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THE SPATIOTEMPORAL DYNAMICS OF LONGEVITY-DEFINING CELLULAR PROCESSES AND ITS MODULATION BY GENETIC, DIETARY AND PHARMACOLOGICAL ANTI-AGING INTERVENTIONS

Topic Editors
Vladimir I. Titorenko and Troy A. A. Harkness

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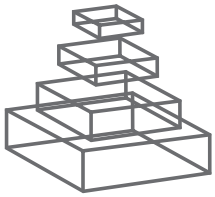

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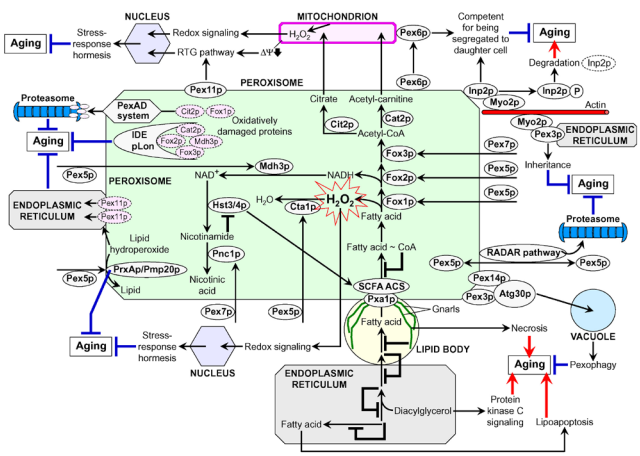

A model for the dynamic integration of peroxisomes into an endomembrane system governing cellular aging. Image from Beach A et al. (2012) Integration of peroxisomes into an endomembrane system that governs cellular aging. *Front. Physio.* 3:283. doi: 10.3389/fphys.2012.00283. Copyright © 2012 Beach, Burstein, Richard, Leonov, Levy and Titorenko.

Aging of multicellular and unicellular eukaryotic organisms is a highly complex biological phenomenon that affects a plethora of processes within cells.

This wide array of longevity-defining cellular processes–which are governed by an evolutionarily conserved signaling network–includes oxidative metabolism and protein synthesis in mitochondria, lipid and carbohydrate metabolism, NAD+ homeostasis, amino acid biosynthesis and degradation, ammonium and amino acid uptake, ribosome biogenesis and translation, proteasomal protein degradation, nuclear DNA replication, chromatin assembly

and maintenance, actin organization, apoptosis, necrosis, autophagy, protein folding, stress response, signal transduction, cell cycle, and cell growth.

The focus of this Frontiers Special Topic Issue is on an important conceptual advance in our understanding of how cells integrate and control these numerous processes and how genetic, dietary and pharmacological anti-aging interventions extend longevity by altering their functional states and spatiotemporal dynamics.

The Issue will highlight the various strategies used by evolutionarily diverse organisms for coordinating these longevity-defining cellular processes in space and time, critically evaluate the molecular and cellular mechanisms underlying such coordination, and outline the most important unanswered questions and directions for future research in this vibrant and rapidly evolving field.

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EDITORIAL
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The spatiotemporal dynamics of longevity-defining cellular processes and its modulation by genetic, dietary, and pharmacological anti-aging interventions

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Aging of multicellular and unicellular eukaryotic organisms is a highly complex biological phenomenon that affects a plethora of processes within cells. This wide array of longevity-defining cellular processes—which are governed by an evolutionarily conserved signaling network—includes oxidative metabolism and protein synthesis in mitochondria, lipid, and carbohydrate metabolism, NAD^+ homeostasis, amino acid biosynthesis and degradation, ammonium and amino acid uptake, ribosome biogenesis and translation, proteasomal protein degradation, nuclear DNA replication, chromatin assembly and maintenance, actin organization, apoptosis, necrosis, autophagy, protein folding, stress response, signal transduction, cell cycle, and cell growth (Fontana et al., 2010; Kenyon, 2010). The focus of this Frontiers Special Topic Issue is on an important conceptual advance in our understanding of how cells integrate and control these numerous processes and how genetic, dietary, and pharmacological anti-aging interventions extend longevity by altering their functional states and spatiotemporal dynamics. Collectively, the articles in this Issue highlight the various strategies used by evolutionarily diverse organisms for coordinating these longevity-defining cellular processes in space and time, critically evaluates the molecular and cellular mechanisms underlying such coordination, and outlines the most important unanswered questions and directions for future research in this vibrant and rapidly evolving field. Iliadi et al. (2012) eloquently review the use of the fruit fly *Drosophila* as an advantageous model organism to study the mechanisms underlying healthy aging. They provide a broad overview of the important advances that such mechanistic studies in *Drosophila* have made to our understanding of the age-related decline in muscle mass and strength, immune response, stress resistance, sexual behavior, and cognitive function. Walton and Pizzitelli (2012) demonstrate that peroxisome-derived oxidative imbalance in replicatively aging human fibroblasts elicits age-related mitochondrial damage and impairs mitochondrial function. Their findings imply that peroxisomal oxidative damage precedes and is causal to the global mitochondrial dysfunction observed in aging human cells entering a senescent state. Jazwinski and Kriete (2012) explore common principles in the ways by which yeast, nematode, fruit fly, mouse, and cultured human cells respond to partial mitochondrial dysfunction by activating retrograde signaling pathways. The authors critically evaluate how an integration of the retrograde response with other signaling pathways and downstream processes establishes a network that supports cell survival following various external perturbations and under a variety of age-related intracellular stresses. Based on a side-by-side comparison of molecular mechanisms underlying the retrograde responses in yeast and mammalian cells, the authors propose a concept in which these responses operate as a double-edged sword by (1) delaying aging and protecting from external stresses upon their short-term activation; and (2) causing cell death and promoting inflammatory disease if chronically activated in aging. Hou and Taubert (2012) provide excellent insights into important roles for lipid signaling and metabolism in defining longevity of the nematode *Caenorhabditis elegans*. The authors dissect molecular and cellular mechanisms through which the spatiotemporal dynamics of unsaturated fatty acids regulates longevity by modulating the Nuclear Hormone Receptor signaling and maintaining the integrity of various organellar membranes. They also explore the mechanistic links between hydrolysis of neutral lipids and several longevity-defining processes, discuss how lipid-derived signaling molecules impact signaling networks central to longevity assurance, and outline the essential roles of mitochondrial membrane lipids in longevity regulation via multiple cellular pathways. Postnikoff and Harkness (2012) offer us a comprehensive and thought-provoking discussion of how numerous direct and indirect interactions between the Anaphase Promoting Complex and the Forkhead box transcriptional factors orchestrate health- and longevity-related processes in evolutionarily distant organisms by balancing cell-cycle progression and ubiquitin-dependent protein turnover with stress responses and longevity. Their discussion demonstrates that the simple yeast model could be of use to untangle the complex web of Forkhead box protein regulation in higher eukaryotes. Brown and Naidoo (2012) outline recent progress in understanding how intrinsic and extrinsic stresses that impair proteostasis in the endoplasmic reticulum (ER) elicit the unfolded protein response (UPR) signaling pathways that have been shown to play a pivotal role in maintaining cellular protein homeostasis and have been implicated in various age-related diseases. The authors critically evaluate the evidence that progressive age specific changes in the ER stress response and the resulting decline in UPR signaling underlie numerous age-related pathologies, including neurodegenerative diseases, cancer, and inflammation. Kyryakov et al. (2012) provide evidence that

the longevity-extending effect of caloric restriction in chronologically aging yeast is due in part to a specific pattern of age-related changes in trehalose concentration elicited by this dietary regimen. They investigate how single-gene-deletion mutations that in chronologically aging yeast alter trehalose concentrations prior to quiescence and following entry into a quiescent state impact lifespan and influence the chronology of oxidative protein carbonylation, intracellular reactive oxygen species, protein aggregation, thermal inactivation of a protein in heat-shocked yeast cells and its subsequent reactivation in yeast shifted to low temperature. Based on their findings, the authors propose a model for molecular mechanisms underlying the essential role of trehalose in defining yeast longevity by modulating protein folding, misfolding, unfolding, refolding, oxidative damage, solubility, and aggregation throughout lifespan. Beach et al. (2012) summarize the evidence that peroxisomes are dynamically integrated into an endomembrane system that governs cellular aging. They discuss various strategies through which peroxisomes are integrated into this endomembrane system, critically evaluate the molecular mechanisms underlying each of these strategies, analyze the age-related dynamics of communications between peroxisomes and other cellular compartments composing the longevity-defining endomembrane system. Communications between peroxisomes and other cellular compartments are explored that influence the development of pro- or anti-aging cellular patterns. Based on the available evidence, the authors propose a model for the integration of peroxisomes into the endomembrane system governing cellular aging. Pallavi et al. (2012) provide insights into molecular and cellular mechanisms underlying longevity-extending and anti-tumor effects of caloric and dietary restriction in mouse models. The authors explore the potential of administering various caloric restriction dietary regimens as a promising way of decelerating the development of breast, colorectal, colon, and ovarian cancers in humans and as a therapeutic approach for cancer treatment in clinical settings.

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Healthy aging – insights from *Drosophila*

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Human life expectancy has nearly doubled in the past century due, in part, to social and economic development, and a wide range of new medical technologies and treatments. As the number of elderly increase it becomes of vital importance to understand what factors contribute to healthy aging. Human longevity is a complex process that is affected by both environmental and genetic factors and interactions between them. Unfortunately, it is currently difficult to identify the role of genetic components in human longevity. In contrast, model organisms such as *C. elegans, Drosophila*, and rodents have facilitated the search for specific genes that affect lifespan. Experimental evidence obtained from studies in model organisms suggests that mutations in a single gene may increase longevity and delay the onset of age-related symptoms including motor impairments, sexual and reproductive and immune dysfunction, cardiovascular disease, and cognitive decline. Furthermore, the high degree of conservation between diverse species in the genes and pathways that regulate longevity suggests that work in model organisms can both expand our theoretical knowledge of aging and perhaps provide new therapeutic targets for the treatment of age-related disorders.

Keywords: *Drosophila*, lifespan, aging, genetics, environment

INTRODUCTION

Aging generally refers to the process of getting chronologically older and it is typically accompanied by senescence, the gradual loss of physiological functions. Both of these processes are to some degree, inevitable for all living organisms. Chronological aging is primarily predetermined by heredity, whereas senescence results from a complex interaction between environmental and genetic factors.

During the last century, advances in medical technology have significantly contributed to extension of human longevity. According to data from the United Nations, US Census Bureau, Statistical Office of the European Communities, and National Institute of Aging (NIA) there are several trends in global aging: (1) *The overall population is aging*. For the first time in history, people aged 65 and over will outnumber children under the age of 5. (2) *Life expectancy is increasing*. Most countries, including developing countries, show a steady increase in longevity over time. (3) *The number of oldest old is rising*. People aged 85 and over are now the fastest growing portion of many national populations. (4) *New economic challenges are emerging*. Population aging will have dramatic effects on social entitlement programs, labor supply, trade, and savings around the globe and may demand new fiscal approaches to accommodate a changing world (Doriansky et al., 2007). Altogether, these findings further emphasize the need to understand how to promote healthy aging rather than just extending lifespan.

Non-genetic factors such as nutrition, environmental quality, psychosocial factors, and lifestyle play an important role in healthy aging. However, experimental studies have indicated that the heritable component has a significant impact on the senescence of invertebrates and mammals and accounts for approximately 35% of the variance in lifespan (see Finch and Tanzi, 1997; Finch and Ruvkun, 2001 for details). Over the past few decades and especially since the completion of the Human Genome Project, a great number of studies have been carried out aimed at identifying the genetic factors that affect human lifespan. Despite the wide range of approaches utilized to identify longevity genes in humans including, linkage analysis (Puca et al., 2001; Tan et al., 2004), candidate–gene association analysis (Park et al., 2009; Lopez et al., 2012), and longitudinal studies (Nybo et al., 2003; Soerensen et al., 2010), progress has been limited due to the fact that these methods can be experimentally intensive, time-consuming, and poorly replicated. In addition, it is very difficult to control for variations in environmental conditions. In contrast, it is easier to minimize the effect of environmental conditions, lifestyle, and genetic background in model organisms. Moreover, while studies of longevity in humans are limited to demographic observations of externally apparent symptoms, model organisms can be genetically manipulated and phenotypically characterized in much greater depth allowing theories of aging and age-related disease to be experimentally tested.

Model organisms have also revealed that diverse organisms may share common biological mechanisms regulating longevity (Guarente and Kenyon, 2000; Helfand and Rogina, 2003; Hekimi, 2006). For example, studies have shown that dietary restriction, without malnutrition, can extend lifespan, and delay the onset of age-related pathologies in a wide range of species including yeast, worms, flies, and mammals (Good and Tatar, 2001; Barger et al., 2003; Koubova and Guarente, 2003; Johnson, 2008; Mair and Dillin, 2008; Skinner and Lin, 2010). Similarly, several metabolic processes and signaling pathways have also been shown

to have an evolutionarily conserved role in aging. For example, the insulin/insulin growth factor (IGF) signaling pathway (Kenyon et al., 1993; Clancy et al., 2001; Tatar et al., 2001; Bluher et al., 2003), histone deacetylases such as rpd3/Sir2 (Kim et al., 1999; Tissenbaum and Guarente, 2001; Rogina et al., 2002), and genes involved in oxidative stress (Phillips et al., 1989; Sohal and Weindruch, 1996; Parkes et al., 1998; Honda and Honda, 1999; Migliaccio et al., 1999; Sun and Tower, 1999; Taub et al., 1999; Tower, 2000), all exert evolutionarily conserved effects on aging and lifespan in a wide range of model organisms. The extent of evolutionary conservation in both the outward signs of aging and the environmental and genetic factors that influence it, suggests that aging itself is an evolutionarily conserved process and not simply an inevitable deterioration of biological systems. As such, studies of both the effects and causes of aging in model organisms can yield valuable insight into the molecular and cellular processes that underlie aging in humans.

Various model systems, including yeast, *C. elegans*, and rodents have been used to study the processes regulating organismal longevity. *Drosophila* also has many advantages to studying the biology of aging. For example, flies represent an optimal compromise between physiological, genetic, and anatomical relevance to humans as well as genetic, physiological, behavioral, and demographic power (Boulianne, 2001; Reiter et al., 2001; Helfand and Rogina, 2003; Jafari et al., 2006; Iliadi and Boulianne, 2010). Fruit flies also have a number of strengths that specifically aid in studies of aging and longevity. For example, while the lifespan of flies is relatively short (60–80 days), flies still exhibit age-related decline in several behaviors (Grotewiel et al., 2005). Furthermore, the demarcation between development and adulthood is much clearer in insects than other model organisms (adulthood being defined as eclosion from the pupal case). Large brood sizes also make it possible to measure survival in large numbers of individuals within each experimental cohort in controlled environments and to test the functional consequences of senescence either longitudinally in individuals or as sampled from the aging population. Finally, since most cells in adult flies are postmitotic (except a few cells in the gut, malpighian tubules, and gonads), the age-related decline in cellular function can be examined without interference from newly dividing cells. In this review, we will discuss how studies in *Drosophila* can provide insight into the mechanisms regulating healthy aging.

PHYSIOLOGY OF SENESCENCE

Aging is a universal process and all species studied show age-related functional declines. However, different species age at different rates likely due to different fitness strategies employed to survive and reproduce in a competitive environment. The rate of aging can also be quite variable between individuals of a given species. Furthermore, while all cells, tissues, and organs show a functional decline over time, not all tissues experience aging at the same rate. Some systems may change slowly, while others decline rapidly, and some may even show periods of increased function (Spirduso et al., 2005). Despite the extensive variability both between species, and within individuals, several tissues exhibit physiological senescence in both invertebrates and mammals, including a decline in muscle strength (Nair, 2005; Augustin and Partridge, 2009; Demontis and Perrimon, 2010), immune response (Hoffmann, 2003; Flajnik and Du Pasquier, 2004), stress resistance (Service et al., 1985; Rose, 1999; Murakami, 2006), reproduction (te Velde and Pearson, 2002; Novoseltsev et al., 2005; Tatar, 2010; Luo and Murphy, 2011), and cognition (Horiuchi and Saitoe, 2005; Grady, 2008).

AGING, MUSCLE STRENGTH, AND LOCOMOTOR FUNCTION

Sarcopenia or loss of muscle mass and function, is perhaps one of the most marked problems associated with aging and has been described for both invertebrates and higher organisms (Fisher, 2004; Augustin and Partridge, 2009). At the cellular level, this disease reflects mitochondrial dysfunction, altered apoptotic and autophagic signaling, as well as trace metal dyshomeostasis (Marzetti et al., 2009). Morphologically, sarcopenia is characterized by a decrease in both the number and size of individual fibers (Larsson et al., 1978) and an increase in the extracellular space and deposition of protein aggregates within the interstitial matrix (Kim et al., 2008). Despite the fact that *Drosophila* and human muscles show essential differences in fiber type, innervation, and regeneration, they both exhibit age-related morphological and functional changes. For example, myofibrils of old flies display reduced sarcomere length, increased *in vivo* interfilament spacing, and increased lattice disorder, showing a loss of ultrastructural integrity and acute sarcopenia (Miller et al., 2008). Interestingly, heart muscle structure and cardiac performance are also progressively impaired with age in flies (Nishimura et al., 2011).

Additionally, recent studies in flies have indicated a role for the well-known longevity-regulating pathways in the coordination of muscle aging. For example, the activation of dFOXO and its target 4E-BP in muscle decelerates aging and reduces the age-related accumulation of protein aggregates, whereas *foxo* mutants accelerate loss of proteostasis (Demontis and Perrimon, 2010). RNAi-mediated knockdown of the mitochondrial superoxide dismutase 2 (SOD2) in muscle tissue decreases locomotion and shortens lifespan (Martin et al., 2009). Likewise, results from another study have shown that overexpression of p38 MAP kinase extends *Drosophila* lifespan in a MnSOD-dependent manner while inhibition leads to early lethality and accelerates age-related motor (muscle-restricted) dysfunction (Vrailas-Mortimer et al., 2011).

Behavioral locomotion assays can offer an accurate way of assessing changes in muscle function. In *Drosophila*, many studies have shown that motor functions are significantly reduced with aging (Le Bourg and Lints, 1984; Fernandez et al., 1999; Simon et al., 2006; Martinez et al., 2007; Rhodenizer et al., 2008). Among various available methods for the assessment of locomotor activity, the startle-induced climbing behavior (negative geotaxis) is a reliable and informative assay. Usually, in these experiments, flies are tapped down to the bottom of their test vial and the distance the flies climb up the vial within a particular period of time is measured. This assay measures a whole complex of different behaviors including the escape reflex in response to mechanical stress, negative geotaxis (an inner orienting response and movement in opposition to gravitational cues), climbing ability, and locomotor activity itself. All of these behaviors reflect the functional status of muscle and locomotor function to varying degrees. Interestingly, detailed studies have revealed that the age-related decline in performance does not depend on the density of animals in the

test vial or the housing conditions (Cook-Wiens and Grotewiel, 2002; Goddeeris et al., 2003) but rather is primarily due to an age-dependent decrease in locomotor speed (Rhodenizer et al., 2008) similar to that seen in humans, suggesting a conserved mechanism. The relative simplicity and reproducibility of these behavioral assays makes locomotor activity a useful biomarker for healthy aging.

AGE-SPECIFIC IMMUNE RESPONSE

The aging of an organism is accompanied by weakening of both the acquired and innate immune response (immunosenescence) to a wide range of pathogens. This process is mainly characterized by a gradual depression of cellular function throughout the immune system that, not only decreases immune response, but can also provoke the development of autoimmune disorders (Prelog, 2006). The innate immune response represents the first line of defense, comprising multiple pathways and systems, which are functionally conserved in both vertebrates and invertebrates (Flajnik and Du Pasquier, 2004). In *Drosophila* an innate immune response consists of several general components including pathogen detection via receptors that activate the Toll or Imd (Immune deficiency) signaling pathways (De Gregorio et al., 2002), hormonal regulation by means of juvenile and 20-hydroxy-ecdysone hormones that antagonistically induce antimicrobial peptide genes (Flatt et al., 2008a), prophenoloxidase cascade resulting in melanization (encapsulation of pathogens) (Tang, 2009), and cellular events such as phagocytosis (Williams, 2007). In contrast, acquired or adaptive immunity is an antigen-specific response that lasts a very long time and can generate "immune memory" to protect an organism against re-exposure to the same antigens. This type of response has been described for vertebrates and represents the second line of defense that is initiated when the non-specific, innate immune response is unable to deal with an invading pathogen.

Several studies suggest that aging has a profound effect on the status of the immune system in insects. For example, reduced phenoloxidase activity was observed in aged bumblebees (Whitehorn et al., 2011) and crickets (Adamo, 2004). Adult scorpionflies show a dramatic decrease in phagocytic capacity with age, even though cell numbers remained fairly constant (Kurtz, 2002). To date, the majority of studies in *Drosophila* have focused on the analysis of genes that exhibit age-related transcriptional changes. Interestingly, the most striking genome-wide (Pletcher et al., 2002; Landis et al., 2004; Sarup et al., 2011) and body-wide (Seroude et al., 2002) age-related increase in expression was found for genes that are involved in the immune response. However, it is unclear why immune response genes are up-regulated during aging. One possible explanation is that organisms increase their expression of immune related genes in response to prolonged exposure to pathogens throughout their life. Alternatively, it may reflect the decline in functional capacity of innate immunity with age (Zerofsky et al., 2005; Ramsden et al., 2008; Sarup et al., 2011).

Studies such as these may be useful for identifying potential biomarkers of immunosenescence, although additional microarray and proteomic studies will be required to identify novel markers and validate genes/proteins previously shown to exhibit age-related expression changes following infection or in the absence of infection. Additional studies will also facilitate the development of appropriate molecular biomarkers of immunosenescence and the possible discovery of a completely novel mechanism that does not involve any of the genes/pathways identified to date. Another approach is to use cellular immunity biomarkers such as hemocytes. In adult flies, hemocytes either freely float within the hemolymph or are sessile (Williams, 2007). They are primarily specialized for phagocytosis and encapsulation. In a recent study, Mackenzie et al. (2011) compared the numbers and activity of the circulating hemocytes in flies of different ages. They found that the hemocyte population, which is responsible for clearing microbes from the hemocoel, becomes less able to phagocytose microbes with age due to fewer cells with phagocytic activity. The number of circulating hemocytes in females also significantly declined with age. Given the similarity between plasmatocyte hemocytes in adult *Drosophila* and the monocyte lineage that gives rise to macrophages in vertebrates, coupled with the relative simplicity of these measurements, this method has the potential to be a valid way of monitoring healthy aging of the fly immune system. Furthermore, using techniques such as this to analyze age-specific survival and the ability to protect against, and clear infection, will improve our understanding of which components of the immune system are responsible for immunosenescence.

AGING AND STRESS RESISTANCE

The rate of living theory is probably the oldest among theories of aging (Pearl, 1928). Pearl formulated his theory based on Rubner's observation of a negative relationship between metabolic rate, body size, and longevity. Pearl's theory postulated that the lifespan of an organism is related to its metabolic rate; such that individuals (within a given species) with a higher metabolic rate (high level of energy consumption) will have a shortened lifespan compared to individuals with a lower rate. Consistent with this theory, observations in *Drosophila melanogaster* and *Musca domestica*, showed that flies raised at lower ambient temperatures have a lower level of metabolic activity and much longer lifespan compared to flies raised at higher temperatures (Miquel et al., 1976; Sohal, 1986; Farmer and Sohal, 1987). The association between metabolic rate and lifespan was also supported by artificial selection experiments (Riha and Luckinbill, 1996; Arking et al., 2002). Although this theory is not universally accepted (Austad and Fischer, 1991; Speakman et al., 2004; Van Voorhies, 2004) it nevertheless formed the basis for a free radical theory of aging (Harman, 1956). According to Harman's theory, free radicals, produced as a by-product of oxidative phosphorylation can damage important biological molecules. Accumulation of this damage over time, accounts for the effects of aging and eventually leads to death. The reactive oxygen-containing species (ROS) can covalently modify biomolecules such as nucleic acids, proteins, and lipids and cause oxidative stress. The level of this stress is determined by the misbalance between the production of ROS on the one hand and the efficiency of the antioxidant defense and repair processes on the other hand. Several studies in *Drosophila* have shown that flies selected for extended longevity exhibit resistance to oxidative stress (Service et al., 1985; Arking et al., 1991). In healthy cells, resistance to oxidative stress is accomplished by means of a complex defense system consisting of both enzymatic and non-enzymatic components, the main role of which is to breakdown and/or to neutralize ROS. This

defense complex includes superoxide dismutases (Cu/Zn SOD and MnSOD), catalase (cat), and glutathione-peroxidase. Firstly, superoxide dismutases convert superoxide to hydrogen peroxide and then catalase and glutathione-peroxidase remove the hydrogen peroxide from the intracellular environment by converting it to water. It is thought that these defense mechanisms become less efficient with aging and in some disease conditions, and ROS damage accumulates within cells in the form of chromosomal aberrations, membrane destabilization, loss of essential protein function, and ATP depletion. In humans, damage caused by ROS is not only thought to contribute to the aging process but has also been linked to numerous neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (Robberecht, 2000) and Alzheimer's disease (Smith et al., 2000).

Several selection experiments have shown that some long-lived *Drosophila* strains express higher than normal levels of SOD (Tyler et al., 1993; Dudas and Arking, 1995; Hari et al., 1998). Interestingly, reverse selection of the long-lived flies for short lifespan restores antioxidant gene expression to control levels (Arking et al., 2000). Studies of the effect of single gene mutations that affect the lifespan of flies may also serve as a confirmation of the relationship between aging and resistance to stress. For example, reduced expression of *methuselah* (encoding a G protein-coupled receptor), causes a significant increase in lifespan and resistance to a number of stresses including starvation, high temperature and paraquat (Lin et al., 1998). In contrast, mutations that affect genes involved in ROS defense such as SOD or catalase have a negative impact on lifespan and show hypersensitivity to a variety of agents that generate ROS including Cu^2, paraquat, ionizing radiation, and hyperoxia (Campbell et al., 1986; Mackay and Bewley, 1989; Phillips et al., 1989). The relationship between oxidative stress and lifespan has also been illustrated by overproducing ROS protective enzymes. Using an inducible expression system based on the FLP recombinase; Tower and colleagues found a positive effect of SOD overexpression on *Drosophila* lifespan (Sun and Tower, 1999; Tower, 2000). A similar effect was seen when human SOD was overexpressed in motorneurons (Parkes et al., 1998). Remarkably, the extension of lifespan did not change the overall metabolic rate of the flies, suggesting that the observed effect was due to increasing oxidative stress resistance. Lifespan was also extended by simultaneous overexpression of SOD and catalase (Orr and Sohal, 1994). Transgenic flies which overexpress these two tandem acting enzymes exhibited a one-third extension of lifespan as well as reduced oxidative damage and a delayed loss of locomotor activity.

Despite the above examples, it is unclear whether the activity of antioxidant enzymes is required to extend lifespan. For example, the activity of several antioxidant enzymes were essentially the same in *Drosophila* lines selected for long or short lifespan. Similarly, the glutathione content (Mockett et al., 2001), SOD allele frequency (Force et al., 1995), and the electrophoretic mobility of SOD enzymes (Luckinbill et al., 1989), were also similar in short and long-lived lines. Furthermore, other studies showed that transgenic manipulations of antioxidant enzyme expression had little or no effect on lifespan (Seto et al., 1990; Reveillaud et al., 1991; Orr and Sohal, 1992, 1993; Orr et al., 2003). These discrepancies may be explained by differences in genetic background, the level of transgene expression, the experimental methodologies used and/or the lack of adequate controls. Overall, these studies suggest that antioxidant defense may not be essential for lifespan extension but rather play a primary role in healthy aging. Unfortunately, there are only a few studies that report the functional consequences of manipulating the levels of antioxidant enzymes in *Drosophila*. Nevertheless, several facts appear to indicate that antioxidant defense affects healthy aging. First, in those studies where behavioral analysis was reported, the increased or reduced/abolished antioxidant enzyme activity was associated with delayed or accelerated functional senescence, respectively (Orr and Sohal, 1994; Ruan et al., 2002; Piazza et al., 2009; Hirano et al., 2012). Second, increased antioxidant enzyme activity increases stress resistance while reduced/abolished activity has the opposite effect, in most studies (see Le Bourg, 2001b for details). Third, the positive effect of increased antioxidant expression on lifespan tends to be most apparent in short-lived genetic backgrounds. Using data from all published studies examining overexpression of antioxidant enzymes, Orr and Sohal (2003) found a clear negative correlation between lifespan extension with overexpression of the antioxidant enzymes and the lifespan of controls. It seems that when an organism is "genetically" under great stress, the enhancement of its antioxidative defense properties can have a beneficial (rescue) effect whereas, under optimal conditions including optimal (wild type) genetic background such enhancement is less effective or even ineffective. Finally, the progression and severity of many age-related pathologies and diseases can be increased or diminished through modulation of the expression of genes involved in oxidative defense. Salmon et al. (2010) summarized the data showing that healthspan rather than lifespan is strongly affected by antioxidant status. Altogether, the data suggests that resistance to oxidative stress can be used as an indicator of healthy aging.

AGING, SEXUAL BEHAVIOR, AND REPRODUCTION

From an evolutionary point of view, there is no doubt that a higher level of reproductive success positively contributes to total fitness (Ehrman and Parsons, 1981). At the same time, life history theory (antagonistic pleiotropy) proposes that higher levels of reproduction are negatively correlated with survival (Williams, 1957). This so-called "cost of reproduction" concept has been widely accepted and demonstrated in a number of experimental studies (see reviews; Rose and Bradley, 1998; Partridge et al., 2005; Flatt and Promislow, 2007; Tatar, 2010). For example, selection experiments in *Drosophila* have shown that direct selection for extended longevity decreases early reproduction (Zwaan et al., 1995), while selection for postponed reproduction leads to increased longevity (Rose, 1991; Partridge et al., 1999). Female flies sterilized by either irradiation or by the sterile ovo^D mutation have reduced age-related mortality (Sgro and Partridge, 1999). However, several experiments have cast doubt on the relationship between lifespan and fecundity. Using *Drosophila* mutants that lack a proliferating germ line, Barnes et al. (2006) have shown that life-long germ line ablation reduced longevity in females and either had no effect, or gave rise to a slight extension of longevity in males. In a similar study, loss of germ cells late in development or in the adult extends lifespan and modulates components of insulin/insulin-like growth factor signaling in *Drosophila* (Flatt et al., 2008b) and

C. elegans (Arantes-Oliveira et al., 2002). Examination of lifespan in various mutant or transgenic animals, also argue against a direct cost of reproduction on lifespan. In *C. elegans*, mutations in the *age-1* and *daf-2* genes result in life extension, without changes in reproductive ability (Johnson et al., 1993; Gems et al., 1998). In *Drosophila*, females bearing mutations in either the insulin-like receptor gene (Tatar et al., 2001), or the insulin-receptor substrate *chico* (Clancy et al., 2001), show decreased fertility and increased lifespan consistent with the cost of reproduction theory. However, the extended longevity and reduced fecundity appear to be unrelated since in combination with the dominant sterile mutation ovo^D (which blocks oogenesis and extends female lifespan), *chico* mutant flies do not live as long as either fertile *chico* heterozygotes or sterile homozygotes (Clancy et al., 2001). Interestingly, several long-lived lines, including *Indy* mutants (Marden et al., 2003), *ecdysone receptor* mutants (Simon et al., 2003), or flies overexpressing the transcription factor *dFOXO* in adult head fat body (Hwangbo et al., 2004), do not exhibit reduced fecundity and, in some cases, have even shown an increase for these traits. It has been demonstrated in a series of *Drosophila* behavioral experiments, that sexual behavior and mating itself may have a profound negative impact on lifespan. For example, males that were supplied daily with virgin females showed decreased lifespan (Partridge and Farquhar, 1981). In females, mating reduces lifespan (Fowler and Partridge, 1989), possibly as a result of the competitive reallocation of limited physiological resources for courtship and egg production (Partridge et al., 1987), or from toxic peptides transferred to females in male seminal fluid (Chapman et al., 1995). However, it is unclear whether all of these factors are relevant in nature. For example, most females in nature are fertilized. They actively reject male courtship in many different ways including decamping behavior (running away, jumping, and flying away from the courting male) that may lead to full loss of contact with the courting male. In addition, female egg production and egg-laying rates greatly depend on ecological context, such as weather and climatic conditions, food availability, and the presence of predators.

In humans, the relationship between longevity and reproduction have been addressed in numerous studies, however, the results have yielded even more conflicting conclusions. The majority of these studies were focused on validation of *The Disposable Soma Theory of Aging*. One of the basic principles Kirkwood's evolutionary theory is that aging occurs as a result of decreased investment of resources in somatic maintenance and repair, to allow for increased allocation of resources toward reproduction (Kirkwood, 1977). This theory was tested using historical data sets collected from the British aristocracy living from the eighth to the nineteenth century (Westendorp and Kirkwood, 1998). It was shown that both the longevity of women, living at least up to 50 years (i.e., after the end of their reproductive life) and the longevity of men were negatively correlated with fertility. Thus, the authors came to the conclusion that these results confirm the existence of a trade-off between longevity and fertility in humans. While this study had a large resonance in the scientific and public press, it has been severely criticized (Gavrilov and Gavrilova, 1999; Le Bourg, 2001a; Gavrilova and Gavrilov, 2005; Mitteldorf, 2010) primarily because of the quality of the database and inadequate statistical approach. In another study, using data from 153 countries, a highly significant positive correlation was observed between lifespan and fecundity (Thomas et al., 2000). Also, in a more detailed genealogical analysis of British aristocrats, a significant correlation between human lifespan and fertility was found when the effects of health and of mortality selection during childbearing ages were considered (Doblhammer and Oeppen, 2003). There are many additional reports that indicate a positive (Muller et al., 2002; McArdle et al., 2006) or inconclusive association (Le Bourg et al., 1993; Lycett et al., 2000) between lifespan and fertility in humans (see also Gavrilova and Gavrilov, 2005 for historical review of the relevant studies). Le Bourg (2007), in his comprehensive review summarizes most of the available data and concludes that, at present, there is insufficient evidence to conclude that longevity requires limited resources to be invested in somatic maintenance, thus reducing the availability of resources for reproduction. In fact, as the author points out, even if a relationship between these variables does exist, it depends more on the population under study than on a general mechanism linking longevity and fertility.

Aging has a negative impact on sexual activity and reproductive function for both sexes. Age-related decline in these functions is caused by numerous physiological changes particularly in the neuroendocrine and reproductive systems. In women these changes are mainly manifested in the reduction in follicle number and ovarian function and, as a consequence, in reduced secretion of estrogens and progesterone (Djahanbakhch et al., 2007). In men, the progressive decline in sexual function is accompanied by changes in levels of testosterone and associated reproductive hormones (Murray and Meacham, 1993). There is a fundamental difference between reproductive senescence in men and women. Women, show an abrupt loss of reproductive potential that marks the end of their "reproductive life," while in men, reproductive function declines more gradually and does not involve an acute drop in fertility.

In contrast to humans, *Drosophila* females continue to produce primary oocytes from stem or progenitor cells throughout their life, reaching a maximum in young females and followed by a steady decline (Novoseltsev et al., 2005). The quality of eggs or level of fertilization is also affected by age (David et al., 1975). Interestingly, detailed analysis of female reproductive senescence revealed that *Drosophila* egg-laying and mortality rates were dependent on mating history (Rogina et al., 2007). Female reproductive life can be divided into three epochs: optimal, vulnerable, and declining terminal. The first epoch of adulthood is characterized by a high rate of egg-laying after mating and has a reversible effect on mortality. During the second epoch, mating does not induce increased egg-laying but does result in an irreversible increase in mortality. Finally, at the terminal stage, females exhibit sharp reductions in egg-laying regardless of their chronological age. Despite the differences between flies and humans as to the effect of age on reproductive behavior, there may be some parallels between the processes that occur in follicles of aging humans and in egg chambers of aged flies under oxidative stress (Tatar, 2010). Cumulative oxidative damage caused by free radicals was suggested to impair the viability of developing follicles in humans (Tatone et al., 2008). In *Drosophila,* overexpression

of the antioxidant enzyme SOD in germ line stem cells leads to increased numbers of these cells in aged females (Pan et al., 2007).

Drosophila males exhibit a peak of sexual activity up to 4 weeks. During this period they show a fairly constant performance in such traits as multiple mating, latency time, duration of copulation, and level of fertility. These characteristics decline in a manner that is similar to the age-dependent course of survivorship (Economos et al., 1979). Male reproductive success can be measured either under competitive or non-competitive mating conditions. Selection for delayed senescence increased the male reproductive success in both competitive and non-competitive conditions. This selection also resulted in significant increases in the ability to recover from exhaustive mating bouts (Service, 1993). Several components of male reproductive behavior show a clear age-dependent decline including the time to begin copulation, duration of copulation, and the proportion of females that were remated (Service, 1993). Moreover, males that had been subjected to selection for delayed senescence were superior to control males (rapid senescence) with regards to one of the components, sperm competition (sperm defense).

Overall, the peculiarities of *Drosophila* reproductive history allow us to conclude that fruit flies exhibit obvious senescence in reproductive functions. The progression of senescence can be monitored by measuring the age-related changes in sexual and reproductive behaviors. Delay in reproductive senescence may contribute to the general extension of lifespan, which is more important in healthy aging.

SENESCENCE OF COGNITIVE FUNCTION

The gradual impairment of cognitive function is one of the main components of the normal aging process. Age-related decline in cognitive function may vary considerably between individuals and in the cognitive processes affected such as attention, memory, or decision-making. Progressive impairment of cognitive function is frequently associated with age-related degenerative brain disorders such as different types of dementia, including Alzheimer's and Huntington's disease, vascular and Parkinson's dementia, and Lewy body disease. These diseases are becoming more and more common amongst aged people across the globe and represent a growing clinical and social issue.

Attention is a key cognitive process that affects virtually all other cognitive functions. For example, it has been suggested that attentive processes may be involved in orientation, concentration, and filtration of distracting information (McDowd and Shaw, 2000). Older people usually have some difficulties with attention tasks that require dividing or switching of attention between multiple inputs (Verhaeghen and Cerella, 2002). An early symptom of Alzheimer's disease also involves problems with selective attention. Specifically, Alzheimer's patients exhibit a proportionally greater deficit in inhibitory and visual search tasks (Levinoff et al., 2004) as well as in tasks that require the inhibition of automatic cognitive ability (Perry et al., 2000).

Memory impairments are among the most commonly recognized cognitive changes in senescent humans. They arise from defects in the encoding, storage, and retrieval of information. In general, these defects can be classified into several major groups: (1) "sensory memory" – retains information only long enough to operate on it (object recognition), and requires the contribution of different sensory inputs; (2) "working memory" also known as "short-term memory" – actively holds new information in the mind allowing temporal tasks such as reasoning and comprehension to be performed, and is required for further information processing; (3) "long-term memory" – where information can be essentially stored for a lifetime. Working memory is impaired in old age (Hertzog et al., 2003; Oberauer et al., 2003), however, the mechanisms underlying this impairment are still under debate. Loss of long-term memory in aged humans may be associated with a number of causes. First of all, there are several differences between the long-term memory defects observed during normal aging and those that are associated with some pathological conditions. In normal aging, the memory defects are mainly the result of inconsistent encoding and retrieval strategies, whereas pathologies such as Alzheimer's disease, are characterized by a selective and severe deterioration in the consolidation and storage of new information. In other words, in normal age-related memory loss, individuals may express a sense of frustration due to an inability to recall specific memories, while in Alzheimer's disease patients forget the memory entirely, including the context in which the memory was formed.

Evidence from a wide range of cross-sectional studies suggests that many of the individual differences in age-related cognitive function are associated with differences in sensory function such as hearing or sight. Moreover, when this variance in sensory function is statistically controlled, the differences in cognitive function disappear (Baltes and Lindenberger, 1997). Many cognitive processes such as problem solving, goal-directed behavior, and decision-making require the integration and processing of information from numerous primary inputs for its effective performance.

The study of learning and memory in fruit flies began in the laboratory of Seymour Benzer more than three decades ago. Using chemical mutagenesis approaches coupled with a reliable method designed to specifically induce learning and memory, several mutants were isolated (Quinn et al., 1974). Studies of these mutants have provided insight into the cellular and biochemical mechanisms underlying learning and different kinds of memory formation (Dubnau and Tully, 1998). Flies have multimodal sensory modalities that gather information about the external world and translate it by means of the nervous system into an appropriate behavioral response. In fact, flies possess a large number of sensory organs that result in the perception of taste, touch, smell, hearing, and vision (Stocker, 2004; Ebbs and Amrein, 2007; Gerber and Stocker, 2007; Kernan, 2007; Ting and Lee, 2007). Many studies clearly demonstrate that fruit flies are capable of both associative and non-associative learning and memory. For example, flies can learn to move toward odors previously associated with reward, or avoid an odor that has been paired with punishment (Tempel et al., 1983; Tully and Quinn, 1985). They can also learn to recognize different visual, tactile, and spatial cues (Wustmann and Heisenberg, 1997; Heisenberg et al., 2001). Interestingly, *Drosophila* males show a plasticity in courtship behavior, through a complex process that may include habituation, sensitization,

operant, and classical learning (Siegel and Hall, 1979; Kamyshev et al., 1999; Griffith and Ejima, 2009). Several recent studies have shown that flies may demonstrate attention-like (van Swinderen, 2007), goal-driven (Pick and Strauss, 2005), and decision-making behaviors (Zhang et al., 2007). To date, *Drosophila* has also been successfully used as a model system to reveal molecular, physiological, and behavioral mechanisms of several human neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's (Chan and Bonini, 2000; Lu, 2009; Bonner and Boulianne, 2011).

Similar to other organisms, *Drosophila* exhibit age-related reductions in learning ability and memory performance. In early studies, Le Bourg (1983) investigated the role of age on non-associative learning using a proboscis extension reflex (PER). This reflex represents a stereotyped response to the activation of chemoreceptors located on the foreleg tarsi by sucrose. Repeated application of sucrose caused habituation to the stimulus whereby the fly stopped extending its proboscis in response to application. In this study, flies did not show any age-related effect on habituation as measured either by the percentage of flies that were able to habituate or the number of training sessions required for habituation. Aged individual flies showed impairment in habituation, specifically in the speed of memory acquisition in this learning task (Fois et al., 1991). Decrease in learning performance was observed between 3 and 35 days, and reached a plateau after 35 days. In another paradigm, the conditioned suppression of PER, flies learned to associate sucrose stimulation with negative reinforcement (Brigui et al., 1990). Both middle-aged and old flies required more training to associate the positive and negative stimuli. Age-dependent effects on PER were also observed in a visual discrimination task. The acquisition of PER suppression was delayed in middle-aged and old flies (Fresquet and Medioni, 1993). Age-related memory impairment in flies was also observed using a Pavlovian olfactory avoidance paradigm (Tamura et al., 2003). In this paradigm, flies learn to associate an electric shock with an odor. Depending on the training regimen induced, memory may persist either for minutes (short-term memory), hours (short and middle-term memory), or even days (long-term memory). Flies exhibited a weak reduction in immediate memory performance (right after training) by about 10 days of age, which did not progress up to the age of 50. However, short-term memory was severely impaired in 20 day old flies and declined to a minimum up to the age of 50 days. Interestingly, aging has differential effects on distinct memory forms. For example, old and young flies perform comparably with respect to protein synthesis-independent anesthesia resistant memory, while the protein synthesis dependent long-term memory is completely abolished in old flies (Mery, 2007).

Several studies on *Drosophila* learning and memory mutants confirm that not all forms of memory are equally affected by age. For example, young *amnesiac* (middle-term memory mutant) flies show similar memory retention to aged wild type flies (Tamura et al., 2003). Expression of this gene does not decrease with age and its overexpression does not suppress age-related memory impairment in a wild type background (Saitoe et al., 2005). Another memory mutant, *DCO,* delays age-related memory impairment without altering lifespan and memory at early ages (Yamazaki et al., 2007). Another method to induce and test learning and memory in flies is conditioned courtship suppression, where male flies learn to attenuate their courtship behavior after a negative experience of courting a fertilized female. This paradigm is based on natural sexual behavior and involves only natural stimuli such as visual and olfactory (pheromones) cues. Interestingly, 30-day-old wild type flies do not show a significant decrease in immediate or short-term memory with this assay, whereas mutants in the kynurenine pathway do (Savvateeva et al., 1999). Thus, natural selection may favor the maintenance of some specific forms of memory in aged flies. Taken together, these studies demonstrate that *Drosophila* can be utilized to identify and characterize the effect of age and the role of single genes in learning and memory and provide insight into core mechanisms of cognitive senescence in many species including humans.

CONCLUSION

It is well-known that advances in medicine and health care have significantly contributed to increased longevity in humans over the last 100 years. There is also a clear trend toward increased life expectancy including an increase in the numbers of people living to an advanced age and the number of people with chronic age-related diseases. These trends emphasize the need to understand the genetic and physiological factors underlying biological aging and particularly, those that promote healthy aging.

According to Arking (2003) there are three ways to extend lifespan: increasing early survival rate, increasing late survival rate, or delaying senescence. Remarkably, the first two do not affect basic aging processes. For example, the first one leads to a significant increase in mean but not maximum lifespan, while the second one leads to change in a maximum but not mean lifespan. Delayed senescence, in turn, leads to a significant increase in both the mean and maximum lifespan (Arking, 2005). This raises the question as to whether healthspan and delayed senescence are inter related. As stated above, while many genes have been shown to extend lifespan, these may have little or no ability to delay physiological senescence. In other words, the period of functional disability before death may increase despite the fact that the total duration of life is increased. Thus, the search for appropriate biomarkers applicable to monitor functional senescence is highly important with regards to healthy aging and age-related diseases.

Finally, it is likely that not all senescent physiological changes revealed in flies can be simply translated to humans. However, flies and humans often show very similar age-related physiological phenotypes suggesting that at least some of the basic biological properties and mechanisms that regulate longevity are conserved amongst species. Clearly, future studies focusing on mechanisms that promote healthy aging rather than lifespan extension could have significant impact on our elderly population and those suffering from age-related disorders.

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Effects of peroxisomal catalase inhibition on mitochondrial function

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Peroxisomes produce hydrogen peroxide as a metabolic by-product of their many oxidase enzymes, but contain catalase that breaks down hydrogen peroxide in order to maintain the organelle's oxidative balance. It has been previously demonstrated that, as cells age, catalase is increasingly absent from the peroxisome, and resides instead as an unimported tetrameric molecule in the cell cytosol; an alteration that is coincident with increased cellular hydrogen peroxide levels. As this process begins in middle-passage cells, we sought to determine whether peroxisomal hydrogen peroxide could contribute to the oxidative damage observed in mitochondria in late-passage cells. Early-passage human fibroblasts (Hs27) treated with aminotriazole (3-AT), an irreversible catalase inhibitor, demonstrated decreased catalase activity, increased levels of cellular hydrogen peroxide, protein carbonyls, and peroxisomal numbers. This treatment increased mitochondrial reactive oxygen species levels, and decreased the mitochondrial aconitase activity by ∼85% within 24 h. In addition, mitochondria from 3-AT treated cells show a decrease in inner membrane potential. These results demonstrate that peroxisome-derived oxidative imbalance may rapidly impair mitochondrial function, and considering that peroxisomal oxidative imbalance begins to occur in middle-passage cells, supports the hypothesis that peroxisomal oxidant release occurs upstream of, and contributes to, the mitochondrial damage observed in aging cells.

Keywords: peroxisome, subcellular organelle, catalase, mitochondria, hydrogen peroxide, redox, aging, senescence

INTRODUCTION

Peroxisomes are ubiquitous subcellular organelles present in almost all eukaryotic cells and house a wide variety of indispensable metabolic reactions, the majority of which produce hydrogen peroxide (H_2O_2) as by-product (reviewed by Schrader and Fahimi, 2006). Under normal circumstances, peroxisomally generated H_2O_2 is quickly degraded to water and oxygen by the primary peroxisomal antioxidant enzyme, catalase. Peroxisomes lack DNA and translational machinery and thus all peroxisomal membrane and matrix proteins are encoded by the nuclear genome, synthesized in the cytosol on free polyribosomes and imported post-translationally (reviewed by Lazarow and Fujiki, 1985). To accomplish this task peroxisomes possess dynamic import machinery, including cystolic receptors, membrane docking and translocation activities, and recycling capabilities.

Previous work has demonstrated that peroxisomes of late-passage cells (>PDL40) display mislocalized catalase and diminished antioxidant capacity as peroxisomal import competency is compromised (Legakis et al., 2002). Furthermore, the progressive mislocalization of catalase has been demonstrated to occur in cells as early as middle-passage (PDL30-40). Importantly, this leads to the disequilibrium between H_2O_2 producing and clearing reactions within the organelle, thus, transitioning the peroxisome into a significant source of reactive oxygen species (ROS) and contributing to the elevated levels characteristic of late-passage cells and tissues (Terlecky et al., 2006).

While the contribution of peroxisomes to cellular aging is a relatively new area of investigation, mitochondria have been implicated in the Free Radical Theory of Aging since its initial development (Harman, 1972). This is primarily because mitochondria are not only a constitutive source of ROS, but also because basic mitochondrial function has a fundamental role in overall cellular metabolism. For this reason age-associated mitochondrial dysfunction has been intensively investigated and is often regarded as a pivotal factor in the aging process (Beckman and Ames, 1998; Atamna et al., 2000; Cadenas and Davies, 2000; Muller, 2009). As peroxisomes share many of these features with mitochondria, the focus of the current study has been to investigate how peroxisomal oxidative imbalance may contribute to mitochondrial dysfunction.

Peroxisomes are a potentially significant source of intracellular ROS under circumstances of inadequate antioxidant protection. Furthermore, this appears to be a naturally occurring (age-associated) event in the sense that cells exhibit a progressive mislocalization of peroxisomal catalase due its endogenous targeting signal, which possesses a relatively poor affinity for the Pex5p cycling import receptor as demonstrated in cell culture (Legakis et al., 2002; Koepke et al., 2007). Supporting this concept in animals, quantitative analysis of hepatic peroxisomes comparing old rats (39 months) to young rats (2 months) revealed a respective decrease in catalase content, yet an increase in urate oxidase levels and peroxisome volume density (Beier et al., 1993). Not only are peroxisomes a significant source of ROS which

may elicit downstream effects on other cellular functions, but they also exhibit an age-associated decline in metabolic function and may contribute to aging and age-associated degenerative diseases (Périchon et al., 1998). Peroxisomes are therefore linked to the "Free Radical Theory of Aging" (Beckman and Ames, 1998; Hagen, 2003). Peroxisome metabolism is particularly important with respect to membrane composition and function and therefore peroxisomal dysfunction is also linked to the "Membrane Theory of Aging" (Shinitzky, 1987; Singh, 1997). Previously it has been demonstrated that the restoration of peroxisomal catalase import in late-passage cells via retroviral expression of catalase-SKL, a version of the enzyme with a more effective targeting signal, results in the rescue of many functions, including age-associated mitochondrial inner membrane depolarization (Koepke et al., 2007). Together with the observations that catalase mislocalization to the cytosol and subsequent peroxisomal oxidative imbalance begins to occur as early as middle-passage, has lead to the hypothesis that peroxisomally generated ROS may be an upstream initiator of age-associated mitochondrial dysfunction.

While peroxisomes and mitochondria have been metabolically linked (reviewed by Schrader and Yoon, 2007; Van Veldhoven, 2010), and cells with defects in peroxisomal biogenesis and/or metabolic pathways have coincident mitochondrial defects (Baumgart et al., 2001; Dirkx et al., 2005), we sought to determine whether these organelles were oxidatively linked as well.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Amplex® Red, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) and MitoTracker® Red CM-H_2XRos were purchased from Invitrogen/Molecular Probes (Eugene, OR, USA). 2,7-Dichlorofluorescin diacetate was purchased from Acros Organics (Fisher Scientific). The catalase inhibitor 3-amino-1,2,4-triazole, titanium (IV) oxysulfate, and anti-aconitase (Aco2) antibodies were obtained from Sigma-Aldich Chemical (St. Louis). All other reagents were obtained from standard sources.

CELL CULTURE

Hs27 diploid human fibroblasts were purchased from ATCC (Manassas, VA, USA). Cells were grown in DMEM (Invitrogen/Gibco) containing 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine while maintained at 37°C in 5% atmospheric CO_2. The catalase inhibitor 3-AT was added to 2 mM.

CATALASE ACTIVITY

Catalase activity was measured by its ability to degrade hydrogen peroxide, as previously described (Storrie and Madden, 1990; Koepke et al., 2008). Hs27 cells grown in 60 mm culture dishes to 90% confluency were trypsinized, pelleted, and resuspended in 2% Triton-X 100 solution on ice for at least 2 min. Cell samples were added to a reaction mixture of 20 mM imidazole buffer (pH 7.0), 1 mg/mL BSA, and 0.01% hydrogen peroxide and incubated on ice for 10 min. The reaction was stopped by addition of saturated Titanium (IV) oxysulfate ($TiOSO_4$) in 1M H_2SO_4, which reacts with hydrogen peroxide to produce a yellow peroxotitanium complex. Absorbance at 410 nm was measured for "cell" and "no cell" samples, whereby the difference yielded a rate expressed as ΔOD_{410}/min. Rates were then adjusted for protein concentration as determined by a BCA protein assay (Pierce Chemical), yielding a ΔOD_{410}/min/mg total protein.

ACONITASE ACTIVITY

Hs27 cells grown in 100 mm culture dishes until 90% confluence were semi-permeabilized in 25 μg/mL digitonin in PBS for 10 min, washed and scraped into 0.1% Triton-X 100 in PBS and left on ice for an additional 5 min. Lysate samples were added to 1 mL of aconitase reaction mix consisting of 50 mM Tris–Cl (pH 7.4), 5 mM sodium citrate, 0.6 mM manganese chloride, 0.2 mM NADP, and 1–2 units of isocitrate dehydrogenase. Absorbance at 340 nm was measured using a (Beckman Coulter DU 640) spectrophotometer over a 1 h time period. Rates of aconitase activity were calculated as ΔOD_{340}/min and adjusted for protein concentration of lysate samples using a BCA protein assay (Pierce Chemical), yielding a ΔOD_{340}/min/mg total protein.

ROS MEASUREMENTS

2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was used as previously described (Legakis et al., 2002) to visualize ROS production in live cells. Briefly, cells growing on glass-bottomed culture dishes (MatTech, Ashland, MA, USA) were washed with PBS and incubated for 5 min at 37°C with 25 μM DCFH-DA. Cells were subsequently washed and incubated with DMEM minus phenol red. The resultant cellular fluorescence due to oxidized DCFH was immediately visualized by confocal microscopy using an excitation wavelength of 488 nm.

PROTEIN CARBONYLATION MEASUREMENTS

Oxidative damage to total cellular protein was measured using the quantitative assay of Reznick and Packer (1994). Cells were grown in 100 mm culture dishes, trypsinized, pelleted, and dissolved in 0.1% Triton-X 100 in PBS. Equal amounts of cellular protein from treated and untreated samples, as determined by a BCA protein assay, were then used to determine protein carbonyl levels. Each sample was divided into two and mixed with either 10 mM 2,4-dinitrophenylhydrazine in 2.5 M HCl or 2.5 M HCl alone and allowed to incubate in the dark for 1 h with vortex mixing every 15 min. Trichloroacetic acid [20% (w/v)] was then added to samples to a final concentration of 10% (w/v), left on ice for 10 min, and then centrifuged for 5 min at 4°C using a table top centrifuge to collect protein precipitates. Supernatant was discarded and a second wash with 10% (w/v) trichloroacetic acid was performed. Samples were then washed three times in ethanol-ethyl acetate (1:1; v/v) to remove free DNPH after which precipitates were dissolved in 6 M guanidine hydrochloride and left for 10 min with intermittent vortexing. Absorbance at 365 nm was then obtained using a (Beckman Coulter DU 640) spectrophotometer. The difference in values from derivatized (DNPH) and control (only HCl) subsamples were then determined. The molar extinction coefficient of dinitrophenylhydrazine (ε of 22,000 M^{-1}) was then used to calculate carbonyl values (nmol/mg protein).

IMMUNOCYTOCHEMISTRY AND PEROXISOMAL QUANTITATION

Peroxisomal numbers in control and 3-AT treated cells were measured by immunostaining and imaging cells for the peroxisomal

membrane protein, Pmp70p, as previously described (Koepke et al., 2008). A quantitative measure of the number of peroxisomes per square unit was performed using Image J software (NIH). Using the digital images, cells of interest were outlined and their image areas (in pixels) were measured. Next, these outlined cells were analyzed for the number of particles greater than 4×4 pixels they contained. These particles were the Pmp70p-positive peroxisomes. To account for the different sizes of the cells, the number of peroxisomes was divided by the image area, to give a measure of the number of peroxisomes per 1000 pixels ($\approx 1.5\,\mu m^2$). Average areas per cell were not significantly different between the treated and control groups. The averages and SD from between 6 and 14 cells were analyzed for each time treatment and treatment group.

MITOCHONDRIAL DYE ASSAYS

Mitochondrial ROS production was measured using MitoTracker Red CM-H2XRos dye. For these assays, cells were first grown on coverslips in appropriate culture medium. Pre-warmed growth medium containing 10 nM MitoTracker probe was then incubated with the cells for 15 min. After staining, cells were washed with PBS and incubated with DMEM minus phenol red. Live cells were imaged using confocal microscopy under identical settings between control and treated groups. Fixing cells with formaldehyde prior to imaging yielded a poorer signal to noise ratio, and an altered mitochondrial morphology.

Mitochondrial membrane potential was determined using JC-1, a cationic dye which accumulates in potential-dependent manner in the mitochondria. Depolarization of the organelle is observed as a fluorescence shift from the red (525 nm) J aggregate (polarized mitochondria), to the green (590 nm) J monomer (depolarized mitochondria). Thus, a decline in the red/green fluorescence intensity ratio is associated with depolarization of the organelle. Cells were grown on glass bottom 30 mm culture dishes, incubated with JC-1 dye in DMEM for 15 min at 37°C, rinsed in PBS, and incubated in pre-warmed DMEM minus phenol red. Cells were then immediately imaged using fluorescent confocal microscopy, under identical settings between control and treated groups. Average pixel intensity of the monomeric JC-1 dye (green) from untreated and 3-AT treated cells was obtained using Image J analyses. Regions used for the analyses were depicted in outline masks, with the threshold set from 34 to 255 on the grayscale, and the lower limit for analyses were performed on objects larger than 300 pixels ($\approx 0.4\,\mu m^2$). The averages and SD from between 7 and 10 cells were analyzed for each treatment group.

STATISTICAL ANALYSES

All statistical analyses were performed using GraphPad Prism software. For experiments with two treatment groups a two-tailed, unpaired Student's *t*-test was used. For experiments with greater than two treatment groups a one-way ANOVA with the Tukey's Multiple Comparison *post hoc* test was employed. Differences between groups were considered statistically significant when p values of <0.05 were measured.

RESULTS

INHIBITION OF PEROXISOMAL CATALASE

3-AT has previously been demonstrated to be an irreversible inhibitor of catalase from a number of eukaryotes (Sheikh et al., 1998). While we have previously demonstrated the inhibition of catalase in human cultured Hs27 cells over a broad range of concentrations and times (Koepke et al., 2007), we sought to study the inhibitory effects of intermediate levels of 3-AT (2 mM) over a 24 h time course. Results (**Figure 1**) indicated that ∼80% of the initial catalase activity in Hs27 cell cultures was lost after 4 h of treatment with 2 mM 3-AT. The time for half of the initial activity to be inhibited was estimated to be just less than 1 h of incubation in the presence of 2 mM 3-AT. No further decrease in catalase activity, beyond that seen at 4 h, was observed at 24 h.

To characterize catalase recovery after 3-AT treatment, wash out experiments were performed after 24 h exposure to 3-AT; thereafter cells were allowed to recover in the absence or presence of 100 μg/mL of cycloheximide, an inhibitor of protein synthesis. Removal of aminotriazole permitted a 50% recovery of catalase activity within 24 h (**Figure 2**), a result originally observed by Hayflick and colleagues (Mellman et al., 1972). As expected, this restoration was repressed by treatment with cycloheximide; indicating synthesis of new protein is required for recovery to occur, owing to the covalent and irreversible interaction of 3-AT with catalase protein (Margoliash et al., 1960).

INHIBITION OF CATALASE RESULTS IN INCREASED LEVELS OF INTRACELLULAR ROS, PROTEIN CARBONYLS, AND PEROXISOMAL NUMBERS

Inhibition of peroxisomal catalase would be expected to result in increased levels of hydrogen peroxide, generated by the peroxisomal oxidase enzymes. As hydrogen peroxide is capable of passing through biological membranes (Bienert et al., 2006; Koopman et al., 2010), we would expect to observe elevated levels of hydrogen peroxide within the cell. As **Figure 3** depicts, increased levels of hydrogen peroxide, as measured by 2,7-DCF staining, could

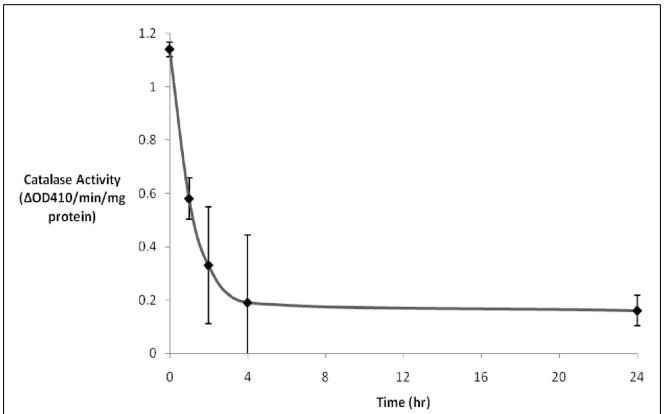

FIGURE 1 | 3-AT inhibits catalase activity. Hs27 fibroblasts were treated with 2 mM 3-AT for varying durations. Catalase activity was determined by adding cell lysates to a 1 mM H_2O_2 solution. The difference in absorbance at 410 nm was due to the remaining H_2O_2 being converted to a yellow peroxotitanium complex by addition of $TiSO_4$ following the 10 min incubation. Within ∼4 h of 3-AT treatment catalase activity was reduced to ∼15% that observed in untreated cells. The residual catalase activity did not diminish between 4 and 24 h of 3-AT treatment.

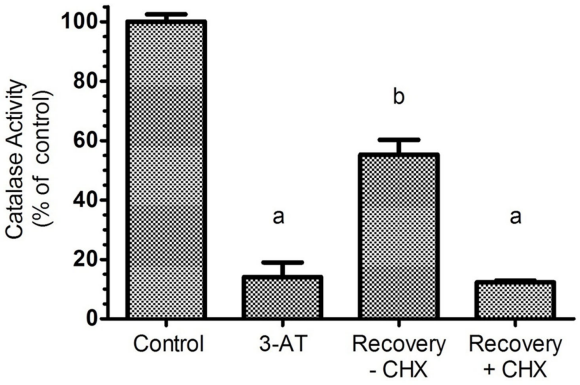

FIGURE 2 | Recovery of catalase activity requires protein synthesis. Hs27 cells were treated with 2 mM aminotriazole for 24 h followed by a 24 h recovery period (in the absence of aminotriazole) with or without 100 μg/mL of cycloheximide, an efficient inhibitor of protein synthesis. Results indicate that aminotriazole is an irreversible inhibitor of catalase, and for cells to recovery activity, synthesis of new catalase protein must occur. Determination of catalase activity is described in Section "Materials and Methods." Letters represent values significantly different from the control and/or each other based on ANOVA analysis where $p < 0.05$.

be observed in 3-AT treated cells within 24 h of treatment. In addition, subcellular structures with mitochondrial morphology (arrowheads) were observed with high levels of 2,7-DCF staining in many of the treated cells. This observation was explored in more detail in **Figure 5**.

We have previously demonstrated oxidative damage to cellular components following treatment of cells with low levels (250 μM) of 3-AT for extended periods of time (Koepke et al., 2007). As can be seen in **Figure 4**, a quantitative measure of protein carbonyls demonstrated a greater than 25% increase in the levels of cellular protein carbonyls in 3-AT treated cells, when compared to untreated fibroblasts.

As a response to a decrease in catalase activity, either in late-passage cells (Legakis et al., 2002; Ivashchenko et al., 2011), diseased cells (Wood et al., 2006), or cells treated with 3-AT (Sheikh et al., 1998; Koepke et al., 2008), cells demonstrate an increase in the number of peroxisomes, although not with a corresponding increase in peroxisomal enzyme activities. In order to determine whether this increase in peroxisomal number occurred shortly after inactivation of catalase with 3-AT, we immunostained control and treated cells for the peroxisomal membrane protein pmp70 after 24 and 48 h of treatment with 3-AT. We employed Image J software to count the number of peroxisomes within cells and normalized the numbers of peroxisomes per 1000 pixels of cell area ($\approx$1.5 μm^2). The results (**Table 1**) indicate that, while there was no significant increase in peroxisomal numbers after 24 h of 3-AT treatment, there was a statistically significant increase in peroxisomal numbers of $\sim$25% after 48 h of 3-AT treatment. While this increase in peroxisomal numbers is less than previously reported, these results demonstrate that cells lacking catalase activity are eliciting a compensatory response very shortly after the levels of hydrogen peroxide begin to increase.

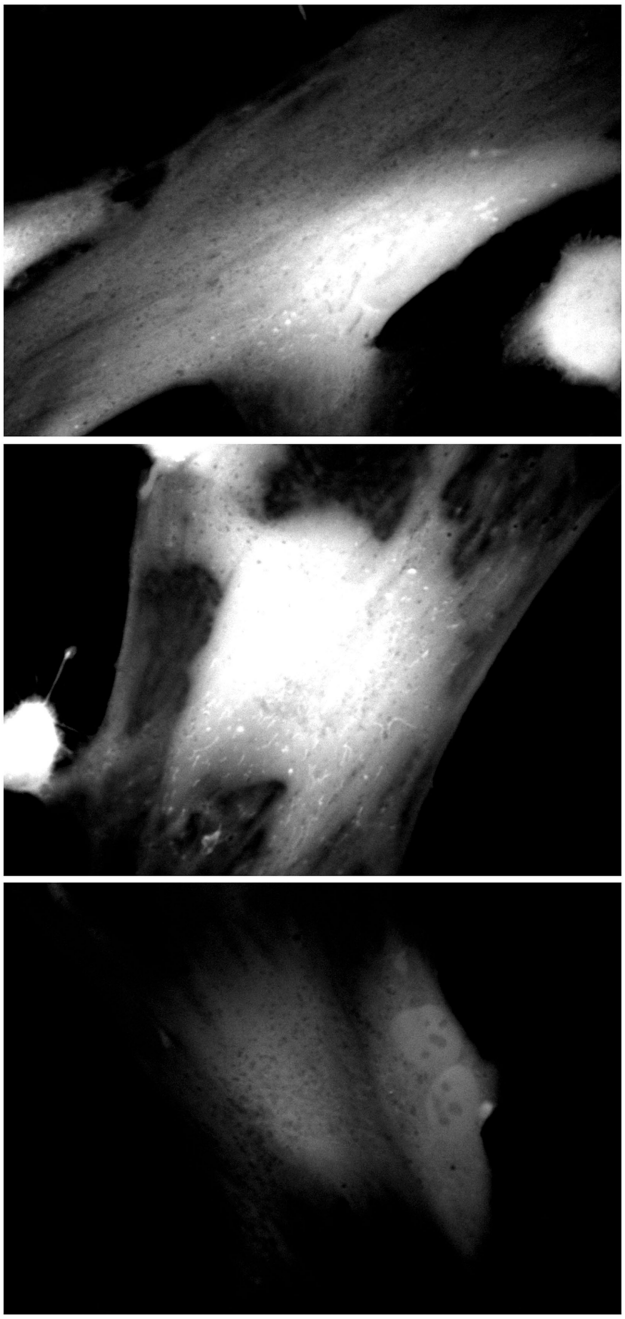

FIGURE 3 | Catalase inhibition increases cellular 2,7-DCF staining. Hs27 fibroblasts were grown in the presence (top two panels) or absence (bottom panel) of 2 mM 3-AT for 24 h, after which they were treated with the ROS-sensitive dye 2,7-DCF (Invitrogen/Molecular Probes). Cells were live-imaged using confocal microscopy and FITC optics. Images are representative of numerous fields, imaged under identical conditions.

INHIBITION OF PEROXISOMAL CATALASE RESULTS IN OXIDATIVE DAMAGE TO MITOCHONDRIA

Having confirmed that treatment of human fibroblasts with 3-AT resulted in the increase in peroxisomally derived ROS and subsequent cellular oxidative damage, we sought to determine the

downstream effects of 3-AT treatment on mitochondria. Results presented in **Figure 3** demonstrate that following the inhibition of peroxisomal catalase activity with 3-AT, 2,7-DCF stained structures with mitochondrial morphology appear in treated cells. In order to confirm whether these ROS staining structures were mitochondria, we double-stained cells following 48 h of 3-AT treatment, staining living cells for both general cellular ROS with 2,7-DCF, and mitochondrial ROS with MitoTracker Red Ros. The results confirmed that the 2,7-DCF staining structures observed following the inhibition of peroxisomal catalase were indeed mitochondria (**Figure 5**). Thus, the peroxisomal oxidative imbalance generated by inhibiting catalase was yielding downstream oxidative effects on mitochondria.

Time course experiments (**Figure 6**), staining for mitochondrial ROS with MitoTracker Red Ros demonstrated increased staining in treated cells beginning 24 h after 3-AT treatment. As was also observed in **Figure 5**, an increased mitochondrial ROS staining was observed at 48 h of 3-AT treatment, this staining appeared to be increased following 5 days of 3-AT treatment. Thus, there appeared to be a progressive accumulation of mitochondrial ROS, beginning ∼24 h after the inhibition of peroxisomal catalase.

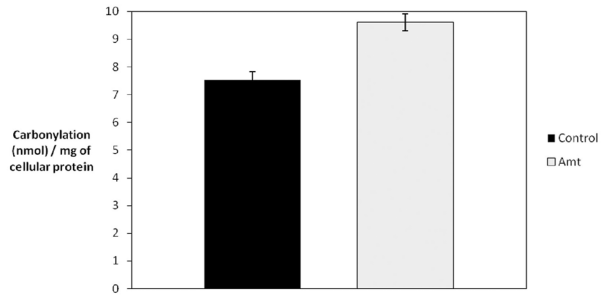

FIGURE 4 | Catalase inhibition results in increased protein carbonylation. Hs27 fibroblasts were grown in the presence or absence of 2 mM 3-AT for 4 days and protein carbonylation was determined. A quantitative spectrophotometric assay involving the derivatization of carbonyl groups with 2,4-dinitrophenylhydrazine was used, as described in Section "Materials and Methods." An ∼7% increase in total cellular carbonyls was observed in cells treated with 3-AT ($p < 0.05$).

INHIBITION OF MITOCHONDRIAL ACONITASE ACTIVITY

In order to determine whether the increase in mitochondrial ROS was reflected in the inhibition of mitochondrial enzyme activities, we chose to study the effects of 3-AT-induced catalase inhibition on mitochondrial aconitase. One of the Krebs Cycle enzymes, aconitase possesses an iron–sulfur cluster at its active site, and as such is very sensitive to inactivation in an oxidizing environment (Gardner et al., 1994). The inhibition of peroxisomal catalase resulted in a greater than 90% inhibition in aconitase activity within 24 h of treatment of cells with 3-AT (**Figure 7**). In order to confirm that 3-AT was not inhibiting aconitase directly, lysates from untreated cells were assayed for aconitase in the presence of 3-AT (**Figure 8**). No decrease in aconitase activity was observed in treated cell lysates when compared with untreated controls. Thus the inhibition of

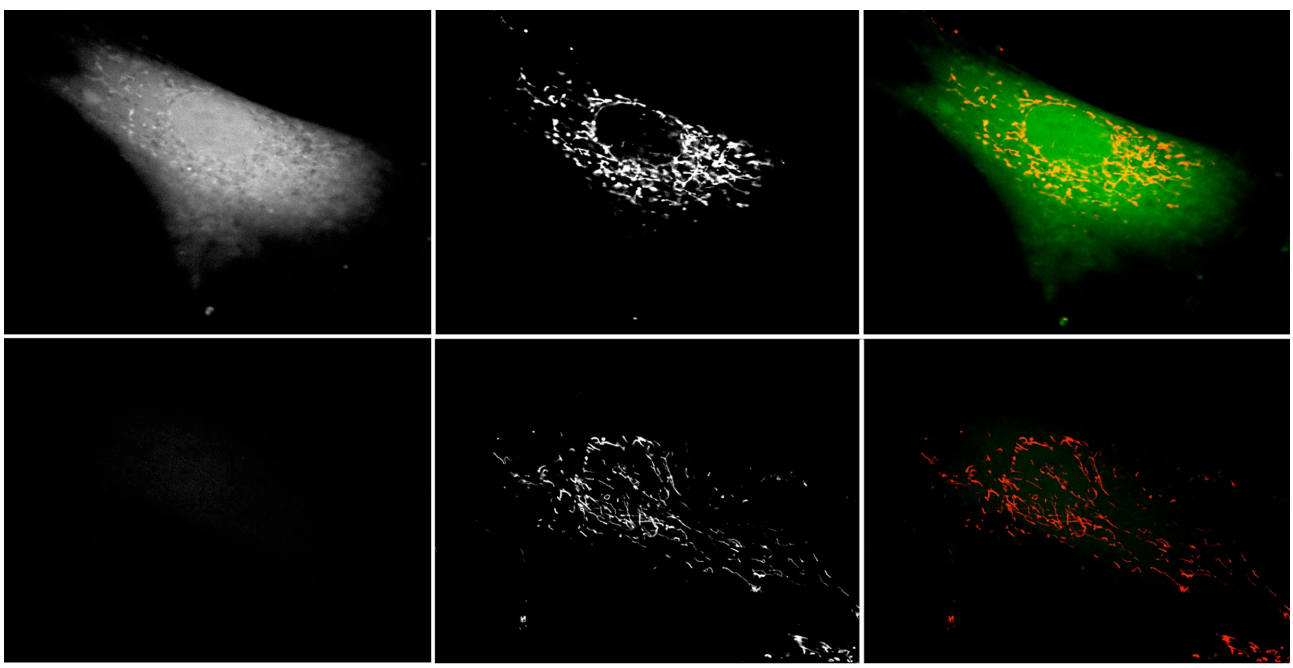

FIGURE 5 | Catalase inhibition increases mitochondrial DCF staining. Hs27 fibroblasts were grown in the presence (top row) or absence (bottom row) of 2 mM 3-AT for 48 h, after which they were treated with the ROS-sensitive dye 2,7-DCF (left column) and the mitochondrial ROS-sensitive dye MitoTracker Red CM-H2XRos (center column). Cells were live-imaged using confocal microscopy employing FITC optics for DCF and Texas Red optics for MitoTracker Red. Right column is a false-color overlay of the DCF and MitoTracker images. Images are representative of numerous fields, imaged under identical conditions.

Table 1 | Effects of 3-AT treatment on pmp70-positive peroxisome numbers per unit area of cell.

Time of treatment (h)	3-AT	Peroxisomes per 1000 pixels
24	−	1.52 ± 0.50
24	+	1.70 ± 0.32
48	−	1.64 ± 0.44
48	+	2.04 ± 0.36

Average numbers of peroxisomes per unit area were not different between treated and control groups at 24 h ($p = 0.45$), but were significantly different at 48 h ($p < 0.05$).

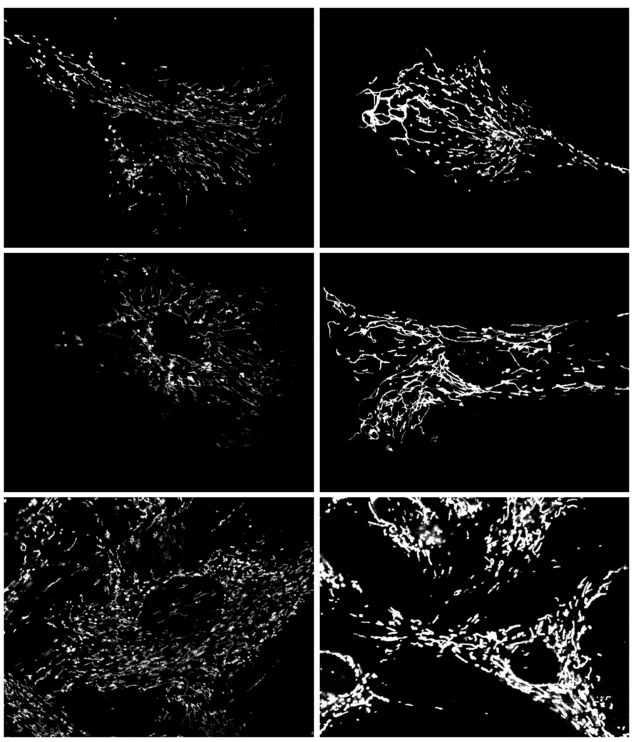

FIGURE 6 | Effects of treatment with 2 mM 3-AT on mitochondrial ROS staining. Control Hs27 cells (left column) demonstrate less mitochondrial ROS staining than 3-AT treated cells (right column) after 24 h (top row), 48 h (middle row), and 5 days (bottom row). In addition, staining in 3-AT treated cells appeared homogeneous throughout the mitochondria. Live cells were imaged under identical conditions, and are representative of numerous fields viewed and imaged.

mitochondrial aconitase was secondary to the effects of 3-AT on peroxisomal catalase.

The recovery of mitochondrial aconitase activity following removal of 3-AT paralleled the recovery of catalase activity, with the restoration of control levels of aconitase activity being observed after 24 h (**Figure 7**). However, in the presence of cycloheximide, aconitase activity failed to recover following the removal of 3-AT. While one possible explanation for these results is that the peroxisomal catalase activity must be restored in order to correct the cellular oxidative balance and thus the mitochondrial aconitase activity, however, inhibition of aconitase by oxidation is known to occur by both reversible and irreversible methods (Bulteau et al., 2003). Therefore, it is also possible that the oxidatively damaged aconitase protein must be replaced in order for the activity to be restored.

EFFECTS OF PEROXISOMAL CATALASE INHIBITION ON MITOCHONDRIAL FUNCTION

We have previously demonstrated the age-dependent decrease in mitochondrial inner membrane potential in late-passage human fibroblasts, which could be restored to normal, early-passage levels by the introduction of catalase bearing the more efficient – SKL peroxisomal targeting signal (Koepke et al., 2008). In light of this correction of mitochondrial function, we sought to determine whether the inhibition of peroxisomal catalase decreased the mitochondrial inner membrane potential in early-passage cells. Results presented in **Figure 9** indicate an increase in the monomeric form of the JC-1 dye, indicative of a decrease in mitochondrial inner membrane potential following treatment of cells with 3-AT, over the 4-day time course. As we have previously observed the loss of mitochondrial inner membrane potential in late-passage cells (Koepke et al., 2007), and in cells treated with 3-AT over 20 population doublings (Koepke et al., 2008), our present results imply that the beginnings of the loss of inner membrane potential begins only a few days following the loss of peroxisomal oxidative homeostasis.

DISCUSSION

Our interest in the order of the formation of cellular oxidants is predicated on two previous observations. First, the process described as "peroxisomal senescence," that being the age-dependant decrease in the import of peroxisomal proteins (Legakis et al., 2002), particularly of catalase, begins in middle-passage cells, before they demonstrate mitochondrial or other peroxisomal dysfunctions. Secondly, the restoration of mitochondrial inner membrane potential that occurs following the targeting of catalase-SKL to peroxisomes in late-passage cells (Koepke et al., 2007) provides strong support to the hypothesis that oxidative damage of peroxisomal origin occurs upstream of that derived from mitochondria. In an attempt to address this order, we sought to determine the effects of the inhibition of peroxisomal catalase on mitochondrial functions in early-passage cells.

The catalase inhibitor, 3-amino-1,2,4-triazole, has been previously demonstrated to make a covalent bond with catalase during its first reactive cycle (Margoliash and Novogrodsky, 1958; Margoliash et al., 1960), forming an irreversible inhibitory complex. This inhibition of catalase activity has been exploited in a number of ways, including the biochemical functioning (Middelkoop et al., 1993) and structure of the enzyme (Kirkman and Gaetani, 1984), and the cellular effects of this inhibition (Sheikh et al., 1998; Koepke et al., 2008). While it remains possible that 3-AT possesses other, direct effects on other mammalian cellular constituents, no effects similar to the direct binding and inhibition of catalase have been reported. Thus, being cognisant of other potential effects of 3-AT, we employed this molecule to study the downstream effects of catalase inhibition on mitochondrial function.

We observed the time and concentration dependent inhibition of peroxisomal catalase, as has been reported in other studies

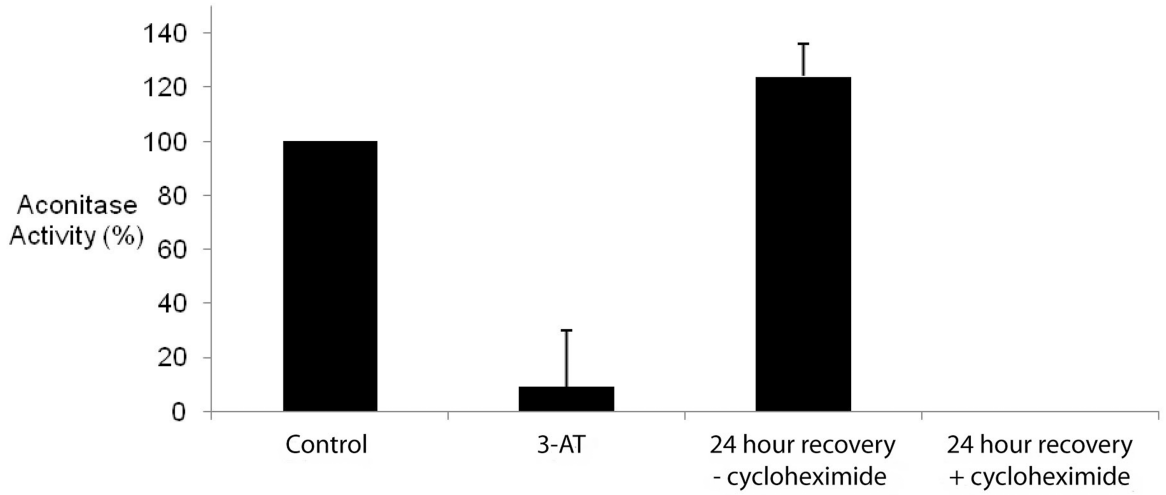

FIGURE 7 | Inhibition of peroxisomal catalase results in the inhibition of mitochondrial aconitase. Hs27 cells were grown in the presence or absence of 2 mM 3-AT for 24 h. Aconitase activity was determined using a linked assay, as described in Section "Materials and Methods." For recovery experiments, Hs27 cells were treated with 3-AT for 24 h followed by a 24 h recovery period (in the absence of 3-AT) with or without 100 μg/mL of cycloheximide, an inhibitor of protein synthesis. Results indicate the inhibition of the peroxisomal antioxidant enzyme, catalase, results in significant inhibition of the oxidatively sensitive mitochondrial enzyme, aconitase ($p < 0.05$). While aconitase activity recovers following the removal of 3-AT, this activity fails to recover and was undetectable in the presence of cycloheximide.

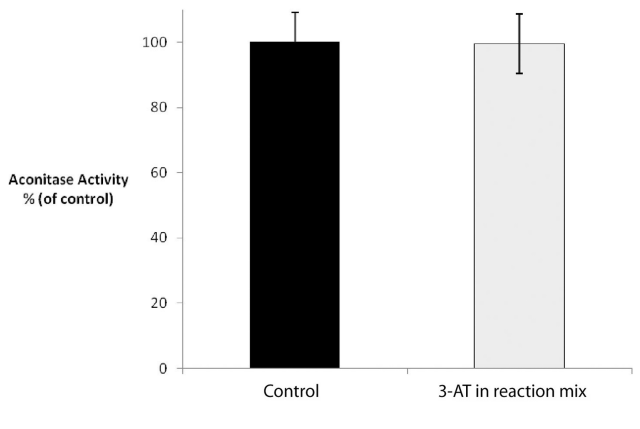

FIGURE 8 | 3-AT does not directly inhibit aconitase activity. Untreated Hs27 cell lysates were assayed for aconitase activity in the presence of absence of 2 mM 3-AT. No significant decrease in aconitase activity was observed in treated cell lysates when compared with untreated controls.

(Sheikh et al., 1998). The inhibition of catalase resulted in the accumulation of ROS within the cells, and the subsequent formation of cellular oxidative damage, including protein carbonyls.

Importantly, we observed oxidation-based changes to mitochondria in the treated cells. We have previously reported increased mitochondrial oxidative changes, as indicated by MitoROS staining, in cells treated with lower levels of 3-AT for longer periods of time (Koepke et al., 2008). However, in this study we demonstrate that the increase in mitochondrial oxidative damage and the decrease in mitochondrial function occur very rapidly following the inhibition of peroxisomal catalase. Within 24 h increases in mitochondrial ROS can be observed, and an inhibition of the oxidation sensitive mitochondrial protein aconitase can be measured. Thus, we demonstrate a linkage between the loss of the peroxisomal oxidative balance and the loss of oxidation sensitive components of the mitochondria. This places peroxisomal dysfunction upstream of subsequent mitochondrial effects. Recent results from Fransen and coworkers (Ivashchenko et al., 2011) have employed redox sensitive probes and demonstrated that excess ROS generated within peroxisomes disturbs mitochondrial redox balance within a few minutes.

It has been established that the inhibition of catalase with 3-AT for 20 cell passages results in elevated levels of ROS, creating an oxidizing intracellular environment (Koepke et al., 2008). Early-passage treated cells exhibited increased staining for the ROS-sensitive dye, 2,7-DCF, as well as increased general levels of protein carbonylation both of which are in agreement with previous long-term results reported by Koepke et al. (2008). A novel finding of the present study was the observation that 3-AT treated cells displayed not only a general increase in 2,7-DCF staining but also specific sub-cellar regions of intensified staining which resembled mitochondrial morphology. These structures were confirmed as mitochondria by co-staining with 2,7-DCF and the MitoTracker Red CM-H2XRos probe. Thus, these data support the hypothesis that peroxisomally derived ROS elicited a downstream effect causing an increase in mitochondrial ROS production or accumulation. Indeed, mitochondria of 3-AT treated cells were shown to accumulate ROS. This chain-reaction like effect is potentially significant as it demonstrates how the uncoupling of antioxidant defense of one cellular organelle, the peroxisome, leads to the uncoupling of another, the mitochondria. 3-AT treated mouse embryonic fibroblast cells have also been demonstrated to show a dysregulation in mitochondrial redox status (Ivashchenko et al., 2011). How exactly this effect is taking place is currently unclear

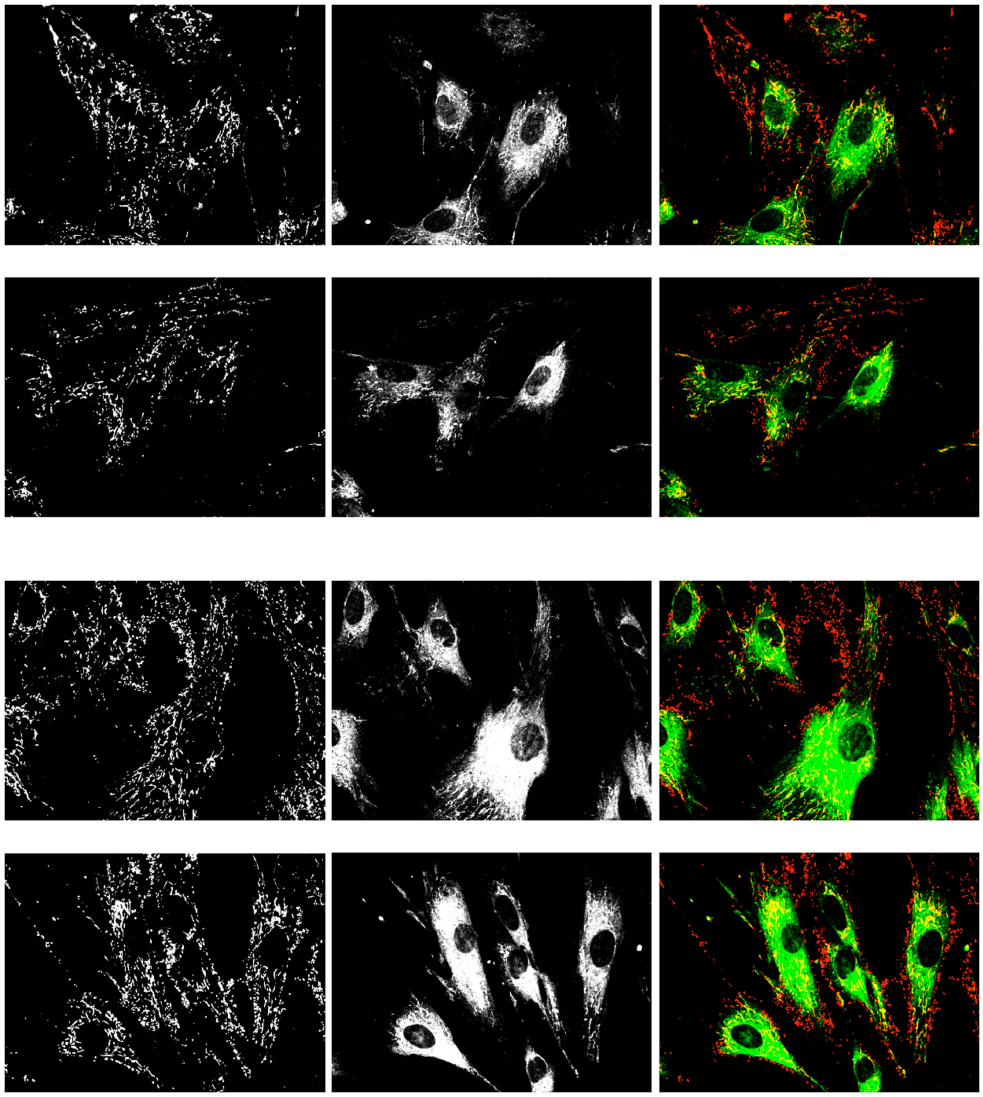

FIGURE 9 | Effects of treatment with 2 mM 3-AT for 4 days on mitochondrial inner membrane potential. Control Hs27 cells (upper two rows) and 3-AT treated cells (lower two rows) were incubated with JC-1 dye and imaged for the presence of the aggregate (left column, red) and the monomeric (center column, green) forms of the dye. Live cells were imaged under identical conditions, and are representative of numerous fields viewed and imaged. The increase in the monomeric, green form of the dye is indicative of a depolarization of the mitochondrial inner membrane. Treated cells had a 30% higher mean pixel intensity than control cells (143 ± 14 vs. 110 ± 10) in the monomeric form of the dye ($p < 0.05$).

but a straightforward explanation may simply be that the mitochondrial antioxidant defense system becomes overwhelmed in the presence of additional oxidative burden contributed by peroxisomally derived ROS. In particular, H_2O_2, expected to be the dominant species of ROS generated through catalase inhibition is freely diffusible across biological membranes and thus capable of "spilling" out into other cellular compartments (Bienert et al., 2006; Koopman et al., 2010). Hydrogen peroxide is not only diffusible through biological membranes, regulated in part by lipid composition, but may also pass through aquaporin (8) channels present in the plasma and mitochondrial membranes (Bienert et al., 2006). Hydrogen peroxide is also the enzymatic product of superoxide dismutase. Therefore the accumulation of hydrogen peroxide may be altering this reaction's equilibrium, according to the Le Chatelier's Principle, resulting in an increase in mitochondrial superoxide concentration. It has also been suggested that oxidative damage to the mitochondrial inner membrane proteins that comprise the electron transport chain alters their efficiency in electron transfer. This is proposed to lead to increased leakage in electron flow to the terminal electron acceptor cytochrome C, thus resulting in an increase in O_2^- and hydrogen peroxide generation (Bandy and Davison, 1990). Indeed it has been demonstrated that Complex I deficiency results in a 2- to 10-fold increase in hydroxyl radical production under basal conditions (Luo et al., 1997). Furthermore, complexes I and IV show selectively diminished activities in aged rat brain and liver (Navarro, 2004) and

are known to be dysfunctional in various age-associated neurological disorders (Navarro and Boveris, 2007). Experiments in transgenic mice have demonstrated that targeting catalase to the mitochondria increases the lifespan of these animals (Schriner et al., 2005). Our results indicate that this localization of catalase may ameliorate the mitochondrial effects of the excess hydrogen peroxide.

Based on previous and current work, the following model as to how peroxisomes may contribute to mitochondrial dysfunction in the cellular aging process is proposed. In this scenario the relatively weak affinity of the PTS1 import cycling receptor, Pex5, for the divergent (-KANL) targeting signal of catalase leads to a pre-disposed disequilibrium between the import of catalase and more effectively targeted, – SKL bearing, oxidases. As the organelle slowly loses the ability to clear generated ROS, the peroxisomal import machinery situated within the organelle's membrane becomes compromised. With the reduced functioning of the import machinery catalase import becomes even more dramatically affected perpetuating a "negative spiral-like" effect. This is supported by the observation that PTS1 import shows reduced functionality in late-passage cells and while – SKL mediated import is maintained (albeit with reduced efficiency) catalase import is practically non-existent (Legakis et al., 2002; Koepke et al., 2007). This progressively transitions the peroxisome into a significant source of intracellular ROS, contributing to elevated levels observed in late-passage cells. As the primary peroxisomal ROS, hydrogen peroxide, is freely diffusible, it elicits oxidative damage throughout the cell and in particular to mitochondria. The resulting excessive oxidative burden may overwhelm the mitochondrial antioxidant defense system and/or decrease the efficiency of ETC complexes causing it to generate more ROS. At the same time the oxidatively sensitive mitochondrial enzyme, aconitase, becomes inactive as its active site is disassembled by oxidative attack. This negative effect of peroxisome-derived oxidants on mitochondrial function occurs within a very short time course, certainly within a few hours. This is potentially detrimental in two ways; the first being that loss of aconitase activity may compromise functionality of the tricarboxylic acid cycle which may contribute to mitochondrial dysfunction over sufficient time and secondly that its inactivation may result in an increase in free iron, which is capable of escalating oxidative insult by catalyzing the production of the more reactive hydroxyl radical through the Fenton reaction. After prolonged incubation this process is believed to culminate in the loss of mitochondrial inner membrane potential and subsequent impaired oxidative phosphorylation, which is characteristic of late-passage and aged cells as well as certain age-related pathologies. The loss of peroxisomal oxidative balance may also mitigate some of the important protective "anti-aging" mitochondrial functions, as reviewed by Titorenko and Terlecky (2011). Coupled with the long-term effects of catalase inhibition on mitochondrial function (Koepke et al., 2008) and the restoration of mitochondrial inner membrane potential observed in late-passage cells expressing peroxisomally targeted catalase-SKL (Koepke et al., 2007) our present short-term results support the hypothesis that peroxisomal oxidative damage is upstream of, and contributes to, the mitochondrial damage observed in the aging process.

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The yeast retrograde response as a model of intracellular signaling of mitochondrial dysfunction

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Mitochondrial dysfunction activates intracellular signaling pathways that impact yeast longevity, and the best known of these pathways is the retrograde response. More recently, similar responses have been discerned in other systems, from invertebrates to human cells. However, the identity of the signal transducers is either unknown or apparently diverse, contrasting with the well-established signaling module of the yeast retrograde response. On the other hand, it has become equally clear that several other pathways and processes interact with the retrograde response, embedding it in a network responsive to a variety of cellular states. An examination of this network supports the notion that the master regulator NFκB aggregated a variety of mitochondria-related cellular responses at some point in evolution and has become the retrograde transcription factor. This has significant consequences for how we view some of the deficits associated with aging, such as inflammation. The support for NFκB as the retrograde response transcription factor is not only based on functional analyses. It is bolstered by the fact that NFκB can regulate Myc–Max, which is activated in human cells with dysfunctional mitochondria and impacts cellular metabolism. Myc–Max is homologous to the yeast retrograde response transcription factor Rtg1–Rtg3. Further research will be needed to disentangle the pro-aging from the anti-aging effects of NFκB. Interestingly, this is also a challenge for the complete understanding of the yeast retrograde response.

Keywords: retrograde response, *RTG* genes, NFκB, metabolism, stress, mitophagy, *Saccharomyces cerevisiae*, replicative lifespan

INTRODUCTION

Mitochondrial dysfunction underlies the spectacular manifestations of a class of diseases known as mitochondrial encephalomyopathy. It is found in cardiac hypertrophy and neurological disorders such as Parkinson's disease. Mitochondrial dysfunction is also a hallmark of cancer (Wallace and Fan, 2010). It would seem that the mitochondrial deficits that contribute to the above disorders would invariably prevent any cell survival. However, cells often respond to mitochondrial stress with specific responses, allowing them to survive in some cases. Thus, adaptations to loss of mitochondrial function are widespread (Jazwinski, 2012). It has become clear over the past decade that these adaptations or compensations are also evident during normal aging, and they may in part determine lifespan. The best known of these cellular responses to mitochondrial dysfunction is the yeast (*Saccharomyces cerevisiae*) retrograde response (Liu and Butow, 2006), which plays a role in determining replicative lifespan (Kirchman et al., 1999). However, similar responses have been described in *Caenorhabditis elegans*, *Drosophila melanogaster*, and the mouse (Jazwinski, 2012). They have even been found in human cells in tissue culture, demonstrating their pervasiveness. We review these developments here to search for common principles in the response to mitochondrial dysfunction.

The yeast retrograde signaling pathway has been elucidated in great detail (Liu and Butow, 2006). The retrograde signaling pathway cross talks with other signaling pathways in the cell. Thus, it is embedded in a mesh of signal transduction events that adapt the cell to a variety of internal and external environments. This range of interactions has recently expanded, and it is our purpose to review them here to provide clues to the signaling modules that may operate in the response of mammalian cells to mitochondrial dysfunction. This augments the bioinformatics approach we have taken earlier to identify the retrograde response transcription factors in mammalian cells (Srinivasan et al., 2010). As that analysis suggested, the "master regulator" NFκB has likely taken on in metazoans the role of the yeast retrograde transcription factor Rtg1–Rtg3. This conclusion gathers further support from the current review.

THE YEAST RETROGRADE RESPONSE

Yeast cells missing mtDNA (rho^0) display a wide variety of changes in nuclear gene expression compared to rho$^+$ cells (Epstein et al., 2001; Traven et al., 2001). The activated genes encode metabolic and stress proteins destined for the mitochondrion, the cytoplasm, and the peroxisome, and they portend a realignment of metabolism that compensates for the mitochondrial dysfunction. The loss of respiratory ability in rho^0 cells eliminates the tricarboxylic acid (TCA) cycle as a source of glutamate for biosynthesis, because the activity of succinate dehydrogenase is compromised. However, the first three reactions of the TCA cycle remain intact, and this part

of the TCA cycle can give rise to α-ketoglutarate, the precursor of glutamate, as long as a supply of citrate is available. This citrate is provided by the activation of the glyoxylate cycle, which utilizes oxaloacetate and acetyl-coenzyme A to generate citrate, but unlike the TCA cycle retains the carbons of acetate rather than ultimately releasing them as carbon dioxide. This central feature of the metabolic adaptation in rho^0 cells is surrounded by additional niceties that allow the cell to function without an active electron transport chain. The oxidative phosphorylation that is abrogated in these cells is easily supplanted by the glycolytic production of ATP. The phenotypic changes described above are together termed the retrograde response.

The key event in retrograde signaling is the translocation of the retrograde transcription factor from the cytoplasm to the nucleus (Rothermel et al., 1995; Rothermel et al., 1997; Sekito et al., 2000). The retrograde transcription factor is a heterodimer of two basic helix-loop-helix/leucine zipper proteins, Rtg1 and Rtg3, which binds to the sequence GTCAC (R box; Liao and Butow, 1993; Jia et al., 1997). Of the two, Rtg1 is atypical for such proteins because it possesses a truncated basic domain with no apparent transcriptional activation domains. Yet, only as a heterodimer can Rtg3 bind the R box and activate transcription. A hierarchical analysis of transcription factor regulatory networks in yeast has shown that Rtg1 is in the top layer, because it is not regulated by any other transcription factor (Jothi et al., 2009). Top layer transcription factors are comparatively abundant, long-lived, and noisy in terms of expression from cell to cell. This variability may allow at least some members of a yeast clone or population to respond to one or another environmental challenge by launching a response whose precision is maximized by the tightly regulated transcription factors in lower layers.

Translocation of Rtg1–Rtg3 requires the Rtg2 protein (Sekito et al., 2000), which has no known homologs in higher organisms (**Figure 1**). Rtg2 promotes the dephosphorylation of Rtg3 by binding Mks1 and preventing Mks1 from forming a complex with the 14-3-3 protein Bmh1 or Bmh2, a complex which maintains

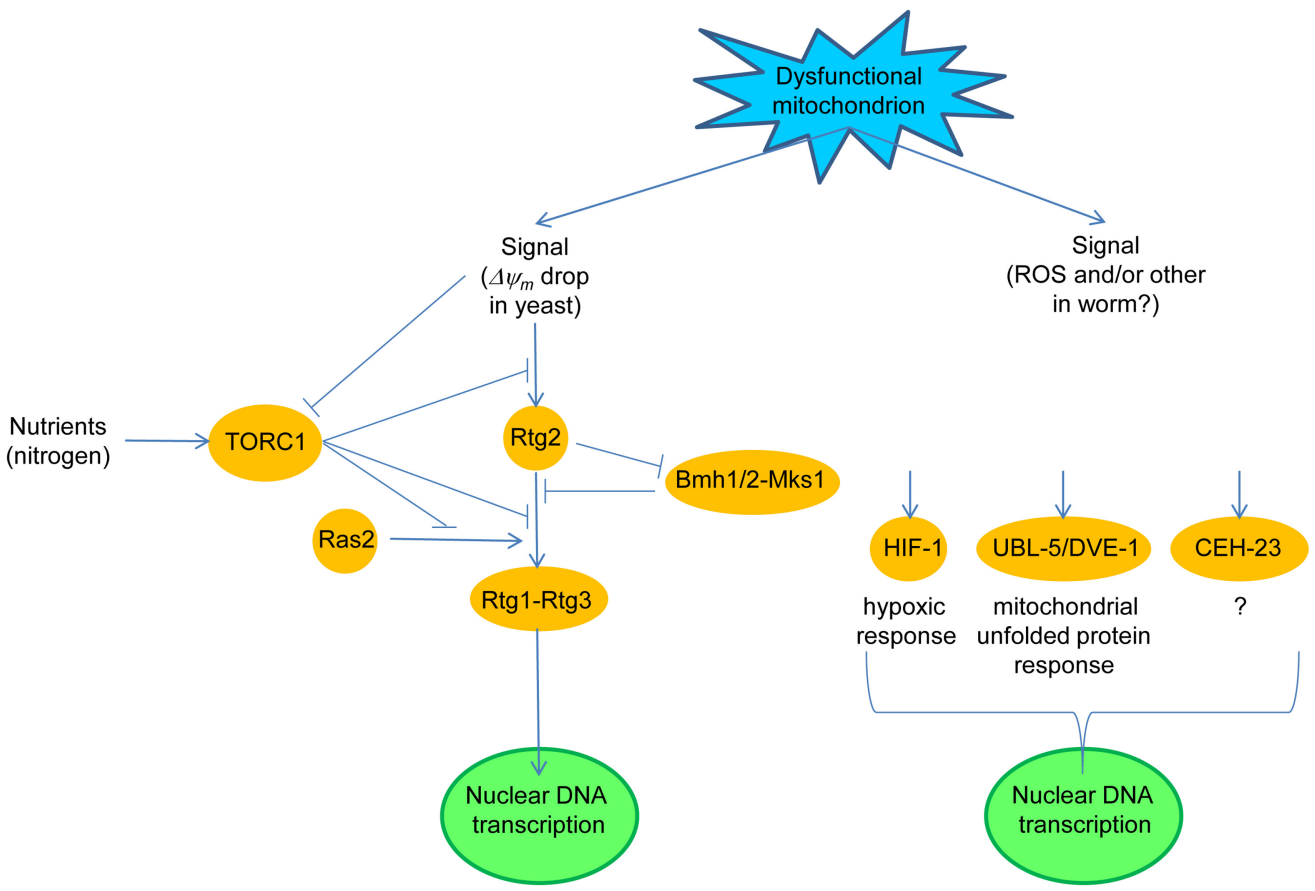

FIGURE 1 | Dysfunctional mitochondria trigger a retrograde response in yeast and in round worms. In yeast, a drop in mitochondrial membrane potential ($\Delta\Psi_m$) initiates retrograde signaling through Rtg2, by preventing the Mks1-Bmh1/2 complex from inhibiting the partial dephosphorylation of Rtg3 in the Rtg1–Rtg3 retrograde transcription factor which is stimulated by Rtg2. Rtg1–Rtg3 translocates from the cytoplasm to the nucleus where it activates the expression of retrograde response target genes. The nutrient-responsive, target of rapamycin (*TOR*) complex 1 (TORC1) blocks the retrograde response both upstream and downstream of Rtg2. TORC1 also suppresses the stimulatory effect of Ras2 on retrograde signaling. In the worm, a signal(s) elicited by dysfunctional mitochondria activate anyone of at least three retrograde signaling pathways defined by the transcription factor which is activated. The transcription factor can be HIF-1, activated by reactive oxygen species (ROS), UBL-5 and DEV-1, or the putative transcription factor CEH-23.

Rtg3 in a hyperphosphorylated state (Sekito et al., 2000; Dilova et al., 2002; Sekito et al., 2002; Liu et al., 2003; Dilova et al., 2004). Partial phosphorylation of Rtg3 is necessary, however, to expose its nuclear localization signal and thus to render the Rtg1–Rtg3 capable of activating retrograde target genes. Mks1 is removed by ubiquitin-mediated degradation promoted by the ubiquitin ligase component Grr1 (Liu et al., 2005). Thus, Grr1 is a positive regulator of the retrograde response, while Mks1 is a negative regulator.

Target of rapamycin complex 1 is also a negative regulator of the retrograde response. TORC1 appears to act both upstream and downstream of Rtg2 (Komeili et al., 2000; Giannattasio et al., 2005; Breitkreutz et al., 2010). One of the components of TORC1 is the WD-protein Lst8, which in genetic studies was shown to act upstream and downstream of Rtg2 depending on the identity of the mutation in Lst8 which was examined (Liu et al., 2001; Chen and Kaiser, 2003). This coincides well with the fact that TORC1 impinges upon the retrograde response at multiple points. The regulation of the retrograde response by TORC1 ensures that it is not active when nutrients, such as glutamate, are plentiful.

Target of rapamycin (*TOR*) complex 1 is subject to negative feedback from dysfunctional mitochondria, because TORC1-mediated phosphorylation of Sch9, an AGC protein kinase, is down-regulated in rho^0 cells (Kawai et al., 2011). Phosphorylated Sch9 antagonizes stress responses under the control of the Msn2–Msn4 transcription factor and promotes ribosome biogenesis (Urban et al., 2007). It also inhibits protein kinase A activity, balancing cell growth and metabolism with stress resistance (Zhang et al., 2011). This occurs because protein kinase A negatively regulates Msn2–Msn4 mediated stress responses and because it feedback inhibits its own activation, which likely prevents an exaggerated response to the feedback inhibition of TORC1 by dysfunctional mitochondria. Osmotic stress also reduces Sch9 phosphorylation by TORC1, but only transiently (Urban et al., 2007). Osmotic stress is known to recruit the Rtg1–Rtg3 transcription factor (Pastor et al., 2009). Thus, retrograde signaling responds not only to metabolic stress but to other types of stress as well.

Ras2 is a positive regulator of the retrograde response (Kirchman et al., 1999); however, it is not clear at which point in the retrograde signaling pathway Ras2 exerts its effect. Interestingly, *MKS1* was originally identified as a negative regulator of the Ras2–cAMP pathway (Matsuura and Anraku, 1993). This, together with the effects of TORC1 on protein kinase A suggests that it is the Ras2–cAMP pathway that contributes to the retrograde response. However, this interpretation is complicated. Activation of the retrograde response extends yeast replicative lifespan, which is measured by the number of times an individual cell divides (Kirchman et al., 1999). Ras2 also extends replicative lifespan (Sun et al., 1994). However, it does so via a cAMP-independent pathway. Thus, it is not clear which of the Ras2 pathways impacts the retrograde response, and indeed both the cAMP-dependent and independent pathways may be involved.

Rtg2 plays multiple roles in the cell. As discussed above, it is a positive regulator of the retrograde response, by promoting dephosphorylation of Rtg3 in the cytoplasm. In addition, it has at least two other roles in the nucleus. Rtg2 is an integral component of the transcriptional co-activator SAGA-like (SLIK) complex that contains the histone acetyltransferase Gcn5 (Pray-Grant et al., 2002). SLIK is required for the induction of the retrograde response target gene *CIT2*, and it has been shown to bind to the *CIT2* promoter. The other role Rtg2 plays in the nucleus is promotion of genome stability (Bhattacharyya et al., 2002; Borghouts et al., 2004). The mechanism by which it extends this protection is not known, except that it does not involve the participation of an intact SLIK complex (Kim et al., 2004).

The retrograde signal transducer proximal to the dysfunctional mitochondrion is Rtg2 (Liu and Butow, 2006). However, the nature of the mitochondrial signal that triggers the retrograde response has not been clear until recently. One of the candidates was the drop in membrane potential ($\Delta\Psi_m$) in dysfunctional mitochondria. Manipulation of $\Delta\Psi_m$ genetically, irrespective of the presence or absence of mtDNA, has shown that loss of $\Delta\Psi_m$ is necessary and sufficient to activate the retrograde response. However, the loss of mtDNA can augment this effect (Miceli et al., 2011). The question now becomes how this signal is read by Rtg2. A ROS scavenger does not block the signal, and it does not appear that a drop in cellular ATP levels is involved. Thus, the loss of $\Delta\Psi_m$ itself must be relayed to Rtg2. Even though they are not part of retrograde regulation, mitochondrial ROS somehow signal increased chronological lifespan (survival in stationary phase) in yeast cells in which TORC1 signaling is attenuated (Pan et al., 2011). Thus, mitochondrial ROS can perform a signaling function in some instances in yeast.

There is a gradual loss of $\Delta\Psi_m$ as yeasts replicatively age, which occurs without loss of mtDNA, and this is accompanied by a progressive activation of the retrograde response (Lai et al., 2002; Borghouts et al., 2004). Thus, it appears it is loss of $\Delta\Psi_m$ that triggers the retrograde response during the yeast replicative lifespan. In fact, the activation of the retrograde response may allow yeasts to live as long as they do. Indeed, the greater the forced induction of the retrograde response at the beginning of their lifespans is the greater the lifespan extension (Jazwinski, 2000). This indicates that the retrograde response is a compensatory mechanism for mitochondrial dysfunction.

There are two other pathways that signal mitochondrial dysfunction and extend replicative lifespan that have recently been described in yeast. Mitochondrial back-signaling is activated upon deletion of the *AFO1*/*MRPL25* gene, which encodes a protein found in mitochondrial ribosomes, and this activation extends replicative lifespan (Heeren et al., 2009). This requires an active TORC1 and the transcription factor Sfp1, which activates expression of cytoplasmic ribosomal proteins. This pathway is activated only in rho^0 cells. However, this occurs during growth on glucose which represses the retrograde response in the yeast strain studied. The deletion of nuclear genes that encode components of the mitochondrial translation complex (MTC), which activates translation of mtDNA-encoded proteins, also extends yeast replicative lifespan in a Sir2-dependent manner (Caballero et al., 2011). It had been known for quite some time that interruption of mitochondrial translation with erythromycin extends yeast replicative lifespan (Holbrook and Menninger, 2002). The relationship of mitochondrial back-signaling and the MTC to the retrograde response is of interest, but it is not known at present.

RETROGRADE RESPONSE IN OTHER ORGANISMS

The glyoxylate cycle is upregulated in *C. elegans* as a function of age and also in certain mutants that display an increased lifespan (Vanfleteren and De Vreese, 1995). This bears much resemblance to the yeast retrograde response. It has been shown that knockdown of respiratory chain components in this worm can extend lifespan (Dillin et al., 2002; Lee et al., 2003). Furthermore, a systematic search for lifespan extending genes has led to the conclusion that there is a retrograde response that extends worm lifespan (Cristina et al., 2009).

Recent studies have addressed the pathways that comprise the worm retrograde response (**Figure 1**). Knockdown of *cco-1*, among several respiratory chain components, extends life span, and activates the hypoxia-inducible transcription factor HIF-1 (Lee et al., 2010a). Parenthetically, activation of HIF-1 involves ceramide signaling and the *hyl-2* encoded ceramide synthase, a homolog of the yeast longevity assurance gene *LAG1* (Mehta et al., 2009; Menuz et al., 2009), which will have significance below. The mitochondrial signal in this *cco-1* knockdown appears to be the ROS generated during mitochondrial stress, but it is not known whether this follows changes in $\Delta\Psi_m$. In another study, down-regulation of COX4 (*cco-1*) by RNAi extended lifespan and concomitantly activated the mitochondrial unfolded protein response, which recruited the transcription factors UBL-5 and DVE-1 (Durieux et al., 2011). This response was cell-non-autonomous implying the secretion of a "mitokine" by certain cells, to which other cells respond. In yet another study, an RNAi screen for reduced mitochondrial electron transport chain function identified CEH-23, a predicted transcription factor, in the longevity increase (Walter et al., 2011). In each of the above studies, the respective transcription factors were shown to be necessary and sufficient for life span extension. Thus, the mutual relationships of the "retrograde responses" uncovered in these studies is not clear at present.

This discussion of the worm retrograde response suggests a marked heterogeneity of the responsible signaling pathways, as compared to the yeast retrograde response. However, recent studies in yeast also point to more than a single, comprehensive response to mitochondrial dysfunction. The worm signaling pathways appear more disparate, and they may reflect the increased complexity of this metazoan. *C. elegans* does not possess an NFκB homolog, which may have evolved as a master regulator along with the appearance of more complex immune systems (Srinivasan et al., 2010). Thus, NFκB may have gathered together the capacity to respond to a variety of inputs with a variety of outputs, including the equivalent of a retrograde response as we discuss below.

There may in fact be more than one type of mitochondrial "retrograde response" in the worm. Recent work has demonstrated that the location of the respiratory chain disruption that extends *C. elegans* longevity affects the associated phenotypic manifestations (Yang and Hekimi, 2010b). Evidence has also been presented that mitochondrial ROS serve a signaling function in lifespan extension, in a pathway distinct from any known lifespan extending mechanism in the worm (Yang and Hekimi, 2010a). Curiously, this novel ROS pathway may have bile acid-like mediators that perform a hormonal function (Liu et al., 2012).

A retrograde response has been characterized in *D. melanogaster* in a variety of RNAi strains in which anyone of several respiratory chain components were knocked down (Copeland et al., 2009). Many of these strains displayed an increase in life span. A mutant in the *sbo* gene involved in coenzyme Q biosynthesis also demonstrated extended lifespan (Liu et al., 2011). The identity of the signaling pathway(s) involved here in lifespan extension is not known.

In mice, reduced activity of *MCLK1*, involved in coenzyme Q biosynthesis and resulting in a defective electron transport chain, markedly extended life span, with no apparent tradeoff in growth or fertility (Lapointe and Hekimi, 2008). These mouse studies followed up on early work on the *C. elegans clk-1* gene, mutants of which extended worm longevity. In a *SURF1* knockout mouse, mitochondrial complex IV assembly is disrupted, and this also results in substantially increased longevity (Dell'Agnello et al., 2007). It is not clear which signal transduction proteins are utilized in these mouse retrograde responses, but they are likely to differ from the yeast *RTG* genes.

Mitochondrial respiratory defects elicit expression of nuclear genes in mammalian cells (Heddi et al., 1993). Calcium-signaling and NFκB signaling are among the potential signaling pathways that have been implicated in these "retrograde responses" (Butow and Avadhani, 2004). NFκB is a conserved master regulator that responds to a wide range of stress signals including ROS (Srinivasan et al., 2010). It is also associated with mitochondrial biogenesis. A mitochondrial stress response to aggregated ornithine decarboxylase in mammalian cells may be similar to the mitochondrial unfolded protein response in *C. elegans*, described above; however, it specifically involves the CHOP transcription factor and not UBL-5 and DVE-1 homologues (Zhao et al., 2002). CHOP is a target for activation by ceramide generated by the *LASS6*-encoded ceramide synthase in human cells, which protects the cells from endoplasmic reticulum stress-induced apoptosis (Senkal et al., 2010). This connection to ceramide signaling will become more significant in the discussion below.

Gene expression differences in rho^+/rho^0 pairs of three different human cell types are heterogeneous between cell types and may reflect cellular pathology (Miceli and Jazwinski, 2005). However, the gene expression changes consistent across cell types reflected an adaptation to loss of respiratory function by stimulation of glycolysis, enhanced protection from ROS, and compensation for genome instability. These gene expression changes reflect the physiologic events in yeast cells in which the retrograde response is activated. One of the genes commonly activated was c-Myc, a basic helix-loop-helix/leucine zipper transcription factor. The Myc–Max heterodimer is homologous to Rtg1–Rtg3 (Srinivasan et al., 2010). Significantly, NFκB has two binding sites in the Myc promoter, suggesting that the mammalian retrograde response may involve both NFκB and Myc (Duyao et al., 1990). NFκB would be upstream of Myc in this scenario. This is further consistent with the observation of both increased NFκB activity and elevated levels of Myc activity with age, which is suppressed by Cdk2 in a cell senescence model (Semsei et al., 1989; Campaner et al., 2010). Acute mitochondrial respiratory stress activates NFκB through a novel calcineurin-dependent pathway (Biswas et al., 2008). Stress-activated calcineurin participates in TORC2

regulation of ceramide synthase, as discussed below. This protein phosphatase also responds to the rise in cytoplasmic calcium found in mammalian rho^0 cells and to which NFκB is known to respond (Butow and Avadhani, 2004). Despite these similarities between the yeast and mammalian retrograde responses, homologues of Rtg2 have not yet been found in mammals. Thus, this particular link to mitochondrial dysfunction in mammalian retrograde signaling is still missing.

There also appears to be a link between mitochondrial dysfunction in mammalian cells and cell lifespan (Passos et al., 2007). Mild mitochondrial uncoupling with dinitrophenol delays the replicative senescence of normal human diploid fibroblasts, while lowering ROS production, reducing telomere shortening, preventing the appearance of DNA repair foci in the nucleus, and inducing a variety of gene expression changes. Thus, mammalian cells show many of the molecular features of yeast retrograde signaling, and they also display the extended life span characteristic of the retrograde response. It will be important to identify the relevant signaling pathways.

CELLULAR QUALITY CONTROL AND THE RETROGRADE RESPONSE

General autophagy is a gene regulated process that is non-selective for removal of proteins and organelles through their degradation in the lysosome (Nakatogawa et al., 2009). This process can become selective, however. In the case of mitochondria, the Atg32 protein tags yeast mitochondria for selective removal by autophagy, in a process termed mitophagy (Kim et al., 2007; Kissova et al., 2007). This can serve to channel nutrients to starving cells. It can also allow remodeling of the cell to adapt to changing metabolic needs, as well as to remove damaged and dysfunctional organelles. The mitochondrial fission–fusion cycle plays a role in mitophagy, since deletion of *DNM1* which is required for fission attenuates mitophagy without entirely eliminating it (Kanki et al., 2009). Interestingly, deletion of this gene extends yeast replicative lifespan, suggesting that some but perhaps not excessive mitophagy is consistent with long life (Scheckhuber et al., 2007).

Stationary phase mitophagy requires the *AUP1* gene in yeast (Journo et al., 2009). The Aup1 protein is located in the mitochondrial intermembrane space, and it specifies a protein phosphatase. During stationary phase mitophagy, the retrograde response is activated, and this requires *AUP1* (Journo et al., 2009). Furthermore, deletion of *RTG3* prevents this activation of the retrograde response and mitophagy, and Aup1 affects the phosphorylation status of Rtg3. Thus, the signal generated by mitochondria appears somehow to be relayed to Rtg3 during stationary phase to elicit retrograde target gene expression and induce mitophagy. These activities are consistent with the extensive remodeling of metabolism as yeast cells adapt to survival in stationary phase.

General autophagy is inhibited by TORC1, which phosphorylates Atg13 a component of the Atg1 kinase that is essential for autophagy (Kamada et al., 2010). Deletion of the genes required for the synthesis of complex sphingolipids in yeast (*IPT1* and *SKN1*) augments autophagy induced by nitrogen starvation (Thevissen et al., 2010). Sphingosine and ceramide are the substrates for complex sphingolipid biosynthesis, but sphingosine-1-phosphate and ceramide stimulate autophagy in mammalian cells (Lavieu et al., 2008). Together these observations suggest that a delicate balance exists in the sphingolipid biosynthetic pathway that can tip the scale from quality control through limited autophagy to full blown degradation. Thus, sphingolipid signaling has a modulatory effect on autophagy, while TORC1 may play the primary role in its regulation.

TORC2 also may regulate autophagy in yeast by stimulating ceramide synthase activity through the activation of Ypk2, another AGC protein kinase (Aronova et al., 2008). It does this in the presence of nutrients. The stimulation of ceramide synthesis would provide the substrate for complex sphingolipid synthesis and thus attenuation of autophagy. On the other hand, ceramide synthase is inhibited by the stress responsive protein phosphatase calcineurin, which is dependent on calcium/calmodulin (Aronova et al., 2008). In this way, stress would enhance autophagy. It appears that TORC2, through its effects on sphingolipid signaling is a potential modulator of autophagy.

The response to mitochondrial dysfunction may be linked to ceramide synthase activity. Ceramide synthase activity in yeast is encoded by the longevity assurance gene *LAG1* and its homolog *LAC1*, and this enzyme is located in the endoplasmic reticulum membrane (D'Mello et al., 1994; Jiang et al., 1998; Guillas et al., 2001; Schorling et al., 2001). The *LAG1*/*LAC1* orthologs in human, *LASS1–6*, encode the six human ceramide synthases, Lass1-6, which are also located in the endoplasmic reticulum (Venkataraman et al., 2002; Guillas et al., 2003; Teufel et al., 2009). A physical junction between the mitochondrion and the endoplasmic reticulum has been identified, and it has been proposed to regulate mitochondrial biology (Kornmann and Walter, 2010). This regulation could involve ceramide signaling, which plays an important role in processes such as cell cycle control and stress resistance (Dickson, 2010). Such a role would require exquisite balance. Perhaps this explains why the expression of the *LAG1* ceramide synthase gene in yeast, which determines yeast replicative lifespan, is so finely tuned (Jiang et al., 2004). In mammalian cells, mitofusin 2 plays a decisive role in bridging the endoplasmic reticulum and mitochondria (de Brito and Scorrano, 2008). It thus impacts metabolism, apoptosis, and cell cycle progression. Importantly, the tethering of mitochondria to the endoplasmic reticulum is required for efficient mitochondrial calcium uptake (de Brito and Scorrano, 2008), which may impact NFκB activation in cells in mitochondrial respiratory stress as discussed earlier (Biswas et al., 2008). One of the hallmarks of mtDNA-less mammalian cells is an increase in cytoplasmic calcium levels, which leads to activation of calcineurin (Biswas et al., 1999), which would inhibit ceramide synthase and thus alter the balance of autophagy.

In addition to the physical connection between the mitochondrion and the endoplasmic reticulum, there are other ways in which the activities in these cell compartments may be coordinated. The *LAC1* gene is coordinately regulated with the multi-drug resistance family of membrane transporters in yeast (Kolaczkowski et al., 2004). These transporters are activated in rho^0 cells by retrograde signaling and by mechanisms independent of the *RTG* genes in the presence of other mitochondrial defects (Hallstrom and Moye-Rowley, 2000; Moye-Rowley, 2005). All in all, it appears that the cellular response to mitochondrial damage

in the form of the retrograde response and autophagy/mitophagy is coordinated.

THE *PINK1*/*PARKIN* CONNECTION

The PINK1 protein kinase, together with the ubiquitin ligase Parkin, plays a crucial role in the elimination of dysfunctional mitochondria by mitophagy (Deas et al., 2011; Kawajiri et al., 2011). The mitochondrial signal that triggers this process is the loss of $\Delta\Psi_m$ (Narendra et al., 2009). This process is defective in Parkinson's disease, which can be caused by mutations in *PARKIN*, resulting in its aggregation, as well as by mutations in *PINK1* (Deas et al., 2011; Kawajiri et al., 2011). PINK1 is localized on the mitochondrial outer membrane. Mitochondrial depolarization results in the translocation of Parkin to mitochondria, and this requires PINK1. One of the targets of polyubiquitination by Parkin is the voltage-dependent anion channel (VDAC1; Geisler et al., 2010). The mitochondrial outer membrane protein Miro is a PINK1 substrate, and it is degraded via Parkin-mediated polyubiquitination (Wang et al., 2011). This detaches the dysfunctional mitochondria from microtubules preventing their trafficking, and likely aiding in their removal by mitophagy. There are no PINK1 or Parkin homologues in yeast. However, Gem1, the yeast Miro homolog, is found in mitochondria-endoplasmic reticulum contact sites and regulates the association of the two organelles (Kornmann et al., 2011), which suggests that Miro degradation would also release mitochondria from the endoplasmic reticulum. It will be of interest to determine whether ceramide signaling is engaged in the processes described here, because of the potential effects of the mitochondria-endoplasmic reticulum junctions on ceramide synthase activity.

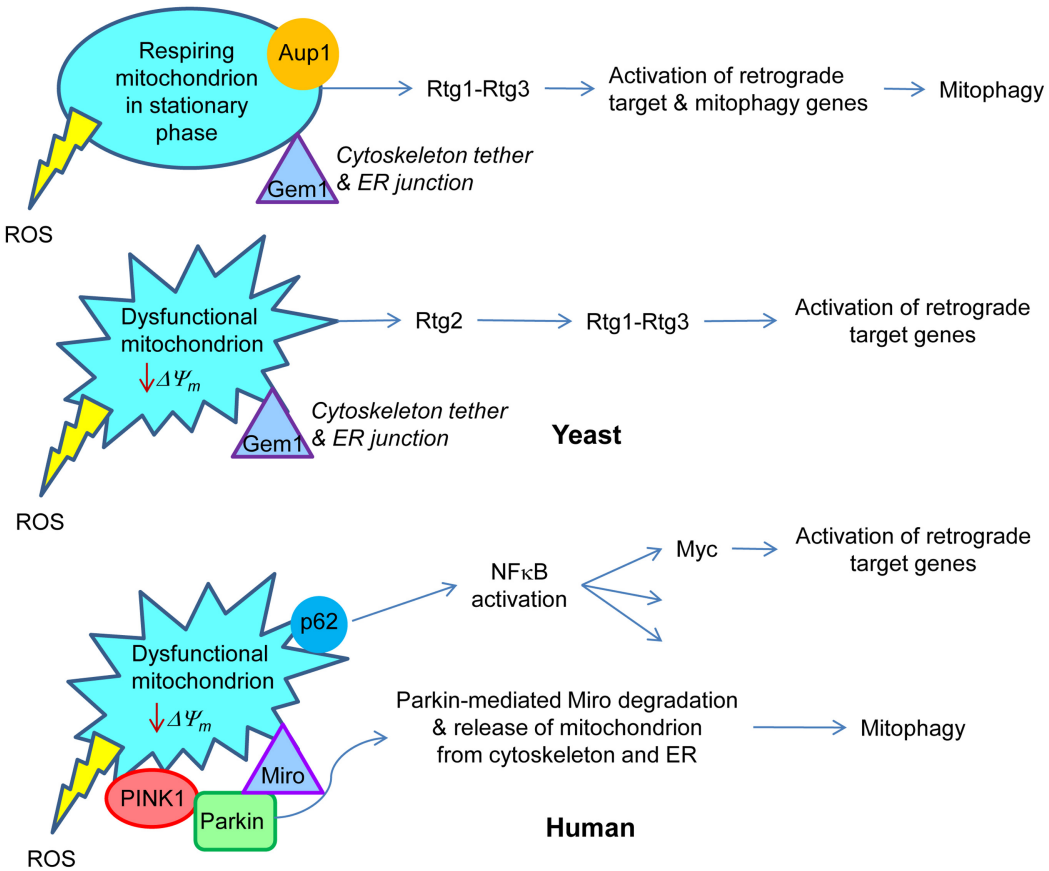

FIGURE 2 | Retrograde signaling in yeast and human. In yeast, respiring mitochondria in non-dividing, stationary phase cells signal the retrograde response that activates both retrograde response target genes, similar to those in dividing cells, and mitophagy genes. This results in the metabolic adaptation to stationary phase. Aup1, a protein phosphatase in the intermembrane space in mitochondria, is essential for this gene induction. Rtg1–Rtg3 is the retrograde transcription factor. On the other hand, dysfunctional mitochondria in growing cells trigger the classical retrograde response with activation of retrograde response target genes. Rtg2 plays an essential role in this process. Gem1 is a Miro homolog in yeast which is important for maintaining junctions between mitochondria and the endoplasmic reticulum. By analogy with mammalian cells, it would also tether the mitochondria to the cytoskeleton. In human cells, a drop in mitochondrial membrane potential ($\Delta\Psi_m$) recruits Parkin by the PINK1 protein kinase to the mitochondrial membrane. Parkin mediates ubiquitylation of Miro, which releases the mitochondria from the cytoskeleton and also, presumably, from the endoplasmic reticulum. This facilitates the removal of dysfunctional mitochondria by mitophagy. Sequestosome 1 (p62) aggregates proteins polyubiquitinated by Parkin on the surface of mitochondria. p62 is known to stimulate NFκB, which among its many target genes has Myc. The Myc–Max dimer is homologous to Rtg1–Rtg3. Transcription of Myc is activated in human cells devoid of mtDNA, and Myc itself activates the transcription of metabolic genes, typical for the retrograde response. The production of reactive oxygen species (ROS) by the mitochondria may elicit responses as well.

Sequestosome 1 (p62) can aggregate ubiquitylated proteins and accumulates on mitochondria that have undergone Parkin-mediated polyubiquitination (Geisler et al., 2010). It is not clear whether or not p62 is required for mitophagy. However, its role in the response to mitochondrial damage may be related to its activation of the NFκB pathway, as discussed below. The similarities and differences between yeast and human cells in the activation of some of the responses to changes in mitochondrial metabolism are highlighted in **Figure 2**. We have discussed those aspects related to mitophagy. We turn our attention below to the master regulator NFκB, which incorporates the roles of the retrograde transcription factor Rtg1–Rtg3 into its repertoire.

NFκB

The components of NFκB heterodimers are diverse, with roles in innate immunity, inflammation, and anti-apoptosis, and they respond to a wide diversity of stressors. NFκB forms a class of transcription factor with several members including RelA and p50, and their activation relies on IKK kinases phosphorylating inhibitory IκB proteins. Phosphorylated subunits of IκB are short-lived and are degraded by the proteasome, through the ubiquitin proteasome system (UPS) pathway. In mammalian cells, NFκB is localized at the mitochondrial membrane (Cogswell et al., 2003), and it participates with mitochondria in innate immune responses (West et al., 2011). The cluster formed by IKK, IκB, and NFκB resembles pathway connections and mediators known for the *RTG* genes (**Figure 3**). One of the constitutive mechanisms of NFκB activation is oncogenic *RAS*, utilizing Raf-dependent and independent MAPK signaling pathways (Norris and Baldwin, 1999). NFκB can be activated by a number of atypical mechanisms including the endoplasmic overdose response (Pahl and Baeuerle, 1997) and aging (Kriete and Mayo, 2009), making NFκB a universal stress response sensor.

We have discussed above the similarities of the *RTG* genes and NFκB in metabolic regulation. One modulator of the NFκB pathway is mTOR. Inhibition of mTOR, involving separation of *TOR* from LST8, activates autophagy (Diaz-Troya et al., 2008). While activation of NFκB in some cases may depend on the degradation of IκB inhibitory proteins by autophagy independent of the proteasome (Jia et al., 2012), the role of mTOR and autophagy in NFκB activity is complex and involves other pathways including protein kinase B (Akt; Dan et al., 2008) and HSP90 (Qing et al., 2007). A related conundrum is the finding that IKK complexes can activate autophagy (Criollo et al., 2010, 2012). In a

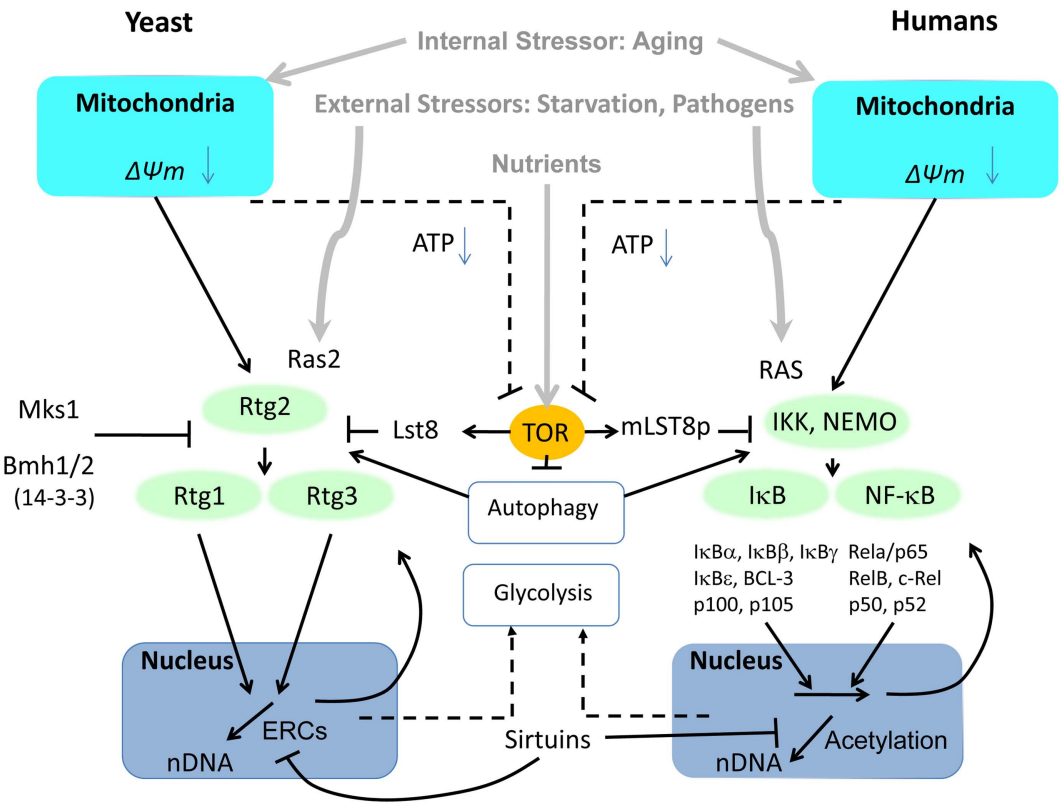

FIGURE 3 | Side-by-side comparison of the retrograde response controlled by the heterodimeric transcription factors Rtg1–Rtg3 in yeast and NFκB in mammalian cells. In contrast to the three Rtg proteins in yeast, NFκB activators, inhibitors and transcription factors have evolved into a wide spectrum of subunits to elicit specific response patterns to a variety of stressors. Common to both pathways is their activation by mitochondrial dysfunction involving reactive oxygen species (ROS) and changes in mitochondrial membrane potential ($\Delta\Psi_m$). Another common activator that responds to external stressors is RAS (Ras2 in yeast). Furthermore, both pathways are modulated by TOR through LST8. LST8 dissociates from mTOR under stress, although it is not known whether this is true of TOR in yeast. Regulation of autophagy by TOR impinges on both pathways, as well. The adaptive response of both pathways to mitochondrial dysfunction includes upregulation of glycolysis to compensate for energy deficiency.

study involving treatment of B-cell lymphoma cells, bortezomib led to proteasome down-regulation and accumulation of polyubiquitinated proteins (Jia et al., 2012). Hereby, the autophagy adapter protein Sequestosome 1 (p62) recruits LC3-II and ubiquitinated proteins including IκBα for subsequent transport and degradation to autophagosomes. p62 can promote tumorigenesis by activating ROS scavenging via the NFκB pathway (Moscat and Diaz-Meco, 2009). It also associates with TRAF6, and in this way regulates NFκB signaling in bone homeostasis (Moscat and Diaz-Meco, 2009). p62 interacts with atypical protein kinase C proteins, such as PKCζ (Moscat and Diaz-Meco, 2009), which control NFκB (Duran et al., 2003) as well as inflammatory cytokine production in adipocytes (Lee et al., 2010b). This can lead to insulin resistance even in the absence of obesity, which has implications for aging. p62 also could be relevant for TNF-α activation of NFκB. Recent studies show that p62 interacts with Raptor and is required for TORC1 activation by amino acids, in turn downregulating autophagy (Duran et al., 2011). Thus, the regulation of autophagy and NFκB-mediated inflammation are related.

NFκB activity is controlled by ceramide signaling in some contexts. LPS-stimulated prostaglandin E_2 synthesis in macrophages is mediated by the upregulation of *COX2* transcription by ceramide (Wu et al., 2003). The transcription factor responsible is NFκB, whose activation is greater in old mice than in young ones. This effect is due to greater degradation of IκB in macrophages from old animals. Constitutively activating NFκB in p65 knock-in mice results in aberrant systemic inflammation involving TNFα signaling and signs of premature aging (Dong et al., 2010).

MAMMALIAN EQUIVALENT OF THE GLYOXYLATE CYCLE

A key feature of the yeast retrograde response is activation of the glyoxylate cycle, which allows the truncated TCA cycle to serve as a source of biosynthetic intermediates (**Figure 4**). Tumor cells often possess mutations in mitochondrial components, which disable the electron transport chain (Mullen et al., 2012). Such cells can grow readily by generating ATP through glycolysis. However, they need a source of biosynthetic intermediates. It has recently been shown that this source is the reductive carboxylation of α-ketoglutarate derived from glutamine, which yields both acetyl-coenzyme A and the four-carbon TCA cycle intermediates that are used in various biosyntheses (Mullen et al., 2012). The key reductive carboxylation is catalyzed by isocitrate dehydrogenase-1 (*IDH1*) in the cytoplasm (**Figure 4**), although some synthesis may occur in mitochondria. The same situation occurs in normal cells under hypoxic conditions, and HIF-1α and HIF-2α both appear to play a role in the metabolic switch (Metallo et al., 2012). It is

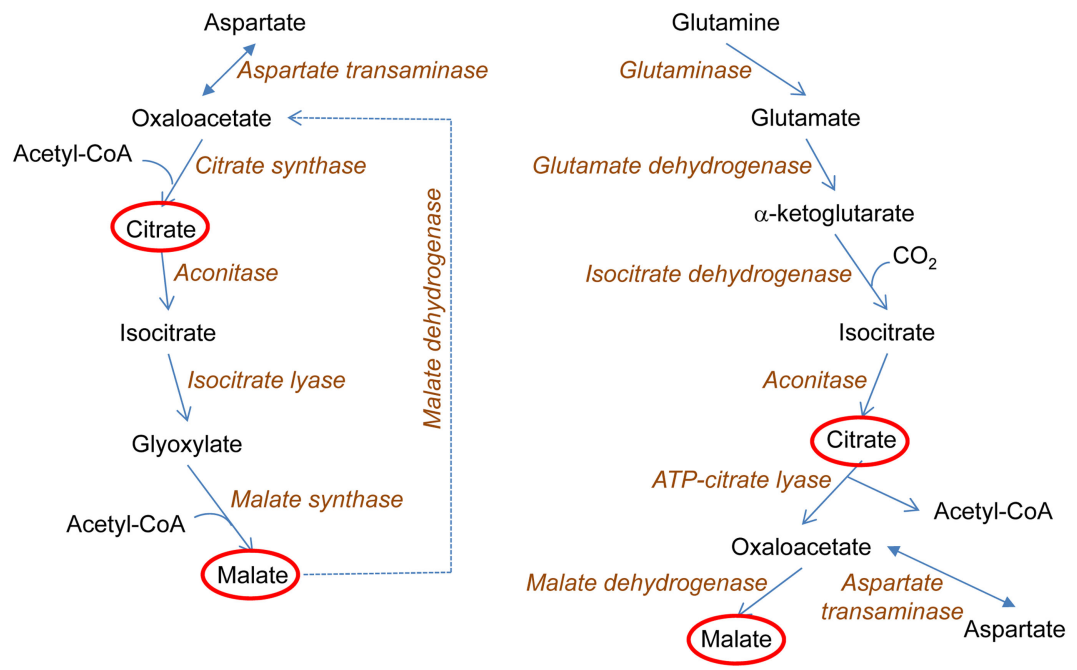

FIGURE 4 | Metabolic adaptations to loss of biosynthetic intermediate production by a truncated tricarboxylic acid (TCA) cycle. The loss of the electron transport chain interrupts the TCA cycle at the succinate dehydrogenase reaction. This prevents the utilization of the TCA cycle for production of biosynthetic intermediates. In yeast, the glyoxylate cycle is induced. This allows acetyl-coenzyme A (acetyl-CoA) to be used for the synthesis of the TCA cycle metabolites citrate and malate, in reactions that conserve the two carbons of acetate. This, in turn, allows the first three reactions of the TCA cycle to proceed with the synthesis of α-ketoglutarate, which can be converted to glutamate, the ultimate source of nitrogen in biosynthesis (not shown here). In human cells, a related metabolic adaptation occurs. This adaptation is the reductive carboxylation of α-ketoglutarate to yield isocitrate, which in turn is a source of TCA cycle intermediates citrate and malate at the same time generating acetyl-CoA for lipid biosynthesis. The ultimate source of α-ketoglutarate in these reactions is glutamine, which allows the use of glucose for production of energy in glycolysis as well as for biosynthetic reactions. In both yeast and human, TCA cycle metabolites are used as macromolecular precursors.

not known whether ceramide regulates these transcription factors as it does HIF-1 in *C. elegans* (Mehta et al., 2009; Menuz et al., 2009). If it does, the parallel between the effects of mitochondrial dysfunction on metabolism in human and in yeast and worms would be striking.

There are additional hypoxia-driven changes that lead to reductive glutamine metabolism. HIF-2α promotes Myc transcription (Gordan et al., 2007). This enhances glutamine catabolism, among others by activating glutaminase expression (Gao et al., 2009). Furthermore, Myc upregulates genes involved in glycolysis, promoting the generation of lactate and ATP by substrate level phosphorylation (Collier et al., 2003), a response important in rho^0 mammalian cells (Miceli and Jazwinski, 2005).

CONCLUSION

The retrograde response in yeast and related pathways in higher organisms share the common adaptive function of supporting cellular survival. Activated by external perturbations like heat and osmotic shock, bacterial pathogens, UV radiation, starvation, and related mitochondrial dysfunction, they protect the cell transiently. Environmental challenges that perturb mitochondrial function are equally likely to be encountered across species, from yeast to mammalian cells. It comes therefore as no surprise that many responses exhibit similar features as shown here for *RTG* and NFκB stress responses. The decisive difference between the aging process and acute environmental perturbations, regardless of the biological system, is a rather slow accumulation of damage and dysfunction in the former. Although mechanisms like the retrograde response compensate for mitochondrial deficiencies and extend lifespan, they cannot escape aging in the long term. The retrograde response was not likely selected as an anti-aging mechanism, as it operates as a double-edged sword. In yeast, its activation extends lifespan, but it also reduces genome stability which can ultimately contribute to cell demise. In mammalian cells, NFκB, optimized under evolutionary pressure to respond to acute challenges like infections, promotes inflammatory disease states when chronically activated in aging.

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Function and regulation of lipid biology in *Caenorhabditis elegans* aging

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Rapidly expanding aging populations and a concomitant increase in the prevalence of age-related diseases are global health problems today. Over the past three decades, a large body of work has led to the identification of genes and regulatory networks that affect longevity and health span, often benefiting from the tremendous power of genetics in vertebrate and invertebrate model organisms. Interestingly, many of these factors appear linked to lipids, important molecules that participate in cellular signaling, energy metabolism, and structural compartmentalization. Despite the putative link between lipids and longevity, the role of lipids in aging remains poorly understood. Emerging data from the model organism *Caenorhabditis elegans* suggest that lipid composition may change during aging, as several pathways that influence aging also regulate lipid metabolism enzymes; moreover, some of these enzymes apparently play key roles in the pathways that affect the rate of aging. By understanding how lipid biology is regulated during *C. elegans* aging, and how it impacts molecular, cellular, and organismal function, we may gain insight into novel ways to delay aging using genetic or pharmacological interventions. In the present review we discuss recent insights into the roles of lipids in *C. elegans* aging, including regulatory roles played by lipids themselves, the regulation of lipid metabolic enzymes, and the roles of lipid metabolism genes in the pathways that affect aging.

Keywords: *C. elegans*, lipids, lipase, ascarosides, fatty acids, nuclear hormone receptors, mitochondria, *N*-acylethanolamine

INTRODUCTION

One of the most desirable goals in biomedical research is to understand the molecular mechanisms that promote healthy aging. Identifying the processes that lead to extended longevity in laboratory settings may allow the exploitation of that knowledge to generate pharmacological treatment regimens that delay the onset and/or reduce the severity of age-associated diseases, or even extend life span *per se*. In model organisms, several genetic and nutritional conditions have been identified that not only extend mean and/or maximal life span but also postpone the onset of phenotypes associated with aging, such as a loss of mobility, a decline in cognitive ability, and others.

It has been almost 30 years since single gene mutations were first found to affect aging in the nematode *Caenorhabditis elegans* (reviewed in Kenyon, 2010a). Since then, *C. elegans* has become a powerhouse for studies on the mechanisms that affect longevity. Numerous labs are identifying an ever-increasing number of genes, molecules, and regulatory networks that influence aging. Several excellent reviews summarize recent progress in this field and the reader is referred to these (Fontana et al., 2010; Gallo and Riddle, 2010; Kenyon, 2010b; Zhou et al., 2011).

Despite the identification of several discrete pathways that affect longevity, the molecular mechanisms that actually result in life span extension remain obscure. Of note, many pro-longevity signaling pathways affect lipid biology and/or nutrient sensing/signaling. For example, insulin/insulin-like growth factor (IGF) signaling (IIS), which affects aging in many model organisms, integrates nutritional cues, and regulates fat storage in many animals (Fontana et al., 2010; Kenyon, 2010b). Other pathways known to influence aging and lipid biology and/or nutrient sensing include dietary restriction mechanisms including intermittent fasting paradigms; the electron transport chain (ETC) of the mitochondria; signaling following genetic or physical germline removal; target of rapamycin (TOR) signaling, which governs nutrient sensing and is linked to dietary restriction; and translation inhibition, which interacts with TOR and dietary restriction in yet poorly understood ways.

One simple explanation would be that all longevity-controlling pathways equivalently affect storage lipid levels, thus evoking similar overall changes in energy balance to extend life span. This is clearly not the case, as mutants in various aging pathways differentially alter overall fat storage. Thus, some *C. elegans* mutants are long-lived and exhibit increased levels of triacylglycerides (TAGs, i.e., stored fat); these include worms carrying a mutation in the *daf-2* gene, which encodes the *C. elegans* insulin/IGF-1 receptor (Kenyon et al., 1993; Ashrafi et al., 2003), germline-less *glp-1* mutants (Arantes-Oliveira, 2002; O'Rourke et al., 2009), and *tub-1* mutants (Mukhopadhyay et al., 2005). In contrast, *eat-2* mutants,

which experience dietary restriction due to mechanically reduced pharyngeal pumping, are long-lived but have reduced fat storage (Lakowski and Hekimi, 1998; Srinivasan et al., 2008; Brooks et al., 2009). Thus, long-lived mutants can be high or low in fat. Moreover, in the *tub-1* mutants, two genetically distinct pathways influence fat storage and aging (Mukhopadhyay et al., 2005). Therefore, there is no strict link between aging and global fat storage, at least when assessing fat deposition by techniques that monitor global lipid stores in whole animals or whole animal extracts. Clearly, if lipids indeed modulated longevity, their role must be more diverse and complex than initially anticipated.

THE CENTRAL ROLES OF LIPIDS IN CELLULAR AND MOLECULAR BIOLOGY

Lipids are broadly defined as hydrophobic or amphiphilic molecules formed in whole or in part by ketoacyl and/or isoprene groups (Fahy et al., 2009; Subramaniam et al., 2011). Lipids play a central role in metazoan physiology, as they affect cellular and organismal functions in three principal, non-exclusive ways: (i) in signaling cascades, both directly as signaling molecules (e.g., diacylglycerol, fatty acids, phosphatidylinositols, sterols, ceramides, and sphingolipids), and indirectly by reversibly and irreversibly tethering signaling proteins to cellular membranes (e.g., via prenylation or palmitoylation); (ii) as structural elements in cellular membranes, providing cellular, and subcellular compartmentalization; and (iii) as key molecules in energy metabolism. Lipids are also integral components in lipophilic vitamins, which influence a large number of cellular processes, and it is conceivable that vitamin bioavailability may influence longevity. In line with their central role in physiology, lipids, or at least genes directly involved in lipid metabolism, have recently been mapped into several signaling pathways that affect longevity; yet, the role of lipids in the modulation of aging remains enigmatic (Ackerman and Gems, 2012). Here, we review recent reports that suggest important roles for lipids and lipid metabolizing enzymes in aging.

LIPID SIGNALING IN AGING: NUCLEAR HORMONE RECEPTOR ACTION

Lipids and their derivatives can act in various signaling pathways to affect aging, including cell membrane receptor signaling or Nuclear Hormone Receptor (NHR) signaling (Germain et al., 2006). NHR signaling is intriguing, as lipids do not only modulate NHRs but some NHRs in turn affect lipid biology through their downstream regulatory actions. NHRs are ligand-gated transcription factors that are conserved in metazoans, and they play important roles in the regulation of physiology and development (Germain et al., 2006).

The *C. elegans* genome encodes a large family of 284 NHRs, far exceeding the number of NHRs in the human, mouse, or fly genomes (Taubert et al., 2011). The vast majority of these NHRs appear to be derived from an ancestor related to Hepatocyte Nuclear Receptor 4 (HNF4; Robinson-Rechavi et al., 2005), which plays important functions in the development and function of liver and pancreas in mammals (Maestro et al., 2007; Gonzalez, 2008), whereas fly HNF4 regulates lipid mobilization and fatty acid β-oxidation (Palanker et al., 2009). Multiple studies suggest that mammalian HNF4 can be modulated by interactions with fatty acids and/or fatty acid derivates, suggesting that lipids act as ligands for HNF4 (Hertz et al., 2003; Yuan et al., 2009). Although the *in vivo* consequences of lipid-HNF4 interactions remain controversial, it is possible that lipids may similarly bind and/or regulate some *C. elegans* NHRs. However, no ligands have been identified for any *C. elegans* NHRs save for DAF-12, an NHR unrelated to HNF4. DAF-12 binds a steroid-like ligand with nanomolar affinity (Motola et al., 2006), and regulates development and aging in *C. elegans* (Antebi et al., 1998, 2000). Individual *daf-12* alleles affect aging in distinct ways, depending on whether they cause a complete loss of *daf-12* activity or abrogate only specific molecular functions, e.g., the capacity to bind ligands (reviewed in Gáliková et al., 2011). Notably, DAF-12 is required for the life span extension in worms that lack the germline (see below); these worms are thought to be long-lived due to a concomitant absence of germline-derived signals that restrict life span (Arantes-Oliveira, 2002). Thus, lipid-derived hormones play a key role in this pathway, albeit how DAF-12 acts in this context is still poorly understood.

A ROLE FOR NHR SIGNALING AND FATTY ACID DESATURASES IN AGING

Adding to the role for DAF-12 in longevity, a new study reports a key role for another NHR and for one downstream target, a fatty acid desaturase (Goudeau et al., 2011); this study thus directly links NHRs, lipid metabolism, and long life span. Specifically, in germline-less *glp-1* mutants, NHR-80 is upregulated, and transgenic NHR-80 overexpression further extends animal life span. One key regulatory target of NHR-80 turns out to be *fat-6*, a stearoyl-CoA desaturase that converts stearic acid into oleic acid; like *nhr-80*, *fat-6* is required for the pro-longevity effect of genetic germline ablation (**Figure 1**). In contrast to germline-less mutants, *nhr-80* is dispensable for the longevity of other long-lived mutants, including IIS pathway mutants, mitochondrial mutants, and worms grown under dietary restriction (Goudeau et al., 2011). Thus, NHR-80, DAF-12, and FAT-6 constitute a lipid signaling pathway that is essential for the long lifespan specifically in germline-less animals. However, the pathway is obviously more complex, as oleic acid supplementation rescues the loss of *fat-6*, but cannot complement for the loss of *nhr-80*. This suggests that NHR-80 may regulate other (lipid biology) genes in this context. Similarly, the specific role of oleic acid remains to be determined; Brock et al. (2006) previously showed that *nhr-80* mutants exhibit several changes in their fatty acid profile, likely as a consequence of reduced oleic acid levels. Any of these fatty acid species could in principle be relevant for lifespan extension in germline-less animals.

The specificity of NHR-80 and FAT-6 in this particular pathway is noteworthy. The ability of NHR-80 to drive *fat-6* expression is apparently restricted to germline-less animals, as *nhr-80* single mutants exhibit near wild-type levels of *fat-6* (Brock et al., 2006). In contrast, *nhr-80* mutants show a significant decreased *fat-5* and *fat-7* expression (Brock et al., 2006), whereas in germline-less worms, *nhr-80* mutation does not affect *fat-7* and only mildly affects *fat-5* expression (Goudeau et al., 2011). In line with a specific regulatory effect of NHR-80 on *fat-6*, only *fat-6*, but not *fat-5* or *fat-7* are required for life span extension in germline-less animals. Mechanistically, NHR-80 must assemble distinct regulatory

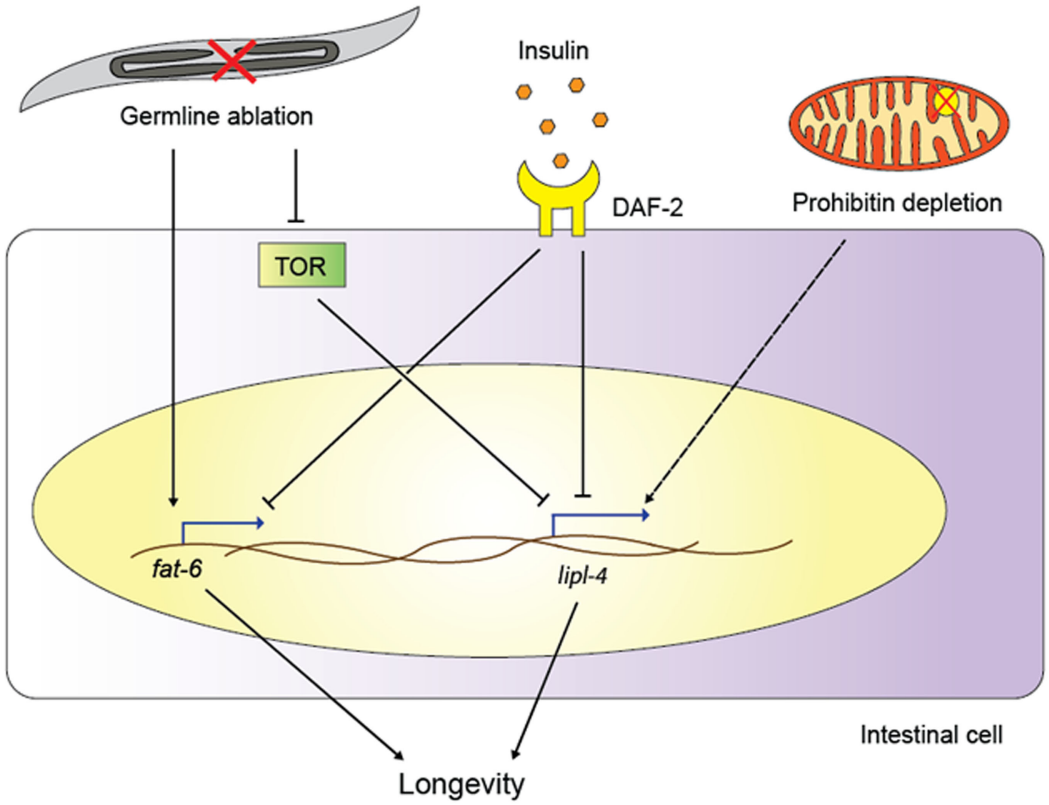

FIGURE 1 | Functions of lipid remodeling enzymes in various aging pathways. Germline ablation activates the transcription of the fatty acid desaturase *fat-6* and, through TOR signaling, of the lipase gene *lipl-4*. Insulin signaling also upregulates *lipl-4* and *fat-6*, and *lipl-4* is required for longevity of both germline-less worms and *daf-2*/insulin receptor mutants. Prohibitin depletion extends the life spans of various *C. elegans* longevity models, possibly also relying on lipase action (as indicated by the dashed line; for details, see main text).

complexes at the *fat-5*, *-6*, and *-7* promoters in the different genetic contexts to achieve appropriate gene expression. Moreover, NHR-80 and the closely related NHR-49 both regulate fatty acid desaturase gene expression (Van Gilst et al., 2005b; Brock et al., 2006), but only NHR-80 targets *fat-6* (Goudeau et al., 2011); due to this difference, NHR-49 is probably not required for the life span extension in germline-less animals, although this has not yet been directly tested using germline-less *nhr-49* mutants.

Goudeau et al. (2011) did not find a requirement for *nhr-80* in other longevity pathways such as IIS or dietary restriction, but fatty acid desaturases have been implicated in several such circuits (**Figure 1**). For example, *fat-1*, *-2*, *-3*, *-6*, and *-7* are all induced in *daf-2* mutants, and this regulation is at least partially dependent on the downstream transcription factor DAF-16, a forkhead box O transcription factor that plays key roles in longevity assurance within multiple pathways (Murphy et al., 2003; Halaschek-Wiener et al., 2005; Budovskaya et al., 2008). These data suggest that increasing the synthesis of mono- and/or poly-unsaturated fatty acids (MUFAs and PUFAs, respectively) may be important for long-lived mutants; indeed, *fat-6* or *fat-7* depletion shortens the life span of long-lived *daf-2* mutants, albeit only mildly (Murphy et al., 2003). Taken together, the above data indicate that at least some fatty acid desaturases contribute to the life span extension downstream of reduced insulin signaling or germline removal.

The roles of fatty acid desaturases and their regulators have also been studied in wild-type worms, and although the roles of these genes in wild-type worms are likely distinct from the lifespan modulation in long-lived strains, the data are nevertheless informative. For example, depletion of *fat-7* by RNA interference (RNAi; Fire et al., 1998) shortens the life span of wild-type worms (Van Gilst et al., 2005b). Similarly, depletion of the transcription factors NHR-49 or SBP-1 (the ortholog of mammalian sterol response element binding protein, a master regulator of lipogenesis and adipogenesis) or their coregulator MDT-15 reduces fatty acid desaturase expression, increases the ratio of stearic acid to oleic acid, and shortens lifespan (Van Gilst et al., 2005b; Taubert et al., 2006, 2008; Yang et al., 2006). In the case of *mdt-15* depletion, the short lifespan can be partially rescued by exogenous PUFAs, suggesting a requirement for PUFAs to maintain normal life span (Taubert et al., 2006). *mdt-15* is also required for the longevity of other long-lived mutants, including IIS pathway mutants, mitochondrial mutants, and the *eat-2* mutants that mimic dietary restriction (Rogers et al., 2011), although it is not clear whether these effects relate to MDT-15's impact on fatty acid metabolism (Taubert et al., 2006; Yang et al., 2006). *fat-3* mutants also

live shorter than wild-type worms, further supporting the notion that reduced levels of PUFAs may shorten life span (Hillyard and German, 2009). Contrasting these studies, *nhr-80* mutants exhibit an increased stearic acid to oleic acid ratio, yet a normal lifespan (Brock et al., 2006; Goudeau et al., 2011). Similarly, *fat-5*, *-6*, or *-7* single mutants do not show a shortened life span, albeit these mutants upregulate other desaturases, perhaps in a compensatory fashion (Brock et al., 2006). Lastly, *fat-4* mutants exhibit an increased lifespan (Lucanic et al., 2011), possibly through their effects on *N*-acylethanolamine (NAE) levels and endocannabinnoid signaling (see below). Clearly, fatty acid desaturases affect the life span of wild-type worms and long-lived mutants alike; yet, the requirements for individual enzymes and their upstream regulators are not the same in all genetic (and perhaps environmental) conditions.

A recent study suggests that fatty acid desaturation *per se* may not be the only determinant of how these molecules affect life span, but that parameters such as chain-length matter as well (Shmookler Reis et al., 2011). The authors used lipid profiling in various long-lived mutants to generate correlations between longevity and fatty acid composition. Several parameters correlate well with longevity; perhaps most strikingly, overall fatty acid oxidation susceptibility (as expressed by the peroxidation index) was strongly anti-correlated with longevity. Although the study focused on a set of IIS pathway mutants with extreme longevity phenotypes (all mutants were either close to wild-type life span, or *very* long-lived, as opposed to representing a more continuous spectrum), these data suggest that high PUFA levels may be disadvantageous for a long life. This somewhat contradicts the findings that, e.g., *nhr-49* mutants, *nhr-49* (*RNAi*), or *fat-7* (*RNAi*) worms are short-lived, as these worms show changes in stearic to oleic acid ratio, but only relatively minor changes in the distribution of other fatty acid species, including most PUFAs (Van Gilst et al., 2005b). However, *nhr-49* mutants, *nhr-49* (*RNAi*), or *fat-7 (RNAi*) worms are severely short-lived, and perhaps the same fatty acid properties do not apply equally in long-lived worms and in severely short-lived worms. In any case, the findings by Shmookler Reis et al. add to an existing body of data suggesting that fatty acid composition is regulated in aging worms and that it may influence life span.

These above studies suggest that fatty acid desaturation is likely important for normal and for extended lifespan, with context-specific requirements for individual regulators and metabolic enzymes. However, the above studies analyzed total fatty acids obtained from whole worm extracts. Thus, the question remains as to whether changes in fatty acid profiles may affect longevity through a role in metabolism, signaling, or perhaps membrane structure (Hulbert, 2011). Given that the observed changes in fatty acid abundance are rather large in some instances, it may seem likely that certain types of unsaturated fatty acids are structurally relevant, e.g., in the membranes of specific organelles, which in turn may affect nematode physiology. Affected organelles could be the mitochondria or the endoplasmic reticulum (ER), both of which can influence lifespan (Wong et al., 1995; Lakowski and Hekimi, 1996; Feng et al., 2001; Henis-Korenblit et al., 2010). Perhaps, membrane lipids may also influence aging by altering the properties of the nuclear envelope, which in turn may influence gene transcription or other nuclear processes. Notably, nuclear structure is linked to aging, as mutations of lamin A cause a progeria syndrome in humans (De Sandre-Giovannoli, 2003), a phenotype that is reflected in *C. elegans lmn-1* mutants (*lmn-1* is the only *C. elegans* lamin a gene), which also exhibit a short life span and altered nuclear architecture (Bank and Gruenbaum, 2011; Bank et al., 2011). Without doubt the elucidation of the mechanisms by which unsaturated fatty acids affect life span will remain a challenging and fascinating topic for future research.

LIPASE ACTION AND A LINK TO AUTOPHAGY

Two recent studies suggest that lipid-remodeling enzymes other than the desaturases can also influence longevity, namely the lipases. Lipases are esterases that catalyze the hydrolysis of fat, thus producing mono- or diglycerides, glycerol, and free fatty acids (Branicky et al., 2010). Wang et al. (2008) found that the lipase LIPL-4 is required for germline-removal induced longevity, suggesting that lipid hydrolysis plays a critical role in life span extension (**Figure 1**). Moreover, LIPL-4 expression is increased in animals without a germline (Wang et al., 2008), and intestinal overexpression of LIPL-4 alone not only reduces fat storage (Wang et al., 2011) but is also sufficient to extend the life span in wild-type worms (Wang et al., 2008).

How does LIPL-4 action contribute to increased life span? Several mechanisms appear possible. For one, lipase action can change overall fat levels, and such changes could alter animal lifespan. However, as pointed out above, fat levels and longevity do not correlate in *C. elegans*, and thus simple reduction of overall storage lipids is unlikely to cause lifespan extension. Instead, given that intestine-specific LIPL-4 is sufficient to extend the lifespan of germline-less animals, it is tempting to speculate that LIPL-4 action could generate lipids that act in endocrine fashion to modulate downstream pathways. In this context we reiterate the role of DAF-12 in the longevity of germline-less animals (Hsin and Kenyon, 1999). Perhaps, LIPL-4 products directly or indirectly contribute to altered abundance of DAF-12 ligands.

Another way for LIPL-4 to modulate lifespan could involve autophagy (Lapierre et al., 2011), a catabolic process that degrades cellular organelles and macromolecular complexes, thus recycling their molecular building blocks (Kundu and Thompson, 2008). Autophagy is a highly regulated process that participates in normal growth, development, and homeostasis, and it is known to modulate aging in *C. elegans* (Meléndez et al., 2003; Hansen et al., 2008). Lapierre et al. found that reduced TOR activity induces autophagy and LIPL-4 lipase activity in long-lived, germline-less animals. Autophagy and lipase action are co-dependent, and hence the authors suggested that LIPL-4 activity promotes autophagy. It will be interesting to determine whether it indeed does so, and if yes, by what mechanism, e.g., by providing a specific type of lipid required for autophagosome formation, or possibly by generating a regulatory ligand for a (membrane or nuclear) receptor that promotes autophagy. As described above, LIPL-4 is required for lifespan extension in IIS signaling mutants (Wang et al., 2008), and autophagy is required for lifespan extension due to impaired TOR signaling, IIS signaling, or mitochondrial function (Hansen et al., 2008; Tóth et al., 2008). Thus, autophagy is emerging as an important player in several longevity pathways, and may perhaps

broadly contribute to lifespan extension in *C. elegans*. This could involve LIPL-4 or possibly other lipases.

Lipases other than LIPL-4 may also contribute to life span modulation and/or are age-regulated in *C. elegans*. Most strikingly, *lips-7* is induced in long-lived *ctbp-1* mutants, and *lips-7* depletion prevents this longevity, whereas another lipase is dispensable; *lips-7* induction also correlates with lower fat levels in *ctbp-1* mutants (Chen et al., 2009). Again, the functional consequence of *lips-7* induction is not clear, but it is possible that *lips-7* induced autophagy contributes to the longevity of *ctbp-1* mutants. *Vice versa*, it would be useful to test whether *lips-7* plays a role in the long life span of germline-less animals. Although both LIPL-4 and LIPS-7 affect overall fat storage, it would be intriguing if they were differentially required for life span extension in different genetic backgrounds.

LIPS-7 and LIPL-4 are members of protein families, and their homologs may also affect aging. In line with this notion, LIPL-1, -2, -5, and -7, and LIPS-4, -14, and -17 expression is altered in aging worms and/or in long-lived mutants (Budovskaya et al., 2008; Golden et al., 2008; McCormick et al., 2012; Youngman et al., 2011), suggesting that they may contribute to lipid remodeling in these contexts. However, whether these regulations are cause or consequence of aging remains to be determined, and none of these lipases have yet been studied genetically to determine their roles in longevity pathways. Nevertheless, taken together with the fact that intestinal LIPL-4 expression is sufficient to extend worm life span, these data suggest that temporal and spatial changes in lipase expression may influence aging. In summary, lipases are likely to act selectively and in a tightly controlled fashion within certain signaling pathways to affect longevity.

SIGNALING BY LIPID-DERIVED SMALL MOLECULES

An exciting recent study has revealed that signaling by lipid-derived small molecules can directly modulate life span in *C. elegans*. Lucanic et al. (2011) identified *N*-acylethanolamines (NAEs) as signaling molecules that link dietary restriction to longevity. NAEs are lipid derivatives that participate in endocannabinoid signaling (Matias and Di Marzo, 2007). In mammals, endocannabinoids have profound effects on energy homeostasis by influencing food intake and by affecting energy metabolism in adipose, liver, pancreas, and skeletal muscle (Banni and Di Marzo, 2010). Lucanic et al. show that NAEs function in the *C. elegans* pharynx to signal nutrient availability; thus, compared to *ad libitum* fed worms, wild-type worms under dietary restriction show reduced NAE levels. Artificial reduction of NAEs under *ad libitum* conditions extends life span, and this effect is refractory to dietary restriction; conversely, exogenous NAEs suppress dietary restriction-induced lifespan extension. Together, these data demonstrate that NAEs and dietary restriction act via overlapping pathways (**Figure 2**). Further demonstrating a direct link between lifespan and lipid biology, Lucanic et al. find that *fat-4* mutants show reduced NAE levels and a concomitant lifespan extension, although molecules other than NAEs may also contribute to this effect. Lastly, the synthesis of one particular NAE species is reduced in long-lived worms carrying a mutation in the ribosomal protein S6 kinase gene *rsks-1*, and pharmacological supplementation with this NAE reverts the mutant's longevity. RSKS-1 is known to influence life span, and it is a critical downstream target of TOR signaling (Hansen et al., 2007; Pan et al., 2007). Thus, the data described in Lucanic et al. also suggest a link between the nutrient sensor TOR and NAE signaling.

The novel role of NAEs in longevity is exciting. Yet, as noted by Lucanic et al. (2011), the *C. elegans* genome lacks obvious orthologs to the endocannabinoid receptors that bind NAEs in mammals (McPartland and Glass, 2003); thus, the molecular mode of NAE action in worms remains unclear. The *C. elegans* genome encodes a large number of G-protein coupled receptors, and it is possible that some of these (or some other membrane receptors) bind NAEs and mediate their effects in paracrine fashion (**Figure 2**). Alternatively, NAEs may act as NHR ligands, akin to the role of two endogenous NAEs as ligands for the mouse NHR peroxisome proliferator activated receptor alpha (PPARα; Fu et al., 2003; Verme, 2005). By activating PPARα, these NAEs modulate feeding behavior. Perhaps, NAEs similarly modulate NHR activity in *C. elegans*; although PPARα orthologs are unrecognizable in the *C. elegans* genome, NHR-49 acts in a PPARα-like fashion to regulate fatty acid β-oxidation and the fasting response(Van Gilst et al., 2005a,b), and it would be interesting to determine whether it participates in NAE signaling and/or dietary restriction (**Figure 2**). Other candidates include the aforementioned NHR-80 (Goudeau et al., 2011), NHR-69, which was recently shown to modulate longevity (Park et al., 2012), and other NHRs that regulate lipid metabolism in *C. elegans* (Ashrafi et al., 2003; Arda et al., 2010; Wang et al., 2011). Clearly, much remains to be discovered about the NAEs and their role in the regulation of metabolism, behavior, and aging.

In addition to the NAEs, another class of lipid-derived small molecules is emerging as candidate life span modulators: the ascarosides, a family of small molecules whose founding members were identified based on their capacity to induce the formation of dauer larvae (also collectively referred to as dauer pheromones). The dauer is a specialized *C. elegans* larva that is long-lived and stress resistant, and its formation is induced by overcrowding, starvation, or high temperatures (Hu, 2007). Parallels between dauer larvae and long-lived mutants had been noted a long time ago: not only are dauer larvae long-lived, but many genetic pathways (e.g., IIS) and dietary stimuli (e.g., starvation) that control dauer formation also affect life span. Recent work has identified the molecular identity of the ascarosides (Jeong et al., 2005; Butcher et al., 2007). These molecules are linked to lipid metabolism in two ways. Firstly, the ascaroside core structure is composed of a sugar (ascarylose) and a lipid moiety. Thus, the abundance of individual ascarosides may directly reflect the availability of certain lipids. Secondly, ascarosides are intricately linked to lipid metabolism through their biosynthetic pathway, as several ascaroside biosynthesis enzymes are involved in fatty acid β-oxidation; these include the acyl-CoA oxidase ACOX-1, the enoyl CoA-hydratase MAOC-1, the β-hydroxyacyl-CoA dehydrogenase DHS-28, and DAF-22, a homolog of sterol carrier protein SCPx (Butcher et al., 2009; Pungaliya et al., 2009; Joo et al., 2010; von Reuss et al., 2012). In line with an important role for these enzymes in fatty acid catabolism, *daf-22*, *dhs-28*, and *maoc-1* mutants all accumulate excess fat; yet, *daf-22* and *dhs-28* mutants are short-lived, whereas worms with depleted *maoc-1* are long-lived (Hansen et al., 2005;

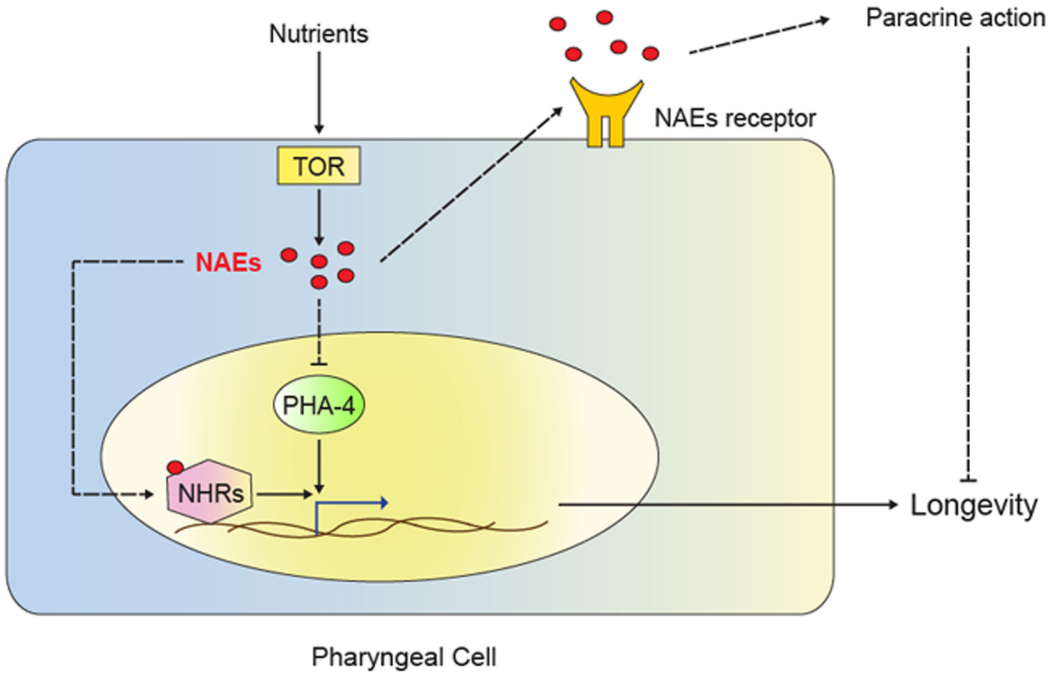

FIGURE 2 | Model for NAE action in dietary restriction-mediated longevity. The TOR signaling pathway senses nutrient availability. Activation of TOR triggers NAE synthesis in the pharyngeal cell, leading to several possible mechanisms of action. NAEs could act in paracrine fashion to stimulate signaling through unidentified receptors, thus promoting growth, reproduction and inhibiting longevity. NAEs could also inhibit the transcription factor PHA-4 and therefore repress genes required for dietary restriction-induced longevity. Lastly, NAEs could act as ligands for nuclear hormone receptors (NHRs) and result in the regulation of genes required for dietary restriction-mediated longevity. Low NAE levels signal dietary restriction and result in activation of the transcription of genes that contribute to extension of lifespan.

Joo et al., 2009; Zhang et al., 2010). As *maoc-1*, *daf-22*, *dhs-28*, and *acox-1* are expected to act in a linear biosynthetic pathway (von Reuss et al., 2012), the differential effects of individual gene deletions on life span is somewhat unexpected. Perhaps, distinct ascarosides differentially affect lifespan, and the enzymes outlined above are not equivalently required for the synthesis of each ascaroside subspecies. Structurally diverse members of the ascaroside family continue to be identified (von Reuss et al., 2012), and thus such functional specialization may not be too surprising. Lastly, it is also conceivable that the enzymes outlined above influence lifespan through a mechanisms that does not involve ascarosides.

In addition to the identification of the ascarosides and relevant biosynthetic enzymes, two recent studies describe the identification of dauer pheromone receptors (Kim et al., 2009; McGrath et al., 2011). Worms carrying mutations in the pheromone receptor genes *srg-36*, *srg-37*, *srbc-64*, and *srbc-66*, or in *gpa-3*, a key downstream effector, are compromised for dauer formation upon ascaroside treatment; alas, none of the studies report life span phenotypes. It will be interesting to test whether these mutants, or other mutants defective for dauer pheromone sensing and/or signaling exhibit an increase or decrease in longevity; perhaps, different pheromone receptor combinations will emerge that specifically affect life span but not other phenotypes, akin to the combinatorial and differential roles of ascarosides in development and behavior (Srinivasan et al., 2012).

MITOCHONDRIAL PATHWAYS TO LONGEVITY AND LIPID METABOLISM

Mutations in mitochondrial ETC genes extend lifespan in *C. elegans* and in other organisms, and lipids play critical structural and energetic roles in mitochondria (Nicholls, 2002; Marchi et al., 2012). Mitochondria produce energy by means of oxidative phosphorylation, which generates a proton gradient across the inner mitochondrial membrane that is used to generate ATP. Oxidative phosphorylation also produces reactive oxidative species (ROS) that react with and can damage macromolecules such as DNA, proteins, and lipids. The mitochondrial free radical theory of aging first proposed by Harman (1956) suggested that ROS-induced damage accumulation would gradually lead to a decrease in many cellular functions and eventually result in aging. However, several studies have cast doubt on this model (reviewed in Hekimi et al., 2011), and a recent study found that mitochondrially produced superoxide signals may in fact contribute to life span extension (Yang and Hekimi, 2010), possibly by signaling the occurrence of damage to relevant repair pathways. In line with this notion, moderate and transient ROS levels trigger autophagy to remove damaged organelles, sustaining cell life, and therefore promoting longevity (Marchi et al., 2012). Alternative explanations for the increased longevity of ETC mutants include the notion that reduced mitochondria function generally slows down metabolism (the rate-of-living theory) or the possibility that specific metabolic changes cause delayed aging (Cristina et al., 2009). Below,

we discuss several recent studies that provide new insights into how mitochondria may affect life span, hinting at roles for energy metabolism, homeostasis, and signaling.

Mitochondria are intricately linked to lipid biology because triglycerides and fatty acids provide acetyl-CoA, the substrate for the citric acid cycle that is directly coupled to mitochondrial oxidative phosphorylation. A recent study elegantly linked fat metabolism and mitochondrial energetics to longevity in *C. elegans* (Artal-Sanz and Tavernarakis, 2009). The authors investigated the prohibitin complex, which is formed at the inner mitochondrial membrane. Depletion of the prohibitins extends life span in several genetic backgrounds, including IIS pathway mutants, mitochondrial mutants, and the *eat-2* mutants mimicking dietary restriction; in contrast, reduced prohibitin levels shorten the lifespan in wild-type animals, suggesting that prohibitins act in a context-specific fashion. Because of the direct link between mitochondria and fat catabolism, the authors assessed fat storage in worms with depleted prohibitins. Strikingly, they found that in the long-lived mutants, prohibitin depletion correlates with lower fat levels, reduced mitochondria content, and increased ATP production, whereas in wild-type worms, prohibitin depletion results in only a slight decrease in fat content, an increase in mitochondrial level, and no change in ATP synthesis. Prohibitin depletion also decreases fat levels and increases life span in *fat-7* and *nhr-49* mutants, which store excess fat. These data suggest that *fat-7* and *nhr-49* are not required for the life span extension in this context, and that prohibitin depletion reduces fat levels and achieves life span extension even in high-fat backgrounds. Although it is not certain that reduced fat storage is causally linked to lifespan extension, these data are especially intriguing in view of the roles for lipases in the long life span of germline-less and IIS pathway mutants (see above). Indeed, it is tempting to speculate that lipases may be required for the increase in life span following prohibitin depletion (**Figure 1**), which would be supported by the concomitant decrease in fat levels.

Prohibitins localize to the inner membrane of the mitochondria, which is the only eukaryotic membrane to contain the bacterial lipid cardiolipin (Marchi et al., 2012). Given the shared localization of prohibitins and cardiolipin it is tempting to speculate that cardiolipin abundance could alter lifespan by affecting the properties of the inner mitochondrial membrane and its associated proteins. A recent study on wild-type worms found that cardiolipin abundance decreases with worm age, as does mitochondrial number (Gruber et al., 2011). Another study found that cardiolipin synthase mutant worms exhibit a decreased mitochondrial membrane potential and developmental phenotypes, but no lifespan phenotype was reported (Sakamoto et al., 2012). It would be informative to find out whether cardiolipin synthase mutation, or perhaps cardiolipin depletion in adult worms, affects aging in wild-type worms, or in long-lived mutants.

In addition to the requirement for prohibitins in several longevity pathways, mitochondrial membrane potential – the driving force for ATP generation – may broadly impact aging. Lemire et al. (2009) found that reduced mitochondrial membrane potential correlates with long life span in several mutants and RNAi treated worms. Moreover, the artificial reduction of membrane potential with a chemical uncoupler – which dissipates the proton gradient across the inner mitochondrial membrane and thus prevents ATP synthesis – directly increases lifespan. Given the effect of chemical uncoupling, one might expect that channels alleviating the protein gradient, such as the uncoupling proton UCP-4, might also positively influence aging. However, *ucp-4* deficiency does not alter the lifespan of wild-type worms (Iser et al., 2005), albeit it is possible that *ucp-4* is required for the long lifespan of certain mutants. Together, the above studies suggest that mitochondria, and perhaps especially membrane potential and the function of the inner mitochondrial membrane, may play important roles in multiple pathways that extend lifespan.

Recent discoveries have also shed new light on the mechanisms by which mitochondrial ETC mutants may achieve an extended life span. An exciting study by Durieux et al. (2011) found that ETC loss is required specifically in the intestine to delay aging. A signal emanating from the intestine – termed a mitokine – is proposed to set the rate of aging throughout the body. The molecular nature of the proposed mitokine is unclear, but ROS come immediately to mind, given the recent identification of superoxide as a signaling molecule in long-lived mitochondrial mutants (Yang and Hekimi, 2010). Lipid-derived molecules could also play such a role, especially given that the *C. elegans* intestine is the major organ involved in lipid metabolism. NAEs, or perhaps ascarosides, could in principle perform such functions. Whatever the molecule, these studies all reinforce the notion that mitochondria play a key role in influencing life span, and provide new evidence that lipids are paramount for the longevity-affecting role of mitochondria.

SPHINGOLIPID AND CERAMIDE SIGNALING

Sphingolipids and ceramides constitute a diverse class of lipids that play important roles in many processes, including cellular proliferation, differentiation, and apoptosis (Kolesnick, 2002). Ceramides are composed of a sphingosine group and a fatty acid, and they represent important components of cellular structures, especially membranes, while also participating in cellular signaling pathways. Notably, one of the first genes found to affect yeast longevity, Longevity Assurance Gene 1 (LAG1), encodes a ceramide synthase (D'Mello et al., 1994; Guillas et al., 2001). LAG1 has four homologs in *C. elegans*, and a recent paper investigated the potential of these genes to influence life span in *C. elegans* (Tedesco et al., 2008). However, neither mutation nor overexpression of *hyl-1*, the closest LAG1 ortholog, results in life span extension, and *hyl-1* depletion only causes a mild lifespan extension. These conflicting results may relate to the fact that different *E. coli* strains were used as food sources in mutant and RNAi studies; alternatively, the RNAi clone may have off target effects. Depletion of two *hyl-1* homologs, *hyl-2* and *lagr-1*, results in a shortened lifespan. In summary, whether and how ceramides and/or sphingolipids affect longevity in *C. elegans* remains obscure and needs to be studied further.

CONCLUSION

Lipids are key for many biological processes, acting in structural, metabolic, and/or signaling capacities. It is thus of little surprise that lipids, and the enzymes involved in lipid synthesis and remodeling, are materializing as key players in aging. We have highlighted recent studies in *C. elegans* that depict exciting new connections between aging and lipid biology, including emerging

roles for lipases and fatty acid desaturases, and the identification of lipid-derived signaling molecules that influence – or may influence – aging. Some of the challenges that lie ahead include defining in which tissues and by what molecular mechanisms these newly discovered molecules act (including the identification of their receptors). It will also be important to determine whether these molecules and enzymes such as lipases act more broadly or in restricted fashion.

The recent identification of novel lipid-derived signaling molecules that affect aging suggests that our insight into lipid signaling is likely incomplete. Similar limitations may apply to our views of structural and metabolic contributions of lipids to aging. To gain better insight into lipid action, and to identify the key molecules/metabolites linked to aging we may benefit from new technologies such as (lipid) metabolomics. Recent publications suggest that metabolomics is a powerful approach to identify novel regulatory relationships (Walker et al., 2011), and to reveal metabolite changes in aging worms (Fuchs et al., 2010). However, metabolomic approaches have to date primarily been used to analyze whole-worm extracts, which provides little insight into tissue-restricted or subcellular roles of individual lipids. Perhaps, organelle purification may be required to yield information about spatially restricted roles for certain lipids. Similarly, sophisticated analysis of individual lipid synthesis pathways as performed by Perez and Van Gilst (2008) can be used to delineate activity changes of certain pathways over time, e.g., in aging worms.

In summary, the studies reviewed here provide exciting new directions for future research on the roles of lipids in aging. Given that many genes and molecules are conserved in mammals, there is a great potential that such pathways may be developed as targets for drugs to ameliorate age-related diseases and/or slow aging.

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Mechanistic insights into aging, cell-cycle progression, and stress response

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The longevity of an organism depends on the health of its cells. Throughout life cells are exposed to numerous intrinsic and extrinsic stresses, such as free radicals, generated through mitochondrial electron transport, and ultraviolet irradiation. The cell has evolved numerous mechanisms to scavenge free radicals and repair damage induced by these insults. One mechanism employed by the yeast *Saccharomyces cerevisiae* to combat stress utilizes the Anaphase Promoting Complex (APC), an essential multi-subunit ubiquitin-protein ligase structurally and functionally conserved from yeast to humans that controls progression through mitosis and G1. We have observed that yeast cells expressing compromised APC subunits are sensitive to multiple stresses and have shorter replicative and chronological lifespans. In a pathway that runs parallel to that regulated by the APC, members of the Forkhead box (Fox) transcription factor family also regulate stress responses. The yeast Fox orthologs Fkh1 and Fkh2 appear to drive the transcription of stress response factors and slow early G1 progression, while the APC seems to regulate chromatin structure, chromosome segregation, and resetting of the transcriptome in early G1. In contrast, under non-stress conditions, the Fkhs play a complex role in cell-cycle progression, partially through activation of the APC. Direct and indirect interactions between the APC and the yeast Fkhs appear to be pivotal for lifespan determination. Here we explore the potential for these interactions to be evolutionarily conserved as a mechanism to balance cell-cycle regulation with stress responses.

Keywords: FoxO3a, FoxM1, Fkh1, Fkh2, Anaphase Promoting Complex

INTRODUCTION

Throughout history humanity has sought to understand the reasons for aging and dying. Relatively recently, genetic and biochemical studies have offered some insight into these complex processes. The health of an organism is directly related to the health of its cellular constituents, with genomic instability being a dominant force that leads to either senescence of stem cells or uncontrolled growth and tumor formation. Advances in our understanding of these processes have been made through the identification of gene products that can increase or decrease cellular health span, influencing the incidence of tissue degeneration and age-related diseases, such as Alzheimer's, diabetes, and cancer (reviewed in Kloet and Burgering, 2011; Stünkel and Campbell, 2011; Ziv and Hu, 2011; Jia et al., 2012; Salminen and Kaarniranta, 2012). Genetic screens in the nematode *Caenorhabditis elegans* identified members of the insulin-signaling pathway as regulators of the aging process. Specifically, decreased activity of the PI3K/AKT pathway, a prominent pathway overactive in many cancer cells, increases longevity in a variety of model organisms (Kloet and Burgering, 2011; Speakman and Mitchell, 2011).

Regulation of growth in conjunction with stress resistance and genomic stability was found to rely on the worm Daf-16, a stress response transcription factor featuring the forkhead box (Fox) DNA-binding domain (Kenyon et al., 1993; Lin et al., 2001; Libina et al., 2003; Murphy et al., 2003). Fox-containing proteins have been identified from yeast to worms and insects to humans (Baldauf, 1999; Mazet et al., 2003). Fox family members regulate diverse biological processes, such as metabolism, embryonic development, differentiation, cell migration, invasion, cell-cycle progression, apoptosis, autophagy, immunity, DNA-damage repair, and toxin scavenging (Tuteja and Kaestner, 2007). A large number of Fox genes have been identified in higher eukaryotic systems (Kaufmann and Knöchel, 1996; Murakami et al., 2010), which makes it very difficult to gain insight into any one Fox protein or subfamily. The brewing yeast *Saccharomyces cerevisiae* contains only four Fox proteins (Fkh1, Fkh2, Fhl1, and Hcm1; Murakami et al., 2010), presenting an opportunity to learn in greater depth how individual Fox proteins are regulated and what they control. It has become apparent that unraveling the regulation, targets, and evolution of the Fox family of transcription factors is crucial for understanding cancer biology and aging, as this group contains suspected oncogenes, as well as *bona fide* tumor suppressors and longevity determining factors. Emerging evidence highlights the importance of these factors in cell-cycle regulation and stress responses, through the regulation of the evolutionary conserved Cyclins, Cyclin Dependent Kinase inhibitors (CDKIs), and the Anaphase Promoting Complex (APC), a ubiquitin-protein ligase. Here we explore the evolutionary conservation of these mechanisms from yeast to humans.

THE EVOLUTIONARILY CONSERVED FOX FACTORS

Fox transcription factors have been identified in a wide range of species from yeast to humans based on the common 110-amino-

acid winged-helix DNA-binding domain, known as the forkhead box (Kaufmann et al., 1995; Kaufmann and Knöchel, 1996). Due to the highly conserved nature of the Fox DNA-binding domain, all Fox proteins bind to the consensus core nucleotide sequence A/CAAC/TA (Lalmansingh et al., 2012). Although Fox genes have been found in animals, as well as yeast and other fungi, plants do not encode these transcription factors. This suggests the proto-Fox gene originated in the animal/fungal ancestor after the evolutionary split of autotrophs and heterotrophs (Baldauf, 1999). Phylogenetic and comparative analyses have identified over 100 Fox genes in humans that can be subgrouped into 19 subclasses (A to S) with further subdivision based on the relationship between vertebrate and invertebrate genes (Kaufmann and Knöchel, 1996; Hannenhalli and Kaestner, 2009; Murakami et al., 2010). Analyses of fungal Fox genes found they were equally related to all animal Fox genes, suggesting only one proto-Fox gene was present at the divergence of animals and fungus (Baldauf, 1999).

A fascinating aspect of Fox gene function is their involvement in many developmental defects and cancer (Lehmann et al., 2003; Myatt and Lam, 2007). This review will focus on two closely related subclasses: the aging and tumor suppressor FoxO and the oncogenic FoxM.

The FoxO subclass is highly conserved throughout animals and is primarily responsible for regulation of G2-M and G1-S cell-cycle checkpoints, as well as for the expression of stress response, DNA repair, and apoptotic genes (Brunet et al., 1999; Medema et al., 2000; Dijkers et al., 2002; Kops et al., 2002; Nemoto and Finkel, 2002; Tran et al., 2002). The FoxO subclass is of special interest as its members seem intrinsic to tumor suppression and lifespan extension. Intense investigation of post-translational regulation of the FoxOs is underway, wherein phosphorylation, acetylation, and ubiquitination (both poly and mono) are known to influence FoxO nuclear shuttling, DNA-binding ability, transcriptional activity, and protein stability (Calnan and Brunet, 2008; Boccitto and Kalb, 2011; Daitoku et al., 2011; Huang and Tindall, 2011; Tzivion et al., 2011; Zhao et al., 2011). Under non-stress conditions, phosphorylation of FoxOs by growth factor (insulin/insulin-like growth factor, TOR2C and Mitogen activated kinase) pathways results in cytosolic (rather than nuclear) localization and proteasome-dependent degradation via ubiquitination by the Skp/Cullin/F-box ubiquitin-protein ligase (E3) complex SCF^{Skp2}. Conversely, stress-induced phosphorylation signals (oxidative stress activated c-Jun N-terminal kinase and MST1, as well as starvation response AMPK) result in nuclear localization and the transcription of specific factors. Furthermore, FoxO acetylation leads to dissociation from DNA, while simultaneously stabilizing FoxO proteins by blocking ubiquitination and subsequent degradation.

FoxM1, the single member of the FoxM subfamily, is found only in proliferating cells, and is also involved in cell-cycle regulation, aging, and cancer (Korver et al., 1997; Mazet et al., 2003; Laoukili et al., 2007; Tang et al., 2008; Pandit et al., 2009; Petrovic et al., 2010; Wang et al., 2010). Cells deficient in FoxM1 show delays in G2/M and G1/S progression, as well as defects in chromosome segregation and cytokinesis (Laoukili et al., 2005; Wang et al., 2005, 2008; Ustiyan et al., 2009). Increased FoxM1 protein has been found in numerous types of cancer and may be involved in early stages of tumorigenesis (Wilson et al., 2011). Like FoxO proteins, FoxM1 is also involved in DNA repair, implicating a need for FoxM1 in genomic stability and survival. Phylogenetic analyses suggest that the FoxM subclass is an offshoot of the FoxO family that split early in vertebrate evolution as no known structural ortholog has been found in modern chordate or invertebrate species (Mazet et al., 2003).

A possible direct connection between FoxO3a and FoxM1 was suggested by microarray analyses of FoxO3a overexpressing cells, which identified FoxM1 as a gene differentially repressed when Fox3a levels were increased (Delpuech et al., 2007). Of the 151 differentially expressed genes (>2-fold up or down), 59 (39.1%) were downregulated. A large percentage of the cell-cycle regulated genes were downregulated, consistent with a role for FoxO3a in cell-cycle inhibition. FoxM1 was previously identified as a Myc target gene containing E-boxes (CACTGT) within the FoxM1 promoter at −1244 and −1091 (**Figure 1**; Fernandez et al., 2003). Myc forms a heterodimer with Max, which binds the E-box to activate gene expression. Contrary to this, Max can also dimerize with Mxi1 to bind E-boxes, resulting in transcriptional repression (Delpuech et al., 2007). Increased FoxO3a expression upregulated members of the Mad/Mxi family, which was predicted to antagonize Myc function. It was concluded that downregulation of FoxM1 following FoxO3a induction may be an indirect effect of Myc inhibition via up-regulation of the Myc antagonists Mxi1 and Mad. It was also observed that increased FoxO3a expression decreased Myc protein levels, providing an additional mechanism for FoxM1 repression. However, it should be noted that FoxM1 contains a TAAACA Fox binding site at position −88 within the FoxM1 promoter (**Figure 1**), indicating that FoxO3a may indeed bind and repress the FoxM1 promoter, perhaps first requiring the displacement of Myc.

The reciprocal interaction of FoxO3a and FoxM1 is critical to stave off cancer (Wilson et al., 2011). Previous work indicated that repression of FoxM1, via FoxO3a, was necessary to suppress the growth of breast cancer cells treated with the EGRF inhibitor Gefitinib (McGovern et al., 2009). It was observed that Gefitinib induced the expression of FoxO3a in association with the repression of FoxM1. Silencing of FoxO3a increased FoxM1 expression in response to Gefitinib, which was associated with increased cell proliferation and reduced cell death. Similarly, increased FoxM1 expression reduced Gefitinib induced cell-cycle arrest.

FoxO3a and FoxM1 also appear to oppose one another in ERα-positive breast cancer cells. FoxO3a was identified as a binding partner for ERα and ERβ, which was important for the suppression of estrogen-dependent breast cancer (Zou et al., 2008). This interaction resulted in the repression of ERα-regulated gene expression (**Figure 2**). Conversely, FoxM1 was shown to drive the expression of ERα via "promoter A" of the two-set ERα promoter (**Figure 2**), and that silencing of FoxM1 completely blocked ERα expression (Madureira et al., 2006). It was also observed that FoxO3a could promote weak expression of ERα through ERα "promoter B." However, both FoxM1 and FoxO3a could bind to both ERα promoter sets. Lastly, although FoxM1 and FoxO3a could be co-immunoprecipitated the relevance of this observation was not pursued. The compiled evidence suggests that FoxM1 and FoxO3a may form a complex that cooperatively regulates ERα expression.

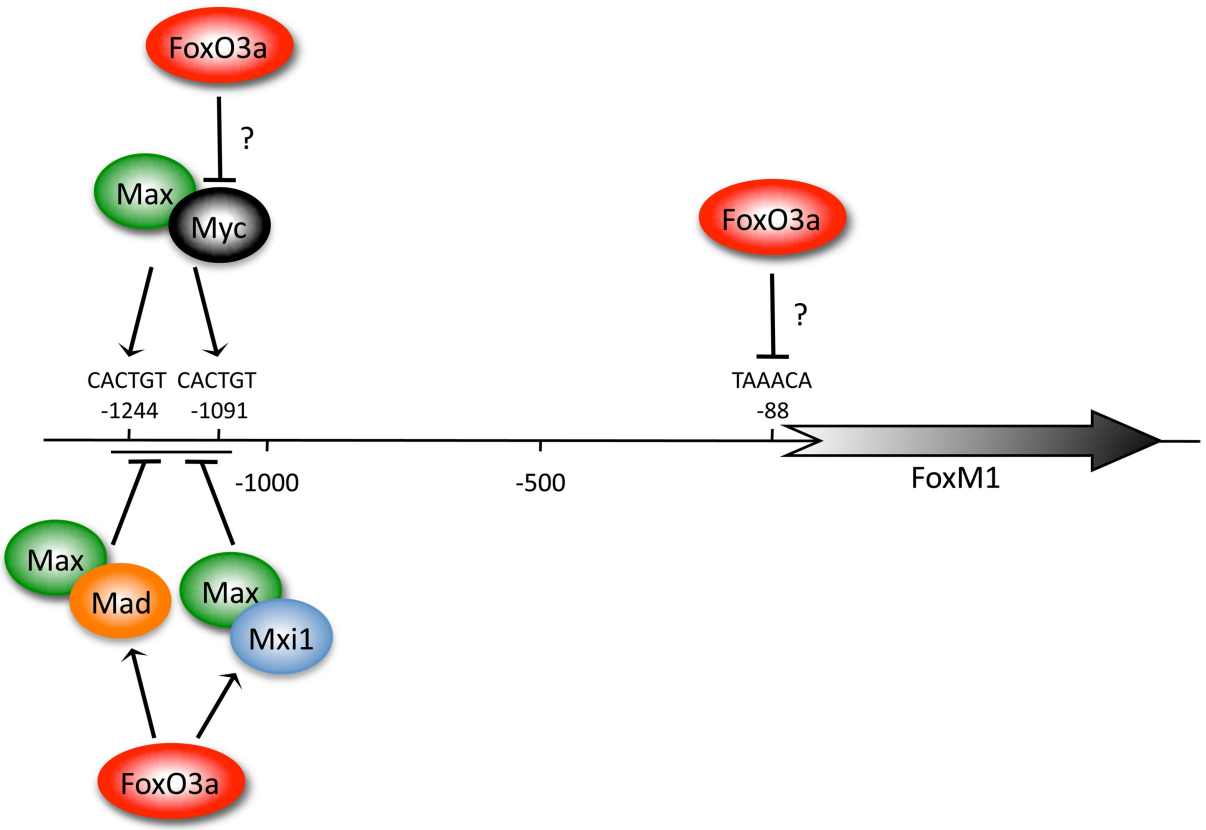

FIGURE 1 | FoxM1 expression is repressed by FoxO3a. FoxO3a may repress FoxM1 expression in one of three ways, which may not be mutually exclusive. First, FoxO3a may bind to a Fox consensus site at position −88 of the FoxM1 promoter. This could lead to FoxM1 repression. Second, expression of the Myc antagonists Mad and Mxi1 are driven by FoxO3a. Mad and Mxi1 compete with Myc to dimerize with Max. The Max/Myc dimer binds to E-boxes (CACTGT) located within the FoxM1 promoter to drive FoxM1 expression, while Mad/Max and Mxi1/Max dimers bind the same E-boxes, but repress expression. Thus, increased expression of Mxi1 and Mad by FoxO3a could inhibit FoxM1 expression by blocking Myc/Max dimerization. Third, Myc protein levels decrease when FoxO3a expression is increased, perhaps through a post-translational mechanism, providing another method to potentially repress FoxM1 expression following FoxO3a activation. This figure is based on work from Delpuech et al. (2007) and Fernandez et al. (2003).

However, these interactions could also be interpreted to imply that FoxO3a can bind FoxM1 at promoters to inhibit FoxM1 activity. Much more work is required to elucidate the mechanisms regulating ERα expression and breast cancer progression.

YEAST FOX PROTEINS

The budding yeast *S. cerevisiae* contains four Fox genes: *FKH1*, *FKH2*, *HCM1*, and *FHL1*. *FHL1* regulates ribosome biogenesis (Rudra et al., 2005), and *HCM1* regulates progression through G2, preparing the cell for mitosis (Pramila et al., 2006). Although both may play a role in lifespan determination, their individual deletion did not influence yeast replicative lifespan (RLS; a measure of how many daughter cells a single mother can produce; Kennedy et al., 1994; Wei et al., 2008). Here we focus on *FKH1* and *FKH2* as they show conserved function with human FoxM/FoxO genes (Murakami et al., 2010; Postnikoff et al., 2012). Genetic redundancy is suggested for these two factors as the combined deletion of both *FKH1* and *FKH2* is necessary to alter growth, stress response, longevity, cell morphology, and gene transcription phenotypes (Hollenhorst et al., 2000; Zhu et al., 2000; Shapira et al., 2004; Sherriff et al., 2007; Voth et al., 2007; Postnikoff et al., 2012). Evolutionary conservation for *FKH1* and *FKH2* with higher Fox genes is suggested by their similar involvement in ROS induced cell-cycle arrest and resistance to oxidative stress during stationary phase (Shapira et al., 2004; Postnikoff et al., 2012), as well as in cell-cycle regulation through both G1 and G2/M gene clusters (Zhu et al., 2000). Finally, we have recently found that deletion of both *FKH1* and *FKH2* reduces lifespan in a manner that inhibits lifespan extension due to caloric restriction, while over-expression of one or both genes extends lifespan (Postnikoff et al., 2012), hallmarks of human FoxO genes.

Fkh1 and Fkh2 are capable of binding the Forkhead box consensus site (TAAACA) first identified for *Xenopus* XFD1, 2, and 3 (Kaufmann et al., 1995). Under normal laboratory conditions Fkh2 primarily occupies known binding sites of the Clb2 gene cluster. Fkh2 forms a complex with the MADS-box transcription factor Mcm1, while Fkh1 does not (Hollenhorst et al., 2001). Cell-cycle specific activation of this complex is initiated by the expression

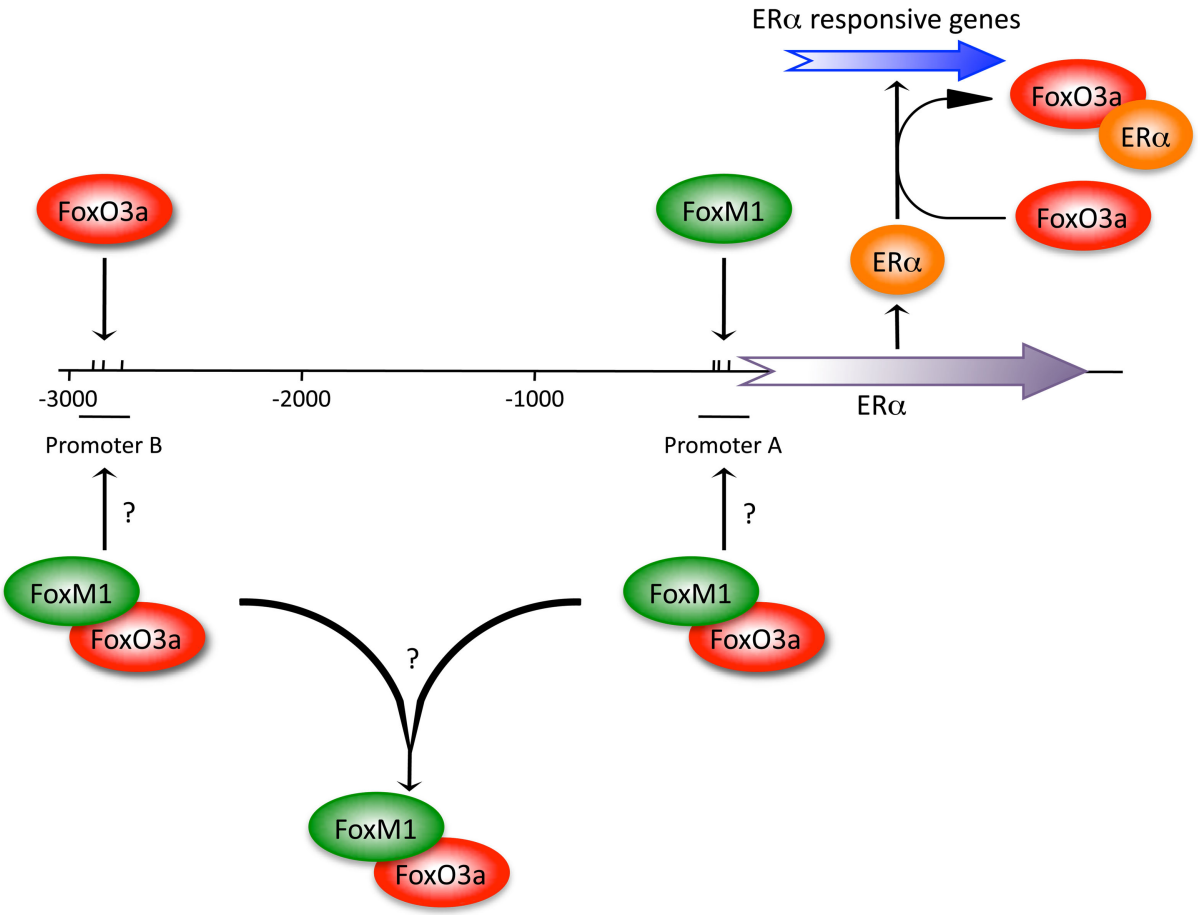

FIGURE 2 | A complex interaction between FoxM1 and FoxO3a controls ERα expression. The ERα promoter contains two clusters of Fox binding sites, one immediately upstream of the ERα start site, "Promoter A" and a second site at approximately position −3000, "Promoter B." ChIP studies showed that FoxM1 and FoxO3a could bind both promoters. Based on RNAi experiments, FoxM1 and FoxO3a were both capable of driving ERα expression, although through Promoters A and B, respectively, with FoxM1 playing a dominant role. A second level of complexity involves a protein/protein interaction between FoxO3a and ERα that blocks ERα from promoting the transcription of ERα responsive genes. A potential FoxO3a/FoxM1 physical interaction provides a third possible layer of complexity. The relevance of the FoxO3a/FoxM1 interaction remains unknown. This figure is based on work by Zou et al. (2008) and Madureira et al. (2006).

and binding of the co-activator Ndd1 to Fkh2, which switches the function of Fkh2 from repressor to activator (Loy et al., 1999; Koranda et al., 2000). Fkh1 may function as a co-regulator of this process, or may function as a primary regulator under alternate growth conditions. In *FKH2* deletion strains, cell-cycle specific expression of Fkh2 targets is not disrupted, as it is in *fkh1*Δ *fkh2*Δ double mutant strains (Zhu et al., 2000; Hollenhorst et al., 2001), suggesting Fkh1 can function at the same loci without Fkh2/Ndd1 occupancy (Reynolds et al., 2003). However the mechanism by which Fkh1 regulates these genes in a periodic cell-cycle dependent manner, in the absence of Fkh2 and Ndd1, requires further investigation.

COMMON BIOLOGY OF THE FKHs AND THE FOXs

Mitotic progression genes are common targets of both yeast and human Fox proteins. As described earlier, the yeast Fkh1 and Fkh2 regulate clusters of genes required for cell-cycle progression, such as the CLN2 and CLB2 gene clusters (Zhu et al., 2000), which include targets (Iqg1, Cdc20, the B type cyclins Clb1, Clb2, Clb5, and the yeast the polo-like kinase Cdc5) and regulators (Clb2, Cdc20, and Cdc5) of the APC (Ko et al., 2007; Sari et al., 2007; Qiao et al., 2010). The APC is a highly conserved ubiquitin-protein ligase (E3) that primarily controls progression through mitosis and G1. The observation that the Fkh proteins control transcription of many genes required for APC function suggests that the APC may be a critical downstream target of the Fkhs (discussed in more detail below). The Fkh proteins also control the transcription of the Histone gene cluster (Zhu et al., 2000). Interestingly, the yeast APC is required for histone protein expression and post-translational modification, which may be a shared feature with the Fkh proteins, as deletion of *FKH1* and *FKH2* in a mutant $apc5^{CA}$ background further impairs histone protein levels (Turner et al., 2010; Postnikoff et al., 2012). In mammals, FoxM1 primarily regulates G2/M genes, such as B type cyclins, the polo-like kinase (PLK-1), Aurora B, Skp2, INCENP, Nek2, and the centromere specific proteins CENP-A, CENP-B, and CENP-F

(Laoukili et al., 2005; Wang et al., 2005). Like in yeast, many of the FoxM1 controlled genes are either required for APC function (PLK-1, B type cyclins), or are targeted for degradation by the APC (Aurora B, Skp2, Nek2, PLK-1, and B type cyclins; Pfleger and Kirschner, 2000; Qiao et al., 2010). An additional level of striking conservation between mammalian and yeast cells is the positive feedback loop that initiates an irreversible commitment to mitosis, wherein Cdk-cyclin B complexes and polo-like kinases phosphorylate FoxM1 and the Fkhs to increase their transcriptional activity (Murakami et al., 2010).

Many of the FoxM1 targets are required for genomic stability (O'Brien et al., 2007; van der Waal et al., 2012). For example, silencing of CENP-F (activated by FoxM1) resulted in the loss of the mitotic checkpoint proteins Mad1 and BubR1, which are required to block APC activity until appropriate (Laoukili et al., 2005). The subsequent inappropriate activation of the APC in the presence of DNA damage could result in potential mitotic catastrophe (D'Arcy et al., 2010; Lara-Gonzalez et al., 2011). Likewise, the Chromosomal Passenger Complex, composed of Aurora B kinase, INCENP, Survivin, and Borealin, regulates the mitotic checkpoint to ensure accurate segregation of mitotic chromosomes (van der Waal et al., 2012). Thus, FoxM1 expression at least in part increases mitotic checkpoint control and APC function, ensuring proper transit through mitosis. Considering that improved mitotic checkpoints should result in increased genomic stability, not in cancer development, this apparent paradox may reflect a mechanism whereby increased FoxM1 expression in cancer cells allows the defective cell to bypass cellular mechanisms aimed at destroying it. FoxM1 itself is a target of the APC^{Cdh1} G1 specific complex (Laoukili et al., 2008; Park et al., 2008). This is an interesting finding since FoxM1 joins the growing list of APC activators that are later targeted by the APC for degradation to complete a negative feedback loop. It is thought that in order to shut down the expression of mitosis-specific genes, FoxM1 must be degraded as cells exit mitosis. Our unpublished data suggests that the targeting of Fox proteins by the APC is indeed a conserved process, as the yeast Fkh1 is targeted by the APC during G1 for degradation (Malo, Postnikoff, and Harkness, unpublished).

Stability of the FoxO and FoxM proteins is controlled antagonistically by the SCF and APC ubiquitin-protein ligase/E3 complexes, respectively. The APC targets FoxM1 for degradation, and indirectly stabilizes FoxO during G1 (Laoukili et al., 2008; Park et al., 2008). The SCF^{Skp2} complex targets phosphorylated FoxO proteins for degradation (Huang et al., 2005; Huang and Tindall, 2011). The APC potentially controls this process through targeted degradation of the F-box protein Skp2 (van Leuken et al., 2008); degradation of Skp2 by the APC^{Cdh1} blocks SCF^{Skp2} function, thereby delaying FoxO degradation and allowing increased FoxO tumor suppressor activity. There appears to be a battle over the control of Skp2 stability in mammalian cells. AKT phosphorylates FoxO proteins, leading to their SCF^{Skp2}-dependent ubiquitination and degradation. AKT also phosphorylates Skp2 (Gao et al., 2009; Lin et al., 2009). AKT phosphorylation of Skp2 is believed to signal cytosolic localization of Skp2, thereby protecting Skp2 from APC-dependent ubiquitination and degradation. However, this mechanism may be cell type dependent, as another study was not able to reproduce these results (Bashir et al., 2010). Nonetheless, the APC and AKT may be competing for Skp2's attention, with the winner perhaps deciding between cell health and death.

Opposing FoxO3a and FoxM1 function exists at the transcriptional target level as well. As already mentioned, FoxM1 is repressed by FoxO3a (**Figure 1**). However, FoxO3a also represses the expression of many cell-cycle specific genes, consistent with its role in blocking cell-cycle progression (Delpuech et al., 2007). The G2/M genes activated by FoxM1, such as UBE2C, NEK2, CENP-F, and Aurora, were repressed by FoxO3a (Laoukili et al., 2005; Delpuech et al., 2007). FoxO3a was more likely to activate the expression of genes required for apoptosis/stress response, transcription, and signaling (Delpuech et al., 2007). This pattern was also observed when asynchronous *fkh1*Δ *fkh2*Δ cells were used for microarray analyses, with downregulation of genes involved in cell stress response (*RNR1*, *ALK1*, *IRC8*, *PHO5*/*11*/*12*, *DSE1*/*2*, *CRG1*; Zhu et al., 2000). Importantly, these genes all contain the Fox TAAACA binding site within 1 kb of the start site. Thus, the yeast Fkh1 and Fkh2 stress response transcription factors appear to possess activities associated with both FoxM1 and FoxO3a.

Another conserved role for these proteins is in the regulation of cell-cycle arrest and check point genes through two different mechanisms: the induction of cell-cycle arresting agents, such as CDKIs, and the repression of cell-cycle regulators, such as cyclins and polo-like kinases. The FoxOs regulate G1/G0 cell-cycle arrest by induction of the CDKIs p27Kip1, p21Cip1, p15Ink4b, p19INK4d, and p19Arf, which inhibit the formation of S-phase entry cyclin-CDK complexes (Medema et al., 2000; Seoane et al., 2004; Gomis et al., 2006; Bouchard et al., 2007; Miyamoto et al., 2007; Katayama et al., 2008). In addition, the FoxOs are involved in increasing the level of expression of the quiescent cyclin G2 while repressing that of cell-cycle entry cyclin D family members (Ramaswamy et al., 2002; Schmidt et al., 2002; Martínez-Gac et al., 2004). Furthermore, the FoxOs prevent the transcriptional activation of cell-cycle entry proteins through the up-regulation of the retinoblastoma protein family member p130 (Kops et al., 2002). Similarly, the FoxOs may be involved in check point arrest through the regulation of GADD45α, a component of the G2 checkpoint and DNA-damage repair systems (Tran et al., 2002; Laoukili et al., 2005).

The yeast Fkhs appear to function in a similar manner to the FoxOs through complimentary mechanisms. As mentioned earlier, Fkh2 *in vivo* is dominant over Fkh1 at promoter-binding sites where it acts as a transcriptional inhibitor until it binds the co-activator Ndd1 (Loy et al., 1999; Koranda et al., 2000; Hollenhorst et al., 2001). Thus, Fkh2 acts to block the expression of G2/M progression genes until the appropriate growth signals regulating the binding of Ndd1 to Fkh2 are present. At this point there is a switch to mitotic progression via the up-regulation of Clb2 and Cdc5, which further activates the Fkh2/Ndd1 complex (Reynolds et al., 2003). The expression of other genes, such as the rest of the Clb2 gene cluster is similarly regulated: this includes the APC subunits/activators and the G1 transcription factors Swi5 and Ace2, as well as the Histone gene cluster (Zhu et al., 2000). Swi5 and Ace2 are responsible for the expression of M/G1 progression genes as well as the yeast CDKI Sic1. In late mitosis the proteasomal degradation of Ndd1 switches Fkh2 to a transcriptional repressor of the Clb2 gene cluster. Interestingly, the Fkhs also bind to

and repress many Swi5/Ace2 targeted promoters, preventing early G1 progression. As it appears Fkh1 is functionally redundant with Fkh2 (Hollenhorst et al., 2000; Zhu et al., 2000; Shapira et al., 2004; Sherriff et al., 2007; Voth et al., 2007; Postnikoff et al., 2012), it can be confidently postulated that the Fkhs follow the same pattern of cell-cycle regulation as the human Fox proteins, switching from cell-cycle repression in G2, to activation in M, followed by early G1 repression.

THE ROLE OF FOX PROTEINS IN LIFESPAN DETERMINATION

The FoxO family of proteins have been reproducibly found to extend lifespan when expression is increased in many model systems studied, including flies, worms, and yeast (Libina et al., 2003; Giannakou et al., 2007; Postnikoff et al., 2012). The *C. elegans* FoxO ortholog DAF-16 was found to serve as a direct downstream target of the worm insulin-signaling pathway (Kenyon et al., 1993). Worm mutants encoding defective *daf-2* insulin receptor genes exhibited a twofold lifespan extension, which was abolished when *daf-16* was mutated. Since the first studies of DAF-16 in worms, inactivation of the insulin-signaling pathway from flies and worms to mice has resulted in extended lifespan (Kenyon et al., 1993; Carter et al., 2002; Blüher et al., 2003; Holzenberger et al., 2003; Rincon et al., 2004). For the most part, lifespan extension through reduced insulin-signaling depends on the evolutionarily conserved FoxO factors. Mutation to the FoxO stress response factors reduces lifespan and stress response in many model systems (Lin et al., 2001; Greer and Brunet, 2008; Moskalev et al., 2011; Yamamoto and Tatar, 2011; Postnikoff et al., 2012). While independent mutation to either yeast *FKH1* or *FKH2* has no effect on replicative or chronological lifespan (CLS; Wei et al., 2008; Postnikoff et al., 2012), a combined deletion of both *FKH1* and *FKH2* in the same cell dramatically impaired CLS and these cells could not respond to severe caloric restriction induced by maintenance in water (Postnikoff et al., 2012). As opposed to RLS, CLS measures how long stationary phase cells can remain metabolically active (Fabrizio et al., 2001; Longo and Fabrizio, 2012). The requirement for Fkh1 or Fkh2 for maintenance of stationary phase metabolic activity indicates that the yeast Fkh1 and Fkh2 proteins are fully active in non-dividing cells. This was supported by experiments demonstrating that *fkh1*Δ *fkh2*Δ cells are far more sensitive to oxidative stress when in stationary phase than when rapidly dividing (Postnikoff et al., 2012). Since yeast cells do not naturally respond to insulin, this indicates that yeast respond to nutrients directly through evolutionarily conserved insulin-like signaling mechanisms. In this respect, insulin may act as a "middle-man" in multi-cellular organisms, indicating to cells that express an insulin receptor that nutrients are available.

A series of studies focused on long-lived human populations have convincingly shown that long-lived individuals express altered insulin-signaling. A study of female human centenarians revealed that these individuals carry a heterozygous mutation in the IGF1 receptor that was over-representative compared to controls (Suh et al., 2008). Transformed lymphocytes generated from these individuals revealed reduced insulin-signaling. Other long-lived human cohorts were found to carry an altered FoxO3a allele that was not generally found in the population (Willcox et al., 2008; Chung et al., 2010; Ziv and Hu, 2011). Taken together, the findings in model systems ranging from yeast to mice clearly show that increased lifespan as a result of diminished insulin-signaling is a trait likely conserved in humans.

FoxM1 also appears to play a critical role in cell survival with respect to cancer cells. FoxM1 is highly expressed in many cancer cells and most weakly expressed in prematurely aging fibroblasts, such as those isolated from individuals suffering from Progeria, a rapid aging phenotype (Laoukili et al., 2007; Zeng et al., 2009; Anders et al., 2011). From these observations, it is clear that FoxM1 expression is associated with the proliferative capacity of the cell, consistent with its role in primarily driving the expression of G2/M specific genes (Laoukili et al., 2005), with associated phenotypic expression of mitotic defects and chromosome aberrations when defective (Wonsey and Follettie, 2005). As such, FoxM1 appears to be tightly linked with inducing the expression of genes required for G2 and mitotic progression, very similar to the yeast Fkh1 and Fkh2 Fox proteins. FoxM1 seems to counter senescence, but primarily to maintain the life of cancer cells.

DOWNSTREAM FOX TARGETS REQUIRED FOR INCREASED LIFESPAN

A major quest over the past decade has been to identify downstream targets of the FoxO proteins specifically required for lifespan extension in model systems. The primary model used for these screens to identify FoxO lifespan targets has been the nematode *C. elegans*, largely due to the emergence of RNAi libraries (Murphy et al., 2003; Murphy, 2006; Oh et al., 2006; Szewczyk et al., 2006; Pinkston-Gosse and Kenyon, 2007; Shmookler et al., 2009; Schuster et al., 2010). An early study performed microarray on a series of long- and short-lived worm mutants over a lengthy time course spanning their overall development (Murphy et al., 2003). The 60 experiments were combined and mined for differentially expressed genes. Genes were found that grouped into distinct clusters representing signaling, stress response, and antimicrobial responses. Many of these genes were then silenced by RNAi in order to determine lifespan. Although many of the silenced genes conferred lifespans that altered from wild type controls, none of the lifespan differences approached those observed with *daf-2* mutants, leading to the conclusion that multiple effector genes involved in longevity determination are regulated by the insulin-signaling pathway. Using chromatin immunoprecipitation (ChIP) 103 specific promoters were identified as bound by DAF-16 (Oh et al., 2006). Again using RNAi, few of these genes were seen to be involved in lifespan in isolation, although several were controlled by insulin-signaling. This study suggested that either (i) multiple proteins are involved in DAF-16 functions, or (ii) DAF-16 may have non-productive promoter interactions. A rigorous examination of microarray data from long-lived worms, flies, and mice that were impaired in insulin-signaling showed a striking lack of convergence between the differentially expressed genes (McElwee et al., 2007). Nonetheless, several functional categories were found to be conserved, such as protein biosynthesis, sugar catabolism, energy generation, and cellular detoxification. A novel approach to identifying DAF-16 targets involved fusing DAF-16 with a bacterial DNA adenine methyltransferase (DAM), which methylates adenine residues within the sequence GATC anywhere within 2 kb of its binding site (Schuster et al., 2010). An original list

of 907 promoters was whittled down to 65 DAF-16 targets. This list was largely enriched in genes involved in signaling, and not in genes required for somatic maintenance processes, suggesting that DAF-16 is more likely activating signaling processes that lead to cellular maintenance and protection, rather than the individual genes themselves. Although dramatic gains have been made in identifying DAF-16 targets required for longevity determination, surprisingly little ground has been made toward defining networks leading from DAF-16 that result in definitive increased lifespan.

THE ANAPHASE PROMOTING COMPLEX, AN EMERGING FOX SPECIFIC TARGET BRIDGING THE LONGEVITY GAP

Emerging evidence suggests that the APC may be an important downstream target necessary for many Fox/Fkh functions (Laoukili et al., 2008; Park et al., 2008; Postnikoff et al., 2012). The APC is a highly conserved multi-subunit ubiquitin-protein ligase (E3) that primarily targets proteins that inhibit chromosome segregation and mitotic exit for ubiquitin- and proteasome-dependent degradation (Qiao et al., 2010; McLean et al., 2011). Initiation of and progression through mitosis is mediated by the APC^{Cdc20} complex, while exit from mitosis and maintenance of G1 is controlled by the APC^{Cdh1} complex, which targets components that drive mitosis, such as Clb2, Cdc5, and Cdc20, for degradation. Cdc20 is activated by the polo-like kinase Cdc5, as well as by the Cdc28/Clb2 Cdk complex prior to chromosome segregation. APC^{Cdc20} targets spindle checkpoint proteins, such as the securin Pds1 in yeast, for proteasomal degradation (Hilioti et al., 2001; Wang et al., 2001). A specific example of such regulation involves the mouse spindle checkpoint protein BubR1. The spindle checkpoint functions in normal mitotic progression to inhibit APC^{Cdc20} as a counter to polo-like kinase activation, ensuring that chromosomes are properly attached to the mitotic spindle, thereby preventing changes in chromosome numbers (D'Arcy et al., 2010; Lara-Gonzalez et al., 2011). Mice deficient in BubR1, either through mutation or as a natural property of aging, show signs of early aging such as kyphosis, cataracts, cardiovascular disease, muscle wasting, and susceptibility to carcinogens (Baker et al., 2004, 2005; Kim and Kao, 2005). Proper regulation of APC^{Cdc20} may increase chromosome segregation fidelity, reducing non-disjunction events, and potentially increasing cellular healthspan. Other non-mitotic functions have now been ascribed to the APC, including maintaining neuronal development and genomic stability, as well as regulating chromatin metabolism and enhancing longevity in yeast and higher eukaryotic organisms (Harkness et al., 2002, 2004, 2005; Baker et al., 2004; Arnason et al., 2005; Turnell et al., 2005; Li et al., 2008; Turner et al., 2010; Eguren et al., 2011; Islam et al., 2011; Puram and Bonni, 2011; Postnikoff et al., 2012). As diverse as APC functions seem to be, regulatory mechanisms controlling APC output remain largely unknown.

Our recent work demonstrates that the APC may serve as a downstream Fkh1/Fkh2 target that regulates Fox-dependent longevity (Postnikoff et al., 2012). The APC and the Fkhs interact genetically and functionally to ensure normal yeast lifespan, and to respond to severe caloric restriction and stress in non-dividing cells. As mentioned above, increased expression of *FKH1* or *FKH2* could increase both RLS and CLS in yeast (Postnikoff et al., 2012). As expected from redundant factors, deletion of both *FKH1* and *FKH2*, rather than either one alone, was required to reduce yeast CLS. However, disruption of both *FKH1* and *FKH2* in cells harboring a temperature sensitive allele of the gene encoding the APC subunit *APC5*, exhibited a CLS that was the same as *fkh1*Δ *fkh2*Δ cells, indicating that *fkh1*Δ *fkh2*Δ CLS is epistatic to $apc5^{CA}$ CLS under normal culture conditions. This indicates that under non-stress conditions Fkh1/2 acts directly upstream of the APC. We believe that this is through the direct transcriptional regulation of APC activators and APC substrates (Zhu et al., 2000).

The $apc5^{CA}$ mutant had a lifespan shorter than *fkh1*Δ *fkh2*Δ cells (Postnikoff et al., 2012). Unlike an earlier *C. elegans* study where RNAi of many DAF-16 targets (determined by microarray analyses) did not generate lifespan alterations as impressive as *daf-2* or *daf-16* mutants (Murphy et al., 2003), our data indicates that the APC may indeed be capable of mediating the lifespan effects of Fkh1/2 (Postnikoff et al., 2012). However, under stress conditions, such as maintenance of stationary phase cells in water, or exposure to either oxidative or heat stress, $apc5^{CA}$ *fkh1*Δ *fkh2*Δ cells grew much slower, were far more sensitive to stress and had a dramatically reduced CLS. While *fkh1*Δ *fkh2*Δ cells do not show the normal increased CLS in water, the triple mutant exhibited a dramatically shortened lifespan. This indicated that the Fkhs and the APC work together in a redundant manner to respond to stress and to ensure prolonged longevity. This is likely coordinated through the transcriptional up-regulation of stress response genes by Fkh1/2 in tandem with the chromatin assembly and histone modification functions of the APC (Harkness et al., 2002, 2005; Arnason et al., 2005; Turner et al., 2010; Islam et al., 2011). Together, the APC and the Fkhs drive response to stress and protect the genome from environmental stressors.

CONCLUSION

A pattern is emerging where pro-mitotic processes act as cell-cycle inhibitors to slow progression through early G1, allowing for preparation of S phase by resetting the transcriptome, repairing cellular damage, or remaining in a non-dividing state until conditions are right for the next cell cycle. Stress and starvation may act to prolong the function of this mechanism, allowing more time for stress response and cell repair. However, growth factors and energy sources inactivate these processes, favoring rapid growth over maintenance of youth. In mammals this process is regulated, at least in part, by the Fox class of transcription factors and the APC. Specifically, FoxM1 and APC^{Cdc20} function together to maintain genomic stability by regulating separation of sister chromosomes and chromatin structure, while the FoxOs and APC^{Cdh1} regulate cellular repair and maintenance, as well as the removal of built up pro-mitotic signals resetting the daughter cells. In yeast, new evidence suggests that this process is co-regulated by Fkh1 and Fkh2, which redundantly function in both mitotic progression and G1 maintenance, acting as both FoxO3a and FoxM1. This suggests a common role for evolutionary conserved proto-Fox proteins that regulates orthologous processes, such as cell-cycle progression and stress response. With this understanding, the resourcefulness of yeast biology and genetics, in addition to the ease of environmental control, could be utilized for untangling the mechanisms of Fox function, especially with regards to cancer and lifespan.

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Caloric restriction extends yeast chronological lifespan by altering a pattern of age-related changes in trehalose concentration

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The non-reducing disaccharide trehalose has been long considered only as a reserve carbohydrate. However, recent studies in yeast suggested that this osmolyte can protect cells and cellular proteins from oxidative damage elicited by exogenously added reactive oxygen species (ROS). Trehalose has been also shown to affect stability, folding, and aggregation of bacterial and firefly proteins heterologously expressed in heat-shocked yeast cells. Our recent investigation of how a lifespan-extending caloric restriction (CR) diet alters the metabolic history of chronologically aging yeast suggested that their longevity is programmed by the level of metabolic capacity – including trehalose biosynthesis and degradation – that yeast cells developed prior to entry into quiescence. To investigate whether trehalose homeostasis in chronologically aging yeast may play a role in longevity extension by CR, in this study we examined how single-gene-deletion mutations affecting trehalose biosynthesis and degradation impact (1) the age-related dynamics of changes in trehalose concentration; (2) yeast chronological lifespan under CR conditions; (3) the chronology of oxidative protein damage, intracellular ROS level and protein aggregation; and (4) the timeline of thermal inactivation of a protein in heat-shocked yeast cells and its subsequent reactivation in yeast returned to low temperature. Our data imply that CR extends yeast chronological lifespan in part by altering a pattern of age-related changes in trehalose concentration. We outline a model for molecular mechanisms underlying the essential role of trehalose in defining yeast longevity by modulating protein folding, misfolding, unfolding, refolding, oxidative damage, solubility, and aggregation throughout lifespan.

Keywords: yeast, cellular aging, longevity, chronological lifespan, caloric restriction, trehalose, proteostasis

INTRODUCTION

Growing evidence supports the view that the fundamental mechanisms of aging are conserved across phyla (Kenyon, 2001; Kirkwood, 2008; Fontana et al., 2010; Kenyon, 2010). The identification of single-gene mutations that extend lifespan in yeast, worms, flies, and mice revealed numerous proteins that regulate longevity (Kenyon, 2005, 2011; Fontana et al., 2010; Kaeberlein, 2010). These proteins have been implicated in a wide array of cellular processes including cell cycle, cell growth, stress response, protein folding, apoptosis, autophagy, proteasomal protein degradation, actin organization, signal transduction, nuclear DNA replication, chromatin assembly and maintenance, ribosome biogenesis and translation, lipid and carbohydrate metabolism, oxidative metabolism in mitochondria, NAD^+ homeostasis, amino acid biosynthesis and degradation, and ammonium and amino acid uptake (Greer and Brunet, 2008; Guarente et al., 2008; Kenyon, 2010; Masoro and Austad, 2011). The spatiotemporal organization of all these numerous cellular processes and their functional states are governed by a limited number of nutrient- and energy-sensing signaling pathways that are conserved across phyla and include the insulin/insulin-like growth factor 1 (IGF-1), AMP-activated protein kinase/target of rapamycin (AMPK/TOR), and cAMP/protein kinase A (cAMP/PKA) pathways (Greer and Brunet, 2008; Narasimhan et al., 2009; Fontana et al., 2010; Kenyon, 2010).

Caloric restriction (CR), a dietary regimen in which only calorie intake is reduced but the supply of amino acids, vitamins, and other nutrients is not compromised, is known to have the most profound longevity-extending effect across phyla and to improve overall health by delaying the onset of age-related diseases (Weindruch and Walford, 1988; Masoro, 2002; Mair and Dillin, 2008; Colman et al., 2009; Anderson and Weindruch, 2010; Fontana et al., 2010). The longevity benefit associated with CR is mediated by a signaling network that integrates the insulin/IGF-1, AMPK/TOR, and cAMP/PKA longevity regulation pathways and governs a distinct group of cellular processes (Mair and Dillin, 2008; Greer and Brunet, 2009; Narasimhan et al., 2009; Fontana et al., 2010; Goldberg et al., 2010). Our recent investigation of how CR alters the metabolic history of chronologically aging yeast suggested that trehalose metabolism is one of these longevity-defining processes (Goldberg et al., 2009). A possible essential role of trehalose in regulating yeast longevity has been also suggested by other recent

studies (Wang et al., 2010; Pluskal et al., 2011). Trehalose is a non-reducing disaccharide that until recently has been considered only as a reserve carbohydrate (François and Parrou, 2001). However, the demonstrated abilities of this osmolyte to protect yeast cells and cellular proteins from oxidative damage caused by exogenously added reactive oxygen species (ROS; Benaroudj et al., 2001) or inflicted in the process of industrial alcoholic fermentation (Trevisol et al., 2011) and to impact stability, folding, and aggregation of bacterial and firefly proteins heterologously expressed in heat-shocked yeast (Singer and Lindquist, 1998a,b) suggested that trehalose may exhibit similar effects on endogenous proteins in cells of yeast and other organisms (Singer and Lindquist, 1998a,b; Elbein et al., 2003; Jain and Roy, 2009, 2010). It is conceivable therefore that trehalose may be involved in modulating cellular protein homeostasis (proteostasis). By maintaining proper synthesis, post-translational modifications, folding, trafficking, degradation, and turnover of proteins within a cell, an evolutionarily conserved proteostasis network governs various cellular activities, influences diverse age-related pathologies, and defines organismal healthspan and longevity (Tavernarakis, 2010; Morimoto et al., 2012).

To evaluate a potential role of trehalose in lifespan extension by CR, in this study we monitored how single-gene-deletion mutations that alter trehalose concentrations in pre-quiescent and quiescent yeast cells affect longevity of chronologically aging yeast under CR conditions. We also elucidated how these mutations influence the chronology of oxidative protein carbonylation, intracellular ROS, protein aggregation, thermal inactivation of a protein in heat-shocked yeast cells and a subsequent reactivation of this protein in yeast shifted to low temperature. Our findings provide evidence that the longevity-extending effect of a CR diet in chronologically aging yeast is due in part to a specific pattern of age-related changes in trehalose concentration elicited by CR. Based on these findings, we propose a model for molecular mechanisms by which trehalose modulates cellular proteostasis throughout lifespan, thereby defining yeast longevity.

MATERIALS AND METHODS

YEAST STRAINS AND GROWTH CONDITIONS

The wild-type (WT) strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and single-gene-deletion mutant strains in the BY4742 genetic background (all from Open Biosystems) were grown in YP medium (1% yeast extract, 2% peptone) containing 0.2% glucose as carbon source. Cells were cultured at 30°C with rotational shaking at 200 rpm in Erlenmeyer flasks at a "flask volume/medium volume" ratio of 5:1.

CHRONOLOGICAL LIFESPAN ASSAY

A sample of cells was taken from a culture at a certain time-point. A fraction of the sample was diluted in order to determine the total number of cells using a hemacytometer. Another fraction of the cell sample was diluted and serial dilutions of cells were plated in duplicate onto YP plates containing 2% glucose as carbon source. After 2 day of incubation at 30°C, the number of colony forming units (CFU) per plate was counted. The number of CFU was defined as the number of viable cells in a sample. For each culture, the percentage of viable cells was calculated as follows: (number of viable cells per ml/total number of cells per ml) × 100. The percentage of viable cells in mid-logarithmic phase was set at 100%. The lifespan curves were validated using a LIVE/DEAD yeast viability kit (Invitrogen) following the manufacturer's instructions.

TREHALOSE CONCENTRATION MEASUREMENT

Preparation of alkali cellular extract and a microanalytic biochemical assay for measuring trehalose concentration were performed as previously described (Lin et al., 2001). To prepare an alkali cellular extract, 2×10^9 cells were harvested by centrifugation for 1 min at 21,000 × *g* at 4°C. The cells were washed three times in ice-cold PBS (20 mM KH_2PO_4/KOH, pH 7.5, and 150 mM NaCl). The cell pellet was quickly resuspended in 200 µl of ice-cold SHE solution (50 mM NaOH, and 1 mM EDTA), and 800 µl of ice-cold SHE solution were added to the cell suspension. The resulting alkali extract was incubated at 60°C for 30 min to destroy endogenous enzyme activities and pyridine nucleotides. The extract was neutralized by adding 500 µl of THA solution (100 mM Tris/HCl, pH 8.1, and 50 mM HCl), divided into 150-µl aliquots, quickly frozen in liquid nitrogen, and stored at – 80°C prior to use. To measure trehalose concentration, 50 µl of alkali extract (recovered from the total of 6.5×10^7 cells) were added to 150 µl of trehalose reagent [25 mM KH_2PO_4/KOH, pH 7.5, and 0.02% BSA; with or without 15 mU trehalase (Sigma)]. The mixture was incubated for 60 min at 37°C. Eight hundred microliters of glucose reagent [100 mM Tris/HCl, pH 8.1, 2 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 0.2 mM $NADP^+$, and mixture of hexokinase (7 U) and glucose-6-phosphate dehydrogenase (8 U; Sigma)] was added and the mixture incubated for 30 min at 25°C. The NADPH generated from $NADP^+$ was measured fluorimetrically (excitation at 365 nm, emission monitored at 460 nm).

HEXOKINASE ACTIVITY MEASUREMENT

Preparation of cellular lysate and a microanalytic biochemical assay for measuring hexokinase enzymatic activity were performed as previously described (Lin et al., 2001). To prepare a cellular lysate, 2×10^7 cells were harvested by centrifugation for 1 min at 21,000 × *g* at 4°C. The cells were washed three times in ice-cold PBS (20 mM KH_2PO_4/KOH, pH 7.5, and 150 mM NaCl). The cell pellet was quickly resuspended in 800 µl of EB buffer (20 mM KH_2PO_4/KOH, pH 7.5, 0.02% BSA, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 25% glycerol, and 0.5% Triton X-100) and incubated for 5 min at 25°C. The resulting lysate was divided into 40-µl aliquots and stored at – 80°C prior to use. To measure hexokinase activity, 4 µl of cellular lysate (recovered from the total of 1×10^5 cells) were added to 996 µl of hexokinase reagent [100 mM Tris/HCl, pH 8.1, 0.05% BSA, 7 mM $MgCl_2$, 5 mM ATP, 5 mM glucose, 0.5 mM DTT, 100 µM $NADP^+$, 0.5% Triton X-100, and 2 U glucose-6-phosphate dehydrogenase (Sigma)]. The mixture was incubated for 1 h at 25°C. The NADPH generated from $NADP^+$ was measured fluorimetrically (excitation at 365 nm, emission monitored at 460 nm). To monitor the extent of thermal inactivation of hexokinase in heat-shocked yeast cells and the efficacy of its reactivation during subsequent incubation of these cells at low temperature, yeast were grown at 29°C, and recovered upon entry into a quiescent state at day 7 or following such an entry at day 13. These cells were treated with cycloheximide for 5 min at

29˚C, heat-shocked for 60 min at 43˚C, then shifted to 29˚C, and incubated for 60 min. Hexokinase enzymatic activity was measured every 15 min of heat shock treatment and every 15 min of the following incubation at 29˚C.

IMMUNODETECTION OF CARBONYL GROUPS IN OXIDATIVELY DAMAGED CELLULAR PROTEINS

Total cell lysates were made by vortexing the cells in ice-cold TCL buffer (25 mM MOPS/KOH, pH 7.2, 150 mM NaCl, 50 mM DTT, and 1% CHAPS) with glass beads three times for 1 min. Lysates were then centrifuged for 5 min at 21,000 × *g* at 4˚C, and the supernatants of total cell lysates were collected. The carbonyl groups of proteins recovered in total cell lysates were derivatized to 2,4-dinitrophenylhydrazones using the OxyBlot™Protein Oxidation Detection Kit (Chemicon), according to the manufacturer's instructions. Briefly, total cellular proteins were denatured by adding 12% SDS to an equal volume of the total cell lysate containing 10 μg of protein. Denatured proteins were incubated with 2,4-dinitrophenylhydrazine for 15 min at room temperature. Proteins were separated by 12.5% SDS-PAGE. Immunoblotting using a Trans-Blot SD semi-dry electrophoretic transfer system (Bio-Rad) was performed as described (Titorenko et al., 1998). The derivatized carbonyl groups were detected with a 2,4-dinitrophenyl-specific antibody (Chemicon) and the Amersham ECL Western Blotting System (GE Healthcare).

ROS MEASUREMENT

Reactive oxygen species were measured in live yeast by fluorescence microscopy of Dihydrorhodamine 123 (DHR) staining according to established procedures (Madeo et al., 1997; Goldberg et al., 2009). Briefly, 5×10^6 cells were harvested by centrifugation for 1 min at 21,000 × *g* at room temperature and then resuspended in 100 μl of PBS. DHR (Sigma) was added to a final concentration of 10 μM. Following incubation in the dark for 60 min at room temperature, the cells were washed in PBS, and then analyzed by fluorescence microscopy. Images were collected with a Zeiss Axioplan fluorescence microscope (Zeiss) mounted with a SPOT Insight 2 megapixel color mosaic digital camera (Spot Diagnostic Instruments). Fluorescence of individual DHR-positive cells in arbitrary units was determined by using the UTHSCSA Image Tool software (Version 3.0). In each of three to six independent experiments, the value of median fluorescence was calculated by analyzing at least 800–1000 cells that were collected at each time-point. The median fluorescence values were plotted as a function of the number of days cells were cultured.

RECOVERY OF INSOLUBLE AGGREGATES OF DENATURED PROTEINS

Insoluble aggregates of denatured proteins were recovered according to established procedures (Parsell et al., 1994; Boukh-Viner et al., 2005), with the following modifications. Total cell lysates were made by vortexing the cells in ice-cold MBS buffer (25 mM MOPS/KOH, pH 7.2, and 150 mM NaCl) with glass beads four times for 1 min. Unbroken cells and cell debris were removed by centrifugation for 3 min at 1,000 × *g* at 4˚C. The supernatants of total cell lysates were collected and normalized by dilution to a final concentration of 1 mg/ml. Equal aliquots of the total cell lysates were supplemented with 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Sigma) to a final concentration of 10 mM. CHAPS is a zwitterionic, non-denaturing, and electrically neutral detergent; although it protects a native state of soluble proteins and efficiently solubilizes intrinsic membrane proteins (including proteins associated with lipid raft membrane domains), it is unable to solubilize aggregates of denatured proteins (Chow and Zukin, 1983; Evans et al., 1986; Boukh-Viner et al., 2005; Tao et al., 2010). After incubation on ice for 30 min, samples were subjected to centrifugation at 100,000 × *g* for 30 min at 4˚C. The pellet fractions of insoluble aggregates of denatured proteins were analyzed by 12.5% SDS-PAGE, followed by silver staining.

STATISTICAL ANALYSIS

Statistical analysis was performed using Microsoft Excel's (2010) Analysis ToolPack-VBA. All data are presented as mean ± SEM. The *p* values were calculated using an unpaired two-tailed *t* test.

RESULTS

LIFESPAN EXTENSION BY CR REQUIRES A SPECIFIC PATTERN OF AGE-RELATED CHANGES IN TREHALOSE CONCENTRATION

To evaluate the effect of trehalose on lifespan extension by CR, we incubated WT strain and several mutant strains, each carrying a single-gene-deletion mutation affecting trehalose biosynthesis or degradation (François and Parrou, 2001), in YP medium initially containing 0.2% glucose. We monitored the chronological lifespans of all these strains and assessed the dynamics of changes in trehalose concentration during their aging under CR conditions.

The *tps1*Δ and *tps2*Δ mutations, which eliminate two different catalytic subunits of the trehalose synthase complex (**Figure 1A**), decreased intracellular trehalose concentration and shortened lifespan (**Figures 1B,D**). Yeast whose trehalose level was increased before they have entered the non-proliferative stationary (ST) growth phase and remained elevated during ST phase – as it was observed in mutant cells lacking the Nth1p isozyme of neutral trehalase – were short-lived (**Figures 1C,E**). Moreover, even if trehalose concentration exceeded the level seen in WT only after yeast have entered ST phase – as it occurred in mutant cells lacking the Nth2p isozyme of neutral trehalase – cells were short-lived (**Figures 1C,E**). Importantly, some genetic manipulations altering trehalose concentration extended lifespan. Specifically, in long-lived mutants lacking the Tsl1p or Tps3p regulatory subunit of the trehalose synthase complex, trehalose concentration exceeded that in WT until the end of post-diauxic (PD) growth phase, but then in ST phase reached a plateau at the level that was 50–70% of that in WT (**Figures 1B,D**). Similar dynamics of age-related changes in trehalose concentration was observed in the long-lived mutant *ath1*Δ lacking acid trehalase (**Figures 1C,E**).

Altogether, these findings imply that the extended chronological lifespan of CR yeast (as compared to that of non-CR yeast) can be further prolonged by genetic manipulations that simultaneously (1) increase trehalose concentration by 70–160% during PD phase, prior to entry into a quiescent state; and (2) reduce trehalose concentration by 60–80% during ST phase, following entry into quiescence. Thus, lifespan extension by a low calorie diet requires a specific pattern of age-related changes in the intracellular level of trehalose.

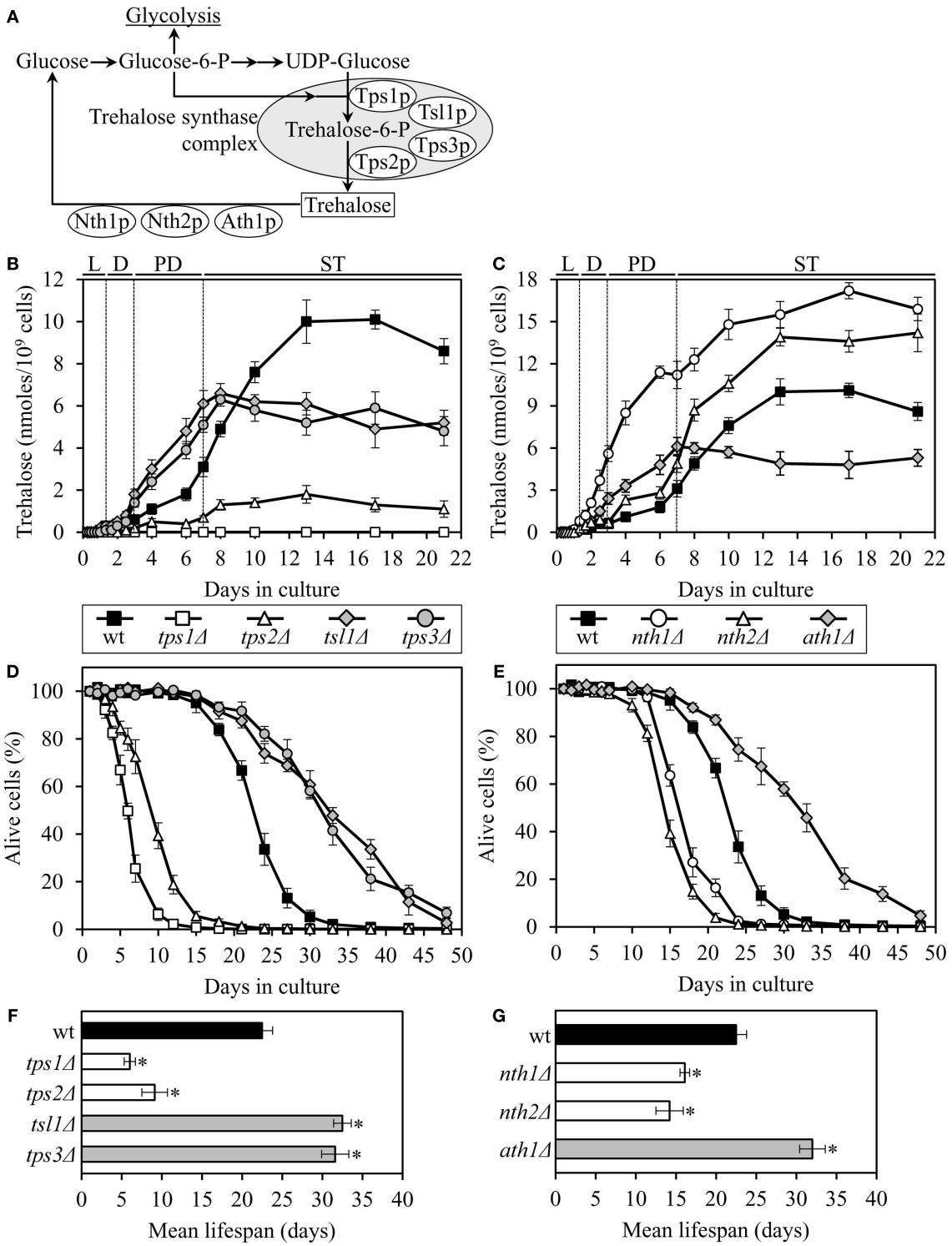

FIGURE 1 | The chronological lifespan of yeast grown under CR conditions can be extended by mutations that simultaneously increase trehalose concentration prior to quiescence and reduce trehalose concentration following entry into a quiescent state. (A) Outline of metabolic pathways of trehalose biosynthesis and degradation. **(B,C)** The dynamics of age-dependent changes in the intracellular levels of trehalose during chronological aging of wild-type (wt) and mutant strains. **(D–G)** Survival **(D,E)** and the mean lifespans **(F,G)** of chronologically aging wt and mutant strains. Each mutant carried a single-gene-deletion mutation that affects trehalose biosynthesis or degradation. Cells were cultured in YP medium initially containing 0.2% glucose. Data are presented as mean ± SEM ($n = 5$–6); $^*p < 0.01$ (relative to the mean lifespan of wt strain). Abbreviations: D, diauxic growth phase; L, logarithmic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase.

MUTATIONS THAT INCREASE TREHALOSE CONCENTRATION PRIOR TO ENTRY INTO QUIESCENCE REDUCE OXIDATIVE DAMAGE TO CELLULAR PROTEINS THROUGHOUT LIFESPAN, IRRESPECTIVE OF THEIR EFFECTS ON LONGEVITY

Trehalose accumulation in exponentially grown yeast cells exposed to elevated temperature or to a proteasome inhibitor has been shown to increase their ability to survive a subsequent treatment with exogenous ROS and to protect cellular proteins from oxidative carbonylation caused by such a treatment (Benaroudj et al., 2001). According to the mitochondrial free radical theory of aging, the gradual accumulation of macromolecular damage caused by mitochondrially produced ROS throughout lifespan accelerates cellular dysfunction and later in life leads to a functional decline and increased mortality (Harman, 1956, 1972). Although a body of evidence does not validate the core statement of this theory on a casual role of ROS generation in aging, the importance of ROS in mediating a stress response to age-related cellular damage is supported by numerous findings (Gems and Doonan, 2009; Pérez et al., 2009; Lapointe and Hekimi, 2010; Ristow and Zarse, 2010; Sanz et al., 2010; Hekimi et al., 2011). To evaluate a potential role of trehalose in linking a ROS-dependent oxidative macromolecular damage to lifespan extension by CR, we assessed how the *tsl1*Δ and *nth1*Δ mutations influence the dynamics of age-related changes in protein carbonylation and ROS in yeast grown under CR conditions.

Both the *tsl1*Δ and *nth1*Δ mutations elevated trehalose concentration (**Figures 1B,C**) and reduced oxidative carbonylation of cellular proteins (**Figure 2A**) during PD phase, prior to entry into a quiescent state. None of these mutations altered ROS levels in pre-quiescent cells (**Figure 2B**). Thus, it is unlikely that the observed reduction of oxidative damage to cellular proteins in pre-quiescent *tsl1*Δ and *nth1*Δ cells was due to the previously proposed by Benaroudj et al. (2001) ability of trehalose, a non-reducing disaccharide, to quench ROS. It is conceivable therefore that prior to quiescence trehalose protects cellular proteins from oxidative carbonylation (**Figure 2A**) by interacting with their carbonylation-prone misfolded and unfolded species. These aberrantly folded protein species are known to be much more sensitive to oxidative carbonylation than their properly folded counterparts (Nyström, 2005; Hipkiss, 2006).

The extent of protein carbonylation reached prior to entry into a quiescent state was not significantly altered in *tsl1*Δ and *nth1*Δ cells following entry into quiescence (**Figure 2A**), likely due to greatly diminished ROS levels observed in quiescent *tsl1*Δ and *nth1*Δ cells (**Figure 2B**). Trehalose concentration in quiescent *tsl1*Δ cells was substantially lower than that seen in quiescent WT cells (**Figure 1B**). In contract, the concentration of trehalose in quiescent *nth1*Δ cells exceeded the level detected in quiescent WT cells (**Figure 1C**). We therefore concluded that genetic manipulations that increase trehalose concentration prior to entry into a quiescent state reduce oxidative damage to cellular proteins throughout lifespan, regardless of their effects on the intracellular concentration of this non-reducing disaccharide following entry into quiescence.

Although both the *tsl1*Δ and *nth1*Δ mutations reduced oxidative carbonylation of cellular proteins throughout lifespan (**Figure 2A**), their effects on longevity differed. The *tsl1*Δ mutation extended yeast lifespan, whereas the *nth1*Δ mutations shortened it (**Figures 1D–G**). Hence, it is unlikely that the observed ability of these genetic manipulations to protect cellular proteins from oxidative damage plays a role in defining yeast longevity under CR conditions.

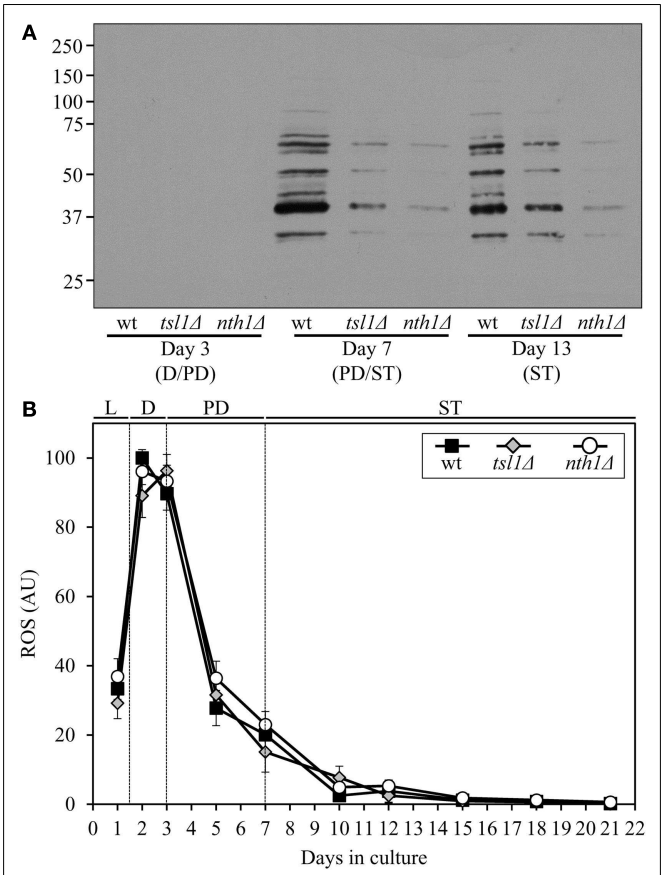

FIGURE 2 | Although mutations that in yeast grown under CR conditions increase trehalose concentration prior to entry into quiescence do not alter ROS levels, they reduce oxidative damage to cellular proteins throughout lifespan. (A) Immunodetection of carbonyl groups in oxidatively damaged cellular proteins in chronologically aging wt and mutant strains. **(B)** The dynamics of age-related changes in intracellular ROS levels during chronological aging of wt and mutant strains. wt, *tsl1*Δ and *nth1*Δ cells were cultured in YP medium initially containing 0.2% glucose. Data are presented as mean ± SEM ($n = 3$–4). Abbreviations: D, diauxic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase.

A PATTERN OF AGE-RELATED CHANGES IN TREHALOSE CONCENTRATION DEFINES THE DYNAMICS OF PROTEIN AGGREGATION THROUGHOUT LIFESPAN

Trehalose has been shown to (1) stabilize bacterial and firefly luciferases in their native (folded) states in heat-shocked yeast cells; (2) prevent aggregation and maintain non-native (misfolded or partially folded) states of these two luciferases, as well as of firefly rhodanese, following their guanidinium-induced denaturation *in vitro* and in yeast cells briefly exposed to elevated temperature; and (3) inhibit the refolding and reactivation of

these pre-denatured bacterial and firefly proteins *in vitro* and in yeast cells by interfering with chaperone-assisted folding of their non-native (misfolded or partially folded) species (Singer and Lindquist, 1998a). It has been predicted that trehalose may exhibit similar effects on the stability, folding, and aggregation of endogenous proteins in cells of yeast and other organisms (Singer and Lindquist, 1998a,b; Elbein et al., 2003; Jain and Roy, 2009, 2010; Mir et al., 2009). Furthermore, our investigation of how a CR diet affects the metabolic history of chronologically aging yeast suggested that the elevated level of trehalose observed prior to entry into quiescence in slowly aging CR yeast (as compared to that seen in rapidly aging non-CR yeast) protects from aggregation proteins that have been completely or partially unfolded and/or oxidatively carbonylated due to their exposure to intracellular ROS (Goldberg et al., 2009). We hypothesized that (1) such protective effect of high trehalose concentrations could contribute to the enhanced survival of CR yeast (as compared to survival of non-CR yeast) following their entry into quiescence; and (2) a dietary or genetic intervention providing yeast with the ability to maintain trehalose concentration at a certain "optimal" level prior and following entry into a quiescent state would extend their longevity (Goldberg et al., 2009). We predicted that at such an "optimal" level trehalose concentration is (1) sufficiently high prior to entry into quiescence to allow this osmolyte to prevent aggregation of proteins that have been completely or partially unfolded and/or oxidatively carbonylated; and (2) sufficiently low following entry into quiescence to reduce the efficiency with which trehalose inhibits the refolding and reactivation of partially unfolded and/or oxidatively carbonylated proteins (Goldberg et al., 2009).

To test the validity of our hypothesis, we assessed how the *tsl1*Δ and *nth1*Δ mutations influence the dynamics of age-related changes in the extent of protein aggregation in yeast limited in calories. We found that both these mutations, which we demonstrated to elevate trehalose concentration (**Figures 1B,C**) and to decrease oxidative protein carbonylation (**Figure 2A**) during PD phase, significantly reduce the extent of aggregation of cellular proteins during this growth phase preceding entry into a quiescent state (**Figure 3**). Following entry into quiescence, the extent of protein aggregation in *tsl1*Δ cells was substantially lower than that seen in quiescent WT cells and especially in quiescent *nth1*Δ cells (**Figure 3**). As we mentioned above, trehalose concentration in quiescent *tsl1*Δ cells was significantly reduced as compared to that in WT (**Figure 1B**) and especially in *nth1*Δ (**Figure 1C**) cells reached reproductive maturation. Furthermore, both the concentration of trehalose (**Figures 1B,C**) and the extent of protein aggregation (**Figure 3**) in quiescent *nth1*Δ cells were significantly higher than that observed in WT cells and especially in *tsl1*Δ cells entered a quiescent state.

In sum, these findings validate our hypothesis in which a genetic intervention will extend longevity of calorically restricted yeast if it (1) elevates trehalose concentration prior to entry into quiescence to allow this osmolytic disaccharide to prevent aggregation of completely or partially unfolded and/or oxidatively carbonylated cellular proteins; and (2) reduces the concentration of trehalose following entry into quiescence to limit its inhibitory effect on the refolding and reactivation of partially unfolded and/or oxidatively carbonylated proteins.

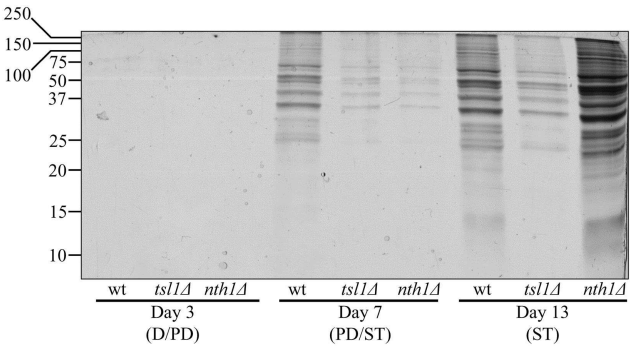

FIGURE 3 | In yeast grown under CR conditions, a pattern of age-related changes in trehalose concentration define the dynamics of protein aggregation throughout lifespan. Total cell lysates were made by vortexing the cells in ice-cold buffer with glass beads. Unbroken cells and cell debris were removed by centrifugation for 3 min at 1,000 × *g* at 4°C. The supernatants of total cell lysates were collected and normalized by dilution to a final concentration of 1 mg/ml. Equal aliquots of the total cell lysates were supplemented with CHAPS, a zwitterionic, non-denaturing, and electrically neutral detergent that protects a native state of soluble proteins and efficiently solubilizes membrane proteins, but is unable to solubilize aggregates of denatured proteins. After incubation on ice for 30 min, samples were subjected to centrifugation at 100,000 × *g* for 30 min at 4°C. The pellet fractions of insoluble aggregates of denatured proteins were analyzed by 12.5% SDS-PAGE, followed by silver staining. wt, *tsl1*Δ and *nth1*Δ cells were cultured in YP medium initially containing 0.2% glucose. Abbreviations: D, diauxic growth phase; PD, post-diauxic growth phase; ST, stationary growth phase.

TREHALOSE CONCENTRATION IN YEAST CELLS DEFINES THE SENSITIVITY OF AN ENDOGENOUS ENZYME TO THERMAL INACTIVATION AND THE EXTENT OF ITS SUBSEQUENT REACTIVATION AT LOW TEMPERATURE

To use a complementary experimental approach for validating our hypothesis on a longevity-defining role of trehalose concentration in maintaining biological activities of proteins in chronologically aging yeast under CR conditions, we assessed how the *tsl1*Δ and *nth1*Δ mutations influence (1) the extent of thermal inactivation of hexokinase, an endogenous enzyme protein, in heat-shocked yeast cells; and (2) the efficacy of its reactivation during subsequent incubation of these cells at low temperature. In these experiments, yeast grown at 29°C and recovered upon entry into a quiescent state or following such an entry were treated with cycloheximide for 5 min at 29°C, heat-shocked for 60 min at 43°C, then shifted to 29°C, and incubated for 60 min (**Figure 4**).

In *tsl1*Δ and *nth1*Δ cells recovered at day 7, upon entry into a quiescent state, the activity of hexokinase synthesized prior to a cycloheximide-induced inhibition of protein synthesis at 29°C was less susceptible to thermal inactivation at 43°C than in identically treated and aged WT cells (**Figure 4C**). Under these conditions, trehalose concentrations in both *tsl1*Δ and *nth1*Δ cells exceeded that in WT cells of the same age (**Figure 4A**). If cells were recovered at day 13, following entry into a quiescent state, hexokinase activity in *tsl1*Δ cells having a lower trehalose concentration than WT cells (**Figure 4B**) was more susceptible to the thermal inactivation at 43°C then in WT cells (**Figure 4D**). In contrast, in *nth1*Δ cells

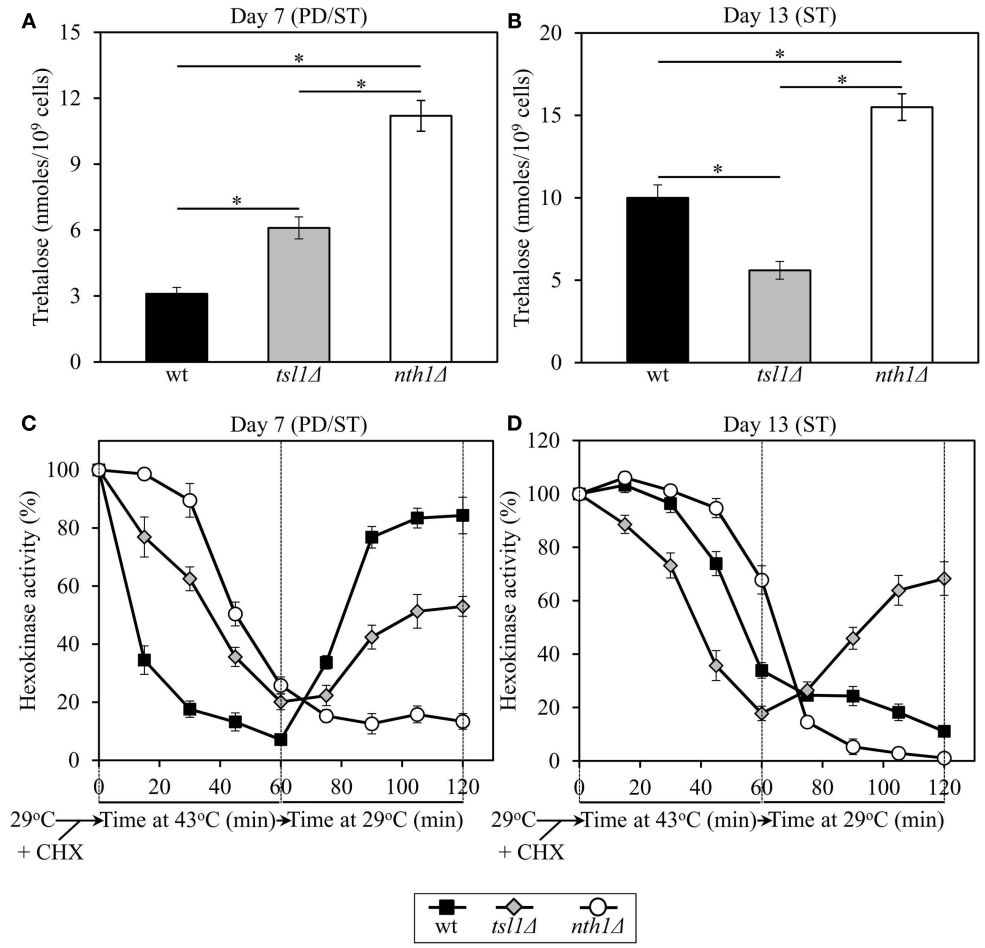

FIGURE 4 | In yeast grown under CR conditions, trehalose concentration define the sensitivity of hexokinase, an endogenous enzyme, to thermal inactivation, and the extent of its subsequent reactivation at low temperature. Yeast cells grown at 29˚C were recovered upon entry into a quiescent state at day 7 or following such an entry at day 13. The cells were treated with cycloheximide for 5 min at 29˚C to inhibit protein synthesis, heat-shocked for 60 min at 43˚C, then shifted to 29˚C, and incubated for 60 min. **(A,B)** The intracellular levels of trehalose prior to cell treatment with cycloheximide. **(C,D)** Changes in hexokinase enzymatic activity following cell exposure to cycloheximide, during heat shock treatment for 60 min at 43˚C, and subsequent incubation for 60 min at 29˚C. wt, *tsl1*Δ and *nth1*Δ cells were cultured in YP medium initially containing 0.2% glucose. Data are presented as mean ± SEM ($n = 3$–5). Abbreviations: CHX, cycloheximide; PD, post-diauxic growth phase; ST, stationary growth phase.

recovered at day 13 and having a higher trehalose concentration then WT cells of the same age (**Figure 4B**) hexokinase activity was less susceptible to such thermal inactivation then in WT cells (**Figure 4D**). These findings imply that in calorically restricted pre-quiescent yeast trehalose preserves biological activities of partially inactivated cellular proteins, perhaps by stabilizing their native (folded) state, preventing their unfolding, and/or inhibiting their subsequent aggregation.

In *tsl1*Δ and *nth1*Δ cells recovered at day 7, upon entry into quiescence, the reactivation of thermally inactivated hexokinase during the subsequent incubation at low temperature occurred less efficient then in WT cells of the same age (**Figure 4C**). Noteworthy, the efficacy of such hexokinase reactivation was inversely proportional to trehalose concentration in yeast cells that reached a transition to a quiescent state (**Figures 4A,C**). If cells were recovered at day 13, following entry into quiescence, the reactivation of thermally inactivated hexokinase during the subsequent incubation at low temperature occurred only in *tsl1*Δ cells having lower trehalose concentration as compared to WT and especially to *nth11*Δ cells of the same age (**Figures 4B,D**). In *nth1*Δ cells recovered at day 13 and having a higher trehalose concentration then WT cells of the same age (**Figure 4B**), thermally inactivated hexokinase was further inactivated during the subsequent incubation at low temperature with the efficiency exceeding that in WT cells (**Figure 4D**). These findings imply that in calorically restricted quiescent yeast trehalose inhibits the reactivation of inactivated cellular proteins, perhaps by interfering with chaperone-assisted folding of their non-native (misfolded or partially folded) species.

DISCUSSION

To investigate whether trehalose homeostasis in yeast cells may play a role in longevity extension by CR, we assessed how single-gene-deletion mutations that in chronologically aging yeast alter

trehalose concentrations prior to quiescence and following entry into a quiescent state impact lifespan. We also examined the effects of these mutations on the chronology of oxidative protein carbonylation, intracellular ROS, protein aggregation, thermal inactivation of a protein in heat-shocked yeast cells and a subsequent reactivation of this protein in yeast shifted to low temperature. Our findings provide evidence that CR extends yeast chronological lifespan in part by altering a pattern of age-related changes in trehalose concentration. Based on our data, we propose a model for molecular mechanisms underlying the essential role of trehalose in defining yeast longevity by modulating cellular proteostasis throughout lifespan (**Figure 5**). This outlined below model adequately explains how genetic interventions altering a pattern of age-related changes in trehalose concentration influence a longevity-defining balance between protein folding, misfolding, unfolding, refolding, oxidative damage, solubility, and aggregation.

Pre-quiescent WT cells proliferating under CR conditions cope with a flow of misfolded, partially folded, and unfolded protein species in the non-native folding state (**Figure 5A**, process

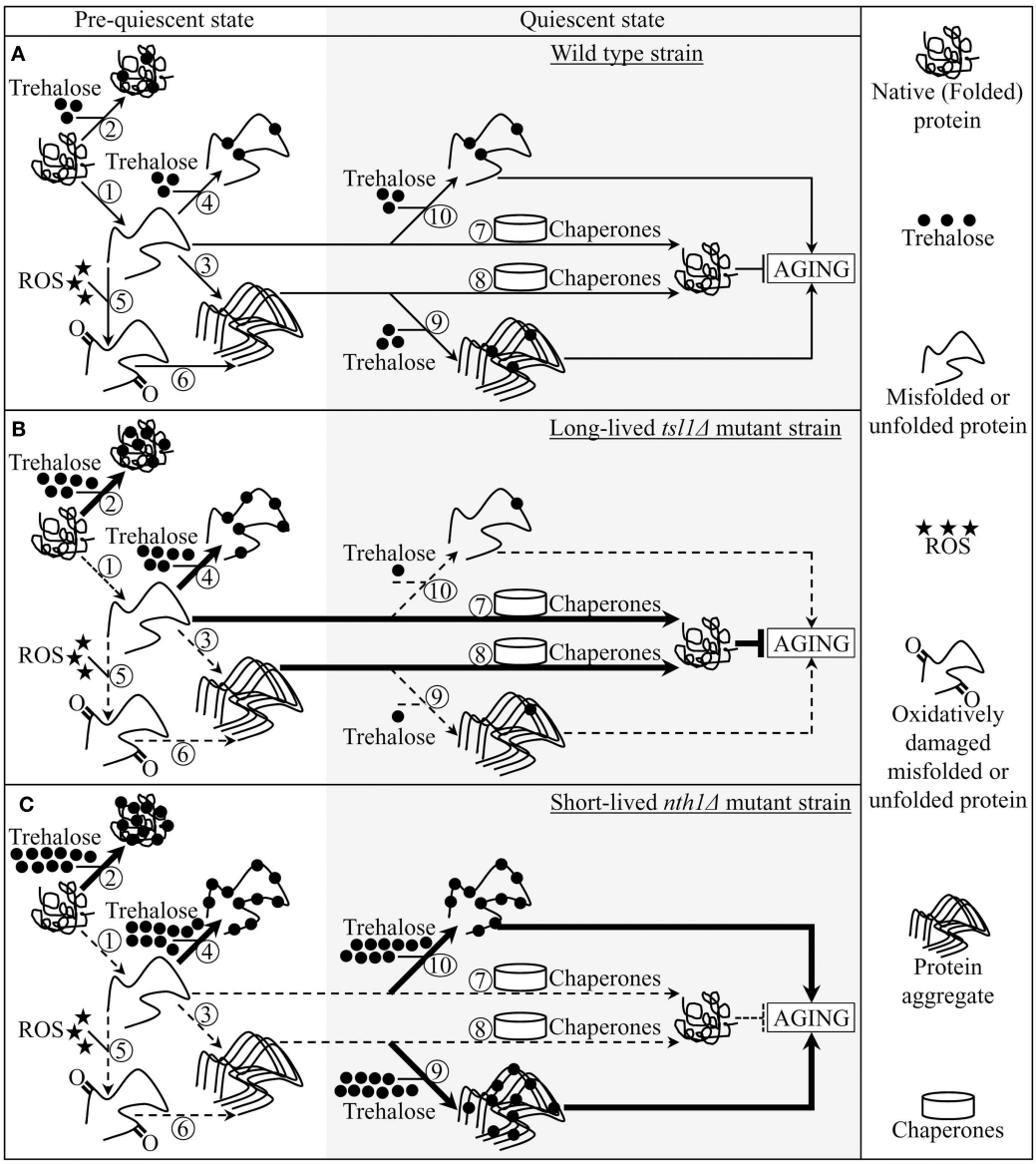

FIGURE 5 | A model for molecular mechanisms underlying the essential role of trehalose in definin yeast longevity by modulating cellular proteostasis throughout lifespan. The outlined model adequately explains how the *tsl1*Δ and *nth1*Δ mutations altering a pattern of age-related changes in trehalose concentration influence a longevity-defining balance between protein folding, misfolding, unfolding, refolding, oxidative damage, solubility, and aggregation. **(A–C)** The effects of trehalose on essential processes governing proteostasis in wild-type **(A)**, *tsl1*Δ **(B)** and *nth1*Δ **(C)** cells prior to and following entry into a quiescent state are outlined. See text for details. The thickness of arrows and T bars correlates with the rates of the processes taking place in chronologically aging yeast prior to entry into a quiescent state and following such an entry under CR conditions. T bars denote inhibition of the process. Abbreviation: ROS, reactive oxygen species.

1). The continuous formation of these protein species within a proliferating cell is due to a number of factors, including (1) macromolecular crowding, which is caused by the very high intracellular protein concentration and leads to inappropriate intermolecular contacts; (2) stochastic fluctuations in protein structure; (3) transcriptional errors; (4) inherited genetic polymorphisms, including gene copy number variations; (5) intrinsic errors in gene expression that may create an excess of unassembled subunits of oligomeric protein complexes; (6) errors in protein translation – such as missense incorporation of amino acids, frame-shifting, stop-codon readthrough, and premature termination; (7) defects in post-translational protein modifications and turnover; and (8) inefficient translocation of secretory and mitochondrial precursor proteins across membranes of their target organelles (Chen et al., 2011; Gidalevitz et al., 2011; Lindquist and Kelly, 2011). In pre-quiescent WT cells, trehalose stabilizes the native state of proteins and thereby reduces the formation of their aberrantly folded species (**Figure 5A**, process 2). The promoted by trehalose shift of a balance between native and non-native protein folding states toward properly folded protein species is amplified by the *tsl1*Δ and *nth1*Δ mutations, both of which significantly elevate trehalose concentration prior to entry into a quiescent state (**Figures 5B,C**, process 2). Our finding that the enzymatic activity of an endogenous hexokinase synthesized prior to a cycloheximide-induced inhibition of protein synthesis at 29˚C in pre-quiescent *tsl1*Δ and *nth1*Δ cells is significantly less susceptible to thermal inactivation at 43˚C than in identically treated and chronologically aged WT cells (**Figure 4C**) supports the role of trehalose in stabilizing the native state of cellular proteins. Moreover, a previously demonstrated ability of trehalose to stabilize bacterial and firefly luciferases in their native states in heat-shocked yeast cells (Singer and Lindquist, 1998a) provides additional support for the validity of our conclusion on the essential role of this osmolyte in shifting a balance between native and non-native protein folding states toward native folding structures. It is conceivable that trehalose may stabilize the native state of proteins in pre-quiescent yeast cells via any of the three recently proposed mechanisms (Jain and Roy, 2009, 2010).

In pre-quiescent WT cells, the aberrantly folded protein species that have not been refolded into functional three-dimensional native conformations or degraded within an elaborate network of molecular chaperones and protein degradation factors (Chen et al., 2011; Gidalevitz et al., 2011; Lindquist and Kelly, 2011) form insoluble aggregates (**Figure 5A**, process 3). In these cells, trehalose reduces the formation of such protein aggregates, perhaps by shielding the contiguous exposed hydrophobic side chains of amino acids that are abundant in misfolded, partially folded, and unfolded protein species and promote their aggregation (**Figure 5A**, process 4). Our finding that the *tsl1*Δ and *nth1*Δ mutations, both of which elevate trehalose concentration prior to entry into quiescence (**Figures 1B,C**), significantly reduce the extent of protein aggregation in pre-quiescent cells (**Figure 3**) supports the essential role of trehalose in preventing the formation of insoluble protein aggregates in these proliferation-competent cells (**Figures 5B,C**, process 4). Moreover, a previously demonstrated ability of trehalose to prevent aggregation and maintain non-native states of bacterial and firefly luciferases, as well as of firefly rhodanese, following their guanidinium-induced denaturation *in vitro* and in heat-shocked yeast cells (Singer and Lindquist, 1998a) further validates our conclusion that trehalose inhibits aggregation of the aberrantly folded protein species accumulating in pre-quiescent yeast.

The misfolded, partially folded, and unfolded protein species present in pre-quiescent WT cells are known to be more sensitive to ROS-driven oxidative carbonylation than their properly folded counterparts (Nyström, 2005; Hipkiss, 2006). These cells accumulate substantial levels of ROS (**Figure 2B**), which oxidatively damage a pool of the aberrantly folded and unfolded proteins (**Figure 2A**) prior to entry into a quiescent state (**Figure 5A**, process 5). Prior to quiescence, trehalose protects cellular proteins from oxidative carbonylation by interacting with their carbonylation-prone misfolded and unfolded species (**Figure 5A**, process 4) but not by quenching ROS (as it has been previously proposed by Benaroudj et al., 2001). In support of this mechanism for the protection of proteins from ROS-elicited oxidative damage by trehalose, we found that in pre-quiescent cells the *tsl1*Δ and *nth1*Δ mutations reduce oxidative carbonylation of cellular proteins (**Figure 2A**) but do not alter ROS levels (**Figures 2B** and **5B,C**, process 5).

The oxidatively carbonylated protein species present in pre-quiescent WT cells are known to have a tendency to form insoluble aggregates that escape degradation and can compromise the cellular proteostasis network by inhibiting the proteasomal protein degradation machinery (Nyström, 2005; Hipkiss, 2006; Taylor and Dillin, 2011). By protecting cellular proteins from oxidative carbonylation (**Figure 5A**, process 5; see above), trehalose reduces the formation of insoluble protein aggregates prior to entry into senescence (**Figure 5A**, process 6). This indirect inhibitory effect of trehalose on protein aggregation supplements its direct inhibition by trehalose (**Figure 5A**, process 3; see above), which could shield the patches of exposed hydrophobic side chains of amino acids tending to promote aggregation of aberrantly folded and unfolded protein species (**Figure 5A**, process 4; see above).

Following entry into a quiescent state, a network of molecular chaperones in WT cells promotes a refolding of misfolded, partially folded, and unfolded protein species, either soluble or extracted from protein aggregates accumulated in pre-quiescent cells (**Figure 5A**, processes 7 and 8). This chaperone-assisted refolding of aberrantly folded protein species is the essential anti-aging process (Kikis et al., 2010; Chen et al., 2011; Lindquist and Kelly, 2011; Taylor and Dillin, 2011). By shielding the contiguous exposed hydrophobic side chains of amino acids that are abundant in misfolded, partially folded and unfolded protein species, trehalose in quiescent WT cells competes with molecular chaperones for binding with these patches of hydrophobic amino acid residues (**Figure 5A**, processes 9 and 10) known to be mandatory for enabling the chaperone-assisted refolding of aberrantly folded protein species (Kikis et al., 2010; Chen et al., 2011; Lindquist and Kelly, 2011; Taylor and Dillin, 2011). By interfering with this essential anti-aging process in quiescent WT cells, trehalose operates as a pro-aging compound (**Figure 5A**). In support of our hypothesis that this mechanism underlies the essential role of trehalose homeostasis in defining longevity of chronologically aging yeast under CR conditions (**Figures 5B,C**, processes 9 and 10) we found

that (1) the *tsl1*Δ mutation reduces trehalose concentration following entry into quiescence (**Figure 1B**), decreases the extent of protein aggregation in quiescent cells (**Figure 3**) and extends yeast chronological lifespan (**Figure 1D**); and (2) the *nth1*Δ mutation elevates trehalose concentration following entry into quiescence (**Figure 1C**), increases the extent of protein aggregation in quiescent cells (**Figure 3**) and shortens yeast chronological lifespan (**Figure 1E**).

The major challenge now is to get a greater insight into the proposed mechanism underlying the essential role of trehalose homeostasis in defining longevity of chronologically aging yeast under lifespan-extending CR conditions. To address this challenge, many important questions need to be answered. What are the identities of oxidatively damaged proteins whose accumulation in pre-quiescent WT cells proliferating under CR conditions is reduced by genetic manipulations that elevate trehalose concentration prior to entry into quiescence (**Figure 2A**)? Are these proteins known for their essential role in defining longevity? Will genetic manipulations eliminating any of these proteins or altering their levels affect the chronological lifespan of yeast? What kind of proteins form insoluble aggregates that accumulate, in a trehalose-dependent fashion, in WT cells prior to and/or following entry into a quiescent state (**Figure 3**)? Are they known to be modifiers of lifespan in yeast? How will genetic manipulations eliminating any of these proteins or altering their levels influence longevity of chronologically aging yeast? Do oxidatively damaged and/or aggregated protein species concentrate in certain protein quality control compartments, such as the juxtanuclear quality control compartment, the insoluble protein deposit compartment and/or aggresome (Ben-Gedalya et al., 2011; Chen et al., 2011; Ben-Gedalya and Cohen, 2012), or are they randomly distributed throughout a cell prior to and/or following entry into quiescence? Does trehalose reside, permanently or temporarily, in any of these protein quality control compartments or is this osmolyte dispersed within a cell before and/or after it enters a quiescent state? What molecular chaperones constitute the proteostasis machinery whose ability to refold aberrantly folded proteins is compromised by trehalose in quiescent cells? We shall have to answer these important questions if we want to understand the complexity of the proteostasis network that defines longevity by sensing the dynamics of age-related changes in trehalose concentration.

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The endoplasmic reticulum stress response in aging and age-related diseases

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The endoplasmic reticulum(ER) is a multifunctional organelle within which protein folding, lipid biosynthesis, and calcium storage occurs. Perturbations such as energy or nutrient depletion, disturbances in calcium or redox status that disrupt ER homeostasis lead to the misfolding of proteins, ER stress and up-regulation of several signaling pathways coordinately called the unfolded protein response (UPR). The UPR is characterized by the induction of chaperones, degradation of misfolded proteins and attenuation of protein translation. The UPR plays a fundamental role in the maintenance of cellular homeostasis and thus is central to normal physiology. However, sustained unresolved ER stress leads to apoptosis. Aging linked declines in expression and activity of key ER molecular chaperones and folding enzymes compromise proper protein folding and the adaptive response of the UPR. One mechanism to explain age associated declines in cellular functions and age-related diseases is a progressive failure of chaperoning systems. In many of these diseases, proteins or fragments of proteins convert from their normally soluble forms to insoluble fibrils or plaques that accumulate in a variety of organs including the liver, brain or spleen. This group of diseases, which typically occur late in life includes Alzheimer's, Parkinson's, type II diabetes and a host of less well known but often equally serious conditions such as fatal familial insomnia. The UPR is implicated in many of these neurodegenerative and familial protein folding diseases as well as several cancers and a host of inflammatory diseases including diabetes, atherosclerosis, inflammatory bowel disease and arthritis. This review will discuss age-related changes in the ER stress response and the role of the UPR in age-related diseases.

Keywords: aging, age-related disease, UPR, BiP/GRP78, endoplasmic reticulum, stress

INTRODUCTION

Average life expectancies have been extended by as much as 30 years in developed countries during the Twentieth Century; a trend that is expected to continue in this century (Vaupel et al., 1998; Oeppen and Vaupel, 2002). The increase in elderly populations has raised interest in health consequences related to the aging process. A multitude of diseases that seemed rare many decades ago, are now amplified in aged individuals. Cases of dementia and Alzheimer's, incurable brain-wasting conditions, are expected to almost double every 20 years to around 66 million in 2030 and over 115 million in 2050 (Alzheimer's Association, 2012).

Evidence has implicated a role for unfolded/misfolded proteins in normal aging and age-related cognitive dysfunction. Age-associated deterioration of cellular machinery leads to an increase in the occurrence of protein misfolding, accumulation and aggregation, due in part to the gradual decay of chaperoning systems (Macario and Conway de Macario, 2002). In the majority of these diseases, proteins or protein fragments are transformed from their native soluble forms into insoluble fibrils or aggregated plaques that accumulate in a variety of organs. This group of conformational disorders, which includes Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease, type 2 diabetes mellitus, and a variety of other lesser known but equally severe conditions, appear later in life and are associated with aging. The fact that under normal physiological conditions, protein aggregates do not accumulate in the cells is partially due to the presence of cellular "quality control" mechanisms.

The endoplasmic reticulum (ER) contains one such system. The ER suppresses aggregation by accurately ensuring transcription and translation, chaperoning nascent or unfolded proteins, and discerning then transporting improperly folded polypeptides through a degradation pathway before they can aggregate (Ellgaard et al., 1999). Under conditions of stress, an adaptive mechanism that includes a set of coordinated signaling pathways termed the ER stress response or the unfolded protein response (UPR) is activated with the goal of returning the ER to its normal functioning state. In this review, we will examine key elements of the ER stress response, their age-related modifications, the effects of prolonged ER stress and the role of the ER stress response in several pathological disorders, many of which have implications for aging.

PROTEIN FOLDING AND QUALITY CONTROL

In general, protein folding is a staggeringly inefficient process where some 30% of the proteins never acquire their fully folded conformation (Romisch, 2004). The ER is a membrane bound

compartment and the ER lumen is topologically equivalent to the extracellular space. Its environment is highly oxidizing, which makes it suitable for protein folding and maturation. In mammalian cells, protein folding occurs in three phases (Naidoo, 2011). First, co-translational and co-translocational folding transpires as proteins traverse the ER membrane. After the release of the completed polypeptide from the ribosome, post-translational folding takes place.

Folding in the ER is the limiting step in the biogenesis of most secretory and transmembrane proteins. Chaperones and folding enzymes are engaged in all three folding stages. Key chaperones and folding sensors in the ER include: glucose regulated proteins 78 (GRP78; also known as Immunoglobulin Binding protein – BiP) and 94 (GRP94), the lectins, calnexin and calreticulin, and the thiol-disulfide oxidoreductases, protein disulfide isomerase (PDI) and ERp57. These components reside in the ER at high concentrations and participate in all stages of folding and in quality control.

Aging reduces the efficacy of many of these chaperones and foldases. Stringent restrictions on quality control within the ER prevent incompletely assembled or improperly folded proteins from exiting the ER and being transported to the cytosol or to downstream organelles and terminal compartments. Several abnormalities, including exposure of hydrophobic regions, unpaired cysteines and the tendency to form aggregates, will result in recognition of nascent proteins by the chaperones and folding sensors, which will cause their retention within the ER. Accumulation of misfolded proteins triggers ER stress and the UPR (Berridge, 2002).

THE ER STRESS RESPONSE

Perturbations that alter ER homeostasis disrupt protein folding and lead to the accumulation of unfolded proteins and/ aggregates that trigger ER stress. ER stress can be provoked by a variety of physiological conditions, including perturbations in calcium homeostasis, glucose/energy deprivation, redox changes, ischemia, hyperhomocystinemia, and viral infections and mutations that impair client protein folding (Kaufman, 2002; Ron, 2002). As a consequence, the cell has evolved an adaptive coordinated response to limit the accumulation of unfolded proteins in the ER. These signaling pathways are collectively termed the ER stress response or the UPR [for more detailed reviews see (Zhang et al., 2001; Harding et al., 2002; Schroder and Kaufman, 2005; Walter and Ron, 2011)]. On a cellular level, the UPR triggers three kinds of protective cellular responses (see **Figure 1** schematic): (i) up-regulation of ER chaperones such as BiP/GRP78 to assist in the refolding of proteins; (ii) attenuation of protein translation which is mediated by the serine-threonine kinase PERK which phosphorylates the initiation factor—eIF2α thereby reducing translation; and (iii) degradation of misfolded proteins by the proteasome by a process called ER associated degradation (ERAD). PERK activation also upregulates an antioxidant response through NF-E2-related factor 2 (Nrf2) (Johnson et al., 2008; Brown and Naidoo, 2010) (**Figure 1**). Nrf2 which belongs to the cap "n" collar (CNC) subfamily of basic leucine zipper transcription factors is known to play a significant role in the adaptive stress response to oxidative stress (Venugopal and Jaiswal, 1996; Itoh et al., 1999; He et al., 2001) and xenobiotic detoxification (Motohashi and Yamamoto, 2004). The three UPR responses are protective measures to limit protein load and alleviate ER stress; however, excessive and/prolonged stress leads to a maladaptive response and apoptosis (Szegezdi et al., 2006).

THE ER STRESS RESPONSE SIGNAL IS TRANSDUCED BY 3 PROXIMAL SENSORS

ER stress signals are transduced across the ER membrane by three proximal sensors of the UPR, inositol requiring element-1 (IRE-1), PKR like ER kinase (PERK) and activating transcription factor 6 (ATF6). All three of these sensors are maintained in an inactive state at the ER membrane by binding to the ER chaperone BiP (Immunoglobulin binding protein).

BiP is a peptide-dependent ATPase and member of the heat shock 70 protein family that binds transiently to newly synthesized proteins translocated into the ER, and more permanently to underglycosylated, misfolded, or unassembled proteins. BiP is also known as GRP78 and under the most recent nomenclature is known as HSP5A (Heat shock protein 5A). Under non-stress conditions BiP is bound to the three sensors ATF6, IRE1 and PERK, preventing them from activating downstream events. Upon accumulation of unfolded/ misfolded proteins, bound BiP dissociates from ATF6, IRE1 and PERK to chaperone the misfolded proteins thereby permitting the activation of one or more of these transducers (Zhang and Kaufman, 2006).

PERK ACTIVATION LEADS TO THE ATTENUATION OF PROTEIN TRANSLATION

PERK is a type I transmembrane serine threonine kinase that appears to be present in most cells. It is held in an inactive monomeric state by binding to BiP. When this binding is disrupted, PERK homodimerizes and phosphorylates itself, becomes active and initiates its eIF2α kinase activity. Phosphorylation of the translation initiation factor, eIF2α, results in the formation of a stalled 43S ternary complex that causes a general decrease in translation of most proteins. However, some selected proteins with internal ribosomal entry sites (IRES), such as ATF4 and BiP, are translated more efficiently (Harding et al., 2000) and hence their protein levels actually increase.

ACTIVATION OF IRE-1 LEADS TO SPLICING OF XBP1

Once activated the cytoplasmic domain of IRE1α gains endoribonuclease activity and excises an intron from the mRNA encoding an UPR-specific transcription factor, X-box binding protein (XBP) 1, generating a spliced variant XBP1s. Spliced XBP1 functions as a potent transcriptional transactivator of genes involved in ER expansion, protein maturation, folding and export from the ER, as well as export and degradation of misfolded proteins (Yoshida et al., 2001; Calfon et al., 2002; Lee et al., 2002, 2003; Yoshida et al., 2003). ER-bound mRNAs are also degraded in an IRE1 dependent manner via a process called RIDD ("regulated IRE1-dependent decay") and may serve to limit protein influx and unfolded protein load into the ER lumen after prolonged UPR induction (Hollien and Weissman, 2006; Pirot et al., 2007; Walter and Ron, 2011). Recent studies indicate an

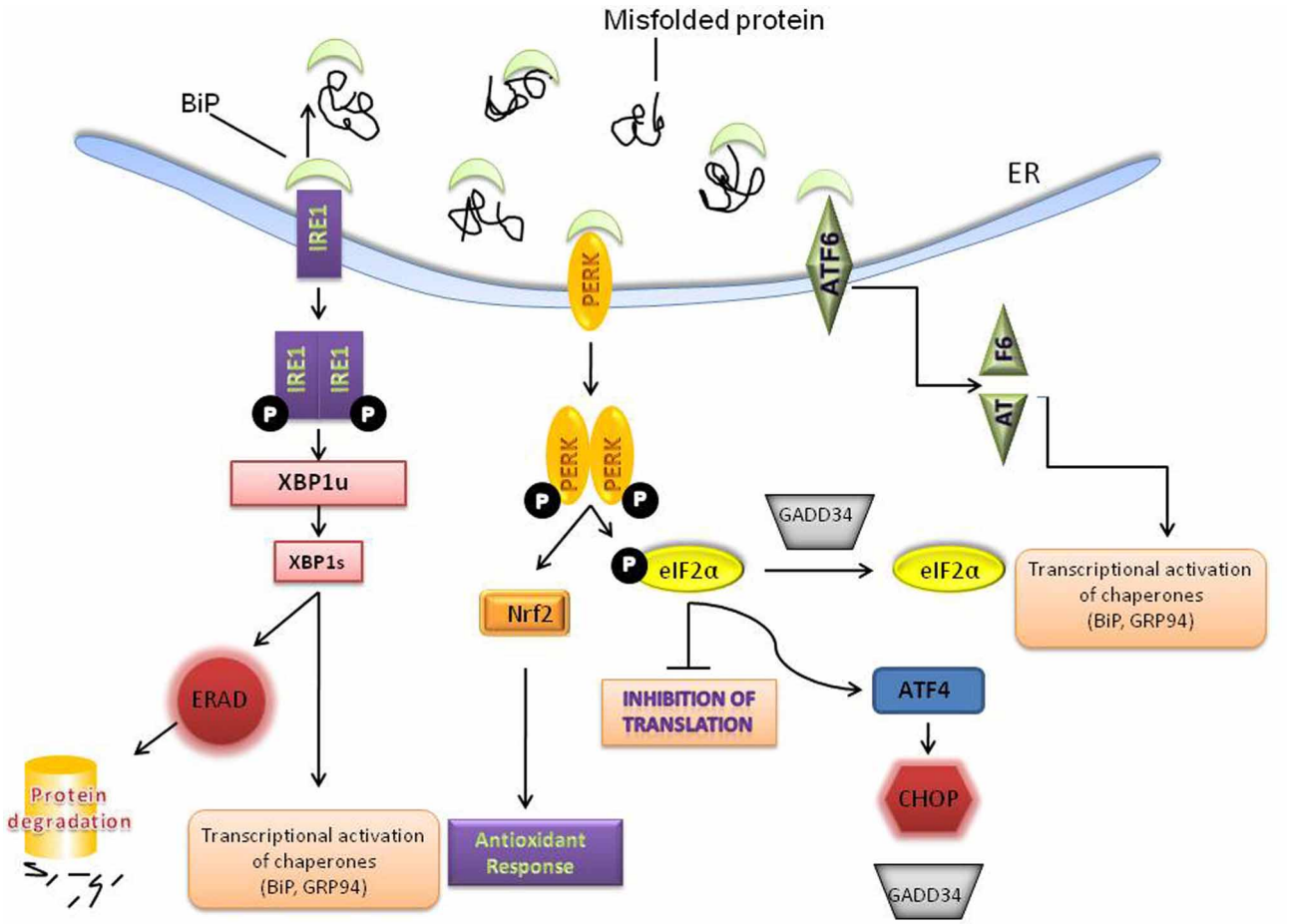

FIGURE 1 | Activation of the unfolded protein response (UPR). Accumulation of misfolded or unfolded proteins in the ER leads to the dissociation of BiP from 3 transducers –PERK, IRE1 and ATF6. PERK homodimerizes and phosphorylates eIF2α to inhibit general protein translation. PERK also regulates several transcription factors including, NRF-2 to up-regulate the anti-oxidant response and ATF4 which can lead to both protective and apoptotic signaling. IRE-1 activation results in the unconventional splicing of XBP-1, which induces the transcription of several molecular chaperones, such as BiP and GRP94 and stimulates protein degradation via ER-associated degradation (ERAD). ATF6 is activated and cleaved and leads to induction of molecular chaperones. The various ER chaperones are part of a protective adaptive response that regulates protein folding and other components of the UPR.

alternative model where IRE1 binds to unfolded proteins directly and that these serve as activating ligands (Walter and Ron, 2011).

ACTIVATION OF ATF6 INCREASES THE TRANSCRIPTION OF ER CHAPERONES

ATF6 is a 90-kDa bZIP protein that is activated by posttranslational modifications. ATF6 activation as part of the UPR leads to its translocation to the Golgi and cleavage by site-1 protease (S1P) and S2P. The 50-kDa cleaved ATF6α translocates to the cell nucleus, where it binds to the ER stress response element CCAAT(N)9CCACG (Yoshida et al., 1998) in genes encoding ER chaperone proteins such as BiP and GRP94. GRP94 is a member of the heat shock90 family of chaperones. This binding results in increases in the level of these proteins and hence increased protein folding activity in the ER (Yoshida et al., 1998; Okada et al., 2002). Other important targets regulated by ATF6 include XBP-1, CHOP, HERP (hyperhomocysteinemia-induced ER stress responsive protein) and PDI (Protein disulfide isomerase).

SUSTAINED ER STRESS LEADS TO AN INFLAMMATORY RESPONSE AND APOPTOSIS

Prolonged ER stress leads to inflammatory signaling while unmitigated and excessive stress leads to apoptosis (Szegezdi et al., 2006; Ron and Walter, 2007). Apoptosis in response to ER stress is specific to metazoan cells (Schroder and Kaufman, 2005). When cell protective changes mediated by the UPR fail to restore folding capacity, a combination of both the intrinsic and extrinsic apoptotic pathways are activated (Schroder and Kaufman, 2005). Apoptosis in response to ER stress is mediated largely by C/EBP homologous protein (CHOP) also known as growth arrest and DNA damage 153 (GADD 153). CHOP which is downstream of the PERK and ATF6 pathways induces the expression of a number of pro-apoptotic factors including Tribbles 3, GADD34

and DR5. Bcl-2 family members (Bak/Bax), caspase-12 and c-jun NH2 terminal kinase (JNK) are other components of the ER stress mediated apoptotic pathway (Wu and Kaufman, 2006) (**Figure 2**).

JNK activation, which occurs through IRE1 dependent signaling, induces the expression of inflammatory genes by phosphorylation of AP1 (transcription activator protein 1) (Davis, 2000; Zhang and Kaufman, 2008). NF-kappa B (NF-κB) dependent transcription is increased two ways during ER stress. First, I kappa K (IKK) which has a shorter half life is reduced when protein translation is attenuated thereby changing the stoichiometric ratio of NF-κB: IKK and freeing NF-κB to translocate to the nucleus (Zhang and Kaufman, 2008). Secondly, the IRE1-TRF2 complex recruits I kappa B (IKB) kinase that phosphorylates IKB and leads to its degradation (Hu et al., 2006).

AGE-RELATED CHANGES IN THE ER STRESS RESPONSE

UPR COMPONENTS DECLINE WITH AGE

During aging, there is a shift in the balance between the protective adaptive response of the UPR and pro-apoptotic signaling; where the protective arm is significantly reduced and the apoptotic arm is more robust (Paz Gavilan et al., 2006; Hussain and Ramaiah, 2007; Naidoo et al., 2008). Key ER resident chaperones and enzymes within the ER such as BiP, PDI, calnexin and GRP94, which are required for proper protein folding, are impaired during the aging process. Chaperones are progressively oxidized with age and this process may contribute to their functional decline. These alterations in oxidation significantly correlate with reductions in enzymatic activity of several chaperones (Nuss et al., 2008).

BiP expression levels are significantly reduced in several species with age. In the cerebral cortex of aged (22–24-month old)

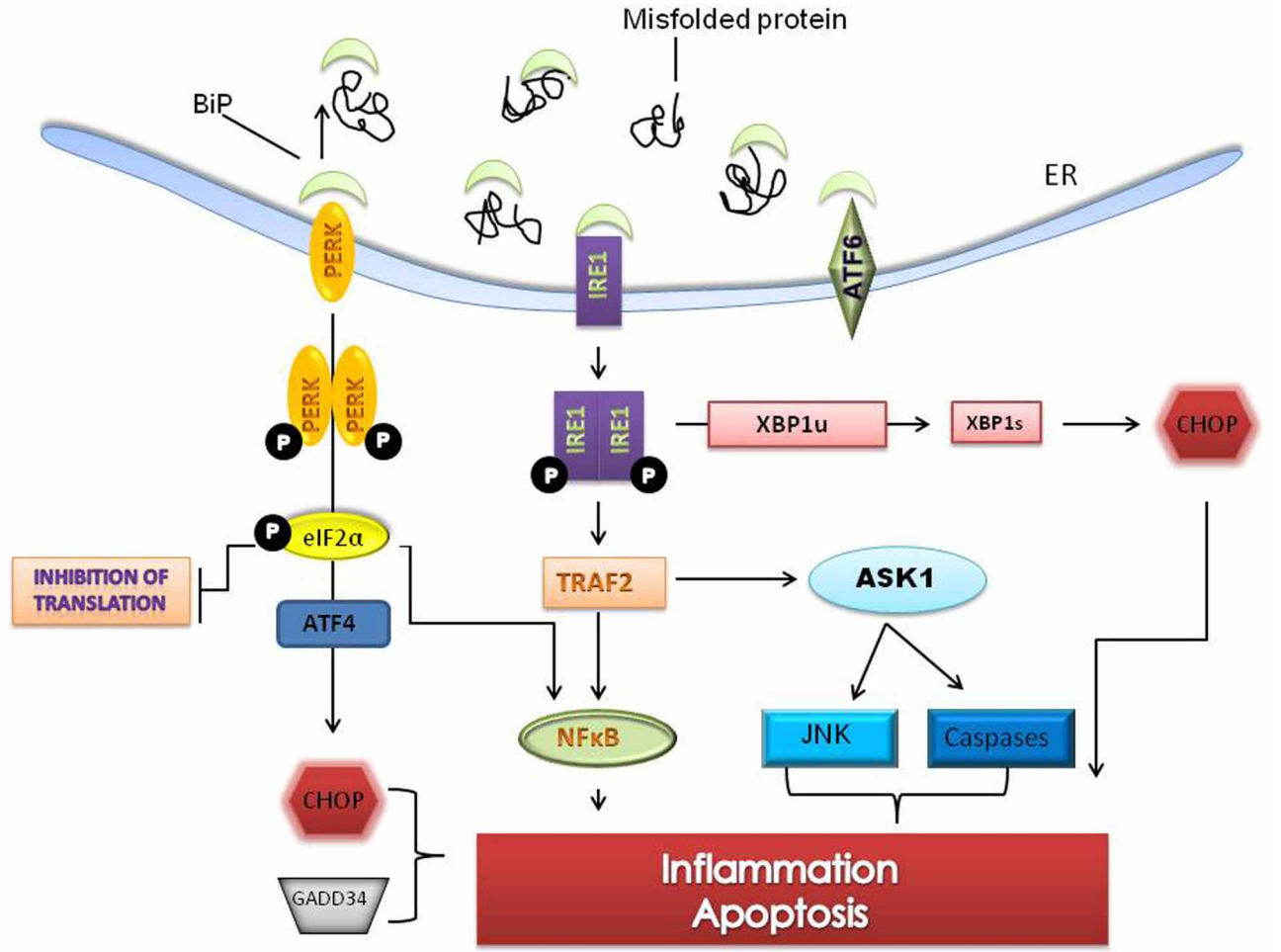

FIGURE 2 | Sustained ER stress leads to pro-apoptotic signaling and cell death. Unresolved ER stress leads to inflammation and cell death pathways involving the PERK and IRE1 branches of the UPR. The IRE1-TRAF2- apoptosis signaling kinase 1 (ASK1) complex upregulates c-jun NH2 terminal kinase (JNK) and caspases and through splicing of XBP1 can activate C/EBP homologous protein (CHOP), a pro-apoptotic transcription factor. PERK signaling, through phosphorylation of eIF2α, can activate ATF4 dependent transcription resulting in activation of nuclear factor-kappaB (NFκB) and increases in CHOP. CHOP and ER specific caspases are thought to directly induce cell death. Several genes that mediate apoptotic function and inflammation are induced by prolonged ER stress.

C57/B6 mice compared with that in young (3-month old) mice, BiP protein levels were decreased 30% (Naidoo et al., 2008). BiP mRNA and protein expression levels are also decreased in the hippocampus of aged (23–26-month old) versus young (4–6-month old) Wistar rats (Paz Gavilan et al., 2006). A study that examined BiP protein expression in the brain (cortex and cerebellum) and in peripheral tissue (lung, liver, kidney, heart, and spleen) of Wistar rats across their life span found that expression of BiP was higher in the young tissue in comparison to the aged tissue (Hussain and Ramaiah, 2007).

Age modifies other components of the UPR in addition to the chaperones and enzymes. PERK mRNA was significantly reduced in the hippocampus of aged rats (Paz Gavilan et al., 2006). Another study reported that the activity of PKR (double stranded RNA-dependent kinase), an eIF2α kinase, was less efficient when isolated from aged rat brain tissue (Hussain and Ramaiah, 2007) than similar tissue isolated from young rats. Accompanying the declines in PERK signaling are increases in GADD34, which removes the translational block imposed by eIF2α phosphorylation. Increases in the expression of GADD34 were found in the cortical tissue of aged mice. Suppression of the translational block by GADD34 allows for the synthesis of pro-apoptotic proteins like CHOP.

Both basal and inducible CHOP expression levels are elevated with age (Kirkland et al., 2002; Ikeyama et al., 2003). Expression of CHOP and caspase-12, another pro-apoptotic molecule, was induced in aged rats that were stressed, but not in the young stressed animals (Paz Gavilan et al., 2006), lending support to the idea that the aged animals are more vulnerable to apoptosis. Studies from our laboratory demonstrate the upregulation of CHOP in aged mouse cortex (Naidoo et al., 2008) as others have shown in aged rat hippocampus (Paz Gavilan et al., 2006) and in aged rat cortex (Hussain and Ramaiah, 2007).

JNK kinases are also upregulated during aging. They are signal transduction proteins that regulate gene expression through the phosphorylation of transcription factors such as c-Jun and ATF-2. JNK is also activated by the kinase domain of IRE1 through the TNF receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK1) (Ichijo et al., 1997; Szegezdi et al., 2006), which contributes to the induction of apoptosis.

STRUCTURAL CHANGES

There are also structural changes in the ER with age. Hinds and McNelly, have demonstrated that the highly ordered parallel cisternae of rough ER characteristic of young neurons seems to become dispersed during aging (Hinds and McNelly, 2005). They have quantitatively measured this dispersion of the aging, rough ER cisternae in both mitral cells in the olfactory bulb and in Purkinje cells and found that there is a highly linear decrease in the measured closeness of rough ER cisternae throughout adult life. The age-related declines of important UPR chaperones, enzymes and ER structure significantly affect the efficiency of managing proper protein folding and ultimately ER homeostasis.

AUTOPHAGY

The UPR activates autophagy in order to remove aggregates of misfolded proteins that cannot be degraded by the ERAD pathway (Ogata et al., 2006). Evidence suggests that autophagy can provide neuroprotection by enhancing clearance of these aggregates. Growing evidence indicates that autophagy also declines with age; the rate of autophagosome formation and maturation and the efficiency of autophagosome/lysosome fusion are reduced (Rajawat and Bossis, 2008). Dysregulation of the autophagic process may lead to neurodegeneration (Nedelsky et al., 2008). There are several excellent reviews that explore the relationship between ER stress, the UPR, autophagy and neurodegenerative disorders (Matus et al., 2008; Doyle et al., 2011).

THE ER STRESS RESPONSE IN AGE-RELATED DISEASES

METABOLIC DISORDERS AND TYPE 2 DIABETES

The ER, which regulates protein synthesis and secretion as well as triglyceride and cholesterol biosynthesis and controls cellular metabolism, has been postulated to be a site for sensing metabolic stress. Recent data from experimental models indicate that ER stress is critical to the initiation and integration of pathways of inflammation and insulin action in metabolic syndrome (Hotamisligil, 2006). Metabolic syndrome which encompasses insulin resistance, reduced glucose utilization and type 2 diabetes are regulated by numerous mechanisms, including the UPR, JNK activation, NF-κB activation and apoptosis (Hotamisligil, 2006).

The two principal inflammatory pathways that disrupt insulin action, JNK–AP-1 and IKK–NF-κB, are linked to IRE-1 and PERK activity during ER stress (Deng et al., 2004). IRE-1 is linked to activation of JNK through a pathway involving TNF-receptor-associated factor 2 (Urano et al., 2000). Activation of both IRE-1 and PERK is also linked to the IKK–NF-κB pathway, through distinct mechanisms as described in the section above.

Insulin-receptor substrate 1 (IRS1), which transmits the effects of insulin through interactions with other cytosolic molecules is phosphorylated by activated insulin receptors on tyrosine residues. It is thought that JNK-mediated phosphorylation of serine residues in IRS1 inhibits the phosphorylation of IRS1 on tyrosine residues and leads to insulin resistance (Aguirre et al., 2002; Zhang and Kaufman, 2008).

SLEEP

It is becoming increasingly evident that sleep disruption leads to the ER stress response. Several studies have shown that acute sleep deprivation induces the up-regulation of BiP/GRP78 in the brains of mice (Naidoo et al., 2005; Mackiewicz et al., 2007), rats (Cirelli et al., 2004), birds (Jones et al., 2008) and fruitflies (Shaw et al., 2000; Naidoo et al., 2007). We have demonstrated that the PERK pathway is activated with six or more hours of sleep loss in the cerebral cortex of mouse brain (Naidoo et al., 2005). PERK cerebellar transcript levels were also found to be higher in wakefulness than in sleep (Cirelli et al., 2004). Other UPR specific transcripts that change with sleep deprivation include DNA-J which is a co-chaperone of BiP, XBP-1, calreticulin, caspase-9, ATF4 and ATF6.

Not only is the ER stress response activated with sleep loss it appears to be involved in the sleep homeostatic response. In Drosophila there is an increase in BiP with sleep loss and a diminution of expression with recovery sleep (Naidoo et al., 2007). BiP protein levels return to baseline levels over 24 h with

recovery sleep following an almost 3-fold increase with 6 h of sleep deprivation. Over expression of BiP through genetic means leads to an increase in the amount of sleep recovered after sleep loss. Whether the altered amounts of recovery sleep when BiP levels are manipulated is due to BiP itself or more indirectly through other effects on the UPR remains to be determined.

Changes with age

Aged animals exhibit more fragmented sleep (Welsh et al., 1986; Shiromani et al., 2000; Naidoo et al., 2008) and display basal levels of ER stress in tissues examined (Naidoo et al., 2011). The adaptive ER stress response to sleep deprivation is impaired in aged mice cerebral cortices (Naidoo et al., 2008). There is little evidence of BiP up-regulation and attenuation of protein translation. Orexin and noradrenergic neurons in aged mice display considerable ER stress with activation of the PERK pathway when compared with similar regions in young mice (Naidoo et al., 2011). Surprisingly, recovery sleep following sleep deprivation is less in older animals than young. This has been shown in humans (Bonnet, 1985; Carskadon and Dement, 1987) and in rats (Mendelson and Bergmann, 2000; Shiromani et al., 2000). It is not known whether the UPR plays a role in the mammalian recovery sleep response to sleep deprivation and is currently being investigated.

NEURODEGENERATIVE DISEASES

ER stress and activation of the UPR has been implicated in abnormal protein processing and neuronal death in age-associated diseases, which subsequently play a role in the pathogenesis of neurodegenerative diseases (see reviews Forman et al., 2003; Johnson et al., 2008). These diseases, which include Alzheimer's disease, Parkinson's disease, ALS and Huntington's disease, normally appear later in life and are thus associated with the aging process. Neuronal loss in both familial and sporadic forms of neurodegenerative disorders is often accompanied by aggregation of misfolded proteins (Selkoe, 2003). Studies have suggested that initial participation of the UPR in neurodegenerative disorders is probably cytoprotective, however, when activation of the UPR is sustained over an extended period of time, apoptotic pathways are upregulated. Accumulation of misfolded proteins that lead to alterations in organelle structure including the ER has been described in transgenic models of ALS, Alzheimer's and Huntington's disease (Reddy et al., 1999; Rao et al., 2002). Like many other signaling pathways, the UPR suffers from age-related impairments and becomes less effective (Naidoo et al., 2008).

Although many studies found activation of the UPR in neurodegenerative diseases, the studies are conflicting on whether there is induction of BiP. One study shows that BiP, along with other UPR markers were increased in the neurons of AD patients versus the non-demented controlled individuals (Hoozemans et al., 2005). Another study, on the other hand, found activation of the UPR, as measured by analyzing XBP-1 mRNA splicing along with activation of pro-apoptotic factors such as CHOP and caspases-3, 4, and 12; however, these authors show no induction of BiP expression (Lee et al., 2010). The conflict in each of these reports could be marked differences in the basal levels of BiP.

Lee et al. showed that UPR activation could be separated from amyloid β (Aβ) burden in the Tg2576 mice. Levels of PDI and BiP were similar in the cortex of aged Tg2576 and wild type mice (Lee et al., 2010). The authors suggest that Aβ burden alone is insufficient for induction of the UPR. This too is controversial, and rather ambiguous, since others have found that amyloid beta (Lee do et al., 2010; Costa et al., 2011), oligomeric not fibrillar, induces ER stress (Chafekar et al., 2007). A Drosophila model showed that neurotoxicity of tau was significantly increased when levels of XBP1 were reduced (Loewen and Feany, 2010). An earlier study, however, found that in an ALS murine model expressing mutant superoxide dismutase, removing XBP1 was neuroprotective, presumably through activation of an autophagic response (Hetz et al., 2009). P-PERK and P-IRE1 were found to be upregulated in response to early tau pathology (Nijholt et al., 2012).

Involvement of the UPR in Parkinson's disease (PD) has been described primarily in cellular models using drugs that mimic certain aspects of PD (Ryu et al., 2002; Smith et al., 2005). Parkin, an ubiquitin-protein ligase, is up-regulated in response to unfolded protein stress and suppresses cell death via its E3 activity. Loss of parkin function results in the misfolding and accumulation of PAEL-R, a substrate of parkin in the ER of substantia nigra neurons, leading to ER stress and cell death (Imai et al., 2001). This process has been proposed to be responsible for neuronal cell death in autosomal recessive juvenile parkinsonism and it suggests a physiological role of Parkin in dealing with ER stress (Imai et al., 2001). In addition, overexpression of wild-type or mutant α-synuclein induces UPR activation in yeast (Cooper et al., 2006). A study performed by Hoozemans and colleagues demonstrated activation of the PERK–eIF2a pathway in dopaminergic neurons in the substantia nigra of PD cases (Hoozemans et al., 2007). These varied studies suggest that targeting the UPR, whether by activation or inhibition, may provide opportunities for therapeutic intervention in several neurodegenerative disorders.

ATHEROSCLEROSIS AND HYPERHOMOCYSTEINEMIA

Evidence suggests that ER stress and the UPR can mediate the pathogenesis of atherosclerosis and vascular inflammation (Zhang and Kaufman, 2008; Santos et al., 2009). Atherosclerosis is a progressive disease of the large arteries where lipids and proteins derived from circulating low-density lipoproteins (LDL) accumulate within the cells and in the extracellular space (Ursini et al., 2002). There are a variety of methods in which ER function may influence the development of atherosclerosis. First, many critical lipid biosynthetic pathways are located in the ER (Gregor and Hotamisligil, 2007). Secondly, ER stress has been found to drive free-cholesterol-induced apoptosis in macrophages in a model of cellular free-cholesterol loading (Tabas, 2009). This suggests that the ER may sense stress related to lipid status and exposure, resulting in the passage of information to signaling pathways involved in inflammation and death in cells that are key in the development of atherosclerosis (Tabas, 2009). Finally, the UPR may have an important function in adaptive immunity. Substantial evidence indicates that autoimmune events mediated by ER stress may contribute to atherosclerosis (for a more detailed commentary on this subject see G. S. Hotamisligil, Nature Medicine, 2010) (Hotamisligil, 2010a,b).

Hyperhomocysteinemia (HHcy) is a common, independent risk factor for the development of cardiovascular disease. Epidemiological studies have found associations between high levels of serum homocysteine and the development of ischemic heart disease and stroke (Eikelboom et al., 1999); however, whether homocysteine is the underlying cause of atherosclerosis and thrombosis is not known. It was reported in cultured vascular endothelial cells that homocysteine induced protein misfolding in the ER by interfering with disulfide bond formation. This leads to activation of the UPR as the induction of several ER stress response proteins, such as BiP, GRP94, CHOP and HERP were found (Outinen et al., 1999; Huang et al., 2001). Homocysteine was also shown to activate apoptosis in an IRE1-dependent manner (Zhang et al., 2001). HHcy activates cleavage of the sterol regulatory element binding protein (SREBP), which leads to intracellular accumulation of cholesterol (Ron, 2001). Overexpression of BiP, which attenuates ER stress, suppresses this activation, implicating the UPR in the process (Kammoun et al., 2009; Basseri and Austin, 2012). The role of ER stress in atherosclerosis and other forms of cardiovascular disease likely involves an integrated network of multiple signaling pathways, including, but not limited to inflammation and lipid metabolism.

THE ROLE OF THE UPR IN CANCER

Cancer rates increase sharply with age in both sexes, with the majority of cases occurring in patients over the age of 65 (Burkle et al., 2007). Recent studies in the cancer field have clearly demonstrated that ER stress and the UPR are robustly upregulated in various tumor types and are closely associated with cancer cell survival and their resistance to anti-cancer treatments (Wang et al., 2010). Researchers are now examining what markers of the UPR pathway are either induced or suppressed in malignancy in the hope of targeting these specific components for treatment. Tumor formation results in the high proliferation of cancer cells. During this process, a burden is placed on the ER that requires increased activities of protein folding, assembly and transport leading to physiological ER stress (Lee, 2007). As the tumor increases, the cancer cells are exposed to nutrient deprivation and hypoxic conditions; which are well known inducers for the accumulation and aggregation of unfolded and/or misfolded proteins in the ER, resulting in activation of the UPR pathways (Lee, 2007; Luo et al., 2009).

Evidence suggest that the adaptive arm of the UPR provides survival signaling pathways that are conducive to the growth of tumors, while suppressing the apoptotic arm of the UPR that would contribute to cell death and normal growth. Cancer cells may evade the apoptotic pathways by differentially activating the UPR branches (Pyrko et al., 2007; Hersey and Zhang, 2008). BiP has been shown to be upregulated in several different types of cancers (Zhang and Zhang, 2010) and is implicated in playing a critical cytoprotective role in oncogenesis (Li and Lee, 2006; Healy et al., 2009). BiP expression levels have been positively correlated with cerebral tumor malignancy, i.e., the higher the BiP levels, the more malignant the tumor (Zhang and Zhang, 2010). Previous reports found that BiP afforded protection against a variety of chemotherapeutic drugs that included: adriamycin, etoposide, 5-FU and temozolomide (Reddy et al., 2003; Fu et al., 2007; Lee, 2007; Pyrko et al., 2007). Later studies found that BiP was able to confer chemoresistance to tumor-associated endothelial cells (Virrey et al., 2008).

It has been reported by several groups that knocking down or interfering with BiP function, sensitizes tumors to treatment. Wang et al found that (-)-epigallocatechin gallate (EGCG), a natural inhibitor of BiP that targets its ATP-binding domain, sensitizes breast cancer cells to taxol and vinblastine, two broad cytotoxic drugs (Wang et al., 2009). Another study found that down-regulation of BiP by small interfering RNA decelerates glioma cell growth (Pyrko et al., 2007).

Other components of the UPR are also activated in cancer. When the spliced variant of XBP1 was expressed in IRE1α dominant-negative expressing cells angiogenesis was restored (Romero-Ramirez et al., 2009), suggesting that signaling through the IRE1α-XBP1 arm of the UPR is essential for angiogenesis in the early stage of tumor development.

The PERK-eIF2α branch of the UPR has been shown to protect cells under conditions of hypoxia; a feature common to solid tumors that is the result of increased demands on energy requirements due to dysregulated cell growth (Wouters and Koritzinsky, 2008). Hypoxic conditions can induce PERK, leading to phosphorylation of eIF2α in tumor cells (Koumenis et al., 2002; Fels and Koumenis, 2006). Evidence supporting the role of the UPR in cancer offers some interesting therapeutic alternatives for cancer treatment. Approaches to induce and/or prevent UPR activation in oncogenesis could have implications for improving therapeutic outcomes.

OTHER AGE-RELATED INFLAMMATORY DISEASES

Besides the well known age–related diseases and disorders discussed above, ER stress has been implicated in several chronic diseases involving inflammation (Hotamisligil, 2010a,b). These include neuromuscular inflammatory diseases, arthritis and spondyloarthropathies, multiple forms of respiratory inflammation and inflammatory bowel diseases (Hybiske et al., 2007; Mhaille et al., 2008; Colbert et al., 2010; McGuckin et al., 2010). In many of these diseases, it is not yet clear whether ER stress is a primary contributor to the disease or a consequence of the condition. It has been suggested that, in some circumstances, ER stress can initiate disease but that inflammation, in some cases owing to infection, is an important exacerbator of ER stress and can be the trigger for the onset of disease in a genetically susceptible individual [for more information see review by (Hasnain et al., 2012)]. Increasing evidence links ER stress to inflammatory bowel disease. Secretory epithelial cells that produce antimicrobial molecules and the mucus barrier, which separate the epithelium from the luminal microbes, are very vulnerable to ER stress (Hasnain et al., 2012). These cells produce the high molecular weight cysteine rich mucins that are prone to misfolding. Genetic studies in rodent models indicate that deficiencies in the UPR pathway lead to spontaneous intestinal inflammation (Heazlewood et al., 2008).

CONCLUDING REMARKS

The ER stress response comprises a complex set of signaling pathways that serves to maintain ER and protein homeostasis

in response to genetic, host (hypoxia, ATP, calcium alterations), microbial and inflammatory stressors. With age, many of the components of the UPR decline and become less efficient thus dampening the response to these stressors. This results in or exacerbates existing diseases. Therapeutics that reduce ER stress by promoting correct folding of proteins, improving the efficiency of ERAD and/or enhancing the detection of misfolded proteins may prove useful in delaying or preventing some of the age-related diseases and disorders discussed in this review. Also, recognizing perturbations in the ER stress response could lead to early detection of a number of age-related pathologies and the development of therapeutics which maintain a normal ER stress response later in life. This represents a hitherto unexplored avenue to disease prevention.

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Integration of peroxisomes into an endomembrane system that governs cellular aging

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The peroxisome is an organelle that has long been known for its essential roles in oxidation of fatty acids, maintenance of reactive oxygen species (ROS) homeostasis and anaplerotic replenishment of tricarboxylic acid (TCA) cycle intermediates destined for mitochondria. Growing evidence supports the view that these peroxisome-confined metabolic processes play an essential role in defining the replicative and chronological age of a eukaryotic cell. Much progress has recently been made in defining molecular mechanisms that link cellular aging to fatty acid oxidation, ROS turnover, and anaplerotic metabolism in peroxisomes. Emergent studies have revealed that these organelles not only house longevity-defining metabolic reactions but can also regulate cellular aging via their dynamic communication with other cellular compartments. Peroxisomes communicate with other organelles by establishing extensive physical contact with lipid bodies, maintaining an endoplasmic reticulum (ER) to peroxisome connectivity system, exchanging certain metabolites, and being involved in the bidirectional flow of some of their protein and lipid constituents. The scope of this review is to summarize the evidence that peroxisomes are dynamically integrated into an endomembrane system that governs cellular aging. We discuss recent progress in understanding how communications between peroxisomes and other cellular compartments within this system influence the development of a pro- or anti-aging cellular pattern. We also propose a model for the integration of peroxisomes into the endomembrane system governing cellular aging and critically evaluate several molecular mechanisms underlying such integration.

Keywords: peroxisome, cellular aging, interorganellar communication, sirtuins, senescence factors, organelle inheritance, proteostasis, autophagy

INTRODUCTION

A growing body of evidence implies that, in addition to the well known roles of the peroxisome in housing fatty acid oxidation and maintaining hydrogen peroxide homeostasis (Poirier et al., 2006; Wanders and Waterham, 2006; Schlüter et al., 2010), this organelle is actively involved in organizing the processes of development, differentiation, and morphogenesis in evolutionarily distant organisms. In mammalian and plant cells, the rate of fatty acid metabolism and the efficiency of reactive oxygen species (ROS) and reactive nitrogen species (RNS) turnover within the peroxisome define the dynamics of changes in the levels of signaling lipids, ROS, and RNS outside this organelle (Desvergne and Wahli, 1999; Corpas et al., 2001; del Río et al., 2006; Nyathi and Baker, 2006). Following their release from the peroxisome, these signaling molecules bind and activate a distinct set of transcription factors that respond by causing global changes in gene expression to initiate certain developmental and differentiation programs (Kersten et al., 2000; Desikan et al., 2001; Hu et al., 2002; Ma et al., 2002; Michalik et al., 2002; Baker et al., 2006; del Río et al., 2006; Michalik and Wahli, 2006; Nyathi and Baker, 2006; Bonekamp et al., 2009; Antonenkov et al., 2010; Ivashchenko et al., 2011; Li et al., 2011; Neher et al., 2012). Thus, the peroxisome functions as an intracellular signaling compartment that can orchestrate important developmental decisions from inside the cell by modulating the extra-peroxisomal concentrations of several potent cellular messengers (Titorenko and Rachubinski, 2004; Terlecky and Titorenko, 2009; Thoms et al., 2009; Dixit et al., 2010). Furthermore, the peroxisome can operate as an organizing platform for several developmental and differentiation programs by compartmentalizing the initial steps of plasmalogen biosynthesis in mammalian and nematode cells, providing acetyl-CoA for the biosynthesis of melanin and glycerol in fungal cells, and carrying out the oxidative decomposition of very long-chain fatty acids, phytanic acid, and pristanic acid in mammalian cells (Powers and Moser, 1998; Motley et al., 2000; Thines et al., 2000; Gould et al., 2001; Kimura et al., 2001; Petriv et al., 2002; Wang et al., 2005; Asakura et al., 2006; Terlecky and Titorenko, 2009; Imazaki et al., 2010; Van Veldhoven, 2010; Goh et al., 2011; Mast et al., 2011; Bhadauria et al., 2012). Moreover, while the peroxisome-associated pools of several bifunctional proteins with dual subcellular localization operate in peroxisome biogenesis and function, their pools in other organelles organize certain processes of development, differentiation, and morphogenesis in mammalian, plant, and yeast cells (Titorenko et al., 1997; Titorenko and Rachubinski, 1998, 2004; Lin et al., 1999;

Footitt et al., 2002; Gavva et al., 2002; Geuze et al., 2003; Lin et al., 2004; Slabas et al., 2004; Karnik and Trelease, 2005; Ashibe et al., 2007; Freitag et al., 2012). In addition, the peroxisome provides a template for the formation of the Woronin body, a specialized subcellular compartment that in the filamentous fungi *Neurospora crassa* and *Aspergillus oryzae* is essential for a multistep process in cell morphogenesis initiated by physical damage to hyphae (Jedd and Chua, 2000; Tenney et al., 2000; Liu et al., 2008; Escaño et al., 2009; Jedd, 2011; Liu et al., 2011). In human cells, the peroxisome can also serve as an intracellular platform for the development of the human immunodeficiency virus and rotavirus (Cohen et al., 2000; Mohan et al., 2002).

Recent findings have broadened a spectrum of complex biological processes that depend on the functional integrity of the peroxisome. Emergent evidence supports the view that such peroxisome-confined metabolic processes as fatty acid oxidation, ROS turnover, and anaplerotic replenishment of tricarboxylic acid (TCA) cycle intermediates play essential roles in defining the replicative and chronological age of a eukaryotic cell (Titorenko and Terlecky, 2011). Peroxisomal fatty acid oxidation has been shown to regulate cellular aging because it operates as a system controller that modulates levels of non-esterified fatty acids and diacylglycerol by governing lipid dynamics in peroxisomes, lipid bodies, and the endoplasmic reticulum (ER) (Goldberg et al., 2009a,b; Titorenko and Terlecky, 2011); non-esterified fatty acids are known to accelerate the age-related necrotic and apoptotic cell death mechanisms, whereas the diacylglycerol-activated protein kinase C signaling sensitizes cells to age-related stresses (Spitaler and Cantrell, 2004; Low et al., 2005; Feng et al., 2007; Aksam et al., 2008; Jungwirth et al., 2008). Furthermore, peroxisomal fatty acid oxidation and anaplerotic reactions have been demonstrated to delay cellular aging by potentiating the mitochondrial retrograde (RTG) signaling pathway of longevity regulation (Chelstowska and Butow, 1995; Kos et al., 1995; Epstein et al., 2001; Traven et al., 2001; Jazwinski, 2005b; Liu and Butow, 2006; Titorenko and Terlecky, 2011; Jazwinski, 2012). Moreover, ROS homeostasis and the extent of macromolecular oxidative damage within the peroxisome govern several anti-aging processes confined to this organelle (Morita et al., 2000; Legakis et al., 2002; Aksam et al., 2007; Koepke et al., 2008; Aksam et al., 2009; Lingard et al., 2009; Mathur, 2009; Sinclair et al., 2009; Titorenko and Terlecky, 2011).

The peroxisome defines the replicative and chronological age of a eukaryotic cell not only by operating as a system controller that modulates levels of non-esterified fatty acids and diacylglycerol, replenishes TCA cycle intermediates destined for mitochondria, and contributes to the maintenance of peroxisomal ROS homeostasis and macromolecular oxidative damage. Recent studies have revealed that this organelle can also regulate cellular aging via its communication with other cellular compartments. This dynamic communication involves the establishment of extensive physical contact between peroxisomes and lipid bodies, maintenance of an ER to peroxisome connectivity system, exchange of certain metabolites between peroxisomes and other cellular compartments, and bidirectional flow of some protein and lipid constituents between peroxisomes and other organelles. In this review we summarize the evidence that peroxisomes are dynamically integrated into an endomembrane system that governs cellular aging. We discuss various strategies through which peroxisomes are integrated into this endomembrane system, critically evaluate the molecular mechanisms underlying each of these strategies, and analyze the age-related dynamics of communications between peroxisomes and other cellular compartments composing the longevity-defining endomembrane system. We also outline recent progress in understanding how communications between peroxisomes and other cellular compartments within this system influence the development of a pro- or anti-aging cellular pattern. Based on the available evidence, we propose a model for the integration of peroxisomes into the endomembrane system governing cellular aging.

A ROLE FOR CYTOSOL-TO-PEROXISOME TARGETING OF Pnc1p IN REGULATING YEAST LONGEVITY

A support for a distinctive mechanism that underlies the essential role of peroxisomes in regulating cellular aging comes from the observation that Pnc1p, a pyrazinamidase/nicotinamidase 1 that converts nicotinamide to nicotinic acid in the NAD^+ salvage pathway (Ghislain et al., 2002), is targeted from the cytosol to the peroxisome in response to CR and various mild stresses (Anderson et al., 2003). CR and all of these other "hormetic" stimuli—the term "hormesis" refers to a beneficial defense response of an organism to a low-intensity biological stress (Gems and Partridge, 2008; Rattan, 2008; Calabrese et al., 2011, 2012)—increase the lifespan of replicatively aging yeast in a Pnc1p-dependent manner (Anderson et al., 2003). Peroxisomal import of Pnc1p under conditions of such longevity-extending hormesis requires the peroxisomal targeting signal 2 (PTS2) shuttling receptor Pex7p and the peroxin Pex6p, but does not rely on the PTS1 receptor Pex5p (Anderson et al., 2003). Such specific peroxisomal targeting of Pnc1p, one of the key regulators of replicative aging in yeast (Lin and Sinclair, 2008), in response to their exposure to various anti-aging exogenous factors suggests that Pnc1p in the peroxisome could modulate some longevity-related processes confined to this organelle. What are these processes?

The established function of Pnc1p in the nucleus—an organelle to which this protein is also sorted from the cytosol in yeast exposed to CR and other hormetic stimuli (Anderson et al., 2003)—provides a useful hint on the nature of peroxisome-confined processes that could be modulated by Pnc1p under these longevity-extending conditions. In the nucleus, Pnc1p depletes the level of nicotinamide, a strong non-competitive inhibitor of the NAD^+-dependent protein deacetylase Sir2p required for lifespan extension in yeast under CR conditions (Bitterman et al., 2002). The resulting Pnc1p-driven activation of Sir2p delays replicative aging by suppressing recombination at the ribosomal DNA (rDNA) locus, thereby decreasing the efficiency of extrachromosomal rDNA circle (ERC) formation in the nucleolus (Lin and Sinclair, 2008). It should be stressed that two of the four members of the Sir2p family of NAD^+-dependent protein deacetylases (*i.e.*, sirtuins) in yeast—called Hst3p and Hst4p for being Homologs of SIR Two proteins—drive the metabolism of fatty acids by activating acyl-CoA synthetases for their short-chain species (Starai et al., 2003). By converting

short-chain fatty acids into their corresponding acyl-CoA forms, acyl-CoA synthetases enable their cellular and intracellular transport and metabolism (Starai et al., 2003). It has been proposed that both Hst3p and Hst4p activate these acyl-CoA synthetases by deacetylating them and cleaving NAD^+ in each reaction cycle (Starai et al., 2003). Because sirtuins are also known for their NAD^+-dependent ADP-ribosylation activity (Haigis and Guarente, 2006; Haigis and Sinclair, 2010), a possibility that Hst3p and Hst4p activate acyl-CoA synthetases for short-chain fatty acids in ADP-ribosylation reactions is also feasible. Altogether, these findings suggest the following hypothesis for a role of cytosol-to-peroxisome targeting of Pnc1p in regulating longevity of replicatively aging yeast (**Figure 1**). In response to their exposure to CR and other hormetic anti-aging stimuli, yeast cells target Pnc1p not only to the nucleus but also to the peroxisome. Following its PTS2- and Pex7p-dependent import into the peroxisome, Pnc1p depletes the level of nicotinamide, a strong non-competitive inhibitor of Hst3p and Hst4p. As a co-substrate in protein deacetylation and/or ADP-ribosylation reactions, each of these sirtuins could use NAD^+ known to be generated by the peroxisomal malate dehydrogenase Mdh3p (Kunze et al., 2006). The Pnc1p-dependent depletion of nicotinamide activates Hst3p and Hst4p; in turn, these sirtuins stimulate acyl-CoA synthetases required for peroxisomal transport and oxidation of short-chain fatty acids (**Figure 1**). We hypothesize that, by depleting the levels of these fatty acids in the cytosol and/or oxidizing them, peroxisomes make an important contribution to the longevity-extending effect of CR and other

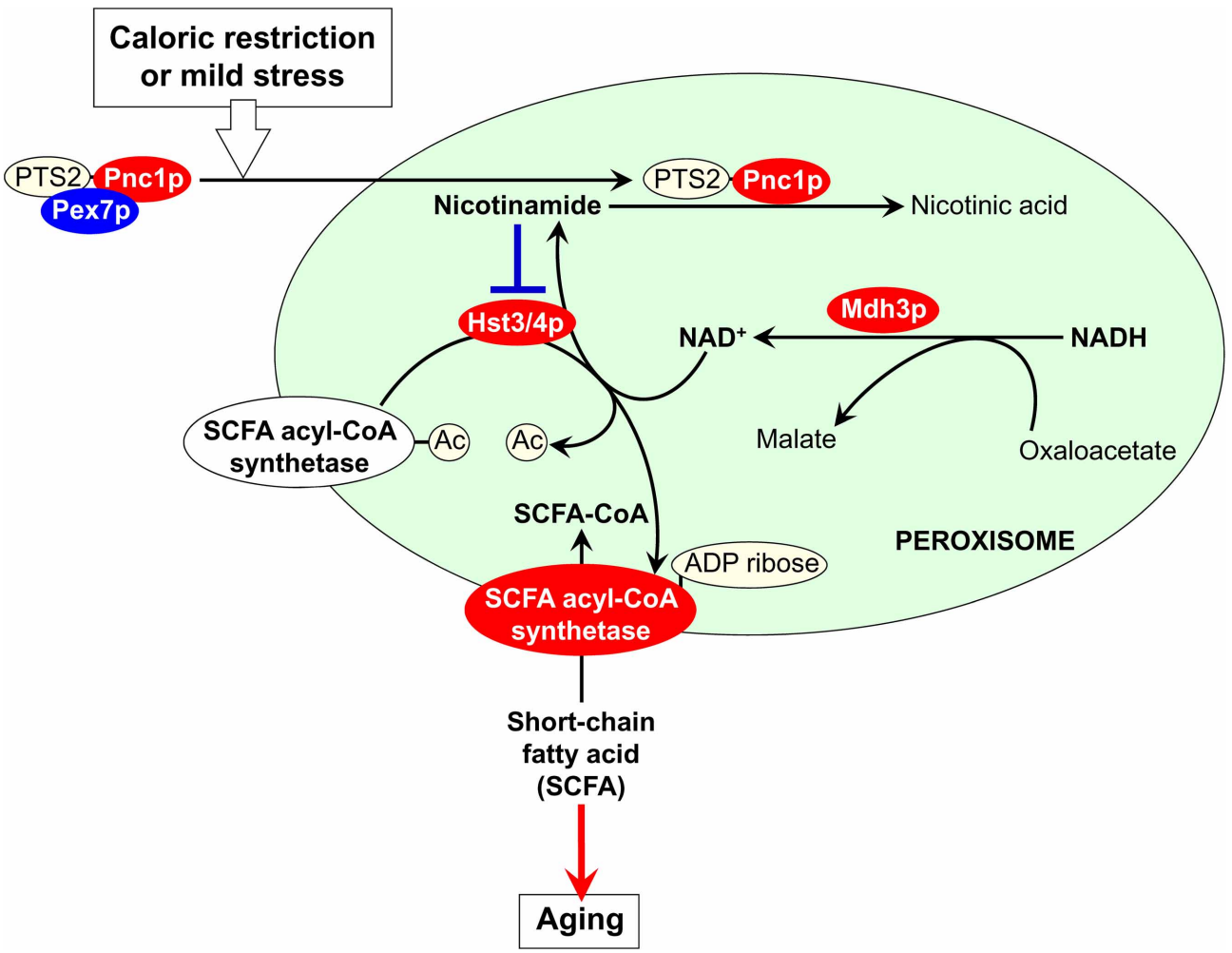

FIGURE 1 | A proposed role for cytosol-to-peroxisome targeting of Pnc1p in regulating longevity of replicatively aging yeast. If exposed to caloric restriction (CR) and other hormetic anti-aging stimuli, yeast cells respond by targeting Pnc1p—a pyrazinamidase/nicotinamidase 1 that converts nicotinamide to nicotinic acid in the NAD^+ salvage pathway—not only to the nucleus but also to the peroxisome. The delivery of Pnc1p to the peroxisome depends on the peroxisomal targeting signal 2 (PTS2) shuttling receptor Pex7p. Inside the peroxisome, Pnc1p activates the sirtuins Hst3p and Hst4p by reducing the concentration of their non-competitive inhibitor nicotinamide. Using NAD^+ generated by the peroxisomal malate dehydrogenase Mdh3p as a co-substrate in protein deacetylation and ADP-ribosylation reactions, the activated Hst3p and Hst4p stimulate acyl-CoA synthetases required for peroxisomal transport and oxidation of short-chain fatty acids (SCFA). By reducing the levels of these fatty acids in the cytosol, peroxisomes contribute to the beneficial effect of CR and other hormetic stimuli on longevity. See text for details.

hormetic stimuli. A critical evaluation of our hypothesis will require testing of the localization of Hst3p and Hst4p to the peroxisome, either permanent or triggered in response to CR and mild stresses. Another key challenge for the future will be to evaluate the ability of peroxisomal acyl-CoA synthetases to undergo reversible deacetylation and/or ADP-ribosylation in an Hst3p- and/or Hst4p-dependent fashion following exposure of yeast to these longevity-extending stimuli.

THE PEROXIN Pex6p CONTRIBUTES TO THE MAINTENANCE OF AGE ASYMMETRY BETWEEN THE MOTHER AND DAUGHTER YEAST CELLS WITH RESPECT TO SEGREGATION OF FUNCTIONAL MITOCHONDRIA

Several peroxisomal proteins are known to possess dual subcellular localization and function (reviewed by Titorenko and Rachubinski, 2004; Mast et al., 2010; Islinger et al., 2012). While the major, peroxisome-bound portion of each of these proteins controls essential processes confined to this organelle, their pools in other organellar compartments govern certain developmental, differentiation, and morphogenetic programs (Titorenko and Rachubinski, 2004; Islinger et al., 2012). The emerged compendium of these bifunctional peroxisomal proteins with dual subcellular localization is on a fast-growing list of the so-called "moonlighting proteins" (Jeffery, 1999, 2011; Shi and Shi, 2004; Kim and Dang, 2005; Gancedo and Flores, 2008; Jeffery, 2009; Flores and Gancedo, 2011). By analyzing the information on dynamic changes in metabolic status and/or organelle functional state within one subcellular location and then moving to other location(s) for initiating an adequate response to such changes, these moonlighting proteins integrate various cellular activities in space and time (Shi and Shi, 2004; Kim and Dang, 2005; Cho et al., 2006; Gancedo and Flores, 2008; Sen et al., 2008; Flores and Gancedo, 2011; Jeffery, 2011).

The peroxin Pex6p is an AAA ATPase (ATPase associated with various cellular activities) whose peroxisome-associated pool has been implicated in peroxisomal protein import (Titorenko and Rachubinski, 2009; Ma et al., 2011; Rucktäschel et al., 2011). In the yeast *Yarrowia lipolytica* Pex6p is a moonlighting protein whose minor portion is confined to the ER (Titorenko et al., 1997; Titorenko and Rachubinski, 1998). The ER-associated Pex6p is an essential component of protein machinery that orchestrates the dimorphic transition from a round yeast form to a filamentous (mycelial) form (Titorenko et al., 1997; Titorenko and Rachubinski, 2004). Pex6p, along with other ER components of this machinery, governs this cell polarization and differentiation program by driving the delivery of mycelium-specific proteins from the ER to the cell surface (Titorenko et al., 1997).

Recently, a list of the "extra-curricular" activities of Pex6p has been updated by including to it the essential role that this peroxin plays in regulating yeast longevity. Because the yeast *Saccharomyces cerevisiae* reproduce by asymmetric cell division, replicatively "young" mother cells retain such "senescence factors" (also called "aging factors") as ERCs, oxidatively damaged proteins, protein aggregates, and dysfunctional mitochondria (**Figure 2A**) (Jazwinski, 2005a; Erjavec et al., 2007, 2008; Henderson and Gottschling, 2008; Steinkraus et al., 2008; Eldakak et al., 2010; Liu et al., 2010; Zhou et al., 2011). Their budding progeny therefore retains the full replicative capacity by not inheriting ERCs or damaged/aggregated proteins and receiving only functional mitochondria (Henderson and Gottschling, 2008; Erjavec et al., 2008; Steinkraus et al., 2008). In replicatively "old" mother cells, this age asymmetry between the mother and daughter cells is lost. As a result, the daughters inherit all four of the known senescence factors (**Figure 2A**) (Jazwinski, 2005a; Henderson and Gottschling, 2008; Steinkraus et al., 2008). It should be stressed that the overexpression of Pex6p suppresses the lack of age asymmetry between mother and daughter cells in a strain carrying a point mutation in the nuclear gene *ATP2* encoding the β-subunit of the F_1 sector of mitochondrial F_0, F_1-ATP synthase (Lai et al., 2002; Seo et al., 2007). Moreover, not only Pex6p—along with yet-to-be-identified cytosolic proteins—facilitates the import of Atp2p into mitochondria, but it also drives the segregation of functional mitochondria to daughter cells (Seo et al., 2007). Therefore, it is conceivable that Pex6p could operate as one of the "filters" sequestering dysfunctional mitochondria in the mother cell and/or segregating functional mitochondria to the daughter cell (**Figures 2B** and **2C**). The challenge remains to define the mechanisms underlying the ability of Pex6p to facilitate mitochondrial import of Atp2p and to maintain the age-related asymmetrical segregation of functional mitochondria between mother and daughter cells. Another key challenge for the future will be to establish the mechanism for delivery of Pex6p from peroxisomes to mitochondria. Importantly, not only the biogenesis of these two organelles is governed by common transcriptional pathways, but they also share several key components of their division machineries and are linked through mitochondria-to-peroxisome vesicular traffic (**Figures 2B** and **2C**) (Liu and Butow, 2006; Neuspiel et al., 2008; Andrade-Navarro et al., 2009; Delille et al., 2009; Jazwinski, 2012; Islinger et al., 2012).

TWO MECHANISMS FOR PREVENTING THE SEGREGATION OF DYSFUNCTIONAL, OXIDATIVELY DAMAGED PEROXISOMES TO THE DAUGHTER YEAST CELL DURING MITOSIS

Not only peroxisomes in replicatively aging yeast contribute to selective segregation of functional mitochondria to the daughter cell, but they also possess a protein machine that governs their own distribution between mother and daughter cells. Recent studies suggested two mechanisms by which this protein machine may operate in preventing the inheritance of dysfunctional, oxidatively damaged peroxisomes by the daughter cell during mitosis.

In *S. cerevisiae*, the inheritance of peroxisomes by daughter cells relies on the peroxisomal protein Inp2p (Fagarasanu et al., 2006). By acting as a receptor for the class V myosin motor Myo2p, Inp2p tags peroxisomes for their segregation to the daughter cell (Fagarasanu et al., 2009). It is conceivable that such Inp2p-dependent tagging of peroxisomes plays a longevity-extending role by enabling the inheritance of only functional peroxisomes by daughter cells. Importantly, the phosphorylation of Inp2p makes it susceptible to degradation, thereby impairing the segregation of Inp2p-less peroxisomes to the daughter cell (Fagarasanu et al., 2009, 2010). One could therefore

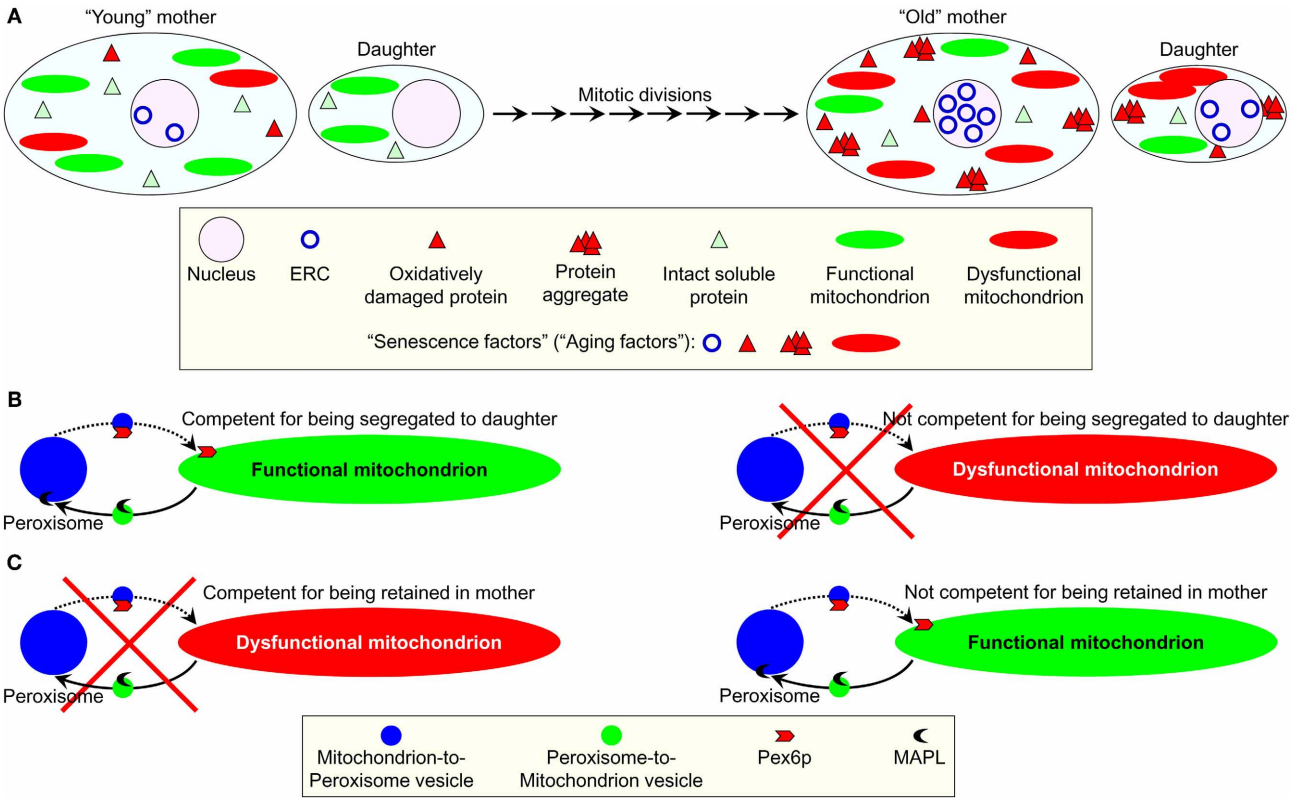

FIGURE 2 | A proposed role for the mainly peroxisomal protein Pex6p in sequestering dysfunctional mitochondria in the mother cell of replicatively aging yeast and/or segregating functional mitochondria to the daughter cell. (A) In the reproducing by asymmetric cell division yeast *Saccharomyces cerevisiae*, the budding progeny of replicatively "young" mother cells retains the full replicative capacity by not inheriting such "senescence factors" (also called "aging factors") as extrachromosomal rDNA circles (ERC), oxidatively damaged proteins, protein aggregates, and dysfunctional mitochondria. In contrast, the daughters of replicatively "old" mother cells inherit these senescence factors. **(B,C)** A peroxisome-associated pool of the peroxin Pex6p has been long known for its essential role in peroxisomal protein import. An "extra-curricular" activity of this protein consists in driving the segregation of functional mitochondria to daughter cells. Pex6p could operate as a "filter" sequestering dysfunctional mitochondria in the mother cell **(B)** and/or segregating functional mitochondria to the daughter cell **(C)**. The mechanism underlying such function of Pex6p may involve a recently discovered vesicular traffic between peroxisomes and mitochondria. See text for details.

speculate that such phosphorylation and degradation target mainly Inp2p on dysfunctional, oxidatively damaged peroxisomes for sequestering them in the mother cell.

In another yeast species, *Y. lipolytica*, the inheritance of only newly formed from the ER template peroxisomes may prevent the segregation of their oxidatively damaged, "old" counterparts to the daughter cell during mitosis (Chang et al., 2009). By possessing a dual role in the formation of new peroxisomes from the ER template and in the recruitment of the class V myosin motor Myo2p to their membranes, the peroxins Pex3p and Pex3Bp may enable the selective segregation of these newly formed peroxisomes to the daughter cell, thereby allowing to retain the entire population of dysfunctional, oxidatively damaged peroxisomes in the mother cell (Chang et al., 2009; Fagarasanu et al., 2010).

The challenge remains to define the molecular mechanisms underlying the proposed selectivity in (1) phosphorylating Inp2p only on dysfunctional, oxidatively damaged peroxisomes; and (2) targeting Myo2p only to the ER-confined pool of Pex3p.

A MODEL FOR THE INTEGRATION OF PEROXISOMES INTO AN ENDOMEMBRANE SYSTEM THAT GOVERNS CELLULAR AGING

A body of evidence summarized here and elsewhere (Titorenko and Rachubinski, 2004; Titorenko and Terlecky, 2011; Islinger et al., 2012) implies that peroxisomes contribute to the regulation of cellular aging via several different mechanisms. In each of these mechanisms, peroxisomes communicate with other organelles by establishing extensive physical contact with lipid bodies, maintaining the ER-peroxisome connectivity, exchanging certain metabolites, and/or being involved in the bidirectional flow of some of their protein and lipid constituents. Thus, peroxisomes are dynamically integrated into an endomembrane system that governs cellular aging. We propose a model for such integration (**Figure 3**). The central tenet of this model is that the age-dependent efficiency of protein import into the peroxisome modulates the dynamics of its communication with other cellular compartments, thereby influencing several longevity regulation pathways that rely on such communication. The overall efficiency of peroxisomal protein import is defined by the efficiencies

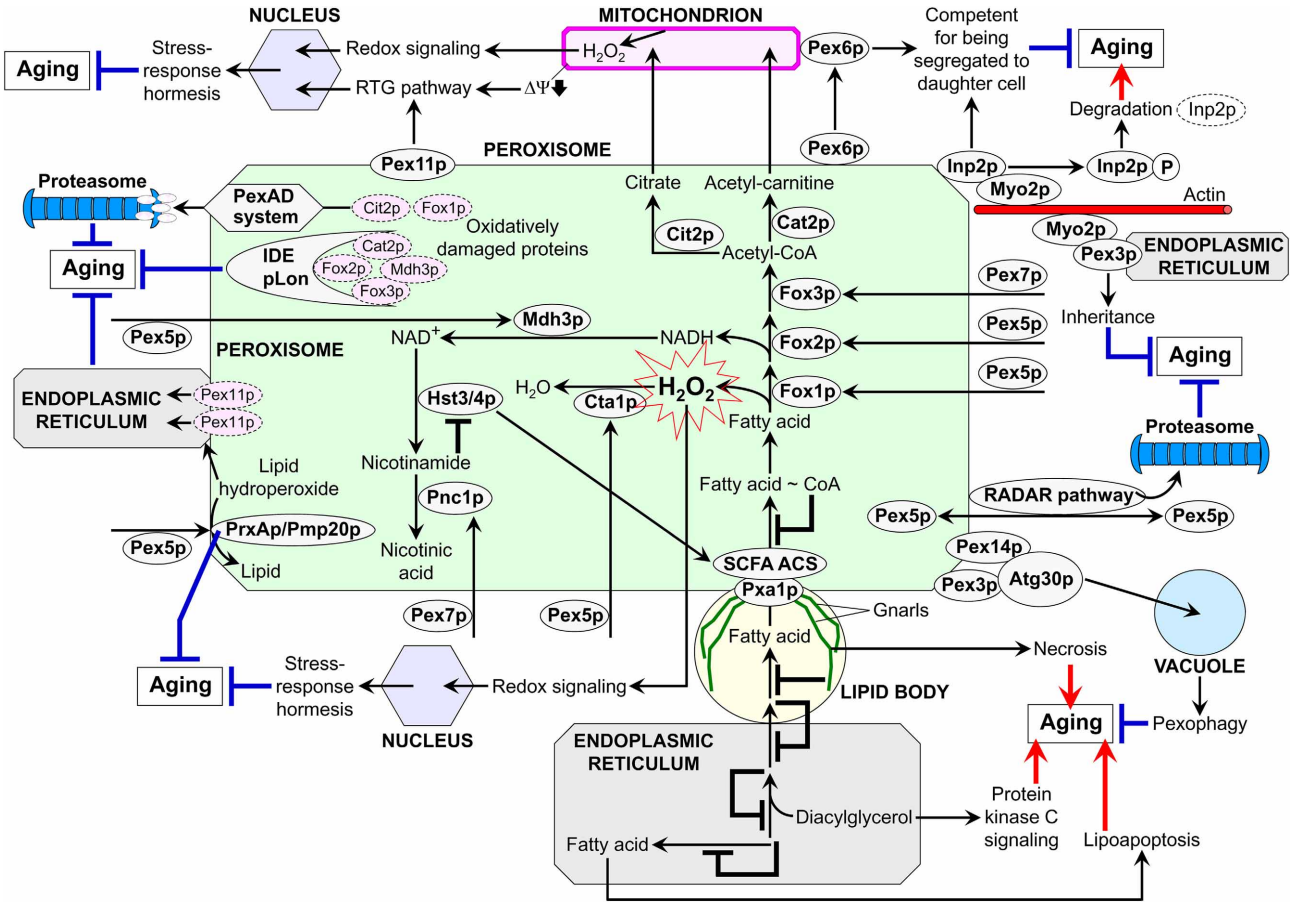

FIGURE 3 | A model for the dynamic integration of peroxisomes into an endomembrane system governing cellular aging. Several mechanisms underlie the essential contribution of peroxisomes to the regulation of cellular aging. Each of these mechanisms relies on a network of communications between peroxisomes and other organelles through the maintenance of the endoplasmic reticulum-peroxisome connectivity, establishment of the extensive physical contact with lipid bodies, exchange of certain metabolites, and/or the bidirectional flow of some of their proteins and lipids. Thus, peroxisomes are dynamically integrated into an endomembrane system that governs cellular aging. By modulating the dynamics of communication between peroxisomes and other cellular compartments, the age-dependent efficiency of peroxisomal protein import influences a compendium of longevity regulation pathways relying on such communication. Peroxisomes promote the development of a pro-aging pattern within an endomembrane system governing cellular aging if the overall efficiency of peroxisomal protein import is actively maintained at a sufficiently high level. Conversely, peroxisomes trigger certain pro-aging processes within this endomembrane system if the overall efficiency of peroxisomal protein import is lower than this critical level. See text for details. IDE, insulin degrading enzyme; PexAD, peroxisome-associated protein degradation; pLon, peroxisomal Lon protease; RADAR, receptor accumulation and degradation in the absence of recycling; RTG, retrograde; SCFA ACS, short-chain fatty acid acetyl-CoA synthetase; ΔΨ, electrochemical potential across the inner mitochondrial membrane.

of binding of Pex5p and Pex7p—the PTS1 and PTS2 cytosolic shuttling receptors, respectively—to their cargo proteins in the cytosol, translocation of the receptor-cargo complexes across the peroxisomal membrane, and receptor recycling (Ma et al., 2011; Rucktäschel et al., 2011). Importantly, the efficiencies of all these processes are reduced with age (Legakis et al., 2002; Terlecky et al., 2006; Titorenko and Terlecky, 2011). In our model, if the overall efficiency of protein import into peroxisomes is actively maintained at a sufficiently high level, these organelles trigger certain anti-aging processes within the endomembrane system governing cellular aging (**Figure 3**). Conversely, if the overall efficiency of peroxisomal protein import is lower than this critical level, peroxisomes promote the development of a pro-aging pattern within this endomembrane system (**Figure 3**).

Our model envisions that the efficient Pex5p-dependent peroxisomal import of the ROS scavenging enzymes catalase (Cta1p in yeast) and peroxiredoxin (PrxAp in mammals and Pmp20p in yeast) in replicatively and chronologically "young" cells aids in minimizing the oxidative damage to peroxisomal proteins and membrane lipids (**Figure 3**; Antonenkov et al., 2010; Titorenko and Terlecky, 2011; Ivashchenko et al., 2011). At the surface of the peroxisome, quality control of the Pex5p-driven protein import in these cells is governed by the receptor accumulation and degradation in the absence of recycling (RADAR) pathway for the recycling of Pex5p (**Figure 3**; Léon et al., 2006; Ma et al., 2011; Titorenko and Terlecky, 2011). Inside the peroxisome, the insulin degrading enzyme (IDE), peroxisomal Lon (pLon) protease, and peroxisome-associated protein degradation (PexAD) system carry

out the degradation of oxidatively damaged peroxisomal matrix proteins that are incapable of supporting the anti-aging processes orchestrated by functionally active peroxisomes (Morita et al., 2000; Aksam et al., 2007, 2009; Lingard et al., 2009). A healthy population of such peroxisomes in "young" cells is also sustained by pexophagy, an autophagy-related process in which dysfunctional peroxisomes carrying oxidatively damaged proteins are selectively degraded following their sequestration by vacuoles of yeast and plant cells or lysosomes of mammalian cells (**Figure 3**; Aksam et al., 2007; Farré et al., 2008; Manjithaya et al., 2010). In plant cells exposed to oxidative stress, the ER-peroxisome connectivity enables the retro-flow of oxidatively damaged matrix proteins as well as of membrane proteins and lipids to the ER—thereby contributing to the maintenance of a healthy population of functionally active peroxisomes (Mathur, 2009; Sinclair et al., 2009).

In our model, peroxisomes in "young" cells not only actively avoid the oxidative damage to their protein and lipid constituents but also operate as a signaling platform that, by maintaining ROS concentration at a certain "optimal" level, delays cellular aging by inducing stress-response hormesis (**Figure 3**; Titorenko and Terlecky, 2011). At such a level, ROS are unable to elicit substantial oxidative damage to cellular macromolecules but can activate several redox signaling networks known to elevate the abundance and/or activity of stress-protecting and other anti-aging proteins (D'Autréaux and Toledano, 2007; Giorgio et al., 2007; Veal et al., 2007).

Furthermore, the PTS1 and PTS2 cytosolic shuttling receptors Pex5p and Pex7p drive peroxisomal import of Fox1p, Fox2p, and Fox3p (Hiltunen et al., 2003). The efficient import of these core enzymes of fatty acid β-oxidation into peroxisomes of "young" cells increases the efficacy with which they decompose fatty acids derived from triacylglycerols that are synthesized in the ER and deposited within lipid bodies (Goodman, 2008; Goldberg et al., 2009a,b; Kohlwein, 2010). Due to such accelerated peroxisomal fatty acid oxidation and the resulting decrease in the concentrations of non-esterified fatty acids and diacylglycerol, "young" cells escape the premature death by resisting lipid-induced necrosis and apoptosis and by sustaining stress resistance through the attenuation of diacylglycerol-activated protein kinase C signaling (**Figure 3**; Goldberg et al., 2009a,b; Titorenko and Terlecky, 2011). Another way for the longevity-extending acceleration of peroxisomal fatty acid oxidation in "young" cells is the governed by sirtuins Hst3p and Hst4p stimulation of acyl-CoA synthetases that are required for peroxisomal transport and oxidation of short-chain fatty acids. This anti-aging process is driven by the efficient Pex5p- and Pex7p-dependent peroxisomal import of Mdh3p and Pnc1p for synthesizing a substrate and decomposing an inhibitor of the sirtuins, respectively (**Figure 3**).

Moreover, the longevity-extending ability of peroxisomes to promote the anti-aging RTG signaling pathway of peroxisomes-mitochondria, mitochondria-nucleus, and nucleus-peroxisomes communications in "young" cells is enhanced by the highly efficient peroxisomal import of Fox1p, Fox2p, Fox3p, Cit2p, and Cat2p in these cells (**Figure 3**; Titorenko and Terlecky, 2011). Fox1p, Fox2p, and Fox3p are involved in the peroxisomal oxidation of fatty acid to acetyl-CoA following their Pex5p- and Pex7p-dependent delivery to peroxisomes, whereas the citrate synthase Cit2p and acetyl-carnitine synthase Cat2p are imported into these organelles with the help of Pex5p to catalyze the anaplerotic conversion of acetyl-CoA to citrate and acetyl-carnitine (**Figure 3**; Epstein et al., 2001; Traven et al., 2001; Hiltunen et al., 2003; Titorenko and Terlecky, 2011). The longevity-extending RTG signaling pathway in "young" cells is further amplified through the Pex11p-driven proliferation of peroxisomes and the resulting increase in the effectiveness with which the confined to these organelles fatty acid oxidation and anaplerotic reactions replenish TCA cycle intermediates destined for mitochondria (**Figure 3**; Jazwinski, 2005b; Liu and Butow, 2006; Titorenko and Terlecky, 2011). It should be emphasized that, by maintaining the functionality of mitochondria in "young" cells, the peroxisome-driven RTG pathway controls the homeostasis of mitochondrial ROS (Titorenko and Terlecky, 2011). This enables the ROS-dependent activation of several redox signaling networks aimed at increasing the levels of stress-protecting and other anti-aging proteins or post-translationally activating some of them (**Figure 3**; D'Autréaux and Toledano, 2007; Giorgio et al., 2007; Veal et al., 2007). In our model, the segregation of functional mitochondria to the "young" daughter cell and/or the sequestration of dysfunctional mitochondria in the "old" mother cell in replicatively aging yeast are/is driven in part by the delivery of the peroxin Pex6p from peroxisomes to mitochondria through a mechanism that remains to be established (**Figure 3**; Lai et al., 2002; Seo et al., 2007).

According to our model, *S. cerevisiae* Inp2p—a peroxisome-specific receptor for the class V myosin motor Myo2p—tags peroxisomes for their segregation to the "young" daughter cell in a process that may play a life-extending role by enabling the inheritance of only functional peroxisomes (**Figure 3**; Fagarasanu et al., 2009, 2010). Furthermore, by possessing a dual role in the formation of new peroxisomes from the ER template and in the recruitment of Myo2p to their membranes, the *Y. lipolytica* peroxins Pex3p and Pex3Bp may enable the inheritance of only newly formed from the ER template peroxisomes thus preventing the segregation of their oxidatively damaged, "old" counterparts to the daughter cell during mitosis (**Figure 3**; Chang et al., 2009; Fagarasanu et al., 2010).

Our model envisions that the overall efficiency of peroxisomal protein import gradually decreases with replicative and chronological age (**Figure 3**). A steady, age-related increase in the concentration of peroxisome-confined proteins that are oxidatively damaged by peroxisomally produced ROS could be the driving force for such deterioration of peroxisomal protein import efficiency. The Pex5p-dependent peroxisomal import of catalase—due to the age-dependent decline in the efficiency of its binding to Pex5p and in the extent of Pex5p recycling—is the most sensitive to oxidative damage peroxisomal process (Legakis et al., 2002; Terlecky et al., 2006). The resulting deceleration of catalase import into peroxisomes increases the extent of oxidative damage to their proteins and lipids, thereby initiating the "deterioration spiral" that eventually lowers the overall efficiency of peroxisomal protein import below a critical level. Consequently, the role of peroxisomes in the regulation of cellular aging is switching from being a platform for activating a compendium of

anti-aging processes within the endomembrane system governing cellular aging to becoming a platform for the development of a pro-aging pattern within this endomembrane system (Titorenko and Terlecky, 2011). Specifically, the RADAR pathway, IDE and pLon proteases, PexAD system, and pexophagy eventually fail due to the progressive, age-dependent accumulation of oxidatively damaged proteins and lipids in peroxisomes. Thus, cellular aging coincides with the build-up of dysfunctional peroxisomes that are unable anymore to support the anti-aging processes within the endomembrane system governing such aging. Among these impaired anti-aging processes are (1) the peroxisome- and mitochondria-driven pathways of stress response hormesis; (2) the Hst3p/Hst4p-dependent stimulation of acyl-CoA synthetases for peroxisomal transport and oxidation of short-chain fatty acids; (3) the RTG signaling pathway of peroxisomes-mitochondria, mitochondria-nucleus, and nucleus-peroxisomes communications; (4) the Pex6p-dependent sequestration of dysfunctional mitochondria in the "old" mother cell and/or segregation of functional mitochondria to the "young" daughter cell; and (5) the Inp2p-, Pex3p-, and Pex3Bp-dependent segregation of functional peroxisomes to the "young" daughter cell (**Figure 3**). Moreover, our model foresees that, by being unable to maintain low levels of non-esterified fatty acids and diacylglycerol, the dysfunctional, oxidatively damaged peroxisomes accumulated in aged cells (1) activate the longevity-shortening necrotic and apoptotic cell death mechanisms induced by non-esterified fatty acids; and (2) are unable to attenuate the diacylglycerol-activated protein kinase C signaling that reduces stress resistance (**Figure 3**).

CONCLUSION

Growing evidence supports the view that peroxisomes govern cellular aging via several different mechanisms involving their dynamic communication with other cellular compartments. An important conceptual advance in our understanding of the inherent complexity of cellular aging is that the age-related dynamics of communications between peroxisomes and various other organelles modulates a compendium of longevity regulation pathways. It is conceivable therefore that the peroxisome is dynamically integrated into an endomembrane system governing cellular aging. Much progress has recently been made in defining how communications between peroxisomes and other cellular compartments influence the development of a pro- or anti-aging pattern within this endomembrane system. The challenge remains to define the molecular mechanisms underlying the integration of peroxisomes into the endomembrane system governing cellular aging. Future work will aim at understanding how peroxisomes switch their role in the regulation of cellular aging from being a platform for activating a compendium of anti-aging processes confined to this endomembrane system in "young" cells to becoming a platform for the development of a pro-aging pattern within this endomembrane system in "old" cells. This knowledge will provide greater insight into the mechanisms underlying longevity regulation and is expected to reveal novel targets for anti-aging pharmaceuticals that can extend longevity by modulating the age-related dynamics of communications between peroxisomes and other cellular compartments.

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Insights into the beneficial effect of caloric/ dietary restriction for a healthy and prolonged life

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Over the last several years, new evidence has kept pouring in about the remarkable effect of caloric restriction (CR) on the conspicuous bedfellows- aging and cancer. Through the use of various animal models, it is now well established that by reducing calorie intake one can not only increase life span but, also, lower the risk of various age related diseases such as cancer. Cancer cells are believed to be more dependent on glycolysis for their energy requirements than normal cells and, therefore, can be easily targeted by alteration in the energy-metabolic pathways, a hallmark of CR. Apart from inhibiting the growth of transplantable tumors, CR has been also shown to inhibit the development of spontaneous, radiation, and chemically induced tumors. The question regarding the potentiality of the anti-tumor effect of CR in humans has been in part answered by the resistance of a cohort of women, who had suffered from anorexia in their early life, to breast cancer. However, human research on the beneficial effect of CR is still at an early stage and needs further validation. Though the complete mechanism of the anti-tumor effect of CR is far from clear, the plausible involvement of nutrient sensing pathways or IGF-1 pathways proposed for its anti-aging action cannot be overruled. In fact, cancer cell lines, mutant for proteins involved in IGF-1 pathways, failed to respond to CR. In addition, CR decreases the levels of many growth factors, anabolic hormones, inflammatory cytokines, and oxidative markers that are deregulated in several cancers. In this review, we discuss the anti-tumor effect of CR, describing experiments done *in vitro* in tumor models and *in vivo* in mouse models in which the tumor was induced by means of radiation or chemical exposure, expressing oncogenes or deleting tumor suppression genes. We also discuss the proposed mechanisms of CR anti-tumor action. Lastly, we argue the necessity of gene expression studies in cancerous versus normal cells upon CR.

Keywords: caloric restriction, dietary restriction, cancer, anti-tumor effect, aging

INTRODUCTION

Growing awareness that diet and environmental factors have a profound effect in the initiation, promotion, and progression of cancer argues that cancer is a preventable disease. In fact, alteration in the eating habit from traditional to westernized diets appears to correlate with the increased risk of many common cancers in both developed and developing countries (Baade et al., 2009; Kolonel et al., 2004). For example, prostate cancer, which was previously prevalent only in developed countries, showed an increased incidence rate in less developed and developing countries, due to westernization of food habits (Baade et al., 2009). Further, studies of ethnic and migrant groups in Hawaii showed adaptation of Japanese immigrant cancer risk incidences to those of native Hawaiian people (Kolonel et al., 2004). These observations reinforce the belief that environment and diet have an impact on cancer development. The increased risk of breast cancer in Japanese women who migrated to US also supports the influence of environment and diet on the pathogenesis of cancer (Probst-Hensch et al., 2000). In addition, observational studies showing a decreased risk of cancer in a population with a dietary habit enriched for plant food, and limited consumption of animal fat and dairy products, also point toward the importance of diet in cancer (Kushi et al., 2006). Obesity due to over eating has been shown to be associated with increased risk of colon, breast (in post-menopausal women), endometrium, kidney, esophagus, pancreas, prostate, gallbladder, and liver cancer (Calle and Kaaks, 2004). It is believed that increased food consumption can influence the expression of genes involved in important cellular functions, such as DNA repair, cell proliferation and differentiation, and apoptosis, by altering the levels of metabolic hormones and growth factors, and can lead to accumulation of damage and mutations and ultimately malignant transformation (Hursting et al., 1999; Calle and Kaaks, 2004). Therefore, it can be assumed that by controlling our diet we might also control cancer risk. Caloric restriction (CR), which can be defined as "under nutrition without malnutrition," has emerged as a robust method to decrease cancer incidence, besides increasing the life span of the individuals (Sell, 2003). CR has been shown to

reduce the levels of many cancer-causing agents like anabolic hormones, growth factors, and reactive oxygen species (ROS) in animal models. Unfortunately, the exact metabolic adaptation through which CR exhibits its anti-tumor effect is not completely understood. However, at least in part, the mechanism responsible for the anti-tumor effect of CR involves a similar metabolic adaptation as seen in the case of its anti-aging effect. The anti-proliferative and pro-apoptotic properties of CR, in addition to its ability to decrease oxidative stress and maintain genomic stability, could be responsible for its anti-tumor activity.

Here, we discuss the existing evidence regarding the anti-tumor activity of dietary/energy restriction and factors and pathways crucial for its effect. We also discuss the putative parallel mechanisms through which CR exerts both anti-aging and anti-tumor activities. An insight into how anti-aging effects may also lead to tumorigenesis is also provided. Finally, we discuss the potential of CR interventions at clinical level.

CALORIC RESTRICTION AS A PROMISING NATURAL APPROACH TO OVERCOME CANCER

Realization that both the environment and the diet of an individual can influence the occurrence of cancer has heightened the idea that cancer is a preventable disease. Epidemiological studies have also shown a correlation between weight of an individual and development of tumors suggesting that controlling the diet may contribute to cancer prevention (Tannenbaum, 1940, 1942; Collaborative Group on Epidemiological Studies of Ovarian Cancer, 2012). In fact, beside the recognized effects that restriction in calorie intake has on aging, increasing evidence also supports a role of CR in inhibiting tumor. The first ever observation on the anti-tumor activity of CR was made in the early 1900's. One of the very first experiments in mice and rats showed that lowering the weight by CR can lower the frequency of various types of spontaneous tumors and other inducible tumors (Mccay et al., 1939; Tannenbaum, 1940). More recently, experiments carried out in tumor susceptible C3H/He female mice showed that a change in diet to 70% of the *ad-libitum* diet could single-handedly suppress the spontaneously occurring mammary tumor, suggesting a new way of restricting tumor growth (Kharazi et al., 1994). The fact that CR is linked to a reduction of the levels of mouse mammary tumor virus (MMTV) RNA and incidences of spontaneous mammary tumor further reinforces this belief (Li et al., 1994). Furthermore, in an attempt to find out differences in tumor biology with age, Pili et al. found that young mice that were otherwise vulnerable to tumor growth and expansion as compared to old mice, when fed on a caloric restricted diet showed a decrease in the growth of transplantable tumors and decreased angiogenesis (Pili et al., 1994). In addition, CR has also been shown to be effective against chemically and radiation induced leukemia, and mammary and liver tumors (Beth et al., 1987; Ruggeri et al., 1989; Fu et al., 1994; Yoshida et al., 1997, 1999, 2006). The anti-tumor ability of CR was also observed in animal models of pancreatic, colon, breast, prostate, and lung tumor, proving it to be active against various kinds of cancer (**Figure 1A**) (Bunk et al., 1992; Roebuck et al., 1993; Mukherjee et al., 1999, 2002; Dirx et al., 2003a,b; Mai et al., 2003; Phoenix et al., 2010; Lashinger et al., 2011). The inhibitory effect of CR appears to depend on a caloric intake restriction ranging from

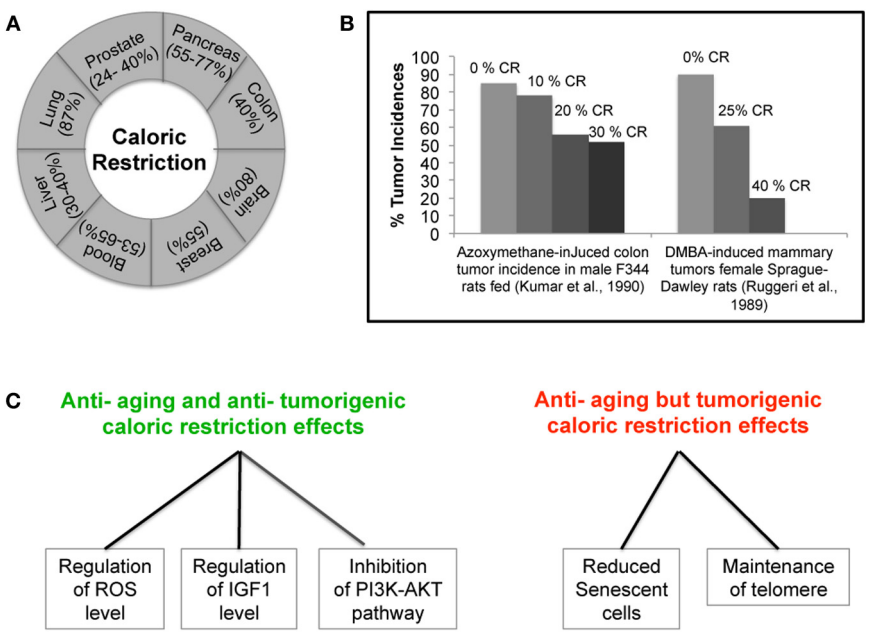

FIGURE 1 | Caloric Restriction (CR) and Cancer. (A) CR effectively inhibits various kinds of cancer in animal models. **(B)** Correlation between decrease in the incidence of tumor and severity of CR. **(C)** Demonstration of parallel and opposing effects of CR on cancer and aging. The ability of CR to decrease the levels of IGF-1 and ROS and inhibit the PI3K-AKT pathway can simultaneously protect cells from aging and cancer. However, CR ability to maintain telomere length and reduce the frequency of senescence can promote cancer but may be also beneficial in delaying aging.

25% up to 60% of *ad-libitum* levels, combined with adequate intakes of essential nutrients. Interestingly, experiments in rats showed that by increasing the degree of CR intervention, the reduction in chemically-induced tumor incidence was intensified (**Figure 1B**) (Ruggeri et al., 1989; Kumar et al., 1990). Most importantly, a very recent study, using a mouse model of post-menopausal obesity, provided the evidence that CR can break the obesity-cancer progression link offering a new hope to women vulnerable to post-menopausal breast cancer (Nogueira et al., 2012). Several studies using mouse models of brain tumor showed CR to be effective not only in non-invasive tumors but also in the most aggressive and invasive forms of brain tumor, proving it to have anti-proliferative, anti-angiogenic, and anti-invasive properties (Mukherjee et al., 2002, 2004; Zhou et al., 2007; Shelton et al., 2010). Recently, CR has also been shown to be beneficial for mice lacking the tumor suppressor p53. In fact, mice lacking p53 develop lymphoma by six months of age and die very early; however, when put on a calorie restriction diet, these mice live longer due to decreased tumor incidence (Hursting et al., 1994, 1997). This is very interesting as p53 is known to be non-functional in almost all types of human cancer, either because of its own mutation or mutation in its regulator/s. This last observation again indicates that CR might constitute an effective intervention for a prolonged healthier life. So far, the only tumor types which failed to respond to calorie restricted diets are the tumors carrying a mutation in either PI3K or PTEN genes, thus leading to the constitutive activation of the PI3K pathway (Kalaany and Sabatini, 2009). **Table 1** summarizes the amount of calorie restriction in percentage or kcal/day or kcal/week in the above mentioned studies.

PROPOSED MECHANISMS FOR THE ANTI-TUMOR ACTIVITY OF CALORIC RESTRICTION

The mechanisms responsible for the observed effect of CR in aging, cancer and other chronic diseases are still under scrutiny. The initial studies indicated the involvement of similar mediators and pathways for both the anti-aging and anti-tumor activity of CR. Accumulation of cellular damage is assumed as one of the initiating events in aging and cancer. ROS, which are natural byproducts of cell normal metabolism and are capable of damaging macromolecular components of the cells, including proteins, lipids and DNA, are considered as the main culprit. In the cells, ROS are neutralized by the action of anti-oxidant enzymes to avoid oxidative damage. However, as the cells age, their capacity to neutralize ROS diminishes, leading to accumulation of macromolecular damage. Moreover, tumorigenesis is also fuelled by accumulation of cellular damage, partly by increased intrinsic ROS stress due to oncogene stimulation, increased metabolic activities, and mitochondrial malfunction (Pelicano et al., 2004). Thus, regulation of oxidative stress could be the mechanism in common between CR anti-tumor and anti-aging activities. In fact, 25–40% CR was shown to maintain the otherwise diminishing levels of anti-oxidant defense systems in aging rodents (Youngman et al., 1992). Further, 30% CR was shown to decrease nitric oxide production in p53 deficient mice and delay tumorigenesis (Hursting et al., 1994, 2001; Mei et al., 1998). The ability of CR to reduce oxidative stress was recently shown to be dependent on SIRT3, a deacetylase (Qiu et al., 2010) and mice deficient in SIRT3 were shown to be prone to both aging and cancer and failed to respond to CR (Kim et al., 2010).

Another highly studied common mediator for the anti-tumor and anti-aging activity of CR is IGF-1 (Insulin Growth Factor 1). The involvement of IGF-1 in aging is supported by the availability of nutrition studies in yeast, nematode, fruit fly and mouse (Gems and Partridge, 2001). Mutants of *Drosophila* that exhibited alteration of insulin/IGF-1 signaling pathways lived longer (Clancy et al., 2001; Tatar et al., 2001). Life span extension in *C. elegans* requires deregulation of Insulin/IGF-1 signaling (Lin et al., 2001). The importance of IGF-1 in life span extension is also observed in genetically modified mice defective for growth hormone (GH) or IGF-1 production (Flurkey et al., 2001). These mice live longer as compared to wild type mice. The production of IGF-1 by the liver was shown to be stimulated by GH (Isaksson et al., 1987). Many of the several biological roles of GH seem to depend on its interaction with the growth hormone receptor/binding protein (GHR/BP). Coschigano and colleagues reported that mice with disruption in GHR/BP, although showing high levels of circulating GH, had lower serum levels of IGF-1 in comparison to wild type, and lived longer (Coschigano et al., 2000). All these studies show the importance of the IGF-1 pathway in aging. CR was invariably shown to decrease the IGF-1 serum levels in animal studies (Weindruch and Walford, 1988; Ruggeri et al., 1989; Hursting et al., 1993; Berrigan et al., 2002). Interestingly, the injection of IGF-1 in the mice reversed the CR effect. Furthermore, the ability of CR to regulate IGF-1 levels and the Insulin/IGF-1 pathway could also be responsible for its anti-tumor effect as an elevated IGF-1 serum level is associated with an increased risk of breast, prostate, colon, and lung cancer in humans (Chan et al., 1998; Hankinson et al., 1998; Schaefer et al., 1998; Wolk et al., 1998; Ma et al., 1999; Yu et al., 1999). Involvement of IGF-1 in cancer is further supported by its ability to enhance the growth of a variety of cancer cell lines (Macaulay, 1992; LeRoith et al., 1995; Singh et al., 1996). The tumorigenic property of IGF-1 is thought to be due to its ability to regulate mitogenic and anti-apoptotic pathways (Resnicoff et al., 1995; Yu and Rohan, 2000). In fact, in a transplantable and spontaneous leukemia mouse model, CR has been shown to reduce serum IGF-1 level and decrease leukemia cell proliferation (Hursting et al., 1993). Interestingly, the anti-proliferative effect of CR on leukemia cells was annulled by restoration of serum IGF-1 concentration (Hursting et al., 1993). Similarly, in p53 deficient mice, restoration of IGF-1 levels reverses the beneficial effect of CR on p-cresidine induced carcinogenesis (Dunn et al., 1997). Hence, it can be assumed that, at least in part, CR modulation of IGF-1 mediates its anti-tumor and anti-aging effects.

Recently, the Forkhead box-O (FOXO) family of proteins has been shown to be required for the anti-tumor and anti-aging activity of CR (Greer et al., 2009; Yamaza et al., 2010). The FOXO family of transcription factors is a direct target of the PI3K-AKT pathway (Lin et al., 1997; Ogg et al., 1997). Activation of the PI3K-AKT pathway leads to the phosphorylation and inactivation of FOXO by AKT. However, its phosphorylation by AMPK enhances its transcriptional activity (Greer et al., 2009). FOXO transcription factors, by modulating specific targets genes (p21,

Table 1 | Summary of the extent of caloric restriction and its effect on tumor growth.

Mice/Rat strain	Control intake (type or kcal/day)	Calorie restriction		Outcome	Reference
		Amount in kcal/day	% Restriction with respect to *ad-libitum* diet		
Female sprague dawley rats	50 kcal/day	35 kcal/day	30	Decreased MNU induced carcinoma	Bunk et al., 1992
Sukling male lewis rats	84 kcal/day	∼75.6 kcal/day, ∼71.5 kcal/day, ∼67.3 kcal/day, ∼58.9 kcal/day	10, 15, 20, 30	CR inhibited Azaserine-induced preneoplastic lesion The inhibition increased with more severe restriction regimens	Roebuck et al., 1993
Balb/c	*Ad-libitum* (standard chow diet) or high energy diet	-	30	CR reduced tumor growth and metastasis in aggressive model of hormone independent breast cancer in syngeneic model using triple negative 66cl4 tumor cells in Balb/c mice	Phoenix et al., 2010
Apc (Min) mouse	*Ad-libitum* AIN-76A diet	-	40	Intestinal polyp was reduced by 57% by CR	Mai et al., 2003
Male FischerX cophenhagen F1 rats	*Ad-libitum* AIN-76A diet (61–69 kcal/day)	∼44.53 kcal/day	(1) 30% total diet restriction (2) 30% carbohydrate restriction (3) 30% lipid restriction	Each of the 3 different restriction diets inhibited R3327 tumor to the same extent	Mukherjee et al., 1999
Adult male SCID mice	*Ad-libitum* AIN-76A diet (15.4 kcal/day)	11.1 kcal/day	(1) 30% total diet restriction (2) 30% carbohydrate restriction (3) 30% lipid restriction	Each of the 3 different restriction diets inhibited LNGP human carcinoma to the same extent	Mukherjee et al., 1999
C57BL/6J and BALBc/J-SCID	*Ad-libitum* PROLAB Chow diet (18 to up to 24 kcal/day)	13 kcal/day	30%	CR reduced intra- cerebral CT-2A tumor growth and angiogenesis in syngeneic CT-2A experimental mouse brain tumor	Mukherjee et al., 2002
C57BL/6J and BALBc/J-SCID	*Ad-libitum* PROLAB Chow diet (12–14 to up to 20–24 kcal/day)	-	40%	CR decreased vascularity (factor VIII) and increased apoptosis in three distinct models of brain tumor (a) A malignant mouse astrocytoma (CT-2A) (b) A human glioma (U87-MG) (c) Mouse ependymoblastoma	Mukherjee et al., 2004
C57BL/6	*Ad-libitum*	-	30%	CR inhibited MMTV-Wnt1 tumor growth in mouse model of post-menopausal obesity	Nogueira et al., 2012
VM/DK (VM)	*Ad-libitum*	-	60%	CR effectively reduce malignant brain tumor growth in VM-M3 GBM model	Shelton et al., 2010

p27, cyclin G2, BIM1, Bcl-6, FasL, GADD45, MnSOD, catalase), promote a variety of cellular responses such as cell cycle arrest, apoptosis, DNA repair and resistance to cellular stress (Greer and Brunet, 2005), hence representing an attractive tumor suppressor candidate. Interestingly, FOXO3 has been found to be deregulated in breast cancer (Hu et al., 2004). Moreover, expression of an active form of FOXO suppresses tumor in transplanted nude mice (Hu et al., 2004; Yang et al., 2005). Further, expression of

a constitutive active form of FOXO has been shown to inhibit tumorigenesis in PTEN-null cells (Ramaswamy et al., 2002). An indication of the involvement of FOXO in longevity comes from a study in mutant worms where the increased longevity, due to mutations in the insulin receptor and PI3K, was reversed by an additional mutation in the FOXO ortholog Daf-16 (Lin et al., 1997; Ogg et al., 1997; Kenyon, 2005). Studies in worm have shown that CR ability to extend life span is dependent on AMPK, which partly acts via FOXO (Greer et al., 2007). The inability of CR to extend life span in the FOXO (daf-16) mutant strongly indicates FOXO as a mediator of its anti-aging activity (Greer et al., 2007). The requirement of FOXO in the antineoplastic effect of CR has been shown using FOXO1 knockout heterozygous mice. In comparison to wild type mice, these mice failed to recapitulate the beneficial effect of CR on tumor development. Wild type mice on CR diet showed lower incidence of tumor and tumor related deaths as compared to FOXO1 knockout heterozygous mice (Yamaza et al., 2010), thus suggesting FOXO as a mediator of CR anti-tumor effects as well.

THE DIVERGENT MECHANISM OF CALORIC RESTRICTION IN AGING AND CANCER

The assumption that the anti-tumor ability of CR is a parallel effect of its anti-aging activity and *vice-versa* is a debatable issue. The regulation of GHs, oxidative stress, DNA damage, and metabolic pathways by CR could simultaneously result in its anti-tumor and anti-aging activities (**Figure 1C**). Recent studies have shown that CR reduces the frequency of senescent cells in the liver and small intestine of mice (Wang et al., 2010). CR effect on cellular senescence, a cause of aging, could be crucial to its anti-aging activity (Goldstein, 1990; Wang et al., 2010). However, this property of CR could also be beneficial to tumor cells where induction of senescence is an effective tumor suppressor mechanism (Lleonart et al., 2009). Further, CR has been shown to maintain telomere length, another process important for tumorigenesis (Feldser and Greider, 2007; Wang et al., 2010). Experiment by Oliverras-Ferraros showed that cancer cell lines can be maintained in culture for several months in the presence of CR mimetics (CRM) (Oliveras-Ferraros et al., 2010). Gene expression analysis suggests the retrogression from a more differentiated state to a stem like primitive step in the presence of CRM (Oliveras-Ferraros et al., 2010). While this finding promises potential applications in the replacement of adult aging tissues, it is, at the same time, a matter of concern as regards tumor biology. Another interesting observation is the regulation of SIRT1 by p53 in response to CR. It seems that the induction of SIRT1 under the condition of nutrient deprivation requires occupancy of its promoter by p53. Any mutation that affects the binding of p53 to the SIRT1 promoter region affects SIRT1 up-regulation in response to CR. Therefore, it appears that the SIRT1-mediated beneficial effect of CR requires an active p53 and its binding on SIRT1 (Naqvi et al., 2010). However, the ability of CR to exert its ant-tumor effect in the absence of p53, further points towards the singular regulation of aging and cancer by CR (Hursting et al., 1997). In this context, it is essential to understand how CR carries out both anti-tumor and anti-aging activities, and whether its effects occurs through the same mechanism but with parallel and opposing results on cancer and aging. Further investigations are required (**Figure 1C**).

OTHER POSSIBLE MECHANISMS FOR THE ANTI-TUMOR ACTIVITY OF CALORIC RESTRICTION

While one of the mechanisms responsible for CR-mediated beneficial effects on cancer has been shown to involve the same metabolic adaptation implicated in its anti-aging effects, the role of other specific mediators and pathways cannot be ruled out. One possibility could be the regulation of oncogenes and tumor suppressor genes by CR. For example, gene expression analysis of liver from mice fed on caloric restricted diet revealed significant changes in the genes involved in p53 dependent cell cycle and apoptosis (Estep et al., 2009). One of the most highly up regulated genes in the liver of CR fed mice was DNA-damage inducible transcript 4 (Ddit4), a p53 controlled negative regulator of the m-TOR pathway (Wei et al., 2006; Estep et al., 2009). Ddit4 is a known tumor suppressor whose expression has shown to be down regulated in a subset of human cancers (Deyoung et al., 2008). CR mediated up-regulation of the Ddit4 transcript could be one of many ways by which CR exerts its anti-tumor effect. Analysis of pancreatic acinar cells from CR-fed Brown Norway Rat revealed reduced expression of the c-Ha-Ras oncogene and reduced mutations in the p53 gene (Hass et al., 1993). Further, study in mouse mammary tumor/v-Ha-ras transgenic mice showed that a restricted diet decreases the tumor incidence in these mice, may be through CR mediated increased levels of the tumor suppressor p53 and scavenging enzymes and decreased levels of c-erbB2 and v-Ha-ras RNA (Fernandes et al., 1995). p27/kip is a cyclin dependent kinase inhibitor whose activity is deregulated in various kinds of cancer (Slingerland and Pagano, 2000; Bloom and Pagano, 2003). It has been proposed that CR inhibits induced mammary carcinogenesis by arresting cell cycle progression *via* up-regulation of the expression of p27/kip (Zhu et al., 1999). These observations further point towards the ability of CR to modulate the expression of oncogenes and tumor suppressor genes. Recently, epigenetic regulation by CR has been proposed as one of many mechanisms through which CR controls aging (Li et al., 2011). In fact, it has been shown that CR, by modulating epigenetic changes such as DNA methylation or histone modification, controls the expression of oncogenes and tumor suppressors (Hass et al., 1993; Li et al., 2010). The ability of CR to hyper-methylate the promoter of proto-oncogenes such as Ras, thus leading to their silencing, could contribute toward cancer prevention (Hass et al., 1993).

A recent study using WI-38 (normal cells) and SV-antigen transfected immortalized WI-38 cells (precancerous cells) showed that glucose restriction displayed an altered regulation of the expression of both hTERT and the tumor suppressor p16 in normal and precancerous cells. In normal cells, glucose restriction leads to increased expression of hTERT and decreased expression of p16, leading to delayed aging. However, in precancerous cells, glucose restriction leads to decreased expression of hTERT and increased expression of p16 leading to apoptosis (Li et al., 2010), suggesting that CR may mediate its anti-aging and anti-tumor activities *via* differential regulation of oncogenes and tumor suppressors by varied chromatin modifications. It can be also

assumed that, through chromatin modulation, CR might bring out differential gene expression in normal and cancerous cells. Therefore, it would be interesting to examine the effect of CR on the gene expression profile of normal and cancerous cells.

CLINICAL IMPLICATIONS OF CALORIC RESTRICTION

Although, reduction in calorie intake has emerged as a most potent broadly acting intervention that prevents cancer in experimental animals, its role at clinical level is yet to be defined. There are limited numbers of designed and controlled studies that are aimed to find out the efficacy of CR in humans. This is mainly because of the unavailability of human volunteers willing to follow a restricted diet regime despite CR claimed ability to provide a healthy prolonged life. However, epidemiological studies and observations from both natural and historical situations have indicated CR to be effective in humans too. Many of these analyses were carried out from the answers to questionnaires filled by human volunteers regarding their history, such as a study involving Spanish nursing residents indicating a beneficial effect of CR (Roth et al., 1999). Interestingly, studies utilizing data from cancer registries have shown a correlation between weight loss during adulthood and occurrence of breast cancer. Women's cohorts who experienced a weight loss in adulthood had reduced risk of developing breast cancer in comparison to the ones who gained weight (Trentham-Dietz et al., 2000; Harvie et al., 2005; Christou et al., 2008; Kawai et al., 2010). Likewise, a retrospective study in Swedish women who suffered from severe anorexia nervosa showed that they had a 53% lower incidence of breast cancer than the Swedish general population (Michels and Ekbom, 2004). Similarly, a decreased incidence of breast cancer was observed in Danish women suffering from anorexia nervosa, and in Norwegian pre-pubertal girls and Dutch women who had been exposed to famine during World War II (van Noord and Kaaks, 1991; Tretli and Gaard, 1996; Mellemkjær et al., 2001). Further, the women of the Okinawa community, who follow a traditional lower calorie diet, have lower incidence of breast cancer compared to other Japanese women (Willcox et al., 2007). These observations indicated that starvation or CR during adolescence and adulthood had clear impact on the development of breast cancer, as observed in the rodents. Most interestingly, the decreased prevalence of cancer and vascular diseases in the Okinawa community, due to less calorie intake habit, is considered to be responsible for their lower mortality rate and for their tumor free longer life (Kagawa, 1978). The questionnaire based study of the Netherlands Cohort, who experienced severe CR as adolescent during the Hunger Winter of World War II, has shown that energy restriction during childhood and adolescence also decreases the risk of colorectal and ovarian cancer (Dirx et al., 2003a,b; Hughes et al., 2009; Schouten et al., 2011). All these studies point toward a role for CR in the modulation of human cancer development.

Apart from these retrospective and historic observations, controlled studies involving cancer patients also indicated a promising effect of CR on cancer. A study involving the enrollment of obese persons in a weight loss program based on CR, showed a reduction in their rectal cell proliferation, a biomarker for colon carcinogenesis, suggesting that CR may prevent colon cancer (Steinbach et al., 1994). Another case report using a ketogenic diet that resulted in low blood glucose levels, as seen in caloric restricted animals, showed a decrease in tumor metabolism (Nebeling et al., 1995). These observations are encouraging and suggestive of the clinical potential of CR and merit further research.

FUTURE DIRECTIONS

Considering the robustness of the data regarding the beneficial effect of CR on a diverse range of ailments, further scrutiny of CR methods/application must be sought. As extensive CR is impractical to achieve in humans, studies directed at understanding the mechanism of action of CR are essential. These kinds of studies are required for the identification of effectors or pathways that could possibly be targeted to achieve the beneficial effect of CR. As discussed above, CR might exert its effect on various human ailments through different mechanisms. However, no direct evidence is available. Therefore, the mechanism of action of CR should not be assumed universal and needs to be examined in each disease condition.

The effect of CR on energy balance should not be ignored. There is growing evidence to suggest the association of energy balance including diet, weight, adiposity, and physical activity with tumorigenesis. It has been shown that increased energy expenditure due to increasing physical activities can reduce obesity and might be beneficial in delaying tumorigenesis, at least in some of the mouse models of mammary tumor (Cohen et al., 1988; Thompson et al., 1995; Thompson, 1997; Jakicic and Otto, 2005). However, as results on the tumor inhibiting ability of energy expenditure by exercise are controversial, a more extensive examination is required (Cohen et al., 1992; Gillette et al., 1997; Thompson et al., 1988). Additionally, a detailed and systematic quantitative analysis of the effect of energy intake, energy expenditure and energy balance on tumorigenesis and aging is warranted for the further scrutiny of this process.

One emerging alternative to CR is the use of CRMs. One of the known CRMs, Rapamycin has been shown to be effective in delaying aging as well as tumor growth, two main features of CR benefit. However, this field is still very undeveloped and requires

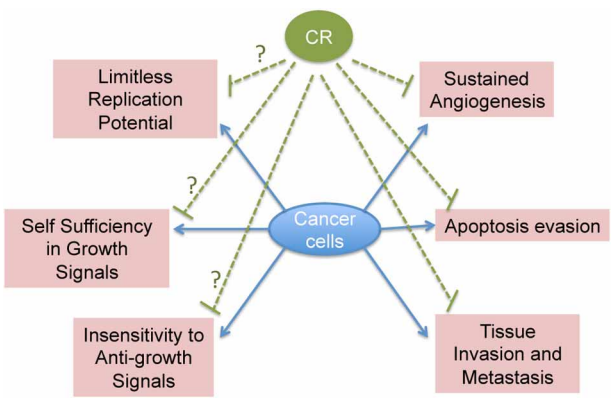

FIGURE 2 | Schematic representation of CR known and potential effects on cancer hallmarks.

more attention. Of course, novel CRMs might be discovered in the course of dissecting CR mechanisms.

Another unexplored area of research is the effect of CR on tumor suppressor and tumor promoter genes. Does CR exert its anti-tumorigenic effect through up-regulation of tumor suppressor genes or down-regulation of oncogenes? Interestingly, the p53 family of tumor suppressors has been shown to negatively regulate the Insulin-like Growth Factor 1 (IGF-1) Receptor (IGFR-1), an important player of the insulin receptor pathway, through which CR has been proposed to exert its action (Bruchim et al., 2009). Though, there is evidence that CR can increase apoptosis in tumor cells and can inhibit angiogenesis and invasive properties of cancer cells, its effect on other hallmarks of cancer is not explored (Mukherjee et al., 2002, 2004, 2008; Zhou et al., 2007; Shelton et al., 2010). Therefore, it would be also interesting to examine the ability of CR to target all the hallmarks of cancer (**Figure 2**).

Another very exciting and unexplored area is that of stem cell biology and CR. Although, a limited number of studies point toward the inhibitory effect of CR on progenitor stem cell proliferation, these need further validation (Yoshida et al., 1997). Finally, studies using human subjects are foremost important for the validation of CR efficacy. In all, we can say that CR is the only natural approach emerging as a conqueror against aging and cancer, and may pave our way toward a healthy prolonged life.

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