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Tissue-specific regulation of cytochrome *c* by post-translational modifications: respiration, the mitochondrial membrane potential, ROS, and apoptosis

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ABSTRACT: Cytochrome *c* (Cyt*c*) plays a vital role in the mitochondrial electron transport chain (ETC). In addition, it is a key regulator of apoptosis. Cyt*c* has multiple other functions including ROS production and scavenging, cardiolipin peroxidation, and mitochondrial protein import. Cyt*c* is tightly regulated by allosteric mechanisms, tissue-specific isoforms, and post-translational modifications (PTMs). Distinct residues of Cyt*c* are modified by PTMs, primarily phosphorylations, in a highly tissue-specific manner. These modifications downregulate mitochondrial ETC flux and adjust the mitochondrial membrane potential ($\Delta\Psi_m$), to minimize reactive oxygen species (ROS) production under normal conditions. In pathologic and acute stress conditions, such as ischemia–reperfusion, phosphorylations are lost, leading to maximum ETC flux, $\Delta\Psi_m$ hyperpolarization, excessive ROS generation, and the release of Cyt*c*. It is also the dephosphorylated form of the protein that leads to maximum caspase activation. We discuss the complex regulation of Cyt*c* and propose that it is a central regulatory step of the mammalian ETC that can be rate limiting in normal conditions. This regulation is important because it maintains optimal intermediate $\Delta\Psi_m$, limiting ROS generation. We examine the role of Cyt*c* PTMs, including phosphorylation, acetylation, methylation, nitration, nitrosylation, and sulfoxidation and consider their potential biological significance by evaluating their stoichiometry.—Kalpage, H. A., Bazylianska, V., Recanati, M. A., Fite, A., Liu, J., Wan, J., Mantena, N., Malek, M. H., Podgorski, I., Heath, E. I., Vaishnav, A., Edwards, B. F., Grossman, L. I., Sanderson, T. H., Lee, I., Hüttemann, M. Tissue-specific regulation of cytochrome *c* by post-translational modifications: respiration, the mitochondrial membrane potential, ROS, and apoptosis. FASEB J. 33, 1540–1553 (2019). www.fasebj.org

KEY WORDS: electron transport chain · ischemia–reperfusion · phosphorylation · reactive oxygen species · signal transduction

Cytochrome *c* (Cyt*c*) plays a pivotal role in making life-and-death decisions for the fate of the cell. It is a small, globular, highly conserved, 104 aa protein with a covalently attached heme group that performs multiple functions and catalytic activities. In the mitochondrial electron transport chain (ETC), Cyt*c* acts as an electron carrier from complex III to complex IV, which are also known as the cytochrome bc_1 complex and Cyt*c* oxidase (COX), respectively. ETC function fully relies on the presence of Cyt*c*, as can be seen in Cyt*c*-knockout mice that die at midgestation (1), when fetal metabolism switches from glycolysis to oxidative phosphorylation (2) to sustain the increasing energy supply of the growing organism. It has been shown with Cyt*c* knockout mouse fibroblasts that Cyt*c* plays a prominent role in supercomplex formation and is essential for the stability and assembly of COX (3). Cyt*c* also plays a key role in apoptosis by acting as a signaling molecule that is released from the mitochondria into the cytosol during cellular stress. In the cytosol, it

ABBREVIATIONS: Apaf, apoptosis activating factor; COX, cytochrome *c* oxidase; Cyt*c*, cytochrome *c*; $\Delta\Psi_m$, mitochondrial membrane potential; Erv, essential for respiration and viability; ETC, electron transport chain; IMAC/nano-LC/ESI-MS, immobilized metal affinity chromatography/nanoliquid chromatography/electrospray ionization mass spectrometry; Mia, mitochondrial intermembrane space import and assembly; PTM, post-translational modification; ROS, reactive oxygen species; WT, wild type

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interacts with apoptosis activating factor (Apaf)-1, leading to the formation of the heptameric apoptosome, which activates downstream caspases and initiates the cell death pathway. Furthermore, early-on during apoptosis, Cyt*c* functions as a peroxidase of cardiolipin, a mitochondria-specific lipid that binds to Cyt*c*. This interaction leads to conformational changes around the heme group, converting Cyt*c* into a pentacoordinated structure from its native hexacoordinated form (4, 5). This conversion enhances cardiolipin peroxidase activity of Cyt*c* in the presence of H_2O_2. This proapoptotic event promotes the dissociation of Cyt*c* from the outer leaflet of the inner mitochondrial membrane and its release into the cytosol, where apoptosis is induced (6). There are 2 distinct binding sites for cardiolipin: the A and C sites. The A site overlaps with the ATP binding site, which is located on the left side of the molecule in the conventional view. The C site allows hydrophobic interaction between cardiolipin and Cyt*c* (7, 8).

Cyt*c* has been referred to as a "moonlighting" protein because of its multifunctional properties in addition to apoptosis and respiration (9, 10). It has been shown to play a role in regulated production of reactive oxygen species (ROS), mainly H_2O_2, by reducing $p66^{Shc}$ (11), a splice variant of $p52^{Shc}/p46^{Shc}$, which are adaptor proteins involved in the Ras signaling pathway (12). $p66^{Shc}$ is a negative regulator of life span with increased survival observed in $p66^{Shc}$ knockout mice and cells (13–15). These knockouts also demonstrate increased resistance to ROS and UV damage. $p66^{Shc}$ is regulated by phosphorylation through the PKC-β signaling pathway (16). Phosphorylation of $p66^{Shc}$ at Ser36 is necessary for its role in ROS production (13, 17, 18). Prevention of Cyt*c* binding by mutation of $p66^{Shc}$ W134F has been shown to attenuate ROS-induced renal toxicity (19–21).

In contrast to the role of Cyt*c* in regulated ROS formation by interaction with $p66^{Shc}$, Cyt*c* is also a well-known ROS scavenger because of its ability to take up and donate electrons quickly. Horse heart Cyt*c* has been shown to act as an H_2O_2 scavenger in rat heart mitochondria linked to reverse electron transfer from succinate to NAD^+ involving complex I (22, 23). In addition, Cyt*c* plays a role in oxidation of superoxide to oxygen, where the electron removed from superoxide is channeled to COX and used for energy production (22, 24). Recently, Cyt*c* was shown to function as an H_2S oxidase and thus as a modulator of H_2S signaling (25).

Cyt*c* also has a rodent testes-specific isoform that is 86% homologous to the somatic form (26). It has been shown that testes-specific Cyt*c* results in 3 times better H_2O_2 scavenging compared to somatic Cyt*c*. However, the testes-specific Cyt*c* also demonstrates significantly higher apoptotic activity, possibly to help maintain sperm integrity (27). In humans, this testes-specific isoform exists as a nontranscribed pseudogene (28, 29). The loss of the testes-specific isoform in humans may at first sight be surprising. However, the loss—engendered by a stop codon at position 49—took place on the primate stem ~65 million years ago (30). The loss of the testes isoform coincided with a period of accelerated evolution of COX in anthropoid primates, such that the COX acceleration was much greater for the residues that are part of the Cyt*c*-COX binding domain than at other COX residues (31). The result is that COX has undergone several amino acid replacements, leading to reduced electrostatic interactions between these 2 proteins in anthropoid primates (31).

In conjunction with its potential as an electron carrier, Cyt*c* is also involved in redox-coupled import of proteins containing twin CX_3C and CX_9C proteins through the Mia40-Erv1 disulfide relay system. These cysteine-rich proteins are transported to the intermembrane space of the mitochondria *via* Mia40-catalyzed oxidation of the 4 cysteine residues of the proteins that enter the intermembrane space through the translocase of the outer membrane proteins. Mia40 is reoxidized by sulfhydryl oxidase Erv1, and Erv1 is reoxidized by transfer of electrons to Cyt*c*, thus connecting this protein import pathway to the ETC (32, 33).

Cyt*c* has also been reported to bind the inositol-1,4,5-triphosphate receptor in the endoplasmic reticulum during apoptosis, which leads to calcium overload in the mitochondria and cytosol, resulting in a positive-feedback mechanism that causes more Cyt*c* release and activation of apoptosis (34–36). In addition, once released, Cyt*c* competes with the Apaf-1 inhibitor 14-3-3ε for binding to Apaf-1, further promoting caspase activation (37). Furthermore, Cyt*c* acts as an oxidizing agent that facilitates conversion of sulfite to sulfate by sulfite oxidase, a molybdenum-dependent mitochondrial protein (38, 39). Sulfite is a harmful metabolic byproduct of the amino acids containing sulfur, methionine and cysteine. Cyt*c* helps reduce sulfite toxicity by interaction with sulfite oxidase in a binding domain similar to that of COX (40).

Cyt*c* can translocate to the nucleus in response to DNA damage, where it has been shown to play a role in apoptosis by inducing chromatin condensation independent of caspase activity (41). Other studies have suggested that nuclear translocation of Cyt*c* takes place before caspase activation. Human Cyt*c* and plant Cyt*c* inhibit the nucleosome assembly function of SET/template-activating factor Ib (SET/TAF-Ib) chaperones in humans and nucleosome assembly protein-1–related protein-1 (NRP-1) chaperones in plants (42, 43). Cyt*c* is a highly positively charged protein that promotes its interaction with low-complexity acidic regions of the above histone-binding chaperones. This inhibitory interaction of Cyt*c* has been proposed to be a cellular life-or-death mechanism, depending on the severity of cell damage (44). Finally, Cyt*c* was recently shown to have yet another catalytic function by acting as a plasmalogenase *in vitro*, cleaving the vinyl–ether linkage of important phospholipids called plasmalogens, which are involved in lipid signaling (45). However, more research is needed to confirm that Cyt*c* acts as a plasmalogenase *in vivo*.

POST-TRANSLATIONAL MODIFICATIONS OF CYT*c* AND THEIR FUNCTIONAL EFFECTS

Considering the multiple roles of Cyt*c* in energy production, ROS balance, and apoptosis (**Fig. 1**), this protein

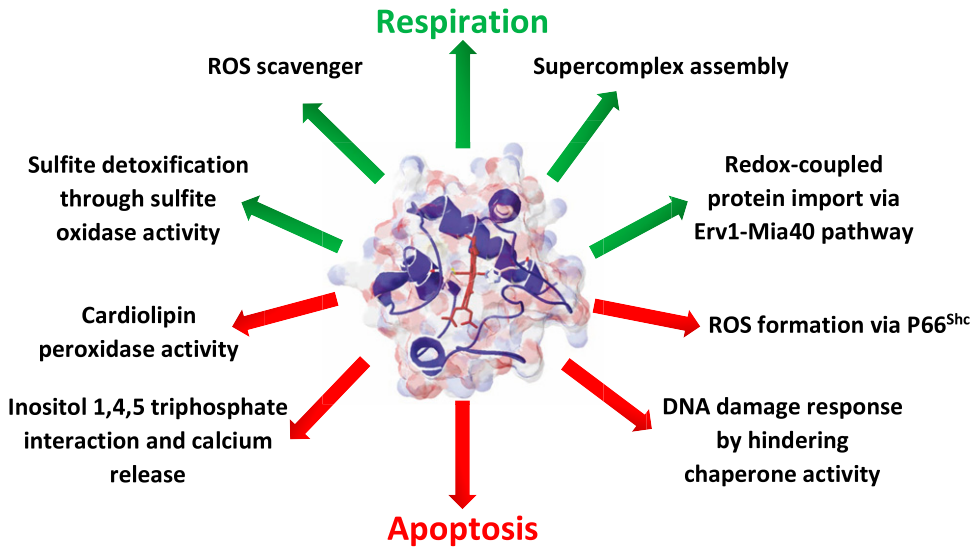

Figure 1. Pro-life (green arrows) and pro-death functions (red arrows) of Cyt*c*.

should be tightly regulated. Regulation of mitochondrial proteins is also necessary because of the differences in the cellular metabolic requirements between tissues and organs. Protein regulation can be achieved by several mechanisms, starting at the gene expression level, through translational regulation, and finally at the post-translational level through post-translational modifications (PTMs) and protein turnover. In multicellular organisms, cell signaling through PTMs is one of the most crucial regulatory pathways. Despite many years of research on Cyt*c*, the fact that it is a target of cell signaling through PTMs was revealed only recently. In this review we focus on the published literature of Cyt*c* modifications such as phosphorylations, methylations, acetylations, nitrations, nitrosylations, and sulfoxidations (**Table 1**). A guiding theme of this review is that the biologic consequences of PTMs should be interpreted with respect to how much of the entire protein pool contains a particular modification. This information is critically important but is missing in most studies. Despite major methodological advances in mapping PTMs to specific residues of a protein, primarily by mass spectrometry, which has become increasingly sensitive, quantitative information is often lacking. If, for example, only 1% of a metabolic enzyme carries a PTM, it is most likely adventitious and unlikely to have a biologic effect. Even Western blot analysis with PTM-specific antibodies can only estimate a relative increase or decrease of the modification, and the same issue remains, despite the often-reported several-fold increase in signal. This point is often overlooked, not just in studies related to mitochondria, but also in publications related to cell signaling in general.

CYT*c* PHOSPHORYLATION

Most reports that studied functional effects of PTMs focused on Cyt*c* phosphorylation. Protein phosphorylation and dephosphorylation are critical cell-signaling mechanisms essential for many cellular functions. In the past, mitochondria were viewed as relatively independent organelles, and not much interest was paid to their regulation by cell signaling. Despite the fact that there are a few long-known examples demonstrating that mitochondrial metabolism is decisively regulated *via* PTMs, such as the pyruvate dehydrogenase complex by pyruvate dehydrogenase kinases, which was discovered ~50 yr ago (46), only recently have mitochondria been recognized as hubs for cell signaling. At the level of the ETC, >100 phosphorylation sites have been mapped, but the functions of most of them remain unknown (47). Of the few sites with a known function, most have been reported for Cyt*c* and its partner COX. Examples for COX include subunit I phosphorylation of Tyr304 mediated by the cAMP-dependent and TNF-α pathways (48, 49), and COX subunit II phosphorylation by Src kinase and receptor tyrosine kinases EGFR and ERBB2, although the sites remain unknown (50–52). On mammalian Cyt*c*, 4 phosphorylation sites have been mapped by mass spectrometry. These are Tyr97, Tyr48, Thr28, and Ser47, which appear to be present in a highly tissue-specific manner and are discussed below in the sequence of their identification. All 4 sites are located on the right side of the molecule in the conventional view (**Fig. 2**) and are conserved in mammals (**Fig. 3**).

Tyr97 phosphorylation

In 2006, our lab reported for the first time that mammalian Cyt*c* is phosphorylated *in vivo* (53). Cyt*c* was isolated from bovine heart under conditions that preserve *in vivo* phosphorylation in the presence of the phosphatase inhibitor vanadate, a nonspecific tyrosine phosphatase inhibitor; fluoride, a nonspecific serine/threonine phosphatase inhibitor; and EGTA, a calcium chelator that prevents the activation of calcium-dependent protein phosphatases. Analysis with immobilized metal affinity chromatography/nanoliquid chromatography/electrospray ionization mass spectrometry (IMAC/nano-LC/ESI-MS) showed that Tyr97 is phosphorylated. Spectral analysis of the protein showed a shift of the characteristic 695 nm peak to 687 nm, which was reversed by alkaline phosphatase treatment,

TABLE 1. *Identified PTMs of Cytc*

Site	Modification	Methodology	Tissue/condition	Primary functional effect	Reference
Lys8	Acetylation	High-throughput Nano-HPLC/MS/MS	Mouse liver mitochondria (unfed state)	Not tested	112
Thr28	Phosphorylation	ESI-MS/MS	Bovine kidney	Lower respiration, membrane potential, and ROS	76
			Human phosphomimetic		77
		High-throughput LC-MS/MS	Human muscle (resting state)	Not tested	74
			Human muscle (after exercise)	Not tested	75
Ser47	Phosphorylation	High-throughput LC-MS/MS	Human muscle (resting state)	Not tested	73, 74
			Human muscle (after exercise)	Not tested	75
			Human phosphomimetic	Lower caspase activity	77
Tyr48	Phosphorylation	IMAC/nano-LC/ESI-MS	Bovine liver	Lower COX activity, abolished caspase-3 activity, and lower cardiolipin peroxidase activity	62, 66
Tyr67	Nitration	ESI-MS	Horse heart (peroxynitrite treatment); osteoclasts	Reduced mitochondrial respiration	135, 137
Lys72 (yeast numbering)	Trimethylation	Radioactive labeling	Fungi/plants	Helps Cyt*c* import into the mitochondria, prevents apoptosis, and improves protein stability against proteolytic degradation	98, 102, 103, 152, 153
Tyr74	Nitration	MALDI-TOF-MS	Recombinant human (peroxynitrite treatment)	Increased cardiolipin peroxidase activity and suppressed caspase-9 activity	124
Met80	Nitrosylation	EPR spectroscopy	Horse heart (reaction with NO)	Increased cardiolipin peroxidase activity	142, 143
Met80	Sulfoxidation	MALDI-TOF-MS	Horse heart (oxidative stress)	Increased cardiolipin peroxidase activity	118, 139
Tyr97	Phosphorylation	IMAC/nano-LC/ESI-MS	Bovine heart; porcine brain (after insulin treatment)	Lower COX activity; reduced Cyt*c* release and apoptosis after ischemia/reperfusion when treated with insulin	53, 59

ESI-MS/MS, electronspray ionization MS/MS; EPR, electron paramagnetic resonance; HPLC/MS/MS, high-performance liquid chromatography tandem mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight-MS.

indicating that the shift is due to phosphorylation. The 695 nm absorption band results from the heme Fe-S (Met80) bond, and a shift suggests a conformational change in the heme environment that potentially affects its function. The absence of the 695 nm peak is pathologic because it suggests a misfolded, dysfunctional conformation of Cyt*c* (54).

The *in vivo* phosphorylated Cyt*c* demonstrated sigmoidal kinetics in the reaction with COX, as compared with the phosphatase-treated dephosphorylated Cyt*c*, which produced a hyperbolic response, shifting the K_m value of COX for Cyt*c* from 5.5 to 2.5 μM. This finding indicates that phosphorylation leads to an inhibition of the reaction with COX when Cyt*c* concentrations are not saturated. However, maximum turnover was similar for both the phosphorylated and nonphosphorylated forms of the protein (53). The differences in enzyme kinetics and spectral changes indicate that phosphorylation of Tyr97, located on the opposite site of the heme crevice (Fig. 2), affects the function of the enzyme.

Tyr97 is located in a relatively hydrophobic area in Cyt*c*, called the "right channel," which contributes to the interaction with lipids (55). The location of Tyr97 between this channel and the heme moiety could provide a pathway for electron transfer when Cyt*c* functions as a cardiolipin peroxidase. Previously identified phosphorylation sites—Tyr304 on subunit I of COX (48, 49) and Tyr97 in cow heart Cyt*c*—share a similar sequence motif, and this phosphoepitope is unique to Cyt*c* and the COX subunit I in the ETC, suggesting that both sites are targeted by the same kinase/phosphatase. Cyt*c* Lys7, a key residue involved in apoptosome formation, is located next to Tyr97 (56). The effect of Tyr97 phosphorylation on apoptosis has not yet been analyzed. However, Tyr97 phosphomimetic Cyt*c* showed only a small reduction in caspase-9 activity *in vitro* (57, 58).

Another study showed that Tyr97 is phosphorylated in the brain after neuroprotective treatment with insulin in the context of brain ischemia–reperfusion injury (59). The study also showed that Cyt*c* is not

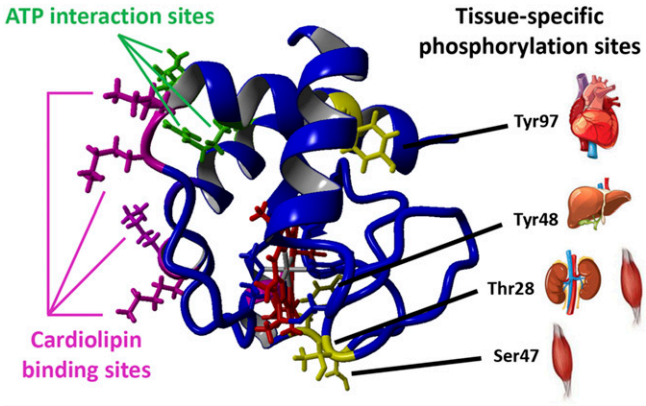

Figure 2. Tissue-specific phosphorylation sites are located on the right side of Cyt*c*, whereas cardiolipin and ATP binding sites are located on the left side. Crystallographic data from oxidized rat Cyt*c* were used (76) and analyzed with the molecular-graphics, -modeling and -simulation program YASARA (*http://www.yasara.org/*). Cyt*c* is shown in the conventional view. The heme group is shown in red, and amino acids known to be phosphorylated in a tissue-specific manner are highlighted in yellow, together with the organs in which they have been identified under baseline condition. Amino acids implicated in ATP binding are Lys88, Arg91, and Glu62 (150) and are shown in green. Residues involved in cardiolipin binding are Lys87, Lys86, Lys73, Lys72 (A site), and Asn52 (C site) (5) and are shown in magenta. A third site for electrostatic interaction of Cyt*c* with phospholipids consists of Lys22, Lys25, Lys27, His26, and His33 (151).

phosphorylated after global brain ischemia, but for brain Cyt*c*, the basal phosphorylation state remains unknown. Insulin-treated rats showed an increase in neuronal survival and reduction of Cyt*c* release from the mitochondria, suggesting that insulin-mediated Tyr97 phosphorylation is a mechanism that limits apoptotic cell death during ischemia-reperfusion (59, 60). These findings are consistent with our model (**Fig. 4**), proposing that Cyt*c* is phosphorylated under normal physiological conditions. During stress, Cyt*c* becomes dephosphorylated, which allows maximum flux in the ETC. However, in the context of ischemia, absence of oxygen leads to a cellular energy crisis, and the ETC becomes primed for hyperactivity, but because of the lack of its terminal substrate, oxygen, the ETC can resume function only when blood flow is restored to the ischemic tissue, leading to mitochondrial membrane potential ($\Delta\Psi_m$) hyperpolarization and excessive ROS production at complexes I and III, which initiates the apoptotic cascade.

Tyr48 phosphorylation

This site was first mapped on Cyt*c* purified from cow liver. Both Tyr48 and Tyr97 are conserved in mammals (Fig. 3) and eukaryotes in general (61), in which tyrosine phosphorylation is an important regulatory mechanism in many processes ranging from hormone responses, to cellular growth, to metabolism. Tyr48 phosphorylation resulted in a ~50% decrease in maximum turnover when analyzed with cow liver COX (62). This result suggests that Tyr48 phosphorylation has a more profound impact on controlling COX activity of Cyt*c* than does Tyr97. Tyr97 phosphorylation produced sigmoidal kinetics, however, maximum turnover rates were similar for the phosphorylated and nonphosphorylated forms (53, 62). In contrast, Tyr48 phosphorylated Cyt*c* produced hyperbolic kinetics. Tyr48 is located on the lower median frontal area of the molecule in proximity to the heme crevice (Fig. 2). It is also important to note that Tyr48 is located in the 40–57 Ω loop, which requires the least free energy to be unfolded (63, 64). Both Tyr48 and Tyr97 residues showed NMR signal intensity changes after cardiolipin binding, whereas Tyr67 and Tyr74 did not, suggesting that changes in Tyr48 and Tyr97 more strongly affect the molecular dynamics of Cyt*c* (65).

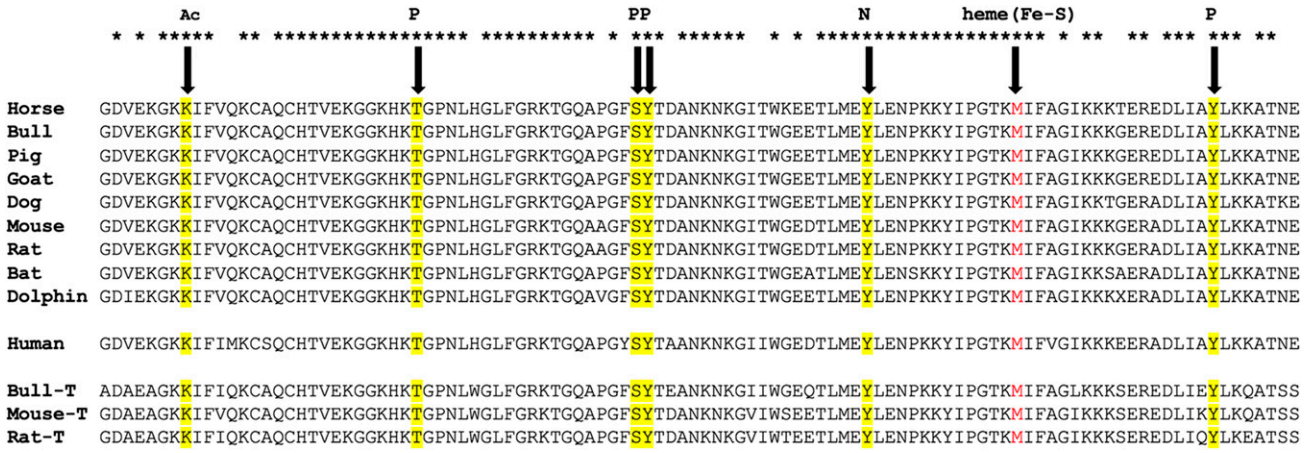

Figure 3. Sequence alignment of Cyt*c* with highlighted post-translational modifications identified in mammals *in vivo*. Top, somatic Cyt*c* from selected mammalian species with matching sequences of the corresponding testes-specific isoforms (T) where available (bottom). Note that humans express only a single ubiquitous Cyt*c* (middle), which shares sequence homology with the somatic and testis isoforms. Ac, acetylation; P, phosphorylation. Met80 of the heme group shown in red is likely involved in sulfoxidation and nitrosylation as a result of nitro-oxidative stress. UniProt (*https://www.uniprot.org/*) Protein sequence accession numbers were F7D4V9 (horse); P62894 (bull); P62895 (pig); A0A0C4Y1X3 (goat); P00011 (dog); P62897 (mouse); P62898 (rat); S7QBN9 (bat); I6VH08 (dolphin); P99999 (human); Q3SZT9 (bull testes); P00015 (mouse testes); and P10715 (rat testes).

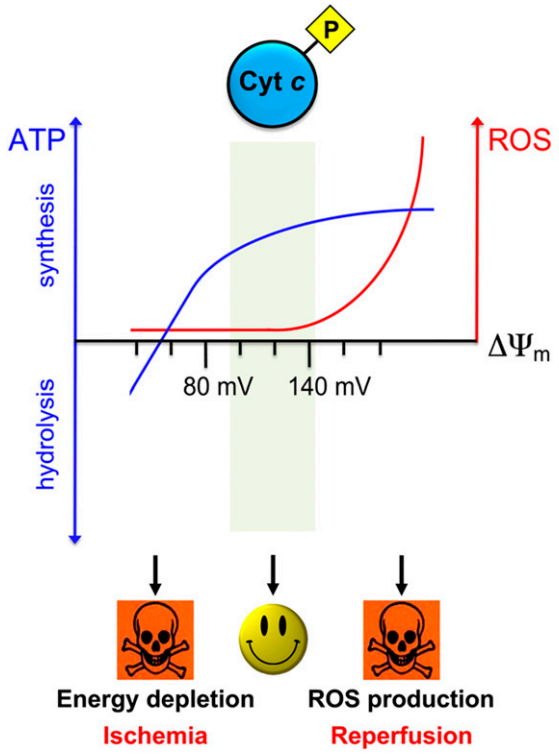

Figure 4. A model of the connection of the phosphorylation state of Cyt*c* in the regulation of electron flux in the ETC, $\Delta\Psi_m$, and ROS production. In normal conditions (middle) Cyt*c* is phosphorylated, which lowers electron flux in the ETC, maintaining healthy intermediate $\Delta\Psi_m$ levels, which are sufficient for effective ATP generation, but limit the production of ROS that are generated exponentially at $\Delta\Psi_m$ levels exceeding 140 mV. During stress conditions, such as ischemia (left), the tissue becomes energy depleted because of the lack of oxygen, leading to dephosphorylation of Cyt*c* and other mitochondrial proteins. This occurrence renders the ETC primed for hyperactivation. When the tissue is reperfused and oxygen re-enters the cells, the ETC generates high $\Delta\Psi_m$ levels, leading to ROS production and initialization of cell death cascades, adding to the damage already generated during the ischemic phase.

The functional effects of Tyr48 phosphorylation were further characterized by generation of a phosphomimetic Cyt*c* mutant, Tyr48Glu, which carries a negative charge similar to that of the phosphate group, and a control mutant Tyr48Phe, which cannot be phosphorylated on this site. Similar to *in vivo* Tyr48 phosphorylation, the phosphomimetic Tyr48Glu mutant showed a reduced maximum turnover rate compared to the wild type (WT), suggesting that it is a useful model (66). The reduction of maximum turnover in the reaction with COX was less pronounced with heart COX than with liver COX, most likely because of the presence of 3 heart-specific COX subunit isoforms (VIa-H, VIIa-H, and VIII-H) (67). Furthermore, a higher K_m was observed, suggesting a lower binding affinity toward heart COX (66). In addition to the commonly used glutamate and aspartate replacements to simulate phosphorylation, Guerra-Castellano *et al.* (68) recently introduced *p*-carboxymethyl-L-phenylalanine as a replacement for tyrosine residues that can be phosphorylated. This substitution not only introduces a negative charge similar to glutamate and aspartate, it also better mimics the tyrosine side chain spatially, because of the presence of the phenyl ring.

The phosphomimetic Tyr48Glu substitution resulted in a lower redox potential by 45 mV, possibly because of the perturbation of the heme crevice and stabilization of the iron in its oxidized state. A midpoint redox potential lower than that of cytochrome c_1 in the bc_1 complex suggests that Tyr48 phosphorylation controls and reduces the electron transfer rate of Cyt*c* at the bc_1 complex. This reduction may cause Cyt*c* to take part in other functions such as ROS scavenging (24). The phosphomimetic Tyr48Glu Cyt*c* demonstrated significant effects on the apoptotic function of Cyt*c* by decreasing cardiolipin peroxidase activity and, strikingly, by abolishing downstream caspase-3 activation (66). A study using the artificial phosphomimetic amino acid, *p*-carboxymethyl phenylalanine, substituted for Tyr48, showed an ~70% reduction in caspase 3 activity, whereas cardiolipin peroxidase activity did not change when data were normalized for baseline peroxidase activity in the absence of cardiolipin (64). This study also concluded that Tyr48pCMF Cyt*c* reduces ETC activity compared to WT protein.

Cyt*c* mutations are very rare in humans and to date, there are only 2 known amino acids with a disease-causing mutation. The first reported mutation causes a Gly41Ser replacement (69), which renders Cyt*c* more susceptible to Met80 oxidation by H_2O_2, generating a Met80 sulfoxide (70). The second reported mutation was Tyr48His in patients from an Italian family (63). Both substitutions cause the same mild disease, thrombocytopenia, which is a condition that results in lower blood platelet levels. In patients with both Gly41Ser and Tyr48His mutant Cyt*c*, mitochondria were morphologically normal, but platelet counts were reduced by about half. The replacement of Tyr48 by the positively charged histidine caused several functional changes, including reduced oxygen consumption rate and increased apoptosis (63). A recent study on the Tyr48His variant showed that the pentacoordinated form of Cyt*c*, which promotes cardiolipin peroxidase activity, is more prevalent in the mutant, further supporting the increased apoptotic activity (71). Molecular dynamics simulations concluded that both Gly41Ser and Tyr48His mutations result in a less stable and more open protein structure, which would promote cardiolipin peroxidase activity (72). At this point, it is unclear why these mutations cause such a cell-type–specific phenotype. For the Tyr48His mutation it is even more surprising that no liver phenotype has been reported, because this mutation would prevent liver Cyt*c* from being Tyr48 phosphorylated and consequently make liver cells more prone to apoptosis.

Thr28 phosphorylation

Thr28 was first identified as being phosphorylated in human skeletal muscle by a high-throughput phosphoproteomics study (73). An independent high-throughput study of the same tissue detected Thr28 and Ser47 phosphorylation (74). Furthermore, in 2015 Hoffman and colleagues (75) found Thr28 and Ser47 phosphorylation in

human skeletal muscle after exercise. However, no functional experiments were conducted in any of these studies.

We recently identified Thr28 phosphorylation in mammalian kidney (76). Over 80% of the Cyt*c* pool isolated from bovine kidney carried this PTM, suggesting that it is biologically relevant. Thr28-phosphorylated and recombinant Thr28Glu phosphomimetic Cyt*c* exhibited inhibition of COX activity, by 50 and 73%, respectively. The finding that phosphomimetic replacement leads to a more pronounced effect may be explained by the presence of some nonphosphorylated Cyt*c* (~20%) when isolated from kidney, whereas the recombinant phosphomimetic protein is homogeneous. Additional cell culture experiments were performed in WT and Thr28Glu Cyt*c* transfected Cyt*c*-knockout mouse lung fibroblasts to further validate these findings. The phosphomimetic Thr28Glu Cyt*c*-expressing cells showed lower intact cell respiration, $\Delta\Psi_m$, and ROS levels. These data suggest that modification of Cyt*c* controls ETC flux and can prevent $\Delta\Psi_m$ hyperpolarization and ROS production. This important discovery is the first indication that a Cyt*c* PTM can regulate the overall flux in the ETC. It fits our model where, in normal conditions, the activity of the ETC is downregulated or "controlled" to prevent $\Delta\Psi_m$ hyperpolarization and consequent ROS production (Fig. 4). Furthermore, AMPK has been identified as the mediator of this site-specific phosphorylation by both *in vitro* and pharmacological approaches, making it one of the few examples where a phosphorylation site has been mapped with a larger fraction of the protein containing the PTM, together with the kinase and functional effects. In contrast to other tissues, AMPK has a higher basal activity in kidney and may account for the finding that Cyt*c* is phosphorylated under basal conditions in this organ.

Thr28 is present in a region of Cyt*c* called the "negative classical gamma turn" (residues 27–29; Figs. 2 and 3) located near the heme crevice and is a part of the COX binding domain of Cyt*c*. Structural analysis of Cyt*c* has shown that the residues present in this turn have the highest root-mean-square deviations of the whole Cyt*c* molecule, suggesting that this is the most flexible element of the protein (76). However, Thr28 phosphorylation does not have an impact on apoptosome activity, because Thr28 is not part of the Apaf-1 binding domain of Cyt*c* (76, 77). It would be interesting to analyze whether Cyt*c* in skeletal muscle is targeted by AMPK (74, 75) or a different kinase. AMPK follows its more traditional role in muscle as an energy sensor and metabolic activator when energy levels drop, and it may seem counterintuitive to trigger this PTM, which partially inhibits the ETC, when increased mitochondrial activity would be needed.

Ser47 phosphorylation

Ser47 phosphorylation of Cyt*c* was also mapped in the above phosphoproteomic studies of human skeletal muscle along with Thr28 (74, 75), but it was not identified in a third study on the same tissue (73). *In vitro* studies with phosphomimetic Ser47Asp Cyt*c* suggest that it prevents caspase activity, opposite to the Thr28Asp phosphomimetic substitution, suggesting that this site is important for the regulation and execution of apoptosis (77).

TISSUE-SPECIFIC REGULATION OF RESPIRATION AND APOPTOSIS THROUGH REVERSIBLE PHOSPHORYLATIONS: AN INTEGRATED MODEL

The fact that basal phosphorylations of Cyt*c* are tissue specific—Tyr97 in heart, Tyr48 in liver, and Thr28 in kidney—may at first seem surprising. All 3 functionally characterized phosphorylations lead to partial inhibition of the reaction between Cyt*c* and COX or "controlled respiration" (Fig. 4). Such a mechanism can help maintain healthy intermediate levels of the $\Delta\Psi_m$ to prevent excessive ROS generation that takes place at high membrane potentials ($\Delta\Psi_m > 140$ mV). Proper regulation of the membrane potential is crucial because it is directly related to the production of ROS. It has been shown experimentally that $\Delta\Psi_m$ levels exceeding 140 mV lead to an exponential increase in ROS production at ETC complexes I and III, whereas mitochondria of resting cells with an intermediate $\Delta\Psi_m$ do not produce significant amounts of ROS (78–80). Studies performed in perfused rat hearts (81), lymphocytes (82), embryonic heart cells (83), intact cultured fibroblasts, and neuroblastoma cells (84) showed $\Delta\Psi_m$ values in the range of 80–140 mV, which may resemble the healthy physiologic $\Delta\Psi_m$ range in intact cells and organs under normal conditions. These results are favorable because maintenance of physiologically intermediate $\Delta\Psi_m$ values prevents excessive ROS generation but provides the full capacity to produce ATP, because maximum ATP synthesis by ATP synthase takes place at a $\Delta\Psi_m$ of 100–120 mV (85). Because the electron transfer reaction from Cyt*c* to COX to oxygen is the proposed rate-limiting step of the ETC under physiological conditions (86–90), it is not surprising that it is highly regulated to prevent $\Delta\Psi_m$ hyperpolarization and ROS generation under normal nonstressed conditions.

However, the situation is different with regard to apoptosis, where some PTMs seem to affect apoptosome formation and downstream caspase activation, whereas others do not. It is noteworthy that Cyt*c* Tyr48 phosphorylation fully protects against caspase activation, as shown with phosphomimetic Cyt*c* (64, 66), whereas Thr28 phosphorylation in kidney has no such effect. Liver cells are constantly bombarded with molecules that the organism takes up through the digestive system and even the lung (91). It may therefore seem reasonable to add another layer of protection, through Tyr48 phosphorylation, to prevent apoptosis in the relatively toxic environment to which liver cells are exposed. Other organs that are not in "direct" contact with the environment and instead face a mostly homeostatic milieu may not need an extra safeguarding mechanism.

CYTc METHYLATION

In histones, the ε-NH_2 group of lysine residues can be mono-, di-, or trimethylated, and such modifications regulate many biological functions, such as gene regulation

and signal transduction (92). In general, lysine, arginine, histidine, and dicarboxylic amino acids are able to accept a methyl group to their side chains. Methylation occurs in a species-specific manner. In mammals, arginine and lysine methylation are found, and in humans they occur at a ratio of ~4:1 for Arg:Lys (93). However, of the 18 lysine and 2 arginine residues present in human Cyt*c* (Fig. 3), none was found to be modified (93). As discussed below, Cyt*c* methylation appears to be important in some organisms other than mammals, where it occurs on lysine residues.

Under physiological conditions the unmodified lysine side chain amino group with a side-chain pK_a of 10.5 is protonated and carries a positive charge, which is essential for ionic interactions between a protein with other molecules including proteins, DNA, RNA, and metabolites. Addition of methyl groups further increases the pK_a modifications of the ε-amino group of lysine residues resulting in a remarkable chemical diversity of ~20 different modifications of this amino acid, including mono-, di-, and trimethylation, acetylation, and ubiquitination (94).

It has been shown in *Saccharomyces cerevisiae* and in plants that apocytochrome *c* expressed from the *CYC1* gene is immediately trimethylated by the Cyt*c* methyltransferase Ctm1p in the cytosol at an evolutionarily conserved site, Lys78, often referred to as Lys72, based on the corresponding homologous residue in vertebrates (95–97). Functionally, it has been shown that the import of methylated yeast Cyt*c* into yeast mitochondria is much greater than the import of nonmethylated Cyt*c* (98). However, this effect was not observed with rat liver mitochondria. It is possible that in yeast Cyt*c* is imported into the mitochondria through a methylation-dependent receptor mechanism. Earlier work showed that, in the fungus class Ascomycetes, methylation of Cyt*c* Lys72 maintains the binding of the hemoprotein to mitochondria and the researchers concluded that methylation of the protein is essential for its physiological functions, including electron transport through the ETC (99). More recently, it has been demonstrated that methylation of yeast Cyt*c* (Cyc1p) increases its interactions with Erv1p and the Cyt*c* heme lyase Cyc3p, further supporting the role of methylation in the import of apocytochrome *c* to yeast mitochondria (100). Metazoan Cyt*c*, methylated at Lys72 after expression in yeast, completely prevented proapoptotic activity of Cyt*c* by lowering the binding affinity toward Apaf-1 (101).

Structurally, methylation of yeast Lys-72 Cyt*c* increases protein stability *in vivo* against proteolytic degradation (102, 103) and may modulate its geometry, isoelectric point, or conformation (104–106). In addition, a structural analysis demonstrated that trimethyllysine 72 is positioned near the heme crevice loop (Ω loop D, residues 70–85) and plays a crucial role in opening the crevice, suggesting a potential role in regulating peroxidase activity during apoptosis (107).

CYTc ACETYLATION

Acetylation of lysine side chains is another important PTM catalyzed by lysine acetyltransferases that use acetyl-CoA as a substrate for transfer of its acetyl moiety to the ε-group of a lysine residue. Acetylation also occurs nonenzymatically, especially in the mitochondrial matrix where high acetyl-CoA concentrations and a more basic pH favor the transfer of the acetyl group and make it the most prevalent PTM (94). Recent studies have confirmed that the mitochondrial proteome is highly acetylated, with more than 60% of proteins analyzed having 1 or more acetylation sites (108–111). Lysine acetylation leads to neutralization of the positive charge related to amide bond formation, increasing hydrophobicity, and spatial layout of the lysine side chain. *In vitro* acetylation of horse heart Cyt*c* was shown to abolish the H_2O_2 scavenging function of Cyt*c* (22). The only reported *in vivo* acetylation site of mammalian Cyt*c* is Lys8 (Fig. 3), which was identified in a high-throughput proteomics study in mouse liver mitochondria when animals were unfed but not when they were fed (112). The extent of acetylation, the functional effect, and specificity—enzymatic *vs.* nonenzymatic acetylation—remain unknown.

CYTc SULFOXIDATION

H_2O_2 is one of the main reactive oxygen species involved in oxidative stress. One established modification of Cyt*c* in response to increased oxidative stress is Met80 sulfoxidation (5). Cyt*c*, when bound to cardiolipin, is more accessible to H_2O_2. Another characteristic change in Cyt*c* upon cardiolipin binding is a significant negative shift in the redox potential (350–400 mV) (113). Typically, a redox potential in the range of 220–270 mV is observed for Cyt*c* in mammals (114, 115). The shift in redox potential caused by cardiolipin binding suggests that it is thermodynamically unfeasible for cardiolipin-bound Cyt*c* to engage as an efficient electron carrier in the ETC. Furthermore, it makes Cyt*c* incapable of acting as a ROS scavenger (113). In Cyt*c*–cardiolipin complexes, submillimolar H_2O_2 concentrations oxidize the sulfur atom of the Met80 axial ligand. Therefore, Met80 sulfoxidation of Cyt*c* significantly enhances peroxidase activity of Cyt*c*, promoting Cyt*c* release and apoptosis (116, 117).

Cyt*c* sulfoxidation is difficult to detect and study *in vivo*. However, Wang *et al.* (118) functionally studied this modification by creating a mutant that models the conformation of Cyt*c* when bound to cardiolipin. To create this mutant, 2 residues (Val83 and Gly84) from the loop containing Met80 were deleted. Through mass spectrometry, this mutant was confirmed to be specifically autoxidized to methionine sulfoxide when purified in the presence of molecular oxygen. Functional changes, such as increased cardiolipin peroxidase activity and Cyt*c* release with regard to Met80 modifications, were also found with the Met80Ala-Cyt*c* mutant (119).

CYTc TYROSINE NITRATION

Tyrosine nitration is a PTM that takes place in the presence of a reaction between superoxide and NO that forms peroxynitrite, which acts as the nitration agent (120). In

contrast to phosphorylation, tyrosine nitration is a stable PTM. It has been estimated that only 1–5 of 10,000 tyrosine residues are nitrated in a protein under inflammatory conditions *in vivo*. Given the very low frequency of this modification of <0.1%, mapping nitrated tyrosine residues has been a challenging process (121). The first protein identified as nitrated *in vivo* was manganese superoxide dismutase (122, 123). Human Cyt*c* has 5 tyrosine residues at sites 46, 48, 67, 74, and 97, of which 4 are evolutionarily conserved in mammals (61, 124). Of the 5 tyrosine residues, only 3 (Tyr67, Tyr74, and Tyr97) are nitrated *in vitro* in the presence of peroxynitrite (125). Tyr67- and Tyr74-nitrated Cyt*c* are less efficient in restoring respiratory capacity in Cyt*c*-deficient mitochondria compared to Tyr97 (125, 126). Tyr48, which is highly conserved, and Tyr46, which is present only in humans and plants, were the least commonly nitrated residues (127).

Tyrosine nitration of Cyt*c* has been reported under several conditions *in vivo*. In a renal ischemia–reperfusion model, Cyt*c* was nitrated 3 h after the ischemic insult, as confirmed by nitrotyrosine immunoprecipitation (128). Furthermore, an increased release of nitrated Cyt*c* was observed in brain tissue sections of rats exposed to ischemia-reperfusion (129). Another study suggested that tyrosine nitration of Cyt*c* was induced by chemoradiotherapies, such as 5-fluorouracil, *cis*-diaminedicholorplatinum, and γ-rays (130). However, the researchers reported that nitration of Cyt*c* alone does not suppress apoptosis in squamous cell carcinoma cells, probably because only a small fraction of Cyt*c* carries the modification. Cyt*c* nitration was also observed in a rat model of chronic allograft nephropathy 4 wk after transplantation, suggesting that nitration of Cyt*c* occurs during kidney injury (131). However, no specific nitration site has been reported under physiological conditions, suggesting that most of these modifications are relatively nonspecific and driven by nitro-oxidative stress. Furthermore, some of the observations reported *in vivo* do not mirror the functional studies performed with nitrated Cyt*c* *in vitro* because most *in vitro* studies conclude that nitration prevents caspase activation (124, 132, 133). This again is likely to be explained by the small portion of Cyt*c* that is nitrated *in vivo*, even under severe stress conditions. Because tyrosine residues can also be phosphorylated, it should be noted that tyrosine nitration and phosphorylation of the same residue in a protein are mutually exclusive events (134).

Tyr67 nitration

Several *in vitro* studies have analyzed peroxynitrite-mediated nitration of Tyr67 and reported changes in redox function, cardiolipin peroxidation, and respiration (135, 136). Furthermore, spectrophotometric studies of Tyr67-nitrated Cyt*c* have shown a loss of the 695 nm band, indicating the disruption of the axial Fe-S (Met80) bond. This finding makes sense because Tyr67 is adjacent to the heme group of Cyt*c* and points toward the Fe-Met80 bond. A study using osteoclasts confirmed Tyr67 nitration by mass spectrometry (137). Tyr67-nitrated Cyt*c* was then proposed to be less prone to apoptosis due to intramolecular hydrogen bonding that promotes stronger interactions with the cardiolipin phosphates, making Cyt*c* release less favorable. Cardiolipin peroxidase activity studies have demonstrated that the Tyr67Phe mutation leads to a partial loss of peroxidase activity, suggesting that this conserved tyrosine could be the reactive tyrosyl radical involved in the oxygenase half reaction of Cyt*c*-cardiolipin peroxidation (65). Finally, studies with human Cyt*c* in which all tyrosines except for Tyr67 were replaced with phenylalanine showed >50% decreased caspase activation at saturating Cyt*c* concentrations after *in vitro* nitration of Tyr67 (126).

Tyr74 nitration

Tyr74 is fully surface exposed and the most commonly nitrated tyrosine residue in Cyt*c*. An *in vitro* study showed that Tyr74 nitration results in increased cardiolipin peroxidase activity (124). However, this mutant also prevents downstream caspase-9 activity. Peroxynitrite-mediated nitration of Tyr74 resulted in a disruption of the axial Fe-S (Met80) bond and was replaced by Lys72 as confirmed by NMR (138). Lys72 is a crucial residue for the interaction of Cyt*c* with Apaf-1, which may explain the low caspase-9 activity (56). *In vitro* studies on Met80 sulfoxidation and Tyr74 nitration suggested that, in the presence of both of these modifications, the binding affinity for cardiolipin was 4 times higher than with WT Cyt*c* (116). Furthermore, Tyr74 nitration leads to a lower midpoint redox potential, interfering with electron transfer (139).

Tyr46 and Tyr48 nitration

There is no current evidence of *in vivo* nitration at Cyt*c* residues Tyr46 and Tyr48, even though they are solvent exposed and located close to the heme group that can catalyze nitration. Tyr46 is only present in human and plant Cyt*c*, whereas Tyr48 is conserved in Cyt*c* across all species. *In vitro* studies showed that Tyr46 and Tyr48 nitration promote the formation of the heme iron pentacoordinated form, which increases peroxidase activity. Nitration of these residues also affects the surrounding heme propionates. Furthermore, the use of monotyrosine mutants of both sites results in rapid degradation of Cyt*c* in Jurkat cell extracts, suggesting that these nitrations are not physiologically favorable (127). The same group also reported that neither nitration has an effect on the rate of electron transfer to COX. However, light-scattering experiments have shown that these modifications lead to poor interactions with Apaf-1, forming a nonfunctional apoptosome and reducing caspase-9 activity (133). Even though Tyr sites 74, 46, and 48 result in a reduction in caspase activity, most *in vivo* studies suggest that Cyt*c* nitration is associated with increased apoptosis and mitochondrial dysfunction (128–131). As discussed above, the low stoichiometries of Tyr nitration in *in vivo* conditions make their biological effect debatable. In fact, Cyt*c* tyrosine nitration may be a good model system to study

the effect of phosphorylation related to the introduction of a negative charge and spatial requirements that are similar to those of a phosphate group. Looking at tyrosine nitration from this angle, it may not be surprising that *in vitro* data for tyrosine phosphorylation and nitration, as well as phosphomimetic substitution, show mostly consistent results.

CYT*c* NITROSYLATION

Nitrosylation involves the addition of an NO group to a free cysteine or a transition metal. Therefore, it takes place in a limited number of proteins *in vivo* (140, 141). Because Cyt*c* has no free cysteines, its only target of nitrosylation is the heme group. A few studies have shown that Cyt*c* can be nitrosylated *in vitro* and in U-937 monocytic cells (142–144). Nitrosylated Cyt*c* was found to be mostly present in the cytosol. However, when antiapoptotic proteins such as Bcl-2 and -XL were overexpressed, most of the nitrosylated Cyt*c* was found in the mitochondria. The researchers concluded that nitrosylation of Cyt*c* may play a role in apoptosis regulation (144). Endogenous heme nitrosylation is a fairly rare event that is observed in only a few other proteins, such as COX and guanylyl cyclase, the only known receptor for NO (145–148). In both COX and guanylyl cyclase, the heme iron is pentacoordinated, unlike in Cyt*c*, where it is hexacoordinated (149). Because Cyt*c* can undergo a conformational change into a pentacoodinated form, it may be nitrosylated in conditions when the Fe-S (Met80) bond is displaced, leading to greater cardiolipin peroxidase activity.

CONCLUSIONS

Cyt*c* plays a significant role in balancing cellular life- and death-related functions. Both the primary functions of Cyt*c*—respiration leading to energy production and apoptosis leading to cell death—are regulated to meet tissue- and organ-specific needs. Therefore, Cyt*c* has tissue-specific phosphorylations that regulate respiration, apoptosis, and ROS production and scavenging. For instance, the liver-specific Tyr48 phosphorylation abolishes apoptosis, which is sensible, given that liver cells are exposed to toxic molecules primarily through food uptake. Therefore, these cells have a higher threshold for cell death commitment, supporting the concept that Tyr48 is an important regulatory site for apoptosis. In contrast, the Thr28 phosphorylation observed in kidney results in the same level of apoptotic activity as unmodified WT Cyt*c*, suggesting that an apoptosis-suppressing mechanism is not needed in this organ. However, Thr28 phosphorylation and all other phosphorylations studied to date indirectly protect the tissues from ROS damage where they are found by reducing mitochondrial respiration and thus the $\Delta\Psi_m$. Maintenance of intermediate $\Delta\Psi_m$ prevents excessive ROS generation but allows efficient energy production. However, during cellular stress such as ischemia-reperfusion, which is found under pathologic conditions in many organs including the heart, kidney, and brain, Cyt*c* becomes dephosphorylated, resulting in $\Delta\Psi_m$ hyperpolarization (>140 mV) during reperfusion and a consequent burst of ROS, adding to the tissue damage and death already generated during the ischemic phase (Fig. 4).

When interpreting the biological significance of PTMs identified on Cyt*c* the stoichiometry has to be considered. Some PTMs such as nitrations of Cyt*c* have been reported under several pathological conditions. These modifications tend to take place in a nonspecific manner and can be attributed as spontaneous chemical reactions to nitro-oxidative stress. In mammals, under normal physiological conditions only tissue-specific phosphorylation of Cyt*c* has exceeded 50% of the Cyt*c* pool. Methylation of Cyt*c*, even though important in yeast and plants, has not been reported to be physiologically relevant in mammalian species. Acetylation could be an interesting regulatory modification given that the mammalian mitochondrial proteome is highly acetylated, and a specific Cyt*c* acetylation site has been identified in mouse liver of unfed animals. In the future, an in-depth understanding of PTMs of Cyt*c* will necessitate the identification of signaling molecules, including kinases, phosphatases, and scaffolding proteins, that mediate cell signaling in a tissue-specific manner. FJ

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AUTHOR CONTRIBUTIONS

H. A. Kalpage, V. Bazylianska, N. Mantena, and M. Hüttemann designed the figures; and all authors wrote, edited, and approved the final version of the manuscript.

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