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Calcium and reactive oxygen species in regulation of the mitochondrial permeability transition and of programmed cell death in yeast

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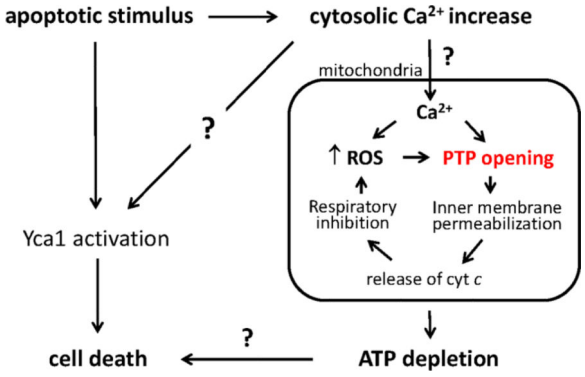
Abstract

Mitochondria-dependent programmed cell death (PCD) in yeast shares many features with the intrinsic apoptotic pathway of mammals. With many stimuli, increased cytosolic [Ca^{2+}] and ROS generation are the triggering signals that lead to mitochondrial permeabilization and release of proapoptotic factors, which initiates yeast PCD. While in mammals the permeability transition pore (PTP), a high-conductance inner membrane channel activated by increased matrix Ca^{2+} and oxidative stress, is recognized as part of this signaling cascade, whether a similar process occurs in yeast is still debated. The potential role of the PTP in yeast PCD has generally been overlooked because yeast mitochondria lack the Ca^{2+} uniporter, which in mammals allows rapid equilibration of cytosolic Ca^{2+} with the matrix. In this short review we discuss the nature of the yeast permeability transition and reevaluate its potential role in the effector phase of yeast PCD triggered by Ca^{2+} and oxidative stress.

Graphical Abstract



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Keywords

Yeast Mitochondria; Programmed cell death; Ca^{2+}; Reactive oxygen species; Permeability transition; F-ATP synthase

1. Mitochondria and yeast programmed cell death

Occurrence of programmed cell death (PCD) in yeast and its role in population dynamics and aging is increasingly understood [1,2]. Many of the signaling events of the mammalian intrinsic (mitochondrial) pathway to apoptosis take place in yeast [3]. It was reported that yeast expresses a BH3 domain-containing protein (Ybh3p, also called Ynl305cp or Bx1p) that upon treatment with lethal stimuli translocates to mitochondria and triggers apoptosis [4]. Ybh3p was initially proposed to be a member of the Bax Inhibitor 1 protein family (Bl-1), due to the presence of a BI-1 transmembrane motif contrasting the death-inducing effect of Bax [5] through a Ca^{2+}-dependent mechanism triggered by ER stress [6]. To date, the physiological function of Ybh3p is still under debate, yet these findings suggest that yeast cells may possess a homolog of the mammalian Bax/Bak pathway for outer mitochondrial membrane (OMM) permeabilization and cell death initiation [7]. Interestingly, *S. cerevisiae* is also endowed with Yca1p, a metacaspase that may be functionally related to mammalian effector caspases [8,9].

Key events of yeast apoptosis include increase of intracellular [Ca^{2+}] and reactive oxygen species (ROS), which are causally involved in yeast PCD induced by oxidative stress itself [10], acetic acid [11-13], pheromone or amiodarone [14,15], ethanol [16] and osmotic [17] or ER stress [18]. As in mammals, increased intracellular [Ca^{2+}] precedes the surge of ROS levels, cristae remodeling, mitochondrial depolarization, ATP depletion, matrix swelling and outer membrane permeabilization eventually leading to release of cytochrome *c* and other proapoptotic proteins [1-3]. In yeast, the link between increased intracellular [Ca^{2+}]/ROS and OMM permeabilization remains puzzling because (i) yeast does not possess a mitochondrial Ca^{2+} uniporter (MCU) [19-21], which in mammals mediates rapid equilibration of Ca^{2+} across the inner mitochondrial membrane (IMM) [22,23]; and (ii) there is no homolog of mammalian Apaf-1 in yeast, so that cytochrome *c* release does not trigger caspase activation while it may certainly contribute to decreased respiration, increased ROS production and ATP depletion.

Matrix Ca^{2+} is essential for opening of the permeability transition pore (PTP), an IMM high-conductance channel that can form under conditions of oxidative stress and mediates a permeability increase (the permeability transition, PT) causing many of the above-mentioned mitochondrial events of PCD in mammals [24]. The defining features of the mammalian pore are high conductance, up to 1.3 nS [25,26], which allows permeation of solutes up to about 1.5 kDa in mass [27]; absolute requirement for matrix Ca^{2+}[27]; inhibition by Mg^{2+} and adenine nucleotides [28]; inhibition (desensitization to Ca^{2+}) by cyclosporin A (CsA) [29,30], an effect exerted through matrix cyclophilin (CyP) D [31]; and requirement for inducing factors such as oxidants and Pi [32] (Table I). Whether a bona fide PTP exists in yeast, and whether it plays a role in yeast PCD, has been the matter of debate [33].

2. The mitochondrial permeability transition pore in yeast

Before discussing the features of the yeast PTP, we would like to mention that yeast mitochondria appear to possess multiple dissipative pathways, a situation that is further complicated by strain-specific differences. These include an ATP-dependent H^+-conductive pathway [34,35] and a variety of both selective [36-39] and unselective channels [40-45].

To the best of our knowledge the first evidence that yeast mitochondria may possess a PTP-like channel was obtained in studies of Pi transport in an industrial baker's yeast strain, Yeast Foam [40]. Mitochondria suspended in a K^+-based medium showed respiration-dependent large-amplitude swelling insensitive to mersalyl (and thus independent of the Pi carrier) and fully inhibited by antimycin A [40]. Subsequent studies demonstrated that a similar pathway was also present in laboratory yeast strains, where it allowed solute permeation with a cutoff of about 1.5 kDa[42,46-49]. At variance from the mammalian PTP, the yeast PTP (i) was unaffected by matrix Ca^{2+} even when uptake of the cation was made possible by the ionophore ETH129 [46]; (ii) was insensitive to Mg^{2+} and ADP [46]; (iii) was insensitive to CsA [46] in spite of the presence of a CsA-sensitive matrix CyP [50,51]; and (iv) was inhibited rather than induced by Pi [46,52]. A PT could be detected in strains lacking VDAC or the adenine nucleotide translocator (ANT), both of which were considered to be essential components of the PTP in mammals (see [33] for a thorough discussion), but the demonstration of a CsA-sensitive PTP in mammalian mitochondria lacking ANT [53] and VDACs [54,55] has refuted the paradigm that these proteins are essential for the PT [24].

Many apparent discrepancies with the mammalian PTP have been resolved in recent years.The Ca^{2+}-dependence of the yeast PTP has now been demonstrated beyond doubt in protocols where Ca^{2+} uptake was permitted by addition of the ionophore ETH129 and the concentration of Pi was optimized to prevent its inhibitory effect on the pore, which would otherwise mask the inducing effects of Ca^{2+} itself [56]. Lack of inhibition by Mg^{2+}/ADP may depend on the experimental conditions because of the presence of additional permeability pathways in yeast mitochondria, one of which is activated by ATP [34-36]. Finally, it is now clear that PTP opening can occur in the absence of CyP D also in mammalian mitochondria, as demonstrated both after genetic ablation of CyP D [57-61] and in cells and tissues where CyP D is expressed at low levels [62], conditions under which pore opening is obviously insensitive to CsA. The recent demonstration that after treatment with Ca^{2+} and oxidants F-ATP synthase forms channels with the features expected of the PTP in *B. taurus* [63], *H. sapiens* [64], *S. cerevisiae* [65] and *D. melanogaster* [66] now provides a unifying frame to address its role(s) in pathophysiology across species [24].

Table I summarizes the channel properties of F-ATP synthases in *S. cerevisiae, D. melanogaster* and *B. Taurus* (as determined by electrophysiology) [63,65,66] and the matching properties of their “PTPs” as determined in isolated mitochondria. It can be appreciated that a unique conductance is a feature of each species. A solute exclusion size of about 1.5 kDa has been defined for the PTP of isolated mitochondria of mammals [67,68] and yeast [46]. The size of the *D. melanogaster* species has not been defined precisely, but it appears to be considerably smaller because Drosophila mitochondria do not swell in sucrose-based media after opening of the “PTP” [69]. In *D. melanogaster* swelling was not

observed even in KCl-based media, suggesting that the channel is selective for Ca^{2+} and H^+, and may be a fast Ca^{2+} release channel rather than an unselective permeability pathway like the classical PTP of mammals [66,69](see [24] for a detailed discussion). In spite of this striking difference with the mammalian and yeast PTPs, we feel that they are all mediated by the same structure, i.e. the F-ATP synthase. This hypothesis is also supported by the finding that expression of human CyP D in Drosophila S_2R^+ cells sensitized the channel to Ca^{2+} without changing the pore size [66].

Inspection of Table I reveals that conserved general features are (i) the requirement for matrix Ca^{2+} and facilitation by oxidants, which affect the pore at discrete sites [70,71]; and (ii) inhibition by Mg^{2+}(which competes with Ca^{2+} for a matrix binding site) and adenine nucleotides [28]. The inducing effect of Pi—a unique feature of the mammalian pore—has always been puzzling because Pi decreases matrix free [Ca^{2+}] [72] and would thus be expected to inhibit rather than promote PTP opening. The finding that Pi inhibits the PTP in *S. cerevisiae* [46,56,65] and *D. melanogaster* [69] suggests that stimulation of PTP opening by Pi in mammals may be due to a specific mechanism that evolved only later in evolution [24].

In mammals Pi increases CyP D binding to the OSCP subunit of the F-ATP synthase resulting in increased sensitivity of the PTP to Ca^{2+} [63,73]. We have proposed that CyP D binding, which is reversed by CsA [63,74], favors access of Ca^{2+} to the catalytic site of F-ATP synthase (which is usually occupied by Mg^{2+}) resulting in a conformational change that is transmitted to the intramembrane portion of the enzyme through the rigid lateral stalk. The conformational change would eventually cause PTP opening at the interface between two F-ATP synthase monomers in a process involving the dimerizations subunits e and g [24,65]. In keeping with this suggestion, in the absence of CyPD (or in the presence of CsA) low concentrations of Pi inhibit the PTP also in mammalian mitochondria [75].

The PTP of *S. cerevisiae* is insensitive to CsA despite the presence of a matrix CyP, Cpr3p [76]. We have shown that genetic ablation of *CPR3* does not affect the PTP, suggesting that Cpr3p does not interact with F-ATP synthase in yeast [65]. We could not detect a matrix CyP in *D. melanogaster*, whose genome potentially encodes several isoforms including one with a putative mitochondrial targeting sequence [77], but expression of the human species in mitochondria did sensitize the PTP to Ca^{2+}[66]. In summary, the key PTP-forming features of F-ATP synthases may have appeared early in evolution, before the regulatory interactions of matrix CyP D developed. The reader is referred to a recent extensive review for further details on the mechanistic and species-specific features of the PTP [24].

3. Calcium homeostasis in yeast

Ca^{2+} ions are fundamental regulators of a wide variety of processes in all eukaryotic cells. Ca^{2+} signaling is triggered by activation of either ion channels or G protein-coupled receptors and affects reactions that range from modulation of enzyme activities to motility, regulation of ion channels and gene transcription. Yeast is no exception, and maintains a tight control of intracellular Ca^{2+} homeostasis through an integrated array of transport systems [78-80]. In addition to cell growth, Ca^{2+}controls mating between *MATa* and *MATα*

cells that secrete specific pheromones able to increase its cytosolic concentration, leading to cellular changes required for agglutination [81]. A key role of Ca^{2+} signaling has also been attributed to the response to an alkaline environment [82] as well as to hypotonic shock through the activation of MAP kinases [83]. Cytosolic free [Ca^{2+}] in yeast is finely regulated and maintained at low levels (50-200 nM) through Ca^{2+}storage in several compartments, i.e. vacuole, endoplasmic reticulum (ER), Golgi apparatus [78-80] and possibly mitochondria. Extracellular Ca^{2+} influx mainly occurs through the plasma membrane voltage-gated Ca^{2+} channel, also referred to as Cch1/Mid1 complex, that is activated by several stimuli such as depolarization, hypotonic shock, pheromone stimulation and unfolded protein response [81,84,85]. Following influx Ca^{2+} binds calmodulin, creating a complex able to activate calcineurin and thus to promote transcription of specific sets of genes required for cell proliferation and for the response to pheromone [86,87]. The signal is terminated by Ca^{2+} sequestration in the vacuole–the major Ca^{2+} store–through the Ca^{2+} ATPase Pmc1 [78] and the high capacity, low-affinity Ca^{2+}/H^{+} exchanger Vcx1 [88], which may link Ca^{2+} homeostasis to regulation of intracellular pH. A contribution of ER/Golgi as alternative Ca^{2+} storage sites has also been recently proposed [89].

4. Mitochondria and Ca^{2+} homeostasis

In mammals, mitochondria play a crucial role in cytosolic Ca^{2+} homeostasis through an array of transport systems [90]. In energized mitochondria the inner membrane MCU complex (which is inhibited by lanthanides [91] and ruthenium red [92]) mediates rapid Ca^{2+}uptake down the cation electrochemical gradient [93]. With a membrane potential of 180 mV (negative inside) the equilibrium Ca^{2+} accumulation is 10^6, which for a cytosolic [Ca^{2+}] of 100 nM would correspond to a matrix [Ca^{2+}] of 100 mM. Thermodynamic equilibration is never attained, however, due to the existence of the $3Na^{+}/Ca^{2+}$ exchanger and of a putative $3H^{+}/Ca^{2+}$ exchanger that extrude Ca^{2+} and thus prevent massive accumulation of Ca^{2+} and Pi (see [94] for a recent review).

Yeast mitochondria do not possess an MCU complex [93] and therefore their potential role in Ca^{2+} homeostasis is usually not given much consideration. However, also in yeast the Ca^{2+} electrochemical gradient favors Ca^{2+} accumulation with the same predicted equilibrium distribution as that of mammalian mitochondria. To quote the original conclusions of Carafoli and Lehninger “We consider it likely that all mitochondria, whatever the cell type, possess the electrochemical capacity for moving Ca^{2+} across the membrane. This capacity cannot be expressed, however, unless a pathway for trans-membrane movement of Ca^{2+} is available, either through the occurrence of a specific Ca^{2+} carrier system or through simple physical permeability of the mitochondrial membrane to Ca^{2+}” [20]. We contend that the driving force is so large that Ca^{2+} uptake could be relevant even if it occured through a leak pathway rather than through a specific transport system (which could be present, however, as further discussed in the following paragraph). Consistently (i) *S. cerevisiae* mitochondria have a Ca^{2+} content of 8-9 ng atoms/mg protein, which is close to that of rat liver mitochondria [20]; (ii) addition of EGTA or Ca^{2+} led to relevant changes of mitochondrial matrix free Ca^{2+} measured with a trapped fluorescent indicator, and mitochondrial Ca^{2+} release could be elicited by antimycin A or uncouplers [95]; and (iii) electrophoretic Ca^{2+} uptake coupled to H^{+} ejection can be easily measured in isolated *S.*

cerevisiae and *C. utilis* mitochondria when the cation is added at concentrations of 1-10 mM [21]. It is of note that respiration-driven uptake is observed with Ca^{2+}, Sr^{2+} and Mn^{2+} but not with Mg^{2+} [21], suggesting that cation accumulation could be taking place through a low-affinity system whose discrimination for the transported species is strikingly similar to that of the MCU [90]. It is also interesting to recall that yeast mitochondria are endowed with a very effective $2H^+/Ca^{2+}$ antiporter activated by fatty acids that mediates mitochondrial Ca^{2+} release [96]. Like in mammals, the antiporter could prevent excessive mitochondrial Ca^{2+} accumulation but also allow rapid mobilization of the matrix Ca^{2+}pool following activation of phospholipases and perhaps other relevant pathophysiological stimuli.

In contrast to the general assumption that yeast mitochondria lack a specific Ca^{2+} transport system machinery, a high-capacity Ca^{2+} uptake system driven by the membrane potential and stimulated by polyamines and ADP has been described in the yeast *E. magnusii* [97,98]. Rather than acting as an inhibitor, and at variance from the mammalian MCU, ruthenium red affected Ca^{2+} transport only marginally or even stimulated it under specific conditions[99]. Taken together, these findings suggest that a specific Ca^{2+} transport system may exist also in yeast mitochondria, and that this putative system could be expressed at varying levels in different yeast strains. Occurrence of Ca^{2+} uptake in *S. cerevisiae* mitochondria combined with the sensitivity of the PTP to oxidative stress may provide the missing link between combined increase of intracellular [Ca^{2+}] and ROS, common triggers of yeast PCD [1-3,10-18], and activation of the intrinsic pathwayof apoptosis.

5. Ca^{2+} and the permeability transition in yeast programmed cell death

As already mentioned, yeast PCD is increasingly recognized as a physiologically relevant event that has intriguing analogies with mammalian apoptosis, which are particularly striking for the mitochondrial pathway [1]. The major mitochondrial changes occurring in yeast apoptosis include cristae remodeling, increased ROS production, matrix swelling and cytochrome *c* release which are often preceded by increased cytsolic [Ca^{2+}] [1-3]. It is remarkable that the mechanistic basis for these changes has not been fully clarified yet, and it is legitimate to wonder whether the striking analogies with the matching consequences of PTP opening in mammals are just a coincidence, or rather underscore occurrence of a *bona fide* PT as also suggested in a relevant study of yeast spheroblasts [100].

We have already mentioned that Ybh3p translocation was reported to cause mitochondrial depolarization and intermembrane protein release, which are expected consequences of PTP opening in yeast [4]. The process also involved the Pi carrier, Mir1p, and the core subunit of ubiquinol-cytochrome *c* oxidoreductase, Cor1p [4]. Whether the PTP could have been involved in this paradigm remains, however, unclear particularly because the site of integration of Ybh3p—whether the IMM or the OMM—was not defined. It is interesting, however, that the stimulus used to trigger apoptosis was acetic acid, which causes increased ROS production [12] and may therefore require a functional respiratory chain and thus Cor1p. The requirement for Mir1p, on the other hand, may depend on the Pi-requirement for mitochondrial Ca^{2+} uptake which is stimulated up to 8-fold by Pi [21]. In this respect it is very intriguing that cell death induced by acetic acid was greatly reduced in ρ_0 cells (where

no mitochondrial respiration hence ROS production takes place) and in *ATP10* mutants (which cannot assemble the F-ATP synthase) [12]. The latter finding acquires a new meaning in the light of the demonstration that yeast F-ATP synthase forms channels with the features of the yeast PTP when subjected to oxidative stress in the presence of Ca^{2+} [65].

One of the most relevant studies on the involvement of mitochondria in yeast PCD was performed using the pheromone α factor or amiodarone, which both cause an increase of cytosolic [Ca^{2+}] and cell death. Cell death occurred by apoptosis and required a functional respiratory chain, as ρ_0 cells were extremely resistant; and was linked to ROS formation, as it could be protected by antioxidants and by low concentrations of the protonophore FCCP [15]. Amiodarone has complex effects on respiration in intact yeast cells, but consistently increased mitochondrial respiration and ROS production which preceded mitochondral depolarization. The Authors suggest that increased respiration was caused by Ca^{2+}-dependent stimulation of NADH dehydrogenase from the intermembrane space, sequentially causing an increase of membrane potential and of ROS production, followed by IMM permeabilization and cell death [15]. We suspect (i) that ROS-dependent depolarization in this and other paradigms was caused by PTP opening induced by oxidative stress and (ii) that this event could be a final common pathway in a variety of forms of yeast PCD. This hypothesis has so far been hard to test due to the lack of a structure for the PTP and to the insensitivity of the yeast PT to CsA. We have already found that genetic ablation of the e and/or g subunits, whose presence is important for F-ATP synthase dimerization [101], confers at least partial resistance to PTP opening [65]. Identification of the cysteine residues responsible for the sensitizing effects of oxidative stress and the availability of novel, CyP-independent PTP inhibitors [102-104] should soon allow a stringent test of this hypothesis.

6. Summary and conclusions

In mammalian cells apoptotic stimuli often lead to increased cytosolic [Ca^{2+}] that is rapidly relayed to mitochondria through MCU-dependent uptake. Under conditions of oxidative stress this Ca^{2+} signal can cause PTP opening and release of cytochrome *c* and other proapoptotic factors that eventually may cause activation of caspase 9 and contribute to execution of cell death (upper part of Figure 1). In spite of the absence of the MCU, yeast mitochondria may accumulate enough Ca^{2+} to undergo the PT when cells are challenged by stimuli that cause yeast PCD, which is accompanied by oxidative stress and increased cytosolic [Ca^{2+}]. Once PTP opening occurs, cytosolic and matrix Ca^{2+} equilibrate stabilizing the PTP in the open conformation, which is followed by osmotic swelling of the matrix, OMM damage and release of intermembrane proteins causing cell death (lower part of Figure 1). Although in yeast cytochrome *c* release is not followed by activation of effector caspases, it does cause decreased respiration and increased ROS production, which stabilizes PTP opening and leads to ATP depletion. This set of events could then contribute to cell death together with activation of metacaspases like Yca1p (see also the graphical abstract). The demonstration that PTP forms from F-ATP synthase provides a unifying framework for future studies, and should allow to assess if the transition of the energy-conserving complex into an energy-dissipating device plays a role in yeast PCD.

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Abbreviations used are

ANT	adenine nucleotide translocator
BI-1	Bax Inhibitor 1
CsA	cyclosporin A
CyP D	cyclophilin D
IMM	inner mitochondrial membrane
MCU	mitochondrial Ca^{2+} uniporter
OMM	outer mitochondrial membrane
PCD	programmed cell death
PT	permeability transition
PTP	permeability transition pore
ROS	reactive oxygen species
VDAC	voltage-dependent anion channel

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Highlights

We discuss the mitochondrial permeability transition in Ca^{2+}-dependent yeast programmed cell death

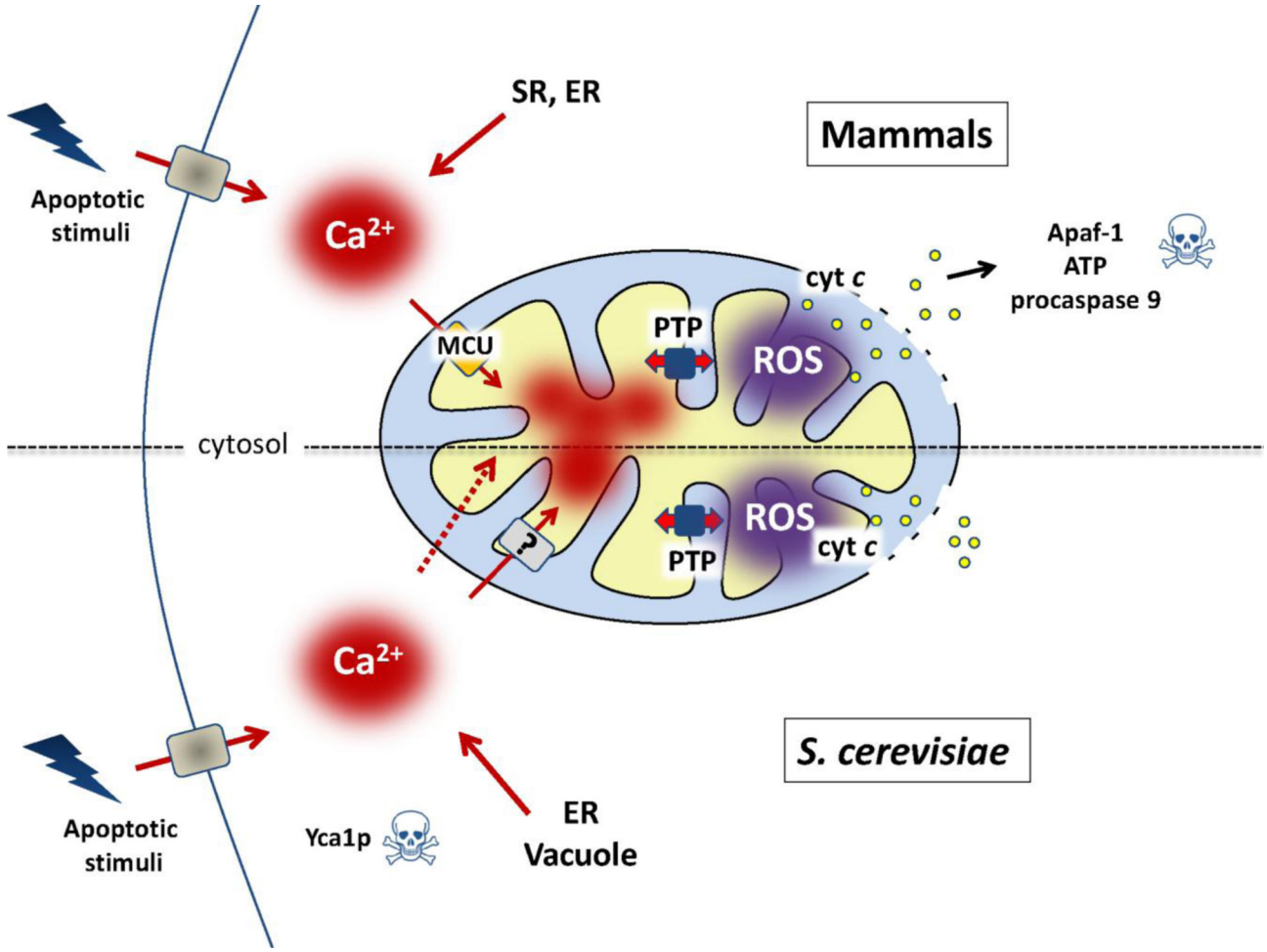

Figure 1. Pathways for Ca^{2+}-dependent cell death in mammals and yeast

Top, increased cytosolic [Ca^{2+}] often takes place in response to apoptotic stimuli in mammalian cells. Mitochondria quickly accumulate Ca^{2+} via the MCU complex, leading to increased matrix [Ca^{2+}] that, together with increased ROS levels, can favor PTP opening leading to release of proapoptotic factors including cytochrome *c*. This eventually results in formation of an active Apaf-1 complex leading to procaspase 9 cleavage and initiation of cell death. Bottom, apoptotic stimuli increase cytosolic [Ca^{2+}] in *S. cerevisiae*. Ca^{2+} can enter mitochondria through a membrane leak pathway (dashed red arrow) or via an unidentified transporter (denoted with a question mark) and, under conditions of oxidative stress, trigger PTP opening. The resulting matrix swelling and rupture of the OMM may cause release of cytochrome *c*, which would further stabilize the open state because of decreased respiratory activity and increased ROS production. The ensuing ATP depletion would add to activation of caspase-like pathways (such as Yca1p) in promoting cell death.

Table I

Properties of the PTP across species

The Table summarizes the conductance of F-ATP synthase channels, the effect of the indicated treatments on the PTP, and the presence of a mitochondrial CyP. For further explanation see text.

	Conductance (pS)	Ca^{2+}, oxidants	Mg^{2+} ADP	Pi	CsA	Matrix CyP
S. cerevisiae	300	Activate	Inhibit	Inhibits	No effect	Yes (Cpr3)
D. melanogaster	53	Activate	Inhibit	Inhibits	No effect	No
B. taurus	500	Activate	Inhibit	Activates	Inhibits	Yes (CyPD)