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HYPOTHESIS

Nuclear cytochrome *c* – a mitochondrial visitor regulating damaged chromatin dynamics

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Over the past decade, evidence has emerged suggesting a broader role for cytochrome *c* (Cyt *c*) in programmed cell death. Recently, we demonstrated the ability of Cyt *c* to inhibit the nucleosome assembly activity of histone chaperones SET/template-activating factor Iβ and NAP1-related protein during DNA damage in humans and plants respectively. Here, we hypothesise a dual concentration-dependent function for nuclear Cyt *c* in response to DNA damage. We propose that low levels of highly cytotoxic DNA lesions – such as double-strand breaks – induce nuclear translocation of Cyt *c*, leading to the attenuation of nucleosome assembly and, thereby, increasing the time available for DNA repair. If DNA damage persists or is exacerbated, the nuclear Cyt *c* concentration would exceed a given threshold, causing the haem protein to block DNA remodelling altogether.

Keywords: chromatin remodelling; cytochrome *c*; DNA damage response; histone chaperone; low-complexity acidic region

When excess DNA damage leads to programmed cell death

Cells are continuously exposed to endogenous (e.g. by-products of cellular metabolism) and exogenous agents (e.g. ionising and ultraviolet radiation) capable of inducing DNA damage [1]. In addition, some DNA lesions originate during DNA replication and by topoisomerase activity. To preserve the integrity of the genome, cells have evolved a set of defence mechanisms collectively known as the DNA damage response (DDR) [2]. The DDR encompasses the recognition of DNA lesions, the initiation of signalling cascades and the modulation of processes such as the cell cycle, transcription, chromatin remodelling, DNA repair and programmed cell death (PCD) [3]. These regulatory functions are performed by different classes of DDR factors acting in coordination to provide an adequate response according to the type of DNA damage [4]. To date, just three sensory proteins are known to detect DNA lesions, all belonging to the family of phosphatidylinositol 3-kinase-related kinases: ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase. Recognition of DNA damage is followed by a sequence of phosphorylation reactions that culminate in the activation of the DDR signalling cascade, which in turn regulates the activity of numerous proteins participating in cell-fate decision-making [5].

Abbreviations

ANP32B, acidic leucine-rich nuclear phosphoprotein 32 family member B; Apaf-1, apoptotic protease-activating factor-1; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; Ch, histone chaperone; Cyt *c*, cytochrome *c*; DDR, DNA damage response; DSB, double-strand break; LCAR, low-complexity acidic region; MDM2, murine double-minute 2; NLS, nuclear localisation signal; NRP1, NAP1-related protein; PCD, programmed cell death; PTM, post-translational modification; SET/TAF-Iβ, SET/template-activating factor Iβ.

During the cell cycle, many minor, relatively harmless DNA lesions occur, which do not require the complete activation of the DDR (Fig. 1A). Thus, the principal function of this protection system seems to be reserved for more perilous situations [4]. Among the various types of DNA damage, double-strand breaks (DSB) are one of the most serious, as inefficient repair can result in cell death or tumour development [1]. DDR factors localise at DSBs sites, forming structures termed DNA repair foci. These foci constitute unique nuclear regions with a specialised protein composition and structure that allows the accumulation of additional DDR factors. Moreover, the foci include large segments of chromatin surrounding DSBs that serve as platforms for the assembly of the repair machinery [4].

Chromatin remodelling plays an essential role in DNA repair by facilitating the entry of DDR factors onto damaged DNA. The dynamic nature of the chromatin is possible thanks to the concerted activity of histone-modifying enzymes, remodelling factors and histone chaperones (Ch). Among the various post-translational modifications (PTMs) to which histones are subjected, acetylation and ubiquitylation are related to DDR-induced chromatin remodelling. Such modifications increase the mobility of histones and facilitate their release from nucleosomes. PTMs can also destabilise chromatin structure, either directly or indirectly by recruiting ATP-dependent chromatin remodellers [6]. These enzymes enhance chromatin accessibility by disrupting contacts between DNA and histones, thus allowing for nucleosome sliding or eviction from damaged DNA, and facilitating the exchange of histone variants [1]. Finally, Chs, which assist the incorporation of histones onto chromatin, are considered essential factors for nucleosome assembly, restoration of chromatin integrity and reactivation of transcription upon DNA damage repair. There are also some Chs which participate in histone eviction from nucleosomes around DSBs. Furthermore, certain Chs may also be involved in both the assembly and disassembly of nucleosomes [6].

Following remodelling of damaged chromatin, DSBs are mostly repaired by either homologous recombination or nonhomologous end joining, depending on the phase of the cell cycle during which DNA lesion occurs [4]. However, if the damage exceeds the DNA repair capacity of the cell or the repair process fails, remaining DSBs can block both replication and transcription. Consequently, chronic DDR signalling triggers cell death by PCD or cellular senescence [2,5].

Programmed cell death – the orchestrated removal of unwanted cells – is an essential mechanism for life, responsible for mediating vital processes such as embryonic development, homeostasis and immune defence [7]. PCD is a highly conserved event, utilised by evolutionarily distant organisms, from plants to humans [8]. In mammals, apoptosis is by far the most common and best-understood form of PCD, and can be initiated via two different pathways: the intrinsic and the extrinsic pathway, both of which converge on the activation of caspases [9]. Cytochrome *c* (Cyt *c*) has long been implicated in the intrinsic pathway of apoptosis, where its association with apoptotic protease-activating factor-1 (Apaf-1) results in the formation of the apoptosome, leading to the activation of caspases-3 and -7, and ultimately cell death. In addition to this well-characterised role of Cyt *c* in apoptosis, there is a growing body of evidence suggesting that the haem protein may regulate novel pathways involved in PCD [10–14]. In plant PCD, the role of Cyt *c* remains largely unclear, although its release from mitochondria is a conserved event [8]. However, the lack of an Apaf-1 plant homologue raises questions as to the function of extra-mitochondrial Cyt *c*.

Cytochrome *c* inhibits the nucleosome assembly activity of low-complexity acidic region-containing histone chaperones

To better understand the role of Cyt *c* in PCD, our group performed two independent proteomic studies – in human and plant cells – aimed at identifying new cytosolic and nuclear protein targets for Cyt *c* [10,11]. A total of 19 novel partners (10 in humans and nine in plants) were found to interact with Cyt *c* both *in vitro* and *in vivo*, all of which were implicated in biochemical pathways directly or indirectly linked to PCD regulation. These findings suggested a wider role for Cyt *c* in regulating cell death by still unknown molecular mechanisms.

Nur-E-Kamal *et al.* [15] were the first to report that Cyt *c* migrates into the cell nucleus upon DNA damage in the absence of caspase activation. They suggested a role for Cyt *c* in chromatin remodelling, but its nuclear function has remained unelucidated. Recently, we confirmed that in response to DSBs Cyt *c* can translocate into the nucleus prior to caspase cascade activation [13,14]. More importantly, we discovered that nuclear Cyt *c* hinders the binding of several Chs – namely, SET/template-activating factor Iβ (SET/TAF-Iβ), acidic leucine-rich nuclear phosphoprotein 32 family member B (ANP32B), nucleolin and NAP1-related protein (NRP1) – to core histones [10–12], thereby inhibiting their nucleosome assembly

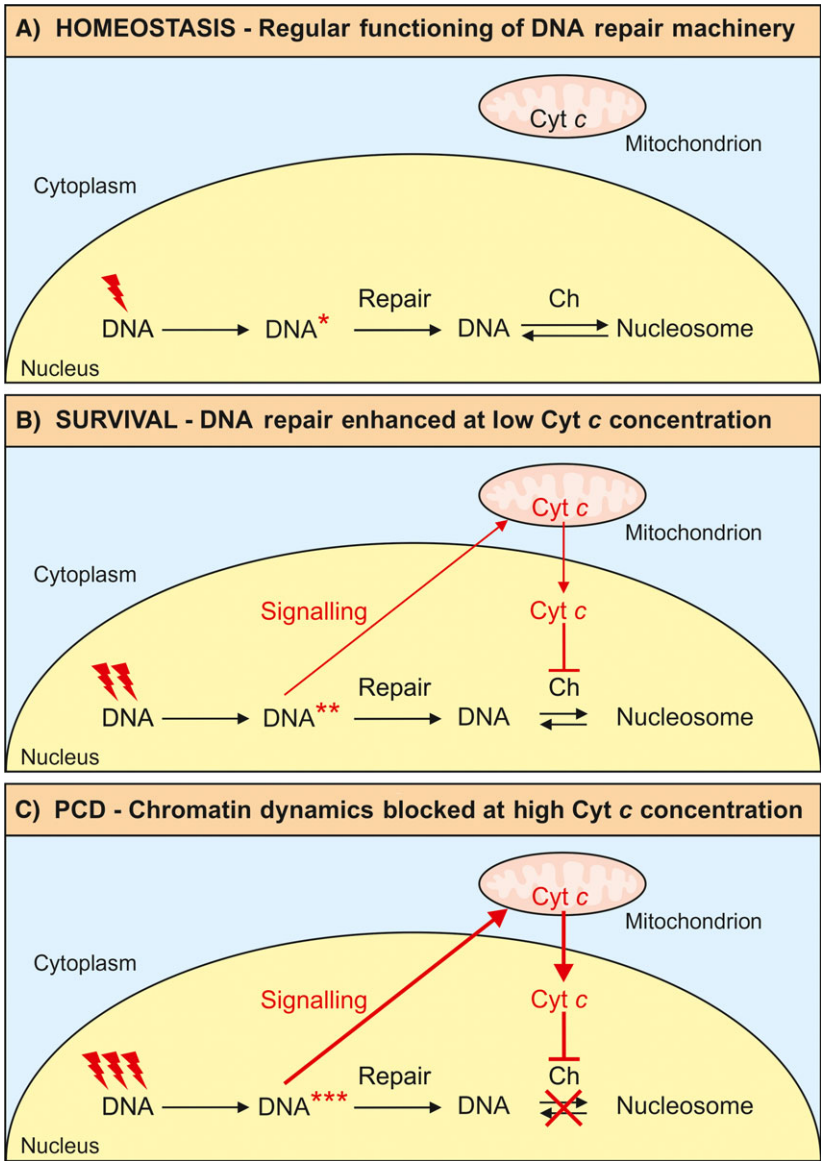

Fig. 1. Nuclear Cyt *c* concentration-dependent effect during DNA damage. (A) DNA suffers minor lesions during the cell cycle that are swiftly repaired without complete activation of the DDR. Under such circumstances, Cyt *c* would remain confined to the mitochondrial intermembrane space. (B) Highly cytotoxic DNA lesions – such as DSBs – trigger Cyt *c* migration into the nucleus. Initially, Cyt *c* translocates at low concentrations, playing a prosurvival role. Cyt *c* binding with histone chaperones (Ch) diminishes their nucleosome assembly activity and could thus prolong the time naked damaged DNA is accessible to repair machinery. (C) If DNA damage persists or is exacerbated, Cyt *c* accumulates in the nucleus surpassing a determined threshold, upon which it can sequester the vast majority of histone chaperones. Consequently, both nucleosome assembly and disassembly are blocked. In this way, Cyt *c* is transformed into a prodeath factor as its nuclear concentration increases. Red thunderbolts represent any endogenous or exogenous agent that causes DNA damage: one for very low doses which does not trigger Cyt *c* release from mitochondria, two for low doses which produce a small liberation of Cyt *c* and three for high doses eliciting a massive release of Cyt *c*. Red asterisks indicate damaged DNA: one for minor lesions, two for low accumulation of serious lesions and three for high accumulation of severe lesions.

activity. Indeed, both Cyt *c* and histones share highly positive isoelectric points, and consequently the ability to interact with the low-complexity acidic regions (LCARs) of the above-mentioned chaperones.

Low-complexity regions in proteins are motifs with little sequence diversity, containing few different or repeating amino acids, which can be either irregularly or periodically positioned [16]. The term LCAR,

normally used to designate a specific domain of ANP32B [17], refers to low-complexity domains mainly composed of acidic residues. Interestingly, Wu *et al.* [18] defined in a recent paper the concept of 'acidic domain' – within which LCARs can be included – as 'a sequence stretch that contains at least 20 acidic residues in 50 consecutive amino acids'. They proposed that acidic domains are the most abundant type of histone-binding motifs because of their presence in numerous chromatin-associated proteins. The ability of these motifs to bind their targets is sequence-independent but directly proportional to length, suggesting flexible or minimal structural requirements for binding [18].

Interactions between Cyt *c* and LCAR-containing Chs are transient and electrostatic in nature. Specifically, the same surface patch of Cyt *c* seems to mediate interactions with both human SET/TAF-Iβ [13] and plant NRP1 [14]. Furthermore, this area, which surrounds the haem cleft, also mediates interactions between Cyt *c* and its partners both within the electron transport chain (Cyt *c* oxidase [19] and Cyt c_1 [20]) and in apoptosis (Apaf-1) [21]. This suggests a conserved molecular recognition mechanism for Cyt *c* binding, which utilises the surface surrounding the haem cleft to interact with many cellular targets.

Our latest papers reveal that the regulation of Chs-mediated chromatin remodelling by Cyt *c* during DDR is well-conserved throughout evolution [13,14]. Human SET/TAF-Iβ and plant NRP1 are highly related proteins from both a functional and structural point of view, sharing a sequence identity of 45%, and almost identical folding [14]. Structurally, the two histone-binding macromolecules form headphone-shaped homodimers with each monomer containing an 'earmuff' domain and a LCAR. From a functional point of view, both SET/TAF-Iβ and NRP1 possess histone-binding and nucleosome assembly activities, and can therefore participate in the restoration of chromatin configuration following DNA repair [13,14]. However, nuclear Cyt *c* can sequester SET/TAF-Iβ and NRP1 (in humans and plants respectively), preventing their association with core histones and possibly modulating DNA repair [13,14]. This regulatory mechanism appears to be widespread, as other LCAR-containing Chs are also targeted by nuclear Cyt *c* [10,11]. Moreover, the LCAR-mediated binding mode is similar to that described in a recent report by other authors on interactions between p53 and LCAR-containing proteins [22].

In addition to the above-mentioned capabilities, SET/TAF-Iβ has also been found at repair foci, where it regulates DNA repair [23]. DNA lesions result in transient and specific transcriptional inhibition of the affected chromatin regions, an event essential in avoiding interference between transcription and DNA repair [24]. It has also been reported that SET/TAF-Iβ promotes transcription by dislodging chromatin-binding proteins that impede the access of RNA polymerase II onto DNA templates [25]. Taken together, the inhibition of SET/TAF-Iβ by Cyt *c* upon DNA damage may help to separate the processes of transcription and DNA repair in a spatio-temporal manner.

Cytochrome *c* may play a dual, concentration-dependent role in damaged chromatin remodelling

Here, we propose that the DSB-dependent release of Cyt *c* and its subsequent role in DNA repair (targeting LCAR modules) is not an all-or-nothing process. In fact, mitochondria-to-nucleus Cyt *c* translocation can occur at low concentrations in the absence of mitochondrial permeability transition pore opening and/or caspase cascade activation [26]. The interaction between nuclear Cyt *c* at low concentration and LCAR-containing Chs could keep naked DNA accessible longer for repair machinery, by slowing down the nucleosome assembly process. Thus, a small and controlled release of Cyt c may benefit DNA repair, avoiding genome instability and cell death (Fig. 1B). On the contrary, if the concentration of liberated Cyt *c* reaches a certain threshold, Cyt *c*-dependent blocking of Chs could disrupt chromatin remodelling and, consequently, gene expression, causing the cell to irretrievably execute DNA damage-induced PCD (Fig. 1C). According to our former proposal [12], the role of extra-mitochondrial Cyt *c* in PCD goes beyond caspase cascade activation; we hypothesised that Cyt *c* could inhibit key cellular processes involved in cell-fate decision-making, both in humans and plants. Accordingly, Cyt *c* binding with human SET/TAF-Iβ and plant NRP1 could be part of an extensive and branched feedback network – probably involving other LCAR-containing targets – whose purpose would be to overcome the Cyt *c* threshold required to activate PCD.

Our hypothesis assumes that highly regulated mechanisms can control both the exit of Cyt *c* from the mitochondria into the cytoplasm and its entrance into the nucleus with no matrix swelling nor outer mitochondrial membrane dismantling. Either of which could compromise cell viability. Specific channel-mediated mechanisms should thus be involved in the selective release of Cyt *c* in response to the level of DNA damage. The proapoptotic proteins Bax and Bak, in particular, could facilitate mitochondrial

release of low levels of Cyt *c* without irreversibly triggering cell death [27,28].

Molecular details on the transport of Cyt *c* through the nuclear membrane are still unknown. Recently, it has been demonstrated that nuclear translocation of Apaf-1 is mediated through its association with the nucleoporin Nup107 [29]. The existence of an analogous nuclear import mechanism for Cyt *c* is therefore conceivable. Intriguingly, nuclear translocation of Apaf-1 occurs in response to DNA damage and, despite being a proapoptotic factor, it exerts a caspase-independent function in the nucleus in a similar manner as we propose for Cyt *c* [30].

The central idea of our hypothesis is that Cyt *c* plays a dual antagonistic role in controlling DDR. It is noteworthy that the tumour suppressor p53 similarly performs opposing tasks during the same process. p53 plays a decisive role in cell-fate choice under DNA damage conditions. During homeostasis, however, p53 is maintained at low levels by its inhibitory partner murine double-minute 2 (MDM2). Upon DNA DSBs, the p53–MDM2 complex is disrupted by the DDR-induced kinases ATM and ATR. These DDR factors contribute to the activation of promoter-specific transcriptional activity of p53 on target genes associated with cell cycle arrest and apoptosis [31]. p53 exhibits greater affinity for binding sites in the promoters of cell cycle arrest genes and so its prosurvival function predominates over its proapoptotic one, as cell cycle arrest favours DNA repair. In contrast, high levels of DNA damage are thought to reverse promoter selection by p53, thereby increasing the expression of apoptotic genes [5]. Interestingly, the transcriptional activity of p53 is also modulated through its interaction with SET/TAF-Iβ. Specifically, SET/TAF-Iβ represses p53 transcriptional activity in unstressed cells. However, acetylation of the C-terminal domain of p53 – an event linked to DNA damage – hinders its association with SET/TAF-Iβ and thus prevents p53 inhibition [22].

To prove our hypothesis, experiments will be necessary to demonstrate the positive effect of Cyt *c* on DNA repair effectiveness, as well as to determine the threshold concentration required to initiate PCD. Supercoiling assays have already been performed, showing that Cyt *c* can inhibit the nucleosome assembly activity of human SET/TAF-Iβ and plant NRP1 [13,14]. How these proteins modulate the evolution of DNA damage over time could thus be followed by monitoring the DSB biomarker γ-H2AX [32]. To this end, Cyt *c* knockout mammalian or plant cells will be transfected with plasmids encoding SET/TAF-Iβ or NRP1 respectively. After induction of DSBs, evolution of DNA repair will be followed by tracking γ-H2AX levels using flow cytometry. Inefficient DNA repair would be expected for Cyt *c* knockout cells, especially at the early DDR stages.

The Cyt *c* threshold concentration can be determined by experiments correlating nuclear Cyt *c* concentrations with cell death. A plasmid containing Cyt *c* fused to the GFP has already been designed to transfect the Cyt *c* knockout cells. A nuclear localisation signal (NLS) could eventually be added at the C-end of the chimera to facilitate its transfer to the nucleus upon proper folding of Cyt *c* in the mitochondria. After treatment with a DSB-inducing agent, nuclear GFP fluorescence will be monitored by time course confocal microscopy. The fluorescence level should be proportional to the amount of Cyt *c* that has been translocated into the nucleus upon DNA damage. On the other hand, cell death levels can be followed by using fluorescent annexin V conjugates, which bind to the early apoptosis marker phosphatidylserine [33]. Additionally, site-directed Cyt c mutants at the interaction area with Chs could be assayed.

In summary, we present here a model for a new role of Cyt *c* in the context of DNA damage. Based on our recent findings, we propose that Cyt *c* exerts an anti-apoptotic effect in the cell nucleus at the onset of the DDR. Such activity would be directly related with its ability to interact with LCAR-containing Chs, resulting in the attenuation of nucleosome assembly and thus providing a greater time frame for repair machinery at damaged DNA sites. We argue that this new function of Cyt *c* would depend on the levels of DNA damage, which would be reflected proportionally in the nuclear concentration of Cyt *c*. If the haem protein exceeded a certain threshold concentration, most of the LCAR-containing Chs would be inhibited. This situation would bring about a strong repression of chromatin remodelling and, therefore, all cellular processes dependent on it (such as DNA replication and transcription), condemning the cell to death.

The enormous and overwhelming complexity of cellular processes regulating cell-fate choice under DNA damage has hampered the establishment of an integrative and exhaustive model defining the spatio-temporal sequence of molecular events. Little by little, new details that advance our knowledge of the intricate mechanisms controlling the delicate balance between cell survival and death are being discovered. With our hypothesis detailing the dual role of Cyt *c* under DNA damage, we intend to contribute to a better understanding of the networks governing cell-fate decisions as well as suggesting new lines of research focused on the adaptive function of Cyt *c* in the cell nucleus.

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Author contributions

ID-M, AD-Q and MAdlR contributed to the original concept of the hypothesis. All the authors contributed equally to further discussion and writing.

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