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Review

Mitochondrial reactive oxygen species: Do they extend or shorten animal lifespan?☆

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ABSTRACT

Testing the predictions of the Mitochondrial Free Radical Theory of Ageing (MFRTA) has provided a deep understanding of the role of reactive oxygen species (ROS) and mitochondria in the aging process. However those data, which support MFRTA are in the majority correlative (e.g. increasing oxidative damage with age). In contrast the majority of direct experimental data contradict MFRTA (e.g. changes in ROS levels do not alter longevity as expected). Unfortunately, in the past, ROS measurements have mainly been performed using isolated mitochondria, a method which is prone to experimental artifacts and does not reflect the complexity of the in vivo process. New technology to study different ROS (e.g. superoxide or hydrogen peroxide) in vivo is now available; these new methods combined with state-of-the-art genetic engineering technology will allow a deeper interrogation of, where, when and how free radicals affect aging and pathological processes. In fact data that combine these new approaches, indicate that boosting mitochondrial ROS in lower animals is a way to extend both healthy and maximum lifespan. In this review, I discuss the latest literature focused on the role of mitochondrial ROS in aging, and how these new discoveries are helping to better understand the role of mitochondria in health and disease. This article is part of a Special Issue entitled 'EBEC 2016: 19th European Bioenergetics Conference, Riva del Garda, Italy, July 2–6, 2016', edited by Prof. Paolo Bernardi.

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1. Introduction

The Free Radical Theory of Ageing, later updated to the Mitochondrial Free Radical Theory of Ageing (MFRTA) was proposed by Denham Harman in 1956 and 1972 respectively [1,2]. The efforts to test MFRTA have been instrumental in gaining a better understanding of the aging process, moreover our knowledge of how free radicals participate in cellular physiology has been extended far beyond the aging field. Primary support for MFRTA comes from descriptive studies which established that ROS production and oxidative damage accumulate with age [3–6], and from correlative studies showing that ROS levels correlate with lifespan in long-lived animals [7–9] or individuals [10–13]. Additionally, excessive ROS levels have been reported in many age-related and degenerative diseases such as Parkinson's disease (PD) [13], diabetes [14] and cancer [15]. In contrast to this, MFRTA has been repeatedly challenged by experimental data, which has demonstrated that neither alteration of antioxidant levels nor direct manipulation of ROS production alter longevity as predicted by MFRTA [16–18]. Administration of antioxidants has repeatedly failed to extend lifespan in several animal models reviewed in [19]. Furthermore, manipulation of endogenous antioxidant levels also did not support MFRTA [20]. A paradigmatic example is *Caenorhabditis elegans*, where suppression of all superoxide dismutase activity by knocking-out all genes encoding superoxide dismutase enzymes fails to reduce lifespan even by a day, despite significantly increasing sensitivity to oxidative stress [21]. Although, *Drosophila melanogaster* or *Mus musculus* are more sensitive to superoxide levels i.e. knock-out of *Sod2* dramatically shortens lifespan to a few days in both animal species, heterozygous *Sod2* knock-out mice have a normal lifespan despite higher levels of oxidative damage [16]. Direct manipulation of ROS produced by the electron transport chain (ETC) does not alter longevity as expected either. Reducing superoxide leak from ETC does not extend lifespan in fruit flies [18], but even more counterintuitive is the fact that low doses of ROS-generating toxins such as rotenone or paraquat, in spite of having different effects on mitochondrial respiration (i.e. only rotenone is a direct inhibitor of respiratory complex I (CI) [22]), extend lifespan in worms in a ROS-dependent manner [17]. Furthermore, mutations in genes encoding subunits of CI increase ROS and extend lifespan in both worms and flies through a ROS dependent mechanism, independently of their effects on mitochondrial respiration

Abbreviations: DCFDA), 2′,7′-dichlorofluorescin diacetate; FCCP), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; CoQ), Coenzyme Q; CI), Complex I; ETC), electron transport chain; H_2O_2), hydrogen peroxide; HE), hydroethidine; LC), liquid chromatography; MS), mass spectrophotometry; mtDNA), mitochondrial DNA; MFRTA), Mitochondrial Free Radical Theory of Aging; mtROS), mitochondrial Reactive Oxygen Species; mtUPR), mitochondrial Unfolded Protein Response; PD), Parkinson's disease.

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[23,24]. The concept of mitohormesis proposes that boosting ROS levels activates a program of responses to stress that over-activate mechanisms of protection (including antioxidants) which compensate for initial damage caused by ROS [25]. Mitohormesis, fails to explain why overexpression of these same mechanisms of protection alone is not sufficient to extend lifespan or why overexpression of these mechanisms suppresses lifespan extension in those models where ROS have been experimentally increased [17,24]. Nevertheless, as mentioned previously, ROS levels have been shown repeatedly to be altered in aging and age-related diseases. In this review, I intend to discuss the role that ROS play in determining animal lifespan, reviewing the latest findings in the field. In the interest of space, I will mainly focus on the three animal models in which the greatest extension in lifespan has been reported: *C. elegans*, *D. melanogaster* and *M. musculus*. For the same reason, I will not discuss ROS generated outside the mitochondrion, although it is clear that they can also contribute to the onset of aging and age-related disease [26,27]. For example, it has recently been shown that lifespan of the long-lived *clk-1* mutant worm is further extended by increasing mitochondrial ROS levels through knock-out of *sod2* [28]. However, increasing cytosolic ROS levels, by knocking out *sod3* and *sod5*, shortened lifespan of this long-lived mutant. This indicates that ROS can have opposing effects on longevity, depending on whether they are produced within or outside of mitochondria. This highlights the importance of understanding where ROS are generated.

2. ROS are not all the same

Mitochondria are the powerhouses of the cell, producing a considerable share of cellular ATP, as well as many other essential cellular components such as iron–sulfur clusters or pyrimidine nucleotides, that the cell requires for survival. In addition, mitochondria participate in calcium metabolism and play a leading role in the initiation of apoptosis and as such are instrumental in maintaining cellular homeostasis acting as important signaling organelles in different tissues [29]. For example, mitochondrial ROS are essential for the elimination of bacteria by the macrophages [30], induction of differentiation of hematopoietic progenitors in fruit flies [31] or control of insulin release in pancreatic β-cells [32]. Most physiological processes taking place in mitochondria (e.g. ATP generation) are to a greater or lesser extent coupled to mitochondrial respiration, which makes mutations, in genes encoding ETC subunits or those proteins involved in mtDNA maintenance, the most common cause of inherited metabolic disorders [33]. Most oxygen consumption occurs during cellular respiration and as a consequence most of the superoxide produced within the cell is generated by the mitochondrion in the majority of, although not all, cell types [34].

Since mitochondria play a central role in cellular function and metabolism, it is not surprising that decreased mitochondrial performance is a hallmark of aging [35]. In fact, mitochondrial malfunction can have terrible consequences as exemplified by those phenotypes associated with mitochondrial disease i.e. inherited mitochondrial disorders that result in progressive neuropathies and myopathies [33]. Thus, many sporadic and age-related diseases such as PD [36] or diabetes are suspected to have a mitochondrial component [37]. It is possible that age-related mitochondrial loss of function is a consequence and not a cause of aging. A recent intriguing paper supports this hypothesis. Siegfried Hekimi's laboratory has shown that controlled disruption of Coenzyme Q (CoQ) biosynthesis, through knock-out of the *Mclk1* gene, severely affects mitochondrial function and dramatically reduces lifespan [38]. Interestingly, restoration of CoQ levels through administration of 2,4-dihydroxybenzoic acid (an analogue of 4-hydroxybenzoic acid, the natural precursor of CoQ) that is only able to partially rescue the mitochondrial phenotype completely rescued the shortened lifespan of *Mclk1* mutant mice. This result is totally unexpected, as chronic mitochondrial dysfunction should cause the accumulation of irreversible damage and a shortened lifespan, if mitochondrial dysfunction is an underlying cause of aging. Conversely, Hekimi's work suggests [38] that mitochondrial dysfunction *per se* does not cause aging, as replacement of damaged mitochondria with functional mitochondria instantly restored a youthful phenotype. This defies the dominant paradigm that states that chronic mitochondrial dysfunction accelerates aging. It would be interesting to test if this is applicable to other models of mitochondrial dysfunction such as mutations in the mitochondrial polymerase (DNA polymerase γ) that has also been shown to accelerate aging [39] through a reduction in mitochondrial function [40], or if this effect is unique to alterations in CoQ synthesis.

A topic highly debated in the field is the role that mitochondrial ROS play in age related and non-age related pathological processes with a mitochondrial component. Are ROS a cause or a consequence of mitochondrial dysfunction? This is a very important question, which needs to be addressed, since it will affect the treatment of those pathologies. Considering all the available evidence, it is plausible to suggest that ROS can have both positive and negative roles depending on the type of the ROS, when, where and how much is produced. Therefore, we can talk about "Good" and "Bad" ROS. "Good" ROS being low reactivity ROS (i.e. superoxide or hydrogen peroxide (H_2O_2)) produced at specific places, at specific times and in moderate amounts and "Bad" ROS being highly reactive ROS (or low reactive ROS as H_2O_2 or superoxide produced at high concentrations) generated continuously and unspecifically. Experimental evidence suggests that boosting ROS production can contribute to the maintenance of cellular homeostasis and positively affect lifespan when induced correctly, whereas if produced in excess or in an unspecific way, they shorten survival and accelerate the onset of age-related disease.

In my opinion, there are two reasons why the role of ROS in aging and in different diseases is not yet fully understood. Firstly, ROS are usually considered as a single entity and are measured using unspecific probes that are prone to experimental artifacts. For example, 2′,7′-dichlorofluorescin diacetate (the popular DCFDA) reacts non-specifically with many types of free radicals that are produced ubiquitously, thus its use is associated with many caveats as for example its propensity to autoxidize [41]. Each ROS has specific properties that are determined by its intrinsic reactivity and relative abundance. The second issue is that the majority of ROS measurements -in the study of aging and age-related diseases- have been performed *in vitro* using isolated mitochondria or cells in culture. I will discuss the first issue now, and will focus on the second in the following section.

As previously mentioned, it is common to see reference to "ROS" without any mention of which specific ROS is being measured or where they are produced. There are many types of ROS but the three most studied in aging and age-related pathologies are superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH), which are the result of the incomplete reduction of oxygen with one, two and three electrons respectively. It is usually accepted that ROS are mainly produced during oxidative phosphorylation, by mitochondria, as a result of the incomplete reduction of O_2 to superoxide. This is true for the majority of cell types but not all, since in some cells other organelles (e.g. peroxisomes or endoplasmic reticulum) or enzymes (e.g. NADPH oxidases, xanthine oxidase, lipoxygenase, cyclooxygenase, cytochrome p450s or nitric oxide synthase) are the main generators of ROS (reviewed elsewhere [42–44]). In normal conditions, generation of superoxide is not particularly problematic for the cell since it is promptly detoxified to H_2O_2 by superoxide dismutase. In fact, neither superoxide nor H_2O_2 are particularly reactive when maintained at low concentrations and are unable to for example cause mutations to DNA by themselves [45]. However, they can both generate more reactive ROS, which are able to cause macromolecular damage including DNA mutations. The main target of superoxide is the iron–sulfur clusters of proteins such as aconitase or respiratory CI and II, which release free iron as a result of superoxide attack [46,47]. This free iron reacts with superoxide and H_2O_2 to form the extremely toxic. ·OH through the Fenton/Haber-Weiss reactions. In addition, superoxide can also react with nitric oxide to form another highly toxic ROS: peroxynitrite ($OONO^-$). Both ·OH and $OONO^-$ can react with and damage all biological components

of the cell [48], and surprisingly there is no specific antioxidant system to detoxify them. This indicates that they are rarely produced under normal conditions or that evolution has not yet been able to come up with a system to neutralize them in an energy efficient manner. Mitochondrial H_2O_2 is particularly important in cellular signaling due to its ability to selectively modify cysteine thiols (reviewed in [49]). These kinds of modifications are reversible, although prolonged periods of high H_2O_2 levels can provoke secondary and tertiary oxidative irreversible modifications. Catalases and peroxiredoxins convert H_2O_2 into H_2O and O_2, and glutathione peroxidases use H_2O_2 to convert glutathione into glutathione disulfide. Peroxiredoxins are preferential targets of H_2O_2 and transduce the H_2O_2 signal through both redox (via thiodisulfide) or non-redox (via protein–protein) interactions [50]. For example, metformin extends *C. elegans*' lifespan by a ROS mediated mechanism that requires PRDX-2 to transmit the signal from the mitochondria to the nucleus [51]. Antioxidant systems are usually able to keep oxidative stress under control in young/healthy cells and in normal conditions oxidation is maintained at safe levels. Based on experimental evidence (see below), we can consider superoxide and H_2O_2 as "Good" ROS involved in cellular signaling and able to extend lifespan if boosted at the right place and time, whilst $^{\cdot}OH$ and $OONO^-$ are "Bad" ROS involved in oxidative damage. As I shall discuss, there are different ways to measure superoxide and H_2O_2, *ex vivo* and *in vivo*, however measurement of highly reactive ROS is much more difficult due to their short half-life and to the best of my knowledge there is currently no reliable method to detect them *in vivo*.

3. Problems measuring ROS: *in vitro* vs *in vivo* measurements

As mentioned previously, one of the biggest caveats associated with data supporting MFRTA is that they have been mainly obtained from *in vitro* experiments using isolated mitochondria. Such experiments are typically performed by feeding mitochondria with saturating concentrations of specific substrates in combination with inhibitors to help dissect the specific locations within ETC where ROS are produced. This approach provides a high resolution that allows precise identification of where ROS are generated, but the trade-off is a loss of physiological relevance resulting from the artificial conditions used, and therefore results obtained using this approach cannot be directly extrapolated to the *in vivo* situation. A non-exhaustive list of problems associated with experiments in isolated mitochondria includes (Fig. 1):

(i) Mitochondria are exposed to oxygen levels that are 4–5 times higher than what they would experience *in vivo*.
(ii) Mitochondria are isolated from their natural environment that includes close associations with the cytoskeleton and other organelles (e.g. the endoplasmic reticulum) that contribute to the determination of mitochondrial morphology and the rate of respiration [52], and therefore have a direct effect on ROS produced by ETC.
(iii) Substrates and ADP are administered at saturating concentrations that would never be encountered *in vivo*.
(iv) ROS are usually measured only during the artificial state 4 (without ADP) or more rarely state 3 (with ADP).
(v) Most studies use either complex I- (e.g. pyruvate + malate) or II-linked (e.g. succinate) substrates e.g. [53,54], whereas *in vivo* electrons are fed simultaneously from several entry points including CI and II, but also glycerol-3-phosphate dehydrogenase or the electron transfer flavoprotein.
(vi) Most studies only measure H_2O_2 released from mitochondria, overlooking that superoxide is the main ROS generated by ETC in animals [55] and that it is largely generated inside the mitochondrion by CI, CII and some Krebs cycle enzymes [56,57]. Measuring H_2O_2 released from the mitochondrion as an indication of the superoxide produced by ETC is misleading since the activity of superoxide dismutase may influence the levels detected [58]. To clarify what happens *in vivo* with superoxide production, more studies specifically measuring mitochondrial superoxide are needed (see below).

A characteristic example of the problems referred to above is the conclusion, by some studies, that only CI produces fewer ROS in long-lived species or individuals [59]. This conclusion is probably biased for two reasons. Firstly, in some of these reports differences are only observed when CI produces ROS working in reverse, so called "revere electron transport" [9,60]. Secondly, other ROS generators as for example complex II and III have been discarded as ROS generators relevant for longevity using concentrations of succinate that are 4–5 times higher than those used for pyruvate; these higher concentrations, much higher than *in vivo*, inhibit production of ROS by CII, compromising the physiological relevance of the results [61]. In fact based on my own experience at the bench, changes in the concentrations of substrates used to feed ETC can alter the conclusions of one specific study dramatically. RET was considered an *in vitro* artifact until recently when it was shown to be responsible for the damage caused by ROS during ischemia–reperfusion [62]. RET occurs as a consequence of the preferential use of $FADH_2$-linked substrates (i.e. succinate, glycerol 3-phosphate or fatty acids), which causes an over-reduction of the ubiquinone pool and generates thermodynamically favorable conditions for ubiquinol to transfer electrons to CI reducing NAD + to NADH [63]. During this process a considerable amount of superoxide is produced by CI. RET is abolished by depolarization (e.g. using uncouplers such as carbonyl cyanide-p-

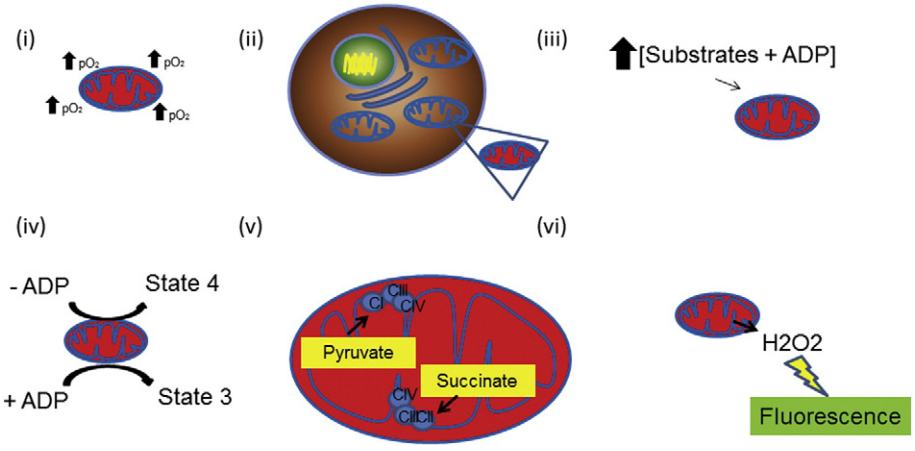

Fig. 1. Problems associated with ROS measurements in isolated mitochondria: (i) non-physiological levels of oxygen, (ii) loss of cellular interactions, (iii) saturating concentrations of substrates and ADP, (iv) measurement of "artificial respiratory" states, (v) use of CI- or CII-linked substrates, (vi) detection of extra-mitochondrial H_2O_2.

trifluoromethoxyphenylhydrazone (FCCP)) or by CI Q-site inhibitors (e.g. rotenone) *in vitro*, and by inhibitors of CII (e.g. malonate) *in vivo* [62]. The role of RET in non-pathological conditions if any remains to be elucidated. In relation to this, it is worth noting that inhibition of CIII and IV or the action of CV working in reverse mode would generate the conditions for RET to occur *in vivo* [64]. In fact, ROS produced via RET have been proposed to be involved in cellular signaling and the activation of mechanisms of protection in response to stress [65].

The use of cells instead of isolated mitochondria negates most (ii, iii, iv, v, vi), but not all (i) the problems outlined above and has other specific drawbacks. Cells are routinely grown under chronic hyperoxic conditions (i.e. 20% O_2 levels) and in most studies, highly glycolytic transformed cells are used. Cells develop non-physiological adaptations when they are continuously cultured under these conditions that may compromise the physiological relevance of results obtained *in vitro*. Two examples related with aging and mitochondrial function perfectly illustrate this problem. Fibroblasts cultured under 20% oxygen levels rapidly develop a senescent phenotype characterized by the accumulation of transversions, a type of mutation that is characteristic of oxidative stress supporting a major role of ROS in this process [66]. However, if oxygen levels are reduced to 3% – closer to the physiological reality – neither senescence or transversions are observed [66]. Similarly, loss of mitochondrial membrane potential triggered by FCCP in cells overexpressing Parkin only induces mitophagy if they are cultured in a glucose-rich medium [67]. However, if cells are forced to use their mitochondria to obtain energy (replacing glucose by galactose), mitophagy does not occur due to a lack of activation of OMA1 and subsequent cleavage of L-OPA1 that prevents mitochondrial fission and therefore mitochondrial turnover. The fact that mitophagy triggered by FCCP only occurs under very restricted conditions *in vitro* (i.e. in cells overexpressing Parkin grown in a glucose-rich medium) demonstrates the limitation of this model, which is considered paradigmatic, to study mitochondrial turnover [67,68] and may explain the lack of physiological effects of *parkin* knock-out in mouse models [69,70]. Using cells cultured in glucose-rich media and under hyperoxic conditions, some studies have discarded the role of ROS as inducers of cellular senescence [71] or regulators of mitophagy [68]. While other studies, using similar growth conditions (i.e. a glucose-rich medium and 20% oxygen levels) but which control better antioxidant concentration in the media (i.e. avoiding excess of antioxidants that can interfere with ROS signaling) or use specific mitochondrial dyes for ROS measurements (e.g. MitoSOX to detect mitochondrial superoxide), have reported a major role of ROS in mitophagy and cellular senescence [72,73].

This shows that experimental conditions clearly influence the output of the experiment, and remind us that *in vitro* experiments must be carefully interpreted and conclusions should not be immediately extrapolated to *in vivo*. Another good example of how problematic studying mitochondrial phenotypes using an *in vitro* approach can be is mitochondrial disease. It is not uncommon that fibroblasts from mitochondrial patients, diagnosed from muscle biopsies, do not display any mitochondrial alterations when cultured *in vitro* [74]. It is unclear whether this is caused by differences in the cell type or the conditions in which cells are cultured [74], in any case, it further demonstrates that conclusions from *in vitro* studies should be made with caution. A solution in some cases has come from reprogramming those fibroblasts into neurons or myocytes, which partially recapitulate the phenotype observed in the patients and can help to clarify whether or not ROS are involved in mitochondrial disorders [75].

4. New approaches for measuring ROS

As a result of the problems referred to above, there have been continuous efforts to develop novel strategies to measure ROS *in vivo*. In many instances ROS levels are inferred indirectly from oxidative damage markers, which is far from optimal since the levels of oxidative damage depend not only upon ROS generation but also upon repair and turnover mechanisms. In addition, effects of ROS signaling on lifespan can be modulated without causing detectable oxidative damage. It is out of the scope of this review to discuss the technical details of different methods to measure ROS *in vivo*, and readers are referred to some excellent reviews, which have recently been published [41,76]. However, I will briefly discuss those approaches that have been used recently to study how mitochondrial ROS affect lifespan. The nature, the when and the where of ROS production is essential when selecting the most appropriate system of detection. The half-life of ROS ranges from nanoseconds to seconds depending on their reactivity and the abundance and existence of antioxidant systems targeted against them. For obvious reasons, fluorescent probes are usually chosen for *in vivo* measurements. The most frequently used probes are either small dyes or genetically encoded fluorescent protein reporters that unfortunately are prone to technical artifacts. As such, confirmation using alternative approaches like overexpression/knockdown of specific antioxidants is highly recommended (Fig. 2).

Protein-based reporters such as reduction–oxidation circularly permuted Yellow Fluorescent Protein (cpYFP), Hydrogen Peroxide sensor (HyPer) or Green Fluorescent Protein (roGFP) have the advantage that they can be engineered to be expressed in a temporally and spatially controlled manner [76]. For example, they can be expressed exclusively in specific tissues or targeted to discreet organelles, which in the study of mtROS is a must have. cpYFP has been presented as a highly selective and sensitive superoxide detector [77], however its ability to detect superoxide has recently come in to question as it has been claimed that it in fact detects changes in pH and not superoxide itself [78,79]. A modified version of cpYFP – Hyper – has been engineered to measure H_2O_2 levels *in vivo* [80]. Hyper is a ratiometric fluorescent that consists of a cpYFP protein inserted into the regulatory domain of an *Escherichia coli* peroxide sensor oxyR. Using hyper, Ursula Jakob's lab has reported that peroxide levels are highest during development, decrease during adulthood and increase again at old ages [81]. Interestingly, in the same study, short-lived *daf-16* and long-lived *daf-2* mutants had more and less ROS respectively compared with controls. Unfortunately, the authors did not measure any markers of oxidative stress or investigate whether effects of ROS levels are mediated through signaling. Therefore it remains unclear if changes in ROS levels are merely correlative or if they may be able to explain differences in longevity. roGFPs are dual-excitation ratiometric probes that were originally developed to detect changes in the redox state of glutathione [82], however they have been converted into probes for specifically detecting H_2O_2 through fusion to the microbial H_2O_2 sensor oxidant receptor peroxidase 1 (Orp1) [83]. Using this approach, the laboratories of Tobias Dick and Aurelio Teleman studied changes in mitochondrial and cytosolic H_2O_2 levels during aging in specific fly tissues using both a mitochondrially- and a cytosolically-targeted versions of Orp1roGPF [84]. Although, they reported important differences between tissues (e.g. gut cells have higher levels of cytosolic H_2O_2 than other cell types) and between different regions of the same tissue (e.g. they reported areas of both high and low ROS levels in fat body), they did not observe any significant increase in mitochondrial H_2O_2 levels with age. However, only young flies (~21 days old) were used, preventing conclusions on increased ROS levels at later ages as other studies using alternative *in vivo* approaches (see work from Mike Murphy's lab below) have reported [85].

Most of aforementioned approaches require the use of optically accessible systems, thus quantification can be difficult and detection of small differences between groups can be technically challenging. The use of liquid chromatography (LC) coupled to fluorescence, electrochemical or mass spectrophotometry (MS) can help to increase specificity and detection of smaller differences. The use of MS to detect superoxide has been applied using hydroethidine (HE) or its mitochondrially-targeted version Mito-HE popularly known as MitoSOX. The specificity of HE (and Mito-HE) is limited by the lack of specificity since HE can be oxidized by molecules other than superoxide. However, HE specifically

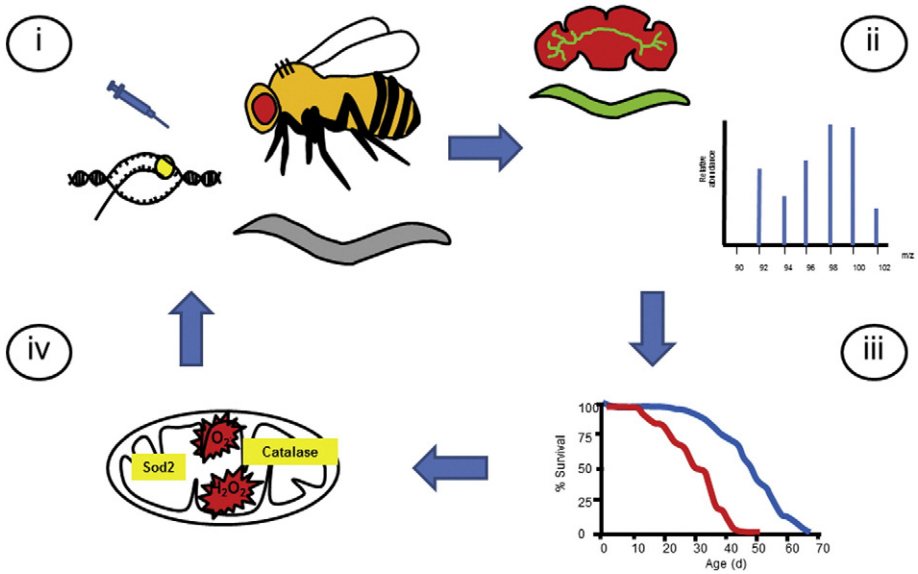

Fig. 2. Four steps to validate the role of ROS in the aging process *in vivo* using the state-of-the art technology to measure ROS. Measurement of mitochondrial ROS levels *in vivo* is required to understand how ROS contribute to aging and age-related diseases. (i) Experimental animals can be injected or fed with small dyes or genetically encoded fluorescent proteins can be expressed both ubiquitously or in a tissue-specific manner. (ii) Small animals can be directly visualized under the microscope (e.g. worms) or tissues can be dissected (e.g. fly brain) allowing study of specific areas or cells using fluorescence reporters. Alternatively, superoxide (using e.g. MitoSOX) or H_2O_2 (using e.g. MitoB) can be extracted from cell or tissue homogenates and quantified using Mass spectrophotometry analysis. (iii) Lifespan is then studied using appropriate controls. (iv) Finally, conclusions can be validated by feeding animals with specific antioxidants (e.g. mitoQ) or through overexpressing/depleting endogenous enzymes.

reacts with superoxide to form 2-hydroxyethidium [86] this is difficult to detect specifically using fluorescence microscopy, but is clearly identified by separating the different oxidation products of HE by LC and then detecting 2-hydroyethidium using a, for example, fluorescence detector [86]. To the best of my knowledge no one has studied superoxide levels during aging measuring MitoSOX coupled to fluorescence or more sensitive MS detection. A similar approach has been developed by Mike Murphy's laboratory to detect mitochondrial H_2O_2 using the radiometric system mitoB/mitoP that controls for artificial oxidation of the probe, variations in its absorption and accumulation in the mitochondrion [85]. Using mitoB/mitoP, a significant increase in the levels of mitochondrial H_2O_2 has been described *in vivo* during aging, however no correlation between mitochondrial H_2O_2 levels and lifespan was found in this study [85]. MitoB/MitoP relies on mass spectrophotometry technology for its detection, which makes the method necessarily invasive since it requires the use of tissues or cell homogenates, making it difficult to differentiate between different cell types and impossible to discern whether distinct mitochondria within the same cell produce different ROS, thus it also complicates the establishment of whether differences in ROS levels are caused by mitochondria which produce more ROS or by an increase in the number of mitochondria. In order to estimate whether differences in ROS levels are caused by changes in number of mitochondria vs mitochondria which are producing more ROS, it is possible to normalize results using classical methods to measure mitochondrial density such as citrate synthase measurements or estimation of mtDNA copy number [87]. For obvious reasons, it is important to understand if changes in ROS levels are caused by more or less mitochondria in order to design strategies that impact ROS levels and can alter lifespan.

5. Is where ROS are produced important?

Another major problem when studying the role of ROS in aging is the lack of resolution of *in vivo* measurements when compared with measurements in isolated mitochondria. Presently, it is virtually impossible to identify which specific respiratory complex or mitochondrial enzyme is producing ROS. As I mentioned previously, *in vitro* studies have singled out CI as the location where ROS most important for aging are produced [59]. An interesting proteomic study from Ulrich Brandt's laboratory which studied changes in the redox state of mitochondrial cysteines in response to changes in ROS levels showed that where (i.e. at which respiratory complex ROS are produced) is as important as the amount of ROS produced in determining if a specific cysteine will be oxidized or reduced [88]. For example, ROS produced by CI only affected the redox state of proteins located within the matrix, indicating that direct targets of CI action can be found in this compartment. Surprisingly, whether some proteins were affected depended on whether CI produced ROS in the forward (e.g. ATP synthase subunit d) or reverse direction (e.g. Succinate dehydrogenase [ubiquinone] flavoprotein subunit), suggesting that depending on how ROS are generated also informs how they alter individual proteins. Similarly, exposure to paraquat or knock-down of *Sod2* increases superoxide levels and dramatically shortens *Drosophila* lifespan [89]. However, only paraquat causes a significant increase in protein carbonylation, indicating that the reduction in survival in *Sod2* mutants could be related more with *Sod2* acting as a signaling transducer than as an antioxidant protecting against oxidative damage.

Brandt's study is subject to all the caveats of experiments performed using isolated mitochondria (see before), but it also highlights the need to increase the resolution of *in vivo* ROS measurements if we want to identify selective targets that are important for aging or age-related diseases. For example, the activity of DJ-1 depends on the oxidation state of a single cysteine [90], and mutations in this gene have been found in familial Parkinson's disease patients [91]. In order to identify how DJ-1 is aberrant in the pathological situation we must identify where and how the ROS that modify the redox state of DJ-1 are generated i.e. we need to increase the resolution of *in vivo* ROS measurements. In flies and worms "resolution" can be increased by feeding animals with specific inhibitors of ETC complexes and/or by altering electron flow using genetic approaches (e.g. RNA interference (RNAi) or CRISPR/Cas9) [92] and then studying the changes in ROS using *in vivo* fluorescent reporters like those described in the previous section.

ROS are now recognized as essential cellular messengers, like other well-known messengers such as NO, Ca^{2+}, cAMP or IP3 [93]. Since ROS are important contributors to redox homeostasis, it has been proposed that aging is a consequence of a pro-oxidizing shift in the redox state of cells that could affect signal transduction and gene regulation

[94,95]. The increase in ROS produced during aging would contribute to altered redox signaling causing a deregulation of redox regulated proteins and feedback systems; however it is unclear how the increase in ROS would occur. It is possible that the increase in ROS is a consequence and not a cause of aging. From this point of view, ROS production could have an adaptive function that is important in order to maintain homeostasis and as such, prevention or suppression of ROS production could have unwanted side effects. "The redox stress hypothesis of ageing" [94] is supported by a redox change to a more pro-oxidative state of both glutathione and NADPH during aging [96,97]. Accordingly, overexpression of enzymes that maintain a high GSH:GSSG or NADPH:NADP+ ratio extend lifespan in fruit flies [98,99]. However, during aging, the ratio NAD+:NADH seems to change in the opposite direction (i.e. to a more reductive state) due to a decline in steady-state NAD+ levels that negatively impacts on the activity of Sirt1 [100] and mitochondrial function [101]. This indicates that changes in redox state to a more reductive state can also cause age-related physiological alterations.

As I discussed earlier, ROS are quite reactive and short-lived, therefore they can exert effects close to their site of generation. Redox-sensitive proteins located at these locations are the most probable candidates in the coordination of ROS signaling. The increase in ROS observed during aging has the potential to disturb this signaling, especially if it is produced ubiquitously and not by specific enzymes (e.g. CI) as seems to be the case in young individuals [102]. According to Brandt's work [88] a change in the location of ROS production would significantly alter cellular signaling as predicted by the redox stress hypothesis of aging [94] contributing to the loss of cellular homeostasis. Several laboratories have initiated a pursuit of the network of proteins implicated in the alteration of redox signaling during aging but there are as yet no conclusive results. An *in vivo* redox proteomic study detected a decrease in the ability to confront redox stress in the muscle of old rats [103], however these results were not replicated in fruit flies where no significant change in the redox state of cysteines was observed during aging [104]. The former approach has the disadvantage that it fails to detect proteins of low abundance that may have a prominent role in redox regulation. Thus, it has only been applied to cell homogenates and so cell- or even organelle-specific changes may remain undetected using this approach. Therefore, we will need to wait until redox proteomic techniques have been improved in order to make definitive conclusions.

Finally, Brandt's study provides evidence that not only where but also how ROS are produced is important when considering downstream effects. From this point of view, the role of the supercomplexes in ROS production should thoughtfully be taken into account. Growing evidence indicates that respiratory complexes are organized into supercomplexes [105]. It has been proposed that supercomplexes contribute to optimize electron transfer [105], through changes in the organization of ETC in response to a whole range of metabolic signals including variations in oxygen levels, substrate supply or calcium [106]. Accordingly ROS production varies depending on how the respiratory complexes are organized [107], and through feedback mechanisms ROS contribute to fine-tune the organization of ETC [108]. Two manuscripts reported a reduction in the amount and organization of the mitochondrial supercomplexes during aging in rat heart [109] and brain cortex [110]. These changes may affect how and where ROS are produced and may contribute to changes in redox signaling reported during aging [90,110]. In conclusion, strategies that aim to alter lifespan through changes in mitochondrial ROS levels must consider whether and how these strategies will affect organization of the supercomplexes. Moreover, strategies that alter mitochondrial supercomplexes with and without altering total ROS levels could be more efficient in altering lifespan than unspecific strategies that affect the total amount of ROS. A handful of targets that do affect the organization of supercomplexes and are susceptible to genetic or pharmacological treatment have been reported [106,108] so it would be interesting to study how modification of the levels of these proteins affects ROS levels and if this has an effect on lifespan.

6. Do changes in ROS production modulate longevity in animals?

Since the discovery of superoxide dismutase by McCord and Fridovich in 1969 [111] ROS have been proposed as the primary cause of many different degenerative diseases [112]. Indeed, an imbalance of ROS levels and the presence of markers of oxidative stress is a hallmark in multiple diseases (reviewed in [113–119]). Interestingly, as I mentioned previously reductive stress can also lead to pathology [120], suggesting that redox alterations can also underlie aging and age-related diseases independently of structural damage caused by ROS [94,95]. Despite thousands of published studies, implicating ROS in many diseases it is still unclear if ROS are a cause, a consequence or both. Moreover, there is no clear mechanism by which ROS are pathological (i.e. oxidative stress, changes in redox signaling, both or other unknown mechanism(s)). Excessive ROS levels have been associated with deleterious effects on health and lifespan. For example, feeding experimental animals with poisons that block ETC and increase ROS levels (e.g. paraquat or rotenone) causes the death of experimental animals in minutes or hours [121], this phenotype is partially rescued by the administration of antioxidants [122,123]. The former indicates that part of the effect is due to ROS accumulation. Similarly, prevention of the surge in ROS produced after ischemia and during reperfusion protects the heart against apoptotic and necrotic cell death [62]. Similarly mutations in key antioxidants genes such as *Sod2* dramatically shorten lifespan in the fly and mouse although intriguingly not in the worm [16,21,124]. This highlights an important difference between worms and other model organisms that should be taken into consideration in longevity research and other fields focused on mitochondrial biology. Surprisingly, no one seems to have noted the importance of understanding how worms are resistant to levels of superoxide that are lethal in other animal species, understanding how this is mediated may help to prevent, for example, the consequences of excessive ROS during ischemia–reperfusion [62].

If the contribution of ROS to the onset of several different diseases (e.g. cancer and diabetes) is clear [125], their role in the progression of normal aging is controversial. For example, it is well known but still surprising that low doses of paraquat, rotenone or piercidine A extend *C. elegans* lifespan via increased ROS levels [17,126]. Similarly, metformin blocks CI and extends lifespan through a ROS mediated mechanism [51]. However, this seems to be something specific to *C. elegans* since feeding metabolic poisons has not been reported to extend lifespan in other model organisms [127,128], and metformin does not increase longevity in fruit flies either [129]. Of course, it is possible that no effect has been found because the right doses have not been used, as most published studies in mice or flies use rotenone to induce a Parkinson's like phenotype and not to extend lifespan [130]. Another substantial difference between worms, flies or mice is how mitochondrial dysfunction alters lifespan. In worms, there is a narrow temporal window where induction of mitochondrial dysfunction is effective in extending lifespan [23,131]. Mitochondrial function may be interrupted during development to efficiently extend lifespan in *C. elegans* [131], thus both mutations and gene knock-down extend lifespan to a similar extent but not through the same mechanisms [23]. In flies, mutations in or strong depletion of ETC components using RNAi dramatically shortens fly lifespan [24,132–134], whilst mild knock-down of ETC subunits has a positive effect on longevity [24,92]. However, and in contrast to what occurs in worms, knock-down must be induced both during development and adulthood in order to extend fly lifespan [92,135]. These differences indicate separate downstream mechanisms that should be carefully considered before planning to translate these strategies into mammalian models or human beings. Interestingly, and as mentioned previously, although mutations and knock-down of specific ETC subunits extend lifespan in worms, they do by distinct mechanisms [23]. Mutations in ETC subunits extend lifespan in worms by increasing mitochondrial ROS levels, since administration of antioxidants suppresses the positive effect on lifespan [17], whereas how lifespan is extended by knocking-

down of ETC subunits remains to be elucidated. It has been proposed that knock-down of ETC subunits or other mitochondrial proteins causes the accumulation of free subunits, which provokes the induction of the mitochondrial unfolded protein response (mtUPR) that would account for the extension in lifespan [24,136]. This seems to be the case at least for mitochondrial ribosomal proteins, whose knock-down results in an imbalance between nuclear and mitochondrial subunits that activates mtUPR and is instrumental for the extension of lifespan in *C. elegans* [136,137]. A recent study, however, shows that induction of mtUPR is not necessary (in models of mitochondrial dysfunction) or sufficient to extend lifespan [138]. Activation of mtUPR has been described as ROS-independent in worms [136] but ROS-dependent in flies [24]. In fact, in fruit flies only knock-down of ETC CI subunits that increase ROS levels and induce mtUPR is able to prolong lifespan [24]. To the best of my knowledge only deletion of the CIV assembly factor, *Surf1*, and heterozygous mutations in *MCLK1* -implicated in the synthesis of ubiquinone- extends lifespan in mice [139,140]. As in flies, the general rule is that mutations in ETC subunits or essential mitochondrial genes are developmentally lethal or dramatically shorten lifespan in mammals [39,141,142]. Extension of lifespan resulting from mutations in genes involved in mitochondrial respiration have been reported to increase ROS levels (*MCLK1*) [143] but also leave them unchanged (*Surf1*) [144]. Unfortunately experiments increasing or decreasing antioxidants have not been performed in these mice, and so no definitive conclusion about the effects of ROS on lifespan can be formulated yet.

In worms, lifespan extension elicited by paraquat is potentiated in a *Sod2* depleted background indicating that superoxide is the ROS responsible for lifespan extension [17]. However, in fruit flies where boosting of ROS levels also extends lifespan, it seems that H_2O_2 and not superoxide is the ROS responsible. For instance, overexpression of *Sod1* and *Sod2*, mutations in *dj-1β* that increase mitochondrial H_2O_2 or feeding flies with H_2O_2 supplemented food have all been reported to extend lifespan in flies [145–148]. Similarly, the extension of lifespan associated with knock-down of CI, which is ROS-dependent, is supressed by overexpression of catalase or glutathione peroxidase [24]. However, some of these results are not always consistent, some reports do not observe extension in lifespan with overexpression of *Sod1* or *Sod2* [149,150] and a shortening of lifespan has been reported in *dj-1β* mutants [151]. A couple of studies from the laboratory of David Gems found that overexpression of *Sod1* prolongs lifespan in worms but increases H_2O_2 and levels of oxidative damage [152,153]. However, the deletion of all superoxide dismutase genes, which should impact on the levels of H_2O_2 [154], did not affect lifespan [21], indicating that the role of H_2O_2 on lifespan in *C. elegans* is complex. In mice, catalase has been reported to extend lifespan when specifically targeted to the mitochondrion [155], however these results have not yet been repeated independently and the lab that generated the transgenic mouse has acknowledged its own difficulties in repeating the results [156]. In flies, by contrast, ectopic expression of catalase in the mitochondrion shortens *Drosophila* lifespan [149], indicating that reducing mitochondrial H_2O_2 can be deleterious for survival. Interestingly, a decrease in the levels of different peroxiredoxins is associated with shorter lifespan in worms, flies and mice [157–159], whilst boosting peroxiredoxin levels has been associated with an extension of lifespan in flies [158,160]. It may seem paradoxical that overexpression of peroxiredoxins and ectopic expression of catalase have opposing effects on lifespan. However, some *in vitro* studies indicate that catalase is much more effective than several peroxiredoxins at detoxifying H_2O_2 [161]. Targeting expression of catalase to the mitochondrion may cause a much more important reduction in H_2O_2 than overexpression of peroxiredoxins 3 and 5 and affect signaling without offering further protection against oxidative stress. This would fit with a model where H_2O_2 levels need to be maintained within a certain range: excessive mitochondrial H_2O_2 would cause oxidative damage (by generation of. OH), and very low levels would interrupt signaling affecting inter-organelle communication. It would be interesting to test this model *in vivo* using the powerful genetic tools available in flies and worms combined with the latest technology to measure ROS and oxidative damage (Fig. 2).

Lifespan studies in flies and worms are affected by diet and genetic background [162], so it is possible that ROS extends lifespan in certain conditions but not in others. Similarly, differences could be related to the nature of expression i.e. where and when the transgene that alters ROS levels is expressed [163]. This indicates that ROS probably regulates lifespan through signaling, and not through the generation of oxidative

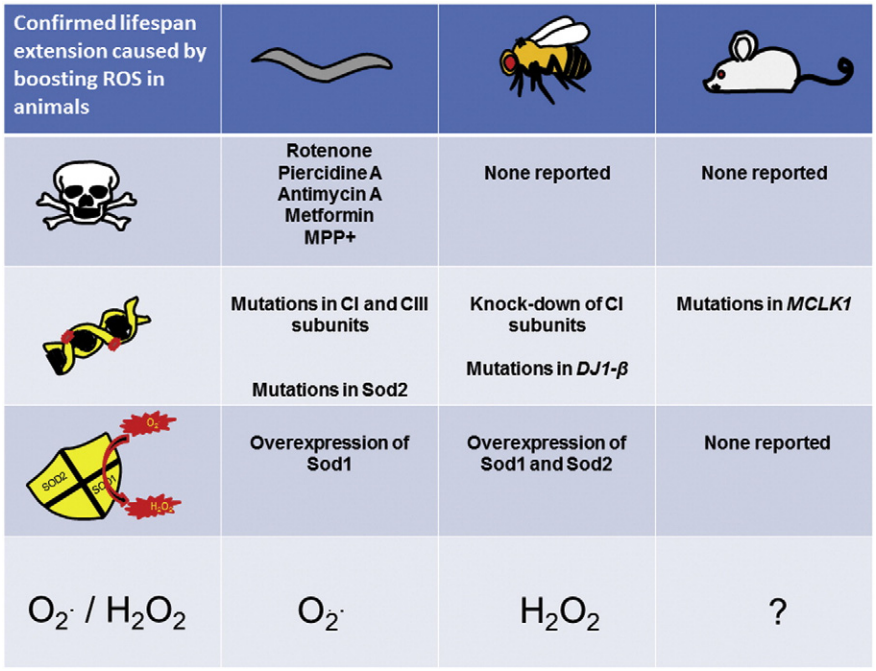

Fig. 3. Summary of the genetic and pharmacological approaches that extend lifespan in animals through a ROS mediated mechanism. Feeding metabolic poisons (e.g. rotenone or paraquat) or mutating ETC subunits that increase superoxide levels extends lifespan in worms. In flies knock-down of CI subunits, mutations in *DJ-1β* or overexpression in Sod1 and Sod2 increase H_2O_2 levels and extends lifespan. Although mutations in *MCLK1* in mice increase ROS levels, there is no definitive evidence that boosting ROS extends lifespan in mammals.

damage, since the positive or negative role of ROS as signaling molecules should depend on the interaction with the environment and the system of expression, whilst if they are causing oxidative damage the effect should be unchanged i.e. negative when oxidative stress is increased and positive when it is attenuated. Unfortunately, almost none of the studies mentioned in this section take advantage of the new techniques previously described to measure ROS *in vivo*. However, an important technical advantage has been introduced in lower organisms, which is that conclusions from most experiments are now being validated by genetic and pharmacological approaches that for example alter antioxidant levels e.g. [24] and [17] (Fig. 2).

7. Conclusions

Presently, we can conclude that mitochondria play an important role in the onset of aging and age-related diseases. However, if mitochondrial deterioration is a cause or a consequence of the aging process remains unresolved. The role of mitochondrial ROS is still controversial, with old data supporting MFRTA and new data challenging MFRTA and indicating that ROS can extend lifespan, at least in lower organisms such as worms or flies (Fig. 3). Superoxide seems to be the key ROS that extends lifespan in worms, whereas H_2O_2 seems to play this role in flies, conclusions in mice will have to wait for more mechanistic studies (Fig. 3). Future work should address which cells and cellular compartments are most important in generating ROS and how, when and where ROS are generated affects lifespan and/or age-related diseases. The new techniques to measure ROS *in vivo* that are already available and those that are on their way, combined with the state-of-art technology to edit the genome will undoubtedly help to achieve this objective.

Transparency document

The Transparency document associated with this article can be found in the online version.

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