis in yeast. *Curr. Opin. Microbiol.* 7: 655-660.

[45] Doran, E., Halestrap, A. P. (2000) Cytochrome c release from isolated rat liver mitochondria can occur independently of outer-membrane rupture: possible role of contact sites. *Biochem. J.* 348 Pt 2: 343-350.

[46] Chalmers, S., Nicholls, D. G. (2003) The relationship between free and total calcium concentrations in the matrix of liver and brain mitochondria. *J. Biol. Chem.* 278: 19062-19070.

[47] Jacobs, E. E., Sanadi, D. R. (1960) The reversible removal of cytochrome c from mitochondria. *J. Biol. Chem.* 235: 531-534.

[48] Jones, S., Holm, T., Mager, I., Langel, U., Howl, J. (2010) Characterization of bioactive cell penetrating peptides from human cytochrome c: protein mimicry and the development of a novel apoptogenic agent. *Chem. Biol.* 17: 735-744.

[49] Sanderson, T. H., Mahapatra, G., Pecina, P., Ji, Q., Yu, K., Sinkler, C., Varughese, A., Kumar, R., Bukowski, M. J., Tousignant, R. N., Salomon, A. R., Lee, I., Hüttemann, M. (2013) Cytochrome c is tyrosine 97 phosphorylated by neuroprotective insulin treatment. *PLoS One* 8: e78627.

[50] Yu, T., Wang, X., Purring-Koch, C., Wei, Y., McLendon, G. L. (2001) A mutational epitope for cytochrome C binding to the apoptosis protease activation factor-1. *J. Biol. Chem.* 276: 13034-13038.

[51] Chandra, D., Bratton, S. B., Person, M. D., Tian, Y., Martin, A. G., Ayres, M., Fearnhead, H. O., Gandhi, V., Tang, D. G. (2006) Intracellular nucleotides act as critical prosurvival factors by binding to cytochrome C and inhibiting apoptosome. *Cell* 125: 1333-1346.

[52] Nakabayashi, J., Sasaki, A. (2006) A mathematical model for apoptosome assembly: the optimal cytochrome c/Apaf-1 ratio. *J. Theor. Biol.* 242: 280-287.

[53] Mei, Y., Yong, J., Liu, H., Shi, Y., Meinkoth, J., Dreyfuss, G., Yang, X. (2010) tRNA binds to cytochrome c and inhibits caspase activation. *Mol. Cell* 37: 668-678.

[54] Brown, G. C., Borutaite, V. (2008) Regulation of apoptosis by the redox state of cytochrome c. *Biochim. Biophys. Acta* 1777: 877-881.

[55] Yuan, S., Topf, M., Reubold, T. F., Eschenburg, S., Akey, C. W. (2013) Changes in Apaf-1 conformation that drive apoptosome assembly. *Biochemistry* 52: 2319-2327.

[56] Villani, G., Greco, M., Papa, S., Attardi, G. (1998) Low reserve of cytochrome c oxidase capacity in vivo in the respiratory chain of a variety of human cell types. *J. Biol. Chem.* 273: 31829-31836.

[57] Kunz, W. S., Kudin, A., Vielhaber, S., Elger, C. E., Attardi, G., Villani, G. (2000) Flux control of cytochrome c oxidase in human skeletal muscle. *J. Biol. Chem.* 275: 27741-27745.

[58] Acin-Perez, R., Bayona-Bafaluy, M. P., Bueno, M., Machicado, C., Fernandez-Silva, P., Perez-Martos, A., Montoya, J., Lopez-Perez, M. J., Sancho, J., Enriquez, J. A. (2003) An intragenic suppressor in the cytochrome c oxidase I gene of mouse mitochondrial DNA. *Hum. Mol. Genet.* 12: 329-339.

[59] Villani, G., Attardi, G. (1997) In vivo control of respiration by cytochrome c oxidase in wild-type and mitochondrial DNA mutation-carrying human cells. *Proc. Natl. Acad. Sci. US* 94: 1166-1171.

[60] Piccoli, C., Scrima, R., Boffoli, D., Capitanio, N. (2006) Control by cytochrome c oxidase of the cellular oxidative phosphorylation system depends on the mitochondrial energy state. *Biochem. J.* 396: 573-583.

[61] Dalmonte, M. E., Forte, E., Genova, M. L., Giuffre, A., Sarti, P., Lenaz, G. (2009) Control of respiration by cytochrome c oxidase in intact cells: role of the membrane potential. *J. Biol. Chem.* 284: 32331-32335.

[62] Hüttemann, M., Lee, I., Pecinova, A., Pecina, P., Przyklenk, K., Doan, J. W. (2008) Regulation of oxidative phosphorylation, the mitochondrial membrane potential, and their role in human disease. *J. Bioenerg. Biomembr.* 40: 445-456.

[63] Covian, R., Balaban, R. S. (2012) Cardiac mitochondrial matrix and respiratory complex protein phosphorylation. *Am. J. Physiol. Heart Circ. Physiol.* 303: H940-966.

[64] Goldberg, E., Sberna, D., Wheat, T. E., Urbanski, G. J., Margoliash, E. (1977) Cytochrome c: immunofluorescent localization of the testis-specific form. *Science* 196: 1010-1012.

[65] Liu, Z., Lin, H., Ye, S., Liu, Q. Y., Meng, Z., Zhang, C. M., Xia, Y., Margoliash, E., Rao, Z., Liu, X. J. (2006) Remarkably high activities of testicular cytochrome c in destroying reactive oxygen species and in triggering apoptosis. *Proc. Natl. Acad. Sci. US* 103: 8965-8970.

[66] Vempati, U. D., Diaz, F., Barrientos, A., Narisawa, S., Mian, A. M., Millan, J. L., Boise, L. H., Moraes, C. T. (2007) Role of cytochrome C in apoptosis: increased sensitivity to tumor necrosis factor alpha is associated with respiratory defects but not with lack of cytochrome C release. *Mol. Cell. Biol.* 27: 1771-1783.

[67] Pierron, D., Wildman, D. E., Hüttemann, M., Letellier, T., Grossman, L. I. (2012) Evolution of the couple cytochrome c and cytochrome c oxidase in primates. *Adv. Exp. Med. Biol.* 748: 185-213.

[68] Ferguson-Miller, S., Brautigan, D. L., Margoliash, E. (1976) Correlation of the kinetics of electron transfer activity of various eukaryotic cytochromes c with binding to mitochondrial cytochrome c oxidase. *J. Biol. Chem.* 251: 1104-1115.

[69] Napiwotzki, J., Shinzawa-Itoh, K., Yoshikawa, S., Kadenbach, B. (1997) ATP and ADP bind to cytochrome c oxidase and regulate its activity. *Biol. Chem.* 378: 1013-1021.

[70] Arnold, S., Kadenbach, B. (1999) The intramitochondrial ATP/ADP-ratio controls cytochrome c oxidase activity allosterically. *FEBS Lett.* 443: 105-108.

[71] Craig, D. B., Wallace, C. J. (1995) Studies of 8-azido-ATP adducts reveal two mechanisms by which ATP binding to cytochrome c could inhibit respiration. *Biochemistry* 34: 2686-2693.

[72] Tuominen, E. K., Zhu, K., Wallace, C. J., Clark-Lewis, I., Craig, D. B., Rytomaa, M., Kinnunen, P. K. (2001) ATP induces a conformational change in lipid-bound cytochrome c. *J. Biol. Chem.* 276: 19356-19362.

[73] Liu, S. S. (1999) Cooperation of a "reactive oxygen cycle" with the Q cycle and the proton cycle in the respiratory chain--superoxide generating and cycling mechanisms in mitochondria. *J. Bioenerg. Biomembr.* 31: 367-376.

[74] Lee, I., Salomon, A. R., Ficarro, S., Mathes, I., Lottspeich, F., Grossman, L. I., Hüttemann, M. (2005) cAMP-dependent tyrosine phosphorylation of subunit I inhibits cytochrome *c* oxidase activity. *J. Biol. Chem.* 280: 6094-6100.

[75] Acin-Perez, R., Gatti, D. L., Bai, Y., Manfredi, G. (2011) Protein phosphorylation and prevention of cytochrome oxidase inhibition by ATP: coupled mechanisms of energy metabolism regulation. *Cell metabolism* 13: 712-719.

[76] Helling, S., Hüttemann, M., Ramzan, R., Kim, S. H., Lee, I., Muller, T., Langenfeld, E., Meyer, H. E., Kadenbach, B., Vogt, S., Marcus, K. (2012) Multiple phosphorylations of cytochrome c oxidase and their functions. *Proteomics* 12: 950-959.

[77] Phillips, D., Aponte, A. M., Covian, R., Balaban, R. S. (2011) Intrinsic protein kinase activity in mitochondrial oxidative phosphorylation complexes. *Biochemistry* 50: 2515-2529.

[78] Hüttemann, M., Helling, S., Sanderson, T. H., Sinkler, C., Samavati, L., Mahapatra, G., Varughese, A., Lu, G., Liu, J., Ramzan, R., Vogt, S., Grossman, L. I., Doan, J. W., Marcus, K., Lee, I. (2012) Regulation of mitochondrial respiration and apoptosis through cell signaling: cytochrome c oxidase and cytochrome c in ischemia/reperfusion injury and inflammation. *Biochim. Biophys. Acta* 1817: 598-609.

[79] Lee, I., Salomon, A. R., Yu, K., Doan, J. W., Grossman, L. I., Hüttemann, M. (2006) New prospects for an old enzyme: mammalian cytochrome *c* is tyrosine-phosphorylated in vivo. *Biochemistry* 45: 9121-9128.

[80] Lee, I., Salomon, A. R., Yu, K., Samavati, L., Pecina, P., Pecinova, A., Hüttemann, M. (2009) Isolation of regulatory-competent, phosphorylated cytochrome *c* oxidase. *Methods Enzymol.* 457: 193-210.

[81] Yu, H., Lee, I., Salomon, A. R., Yu, K., Hüttemann, M. (2008) Mammalian liver cytochrome *c* is tyrosine-48 phosphorylated in vivo, inhibiting mitochondrial respiration. *Biochim. Biophys. Acta* 1777: 1066-1071.

[82] Pecina, P., Borisenko, G. G., Belikova, N. A., Tyurina, Y. Y., Pecinova, A., Lee, I., Samhan-Arias, A. K., Przyklenk, K., Kagan, V. E., Hüttemann, M. (2010) Phosphomimetic substitution of cytochrome c tyrosine 48 decreases respiration and binding to cardiolipin and abolishes ability to trigger downstream caspase activation. *Biochemistry* 49: 6705-6714.

[83] De Rocco, D., Cerqua, C., Goffrini, P., Russo, G., Pastore, A., Meloni, F., Nicchia, E., Moraes, C. T., Pecci, A., Salviati, L., Savoia, A. (2013) Mutations of cytochrome c identified in patients with thrombocytopenia THC4 affect both apoptosis and cellular bioenergetics. *Biochim. Biophys. Acta*.

[84] Morison, I. M., Cramer Borde, E. M., Cheesman, E. J., Cheong, P. L., Holyoake, A. J., Fichelson, S., Weeks, R. J., Lo, A., Davies, S. M., Wilbanks, S. M., Fagerlund, R. D., Ludgate, M. W., da Silva Tatley, F. M., Coker, M. S., Bockett, N. A., Hughes, G., Pippig, D. A., Smith, M. P., Capron, C., Ledgerwood, E. C. (2008) A mutation of human cytochrome c enhances the intrinsic apoptotic pathway but causes only thrombocytopenia. *Nat. Genet.* 40: 387-389.

[85] Liptak, M. D., Fagerlund, R. D., Ledgerwood, E. C., Wilbanks, S. M., Bren, K. L. (2011) The proapoptotic G41S mutation to human cytochrome c alters the heme electronic structure and increases the electron self-exchange rate. *J. Am. Chem. Soc.* 133: 1153-1155.

[86] Zhao, X., Leon, I. R., Bak, S., Mogensen, M., Wrzesinski, K., Hojlund, K., Jensen, O. N. (2011) Phosphoproteome analysis of functional mitochondria isolated from resting human muscle reveals extensive phosphorylation of inner membrane protein complexes and enzymes. *Mol. Cell. Proteomics*: M110.000299.

[87] Garcia-Heredia, J. M., Diaz-Moreno, I., Diaz-Quintana, A., Orzaez, M., Navarro, J. A., Hervas, M., De la Rosa, M. A. (2012) Specific nitration of tyrosines 46 and 48 makes cytochrome c assemble a non-functional apoptosome. *FEBS Lett.* 586: 154-158.

[88] Cassina, A. M., Hodara, R., Souza, J. M., Thomson, L., Castro, L., Ischiropoulos, H., Freeman, B. A., Radi, R. (2000) Cytochrome c nitration by peroxynitrite. *J. Biol. Chem.* 275: 21409-21415.

[89] Jang, B., Han, S. (2006) Biochemical properties of cytochrome c nitrated by peroxynitrite. *Biochimie* 88: 53-58.

[90] Rodriguez-Roldan, V., Garcia-Heredia, J. M., Navarro, J. A., De la Rosa, M. A., Hervas, M. (2008) Effect of nitration on the physico-chemical and kinetic features of wild-type and monotyrosine mutants of human respiratory cytochrome c. *Biochemistry* 47: 12371-12379.

[91] Garcia-Heredia, J. M., Diaz-Moreno, I., Nieto, P. M., Orzaez, M., Kocanis, S., Teixeira, M., Perez-Paya, E., Diaz-Quintana, A., De la Rosa, M. A. (2010) Nitration of tyrosine 74 prevents human cytochrome c to play a key role in apoptosis signaling by blocking caspase-9 activation. *Biochim. Biophys. Acta* 1797: 981-993.

[92] Collins, Y., Chouchani, E. T., James, A. M., Menger, K. E., Cocheme, H. M., Murphy, M. P. (2012) Mitochondrial redox signalling at a glance. *J. Cell Sci.* 125: 801-806.

[93] Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., Lanfrancone, L., Pelicci, P. G. (1999) The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature* 402: 309-313.

[94] Migliaccio, E., Giorgio, M., Pelicci, P. G. (2006) Apoptosis and aging: role of p66Shc redox protein. *Antioxid. Redox Signal.* 8: 600-608.

[95] Suski, J. M., Karkucinska-Wieckowska, A., Lebiedzinska, M., Giorgi, C., Szczepanowska, J., Szabadkai, G., Duszynski, J., Pronicki, M., Pinton, P., Wieckowski, M. R. (2011) p66Shc Aging Protein in Control of Fibroblasts Cell Fate. *Int. J. Mol. Sci.* 12: 5373-5389.

[96] Giorgio, M., Migliaccio, E., Orsini, F., Paolucci, D., Moroni, M., Contursi, C., Pelliccia, G., Luzi, L., Minucci, S., Marcaccio, M., Pinton, P., Rizzuto, R., Bernardi, P., Paolucci, F., Pelicci, P. G. (2005) Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. *Cell* 122: 221-233.

[97] Gertz, M., Steegborn, C. (2010) The Lifespan-regulator p66Shc in mitochondria: redox enzyme or redox sensor? *Antioxid. Redox Signal.* 13: 1417-1428.

[98] Sun, L., Xiao, L., Nie, J., Liu, F. Y., Ling, G. H., Zhu, X. J., Tang, W. B., Chen, W. C., Xia, Y. C., Zhan, M., Ma, M. M., Peng, Y. M., Liu, H., Liu, Y. H., Kanwar, Y. S. (2010) p66Shc mediates high glucose and angiotensin II induced oxidative stress renal tubular injury via mitochondrial dependent apoptotic pathway. *Am. J. Physiol. Renal Physiol.* 299: F1014-1012.

[99] Acin-Perez, R., Hoyos, B., Gong, J., Vinogradov, V., Fischman, D. A., Leitges, M., Borhan, B., Starkov, A., Manfredi, G., Hammerling, U. (2010) Regulation of intermediary metabolism by the PKCdelta signalosome in mitochondria. *FASEB J.* 24: 5033-5042.

[100] Hoyos, B., Acin-Perez, R., Fischman, D. A., Manfredi, G., Hammerling, U. (2012) Hiding in plain sight: uncovering a new function of vitamin A in redox signaling. *Biochim. Biophys. Acta* 1821: 241-247.

[101] Lebiedzinska, M., Duszynski, J., Rizzuto, R., Pinton, P., Wieckowski, M. R. (2009) Age-related changes in levels of p66Shc and serine 36-phosphorylated p66Shc in organs and mouse tissues. *Arch. Biochem. Biophys.* 486: 73-80.

[102] Kagan, V. E., Tyurin, V. A., Jiang, J., Tyurina, Y. Y., Ritow, V. B., Amoscato, A. A., Osipov, A. N., Belikova, N. A., Kapralov, A. A., Kini, V., Vlasova, I. I., Zhao, Q., Zou, M., Di, P., Svistunenko, D. A., Kurnikov, I. V., Borisenko, G. G. (2005) Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nature Chem. Biol.* 1: 223-232.

[103] Garcia Fernandez, M., Troiano, L., Moretti, L., Nasi, M., Pinti, M., Salvioli, S., Dobrucki, J., Cossarizza, A. (2002) Early changes in intramitochondrial cardiolipin distribution during apoptosis. *Cell Growth Differ.* 13: 449-455.

[104] Kagan, V. E., Tyurina, Y. Y., Bayir, H., Chu, C. T., Kapralov, A. A., Vlasova, I. I., Belikova, N. A., Tyurin, V. A., Amoscato, A., Epperly, M., Greenberger, J., Dekosky, S., Shvedova, A. A., Jiang, J. (2006) The "pro-apoptotic genies" get out of mitochondria: oxidative lipidomics and redox activity of cytochrome c/cardiolipin complexes. *Chem. Biol. Interact.* 163: 15-28.

[105] Muenzner, J., Pletneva, E. V. (2013) Structural transformations of cytochrome c upon interaction with cardiolipin. *Chem. Phys. Lipids*.

[106] Sinibaldi, F., Howes, B. D., Droghetti, E., Polticelli, F., Piro, M. C., Di Pierro, D., Fiorucci, L., Coletta, M., Smulevich, G., Santucci, R. (2013) Role of lysines in cytochrome c-cardiolipin interaction. *Biochemistry* 52: 4578-4588.

[107] Balakrishnan, G., Hu, Y., Spiro, T. G. (2012) His26 protonation in cytochrome c triggers microsecond beta-sheet formation and heme exposure: implications for apoptosis. *J. Am. Chem. Soc.* 134: 19061-19069.

[108] Svistunenko, D. A. (2005) Reaction of haem containing proteins and enzymes with hydroperoxides: the radical view. *Biochim. Biophys. Acta* 1707: 127-155.

[109] Tyurina, Y. Y., Kini, V., Tyurin, V. A., Vlasova, I. I., Jiang, J., Kapralov, A. A., Belikova, N. A., Yalowich, J. C., Kurnikov, I. V., Kagan, V. E. (2006) Mechanisms of cardiolipin oxidation by cytochrome c: relevance to pro- and antiapoptotic functions of etoposide. *Mol. Pharmacol.* 70: 706-717.

[110] Kapralov, A. A., Yanamala, N., Tyurina, Y. Y., Castro, L., Samhan-Arias, A., Vladimirov, Y. A., Maeda, A., Weitz, A. A., Peterson, J., Mylnikov, D., Demicheli, V., Tortora, V., Klein-Seetharaman, J., Radi, R., Kagan, V. E. (2011) Topography of tyrosine residues and their involvement in peroxidation of polyunsaturated cardiolipin in

cytochrome c/cardiolipin peroxidase complexes. *Biochim. Biophys. Acta* 1808: 2147-2155.

[111] Rajagopal, B. S., Edzuma, A. N., Hough, M. A., Blundell, K. L., Kagan, V. E., Kapralov, A. A., Fraser, L. A., Butt, J. N., Silkstone, G. G., Wilson, M. T., Svistunenko, D. A., Worrall, J. A. (2013) The hydrogen-peroxide-induced radical behaviour in human cytochrome c-phospholipid complexes: implications for the enhanced pro-apoptotic activity of the G41S mutant. *Biochem. J.* 456: 441-452.

[112] Bergstrom, C. L., Beales, P. A., Lv, Y., Vanderlick, T. K., Groves, J. T. (2013) Cytochrome c causes pore formation in cardiolipin-containing membranes. *Proc. Natl. Acad. Sci. US* 110: 6269-6274.

[113] Muenzner, J., Toffey, J. R., Hong, Y., Pletneva, E. V. (2013) Becoming a peroxidase: cardiolipin-induced unfolding of cytochrome c. *J. Phys. Chem. B* 117: 12878-12886.

[114] Niizuma, K., Endo, H., Chan, P. H. (2009) Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. *J. Neurochem.* 109 Suppl. 1: 133-138.

[115] Fujimura, M., Morita-Fujimura, Y., Murakami, K., Kawase, M., Chan, P. H. (1998) Cytosolic redistribution of cytochrome c after transient focal cerebral ischemia in rats. *J. Cereb. Blood Flow Metab.* 18: 1239-1247.

[116] Sugawara, T., Fujimura, M., Morita-Fujimura, Y., Kawase, M., Chan, P. H. (1999) Mitochondrial release of cytochrome c corresponds to the selective vulnerability of hippocampal CA1 neurons in rats after transient global cerebral ischemia. *J. Neurosci.* 19: RC39.

[117] Rabuffetti, M., Sciorati, C., Tarozzo, G., Clementi, E., Manfredi, A. A., Beltramo, M. (2000) Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-chloromethyl ketone induces long-lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines. *J. Neurosci.* 20: 4398-4404.

[118] Li, M., Ona, V. O., Chen, M., Kaul, M., Tenneti, L., Zhang, X., Stieg, P. E., Lipton, S. A., Friedlander, R. M. (2000) Functional role and therapeutic implications of neuronal caspase-1 and -3 in a mouse model of traumatic spinal cord injury. *Neuroscience* 99: 333-342.

[119] Chan, P. H. (2004) Mitochondria and neuronal death/survival signaling pathways in cerebral ischemia. *Neurochem. Res.* 29: 1943-1949.

[120] Van Horssen, J., Witte, M. E., Ciccarelli, O. (2012) The role of mitochondria in axonal degeneration and tissue repair in MS. *Mult. Scler.* 18: 1058-1067.

[121] Mahad, D., Lassmann, H., Turnbull, D. (2008) Review: Mitochondria and disease progression in multiple sclerosis. *Neuropathol. Appl. Neurobiol.* 34: 577-589.

[122] Wu, D. C., Jackson-Lewis, V., Vila, M., Tieu, K., Teismann, P., Vadseth, C., Choi, D. K., Ischiropoulos, H., Przedborski, S. (2002) Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. *J. Neurosci.* 22: 1763-1771.

[123] Berger, A. K., Cortese, G. P., Amodeo, K. D., Weihofen, A., Letai, A., LaVoie, M. J. (2009) Parkin selectively alters the intrinsic threshold for mitochondrial cytochrome c release. *Hum. Mol. Genet*. 18: 4317-4328.

[124] Yan, M. H., Wang, X., Zhu, X. (2013) Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. *Free Radic. Biol. Med.* 62: 90-101.

[125] Wang, X., Zhu, S., Drozda, M., Zhang, W., Stavrovskaya, I. G., Cattaneo, E., Ferrante, R. J., Kristal, B. S., Friedlander, R. M. (2003) Minocycline inhibits caspase-independent and -dependent mitochondrial cell death pathways in models of Huntington's disease. *Proc. Natl. Acad. Sci. US* 100: 10483-10487.

[126] Wang, X., Zhu, S., Pei, Z., Drozda, M., Stavrovskaya, I. G., Del Signore, S. J., Cormier, K., Shimony, E. M., Wang, H., Ferrante, R. J., Kristal, B. S., Friedlander, R. M. (2008) Inhibitors of cytochrome c release with therapeutic potential for Huntington's disease. *J. Neurosci.* 28: 9473-9485.

[127] Costa, V., Giacomello, M., Hudec, R., Lopreiato, R., Ermak, G., Lim, D., Malorni, W., Davies, K. J., Carafoli, E., Scorrano, L. (2010) Mitochondrial fission and cristae disruption increase the response of cell models of Huntington's disease to apoptotic stimuli. *EMBO Mol. Med.* 2: 490-503.

[128] Bacman, S. R., Bradley, W. G., Moraes, C. T. (2006) Mitochondrial involvement in amyotrophic lateral sclerosis: trigger or target? *Mol. Neurobiol.* 33: 113-131.

[129] Xu, R., Wu, C., Zhang, X., Zhang, Q., Yang, Y., Yi, J., Yang, R., Tao, Y. (2011) Linking hypoxic and oxidative insults to cell death mechanisms in models of ALS. *Brain Res.* 1372: 133-144.

[130] Garcia, M. L., Fernandez, A., Solas, M. T. (2013) Mitochondria, motor neurons and aging. *J. Neurol. Sci.* 330: 18-26.

[131] Birk, A. V., Liu, S., Soong, Y., Mills, W., Singh, P., Warren, J. D., Seshan, S. V., Pardee, J. D., Szeto, H. H. (2013) The mitochondrial-

targeted compound SS-31 re-energizes ischemic mitochondria by interacting with cardiolipin. *J. Am. Soc. Nephrol.* 24: 1250-1261.

[132] Birk, A. V., Chao, W. M., Bracken, C., Warren, J. D., Szeto, H. H. (2013) Targeting Mitochondrial Cardiolipin and the Cytochrome C/ Cardiolipin Complex to Promote Electron Transport and Optimize Mitochondrial Atp Synthesis. *Br. J. Pharmacol.*

[133] Patriarca, A., Polticelli, F., Piro, M. C., Sinibaldi, F., Mei, G., Bari, M., Santucci, R., Fiorucci, L. (2012) Conversion of cytochrome c into a peroxidase: inhibitory mechanisms and implication for neuro-degenerative diseases. *Arch. Biochem. Biophys.* 522: 62-69.

[134] Ji, J., Kline, A. E., Amoscato, A., Samhan-Arias, A. K., Sparvero, L. J., Tyurin, V. A., Tyurina, Y. Y., Fink, B., Manole, M. D., Puccio, A. M., Okonkwo, D. O., Cheng, J. P., Alexander, H., Clark, R. S., Kochanek, P. M., Wipf, P., Kagan, V. E., Bayir, H. (2012) Lipidomics identifies cardiolipin oxidation as a mitochondrial target for redox therapy of brain injury. *Nat. Neurosci.* 15: 1407-1413.

[135] Savino, C., Pelicci, P., Giorgio, M. (2013) The P66Shc/mitochondrial permeability transition pore pathway determines neurodegeneration. *Oxid. Med. Cell Longev.* 2013: 719407.

[136] Su, K., Bourdette, D., Forte, M. (2012) Genetic inactivation of mitochondria-targeted redox enzyme p66ShcA preserves neuronal viability and mitochondrial integrity in response to oxidative challenges. *Front Physiol.* 3: 285.

[137] Endo, H., Saito, A., Chan, P. H. (2006) Mitochondrial translocation of p53 underlies the selective death of hippocampal CA1 neurons after global cerebral ischaemia. *Biochem. Soc. Trans.* 34: 1283-1286.

[138] Sanderson, T. H., Kumar, R., Sullivan, J. M., Krause, G. S. (2008) Insulin blocks cytochrome c release in the reperfused brain through PI3-K signaling and by promoting Bax/Bcl-XL binding. *J. Neurochem.* 106: 1248-1258.

[139] Igney, F. H., Krammer, P. H. (2002) Death and anti-death: tumour resistance to apoptosis. *Nat. Rev. Cancer* 2: 277-288.

[140] Fulda, S. (2010) Evasion of apoptosis as a cellular stress response in cancer. *Int. J. Cell Biol.* 2010: 370835.

[141] Kelly, G. L., Strasser, A. (2011) The essential role of evasion from cell death in cancer. *Adv. Cancer Res.* 111: 39-96.

[142] Koppenol, W. H., Bounds, P. L., Dang, C. V. (2011) Otto Warburg's contributions to current concepts of cancer metabolism. *Nat. Rev. Cancer* 11: 325-337.

[143] Wallace, D. C. (2012) Mitochondria and cancer. *Nat. Rev. Cancer* 12: 685-698.

[144] Schulze, A., Harris, A. L. (2012) How cancer metabolism is tuned for proliferation and vulnerable to disruption. *Nature* 491: 364-373.

[145] Ayyasamy, V., Owens, K. M., Desouki, M. M., Liang, P., Bakin, A., Thangaraj, K., Buchsbaum, D. J., LoBuglio, A. F., Singh, K. K. (2011) Cellular model of Warburg effect identifies tumor promoting function of UCP2 in breast cancer and its suppression by genipin. *PloS One* 6: e24792.

[146] Ko, Y. H., Smith, B. L., Wang, Y., Pomper, M. G., Rini, D. A., Torbenson, M. S., Hullihen, J., Pedersen, P. L. (2004) Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP. *Biochem. Biophys. Res. Commun.* 324: 269-275.

[147] Akers, L. J., Fang, W., Levy, A. G., Franklin, A. R., Huang, P., Zweidler-McKay, P. A. (2011) Targeting glycolysis in leukemia: a novel inhibitor 3-BrOP in combination with rapamycin. *Leuk. Res.* 35: 814-820.

[148] Holohan, C., Van Schaeybroeck, S., Longley, D. B., Johnston, P. G. (2013) Cancer drug resistance: an evolving paradigm. *Nat. Rev. Cancer* 13: 714-726.

[149] Johnson, C. E., Huang, Y. Y., Parrish, A. B., Smith, M. I., Vaughn, A. E., Zhang, Q., Wright, K. M., Van Dyke, T., Wechsler-Reya, R. J., Kornbluth, S., Deshmukh, M. (2007) Differential Apaf-1 levels allow cytochrome c to induce apoptosis in brain tumors but not in normal neural tissues. *Proc. Natl. Acad. Sci. US* 104: 20820-20825.

[150] Barros, S., Mencia, N., Rodriguez, L., Oleaga, C., Santos, C., Noe, V., Ciudad, C. J. (2013) The redox state of cytochrome c modulates resistance to methotrexate in human MCF7 breast cancer cells. *PloS One* 8: e63276.

[151] Plas, D. R., Thompson, C. B. (2002) Cell metabolism in the regulation of programmed cell death. *Trends Endocrinol. Metab.* 13: 75-78.

[152] Bonnet, S., Archer, S. L., Allalunis-Turner, J., Haromy, A., Beaulieu, C., Thompson, R., Lee, C. T., Lopaschuk, G. D., Puttagunta, L., Harry, G., Hashimoto, K., Porter, C. J., Andrade, M. A., Thebaud, B., Michelakis, E. D. (2007) A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer Cell* 11: 37-51.

[153] Vaughn, A. E., Deshmukh, M. (2008) Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c. *Nat. Cell Biol.* 10: 1477-1483.

[154] Ruckenstuhl, C., Buttner, S., Carmona-Gutierrez, D., Eisenberg, T., Kroemer, G., Sigrist, S. J., Frohlich, K. U., Madeo, F. (2009) The Warburg effect suppresses oxidative stress induced apoptosis in a yeast model for cancer. *PloS One* 4: e4592.

[155] Schmidt, T. R., Wildman, D. E., Uddin, M., Opazo, J. C., Goodman, M., Grossman, L. I. (2005) Rapid electrostatic evolution at the binding site for cytochrome c on cytochrome c oxidase in anthropoid primates. *Proc. Natl. Acad. Sci. US* 102: 6379-6384.