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Cytochrome *c* phosphorylation: Control of mitochondrial electron transport chain flux and apoptosis

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Abstract

Cytochrome *c* (Cyt*c*)[1]is a cellular life and death decision molecule that regulates cellular energy supply and apoptosis through tissue specific post-translational modifications. Cyt*c* is an electron carrier in the mitochondrial electron transport chain (ETC) and thus central for aerobic energy production. Under conditions of cellular stress, Cyt*c* release from the mitochondria is a committing step for apoptosis, leading to apoptosome formation, caspase activation, and cell death. Recently, Cyt*c* was shown to be a target of cellular signaling pathways that regulate the functions of Cyt*c* by tissue-specific phosphorylations. So far five phosphorylations sites of Cyt*c* have been mapped and functionally characterized, Tyr97, Tyr48, Thr28, Ser47, and Thr58. All five phosphorylations partially inhibit respiration, which we propose results in optimal intermediate mitochondrial membrane potentials and low ROS production under normal conditions. Four of the phosphorylations result in inhibition of the apoptotic functions of Cyt*c*, suggesting a cytoprotective role for phosphorylated Cyt*c*. Interestingly, these phosphorylations are lost during stress conditions such as ischemia. This results in maximal ETC flux during reperfusion, mitochondrial membrane potential hyperpolarization, excessive ROS generation, and apoptosis. We here present a new model proposing that the electron transfer from Cyt*c* to cytochrome *c* oxidase is the rate-limiting step of the ETC, which is regulated via post-translational modifications of Cyt*c*. This regulation may be dysfunctional in disease conditions such as ischemia-reperfusion

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injury and neurodegenerative disorders through increased ROS, or cancer where post-translational modifications on Cyt*c* may provide a mechanism to evade apoptosis.

Keywords

Cytochrome *c*; cell signaling; phosphorylation; respiration; apoptosis; reactive oxygen species

1. Introduction

The mitochondrial electron transport chain (ETC) consumes oxygen as part of the oxidative phosphorylation (OxPhos) process to produce the majority of cellular ATP, the energy molecule of life. An average human produces 65 kg of ATP per day under resting conditions, and energy production can increase several-fold during exercise (Rich, 2003). Because of its essential role, OxPhos should be tightly regulated for optimal functioning of an organism. The ETC is comprised of four enzyme complexes and two electron carriers: NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), the small non-protein electron carrier ubiquinone, bc_1 complex (complex III), the electron carrier cytochrome *c* (Cyt*c*), and cytochrome *c* oxidase (COX; complex IV) (Fig. 1). Electrons enter the ETC from NADH via complex I. Complex II links the ETC with the citric acid cycle, directly feeding electrons from $FADH_2$ into the ubiquinone/ubiquinol pool. Except for complex II, complexes I, III, and IV pump protons across the inner mitochondrial membrane to generate the proton motive force (p), which consists of the pH gradient (pH) and the mitochondrial membrane potential (Ψ_m), the latter of which is the primary component of p in mammals. The proton gradient is utilized by ATP synthase (complex V) to produce ATP. Dysregulation of the ETC can result in many pathological conditions due to lack of energy or mitochondrial membrane potential hyperpolarization, resulting in the generation of harmful reactive oxygen species (ROS) and the release of Cyt*c*, which triggers apoptosis. Once Cyt*c* is released into the cytosol from the mitochondria, it interacts with the protein apoptosis protease activating factor-1 (Apaf-1), which results in the formation of the apoptosome, leading to downstream caspase activation and cell death (Fig. 1). It should also be noted that Cyt*c*, a small 104 amino acid globular protein in mammals with a covalently attached heme group, has many other functions. These functions either promote cell survival or cell death, including ROS scavenging, redox-coupled protein import via Erv1-Mia40 pathway, ROS formation via $p66^{Shc}$, and cardiolipin peroxidase activity (Hüttemann et al., 2011). Cardiolipin is a mitochondria-specific phospholipid that binds to Cyt*c* at multiple sites (Fig. 2A). This interaction allows Cyt*c* to catalyze cardiolipin peroxidation, which also facilitates Cyt*c* release from the mitochondria to the cytosol. Cyt*c* thus serves as a hub for cellular life and death decisions. Given this crucial role, tight regulation of Cyt*c* is needed to control its pro-life function in the ETC and its pro-death function in apoptosis. We here discuss the regulation of Cyt*c*, focusing on phosphorylation and its effects on respiration and apoptosis. Based on recent data we propose as a new model that Cyt*c* phosphorylation controls overall flux in the ETC and apoptosis, which determines the balance between ATP, ROS, and apoptosis.

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2. Reversible phosphorylations of Cyt*c* and COX

We modified protein purification protocols to preserve the *in vivo* phosphorylations of mitochondrial proteins, which made possible the discovery of novel phosphorylation sites on both COX and Cyt*c* in mammals (Lee et al., 2006; Lee et al., 2009). About 18 phosphorylation sites have been mapped on COX with only a select few having been functionally studied (Helling et al., 2012a; Helling et al., 2012b). One such functionally characterized modification is Tyr304 phosphorylation that was mapped on catalytic subunit I of bovine liver COX, mediated by cAMP-dependent signaling (Lee et al., 2005) or inflammatory signaling via TNFα (Samavati et al., 2008), and resulted in strong enzyme inhibition. Despite decades of research on Cyt*c*, its regulation through cell signaling-mediated phosphorylations was not uncovered until 2006 (Lee et al., 2006). Since then we and others have shown that these phosphorylations take place in a highly tissue-specific manner (Fig. 3). Tyr97 phosphorylation of Cyt*c* was mapped for the first time in bovine heart tissue (Lee et al., 2006), followed by Tyr48, which was mapped in bovine liver (Yu et al., 2008), then Thr28 was mapped in bovine kidney (Mahapatra et al., 2017) and a second phosphorylation site, Thr58, was later also identified in rat kidney (Wan et al., 2019). Ser47 was mapped in both rat and porcine brain under basal conditions (Kalpage et al., 2019b) while Tyr97 was identified in rat and porcine brain post-insulin treatment (Sanderson et al., 2013a). Some of these phosphorylations were identified in high throughput mass spectrometry analyses in mammalian cells but not confirmed or functionally studied (Grimsrud et al., 2012; Hoffman et al., 2015; Lundby et al., 2013; Lundby et al., 2012; Parker et al., 2015; Sacco et al., 2016; Zhao et al., 2014; Zhao et al., 2011). These tissue-specific phosphorylations of Cyt*c* fine-tune the multiple functions of Cyt*c* to specific needs of various tissues and organs as discussed below in the sequence of their discovery and summarized in Table 1. As detailed below, all five reported Cyt*c* phosphorylations lead to functional changes, including altered reaction kinetics with COX. A subset of them also result in a modified ability to trigger downstream caspase activation. Cyt*c* is a small evolutionarily optimized protein, which may explain why modifications, even if they are not directly part of the interaction site based on crystallographic data or docking modeling simulations, may affect or interfere with optimal binding. For example, the interaction between Cyt*c* and COX is mediated primarily by electrostatic interactions of positively charged lysine residues on Cyt*c* and negatively charged residues on COX, in addition to hydrophobic interactions across the binding interface (Roberts and Pique, 1999; Schmidt et al., 2005). Depending on their specific location on Cyt*c*, the introduction of negative charges upon phosphorylation, can affect and interfere with optimal binding of Cyt*c* to COX, lowering the reaction rate. Other contributing factors may be a change of the Cyt*c* redox potential and the attachment of the phosphate moiety, which may cause steric interference or structural changes on Cyt*c*.

2.1. Tyrosine 97 (Tyr97) phosphorylation of Cytc

Lee et al. (Lee et al., 2006) reported for the first time that Cyt*c* is post-translationally modified by phosphorylation. Cyt*c* purified from bovine heart was phosphorylated on Tyr97, which shifted the characteristic 695 nm heme-iron-Met80 absorption band, a marker of Cyt*c* integrity (Dickerson and Timkovich, 1975), to 687 nm. This spectral change in Tyr97-

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phosphorylated Cyt*c* suggests that this phosphorylation affects the heme environment of Cyt*c*. In the reaction with COX, Tyr97-phosphorylated and unphosphorylated Cyt*c* displayed sigmoidal and hyperbolic kinetics, with K_m values for Cyt*c* in the reaction with COX of 5.5 μM and 2.5 μM, respectively (Lee et al., 2006). In another study, no change in redox potential was observed with phosphomimetic Tyr97Glu Cyt*c*, but there was a significant decrease in protein stability of Tyr97Glu Cyt*c* compared to the WT as determined by melting temperature analysis (Garcia-Heredia et al., 2011). This is likely due to the lack of the aromatic ring in Tyr97Glu Cyt*c*, as opposed to the introduction of the negative charge since the control phenylalanine mutant did not show decreased stability (Garcia-Heredia et al., 2011). Tyr97 phosphorylation was later characterized after the replacement of tyrosine with a more realistic phosphomimetic, *p*-carboxymethyl-L-phenylalanine (pCMF), incorporated using the evolved tRNA synthetase method (Guerra-Castellano et al., 2015). However, Tyr97pCMF resulted in increased COX activity in contrast to the *in vivo* Tyr97 phosphorylated Cyt*c*. The Tyr97pCMF phosphomimetic replacement resulted in lower caspase-3 activity while no change in cardiolipin binding or cardiolipin peroxidase activity was observed (Guerra-Castellano et al., 2018). The decrease in apoptotic activity could be due to the close proximity of this residue to Lys7, which is a key residue for apoptosome formation (Yu et al., 2001) (Fig. 2E). Tyr97 is conserved across most species ranging from mammals to plants and microorganisms. There are several fungi that have Leu, Gln, and Phe residues that cannot be phosphorylated in place of Tyr97 (Hampsey et al., 1988; Heller and Smith, 1966). This may be explained by the lack of tyrosine kinase signaling in lower species. The kinase mediating this reaction in mammals is yet to be identified. Sequence alignment of the Tyr97 phospho-epitope with Tyr304 phospho-epitope of COX subunit I revealed that 5 of 10 residues in the alignment are identical, suggesting the possibility that both proteins are targeted by the same kinase (Lee et al., 2006). *In vivo*, insulin treatment of post-ischemic porcine and rat brains resulted in Tyr97 phosphorylation of Cyt*c* (Sanderson et al., 2013a). Furthermore, the induction of Tyr97 phosphorylation after insulin treatment rescued roughly 50% of the CA1 hippocampal neurons in an animal model of global brain ischemia/reperfusion injury, further supporting the anti-apoptotic role of Tyr97 phosphorylation of Cyt*c*. The decrease in neuronal death was associated with a decrease in Cyt*c* release 24 hours post-reperfusion (Sanderson et al., 2013a), suggesting that the phosphorylation state of Cyt*c* may also play a role in Cyt*c* release from the mitochondria to trigger apoptosis. Both cAMP signaling and insulin signaling promote anti-apoptotic mechanisms, suggesting a role of these pathways in the regulation of Cyt*c* (Martin et al., 2005; Sanderson et al., 2009; Sanderson et al., 2008).

2.2. Tyrosine 48 (Tyr48) phosphorylation of Cytc

Tyr48 phosphorylation was primarily found on Cyt*c* purified from cow liver (Yu et al., 2008). Tyr48 is conserved in eukaryotes and mammals (Zaidi et al., 2014). Functional studies using *in vivo* Tyr48-phosphorylated Cyt*c* and a phosphomimetic mutant, Tyr48Glu Cyt*c*, which carries a negative charge similar to the phosphate group, revealed reduced maximal turnover rates in the reaction with liver COX to about 50% (Pecina et al., 2010). Tyr48Glu replacement in Cyt*c* completely abolished caspase-3 activity in a cell-free apoptosis detection system (Pecina et al., 2010), suggesting that this phosphorylation serves as a switch for apoptosis. Furthermore, Tyr48Glu Cyt*c* also resulted in decreased pro-

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apoptotic cardiolipin peroxidase activity. These findings may be explained by the fact that the phosphomimetic Tyr48Glu reduced the binding of Cyt*c* to cardiolipin by about 30% compared to unphosphorylated Cyt*c* (Pecina et al., 2010). Another study that utilized Tyr48pCMF phosphomimetic Cyt*c* further confirmed that this Cyt*c* variant inhibits ETC flux and caspase-dependent apoptosis (Moreno-Beltran et al., 2017). Tyr48 is located at the lower median frontal area at the Ω loop (amino acid residues 40–57), which is close to the heme crevice, and susceptible to unfolding (De Rocco et al., 2014; Moreno-Beltran et al., 2017). Topography of Tyr48 and Tyr97 residues using NMR shows intensity changes when binding to cardiolipin compared to tyrosine residues Tyr67 and Tyr74, suggesting that changes in the former residues have a stronger influence on the molecular dynamics of the protein (Kapralov et al., 2011). Moreover, the midpoint redox potential of the phosphomimetic Tyr48Glu mutant was decreased by 45 mV compared to the WT control (Pecina et al., 2010), which is lower than that of cytochrome c_1 in bc_1 complex, suggesting that Tyr48 phosphorylation reduces the rate of electron transfer from cytochrome c_1 to Cyt*c* at the bc_1 complex, which may also be beneficial for Cyt*c* to function as a ROS scavenger (Pereverzev et al., 2003). Furthermore, a docking model of bc_1 complex and Cyt*c* proposed Tyr48 as a potential interactor with the bc_1 complex (Kokhan et al., 2010), suggesting additional interference with ETC function upon phosphorylation of the site. Interestingly, human Tyr48Glu Cyt*c* displayed an 80 mV reduction of the redox potential compared to WT Cyt*c* (Garcia-Heredia et al., 2011) whereas the decrease was only 45 mV in rodent Cyt*c* (Pecina et al., 2010). Tyrosine phosphorylation is mainly present in higher organisms and involved in mitochondrial and cancer signaling (Carpenter et al., 1978). Therefore, characterization of Tyr48 phosphorylation of Cyt*c* may be an interesting future research direction to better understand the role of Cyt*c* phosphorylation in cancer. It was reported that the Tyr48His mutation causes a mild human disease, thrombocytopenia, resulting in a lower level of blood platelets. The introduction of a positively charged histidine reduced the oxygen consumption rate and increased apoptotic activity (De Rocco et al., 2014). Furthermore, the Tyr48His mutation causes a structural transformation of Cyt*c* from a hexacoordinated form to a pentacoordinated form which promotes cardiolipin peroxidase activity (Deacon et al., 2017). It remains unclear, however, why patients with the Tyr48His mutation present with a mild phenotype that is limited to platelets.

2.3. Threonine 28 (Thr28) phosphorylation of Cytc

A major portion of Cyt*c* (~80%) purified from bovine kidney was found to be phosphorylated on residue Thr28 (Mahapatra et al., 2017). *In vivo* phosphorylated Cyt*c* from the bovine kidney resulted in a 50% decrease in COX activity but there was no significant effect on caspase-3 activity compared to unphosphorylated bovine kidney Cyt*c*. The Thr28Glu phosphomimetic mutant resulted in a 73% decrease in COX activity compared to recombinant unphosphorylated WT Cyt*c* (Mahapatra et al., 2017). Surprisingly, in another study, Thr28Asp phosphomimetic increased COX activity, contrary to what we observed with *in vivo* phosphorylated Cyt*c* and Thr28Glu Cyt*c*. Across evolution, glutamate is permissible at this site of Cyt*c* and found in several plant species, whereas aspartate is not (Zaidi et al., 2014). This suggests that glutamate is the more suitable phosphomimetic model to characterize Thr28 phosphorylation given its functional similarities to *in vivo* phosphorylated Cyt*c*. Importantly, intact Cyt*c* knockout cells expressing Thr28Glu Cyt*c* also

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showed lower respiration rates compared to WT Cyt*c*-expressing cells, mimicking the *in vitro* experiments with purified COX. Furthermore, cells expressing Thr28Glu Cyt*c* had lower mitochondrial membrane potentials and ROS levels. This suggests that phosphorylation of Cyt*c* at Thr28 leads to a partial inhibition of mitochondrial respiration resulting in an optimal intermediate mitochondrial membrane potential for efficient ATP generation with minimal ROS production (Kaim and Dimroth, 1999; Kalpage et al., 2019a). These findings suggested, for the first time, that modifications on Cyt*c* can lead to an overall decrease in ETC flux in intact cells. The decrease in Cyt*c*-COX activity and overall ETC flux with Thr28Glu Cyt*c* may be in part attributed to changes in the midpoint redox potential as well as structural changes of Cyt*c*. This residue is present in the COX binding domain of the protein, interacting with COX subunits I, II and VIIc based on a Cyt*c*-COX docking model (Roberts and Pique, 1999). Furthermore, Thr28 is in close proximity to residue Asp50 of COX subunit I, which is one of the few residues of COX that structurally vary in the COX crystal structure based on its oxidized or reduced state (Sugitani and Stuchebrukhov, 2009). Asp50 is next to Asp51, a residue that was proposed to be involved in the proton pumping mechanism of COX as a proton ejection site (Tsukihara et al., 2003). Therefore, it is possible that Thr28 phosphorylation of Cyt*c* inhibits the electron transfer-coupled proton pumping of COX. Thr28 of Cyt*c* also interacts with Trp104 of COX subunit II, which is the site where electrons enter before reaching the binuclear CuA copper center of COX (Scharlau et al., 2019). Interference of Thr28 phosphorylation with these key residues of COX likely explains its inhibitory function on COX activity and overall ETC flux. Cyt*c* Thr28 is part of the 'negative classical gamma turn' comprised of residues 27 through 29 and is important for the stability of Cyt*c* (Sanishvili et al., 1995).

Thr28 phosphorylation of Cyt*c* represents one of the few examples in the entire OxPhos system for which the corresponding kinase, i.e., AMP kinase (AMPK), has been identified (Mahapatra et al., 2017). AMPK is activated by the widely-used diabetes drug metformin (Zhou et al., 2001). Consistent with the Thr28 phosphorylation study, activation of AMPK with metformin leads to lower respiration in human renal proximal tubular epithelial cells (Takiyama et al., 2011). However, this decrease in OxPhos in the kidney is distinct compared to the generally accepted catabolic (ATP generation-promoting) role of AMPK in other organs (Carling, 2017).The pronounced regulation of kidney function by circadian rhythm oscillations might be a factor promoting anabolic pathways under some conditions (Firsov and Bonny, 2010; Zuber et al., 2009). AMPK has a high basal activity in the kidney (Bhargava and Schnellmann, 2017) likely due to the heavy reliance of this organ on OxPhos. Furthermore, AMPK plays a role in minimizing mitochondrial ROS (Rabinovitch et al., 2017), which was also observed with Cyt*c* Thr28 phosphorylation (Mahapatra et al., 2017). Cyt*c* purified from ischemic kidney was also fully dephosphorylated, similar to Cyt*c* purified from ischemic brain (Kalpage et al., 2019b; Sanderson et al., 2013a), and both organs are known to be highly susceptible to ischemia/reperfusion injury (Kalogeris et al., 2016). Studies have shown that treatment with an AMPK activator, AICAR, prior to ischemia increases cell survival in a rat model of renal ischemia/reperfusion injury (Lempiainen et al., 2012). This supports our model of ischemia-reperfusion injury where we suggest that Cyt*c* phosphorylation is required for the maintenance of optimal intermediate

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membrane potentials, which in turn would minimize ROS production upon reperfusion and thus limit tissue injury (Sanderson et al., 2013b).

2.4. Serine 47 (Ser47) phosphorylation of Cytc

Under basal conditions, Cyt*c* in the mammalian brain was recently shown to be phosphorylated on Ser47 (Kalpage et al., 2019b). This phosphorylation is lost during ischemia, suggesting an important regulatory role of this modification in the brain. The Ser47 phosphorylation site was mapped in both porcine and rat brain tissue. Phos-tag gel electrophoresis showed that about 35% of the Cyt*c* pool was phosphorylated. However, this is likely an underestimation as unavoidable ischemia during tissue harvesting may have started the dephosphorylation process. The role of the Ser47 phosphorylation was characterized using a glutamate phosphomimetic, Ser47Glu Cyt*c*. Both Ser47 phosphorylated *in vivo* and phosphomimetic Cyt*c* showed a 50% decrease in COX activity. Interestingly, Ser47 interacts with Lys58 of COX subunit VIIa based on docking simulations (Roberts and Pique, 1999). Subunit VIIa has a heart/skeletal muscle (heart-type) and liver/brain/kidney (liver-type) isoform pair. However, there was no difference in the relative change of activity of Ser47 phosphomimetic Cyt*c* and the K_m values when analyzed in the reaction with heart and brain COX (Kalpage et al., 2019b), suggesting that subunit VIIa isoforms do not modulate the effect of this Cyt*c* phosphorylation.

In addition, Ser47-phosphorylated and phosphomimetic Cyt*c* both resulted in a significant decrease (~70%) in caspase-3 activity. Furthermore, cardiolipin peroxidase activity was also lower in the presence of Ser47Glu phosphomimetic Cyt*c* compared to unphosphorylated WT Cyt*c*, further supporting the anti-apoptotic role of Ser47 phosphorylation. Ser47Glu phosphomimetic Cyt*c* also demonstrated significantly lower degradation rates of the heme group in the presence of 3 mM H_2O_2, suggesting that when phosphorylated at this site Cyt*c* has a better ability to withstand oxidative stress (Kalpage et al., 2019b). Another study analyzed the role of aspartate phosphomimetic Ser47Asp Cyt*c* and similarly reported a decrease in both caspase-3 activity and cardiolipin peroxidase activity (Guerra-Castellano et al., 2016). Ser47Asp Cyt*c* did not inhibit the reaction with COX as observed with Ser47Glu and *in vivo* Ser47 phosphorylated Cyt*c*. The crystal structure of Ser47Glu Cyt*c* (6N10.pdb) at 1.55 Å suggests that the Ser47-phosphorylated Cyt*c* sidechain and the glutamate sidechain of Ser47Glu spatially arrange in a similar manner, suggesting that Ser47Glu is a better phosphomimetic model than Ser47Asp Cyt*c* (Kalpage et al., 2019b). Overall, Ser47 phosphorylation of Cyt*c* is a tissue protective modification that promotes cell survival under healthy conditions by lowering apoptotic activity. Furthermore, Ser47 phosphorylation of Cyt*c* can prevent pathologically high respiration rates that would lead to hyperpolarization of the mitochondrial membrane potential and a burst of ROS causing cell death, as seen during ischemia/reperfusion injury (reviewed in (Sanderson et al., 2013b)). This suggests that a better understanding of the signaling pathway involved in Ser47 phosphorylation of Cyt*c* may provide a pre- or post-conditional therapeutic target for brain ischemia/reperfusion injury.

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2.5. Threonine 58 (Thr58) phosphorylation of Cytc

The Cyt*c* Thr58 residue is conserved in the somatic Cyt*c* isoform of some mammals but is replaced with an isoleucine residue in their testis isoform, which is also found in apes and humans who only have a single ubiquitous Cyt*c* (Fig. 4). Thr58 phosphorylation was mapped in purified rat kidney Cyt*c* by mass spectrometry. This phosphorylation was also found in a high-throughput phosphoproteomics study of rat kidney (Lundby et al., 2012). Unlike Thr28 phosphorylation, previously identified in five independent kidney preparations (Mahapatra et al., 2017), Thr58 phosphorylation was only found in some preparations. Considering that the kidney contains 26 different cell types (Poornejad et al., 2016), Thr58 phosphorylation may only occurs in a few cell types. Evolutionarily, threonine is the most conserved amino acid at residue 58 of Cyt*c* (Zaidi et al., 2014). Isoleucine is the second most abundant and is present in mammals containing a testes-specific isoform and in humans, who only have a single functional Cyt*c* gene that is ubiquitously expressed. *In vitro* and *in vivo* functional studies revealed that phosphomimetic Thr58Glu Cyt*c* partially inhibits COX activity. Furthermore, the oxygen consumption rate in intact cells expressing Thr58Glu Cyt*c* was decreased, suggesting that Cyt*c* plays a rate-limiting role on overall ETC flux in this cell model. In addition, Thr58Glu replacement reduced the mitochondrial membrane potential and ROS production in intact cells, supporting our model in which Cyt*c* phosphorylation limits membrane potential hyperpolarization, ROS production, and apoptosis. However, during stress conditions such as ischemia, this protective modification is lost, which may be the underlying mechanism explaining reperfusion injury, when oxygen reenters the tissue, and dephosphorylated Cyt*c* drives membrane potential hyperpolarization, bursts of ROS, release of Cyt*c*, and cell death (Sanderson et al., 2013b). In support of this model, the Thr58Glu phosphomimetic substitution also resulted in reduced caspase-3 activity *in vitro*, and intact cells stably expressing Thr58Glu Cyt*c* showed protection from apoptosis when challenged with H_2O_2 and staurosporine. This data further supports an anti-apoptotic role of Thr58 phosphorylation of Cyt*c*. Thr58 is located on the back side of the Cyt*c* heme crevice (Fig. 3). However, electrostatic, steric, and conformational changes in Cyt*c* may explain functional effects due to the small size of the Cyt*c* molecule. Compared to WT, Thr58Glu Cyt*c* was more resistant to heme degradation by H_2O_2. Thr58 was recently reported to be part of a cardiolipin binding site (O'Brien et al., 2015) (Fig. 2A). Cardiolipin peroxidase activity of Thr58Glu was inducible at high cardiolipin concentrations, suggesting that Cyt*c* phosphorylation may stabilize the heme iron-Met80 bond and suppress cardiolipin peroxidation (Pecina et al., 2010), thus providing a safeguard mechanism with which Cyt*c* release and apoptosis are properly regulated. Molecular dynamics simulations of Thr58Glu Cyt*c* suggested that the structural stability of phosphomimetic Cyt*c* is similar to that of Thr58 phosphorylated Cyt*c*. With a lower oxidation rate and a higher reduction rate, phosphomimetic Thr58Glu Cyt*c* reduces the electron transfer rate to COX in the ETC and serves as a better ROS scavenger (Wan et al., 2019).

3. Is Cyt*c* the rate-limiting step of the ETC?

The highly tissue-specific regulation of Cyt*c* and the effects of specific Cyt*c* phosphorylations on ETC and apoptotic function may have far-reaching implications. One question, which has been a subject of much debate, is which step within the ETC controls

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overall electron flux and is thus the bottleneck, or rate-limiting step, of the ETC. We here put forth a new concept and propose that the electron transfer from Cyt*c* to COX at the terminal step of the ETC is the rate limiting step of this vital process in cellular bioenergetics. The terminal step of the ETC is highly regulated by allosteric ATP binding (Fig. 2B), tissue-specific isoforms, and post-translational modifications, which are found in both Cyt*c* and COX. We propose that Cyt*c*, which operates at the intersection of respiration and apoptosis, is an ideal candidate to regulate both processes, in particular through reversible phosphorylations. If this concept holds the test of time it would form the rationale for targeting Cyt*c* to treat pathological conditions caused by mitochondrial dysfunction.

The COX-catalyzed reduction of oxygen to water is essentially irreversible and releases an excess of free energy that is twice as high as for complexes I and III (Hinkle et al., 1991). As discussed in detail below, several studies concluded that COX is the rate-limiting step of the ETC. However, the potential role of Cyt*c*, which binds to and injects electrons into COX catalytic subunit II, has previously not been considered. This may be so in part, because traditional metabolic flux analyses use specific inhibitors of the individual complexes of the ETC such as rotenone, antimycin A, and cyanide for complexes I, III, and IV, respectively. These inhibitors are added at increasing amounts to determine the fraction of the individual complexes that are required to allow unimpeded flux. The lower the fraction needed to reduce overall flux in a given complex the more rate-limiting it is. However, no such inhibitor exists for Cyt*c* so its potential rate-limiting role was never considered. For example, in most cases flux control coefficients of COX were determined by inhibitor titrations with cyanide or azide, which only assesses the contribution of oxygen reduction at the binuclear center of COX. In other words, the potential role of Cyt*c* phosphorylation in overall ETC flux is hidden in the flux control coefficients of bc_1 complex and COX. As discussed in the previous sections, experimental evidence suggests that modifications on Cyt*c* can decisively affect ETC flux in intact cells in addition to regulating apoptosis.

As with biochemical pathways in general, changes in the enzymatic activity of the rate limiting step can result in overall change of flux through the entire pathway and multiple studies have shown this also to be true for the ETC. Since metabolic flux analyses were not conducted for Cyt*c*, we briefly introduce the topic by discussing what is known about COX, which – importantly – includes the electron transfer reaction from Cyt*c* to COX. Several studies concluded that changes in COX activity can profoundly affect ETC flux. The rate-limiting step is often determined by the flux control coefficient, which is the change in relative flux/change in relative enzyme capacity (Gnaiger et al., 1998). Measurement of COX activity or capacity in intact cells through cyanide titrations revealed in a variety of human cell types that only a small excess COX capacity was present above what is required to carry out endogenous respiration (Villani and Attardi, 1997, 2000). A small excess COX capacity suggests a tight regulation of the ETC by COX. This conclusion was further supported by a cell line carrying mitochondria with a COX subunit 1 nonsense mutation. Cells carrying 35% of mutant mitochondrial DNA (mtDNA) resulted in 55% COX activity and 75% endogenous respiration whereas cells carrying 65% of mutant mtDNA resulted in a further decrease of COX activity (15%) and endogenous respiration (10%), suggesting that overall ETC flux is controlled by COX (Bruno et al., 1999). Respiration of human muscle fibers measured in the presence of glutamate and malate as substrates resulted in a 2.3-fold

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excess COX capacity. In the presence of both complex I and complex II substrates glutamate/malate and succinate, which further increases ETC flux, the excess COX capacity was decreased to 1.5-fold. Similarly, a lower excess COX capacity was present in skeletal muscle fibers of two ophthalmoplegia patients who carried deletions in 11% and 49% of their mtDNA. Both patients' muscle fibers showed an increase in the flux control coefficient as predicted by lower excess COX capacity, further supporting a rate-limiting function of COX (Kunz et al., 2000). Metabolic flux control analyses in intact cells showed that COX has a lower excess capacity than that reported in isolated mitochondria (Piccoli et al., 2006). Metabolic flux control analysis of saponin-treated muscle fibers showed that COX activity is affected by oxygen availability to the tissue. Saponin-treated fibers are a model for mitochondrial function of muscle as it allows the extramitochondrial medium to be precisely controlled. Consequently, saponin-treated muscle fibers led to higher flux control coefficients indicating the importance of oxygen availability, the substrate of COX (Wiedemann and Kunz, 1998). Using normoxic (190 μmolar) media, a 50% reduction in COX activity did not affect overall respiration rates. However, under lower oxygen concentrations COX activity became more rate-limiting, likely explaining the pathological phenotypes observed in muscle COX deficiencies associated with mitochondrial diseases, neurodegenerative diseases, and the aging phenotype (Korzeniewski, 1997; Wiedemann and Kunz, 1998).

A mouse cell line carrying the COX subunit I Val421Ala mutation resulted in a 43–65% decrease in COX activity and a lower overall respiration rate in intact or permeabilized cells compared to the controls (Acin-Perez et al., 2003). These data suggest that excess COX capacity is only between 25–40% (1.2–1.4 fold) in this cell culture model, suggesting that this step can be rate-limiting for ETC flux. In order to address the role of tissue specificity on OxPhos regulation by COX, the flux control coefficient and excess COX capacity was determined in human saponin-permeabilized muscle fibers and digitonin-treated hippocampal homogenates. These tissue preparations mimic *in vivo* mitochondrial function. Skeletal muscle homogenates resulted in a higher flux control coefficient of 0.24 ± 0.07 compared to brain homogenates (0.12 ± 0.05). Furthermore, the muscle homogenates had a lower COX excess capacity of 1.9±0.2 compared to a higher COX excess capacity of 3.9±0.6 in the brain homogenates (Kudin et al., 2002). These results suggest that human muscle COX is more rate-limiting in OxPhos compared to human brain COX. Consistent with the above report, the COX respiratory threshold value, which is defined as the %-inhibition value of COX activity that has an effect on the overall rate of endogenous respiration, was measured in mitochondria isolated from 5 different rat tissue types. Both muscle and heart mitochondria had a lower COX respiratory threshold compared to liver, kidney and brain mitochondria (Rossignol et al., 1999). These differences in metabolic control may be attributed to the presence of 3 different tissue-specific isoforms in COX subunits VIa, VIIa, and VIII in heart and muscle COX ("heart-type COX") compared to liver, kidney, and brain COX ("liver-type COX"). Several studies showed that liver-type COX has a significantly higher basal activity compared to heart-type COX, which provides a compensatory mechanism for tissues with less mitochondrial capacity and may explain the lower metabolic control of the brain isozyme compared to the heart isozyme of COX (Kalpage et al., 2019b; Sinkler et al., 2017; Vijayasarathy et al., 1998).

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The role of the electrical component (Ψ_m) and the chemical component (pH) of the mitochondrial electrochemical gradient on COX activity and overall respiration was investigated in intact human hepatoma HepG2 cells. The flux control coefficient of COX in the presence of nigericin, a potassium/proton antiporter, when Ψ_m is maximal, and in the presence of both nigericin and valinomycin (a potassium ionophore) when the electrochemical gradient is abolished, was comparable to the basal flux control coefficient of 0.73 (Dalmonte et al., 2009). In contrast, upon addition of valinomycin, which removes Ψ_m, a lower COX control coefficient of 0.30 was found, suggesting that it is primarily the Ψ_m component of the electrochemical gradient that is responsible for the tight regulation of COX in the overall process of the ETC (Dalmonte et al., 2009). Furthermore, COX activity is limited by the Cyt*c*/COX molar ratio which has been reported to be 1.08 in hepatocytes (Jones et al., 1979) and 1.62 in rat cardiac myocytes (Kennedy and Jones, 1986). Finally, Jurkat cells treated with an anti-Fas antibody and digitonin led to Cyt*c* release and induced apoptosis following a significant decrease in cellular respiration pertaining to the role of Cyt*c* in the rate-limiting step of the ETC (Hajek et al., 2001). Our data, using Thr28Glu and Thr58Glu Cyt*c* in intact cells, support the idea that a small modification on Cyt*c* affects overall ETC activity. Future work should establish the role of Cyt*c* on metabolic flux of the ETC under conditions that are more physiological and consider and maintain post-translational modifications of the protein found in tissues *in vivo* but that are lost when traditional mitochondrial purification protocols are used. In addition, Cyt*c*-COX activity assays should omit tetramethyl-*p*-phenylenediamine (TMPD), which is often used as an electron transfer catalyst, and which renders the reaction unphysiological because TMPD can reduce Cyt*c* when it is bound to COX (Cooper, 1990).

4. Conclusions

It appears to be crucial for cells to maintain a tightly regulated activity range of the last step of the ETC, catalyzed by Cyt*c* and COX, to efficiently produce ATP and minimize ROS production under physiological conditions. This concept is supported by the fact that mammalian Cyt*c* and COX are the only components of the OxPhos machinery that demonstrate all three regulatory mechanisms of metabolic enzymes: tissue-specific isoforms, allosteric regulation via the ATP/ADP ratio, and regulation by post-translational modifications (Fig. 3). The tissue-specificity of the mapped phosphorylation sites to date may seem unexpected. All phosphorylations lead to a reduction of the activity of the reaction catalyzed by Cyt*c* and COX, which supports our concept that intermediate ETC flux and thus intermediate Ψ_m levels are optimal for efficient energy production while limiting ROS generation. However, their effect on the apoptotic pathway are distinct. As an example, Tyr48 phosphorylation, found in the liver, abolishes caspase 3 activation, which may be a safeguard mechanism of a detoxification organ that is constantly exposed to potentially harmful substances. Another example is Ser47 phosphorylation of Cyt*c*, which also profoundly reduces the ability of Cyt*c* to trigger apoptosis and is present in the brain, an organ with limited capacity for regeneration. Other mechanisms underlying tissue-specific phosphorylation of Cyt*c* may be that certain kinases are only expressed in some tissues or their basal activity is high in a given organ, as we have found for AMPK-mediated Thr28 phosphorylation of Cyt*c* in the kidney.

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Cyt*c* and COX have undergone significant adaptations during evolution of anthropoid primates to optimally meet species-specific energy demands. Of the 1500 amino acids in COX, about 300 were replaced in anthropoid primates compared to other mammals. Some of the charged residues in the Cyt*c* binding site of COX were replaced by uncharged hydrophobic residues (Pierron et al., 2012; Schmidt et al., 2005). This adaption decreases the binding affinity between Cyt*c* and COX (Fig. 2C), further supporting the rate-limiting function of this interaction. At the level of Cyt*c*, the testes-specific isoform present in certain mammals became a pseudogene during primate evolution (Pierron et al., 2011) (Fig. 4). We propose that reversible phosphorylations of Cyt*c* mediated by cell signaling pathways are primary regulatory mechanisms in higher organisms. This in turn determines ETC flux, Ψ_m, ATP production, and ROS generation, linking OxPhos to human disease through a lack of energy, ROS production, Cyt*c* release, and activation of apoptosis. The recent discovery of novel regulatory phosphorylation sites on Cyt*c* has advanced the understanding that Cyt*c* plays a crucial role in regulating overall ETC flux and thus mitochondrial respiration, Ψ_m, ATP, and ROS.

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Abbreviations:

Cyt*c*	cytochrome *c*
COX	cytochrome *c* oxidase
$\boldsymbol{\Psi_m}$	mitochondrial membrane potential
ETC	electron transport chain
OxPhos	oxidative phosphorylation
ROS	reactive oxygen species
TMPD	tetramethyl-*p*-phenylenediamine
WT	wild-type

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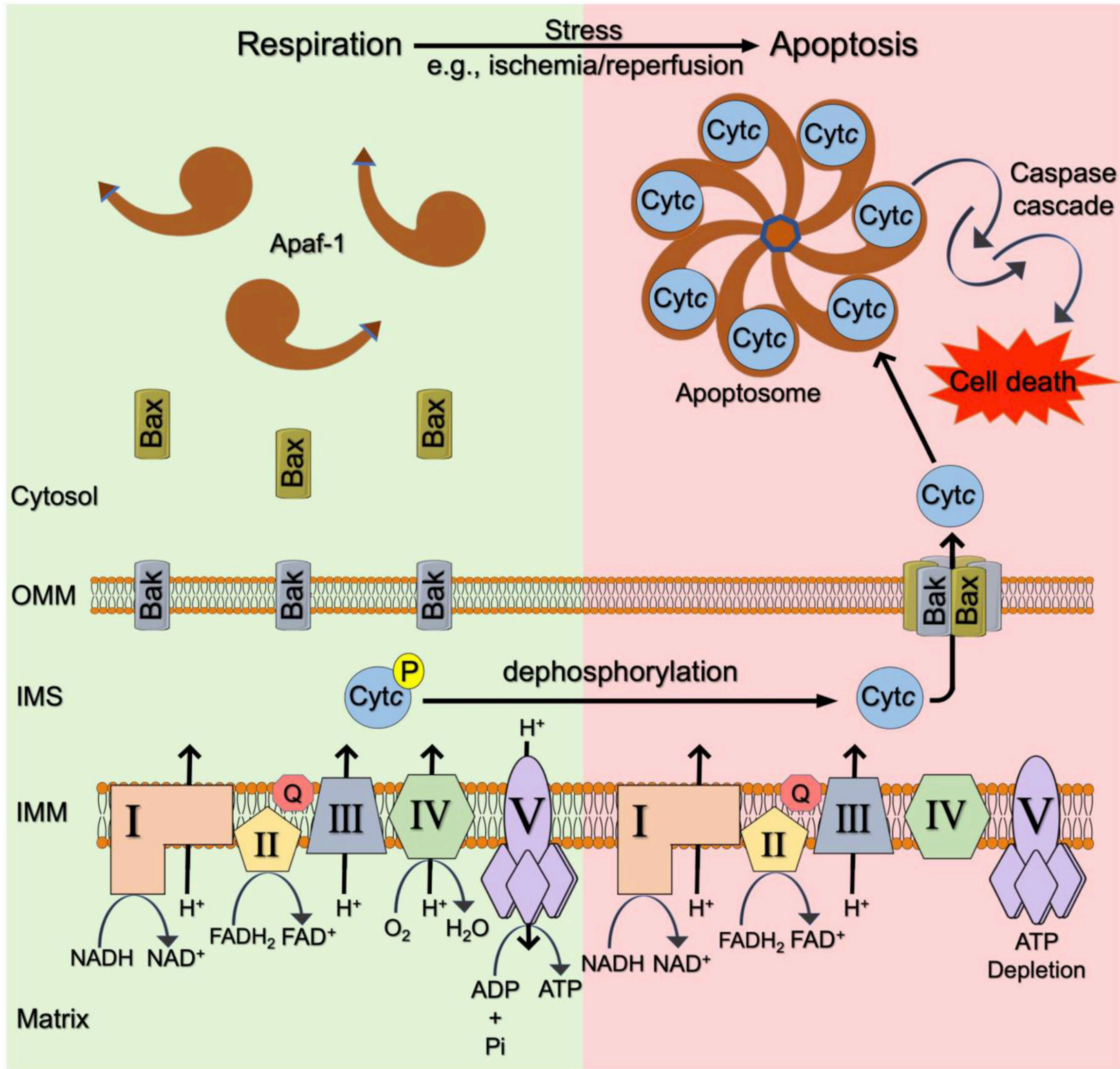

Fig. 1. Proposed pivotal role of Cyt*c* in respiration and apoptosis.

Cyt*c* acts as an electron carrier between complex III (bc_1 complex) and complex IV (cytochrome *c* oxidase) in the ETC resulting in efficient ATP production at complex V (ATP synthase). Under basal conditions, Cyt*c* is phosphorylated in a tissue-specific manner. Phosphorylated Cyt*c* is less likely to get released from the mitochondria and activate apoptosis in the cytosol. Under conditions of cellular stress, such as ischemia-reperfusion injury, dephosphorylated Cyt*c* is eventually released from the mitochondria via channels including Bax/Bak pores. In the cytosol dephosphorylated Cyt*c* interacts with Apaf-1 forming the apoptosome, which then activates capase-9 and the downstream caspase cascade, executing cell death. OMM; outer mitochondrial membrane, IMS; intermembrane space, IMM; inner mitochondrial membrane.

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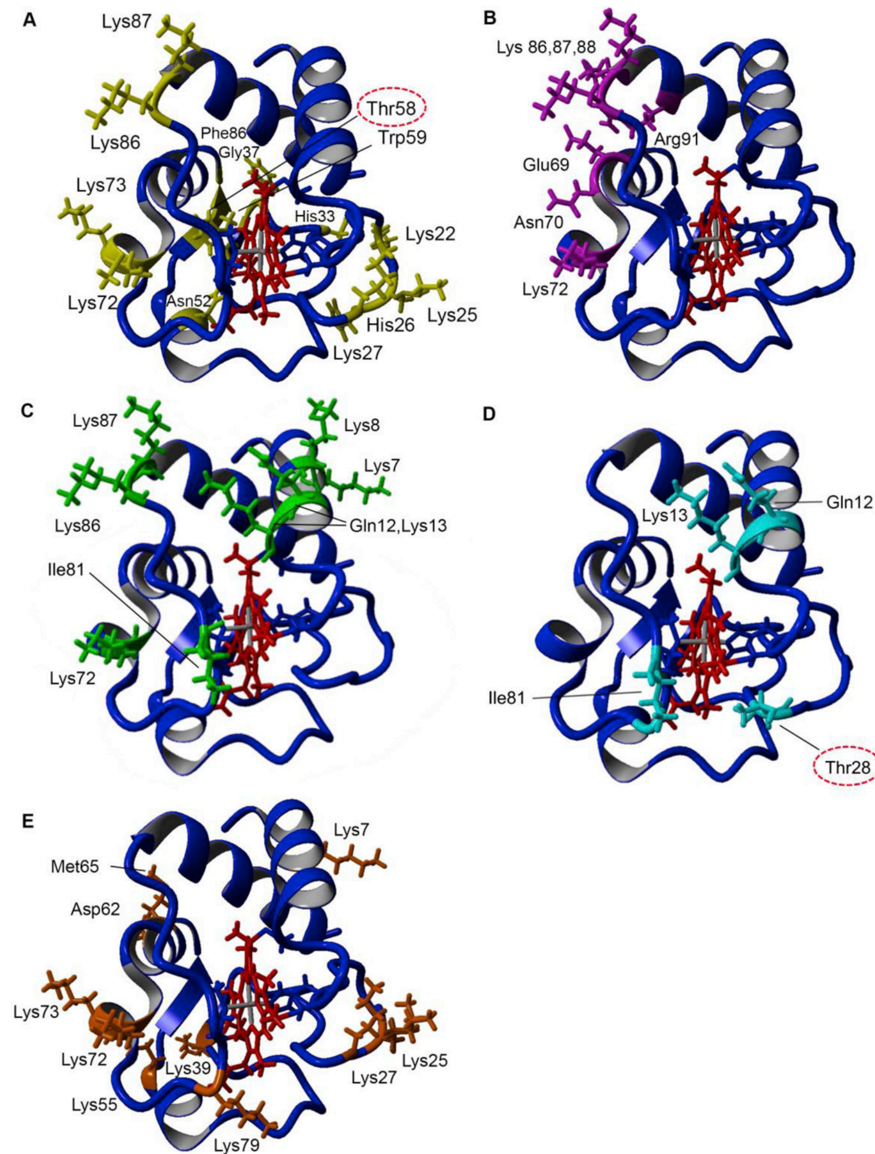

Fig. 2. Proposed Cyt*c* binding sites of cardiolipin, ATP, *bc*$_1$ complex, COX, and Apaf-1.

A: Cardiolipin binding sites of Cyt*c* (yellow). Lys72, Lys73, Lys86, Lys87 (A-site) (Kagan et al., 2009), Lys22, Lys25, His26, Lys27, His33 (L-site) (Kagan et al., 2009), Asn52 (C-site) (Rytomaa and Kinnunen, 1994), Phe36, Gly37, Thr58, Trp59, and Lys60 (N-site) (O'Brien et al., 2015). B: ATP binding pocket of Cyt*c* (magenta). Arg91 (Craig and Wallace, 1993; Tuominen et al., 2001), Glu69, Asn70, Lys72, Lys86, Lys87, Lys88, (McIntosh et al., 1996). C: COX binding sites of Cyt*c* (green) were selected if defined as binding residues in at least two out of four publications, two docking simulations and two kinetic studies. Lys7, Lys8, Gln12, Lys13, Lys72, Ile81, Lys86, Lys87 (Roberts and Pique, 1999; Ferguson-Miller et al., 1978; Sato et al., 2016; Scharlau et al., 2019). D: *bc*$_1$ complex core binding sites of Cyt*c* (cyan) based on the yeast *bc*$_1$-Cyt*c* crystal structures and converted to mammalian Cyt*c* residues. Gln12, Lys13, Thr28, Ile81(Lange and Hunte, 2002; Solmaz and Hunte, 2008). E: Apaf-1 binding sites of Cyt*c* (orange), Lys7, Lys25, Lys39, Asp62, Met65 (Yu et al., 2001),

Lys72 (Kluck et al., 2000), Lys27, Lys55, Lys73, Lys79 (Cheng et al., 2016). Amino acids that have been identified to be phosphorylated and are part of a binding site are circled.

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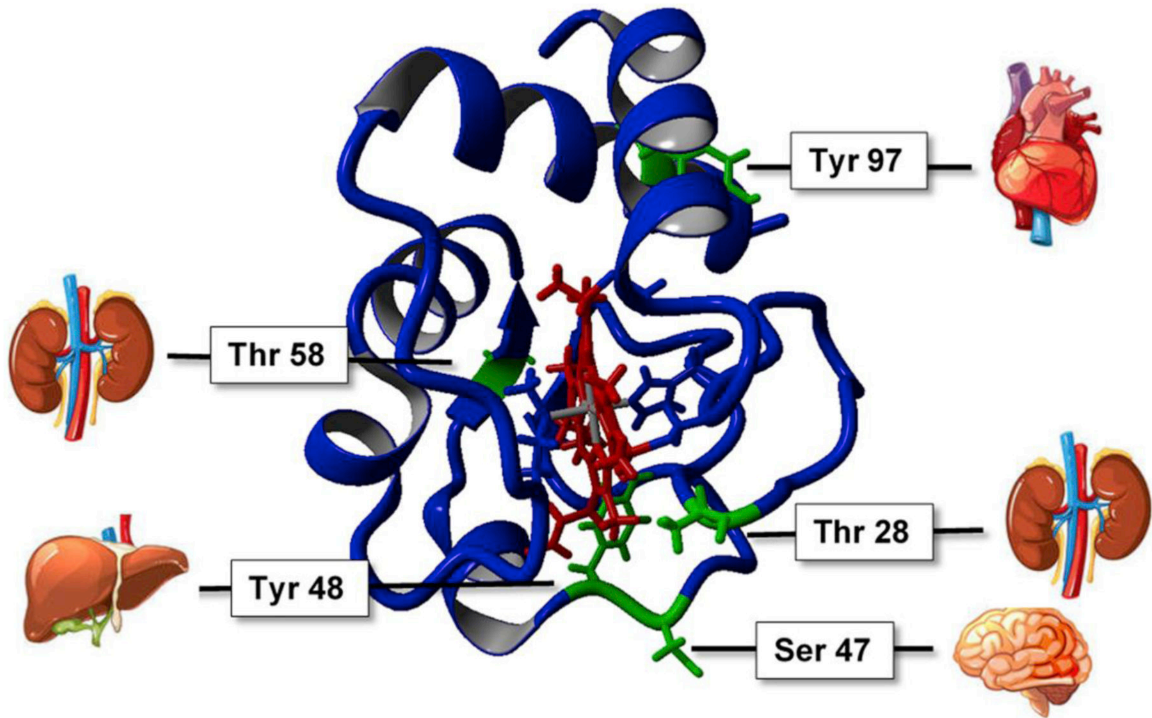

Fig. 3. Tissue-specific phosphorylation sites of Cyt*c* (green) mapped in mammalian tissues under basal conditions.

Sequence of their discovery: Tyr97 (heart), Tyr48 (liver), Thr28 (kidney), Ser47 (brain), Thr58 (kidney). The heme group is highlighted in red.

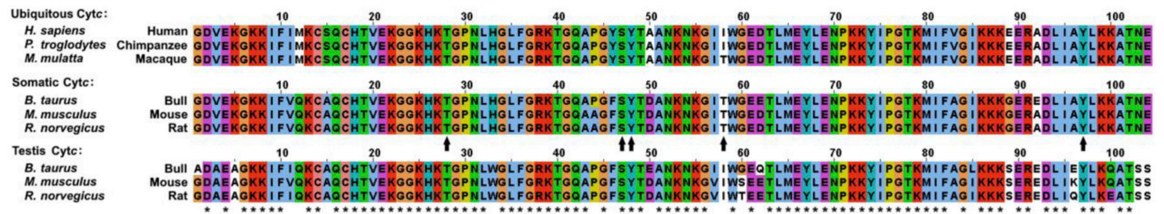

Fig. 4. Alignment of ubiquitous Cyt*c* from anthropoid primates (human, chimpanzee, macaque) and somatic and testes-specific Cyt*c* isoforms of non-anthropoid mammals (bull, mouse, rat).
Stars indicate conserved residues. Arrows indicate mapped and functionally studied phosphorylation sites in mammals.

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Table 1.

Summary of functionally characterized phosphorylation sites of Cyt*c*

Phosphorylation site	Low-throughput identification	Experimental models	Functional and structural characterization
Threonine 28	Electrospray ionization-mass spectrometry (bovine kidney) (Mahapatra et al., 2017).	*In vivo* phosphorylated protein purified from bovine kidney, phosphomimetic T28E recombinant protein, mouse lung fibroblast cells expressing T28E Cyt*c* (Mahapatra et al., 2017); phosphomimetic T28D recombinant protein (Guerra-Castellano et al., 2016).	Lower Cyt*c*-COX activity, lower redox potential, higher rate of reduction. Lower respiration, membrane potential and ROS production in intact cells. Phosphorylated by AMPK (Mahapatra et al., 2017). Lower binding affinity with Cytc_1 in bc_1 complex (Guerra-Castellano et al., 2016).
Serine 47	Electrospray ionization-mass spectrometry (porcine brain tissue) (Kalpage et al., 2019b).	*In vivo* phosphorylated protein purified from porcine brain, phosphomimetic S47E recombinant protein (Kalpage et al., 2019b); phosphomimetic S47D recombinant protein (Guerra-Castellano et al., 2016).	Lower Cyt*c*-COX activity, lower caspase-3 activity, lower cardiolipin peroxidase activity, reduced heme degradation (Kalpage et al., 2019b).
Tyrosine 48	Electrospray ionization-mass spectrometry (bovine liver) (Yu et al., 2008).	*In vivo* phosphorylated protein purified from bovine liver (Yu et al., 2008), Phosphomimetic Y48E recombinant protein (Garcia-Heredia et al., 2011; Pecina et al., 2010), Y48pCMF phosphomimetic Cyt*c* (Moreno-Beltran et al., 2017).	Lower Cyt*c*-COX activity (Pecina et al., 2010; Yu et al., 2008), abolished caspase-3 activity, reduced cardiolipin peroxidase activity, reduced cardiolipin binding, lower redox potential (Pecina et al., 2010). Lower caspase-9 activity (Moreno-Beltran et al., 2017).
Threonine 58 (replaced with isoleucine in humans)	Electrospray ionization-mass spectrometry (rat kidney) (Wan et al., 2019).	Phosphomimetic T58E recombinant protein, mouse lung fibroblasts expressing T58E Cyt*c* (Wan et al., 2019).	Lower Cyt*c*-COX activity, lower caspase-3 activity, lower redox potential, lower rate of oxidation, lower cardiolipin peroxidase activity, higher rate of reduction, lower respiration, membrane potential and ROS production in intact cells. Decreased cell death when treated with H_2O_2 and staurosporine (Wan et al., 2019).
Tyrosine 97	Electrospray ionization-mass spectrometry (bovine heart, insulin-treated porcine brain).	*In vivo* phosphorylated protein purified from bovine heart (Lee et al., 2006), phosphomimetic Y97E recombinant Cyt*c* (Garcia-Heredia et al., 2011); Y97pCMF phosphomimetic Cyt*c* (Guerra-Castellano et al., 2018); insulin-treated rat model of global brain ischemia (Sanderson et al., 2013a).	Lower Cyt*c*-COX activity, shift of the characteristic 695 nm peak to 687 nm (change in heme environment) (Lee et al., 2006), less thermally stable (Garcia-Heredia et al., 2011), lower caspase-3 activity (Guerra-Castellano et al., 2018), lower Cyt*c* release from mitochondria, decreased neuronal cell death (Sanderson et al., 2013a).

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