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REVIEW

Cytochrome *c*: the Achilles' heel in apoptosis

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Abstract Cytochrome *c* is a well-known mitochondrial protein that fulfills life-supporting functions by transferring electrons to the respiratory chain to maintain ATP production. However, during the activation of apoptotic machinery, it is released from mitochondria and, being in the cytosol, it either triggers the activation of the caspase cascade in intrinsic apoptotic pathway, or it is involved in the amplification of extrinsic apoptotic signaling. Accumulating evidence suggests that only unmodified holocytochrome *c* is efficient in the stimulation of apoptosis. Considering the importance of cytochrome *c* in both life and death, it was of significant interest to investigate the complete or partial cytochrome *c* deficiency in vivo. Here, we discuss the importance of distinct amino acid residues for various functions of cytochrome *c* in cells and mice with targeted cytochrome *c* mutations.

Keywords Cytochrome *c* · Mutagenesis · Knockout · Respiration · Apoptosis

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Introduction

Cytochrome *c* is a highly conserved small soluble heme-containing protein with a molecular weight of about 12 kDa (Fig. 1). It was described for the first time by Charles A. MacMunn at the end of the 19th century and was rediscovered by David Keilin in 1925 [1]. The crystal structure of cytochrome *c* was reported by Dickerson and colleagues in 1971 [2] and since that time cytochrome *c* remains among the most intensively studied proteins because of its vital function in living organisms. Consisting of 104 amino acids in humans, horses, and mice, cytochrome *c* is a redox-active molecule that is encoded in the nucleus and synthesized as apocytochrome *c*. It is transported across the outer mitochondrial membrane (OMM) into the intermembrane space, where it is converted into holocytochrome *c* by holocytochrome-*c* synthase to become the mature protein with the heme group covalently linked to Cys-14 and -17.

Cytochrome *c* plays an essential role in mitochondrial respiration by shuttling electrons between complex III and IV of the respiratory chain located in the inner mitochondrial membrane (IMM). However, when cells undergo apoptosis, the localization and function of cytochrome *c* change radically. During the search for mechanisms of early massive cell death induced by ionizing radiation in radiosensitive tissues in the middle of the last century, the suppression of oxidative phosphorylation in mitochondria

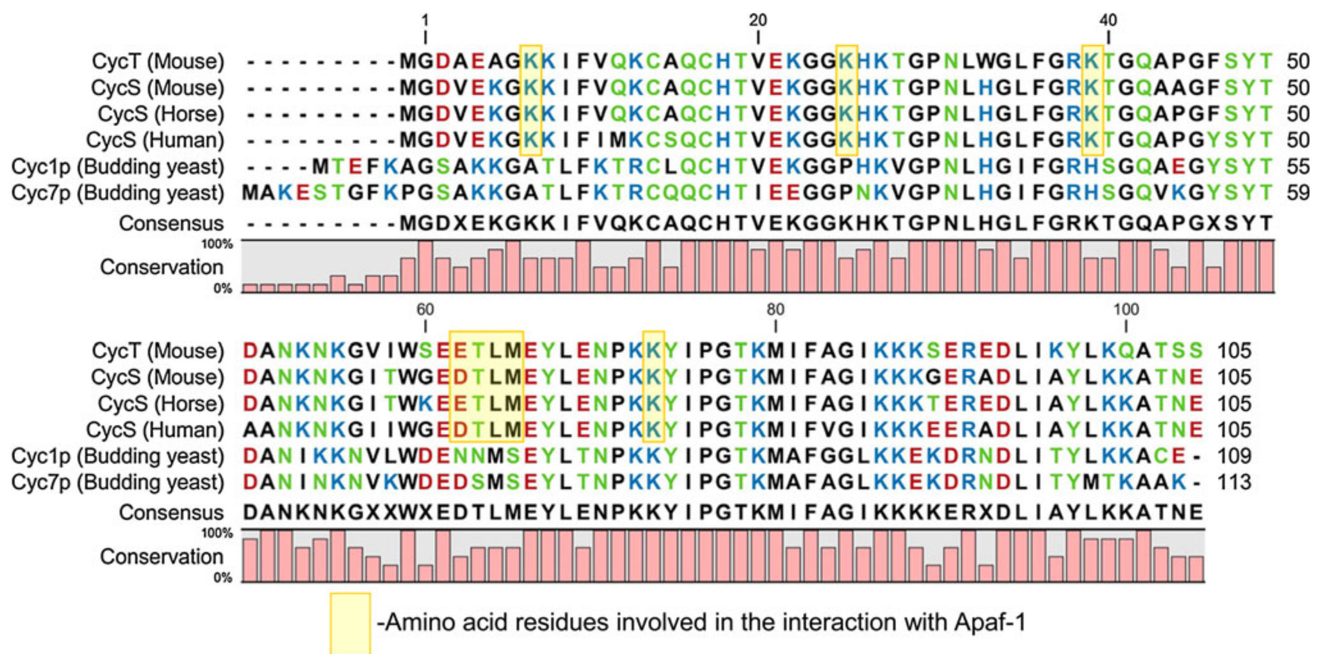

Fig. 1 Alignment of cytochrome *c* from various sources

from the thymus and spleen was described [3]. This phenomenon was not seen in the mitochondria from radioresistant tissues [4]. The suppression of oxidative phosphorylation was observed shortly after whole-body X-radiation of rats with relatively low doses of radiation (50 ± 100 cGy) [5]. Importantly, this suppression of oxidative phosphorylation was associated with the formation of pyknotic nuclei [6], suggesting the link between the deterioration of mitochondria and cell death. More-detailed analysis revealed that a slowing down of electron transport occurred between cytochromes *b* and *c* and was characterized by a significant decrease in the level of cytochrome *c* after irradiation [7]. Thus, the radiation lesion was believed to be due to the looser binding of cytochrome *c* to the inner membrane of the mitochondria after X-irradiation. This observation was in accordance with the previous finding that the addition of exogenous cytochrome *c* could stimulate oxidative phosphorylation in mitochondria isolated from radiosensitive, but not radioresistant tissues of irradiated rats [4, 8]. The loss of cytochrome *c* was not a result of its simple escape from the mitochondria, since additional washing of the mitochondrial fraction with isotonic buffer did not increase the "cytochrome *c* effect" [5], nor did in vitro irradiation of isolated mitochondria cause increased enzyme release [9]. Thus, it was suggested that the perturbation of mitochondrial electron transfer in radiosensitive tissues was based on a controlled release of cytochrome *c* from the mitochondria and the appearance of the hemoprotein in the cytosol [7, 10]. In strong support of these earlier observations were several reports in the late 1990s on the release of cytochrome *c* from mitochondria in cells undergoing radiation-induced cell death [11, 12].

In 1996, the loss-of-function of cytochrome *c* was described in Jurkat cells upon treatment with anti-Fas antibody [13]. Finally, experiments performed by Xiaodong Wang and colleagues led to the discovery that cytochrome *c* is one of cytosolic factors that are able to induce proteolytic processing and activation of the caspase cascade, which is essential for proper development of the apoptotic process. They further demonstrated that some cytosolic proteins can collaborate with cytochrome *c* in caspase-3 processing and activation in vitro [14]. The release of cytochrome *c* is thus believed to be a crucial pro-apoptotic signal [15, 16]. In the cytosol, cytochrome *c* binds to protein Apaf-1 in a dATP/ATP-dependent manner, causing Apaf-1 oligomerization and recruitment of pro-caspase-9, leading to the formation of a heptameric multimolecular complex, known as an apoptosome. Within this complex, pro-caspase-9 undergoes processing and activation [17, 18]. The crucial role of cytochrome *c* in the induction of apoptosis was further demonstrated in experiments where the microinjection of this protein into the cytosol of various cells caused apoptotic cell death [19]. Importantly, heat denaturation abolished the pro-apoptotic activity of cytochrome *c* [20]. The heme moiety was apparently required, as apocytochrome *c*, which lacked the heme group, possessed no pro-apoptotic activity [21]. Substitution of cytochrome *c* by other heme-containing proteins was also ineffective in apoptosis induction, as was in the case for biotinylated cytochrome *c* [22]. It seems that

only full-length and unmodified cytochrome *c* is efficient in the stimulation of apoptosis.

How is cytochrome *c* released from mitochondria?

There are currently several mechanisms that explain the release of cytochrome *c* from the mitochondria. All of them require OMM permeabilization. The first, which can be engaged in both necrotic and apoptotic cell death, involves the induction of mitochondrial permeability transition (MPT). This phenomenon was demonstrated some 30 years ago [23]. It was shown that Ca^{2+} uptake by mitochondria stimulates drastic changes in mitochondrial morphology and functional activity due to the opening of a non-specific pore, commonly known as the MPT pore, in the IMM. This is followed by osmotic swelling of mitochondria and rupture of the OMM, causing the release of intermembrane space proteins, including cytochrome *c*. This process can be facilitated by inorganic phosphate, the oxidation of pyridine nucleotides, ATP depletion, low pH, and ROS. According to the modern view on MPT pore machinery, it is a multimeric complex composed of a voltage-dependent anion channel (VDAC) located in the OMM, adenine nucleotide translocase (ANT), which is an integral protein of the IMM, and a matrix protein, cyclophilin D (CypD). This complex is located in contact sites between the OMM and IMM. In addition, other proteins may bind to the pore complex, in particular, protein kinases (e.g., hexokinase, creatine kinase) (for a review, see [24]). Not all the components of the pore contribute equally to MPT induction. Recently, various attempts have been made to clarify the importance of each component of the pore for its functions. Thus, deletion of the three mammalian VDAC genes did not affect a Ca^{2+} and oxidative stress-induced MPT. Moreover, wild-type and VDAC-deficient cells exhibited similar cytochrome *c* release, caspase cleavage, and cell death in response to the pro-apoptotic members of Bcl-family proteins, Bax and Bid. These results suggest that VDACs are dispensable for both MPT and Bcl-2 family member-driven cell death [25].

A similar conclusion was drawn about the role of ANT. Genetic inactivation of two isoforms of ANT in mouse liver did not affect permeability transition and cytochrome *c* release. Moreover, hepatocytes without ANT remained competent to respond to various initiators of cell death. The authors conclude that ANT is non-essential structural components of the MPT pore, although after inactivation of ANT more Ca^{2+} than usual was required to stimulate MPT [26].

Analysis of a critical role of CypD showed that mice lacking CypD revealed resistance to ischemia/reperfusion-induced cell death in vivo. Primary hepatocytes and fibroblasts isolated from mice lacking CypD were largely protected from Ca^{2+} overload and oxidative stress-induced cell death. In addition, mitochondria isolated from the various organs of mice lacking CypD are resistant to mitochondrial swelling and permeability transition in vitro. In contrast, CypD-overexpressing mice demonstrated mitochondrial swelling and spontaneous cell death, suggesting the role of CypD for MPT-induced cell death [27].

Another mode of OMM permeabilization involves pro-apoptotic members of the Bcl-2 family of proteins. Today, more than 30 members of the Bcl-2 family and related proteins have been identified. They can be divided into three subgroups: Bcl-2-like anti-apoptotic, multidomain pro-apoptotic proteins (such as Bax, Bak, and the much-less-studied Bok) and BH3-only proteins [28]. It was shown that permeabilization of the OMM requires oligomerization of Bax or Bak. The oligomerization of Bax is a result of its binding to the truncated form of the BH3-only pro-apoptotic protein Bid (tBid) cleaved by caspase-8 or caspase-2. Cells deficient in both Bax and Bak, but not cells lacking only one of these proteins, were found to be resistant to tBid-induced cytochrome *c* release and apoptosis [29]. Moreover, Bax and Bak-deficient cells were also resistant to a variety of apoptotic stimuli that act through the mitochondrial pathway. Thus, activation of a "multi-domain", pro-apoptotic Bcl-2 family member, Bax or Bak, appears to be a principal gateway for mitochondrial release of the pro-apoptotic proteins required for cell death in response to diverse stimuli. In some cases, activation of Bax can be achieved by reactive oxygen species (ROS). This assumption was based on the finding that oxidative dimerization of Bax promotes its translocation to mitochondria [30], and that Cys-62 of Bax is critical for its conformational activation and proapoptotic activity in response to H_2O_2 [31]. Anti-apoptotic proteins, for example, Bcl-2, Bcl-X_L, Mcl-1, and Bcl-w, interact with the pro-apoptotic proteins Bax and Bak in order to prevent their oligomerization. A critical factor triggering apoptosis is disturbance of the balance between anti- and pro-apoptotic Bcl-2 family members in favor of the latter. This balance is regulated by the BH3-only proteins, which bind to and sequester the anti-apoptotic proteins, thereby liberating Bax and Bak, or changing their conformational status, and permitting the formation of pores. In addition to cytochrome *c*, OMM permeabilization causes release of various intramembrane space proteins, such as apoptosis inducing factor (AIF), Smac/Diablo, Omi, and others.

Being a globular, positively charged and polar protein, cytochrome *c* is attached to the inner membrane via negatively charged anionic phospholipids, such as cardiolipin [32]. These authors proposed that the cytochrome *c* pool is bound to cardiolipin molecules, which have a lateral and rotational diffusion in the bilayer that is adequate to account for electron-transport rates. In eukaryotic cells,

cardiolipin is only present in mitochondria and is primarily found in the IMM. Because of its unique structure among phospholipids, cardiolipin confers fluidity and stability to the mitochondrial membrane. The molecular interaction between cardiolipin and cytochrome *c* involves both electrostatic and hydrophobic interactions. In order to explain the binding mode of the latter, it was postulated that one of the acyl chains of cardiolipin could be inserted into a hydrophobic pore in cytochrome *c*, while the others extend into the phospholipid bilayer [33]. However, another model for the cardiolipin–cytochrome *c* interaction was recently reported [34], postulating the presence of two distinct regions in cytochrome *c*, each characterized by different affinity for phospholipids [35], which might bind cardiolipin. It was suggested that deprotonated phosphate group of cardiolipin could electrostatically interact with the Lys-72 and Lys-73 residues of cytochrome *c* [36]. Interestingly, Lys-72 is one of the key amino acids through which cytochrome *c* carries out its apoptotic functions (see below). In vitro mutagenesis of Lys-72 attenuates the apoptotic activity, which might be caused by an improper binding to cardiolipin.

In addition to a soluble pool of cytochrome *c*, the size of which is determined by the ionic strength in the intermembrane space [37], a certain amount of cytochrome *c* is bound to the IMM. The experiments with isolated mitochondria revealed that after mitochondrial swelling and cytochrome *c* extraction in isotonic KCl (150 mM), about 15% of cytochrome *c* is still bound to the membrane [38]. However, in situ, the pool of the membrane-bound cytochrome *c* might be larger. The amount of endogenous membrane-bound cytochrome *c* depends on the bulk concentration of soluble protein in the intermembrane space of intact mitochondria. It has been shown that exposure of IMM to various concentrations of cytochrome *c* followed by extensive washing at physiological ionic strength increased the pool of membrane-bound proteins over the endogenous level [39]. It cannot be excluded that low amount of the membrane-bound cytochrome *c* in the experiments with swollen mitochondria is a consequence of extensive dilution of intermembrane space content after the OMM rupture. Apparently, simple permeabilization of the OMM by oligomeric Bax in a low-ionic strength medium is insufficient for considerable release of cytochrome *c* from mitochondria [40]. It appears that the electrostatic and hydrophobic interactions between cardiolipin and cytochrome *c* must be "breached" in order to enhance cytochrome *c* release from the organelles. It was previously found that oxidation of cardiolipin decreases its binding affinity for cytochrome *c* (for a review see [41]). Release of cytochrome *c* from the mitochondria compromises the activity of the respiratory chain, leading to suppression of oxidative phosphorylation. In addition, this will enhance mitochondrial production of ROS, since electron transfer between complexes III and IV will be partially blocked and electrons will start leaking from the respiratory chain producing the superoxide radical [42].

Various functional aspects of the cytochrome *c* complex with cardiolipin were extensively studied. Kagan and colleagues revealed an additional function of cytochrome *c* as a cardiolipin oxygenase [43]. After the induction of apoptosis, cytochrome *c* oxidizes cardiolipin. This facilitates the detachment of cytochrome *c* from the IMM and its release from the mitochondria. The peroxidase function of the cardiolipin–cytochrome *c* complex is compatible with the proposed 'two-step' hypothesis of cytochrome *c* release and provides a plausible explanation for the protective effects reported for multiple mitochondrial antioxidant enzymes [40]. It also emphasizes the importance of mitochondrial ROS production as an integrated component of the apoptotic program. For many years, the site(s) for ROS generation during the initial phase of the apoptotic process was/were unknown. Work from Pelicci's group has shown that redox protein, p66Sch, generates mitochondrial ROS (hydrogen peroxide) as signaling molecules for apoptosis [44]. It utilizes reducing equivalents of the mitochondrial electron transfer chain through the oxidation of cytochrome *c*. The increase in ROS production via p66Shc as a part of the apoptotic pathway could provide cytochrome *c* with peroxide equivalents essential for cardiolipin oxidation. In support of this possibility, recently the correlation between cytochrome *c* release and H_2O_2 production activity of p66Shc was shown [45]. Redox-defective mutants of p66Shc are unable to induce mitochondrial ROS generation and swelling in vitro or to mediate mitochondrial apoptosis in vivo [44].

Cytochrome *c*-mediated oxidation involves a tyrosine radical. Two tyrosine phosphorylation sites were previously detected in cytochrome *c*, Tyr-97 in bovine heart and Tyr-48 in bovine liver [46, 47]. Conserved Tyr-97 (Fig. 1) is connected to the heme moiety through a hydrophobic channel that allows the anchoring of cytochrome *c* with one of the acyl moieties of cardiolipin [2]. Thus Tyr-97 is capable of electron transfer, as required for cardiolipin redox chemistry. It was speculated that Tyr-97 phosphorylation may affect the role of cytochrome *c* in apoptosis (Table 1) because Lys-7, an essential residue for the interaction with Apaf-1, is spatially located next to Tyr-97 and may possibly form a salt bridge neutralizing the positive charge on the lysine residue, which may alter or prevent induction of apoptosis [48].

Similarly to Tyr-97, phosphorylated Tyr-48 might reduce maximal turnover of cytochrome *c* in the reaction with cytochrome *c* oxidase [47]. Nothing was known about

Table 1 Consequences of amino acid substitutions for cytochrome *c* function in apoptosis

Amino acid residue	Substitution	Reason of model	Direct effect on apoptosis	Other in vitro and in vivo effects
Tyr-97	Tyr97Glu	Phosphomimetic substitution similar to bovine heart phospoTyr97	Unknown	Substitution Tyr97Glu caused partial decrease of mitochondrial inner membrane potential by the inhibition of reaction with cytochrome *c* oxidase
Tyr-48	Tyr48Glu Tyr48Phe	Phosphomimetic substitution Tyr48Glu is similar to bovine liver heart phospoTyr48 Tyr48Phe is similar to wild-type	Tyr48Glu could not activate caspase-3 and caspase-9 in cell-free system	Substitution Tyr48Glu decreases binding affinity to cardiolipin and shows inhibition in the reaction with isolated cytochrome *c* oxidase
Gly-41	Gly41Ser	Natural human mutation in Cyt *c* gene observed in New Zealand population	Proapoptotic, leads to increased caspase-3 and caspase-9 activation	Mutation Gly41Ser in heterozygous state is associated with familiar thrombocytopenia
Lys-72	Lys72Ala Lys72Trp Lys72Arg Lys72Gly Lys72Glu Lys72Leu	Trimethylation of horse Cyt *c* lysine 72 residue in yeast expression system causes loss of apoptotic function, similar to different amino acid substitution in this site	All antiapoptotic, lead to diminished caspase-3 and caspase-9 activation with different efficiency	Knock-in homozygous mice Lys72Ala and Lys72Trp show moderate embryonic lethality and different abnormalities in newborn pups, including cachexia and hydrocephalus. Cellular respiration and electron transfer are the same as in wild-type mice
Met-80	M80A	Disruption of methionine 80 (M80)-Fe ligation of cytochrome *c* under nitrative stress	Antiapoptotic, since other pro-apoptotic proteins are not released from mitochondria along with mutant cytochrome *c* and since M80A does not efficiently activate the apoptosome	Subcellular localization of cytochrome *c* may be regulated not only by Bcl-2 family members, the MPTP, and/or serine proteases, but also by the M80-Fe ligation of cytochrome *c* (at least in response to nitrative stress) [74]

its role in apoptosis, but it was proposed that Tyr-48 phosphorylation might affect the cardiolipin peroxidase activity of cytochrome *c*. In order to understand how the modification of cytochrome *c* affects its affinity to cardiolipin, a model with phosphomimetic substitution in Tyr-48 of cytochrome *c*, resulting in major changes in cytochrome *c* behavior, has been proposed [36].

The substitution Tyr48Glu led to multiple malfunctions, such as decreasing cell respiration, diminishing the binding to cardiolipin and also abolishing the cytochrome *c*-mediated caspase activation (Table 1). The binding affinity of the Tyr48Glu mutant cytochrome *c* to cardiolipin was decreased by about 30% compared to the wild-type or the Tyr48Phe variants, and peroxidase activity of the Tyr48Glu mutant cytochrome *c* was only observed at high cardiolipin concentrations compared to the wild-type cytochrome *c*. Thus, a decrease in the stability of the cytochrome *c*–cardiolipin complex could lead to easier dissociation of cytochrome *c* and its release from the mitochondria into the cytosol. However, Tyr48Glu variant of cytochrome *c* lacks the ability of apoptosome formation and could not be involved in caspase-9 activation. Because glutamate residue is not equivalent to phosphotyrosine, the predictive value of this model appears controversial.

Several attempts have been made to elucidate the role of the redox state in the pro-apoptotic properties of cytochrome *c*. Thus, using cytoplasmic extracts, Hampton and colleagues demonstrated that a reduction of cytochrome *c* had no influence on the activation of caspases, which indicates that both the oxidized and reduced forms of cytochrome *c* are competent for pro-apoptotic signaling [22]. Moreover, the redox capacity of holocytochrome *c* is not required for apoptotic activity, as redox-inactive zinc and copper-substituted cytochrome *c*, which are structurally similar to Fe-cytochrome *c*, exhibited strong pro-apoptotic activity in a *Xenopus* cell-free system [20]. Although a later study postulated that only oxidized cytochrome *c*, and not its reduced form promotes apoptosome assembly [49], this issue should be further investigated, since most cellular compartments are associated with reducing environments, and a rapid reduction of oxidized cytochrome *c* could be expected.

The release of cytochrome *c* was independent of its oxidation state that is consistent with a simple model of cytochrome *c* passively diffusing down a concentration gradient through a pore or rupture in the outer membrane [50]. Thus, the redox state of cytochrome *c* probably affects its affinity towards apoptosome proteins, but apparently it plays no role in its passage through the OMM. This was demonstrated by real-time changes in the oxidation state of cytochrome *c* using visible spectroscopy during mitochondrial outer membrane permeabilization (MOMP) initiated by anisomycin in HL-60 cells. It was

shown that the cytochrome *c* was oxidized by ≈62% before MOMP and by ≈70% after MOMP. In contrast, the cytosolic pool of cytochrome *c* was found to be almost fully reduced [22]. It is interesting to note that radiation-induced damage to cytochrome *c* in vitro was appreciably less when it was in the fully reduced state rather than partially oxidized or undergoing alternative oxidation/reduction changes [9].

Consequence of cytochrome *c* deficiency

Considering the importance of cytochrome *c* in both life and death, it was of significant interest to investigate the complete or partial cytochrome *c* deficiency in vivo. Knockout or knock-in mouse and cellular models were established in several studies. Not unexpectedly, a complete deficiency of the somatic isoform of cytochrome *c* resulted in embryonic lethality at the mid-gestation stage, implying that defects in mitochondrial respiration are life-incompatible [51]. However, mouse embryonic fibroblasts (MEFs) homozygous for the knockout mutation could be obtained from heterozygous females and cultured under special conditions. Thus, cytochrome *c*-deficient MEFs, as well as MEFs homozygous for mutant forms of this protein (see below), represent useful cellular models for studying the consequences of cytochrome *c* modifications in vitro and such cells have been used in a plethora of studies of cell death mechanisms.

Importantly, in some cases, the MEFs derived from mice with a complete deficiency in somatic cytochrome *c* started to abnormally express the testicular gene for cytochrome *c* [43]. The existence of two different isoforms of cytochrome *c* in rodents, somatic and testicular, which are encoded by *CycS* and *CycT* genes, respectively, was previously reported [52, 53]. These isoforms found in mice are not identical and contain 14 amino acid variations (see Fig. 1). Thus, the expression of testicular cytochrome *c* could potentially compensate for the deficiency in somatic cytochrome *c* functions, as demonstrated for the CRL 2613 cell line [54]. At the same time, the testis-specific cytochrome *c*-null mice developed normally and were fertile, but they underwent early testicular atrophy [55], which proves the local action of testicular cytochrome *c* in the case of normal function of both somatic and testicular forms. It is also possible that the somatic form of cytochrome *c* could at least partially compensate for the function of testicular cytochrome *c*. Indeed, analysis of the UniGene database reveals almost equal amounts of *CycS* and *CycT* expressed sequence tags (ESTs) in the murine testis cDNA library, suggesting that they have typically similar expression levels, although immunofluorescence technique reveals that only cells on late stages of spermatogenesis express testicular form of cytochrome *c*. Neither spermatogonia, nor Sertoli cells and interstitial cells produce *CycT* [56]. Obviously, there is cytochrome *c* expression switch during mouse spermatogenesis, but regulation of this switch remains unknown. In order to correctly interpret the data, the role of testicular cytochrome *c* should be investigated more precisely, and studies that did not include such an analysis of the testicular form of this gene should be considered with caution. Surprisingly, the pro-apoptotic activity of the testicular isoform appeared to be even higher than that of the somatic one (for instance, 0.2 μM of the testicular isoform stimulated caspase activity to the same extent as the somatic isoform at 0.5 μM), indicating the significance of at least some of the amino acid variations. One can speculate that the higher pro-apoptotic activity of the testicular isoform could be due to its higher affinity to Apaf-1 and other cellular compounds. Interestingly, the testicular isoform of cytochrome *c* was able to reduce hydrogen peroxide three times faster than the somatic isoform, thus providing a better defense against ROS [57].

The interaction between cytochrome *c* and Apaf-1 might be influenced by amino acid mutations. Genetic studies identified the only residue related to the increased apoptotic activity of human cytochrome *c*. Morrison and colleagues reported a genetic linkage between autosomal dominant thrombocytopenia and a mutation in the gene encoding human somatic cytochrome *c* (*CycS*), which causes substitution of a conserved glycine by serine at residue 41 (G41S) [58]. This residue belongs to a highly variable region of the primary sequence, which is located on the surface of the protein [59]. Investigations into an X-ray structure of the reduced G41S cytochrome *c* at a 2.7-Å resolution did not demonstrate any large-scale structural alterations in this protein. Analysis of the functional and structural properties of the recombinant wild-type and the G41S cytochrome *c* revealed that the latter had the same superoxide oxidase activity as the wild-type protein, and mitochondria with mutated cytochrome *c* revealed the same oxygen consumption rate. However, the G41S form was significantly more effective than the wild-type in triggering cleavage of the caspase-3 substrate DEVD-AMC in a cell-free assay. Thus, the G41S mutation results in cytochrome *c* variants with enhanced apoptotic activity in vitro, but normal redox activity. Glycine 41 is located close to lysine 39, a residue involved in the cytochrome *c*-Apaf-1 binding interface [48], further suggesting that the altered pro-apoptotic activity of the G41S cytochrome *c* is related to the step of apoptosome assembly [60], which was also determined by molecular modeling [61]. The authors postulated that the alteration in the affinity of cytochrome *c* for Apaf-1 may explain the increased caspase-stimulatory function of the G41S mutant.

Liptak and colleagues reported that the pro-apoptotic G41S mutation, which causes improper folding of

cytochrome *c*, might alter the electronic structure of heme and increase the electron self-exchange rate [62]. The cause of the enhanced pro-apoptotic activity of the heterologically expressed G41S form of cytochrome *c* mentioned above is unclear and remains to be elucidated.

The best-studied mutation in cytochrome *c* is Lys-72 (Table 1), which represents another interesting spot for understanding the mechanisms of cytochrome *c* function in apoptosis. Structurally, this residue belongs to the fourth helix, including residues 71–75, and was shown to participate in interactions with other proteins [63]. The interest in this residue comes from observations that although cytochrome *c* from horses, bovines, tuna and pigeons could activate caspases in the *Xenopus* cell-free system and in mammalian cytosols with equal efficacy, the yeast-expressed iso-1 (gene *Cyc1p*) and iso-2 (gene *Cyc7p*) cytochrome *c* could not, thus implicating a lack of pro-apoptotic activity. By performing sequence comparisons, the authors pin-pointed several differences between the yeast-expressed isoforms and the pro-apoptotic cytochrome *c;* among them was trimethylated Lys-72, positioned in the most highly conserved region and being one of the critical residues for binding to redox partners, including COX and the cytochrome *bc1* complex [64] (Fig. 1).

In certain eukaryotes, including fungi and plants but not in animals, specific lysine residues in cytochrome *c* are methylated. Methylation is a widespread mechanism for modifying biological functions of proteins. For many years it has been known that yeast cytochrome *c* contains trimethyl-lysine at the single position of 72 [65], which is determined by an aromatic amino acid in position 74 [66]; however, the importance of this amino acid for the pro-apoptotic activity of cytochrome *c* was not evident.

As mitochondrial oxygen consumption was not significantly altered by trimethylation, distinct mechanisms involved in the respiration and pro-apoptotic activities of cytochrome *c* were postulated. How can Lys-72 trimethylation block pro-apoptotic activity? It is believed that the presence of three methyl groups might provide steric hindrances sufficient for interfering with the binding to Apaf-1. To prove this idea, the analysis of differences between yeast cytochrome *c,* which cannot bind Apaf-1, and horse cytochrome *c,* which is typical of mammals, was performed [48]. Experiments on recombinant cytochromes with interspecies swaps of amino acids 7, 25, 39, 62–65 and 72 (nomenclature corresponding to the mammalian cytochrome *c* amino acid sequence, see Fig. 1) of the yeast protein with the corresponding residues from horse cytochrome *c* demonstrated highly increased caspase-9 activation. Thus, these residues are considered to be responsible for the binding with Apaf-1. This is consistent with the observation that cytochrome *c* redox activity, essential for respiration, is not required for apoptosis [20, 22]. However, in yeast there is no known apoptotic pathway downstream of cytochrome *c* that can be inhibited by trimethylation. Thus, possible functions for cytochrome *c* methylation in yeast and plants remain unknown.

The most powerful tool for investigating protein functions in vivo is the modern technology of gene mutagenesis in mice. Based on previously described features of cytochrome *c*, mice with the mutated *CycS* gene resulting in K72A substitution were generated [67]. Unlike cytochrome *c*-deficient mice, some of these knock-in mice were viable, although with complex phenotypic features, that appeared variable and not easy to interpret. First, newborn mice homozygous for the mutation were significantly underrepresented in the offspring from heterozygous parents (12% vs. expected 25%). After birth, they demonstrated a high level of perinatal lethality associated with forehead protrusions and with abnormal clusters of proliferation observed in their brains. Additionally, the surviving homozygous juvenile mice suffered from cachexia, hydrocephalus, and lymphopenia. More than half of the mice demonstrated cachexia, and a fraction of them became moribund at the age of 3–4 weeks, characterized by rapid and severe body weight loss before death. Other mice developed hydrocephalus and displayed thymic atrophy and lymphopenia, linked with hydrocephalus. The total numbers of lymphocytes in the spleen, thymus, and lymph nodes of these mice were decreased by two orders of magnitude compared to the littermate controls. Thus, in vivo the K72A substitution in cytochrome *c* was implicated in normal brain development and in lymphocyte homeostasis in mice during ontogenesis. The possible mechanisms of cachexia and hydrocephalus phenotypes may be due to abnormalities in the survival of immune cells causing inflammatory cytokine overproduction and resulting in cachexia, similar to the cachexia of cancer patients. Alterations in cell death pathways may lead to atresia of the aqueduct between the third and fourth brain ventricles, which can disrupt the circulation of cerebrospinal fluid and cause hydrocephalus; otherwise, cerebrospinal fluid adsorption may be decreased due to an abnormality in glial cell development. The reason for this abnormality could be due to improper folding of the K72A cytochrome *c*, leading to its malfunction. The folding of small proteins generally requires a two-stage (unfolded–native) mechanism, whereas folding of larger proteins (i.e., those with >100 residues), such as cytochrome *c*, goes through pathways involving a number of intermediates that could be responsible for the onset of pathology, as has been previously demonstrated for various neurodegenerative processes [68]. The heterogeneity found in phenotypes between individual mice may reflect threshold signaling phenomena in development or it may simply be due to an incomplete genetic backcrossing in the original study,

implicating the involvement of modifier genes. Another explanation for this heterogeneity could be a variable compensatory expression of the testicular form of cytochrome *c* in individual mice. Yet another possibility would implicate a compensatory contribution of cytochrome-independent cell death pathways. All of these possibilities can be tested experimentally.

The K72A substitution was also studied at the cellular level, specifically in thymocyte cultures and in MEFs obtained from knock-in mice. A careful analysis of the respiratory activity of mitochondria from cytochrome *c* mutant mice revealed a slight suppression in succinate-driven complex II activity [67]. Interestingly, complex II was shown to have tumor suppressor activity by limiting the accumulation of succinate in the cells [69]. Therefore, a possible link between this form of suppression and lymphopenia is intriguing and remains to be investigated.

The effect of K72A substitution on apoptosis induction was also investigated and showed some interesting differences between the tissues. Fibroblasts from the KA/KA mice were resistant to apoptosis induced by diverse treatments; however, their thymocytes responded equally efficiently to a broad range of apoptotic stimuli as wild-type cells. Since both cytochrome *c* and Apaf-1 are essential for apoptosome activation, the role of genetic modification of both proteins in apoptosis activation was compared. Again, KA/KA MEFs were nearly as resistant to apoptotic inducers as Apaf-1$^{-/-}$ MEFs due to their impaired Apaf-1 oligomerization and caspase activation. Interestingly, although KA/KA thymocytes also failed to show Apaf-1 oligomerization in response to gamma-radiation, they were able to retain the ability to efficiently activate caspases. In fact, they were much more sensitive to death than Apaf-1$^{-/-}$ thymocytes. Thus, a pathway of apoptosome-independent caspase activation appears to exist in thymocytes, but it does not operate in the MEFs.

As mentioned above, the K72A mutation resulted in both intrinsic and extrinsic defects in lymphocyte homeostasis. This intrinsic defect was characterized by splenomegaly and lymphadenopathy. The delay in lymphocyte accumulation in Rag-1$^{-/-}$ KA/KA chimeric mice might be explained by the presence of targets other than cytochrome *c* upstream of mitochondria, or by the activation of a caspase-independent pathway in the absence of the wild-type cytochrome *c* [67]. The extrinsic defect in lymphocyte homeostasis was characterized by thymic atrophy and lymphopenia in KA/KA mice, as determined in Rag-1$^{-/-}$ KA/KA chimeric mice. As the authors suggested, this might be partly explained by the reduced production of growth hormone. However, since lymphopenia in almost half of the surviving KA/KA mice was not prevented by addition of growth hormone; it could be that additional factors involved in regulation of these processes may exist.

Several attempts were undertaken to compare the effects of various K72 substitutions on cytochrome *c* functions. A number of horse and murine cytochrome *c* single-point mutants with distinct substitutions at position 72 were quantitatively assayed in living cells after electroporation or in cellular extracts [70, 71]. In this system, K72W substitution demonstrated a higher drop in pro-apoptotic activity than that induced by K72A [72]. It should be noted that in these experimental settings, the cytochrome *c* mutants were expressed in a heterologous system and may have lacked some post-synthetic modifications. Based on these findings K72W knock-in mice and cells were generated and studied. MEFs with K72W mutation showed normal respiration but were partly resistant to staurosporine-induced apoptosis [73]. These mice also demonstrated complex and variable phenotypes, including hydrocephalus and cachexia (unpublished data). However, some of K72W mice survived to adulthood, thus offering the possibility of more extensive studies of cytochrome *c* functions in various compartments in vivo. Thus, in contrast to expectations, the K72W knock-in mice showed a phenotype that was comparable, although not identical, to K72A mice. The reasons for the phenotypic differences between K72A and K72W knock-in mice remain to be explained, especially, since MEFs from these two mouse models were found to be similar in several pro-apoptotic tests. Thus, the availability of several in vivo and cellular models with mutant cytochrome *c* offers the possibility of future in-depth investigations on the role of cytochrome *c* in different aspects of life and death. It would be particularly interesting to evaluate the effects of such mutations in distinct organs and histological compartments that may have different requirements for intrinsic or extrinsic apoptotic pathways or homeostatic programs.

Conclusions

Cytochrome *c* is a multifunctional protein with an important role in the electron transport chain, antioxidant defenses, and cell death. Inactivation of the somatic isoform of this protein, as expected, led to early embryonic lethality, confirming its vital role in maintaining mitochondrial respiration. Based on early observations, a key role of cytochrome *c* in intrinsic apoptosis was postulated. Interestingly, the intrinsic apoptotic pathway is functional during embryonic development. However, studies with knock-in mice and cells revealed that not all developmental programs involving apoptosis require either cytochrome *c* or another important player for apoptosome formation, Apaf-1. Moreover, apoptosis that is mediated by

cytochrome *c* has not been observed in any organisms except vertebrates. Targeted disruption of the cytochrome *c*-dependent apoptotic pathway also revealed the existence of apoptosome-independent caspase activation in thymocytes at least. The first mutation (G41S) identified in humans showed increased apoptotic activity of cytochrome *c*. In contrast, all engineered mutations of cytochrome *c* either did not affect apoptotic activity or even decreased it. The reason for this discrepancy requires clarification. Changes in the cytochrome *c* sequence that result in enhanced caspase activation should make the cell death process more efficient. In this case, it is unclear whether these changes should also cause developmental or physiological outcomes. The observations on premature platelet release in vivo and early platelet formation in vitro strongly argued for the role of a G41S mutation in the development of familiar thrombocytopenia [58]. However, members of the affected families who carried this mutation appeared to be healthy and had a normal life span, suggesting the absence of a direct link between apoptotic outcome in some cell types (i.e., megakaryocytes during platelet maturation) and most organs during ontogenesis and adult life. Again, the reason for these different responses should be further investigated. Another interesting future experiment would be a knock-in mouse with the testicular form of cytochrome *c* placed into the position of the *CysS* gene, which would allow the significance of sequence variations to be directly assessed. It is clear that although recent developments within this field have helped us to understand many of the Achilles' heels of cytochrome *c* during apoptosis in distinct cell types, the real role of functional modifications in some other cells, as well as in fundamental physiological processes, still remains to be addressed.

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