The role of mitochondria in yeast programmed cell death

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The unicellular yeast Saccharomyces cerevisiae has been established as a good model to elucidate molecular mechanisms underlying programmed cell death (PCD) pathways. S. cerevisiae PCD shares many morphological and biochemical features with apoptosis, the major form of mammalian PCD, although there are some peculiar differences. PCD have been described to occur in yeast in different physiological scenarios (Carmona-Gutierrez et al., 2010). Indeed, chromatin condensation, nuclear DNA fragmentation and phosphorylation of externalization onto the cell surface are general markers of both mammalian and yeast PCD cells. A characteristic feature of mammalian apoptosis is the activation of caspases, proteases that initiate and execute cell death through degradation of cell components. Yeast contains only one gene homolog of caspases, named YCA1, encoding for yeast metacaspase (Madeo et al., 2002) which has substrate specificity different from caspases (Wilkinson and Ramsdale, 2011). Glyceraldehyde-3-phosphate dehydrogenase has been identified as the first YCA1-dependent yeast PCD pathway in yeast (Silva et al., 2011), but yeast PCD mechanisms occurring both in YCA1-dependent and independent manner as well as the role of other proteases in yeast PCD remain to be established (Madeo et al., 2009; Wilkinson and Ramsdale, 2011).

Both in yeast and in mammalian PCD mitochondria play a major role in final pro-survival or pro-death decision. Accordingly, the mitochondria-mediated PCD pathway in yeast resembles the mammalian intrinsic pathway, and shows remarkable complexity with respect to different proteins and pathways involved (Eisenberg et al., 2007; Pereira et al., 2008). Alterations in mitochondrial structure and function during PCD depend on a variety of specific triggers, respiratory or fermentative growth conditions, and on overall cell metabolism. First evidence for a mitochondria-dependent yeast PCD pathway was obtained in acetic acid-induced PCD (AA-PCD), with cells showing cytochrome c (cyt c) release into the cytosol and production of mitochondrial reactive oxygen species (ROS). Mitochondrial dysfunction occurs as shown by mitochondrial depolarization, and a large decrease in cyt c oxidase (COX) activity together with higher resistance to AA-PCD of respiratory-deficient cells, lacking either mtDNA or unable to form active cyt c or ATP synthase (Ludovico et al., 2002). Key regulators of mitochondrial metazoan apoptosis are the Bcl-2 family proteins which include both pro-apoptotic and anti-apoptotic members harboring multiple or single Bcl-2 homology (BH) domains (BH1-4). These proteins regulate mitochondrial outer membrane permeabilization (MOMP) followed by the release of pro-apoptotic factors including cyt c (Wang and Youle, 2009; Wasiukiewski and Scorrano, 2009). Recent discovery of a yeast BH3-only protein (Ybhlp) mediating both AA- and H2O2-induced PCD (Buttner et al., 2011) supports the hypothesis of the origin of the eukaryotic PCD systems through acquisition of several PCD effectors as a consequence of mitochondrial endosymbiosis (Koonin and Aravind, 2002). Indeed, yeast Ybhlp translocates to mitochondria inducing PCD and mitochondrial membrane depolarization through interaction with the mitochondrial phosphate carrier (Mitp) and a core subunit of the respiratory complex III (Cor1p; Buttner et al., 2011). Thus, Ybhlp resembles mammalian Bax that can permeabilize mitochondria, whereas mammalian BH3-only proteins require Bak and Bak to release cyt c, suggesting that the most ancestral function of the BH1-like proteins may be to trigger changes in the IMM (Oettinghaus et al., 2011).

Whether yeast PCD does resemble and/or predate apoptotic death in multicellular organisms or is a distinct form of PCD in itself is still a matter of investigation. Indeed, it remains controversial as to whether metacaspases are distant relatives of caspases.

Mammalian apoptosis and yeast programmed cell death (PCD) share a variety of features including reactive oxygen species production, protease activity and a major role played by mitochondria. In view of this, and of the distinctive characteristics differentiating yeast and multicellular organism PCD, the mitochondrial contribution to cell death in the genetically tractable yeast Saccharomyces cerevisiae has been intensively investigated. In this mini-review we report whether and how yeast mitochondrial function and proteins belonging to oxidative phosphorylation, protein trafficking into and out of mitochondria, and mitochondrial dynamics, play a role in PCD. Since in PCD many processes take place over time, emphasis will be placed on an experimental model based on acetic acid-induced PCD (AA-PCD) which has the unique feature of having been investigated as a function of time. As will be described there are at least two AA-PCD pathways each with a multifaceted role played by mitochondrial components, in particular by cytochrome c.

Keywords: yeast, programmed cell death, mitochondria, acetic acid, cytochrome c, protein trafficking, intracellular signaling
or are more closely related to other classes of proteases. Moreover even if yeast encodes a BH3-only protein as recent studies suggest, yeast homologs of Bcl-2 proteins on which BH3-only proteins act are still unknown. Notwithstanding this, the central role of mitochondria in yeast PCD underlines the importance of dissecting the PCD process in this unicellular organism.

In this review we consider the mitochondrial proteins involved in yeast PCD execution and regulation (see Table 1). Most of them are involved in either electron transfer along the respiratory chain and oxidative phosphorylation, or mitochondrial dynamics, or mitochondrial permeabilization and protein trafficking from mitochondria to cytosol and vice versa. These points will be dealt with separately.

**ELECTRON TRANSFER ALONG THE RESPIRATORY CHAIN AND OXIDATIVE PHOSPHORYLATION**

Yeast internal NADH dehydrogenase (NDI1) is the homolog of metazoan AMID, the apoptosis-inducing factor (AIF)-homologous mitochondrion-associated inducer of death. Ndi1p overexpression can cause PCD, probably due to ROS production in mitochondria, only when cells are grown in glucose-rich media. However this occurs in yeast cells lacking mitochondrial superoxide dismutase both during fermentative and respiring growth (Li et al., 2006). Yme1p is a mitochondrial AAA-type protease involved in the coordinated assembly of COX. Yme1p activation results in a decrease of COX level and route to Bax-induced cell death, however since under fermentative conditions, when COX activity is strongly repressed, Yme1p deletion slightly delays Bax-induced cell death, some other unidentified Yme1p substrate could also play a role in this process (Manon et al., 2001). Analysis of the effect of oxidative phosphorylation inhibitors on yeast PCD has shown conflicting results depending on the PCD trigger. Although AA-PCD is insensitive to antimonycin or oligomycin, mycothiol and cyanide prevented amidorodin-a-factor-induced PCD (Ludovico et al., 2002; Pozniakovsky et al., 2005; Guaragnella et al., 2011b). Yeast cells grown in the presence of both antimonycin and oligomycin and subsequently treated with acetic acid in the mitochondrial intermembrane space whose specific enzymatic activity remains unknown (Sevrioukova, 2011). AIF is a FAD-containing oxidoreductase localized in the mitochondrial intermembrane space whose specific enzymatic activity remains unknown (Sevrioukova, 2011). AIF is a functional homolog of anti-apoptotic Bcl-2 family proteins (Cheng et al., 2008a) and, together with Ybk1p (Büttner et al., 2011), is a component of an ancestral mitochondrial PCD pathway. The pro-survival role of FIS1 was confirmed in studies using different apoptotic triggers, such as virus-encoded toxin, ethanol, and fungicidal derivative BAR0329 (Ivanovskaya and Hardwick, 2005; Kitagaki et al., 2007; Bink et al., 2010). However, FIS1 may have an additional long-term survival function which appears to be independent of DNMI and MIV2. Indeed, FIS1 deletion results in acquisition of a secondary mutation in the stress-response gene WHI2 that confers sensitivity to cell death (Cheng et al., 2009b).

Genetic screens have revealed the existence of two novel genes, named yeast suicide protein 1 (YSP1) and yeast suicide protein 2 (YSP2), required for mitochondrial fragmentation en route to amidorodin-induced PCD (Pozniakovsky et al., 2005; Sokolov et al., 2006). It has been proposed that Ysp2p acts downstream of ROS production due to intracellular acidification, following AA-PCD induction (Sokolov et al., 2006). No homologous genes have been found in higher organisms.

**MITOCHONDRIAL DYNAMICS**

MTOR and OXIDATIVE PHOSPHORYLATION

In yeast, the mitochondrial role in healthy cells (Hangen et al., 2010). Similarly to AIF, the yeast homolog Aif1p translocates to the nucleus in

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Frontiers in Oncology  Molecular and Cellular Oncology  July 2012  Volume 2  Article 70  2

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| Gene (protein) | Mammalian homolog | PCD trigger | Role in PCD | Reference |
|---------------|-------------------|-------------|-------------|-----------|
| AAC1/AAC2/AAC3 (ADP/ATP carrier isoforms) | ANT | Acetic acid, diamide, H2O2 | MOMP | Pereira et al. (2007) |
| AIF (apoptosis-inducing factor) | AIF | Acetic acid, bostrycin, H2O2 | Pro-apoptotic released factor translocating to the nucleus | Wissing et al. (2004), Xu et al. (2010) |
| ATP30 (ATP synthase assembly factor) | ATP synthase | Acetic acid | Pro-apoptotic factor | Ludovico et al. (2002) |
| CFI1 (citrate synthase) | CS | Aging, heat | GSH biosynthesis, antioxidant activity | Lee et al. (2007) |
| COR1 (complex III core subunit III) | OCR1 | Acetic acid + Ybh3 overexpression, Cytochrome c, amiodarone, ethanol, heat shock, H2O2 | ETC, YBH3 interaction | Büttner et al. (2011) |
| CYC1/CYC7 (cytochrome c isoforms 1, 2) | Cyt c | Acetic acid, amiodarone, ethanol, heat shock, H2O2 | Pro-apoptotic released factor, ETC electron donor, ROS scavenger | Fannjiang et al. (2004), Kitagaki et al. (2007), Bink et al. (2010) |
| CYC2 (cytochrome c heme lyase) | CCHL | Acetic acid, amidoarone, heat shock | Cyt c holoenzyme formation | Ludovico et al. (2002), Silva et al. (2005) |
| FIS1 (mitochondrial fission protein) | NFIS | Acetic acid, BAR0329, ethanol, heat shock, H2O2 | Mitochondrial dynamics | Fannjiang et al. (2004), Kitagaki et al. (2007), Bink et al. (2010) |
| L44-A (mitochondrial 60S ribosomal protein) | – | Grapefruit seed extract | Unknown | Cao et al. (2012) |
| MIF1 (mitochondrial phosphate carrier) | PHC | Acetic acid + Ybh3 overexpression | Energetic metabolism, YBH3 interaction | Büttner et al. (2011) |
| ND1 (internal NADH dehydrogenase) | AMID | ND1 overexpression | ROS production | Li et al. (2006) |
| NUC1 (mitochondrial nuclease) | Endo G | Acetic acid, amidoarone, ethanol, H2O2 | Pro-apoptotic released factor translocating to the nucleus | Büttner et al. (2007), Kitagaki et al. (2007) |
| POR1 (porin) | VDAC | Acetic acid, H2O2, diamide | Anti-apoptotic factor | Pereira et al. (2007) |
| RSM23 (mitochondrial 40S ribosomal protein) | hDAP-3 | YCA1 overexpression | Pro-apoptotic factor | Madeo et al. (2002) |
| TIM18 (translocase of the inner mitochondrial membrane) | – | Arsenite | MOMP | Du et al. (2007) |
| YME1 (catalytic subunit of i-AAA protease complex) | – | Heterologous expression of Bac | Complex IV degradation | Manon et al. (2001) |
| YSP1 (yeast suicide protein 1) | – | α-Factor, amidoarone | Mitochondrial dynamics | Pozniakovsky et al. (2005) |
| YSP2 (yeast suicide protein 2) | – | Acetic acid, amidoarone | Mitochondrial dynamics | Sokolov et al. (2006) |

The S. cerevisiae mitochondrial proteins reported in this table have been implicated in PCD induced by different triggers through biochemical and/or genetic studies. ANT, adenine nucleotide translocator; MOMP, mitochondrial outer membrane permeabilization; CS, citrate synthase; GSH, glutathione; OCR1, ubiquinol-cytochrome c reductase core protein; YBH3, yeast BH3-only; ETC, electron transport chain; Cyt c, cytochrome c; NFIS, human homolog of FixLp; BAR0329, 4-(3-(4-chlorobenzyl)-2-methylpyrrol-1-yl)methylpiperazine-1-carboxamide; PHC, phosphate carrier; AMID, apoptosis-inducing factor homologous mitochondrion-associated inducer of death; Endo G, endonuclease G; VDAC, voltage-dependent anion channel; NOA3, human death associated protein.
response to apoptotic stimuli (Wissing et al., 2004). AIF1 disruption rescues yeast cells from oxygen stress and delays age-induced PCD. Conversely, overexpression of AIF1 strongly stimulates H2O2-induced PCD; this effect is attenuated by disruption of YCA1. Contrarily, AIF1-dependent bortezomib-induced cell death was shown to be independent of YCA1 (Xu et al., 2010).

Nucp is the yeast homolog of metazoan endonuclease G (EndoG), a mitochondrial protein with DNase/RNase activity involved in apoptotic DNA degradation (Li et al., 2001). Over-expression of Nucp promotes yeast PCD. Nucp-mediated PCD is shown to be AIF1- and YCA1-independent, which favors the existence of multiple, redundant pathways regulating cell death. Nucp is unable to activate caspasas in cytosolic extracts from metazoan cells (Kluck et al., 2000; Bender et al., 2012). Thus, some questions need to be answered: which events triggers cyt c release? Is cyt c released from damaged mitochondria? What is the role of the released cyt c en route to PCD, and is it strictly required for PCD to occur? In this regard, the definition of the sequence of events leading to the death cascade turns out to be useful.

After the discovery of the occurrence of AA-PCD in yeast (Ludovico et al., 2001, 2002), in a series of papers a detailed time course of certain events was investigated (Giannattasio et al., 2005, 2008; Guaragnella et al., 2006, 2007, 2008, 2010a, Ribeiro et al., 2006; Pereira et al., 2007). These events can be classified as pre- and post-cyt c release (Figure 1). Loss of cell viability is complete after 200 min of acetic acid treatment with accumulation of cells with fragmented nuclear DNA. The earliest event (15 min) following acetic acid challenge is ROS production, with a different role for H2O2 and superoxide anion, whose levels are modulated by catalase and superoxide dismutase. En route to death cyt c starts to be released at 60 min from coupled and intact mitochondria; maximum release is reached at 150 min. Later on cyt c is degraded, possibly by yet unidentified proteasas. The latest event of AA-PCD is caspase-like activation occurring at 200 min from death induction. Mitochondria are functionally implicated in this death scenario. In fact, up to 150 min released cyt c can act both as an electron donor as well as a ROS scavenger. However, en route to death a progressive impairment of mitochondrial functions, evidenced by a decrease of the respiratory control index, a collapse of the mitochondrial membrane potential, a decrease in COX activity and in cytochromes a+a3 levels, have been observed.

The AA-PCD time course clearly shows that ROS accumulation and caspase-like activation occur upstream and downstream of cyt c release, respectively. Functional genomics and biochemical studies on knock-out cells lacking YCA1 and/or the genes encoding the two yeast cyt c isoforms allowed the elucidation of casual relationships among ROS levels, cyt c release and caspase-like activation and two separate pathways activated by acetic acid have been identified. Particularly, it has been found that ROS and YCA1 are required for cyt c release, since both prevention of ROS production by the antioxidant N-acetyl cysteine (NAC) and YCA1 disruption result in the inhibition of cyt c release (Guaragnella et al., 2010a,b). How YCA1 is related to cyt c release remains to be elucidated. Nevertheless, a recent report suggests that YCA1 has a role in mitochondrial respiratory functions (Lefevre et al., 2012). Interestingly, AA-PCD still occurs, although with a lower death rate compared to wild type cells, without cyt c release in ADP/ATP carrier as well as YCA1 and/or cyt c knock-out cells (Pereira et al., 2007; Guaragnella et al., 2010b). This confirms on one hand that

**A CASE STUDY: THE ROLE OF CYTOCHROME c IN YEAST PCD**

Although cyt c release occurs en route to yeast PCD, so far in *S. cerevisiae* there is no evidence of the existence of a functional homolog of the apoptosome (Huttemann et al., 2011). Accordingly, yeast cyt c is unable to activate caspasas in cytosolic extracts from metazoan cells (Kluck et al., 2000; Bender et al., 2012). Thus, some questions need to be answered: which events triggers cyt c release? Is cyt c released from damaged mitochondria? What is the role of the released cyt c en route to PCD, and is it strictly required for PCD to occur? In this regard, the definition of the sequence of events leading to the death cascade turns out to be useful.

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FIGURE 1 | Cytochrome c and mitochondrial dysfunction in AA-PCD pathways. An extracellular acidic pH value exerts acid and enters yeast cells and dissociates into acetic and proteins causing intracellular acidification. In NAC-sensitive AA-PCD (red dashed arrows) hydrogen peroxide \(\text{H}_2\text{O}_2\) accumulates early; superoxide dismutase (SOD) activity increases, while catalase activity is undetectable; cytochrome c \(c\) is released to the cytosol in a YCA1-dependent manner as a functional protein, acting as an electron donor \(c\rightarrow c_2\) to the electron transport chain and as a superoxide anion \(O_2^{-}\) scavenger; in a late phase, mitochondrial functions progressively decline, as revealed by a decrease in mitochondrial membrane potential (ΔΨ opened in cytochrome oxidase activity). Caspase-like activity increases and DNA fragmentation occurs. The NAC-insensitive (blue dashed arrows) AA-PCD takes place in a YCA1-independent manner without cytochrome c release, yet caspase-like activation and DNA fragmentation occur in a late phase. In cells expressing a catalytically inactive form of iso-1-cyt c \(\text{W65Scyc1}\), no release of mutant \(c\) occurs with inhibition of AA-PCD, and there is a decrease in \(\text{H}_2\text{O}_2\) production. Possible involvement of certain signaling pathways in the interplay between PCD and cell adaptation is also shown: intracellular acidification caused by AA-PCD induction may stimulate RAS–cAMP–PKA signaling pathway, causing mitochondrial dysfunction, which can activate retrograde (RTG) pathway. The RTG pathway is positively and negatively regulated by Ras and TOR pathways, respectively. The TOR pathway is found at the crossroad of AA-PCD and RTG signaling, which may play a role in AA-PCD resistance.

YCA1 and cyt c act as pro-apoptotic proteins in yeast AA-PCD, but on the other hand that they are dispensable for PCD occurrence, showing the existence of YCA1/cyt c-independent AA-PCD pathway (Figure 1). In this pathway ROS accumulate early, caspase-like activity increase, and DNA fragmentation occurs. Importantly, YCA1/cyt c-independent AA-PCD is insensitive to NAC. This evidence suggests that cyt c still present in mitochondria might play a role in AA-PCD. Recent studies performed on yeast cells expressing a stable but catalytically inactive iso-1-cyt c \(\text{W65Scyc1}\) unable to reduce COX have shown inhibition of AA-PCD, with a decrease of ROS production, no cyt c release, this being independent of electron flow impairment, and an increase in caspase-like activation (Figure 1). Thus, cyt c release does not depend on cyt c function as an electron carrier and when still associated to the mitochondrial membrane, cyt c in its reduced form has a role in AA-PCD by regulating ROS production and caspase-like activity (Guaragnella et al., 2010a,b, 2011b). Regulation of ROS production by mitochondrial cyt c during AA-PCD may be exerted either directly by the cyt c peroxidase system able to scavenge both superoxide anion and \(\text{H}_2\text{O}_2\) (Korshunov et al., 1999) or by a change in cyt c-cardiolipin interaction or inefficient cardiolipin peroxidation by ROS (Kagan et al., 2005; Bayre et al., 2006; Sinibaldi et al., 2010; Huttemann et al., 2011). These issues require further investigations.

CONCLUSIONS AND PERSPECTIVES

In the light of results emerging from research into yeast PCD we feel that there is consensus that the response to any stimulus...
Mitochondrial dysfunction and the mode of cell response to it underlie different pathological conditions such as neurodegeneration. Alterations in mitochondrial functions have long been observed also in cancer cells and targeting mitochondria as an anti-cancer therapeutic strategy has gained momentum recently (Godavade et al., 2019). Since yeast shares with cancer cells the metabolic features identified as the underlying causes of the Warburg effect (Ruckenstuhl et al., 2009; Diaz-Ruiz et al., 2010), it is a suitable model organism to identify cell compartments responsible for tumorigenesis for development of targeted cancer drugs.

ACKNOWLEDGMENTS

This work was financed by Fondazione Cassa di Risparmio di Puglia to Nicoletta Guaragnella, CNR project MERIT to Ervilia Marra, and a grant from the Italian Ministry of Economy and Finance to the CNR for the Project FaReBio di Qualità to Sergio Giannattasio. Maìa Ždráleková is a recipient of a CNR FISL Fellowship in “Biology and Biotechnologies,” Università del Salento, Italy. Lucia Antonacci is a recipient of a CNR contract granted by Fondazione Cassa di Risparmio di Puglia. We thank Professor Shawn Doonan for critically reading of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received 05 April 2012; accepted 14 June 2012; published online: 03 July 2012. Citation: Guaragnella N, Ždralević M, Antonacci L, Passarella S, Murri E and Guarnaccia S (2012) The role of mitochondria in yeast programmed cell death. Front. Oncol. 2:70. doi: 10.3389/fonc.2012.00070

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