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REVIEW

The path from mitochondrial ROS to aging runs through the mitochondrial permeability transition pore

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Summary

Excessive production of mitochondrial reactive oxygen species (mROS) is strongly associated with mitochondrial and cellular oxidative damage, aging, and degenerative diseases. However, mROS also induces pathways of protection of mitochondria that slow aging, inhibit cell death, and increase lifespan. Recent studies show that the activation of the mitochondrial permeability transition pore (mPTP), which is triggered by mROS and mitochondrial calcium overloading, is enhanced in aged animals and humans and in aging-related degenerative diseases. mPTP opening initiates further production and release of mROS that damage both mitochondrial and nuclear DNA, proteins, and phospholipids, and also releases matrix NAD that is hydrolyzed in the intermembrane space, thus contributing to the depletion of cellular NAD that accelerates aging. Oxidative damage to calcium transporters leads to calcium overload and more frequent opening of mPTP. Because aging enhances the opening of the mPTP and mPTP opening accelerates aging, we suggest that mPTP opening drives the progression of aging. Activation of the mPTP is regulated, directly and indirectly, not only by the mitochondrial protection pathways that are induced by mROS, but also by pro-apoptotic signals that are induced by DNA damage. We suggest that the integration of these contrasting signals by the mPTP largely determines the rate of cell aging and the initiation of cell death, and thus animal lifespan. The suggestion that the control of mPTP activation is critical for the progression of aging can explain the conflicting and confusing evidence regarding the beneficial and deleterious effects of mROS on health and lifespan.

Key words: aging; calcium; mitochondria; NAD; permeability transition; reactive oxygen species.

Introduction

Oxidative stress in animals is strongly correlated with aging and lifespan, as predicted by the free radical theory of aging (FRTA; Harman, 1956; Sohal & Allen, 1985; Beckman & Ames, 1998; Barja, 2014). Because most reactive oxygen species are generated in the mitochondria (mROS), in close proximity to mtDNA and the mitochondrial oxidative phosphorylation system, it was suggested that oxidative damage to mtDNA, mitochondrial proteins, and phospholipids is the direct cause of aging and determines lifespan (Harman, 1972). This more specific version of FRTA was named the mitochondrial free radical theory of aging (mFRTA; Barja, 2014; Dai *et al.*, 2014). The evidence supporting mFRTA is extensive (reviewed in Dai *et al.*, 2014). For example, accumulation of mtDNA mutations in the polymerase G-deficient mice accelerated cardiac aging and this was mitigated by catalase, specifically expressed in the mitochondria (Dai *et al.*, 2010), whereas in wild-type, overexpression of catalase, specifically in the mitochondria, extended mouse lifespan (Dai *et al.*, 2017). As predicted by mFRTA, maximal lifespan of vertebrate homeotherms is inversely correlated with mROS production (Lambert *et al.*, 2007). In addition, many age-dependent degenerative diseases appear to be driven by excess production of mROS (Dai *et al.*, 2014). However, recent evidence suggests that it is the oxidative damage to other cellular components, nuclear DNA in particular, that largely determines the rate of aging and lifespan of animals (Schaar *et al.*, 2015; Fang *et al.*, 2016; Ma *et al.*, 2016). Oxidative damage to nuclear DNA triggers the DNA damage response, DDR, which induces both pro-apoptotic signaling (Nicolai *et al.*, 2015) and protective pathways (Fang *et al.*, 2016; Sun *et al.*, 2016) in postmitotic cells, and leads to senescence in mitotic cells (Campisi & Robert, 2014). Among the most important protection pathways are those that depend on induction of PARP1, which repairs damaged DNA in an NAD^+-dependent manner (Golia *et al.*, 2015), and on the induction of NAD-dependent deacetylases, sirtuins (Merksamer *et al.*, 2013), in particular sirt 1 (Mouchiroud *et al.*, 2013; Imai & Guarente, 2014; Fang *et al.*, 2016). Histone demethylases also contribute to stress-induced protection (Merkwirth *et al.*, 2016). Moreover, it is now evident that mROS or mROS-induced mitochondrial damage initiate signals that activate several pathways that protect mitochondria from stress, slow aging, inhibit cell death, and may result in lifespan extension (Sun *et al.*, 2016; Desjardins *et al.*, 2017). The mitochondrial sirtuins, particularly sirt3, are critical in the protection of mitochondria (Ansari *et al.*, 2017). Among the protective pathways are the mitochondrial unfolded protein response, UPR(mt), which enhances mitochondrial homeostasis (Pellegrino *et al.*, 2013; Tian *et al.*, 2016), and the nrf2 antioxidant response, which protects against mROS-induced mitochondrial damage and cell death (Strom *et al.*, 2016). Mitochondria that have been damaged induce the PINK1/Parkin pathway, which initiates mitophagy to remove damaged mitochondria (Durcan & Fon, 2015) and stimulates induction of the PGC-1α pathway to initiate mitochondrial biogenesis and replace damaged mitochondria (Austin & St-Pierre, 2012). Manipulations of a large number of antioxidant enzymes did not impact mouse lifespan, despite moderate nuclear and cellular oxidative damage (Pérez *et al.*, 2009), apparently because the induction of DDR by moderate oxidative damage is sufficient to protect the cells. By contrast, knockout of SOD1, which resulted in more extensive oxidative damage to DNA, did shorten lifespan significantly (Pérez *et al.*, 2009). It was recently suggested that the effect on lifespan of SOD1 knockout resulted from the extensive nuclear DNA damage that drove a large number of cells to senescence

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(Zhang *et al.*, 2017). In regenerating tissues, progenitor cells and stem cells, aging-associated mitochondrial stress and particularly excess mROS also lead to senescence (Ziegler *et al.*, 2015). Senescent cells may release inflammatory factors that lead to inflammation (Campisi & Robert, 2014). Chronic inflammation is associated with aging and contributes to a number of age-associated diseases, and is an important determinant of lifespan (De Martinis *et al.*, 2005). Therefore, even if organismal aging, degenerative disease, or lifespan is largely dependent on senescence and not on the aging and death of postmitotic cells (as believed by many), mitochondrial stress appears to be the driving force in these processes as well.

The mitochondrial permeability transition pore (mPTP) is an inner membrane protein complex that can be induced to form a nonselective channel. The channel is voltage-gated, activated by matrix calcium overloading and reactive oxygen species (ROS), and controlled additionally by a number of associated proteins and the post-translational modifications and binding of ions to these proteins and to the channel itself (Bernardi *et al.*, 2006). The channel exhibits several conducting states that can open for short (ms) or long (s) periods, and with different permeabilities (Biasutto *et al.*, 2016). Full opening of the mPTP results in increased production of mROS and release of most matrix metabolites (up to 1500 kDa) including mROS, calcium, NAD^+, and glutathione. As a result, the mitochondrial membrane potential collapses, oxidative phosphorylation and mitochondrial metabolism are inhibited, the matrix swells, and on prolonged opening the outer membrane ruptures, releasing intermembrane space proteins. Moreover, the release to the cytosol of ROS, calcium, NAD^+, glutathione, and other metabolites disrupts cellular homeostasis and increases oxidative damage to proteins, nuclear DNA, ion channels, transporters, and membrane phospholipids (Bernardi *et al.*, 2006; Zorov *et al.*, 2014). Prolonged pore opening in a large number of mitochondria in the cell can lead to cell death by necrosis or similar pathways (Di Lisa *et al.*, 2001; Petronilli *et al.*, 2001; Kim *et al.*, 2003; Vaseva *et al.*, 2012; Hou *et al.*, 2016; Izzo *et al.*, 2016). Several mitochondrial proteins were found to control pore opening including cyclophilin D (CypD), the adenine nucleotide translocator (ANT), the outer membrane anion channel (VDAC), and the phosphate translocator (PiC), and these, together or in various combination, were suggested to form the mPTP (cf. Bernardi *et al.*, 2006). However, more recent studies, and particularly knockout models of each of these proteins, suggest that all these proteins are not indispensable for mPTP activity. More recently, it was suggested that the conducting core of mPTP is subunit c of ATP synthase (Alavian *et al.*, 2014; Bonora *et al.*, 2015), but it appears more likely that it is the large ATP synthase (FoF1) dimer that forms an alternative structure that constitutes the conducting core of the mPTP (Bernardi *et al.*, 2015*).* Nevertheless, the precise identity of the pore components has not yet been elucidated (Biasutto *et al.*, 2016; Izzo *et al.*, 2016)*.* It cannot be excluded that in addition to a core-conducting structure that is necessary for pore opening, several alternative structures that include additional components exist under some conditions or in some types of cells.

Frequent and extended opening of the mPTP, with its associated bursts of mROS, can overwhelm the cell's antioxidant systems resulting in extensive DNA damage. As a result, the greatly enhanced PARP1 activity leads to a decline in the concentration of its substrate NAD^+, and because NAD^+ is also required for deacetylation reactions catalyzed by the sirtuins, the balance is shifted from sirtuin-dependent protective mechanisms to pro-apoptotic pathways (Gomes *et al.*, 2013; Imai & Guarente, 2014; Fang *et al.*, 2016). Moreover, because NAD^+ exits the mitochondrial matrix and is hydrolyzed by CD38 in the intermembrane space when the mPTP is activated (Di Lisa *et al.*, 2001), the mitochondrial protection that is provided by the mitochondrial sirtuins, particularly sirt 3 (Brown *et al.*, 2013), is also diminished. This mPTP-dependent hydrolysis of NAD^+ contributes directly to the loss of cellular NAD^+. A more moderate ROS production by mitochondria may not lead to strong pro-apoptotic signals but is sufficient to trigger various mechanisms that adjust cellular processes and protect the mitochondria and the cell from damage (Patterson *et al.*, 2015; Reczek & Chandel, 2015; Wei & Kenyon, 2016), without causing indiscriminate oxidative damage. This level of ROS formation is mostly contained by antioxidant systems (Forkink *et al.*, 2015; Araki *et al.*, 2016). When their capacity is exceeded, the increased oxidative stress activates the mPTP. While short, infrequent, opening of the mPTP also triggers protective pathways (Hou *et al.*, 2014a), increasing the frequency and duration of the mPTP is associated with more persistent oxidative damage that may result in aging and even cell death. Damage by mROS to mitochondrial matrix proteins, DNA, and phospholipids results in inhibition of oxidative phosphorylation and a further increase in production of mROS (Barja, 2014; Dai *et al.*, 2014; Zorov *et al.*, 2014). However, this damage, while extensive, is partially mitigated by the numerous mechanisms that protect the mitochondria and replace damaged mitochondria with new mitochondria as described above. By contrast, oxidative damage to cytosolic enzymes, calcium transporters, and nuclear DNA in postmitotic cells is more cumulative, increasing cell aging and eventually leading to cell death (Schaar *et al.*, 2015; Fang *et al.*, 2016). While stem cells that can replace damaged cells are emerging as important for healthy aging (Goodell & Rando, 2015), they are also constrained by aging-induced mitochondrial dysfunction (Min-Wen *et al.*, 2016). mPTP opening mediates toxic insults on stem cells (Wang *et al.*, 2015; Chen *et al.*, 2016), inhibits proliferation of progenitor cells (Hou *et al.*, 2014b), and inhibits liver regeneration (Antony *et al.*, 2016). Sirt3, which inhibits both mROS production and mPTP activation, is critical for proliferation of stem cells (Shin *et al.*, 2015), and therefore, the inhibition of sirt3 in aging (Brown *et al.*, 2013) as well as depletion of its substrate NAD^+ in stem cells (Zhang *et al.*, 2016) is predicted to enhance mPTP activation in aging stem cells and inhibit proliferation. This is likely to also drive the aging-induced conversion of proliferating cells to senescent cells (Campisi & Robert, 2014). This process is driven by excess mROS and by several additional mitochondrial dysfunction drivers (e.g., calcium overloading, loss of membrane potential; Ziegler *et al.*, 2015), suggesting that this effect, too, is mediated by the mPTP.

Because it is difficult to untangle the protective effects of mROS from its deleterious effects, the concept of FRTA has not been widely accepted (cf. Lapointe & Hekimi, 2010; Stuart *et al.*, 2014). Instead, a consensus is emerging in which the balance between mROS-induced protective pathways and cell damage-induced apoptotic pathways is somehow integrated in the mitochondria to determine the progression of aging and ultimately cell death (Wang & Hekimi, 2015; Bhola & Letai, 2016; Min-Wen *et al.*, 2016; Riera *et al.*, 2016; Sun *et al.*, 2016). Here, we propose that these contrasting signals are integrated at the level of the mPTP, which largely determines the rate of aging and ultimately lifespan by the frequency and duration of pore openings. This is illustrated schematically in Fig. 1.

The hypothesis that mPTP is the driver of aging can be considered a refinement of mFRTA as it is proposed that much of the oxidative damage to the mitochondria itself results from the activation of mPTP and that most of the effects of 'mitochondrial dysfunction' and mROS on aging and lifespan are mediated through activation of the mPTP. By controlling

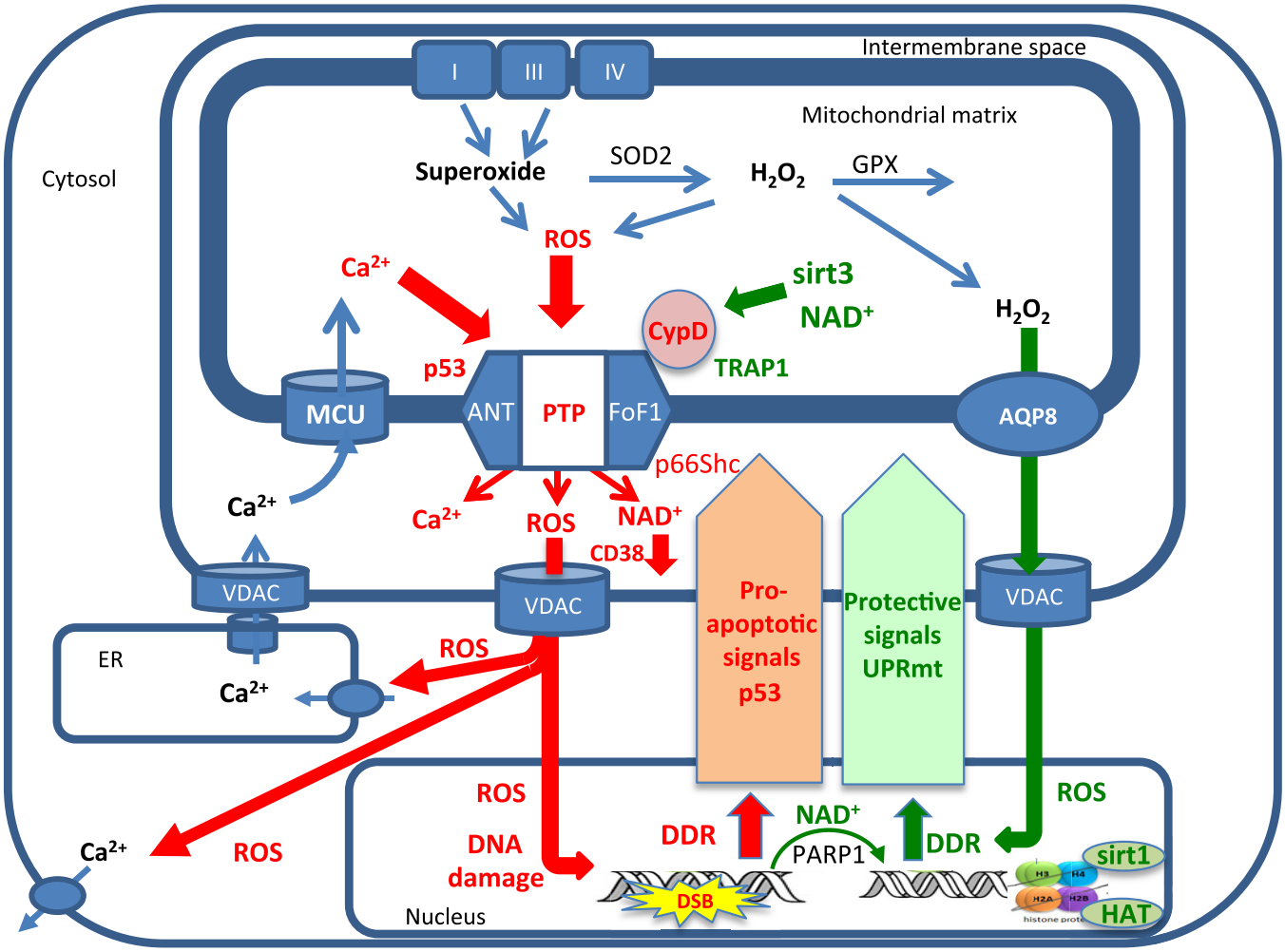

Fig. 1 mPTP integration of protective and apoptotic signals determines the rate of cell aging. In cells from young animals, the opening of the mPTP is infrequent and most metabolism-related increase in mROS production induces mROS signaling to the nucleus, largely through AQP8 (green arrow from mitochondria to nucleus), activating the DNA damage response (DDR) that triggers PARP1 and a number of mitochondria protective pathways (green arrow from nucleus to mitochondria), such as the antioxidant defenses (Nrf2), the mitochondrial unfolded protein response (UPRmt), and sirtuins (e.g., Sirt1, Sirt3), all of which inhibit the mPTP directly and indirectly and prevent more frequent opening of mPTP. Nevertheless, with time, oxidative damage resulting from the frequent and prolonged activity of mPTP (red arrows) damages both mitochondrial electron transport complexes and calcium transporters, particularly in the ER, resulting in increased mROS production and mitochondrial calcium overloading that further enhance mPTP opening. Moreover, the increased release of mROS by the mPT also increases oxidative damage to nuclear DNA resulting in increased pro-apoptotic signaling (red arrows from nucleus to mitochondria) inducing transfer of P53 and p66sch and other pro-apoptotic proteins to the mitochondria where they enhance mPTP opening. As aging progresses, the increased mPTP opening also depletes mitochondrial NAD^+, inhibiting the protective effect of Sirt3 and further stimulating mPTP opening. Finally, the increased oxidative damage to nuclear DNA increases PARP1 activity leading to nuclear NAD^+ depletion, which inhibits the nuclear sirtuins (e.g., Sirt1), weakening the mitochondrial protective pathways and further enhancing mPTP activation. This cycle continues, leading to longer and more frequent mPTP activation events, weaker and weaker protection signaling and stronger and stronger pro-apoptotic signaling, eventually leading to irreversible opening of mPTP, mitochondrial swelling, rupture of the outer membrane, and cell death. The mROS damaging effects that enhance mPTP activation are in red and mROS signaling and the protective pathways are in green (see text for more details).

both the depletion of cellular NAD^+ and the induction of a strong DDR, mPTP can drive aging and death of postmitotic cells as well as senescence in mitotic cells. Moreover, it is likely that mPTP opening also mediates mROS-driven inflammation (Rimessi *et al.*, 2016), because the formation of the NPLR3 inflammasome appears to depend on opening of the mPTP (Murakami *et al.*, 2012; Iyer *et al.*, 2013) and chronic activation of the mPTP (by deletion of MICU1) was found to extend the pro-inflammatory response to liver resection (Antony *et al.*, 2016).

In this review, we describe the evidence that the mPTP is enhanced in aging and aging-driven degenerative diseases, and discuss the mechanisms by which aging enhances mPTP activation and the mechanisms by which the mPTP drives the aging process.

mPTP activation is enhanced by aging and in aging-dependent degenerative diseases

We first reported two decades ago that the activation of the mPTP is enhanced in lymphocytes from old mice (Rottenberg & Wu, 1997). In these lymphocytes, the mitochondrial membrane potential and respiration rates were lower than in young mice but could be restored to normal values by the mPTP inhibitor cyclosporine A (see below). We later showed that most of the lymphocytes with activated mPTP are memory T cells (Mather & Rottenberg, 2002). It was also reported that the calcium-induced swelling of isolated liver mitochondria (which follows extended opening of the mPTP) is faster in old mice than in young mice (Goodell &

Cortopassi, 1998). Similarly, in isolated mitochondria from both liver and brain of old mice, the calcium-induced calcium release (CICR), which results from the calcium-induced opening of the mPTP, can be triggered by lower amounts of calcium than in mitochondria from young mice (Mather & Rottenberg, 2000). Aging was also reported to impair calcium loading in rat heart mitochondria (Jahangir *et al.*, 2001). These early observations suggested that mPTP activation is enhanced by aging in several tissues of mice and rats. Indeed, more recently, many more investigators reported similar observations in different tissues of humans, mice, rats, *C. elegans*, and fungi. Some of these studies were reviewed previously and the possible role of mPTP in aging was discussed (Crompton, 2004; Di Lisa & Bernardi, 2005; Toman & Fiskum, 2011; Paradies *et al.*, 2013). There are differences in the properties of the mPTP and the effects of aging between mitochondria from different organisms, different tissues (cf. Mather & Rottenberg, 2000), different types of cells from the same tissue (cf. LaFrance *et al.*, 2005; Bambrick *et al.*, 2006; Picard *et al.*, 2011; Lores-Arnaiz *et al.*, 2016), and even between mitochondria from different locations in the cell (Brown *et al.*, 2006). This variability probably results from the large differences in the expression and activity of the proteins that control activation of the mPTP such as ANT1 (Stepien *et al.*, 1992), CyPD (Hazelton *et al.*, 2009), the MCU complex (Paillard *et al.*, 2017), and probably other proteins. Nevertheless, there is strong evidence that in mitochondria from cells that play a role in aging (e.g., intrafibrillar heart mitochondria, brain cortex synaptic mitochondria), activation of the mPTP is enhanced by aging. A sampling of the evidence for the enhanced activation of mPTP by aging in different tissues and organisms is presented in Table 1.

Most of the studies that demonstrated enhanced activation of the mPTP in aged animals were conducted with isolated mitochondria or cell suspensions and did not allow observation of discrete, individual mPTP opening events, in situ. Moreover, using one specific assay, for example, calcium-induced activation of the mPTP (CICR), measures only one attribute of the mPTP, namely the calcium threshold, and may not reflect other important changes in the susceptibility of the mPTP to opening (e.g., sensitivity to oxidants, membrane potential threshold). Also, as a control, most assays rely on cyclosporine, which inhibits activation of the mPTP by CypD (see below). However, cyclosporine is only a partial inhibitor of the mPTP (Novgorodov *et al.*, 1992). There are several techniques that facilitate measurement of mPTP activation in living cells (cf. Bonora *et al.*, 2016) and these should be preferred in future studies of the relationship between aging and mPTP. Recent studies of the phenomenon of 'mitoflashes', which are observed by fluorescence microscopy in individual cells and are interpreted as short openings of the mPTP (Wang *et al.*, 2008, 2016a; Hou *et al.*, 2013, 2014b; Wu *et al.*, 2016), may provide a better way to correlate directly the frequency and duration of mPTP opening with the progression of aging. Although the interpretation of the signal of the mitochondria-directed fluorescence probe cpYFP is controversial (Schwarzlander *et al.*, 2014; Wang *et al.*, 2016a,b), there is little doubt that these and other mitoflashes are associated with mPTP opening. A recent study with *C. elegans*, utilizing cpYFP, demonstrated that the frequency of mPTP opening is age dependent (Shen *et al.*, 2014).

There are numerous reports that mPTP is activated in aging-dependent degenerative diseases and contributes to their pathology (reviewed in Paradies *et al.*, 2013). Indeed, it is well established that redox stress is a major driver of most aging-dependent degenerative disease (cf. Dai *et al.*, 2014). mPTP opening plays a major role in ischemic heart damage (discussed below) and also contributes to other aging-dependent cardiac diseases (Zulian *et al.*, 2016), diabetes (Riojas-Hernandez *et al.*, 2015), and multiple neurodegenerative diseases (Martin, 2012). A sampling of the evidence for the role of mPTP in aging-dependent degenerative diseases is listed in Table 2. The fact that inhibition of the mPTP slows the progression of many aging-dependent degenerative diseases (Table 2) supports the conclusion (see below) that mPTP opening accelerates the progression of aging. To the extent that disease pathology depends on the death of particular types of cell (e.g., Parkinson's disease), the critical role of the mPTP in oxidative stress-induced cell death confers a decisive role for mPTP opening in the pathology of that disease. Moreover, the effects of physical exercise on improving health and protection from age-dependent disease, which depends largely on the activation of mitochondrial biogenesis by PGC-1α (Austin & St-Pierre, 2012), were shown to result in inhibition of mPTP (cf. Marcil *et al.*, 2006; Goncalves *et al.*, 2016). Whether there are degenerative disease-specific modulations of mPTP is not yet known.

Aging is also the most important risk factor for carcinogenesis, and aging-associated excess mROS appear to play a major role in this process (Park *et al.*, 2011). It is likely that the aging-associated increase in DNA

Table 1 mPTP activation is enhanced by aging

Species	System	References
Mouse	Lymphocytes	Rottenberg & Wu (1997), Mather & Rottenberg (2000)
Mouse	T cells	Mather & Rottenberg (2002)
Mouse	Liver mitochondria	Goodell & Cortopassi (1998), Mather & Rottenberg (2000)
Mouse	Brain mitochondria	Mather & Rottenberg (2000), Lores-Arnaiz *et al.* (2016)
Rat	Brain mitochondria	Brown *et al.* (2004), LaFrance *et al.* (2005), Krestinina *et al.* (2015)
Mouse	Heart mitochondria	Fernandez-Sanz *et al.* (2015)
Rat	Heart mitochondria	Petrosillo *et al.* (2010), Escobales *et al.* (2014)
Rat	Skeletal muscle mitochondria	Marzetti *et al.* (2008), Picard *et al.* (2011)
Rat	Myocytes	Liu *et al.* (2011)
Human	Permeabilized myofibrils	Gouspillou *et al.* (2014)
Mouse	Osteocytes	Shum *et al.* (2016)
P. anserine (Fungi)	Organism	Brust *et al.* (2010), Kramer *et al.* (2016)
C. elegans	Pharyngeal muscle cells	Shen *et al.* (2014)

Table 2 mPTP is activated in aging-dependent degenerative disease

Alzheimer	Du & Yan (2010), Valasani *et al.* (2016), Chen *et al.* (2016)
Parkinson's disease	Thomas *et al.* (2012), Martin *et al.* (2014a), Rasheed *et al.* (2017)
Huntington's disease	Brustovetsky *et al.* (2005), Quintanilla *et al.* (2013, 2017)
Amyotrophic lateral sclerosis (ALS)	Kawamata & Manfredi (2010), Martin *et al.* (2014b)
Multiple sclerosis	Warne *et al.* (2016), Savino *et al.* (2013)
Diabetes mellitus	Taddeo *et al.* (2013), Riojas-Hernandez *et al.* (2015), Yan *et al.* (2016)
Heart disease	Hafner *et al.* (2010), Gordan *et al.* (2016), Zulian *et al.* (2016)
Osteoporosis	Zhen *et al.* (2014), Shum *et al.* (2016)

mutations drives the evolution of cancer in the old (Hanahan & Weinberg, 2011). However, once a mutated cell has converted to a neoplastic phenotype, its antiapoptotic signaling capacity is greatly activated and the mPTP becomes resistant to activation by calcium and ROS (Rasola & Bernardi, 2014; Bonora & Pinton, 2014). This is then the conundrum that confronts anticancer intervention: For cancer prevention, it should be helpful to inhibit the mPTP before any cell in the body has converted to a cancer cell, but it is necessary to activate the mPTP in order to kill cells that already have converted to the neoplastic phenotype. Below, we discuss how mPTP opening may accelerate aging and how aging, in turn, can accelerate mPTP activation.

mPTP opening contributes critically to age-dependent NAD depletion, which in turn further enhances the activation of mPTP

The frequent and prolonged mPTP opening in aged cells, as described above, contributes to aging-dependent depletion of cellular NAD^+ by the release of matrix NAD^+ and its hydrolysis by the mitochondrial NADase, CD38, as observed in postischemia–reperfusion of myocytes (Di Lisa *et al.*, 2001). In addition, in myocytes, mROS released by mPTP opening induce NAD^+ depletion by activation of PARP1, and this prolongs the opening of the mPTP, leading to further damage and NAD^+ depletion (Schriewer *et al.*, 2013). It was also shown that in cortical neurons the depletion of NAD^+ during anoxia and/or glucose deprivation depends on both mPTP opening and PARP activation (Kahraman *et al.*, 2015). It was recently shown that aging-related NAD^+ depletion results largely from the age-dependent increase in both the expression and activity of CD38, as the effect of aging on cellular NAD^+ concentration was absent in the CD38-knockout mouse (Camacho-Pereira *et al.*, 2016). Although most of the CD38 is located on the cell surface, a fraction has been found to be localized to the mitochondria and the nucleus (Di Lisa *et al.*, 2001; Camacho-Pereira *et al.*, 2016). This finding supports the suggestion that the hydrolysis of NAD^+ by the mitochondrial CD38 following mPTP opening contributes critically to aging-induced depletion of cellular NAD^+. It is possible that the increased expression of CD38 results from an aging-induced chronic inflammation (Lee *et al.*, 2012), which also partially depends on mPTP activation. Perhaps as critical as the decrease in cellular NAD^+ is the direct effect of loss of NAD^+ from the mitochondrial matrix during mPTP opening. The Km for NAD^+ of the mitochondrial sirt 3 and sirt 5 is one order of magnitude higher than that of the nuclear sirtuins and in the same range as matrix concentrations of NAD^+ of 3–5 mM (Canto *et al.*, 2015). This suggests that even a modest loss of NAD^+ from the mitochondrial matrix could decrease the activity of mitochondrial sirtuins and thus inhibit the protection afforded by these sirtuins against oxidative stress and mPTP activation (see below).

The exit of excess mROS from mitochondria to cytosol may depend on mPTP opening

While much has been learned in recent years about the sites, mechanisms, and rates of mitochondrial ROS production under different metabolic conditions (Figueira *et al.*, 2013; Zorov *et al.*, 2014; Goncalves *et al.*, 2015 Brand, 2016), little effort was devoted to elucidating the routes by which mROS leave the mitochondria and reach the cytosol. It is generally accepted that the inner mitochondrial membrane is impermeable to all ions and small solutes except for very lipophilic molecules that can dissolve in the phospholipid membrane. Although a number of ROS and RNS are formed within the mitochondrial matrix, only a few reactive species are known to be transported out of the mitochondria. Quantitatively, the most important are H_2O_2 and superoxide. H_2O_2 is a highly polar molecule (D = 84.2), while superoxide is both polar and charged and neither of these readily permeates phospholipid membranes. The mPTP is permeable to all solutes smaller that ~1500 kDa and therefore to all ROS and RNS. The only inner membrane channel that transports ROS is aquaporin 8 (AQP8), which can transport H_2O_2 (Bienert & Chaumont, 2014; Chauvigne *et al.*, 2015). Little is known about the relative contributions of mPTP and AQP8 to H_2O_2 release from the matrix. It is likely that in young cells, AQP8-mediated diffusion is the dominant pathway. However, as the frequency of mPTP opening increases with age, the mPTP contribution probably becomes more significant. It was reported recently that oxidative stress inhibits the transport of H_2O_2 through AQP8 (Medrano-Fernandez *et al.*, 2016), which suggests that in aged cells most of the matrix-produced H_2O_2 is released through the mPTP. This suggestion is compatible with the observation that ablation of AQP8 enhances the activity of the mPTP (Marchissio *et al.*, 2012; Chauvigne *et al.*, 2015). AQP8 releases H_2O_2 into the mitochondrial intermembrane space (IMS), where additional mitochondrial superoxide is produced directly (Brand, 2016). Most of the superoxide in the intermembrane space is consumed by oxidized cytochrome *c* (Zhao *et al.*, 2003) and some is converted to H_2O_2 by SOD1. It has been shown that the outer membrane anion channel, VDAC, releases superoxide that is generated by complex III in the IMS (Han *et al.*, 2003) and this presumably also applies to H_2O_2 and other ROS/RNS species, because VDAC also allows for transport of solutes up to ~1500 kDa (Shoshan-Barmatz *et al.*, 2010). Most of the superoxide that exits the matrix to the cytosol also passes through VDAC as the VDAC inhibitor DDI inhibited the exit of matrix-produced superoxide from isolated mitochondria (Lustgarten *et al.*, 2012). Although VDAC is not required for mPTP opening (Baines *et al.*, 2007), there are many reports of VDAC involvement in mPTP opening (cf. Tomasello *et al.*, 2009; Hou *et al.*, 2016). It is possible that VDAC associates with the mPTP to form a continuous pore from the matrix to the cytosol. If the mPTP can open either directly to the cytosol or to the intramembrane space, depending on the circumstances, it would allow an additional level of control of the outcome of mPTP opening.

In aging cells, mPTP opening enhances the production of mROS that in turn further enhances mPTP activation

Excessive production of mROS triggers the opening of the mPTP (Bernardi *et al.*, 2006), and there is little doubt that aging increases the production of mROS (Barja, 2014; Dai *et al.*, 2014). Inhibition of electron transport can result in increased production of ROS by complexes I, II, and III (cf. Forkink *et al.*, 2015). Therefore, the increased mROS production with age was attributed to oxidative damage to electron transport complexes and/or to mtDNA, which codes for core peptides of complexes I, III, and IV, resulting in the production of defective, ROS-producing complexes. The latter process was suggested to create a 'vicious cycle' that results in increasingly damaged electron transport complexes with age (Dai *et al.*, 2014). Additional 'vicious cycles' can be suggested in which the aging-dependent enhancement of mPTP-induced mROS production (Zorov *et al.*, 2014), mainly by complex I (Batandier *et al.*, 2004) and Krebs cycle enzymes (Bonke *et al.*, 2016), causes much of the increased mitochondrial oxidative damage observed in aged cells (Takeyama *et al.*, 1993). Moreover, recent evidence suggests that the protective signaling induced by mROS, which normally prevents sustained mROS production and protects OX PHOS enzymes, is inhibited in aged cells, largely due to NAD depletion, as discussed above.

Therefore, the age-dependent decrease in this protection may be another reason for excess mROS production resulting in more persistent damage to OX PHOS enzymes and mtDNA in aged cells. While inhibition of mitochondrial electron transport in aged cells was reported by many investigators, it should be emphasized that it is not always clear whether this observation is not simply a reflection of an enhanced activation of the mPTP (which inhibits electron transport). In lymphocytes from old mice, electron transport rates were inhibited but were completely restored to those of young mice by the addition of cyclosporine A (Rottenberg & Wu, 1997). Thus, in this case, at least, aging did not lead to inhibition of electron transport and the apparent inhibition was simply the result of enhanced activation of the mPTP.

Most of the proteins that are known to control mPTP opening and the protein complex that forms the pore itself (presumably ATP synthase) carry -SH groups, and some of these groups are oxidized in aged animals (Tajeddine, 2016) as the matrix redox balance is shifted toward a more oxidative state (Dan Dunn et al., 2015, Sies *et al.*, 2017). However, it is still not clear which of these sulfhydryl sites control the activity of the mPTP. Because mROS also cause oxidative damage to membrane phospholipids, it has been suggested that oxidized phospholipids, and in particular oxidized cardiolipin, activates the mPTP (Petrosillo *et al.*, 2006). Indeed, it has been argued that oxidation of membrane phospholipids is critical for the progression of aging (Pamplona, 2008; possibly through activation of the mPTP). In addition, oxidative stress triggers the transfer of proteins that enhance mPTP activation such as P53, which is transported to the mitochondrial matrix, or P66Shc, which is transported to the mitochondrial intermembrane space where its cytochrome *c*-dependent ROS production, in turn, activates the mPTP (Savino *et al.*, 2013; Priami *et al.*, 2015; Di Lisa *et al.*, 2017). Moreover, oxidized pyridine nucleotides also activate the mPTP (Chernyak & Bernardi, 1996; Ronchi *et al.*, 2015) and oxidative stress is manifested in increased oxidation of pyridine nucleotides (Sies *et al.*, 2017). In summary, oxidative stress, which increases in aged cells (Dai *et al.*, 2014), enhances mPTP opening by a variety of mechanisms and is, in turn, further increased by the enhanced activation of mPTP.

Disruption of calcium homeostasis in aging increases the frequency of calcium-triggered mPTP opening

Calcium overloading of mitochondria is an essential trigger of mPTP opening (Bernardi *et al.*, 2006; Tajeddine, 2016; Hurst *et al.*, 2017). Mitochondrial calcium accumulation mediated by the calcium uniporter (MCU) depends critically on the elevation of cytosolic calcium concentration (cf. Rottenberg & Marbach, 1990; Antony *et al.*, 2016), although often in restricted spatial domains, such as those maintained between the ER and mitochondrial outer membranes. It is well established that aging disrupts calcium homeostasis (cf. Tsai *et al.*, 1997; Mattson, 2007) and may disrupt the local association between ER and mitochondria (Fernandez-Sanz *et al.*, 2014). While calcium signaling pathways may be modulated by aging in different ways, depending on the tissue and cell type, a general observation is that calcium buffering power in the cytosol is diminished (cf. Tsai *et al.*, 1997; Gant *et al.*, 2015; Pandya *et al.*, 2015) and this could lead to an increase in mitochondrial calcium accumulation. The effect of aging on calcium homeostasis is believed to be mostly due to oxidative damage to calcium transporters and channels (cf. Andersson *et al.*, 2011; Cooper *et al.*, 2013), which increases the leak of calcium into the cytosol and increases calcium overload of mitochondria. Calcium overload of the matrix is also reported to be enhanced by increased direct transfer of calcium from ER to mitochondria (Calvo-Rodriguez *et al.*, 2016). In addition, aging downregulates the expression of the calcium buffering proteins regucalcin (Vaz *et al.*, 2015) and FKBP1b (Gant *et al.*, 2015). MICU1 is a subunit of the mitochondrial calcium transport complex located in the intermembrane space that controls opening of MCU. Under normal conditions, MICU1 limits mitochondrial calcium accumulation to concentrations above the normal resting cytosolic calcium range, thus preventing Ca overloading and activation of the mPTP (Antony *et al.*, 2016; Liu *et al.*, 2016). It was also reported that another MCU subunit, MCUR1, directly controls the calcium-dependent activation of mPTP (Chaudhuri *et al.*, 2016). Very recently, it was reported that MCU itself is modified under oxidative stress by S-glutathionylation of cysteine 97, which enhances calcium overload-induced cell death (Dong *et al.*, 2017). In aging cells, the cytosolic free calcium is frequently above the MICU1-set threshold for calcium uptake, and the calcium threshold for mPTP activation is below the normal threshold (Mather & Rottenberg, 2000: and see Table 1); therefore, more frequent calcium-induced mPTP openings are likely to be observed in aged cells.

Mitochondrial membrane potential and the potential threshold for mPTP activation may be lower in aged cells

The mPTP is voltage-gated and lowering of the mitochondrial membrane potential enhances the activation of the mPTP (Bernardi, 1992). There are many reports that the mitochondrial membrane potential is lower in aged cells (cf. Sugrue & Tatton, 2001). Oxidative damage is probably the most important factor that contributes to lowering the mitochondrial membrane potential in aged cells. Oxidative damage to membrane phospholipids is known to increase the membrane permeability to ions (cf. Runas & Malmstadt, 2015), and it is well documented that aging increases oxidative damage to mitochondrial phospholipids (Pamplona, 2008). However, as mPTP opening collapses the membrane potential, some of the reported effects of aging on membrane potential probably reflect the fact that aging enhances mPTP activation. In these cases, the mitochondrial membrane potential can be restored by the inhibition of mPTP by cyclosporine (cf. Rottenberg & Wu, 1997). Aging most probably also lower the potential threshold for mPTP opening. It was recently shown that this threshold depends on the expression of the adenine nucleotide translocator ANT1 (Doczi *et al.*, 2016) and that ANT1 overexpression inhibits the mPTP (Klumpe *et al.*, 2016). Inhibition of the mPTP by ANT depends on locking ANT in its matrix-facing M configuration by ADP (Rottenberg & Marbach, 1990; Bernardi *et al.*, 2006). This effect was suggested to result from the increase in the negative surface charge on the matrix face of the inner membrane (Rottenberg & Marbach, 1990), which is compatible with the suggestion that ANT determines the voltage threshold for mPTP activation. GSK-3β also controls the mPTP and it was suggested that phosphorylation of ANT by GSK-3β may contribute to aging-dependent activation of the mPTP (Zhu *et al.*, 2013). ANT was reported to be oxidized in aged cells (Le Bras *et al.*, 2005), and oxidation of SH resides was reported to lower the threshold for mPTP opening (Petronilli *et al.*, 1994). Therefore, it is very likely that the potential threshold for mPTP activation is lowered in aged cells.

The enhancement of mPTP opening by aging is mediated by aging-induced modifications of cyclophilin D

Cyclophilin D (CypD) interacts with the mPTP complex (presumably FoF1) to sensitize the mPTP to activation by ROS and Ca^{2+} (Bernardi *et al.*, 2006). This activation depends on masking an mPTP inhibitory

phosphate binding site by CypD (Basso *et al.*, 2008). Several proteins that are known to regulate mPTP, as well as CypD itself, are subject to numerous post-translational modifications that modulate their activity. Increased expression of CypD enhances activation of the mPTP (Lam *et al.*, 2015) and knockout of the gene inhibits the mPTP (Shum *et al.*, 2016). It was reported recently that the concentration of CypD increases in the brain of old mice (Gauba *et al.*, 2017) and this effect alone should enhance mPTP opening. In young animals, CypD is largely inhibited as a result of a number of post-translational modifications (Elrod & Molkentin, 2013). CypD also interacts with inhibitory proteins such as HSP90 (TRAP1 in humans), which was reported to aggregate CypD (Lam *et al.*, 2015) and prevent its interaction with activating proteins (e.g., P53) and the mPTP (Lebedev *et al.*, 2016). In aged cells, the UPRmt is suppressed, inhibiting HSP90 synthesis and its translocation to the matrix, while P53 is translocated to the matrix to further enhance CypD activation (Vaseva *et al.*, 2012; Priami *et al.*, 2015). Aging reverses several of the post-translational modifications that inhibit CypD resulting in a more active CypD. Inhibition of the deacetylation of a lysine residue by sirt3 (Hafner *et al.*, 2010) is, most likely, the strongest effect of aging on CypD activity as the concentration of NAD^+ is lower in old animals, it being lost both through PARP1 activation and through mPTP opening, as discussed above. Moreover, the concentration of sirt 3 itself is also lower in old animals (Kwon *et al.*, 2015). Sirt 3 also inhibits ROS production so that the weaker activity of sirt 3 with age enhances mPTP activation, both directly and indirectly (Brown *et al.*, 2013; Kincaid & Bossy-Wetzel, 2013; Ansari *et al.*, 2017). The thioredoxin/glutathione system is in equilibrium with SH groups on CYPD, so that the increased redox stress in old animals will oxidize the SH groups of CypD (Folda *et al.*, 2016). This is possibly one of the redox sites that activate the mPTP (Nguyen *et al.*, 2011). It is also believed that in aged animals, GSK-3β phosphorylates CypD, thereby further enhancing CypD activity (Zhu *et al.*, 2010, 2013). Other proteins that control mPTP opening may also work, at least partially, through their interaction with CypD. Thus, ANT was reported to interact with CYPD and control the activation of the mPTP (Crompton *et al.*, 1998; Woodfield *et al.*, 1998). CypD can be inhibited directly by the drug cyclosporine A, which is the most frequently used inhibitor of mPTP (Crompton *et al.*, 1988). There are marked differences between mitochondria from different organisms, tissues, and ages in the extent to which cyclosporine inhibits the mPTP (cf. Bambrick *et al.*, 2006; Liu *et al.*, 2011). It was observed that cyclosporine A does not inhibit ANT-dependent stimulation of the mPTP in heart mitochondria of aged animals (Garcia *et al.*, 2009; Liu *et al.*, 2011). This probably reflects the independent activation of mPTP by ANT, as discussed above. Similarly, in the presence of ADP, mouse brain mitochondria are more resistant to Ca^{2+}-induced mPTP opening than liver mitochondria and require a larger amount of calcium to trigger mPTP opening (Mather & Rottenberg, 2000). Thus in brain mitochondria, but not in liver, locking the ANT in the M configuration is sufficient to strongly inhibit mPTP. As a result, in brain mitochondria, cyclosporine A only slightly increases the Ca^{2+} loading threshold (~20%), while in liver mitochondria cyclosporine A increases the threshold to a much larger extent (~400%; Mather & Rottenberg, 2000). In aged mice, the cyclosporine effect was larger than in young mice (by 25–30%) both in liver and in brain mitochondria, indicating more active CypD in both brain and liver of old mice. Because aging in general decreases antiapoptotic pathways and increases pro-apoptotic pathways, other mitochondrial proteins that are known to inhibit mPTP are probably also reduced by aging; these may include HSP75 (Wang *et al.*, 2015), HSP60 and HSP90 (TRAP1; Altieri, 2013), which are induced by the UPR(mt), and antiapoptotic proteins, such as BCL-2 and BCL-x_L (Jonas *et al.*, 2014). Moreover, an increasing number of metabolites were reported to control the mPTP directly or indirectly, for example, mitochondrial cAMP and cyclic nucleotide phosphodiesterase (CNP; Baburina *et al.*, 2015), polyphosphate (Baev *et al.*, 2017), spermine (Wei *et al.*, 2016), and H_2S (Li *et al.*, 2015). These effects are probably also modulated by aging. For instance, it was reported that the enhanced activation of the mPTP in aged neurons partially results from a reduction in the activity of CNP and increased cAMP (Krestinina *et al.*, 2015; Wang *et al.*, 2016a,b).

Preconditioning in ischemia–reperfusion (IRPC) is greatly diminished by old age

Ischemic heart damage (as well as ischemia in brain, kidney, and other tissues) is largely the result of the reperfusion that follows ischemia. Reperfusion floods the cells with calcium, which triggers massive mPTP opening leading to extensive cell death (Bernardi & Di Lisa, 2015). Aging is associated with vascular damage, which increases the incidence of ischemia in the old and the aging-dependent activation of mPTP results in increased ischemic damage in the old (Petrosillo *et al.*, 2010). In the young, mild ischemic events protect the tissues from further ischemic damage by triggering the mitochondrial protection pathways—a process called ischemia–reperfusion preconditioning (IRPC). The mitochondrial protection pathways increase the resistance of the mPTP to opening, both directly and indirectly, although it is not clear which mechanism is dominant in IRPC (Bernardi & Di Lisa, 2015; Halestrap & Richardson, 2015). Aging greatly attenuates IRPC (Boengler *et al.*, 2009; Fernandez-Sanz *et al.*, 2015). Similarly, cyclosporine A protects from ischemia–reperfusion damage in the young but not in the old (Liu *et al.*, 2011; Pottecher *et al.*, 2016). It was suggested that the deacetylation of CypD by sirt3 is an important contributor to IRPC (Bochaton *et al.*, 2015) and this protection is lost in aged cells because of NAD^+ depletion, as discussed above. However, the loss of IRPC in old age appears to be mediated by other mechanisms as well. One suggestion is the age-dependent activation of phosphorylation of ANT (and perhaps other proteins) by GSK-3β (Zhu *et al.*, 2013). Aerobic exercise also induces protection of myocytes from mPTP opening (cf. Marcil *et al.*, 2006; Lumini-Oliveira *et al.*, 2011). As the health benefits of aerobic exercise largely depend on reducing the risk of ischemia, and to the extent that activation of the mPTP causes the damage of IR, the increased risk of heart disease in the aged may reflect the aging-dependent activation of mPTP.

Lifespan extension by mutations and caloric restriction most likely depends on pathways that inhibit mPTP opening, directly or indirectly

The fact the lifespan can be extended experimentally in several animal models of aging (e.g., *C. elegans*, *Drosophila*, and mice), and the findings that in many cases lifespan extension appears to depend on mROS signaling are often cited as the strongest evidence against mFRTA, as discussed in the introduction. Evidently, in these cases, mROS initiate the mitochondria protection pathways at an early age and this leads to lifespan extension. The mitochondrial protection pathways invariably lead to inhibition of the mPTP, whether indirectly by inhibition of mROS production, increased antioxidant protection, increased mitophagy, and increased mitochondrial biogenesis, or by direct inhibition of mPTP activation, as discussed above. It has been shown that lifespan extension by caloric restriction is associated with inhibition of the mPTP (cf. Kristal

& Yu, 1998; Hofer *et al.*, 2009; Amigo *et al.*, 2017) and that lifespan extension by genetic manipulations is often associated with the induction of the UPRmt (cf. Bennett & Kaeberlein, 2014; Pulliam *et al.*, 2014). A recent study on lifespan extension in *C. elegans* by germline loss shows that lifespan extension in this case depends on two independent pathways, one of which is mROS dependent (UPRmt) and the other not (the H_2S pathway; Wei & Kenyon, 2016). However, H_2S, similar to UPRmt, also inhibits the mPTP (Li *et al.*, 2015). In a study of a very large number of *C. elegans* lifespan modulations by mutations and environmental manipulations, it was shown that lifespan correlates negatively with the frequency of 'mitoflashes' at an early adult age (Shen *et al.*, 2014). If one accepts the interpretation that 'mitoflashes' signal the opening of the mPTP (Wang *et al.*, 2016a), it could be argued that in all these cases lifespan extension is the result of inhibition of mPTP opening in early adulthood. Metformin, the first drug approved for clinical trials for retarding the progress of human aging, was shown to inhibit the mPTP (Guigas *et al.*, 2004; Bhamra *et al.*, 2008). Thus, it is likely that in most, if not all, manipulations that extend animal lifespan, the mPTP is inhibited, directly or indirectly.

Conclusions

Although ROS has been suspected for more than half a century to be the driving force of aging, as rationalized first by FRTA, and more recently by mFRTA, and although the association between ROS, aging, age-related degenerative disease, and lifespan was proven to be robust, it has been more difficult to prove that ROS actually drives the progression of aging. The recent discoveries that mROS signaling triggers a large number of pathways that protect the cell, and mitochondria in particular, against oxidative damage, inhibit mROS production, slow aging and even increase lifespan, appear to directly contradict mFRTA. Nevertheless, because mROS signaling originates in the mitochondria and most of the protection pathways triggered by mROS are directed at the mitochondria, it became evident that the control of the progression of aging must reside in the mitochondria. These organelles must, somehow, integrate the protection signals as well as the stress-induced pro-apoptotic signals to determine the progression of aging. It is well accepted that oxidative stress-induced cell death is driven by massive opening of the mPTP, but the cumulative effects of a more moderate opening of the mPTP have not been fully appreciated. Reviewing the large number of recent studies that show that the mPTP is enhanced in aging and in aging-associated degenerative disease, and that inhibition of the mPTP can slow aging and degenerative diseases, we suggest that the mPTP itself is the elusive site of integration of the contrasting pro- and antiapoptotic signals that determine the rate of progression to aging. While many processes upstream of the mPTP (e.g., oxidative phosphorylation, electron transport, mROS production, mitochondrial antioxidant defense, mitophagy, mitochondrial biogenesis) are also affected by the various protection mechanisms, it is likely that these upstream processes affect aging largely through their effects on mPTP activation. There is still much to be learned about the composition and structure of the mPTP, the mechanisms that control mPTP opening, the various activation states of the mPTP, the extent and types of ions and metabolites that are released, and how the progression of aging affects these processes. The progression of aging to death does not follow a uniformly shaped curve in all animals (Jones *et al.*, 2014). An animal's lifespan can be determined by the failure of one particular critical organ, by either postmitotic or mitotic cells, and differences between the control of the mPTP in different organs, and different types of cells, may account for some of the differences between species. Further studies of the control of mPTP in aging can open the door to a much better understanding of the determinants of longevity.

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Conflict of interest

Both authors report no conflict of interest.

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