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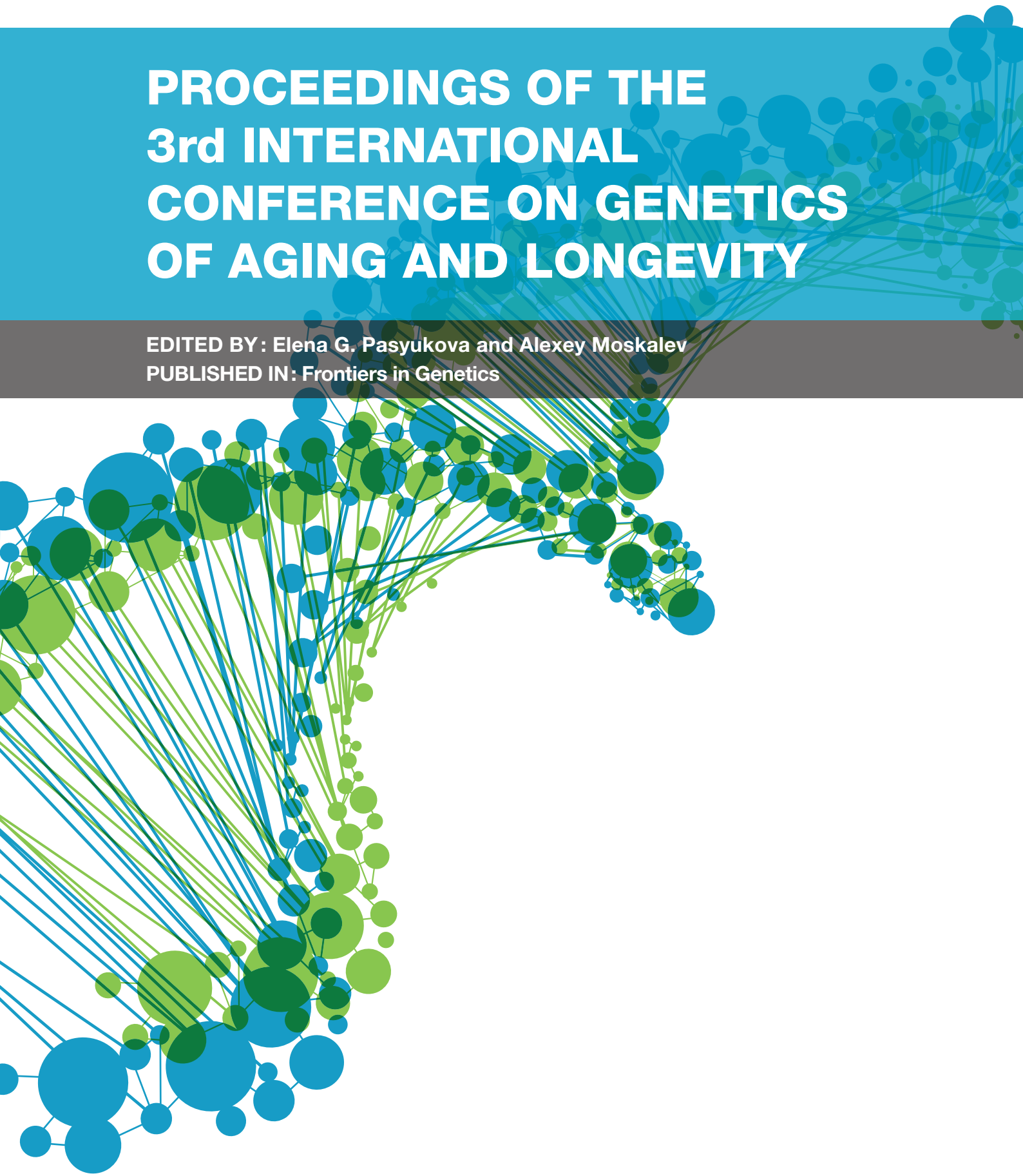

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PROCEEDINGS OF THE 3rd INTERNATIONAL CONFERENCE ON GENETICS OF AGING AND LONGEVITY

Topic Editors:
Elena G. Pasyukova, Russian Academy of Sciences, Russia
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In this book, we present a collection of articles covering a wide range of current aging research and highlighting its prospects and future directions. These articles are based on or related to the topics of the 3rd International Conference «Genetics of aging and longevity». The Conference took place 6-10 April, 2014 in Sochi, the city located on the Black Sea coast near the Caucasian mountains, in Russia. Top gerontologists and geneticists from 31 countries around the world came together to discuss current problems in many areas related to the genetics of longevity and mechanisms of aging. We would like to thank those of them who contributed to this e-Book by sharing latest achievements, ideas and hypotheses. We hope that this e-Book will come to notice of scientists interested in the development of genetics of aging and longevity and in the search for life-beneficial environments and life-prolonging interventions.

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EDITORIAL
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Editorial: Proceedings of the 3rd International Conference on Genetics of Aging and Longevity

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Keywords: redox, telomere shortening, geroprotectors, nervous system, polymorphisms

The Editorial on the Research Topic

Proceedings of the 3rd International Conference on Genetics of Aging and Longevity

In April 2014, the 3rd International Conference ≪Genetics of aging and longevity≫ took place in Sochi, Russia. We are pleased to present a collection of articles based on or related to the topics of the Conference and published in Frontiers in Genetics of Aging in 2015. They reflect understanding complex interactions between genes and genetic pathways underlying aging that is of utmost significance for the future life of humanity. Accordingly, genetic basis of longevity remained the most important topic of the Conference. The role of genes involved in energy supply and mitochondrial integrity and function deserved a close attention in two reviews published in this collection (Morrow and Tanguay; Rogers and Rogina). The importance of redox homeostasis in aging was considered in the paper by Klichko et al. Undoubtedly, energy metabolism governed by various genes and environmental factors, such as calorie intake, represents one of the key players in longevity control. Klichko et al. also demonstrated that temporal regulation of redox status is related to the aging-associated loss of a proper circadian modulation with age. Having joined an interesting extramural discussion, Koliada et al. reviewed the current views on the telomere length as a marker and/or a cause of aging and came to the conclusion that telomere may not serve as a "clock" counting cell divisions, but that telomere shortening, rather, reflects the history of exposure to oxidative stress in cell lineages. Thereto, another article described age-associated alterations in the chromosome morphology in a particular type of cells and indicated molecular mechanisms underlying these changes (Lebedeva et al.).

Aging is defined as a gradual loss of physiological functions accompanied by decreasing fertility and increasing risk of mortality. An important and challenging question for the world-wide scientific community is whether there are means allowing to combat aging and to prolong health span and life span. Therefore, it is not surprising that anti-aging interventions attracted much attention at the Conference and, therefore, a number of articles in this collection were concentrated on possible pharmacological agents aimed to slow down aging (Carretero et al.; Johnson et al.; Semenkov et al.; Sycheva). Of note, effects of treatments on age-related traits, such as weight, as well as on some pathological states, rather than on aging and longevity were often considered (Johnson et al.; Semenkov et al.; Sycheva). In two research papers (Johnson et al.; Semenkov et al.), anti-aging interventions were to apply chemical (rapamycin) and physical (temperature, UV light) agents targeted at TOR signaling and ROS production; the role of vitamins in reducing cellular damage was regarded by Sycheva; five main pharmacological classes of compounds known

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to extend lifespan were considered in the review written by Carretero et al.). Arguments supporting the idea that the nervous system is an important target for anti-aging prophylaxis were also discussed by Omelyanchuk et al. The wide spectrum of agents and targets considered in a restricted number of articles once again sends us a trivial, albeit important message: modulation and fine tuning of multiple molecular mechanisms are needed to ensure longevity. Likewise, analysis of genome-wide screens for polymorphisms significantly associated with human longevity (Yashin et al.) brought authors to the conclusion that genes involved in the life span control affect various molecular functions but, eventually, these functions converge in the development of aging and key age-related pathologies. In the context of the search for means that are able to prolong the life span, Baranova and Willett suggested to pay a close attention to "the world of metabolites," arguing that metabolome might be the most effective target for anti-aging interventions.

Overall, articles from this collection reflect a wide range of current aging research and highlight its prospects and future directions. We hope that they will attract the attention of a broad scientific readership.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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The role of INDY in metabolism, health and longevity

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Indy (I'm Not Dead Yet) encodes the fly homolog of a mammalian SLC13A5 plasma membrane transporter. INDY is expressed in metabolically active tissues functioning as a transporter of Krebs cycle intermediates with the highest affinity for citrate. Decreased expression of the *Indy* gene extends longevity in *Drosophila* and *C. elegans*. Reduction of INDY or its respective homologs in *C. elegans* and mice induces metabolic and physiological changes similar to those observed in calorie restriction. It is thought that these physiological changes are due to altered levels of cytoplasmic citrate, which directly impacts Krebs cycle energy production as a result of shifts in substrate availability. Citrate cleavage is a key event during lipid and glucose metabolism; thus, reduction of citrate due to *Indy* reduction alters these processes. With regards to mammals, mice with reduced *Indy* ($mIndy^{-/-}$) also exhibit changes in glucose metabolism, mitochondrial biogenesis and are protected from the negative effects of a high calorie diet. Together, these data support a role for *Indy* as a metabolic regulator, which suggests INDY as a therapeutic target for treatment of diet and age-related disorders such as Type II Diabetes and obesity.

Keywords: aging, *Drosophila melanogaster*, *Indy*, caloric restriction, longevity genes

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Introduction

The *Drosophila I'm Not Dead Yet (Indy)* gene encodes a plasma membrane transporter of Krebs cycle intermediates with highest affinity for citrate (Rogina et al., 2000; Knauf et al., 2002, 2006). In flies INDY is predominantly expressed in the midgut, which is important for food absorption; the fat body, which modules glycogen and fat storage, and oenocytes (fly liver), which is the site of lipid mobilization and storage (Rogina et al., 2000; Knauf et al., 2002; Frankel and Rogina, 2012; Rogers and Rogina, 2014).Thus, reduction in INDY reduces uptake, synthesis and storage of nutrients and affects metabolic activity. Reduction of *Indy* expression in both flies and worms extends longevity by a mechanism that is reminiscent of calorie restriction (CR), which is an environmental manipulation that extends longevity in a variety of species (McCay et al., 1935; Fei et al., 2003; Wang et al., 2009). Flies with reduced INDY levels experience many of the physiological changes that are commonly observed in CR flies. Such changes include altered lipid metabolism and insulin signaling, as well as enhanced mitochondrial biogenesis and spontaneous activity (Wang et al., 2009; Rogers and Rogina, 2014).

Studies investigating the function of mammalian *Indy* (*mIndy*) show the highest levels of expression in the liver and brain (Inoue et al., 2002). Similar to the trend of *Indy* expression in flies, mRNA levels were found to change during starvation in rat hepatocytes and mice liver. Furthermore, studies in $mIndy^{-/-}$ mice show similar effects in mitochondrial function, as well as lipid and glucose metabolism in the liver as those previously described in less complex organisms and in mice on CR (Fei et al., 2004; Wang et al., 2009; Birkenfeld et al., 2011). Together, these data

suggest that the level and location of INDY serves to regulate and possibly mediate metabolic responses to nutrient availability during aging.

The SLC13 Family of Transporters

INDY is a member of the SLC13 family of transporters in mammals, invertebrates, plants, and bacteria (Pajor, 2006, 2014). This class of transporters has variation in function with three members serving as sodium-coupled transporters for dicarboxylates/citrate (SLC13A2, SCL13A3, SLC13A5) and two members, which transport sulfates (SLC13A1 and SLC13A4). In mammals, SLC13A2 is mostly expressed on the apical membranes of the renal proximal tubule and small intestinal cells. Its primary function is to provide the energy required for normal cell function and balance of urinary citrate levels. SLC13A3 is expressed in a variety of tissues such as liver, brain, placenta, kidney, eye, and pancreas. SLC13A3 has a primary role in nutrient absorption, as well as drug and xenobiotic excretion. Finally, SLC13A5 (*mIndy*) has the highest sequence and functional similarity to *Drosophila Indy*. There is 34% identity and 50% similarity between the predicted INDY protein in flies and the human and rat sodium dicarboxylate transporter. In flies INDY is mainly expressed in the midgut, fat body and oenocytes (Rogina et al., 2000; Knauf et al., 2002; Frankel and Rogina, 2012; Rogers and Rogina, 2014). Similar to the metabolic tissue expression patterns found in fly tissue, the worm homolog of INDY is most robustly expressed throughout the intestinal tract (Fei et al., 2004). In mammals, INDY is predominantly expressed in the liver, testis, and brain, although expression is also found in the testis, placenta and kidneys (Yodoya et al., 2006; Pajor, 2014).

Physiological studies in Xenopus oocytes indicate that fly INDY is a plasma membrane exchanger for Krebs cycle intermediate with highest affinity for citrate, but can also transport succinate, oxaloacetate, fumarate, and α-ketoglutarate (Knauf et al., 2002, 2006). In flies, INDY can exchange dicarboxylates for citrate and a proton during an electro-neutral and Na^+-independent processes (Knauf et al., 2006). On the other hand, worm, bacterial and mINDY mediate transport of citrate in exchange for Na^+ (Pajor, 2014). Stoichiometric analysis of mINDY reveals 11 transmembrane domains with an exchange rate between Na^+:citrate of 4:1 (Inoue et al., 2003; Pajor, 2014).

Mutational analysis identify specific highly conserved amino acid motifs required for Na^+ ion binding, which is subsequently essential for citrate binding. Mutations in this region in either of the two sodium-binding domains of SLC13A5 are associated with autosomal-recessive epileptic encephalopathy with seizures in neonates (Thevenon et al., 2014). It has been speculated that such a severe phenotype is most likely due to the inability of mutated SLC13A5 to bind sodium, which is required for transportation of citrate across the plasma membrane (Thevenon et al., 2014). Similarly, the crystal structure of a bacterial INDY homolog demonstrates that a 1:1 interaction between Na^+ and citrate facilitate binding to conserved amino acids motifs. Binding induces conformational changes that mediate substrate translocation across the membrane (Mancusso et al., 2012).

INDY Reduction Extends Longevity

INDY reduction in flies and two different worm homologs extends longevity (Rogina et al., 2000; Fei et al., 2003, 2004; Toivonen et al., 2007; Wang et al., 2009; Rogina and Helfand, 2013; Rogers and Rogina, 2014). We have described effects of a P-element or a GFP protein-trap insertion on fly longevity in twelve $Indy^{206}$ alleles and described a relationship between longevity extension and the degree of *Indy* mRNA reduction (Rogina et al., 2000; Wang et al., 2009; Rogina and Helfand, 2013; Rogers and Rogina, 2014). In several of the *Indy* alleles, the P-element is inserted within the Hoppel element in the first intron of the *Indy* gene, upstream of the putative translation start site. Several other *Indy* alleles have the P-element inserted upstream from the putative transcriptional start site, which reduce *Indy* transcription and also yield longevity extension (Rogina et al., 2000; Wang et al., 2009; Rogina and Helfand, 2013; Rogers and Rogina, 2014). Further investigation with various *Indy* alleles revealed the extent to which *Indy* alleles were capable of extending lifespan was dependent upon the degree of *Indy* mRNA reduction. When *Indy* levels are reduced about 50% as in $Indy^{206}$ or $Indy^{YC0030}$ heterozygous flies, life of the flies is maximally extended, by up to 100%. Accordingly, moderate *Indy* reduction has modest beneficial effect on longevity of ∼17%, as seen in $Indy^{EY1442}$, $Indy^{EY01458}$, and $Indy^{EY13297}$ heterozygous male flies (Rogina and Helfand, 2013). Interestingly, there appears to be a threshold for *Indy* reduction as dramatic reduction of *Indy* mRNA, as in $Indy^{206}/Indy^{206}$ homozygous flies, reduces beneficial effects on longevity to about 20%, which is thought to induce a state of starvation (Wang et al., 2009). Such longevity extension was observed in multiple genetic backgrounds but not all, stressing the importance of genetic background in longevity studies (Toivonen et al., 2007; Wang et al., 2009; Rogina and Helfand, 2013).

Recent reports extended studies on the effects of *Indy* reduction to natural populations (Zhu et al., 2014). The authors found that natural population of flies heterozygous for insertion of the Hoppel element experience beneficial effects on fly reproduction and longevity (Zhu et al., 2014). This is consistent with data showing that under standard laboratory conditions heterozygous $Indy^{206}$ and $Indy^{302}$ flies laid more eggs during their life compared to control (Marden et al., 2003). Furthermore, INDY reduction does not affect maximal flight velocity, negative geotaxis or resting metabolic rate in heterozygous $Indy^{206}$ and $Indy^{302}$ flies (Marden et al., 2003). Together these data highlight the varied and diverse benefits associated with INDY reduction.

Reduced INDY Mimics Calorie Restriction

Many phenotypes associated with *Indy* reduction are reminiscent of physiological changes induced by CR, which range from starvation sensitivity to changes in fecundity (Bross et al., 2005; Guarente, 2008; Wang et al., 2009; Birkenfeld et al., 2011; Rogers and Rogina, 2014). In that likeness, it has been shown that caloric content of fly diet affects *Indy* mRNA levels showing reduced *Indy* during CR and no further longevity extension when *Indy* mutant flies are on CR (Wang et al., 2009; Rogers and

Rogina, 2014). Similar to findings in flies with reduced INDY, *mIndy*$^{-/-}$ mice have increased hepatic mitochondrial biogenesis, increased insulin sensitivity, and are protected from the adiposity when kept on high fat diet. Whole-genome microarray analysis comparing *mIndy*$^{-/-}$ and *mIndy*$^{-/+}$ revealed that 80% of the differences that were observed in these mice were not only related to transcriptional regulatory pathways but also strikingly similar to differences previously described in CR and *ad libitum*-fed mice (Birkenfeld et al., 2011).

Further support that links INDY reduction and CR-mediated longevity is related to the nutrient sensing insulin-signaling pathway. Under standard conditions, insulin signaling is active and allows for downstream activation of FoxO via phosphorylation. When nutrients are scarce, as in CR, insulin signaling is down regulated, which prevents FoxO phosphorylation and allows for nuclear translocation. Similar to CR, flies with INDY reduction have predominantly nuclear FoxO localization (Wang et al., 2009). Moreover, *Indy* mutants show reduced levels of *Drosophila* insulin-like peptides (Dilps) dilp2, dilp3, and dilp5 when kept on high caloric diet (HCD), with levels similar to genetic control flies on a CR diet (Wang et al., 2009). Furthermore, *mIndy*$^{-/-}$ mice have increased insulin sensitivity, and are protected from adiposity when kept on high fat diet, which supports a conserved role for INDY in metabolic regulation. The finding that INDY may be interacting with insulin signaling links INDY to a key signaling pathway known to influence aging and metabolic disorders in a variety of species (Kenyon, 2010). Nevertheless, additional studies are merited to connect insulin signaling to longevity that is observed in flies with *Indy* reduction.

The Role of INDY in Metabolism

INDY functions as a citrate transporter during intermediary metabolism, thus its transport activity directly influences downstream events related to citrate metabolism. When citrate levels are high, glycolysis and fatty-acid β-oxidation pathways are down regulated and fatty-acid synthesis pathway is active. Cytoplasmic citrate is cleaved to oxaloacetate and acetyl-CoA by ATP-citrate lyase. Acetyl-CoA can be used for the biosynthesis of fatty acids, triglycerides, low-density lipoproteins and cholesterol. Additionally, cytoplasmic oxalacetate can be converted to malate and transported by the malate transporter to the mitochondria and used for energy production in the Krebs cycle. Cytoplasmic citrate levels are also regulated by the mitochondrial citrate carrier (CIC, SLC25A1), which transports citrate from the mitochondria thereby contributing to cytoplasmic citrate regulation (Gnoni et al., 2009).

Reduced INDY activity in flies and mice alters availability of these substrates during intermediary metabolic processes by reducing citrate transport, subsequently causing reduction of ATP levels (Birkenfeld et al., 2011). Augmenting the ATP/ADP ratio activates AMPK, which, by activating mitochondrial transcriptional co-activator *spargel/dPGC-1*, increases mitochondrial biogenesis to maintain cell energetic requirements (**Figure 1;** Gershman et al., 2007; Guarente, 2008; Neretti et al., 2009; Birkenfeld et al., 2011; Rogers and Rogina, 2014). This is supported by the presence of increased *PGC-1* mRNA levels and mitochondrial biogenesis in *mIndy*$^{-/-}$ mice and the midgut of flies with reduced INDY levels (Birkenfeld et al., 2011; Rogers and Rogina, 2014). Consistent with the role of activated AMPK in insulin sensitivity and lipid metabolism, *mIndy*$^{-/-}$ mice have higher lipid oxidation, reduced lipogenesis and increased insulin sensitivity (Birkenfeld et al., 2011).

Environmental manipulations can mimic aging, such as exposure to HCD or paraquat. It was recently shown that such manipulations also increase levels of *Indy* mRNA in the fly midgut, while CR had the opposite effect (Rogers and Rogina, 2014). Given that INDY transports metabolic intermediates, this finding likely represents a response to higher energetic demands for tissue repair during aging or paraquat treatment so that necessary metabolites can more readily reach the TCA or mitochondria for processing. Further evidence that nutrient availability affects *Indy* gene expression has recently been provided showing altered levels of *mIndy* gene expression in rat hepatocytes following glucagon release during early starvation (Neuschafer-Rube et al., 2014). Glucagon binds to the cAMP-dependent and cAMP-responsive element-binding protein (CREB)-binding site in the promoter region of *mIndy* (Neuschafer-Rube et al., 2014). Overnight fasting induces *Indy* mRNA expression, while prolonged starvation decreases expression levels. This is most likely due to the short half-life of glucagon and the subsequent activation of downstream regulatory feedback loops (Neuschafer-Rube et al., 2014). These findings are consistent with decreased levels of *Indy* mRNA found in the livers of mice after 36 h of starvation. Furthermore, increased INDY expression was found in the rat liver after force-feeding large amounts of olive oil, thus supporting a regulatory role for INDY during metabolic activity (Martinez-Beamonte et al., 2011).

INDY Reduction Preserves Intestinal Stem Cell Homeostasis

Aging is a complex process characterized by a loss of homeostasis (Biteau et al., 2010). Preservation of intestinal stem cell (ISC) homeostasis has become an important factor in extending longevity due to a central role for ISCs in preserving normal midgut functions such as food absorption and protection from microorganisms and toxins. Environmental and genetic manipulations that preserve ISC homeostasis promote healthy aging and extend longevity in flies (Biteau et al., 2010). ISC proliferation is significantly increased in the aged intestine due to age-related ROS accumulation, chronic stress, or injuries, which trigger signaling pathways responsible for initiating cell division. However, in the aging midgut, ISCs differentiate at a slower rate, generating non-functional cell populations that are unable to replace damaged midgut cells.

Recently we described a role for INDY in the midgut during intestinal regeneration. INDY is robustly expressed in midgut tissue. Reduce *Indy* levels are associated with dramatic extension of lifespan accompanied by enhanced metabolic activity. We have shown that reduction in *Indy* mediates changes in intermediary metabolism that preserves ISC regenerative homeostasis and intestinal integrity by modulating *dPGC-1*

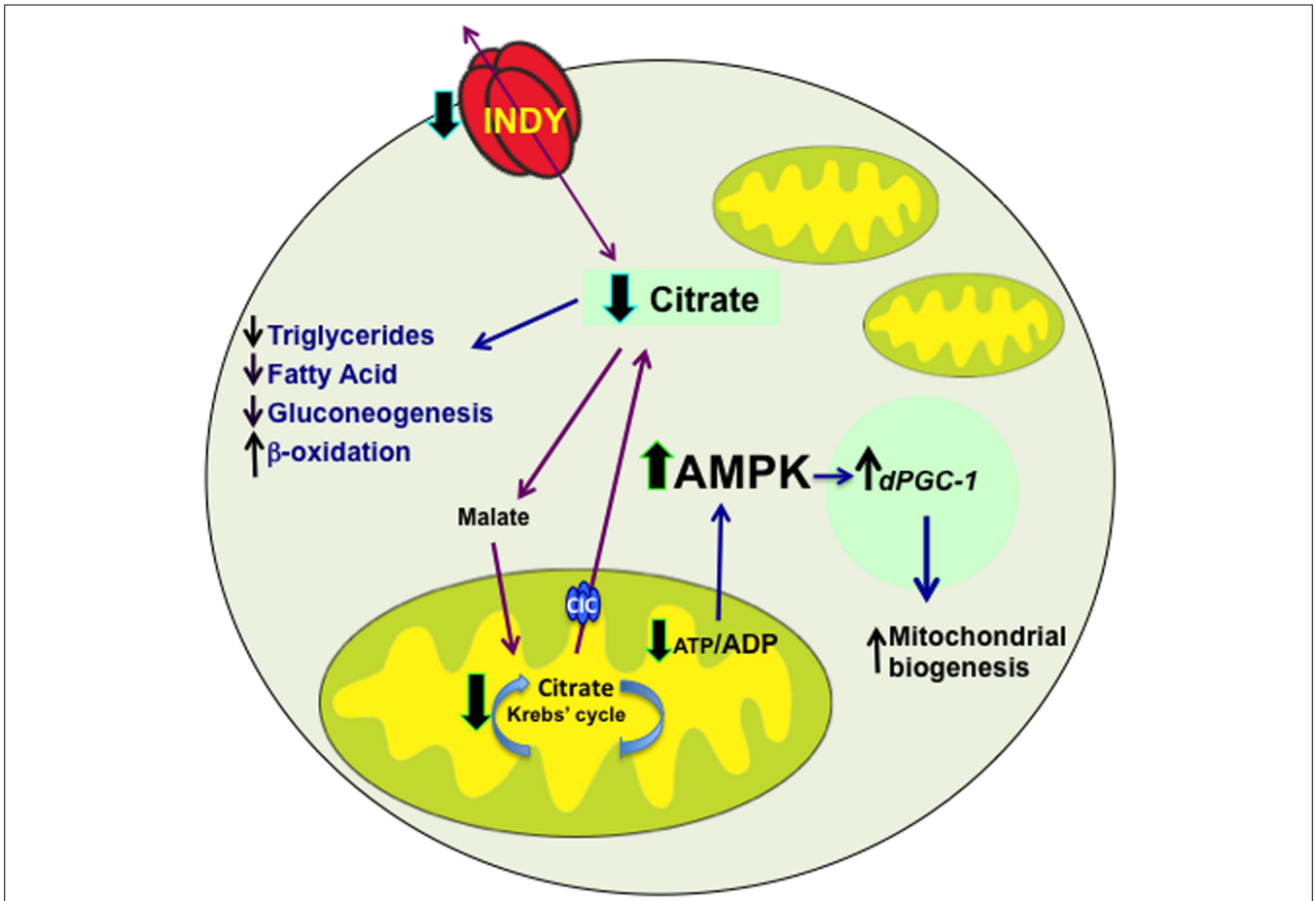

FIGURE 1 | INDY transport activity affects metabolism. INDY reduction decreases cytoplasmic citrate levels, which results in decreased synthesis of triglycerides, fatty acids and reduced gluconeogenesis, but increased β -oxidation. Citrate is converted to malate and transported to mitochondria, where when broken down to oxaloacetate, can enter Krebs' cycle. The Citrate Carrier (CIC, SLC25A1), which is located on the inner mitochondrial membrane transport mitochondrial citrate to cytoplasm. Reduced levels of INDY decrease production of ATP, which activate AMPK. Activated AMPK promotes mitochondrial biogenesis by increasing *dPGC-1* transcription. Blue arrows indicate downstream effects; whereas, black arrows represent changes in expression, red arrows indicate transport of citrate and malate.

activity (Rogers and Rogina, 2014). Flies with reduced *Indy* mRNA levels have significantly higher *dPGC-1* mRNA levels in the midgut throughout lifespan compared to genetic controls, which exhibit an age-related reduction in the levels of *dPGC-1*. Not only do these conditions promote oxidative stress resistance, but also preserve the redox environment of ISCs, which subsequently preserves proliferative homeostasis and intestinal integrity. Without functional *dPGC-1*, *Indy* mutant flies do not show beneficial changes in mitochondrial physiology, ROS production or intestinal integrity, suggesting that *dPGC-1* mediates some of the regulatory effects of *Indy*-mediated healthspan.

The relationship between *dPGC-1* and *Indy* also has a large impact on *Drosophila* longevity. We showed that *Indy* and *dPGC-1* longevity pathways overlap, which was supported by the observation that flies hypomorphic for *Indy* and *dPGC-1* have a lifespan similar to controls (Rogers and Rogina, 2014). Such findings confirm that *dPGC-1* must be present and functional in order to confer lifespan extension in *Indy* alleles. This is consistent with recent work that has revealed a role for the *dPGC-1* in ISC homeostasis, establishing a connection between mitochondrial function and tissue homeostasis (Rera et al., 2011; Rogers and Rogina, 2012). Our work has extended this finding by suggesting INDY as an upstream regulator of *dPGC-1* activity (Rogers and Rogina, 2014). We suggest that reduced *Indy* increases *dPGC-1* activity, which promotes mitochondrial biogenesis and changes the redox environment of the *Indy* mutant midgut. Such changes preserve tissue homeostasis and ultimately mediate lifespan extension.

Summary

Longevity studies in worms and flies demonstrated that reduction in *Indy* gene activity is comparable to CR and associated with a longer and healthier life by affecting energy production. Similarly, knockdown of $mIndy^{-/-}$ in mice mimics CR by altering metabolic activity in the liver (Birkenfeld et al., 2011). The recent work completed by our lab and others support a role for

INDY as a regulator of metabolism whose transcriptional levels change in response to calorie content of the food, as well as in response to energetic requirements of the organism (Willmes and Birkenfeld, 2013; Rogers and Rogina, 2014). The similar effects of INDY reduction on metabolism in flies, worms, and mice suggest an evolutionary conserved and universal role of INDY in metabolism. Together, these findings suggest that INDY could be potentially used as a drug target for treatment of obesity and Type II Diabetes in humans. Further investigation on the mechanism of INDY reduction could provide valuable information regarding the means to a healthier and more productive life.

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Drosophila melanogaster Hsp22: a mitochondrial small heat shock protein influencing the aging process

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Mitochondria are involved in many key cellular processes and therefore need to rely on good protein quality control (PQC). Three types of mechanisms are in place to insure mitochondrial protein integrity: reactive oxygen species scavenging by anti-oxidant enzymes, protein folding/degradation by molecular chaperones and proteases and clearance of defective mitochondria by mitophagy. *Drosophila melanogaster* Hsp22 is part of the molecular chaperone axis of the PQC and is characterized by its intra-mitochondrial localization and preferential expression during aging. As a stress biomarker, the level of its expression during aging has been shown to partially predict the remaining lifespan of flies. Since over-expression of this small heat shock protein increases lifespan and resistance to stress, Hsp22 most likely has a positive effect on mitochondrial integrity. Accordingly, Hsp22 has recently been implicated in the mitochondrial unfolding protein response of flies. This review will summarize the key findings on *D. melanogaster* Hsp22 and emphasis on its links with the aging process.

Keywords: Hsp22, *Drosophila melanogaster*, aging, mitochondria, mitochondrial unfolding protein response

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Introduction

Aging is associated with a decline in protein homeostasis (proteostasis) that leads to the accumulation of deleterious protein damages. Mitochondrial proteins are particularly prone to accumulate damages due in part to the close proximity of the ETC. To circumvent deleterious accumulation of protein damages, three types of mechanisms are involved in mitochondrial PQC (reviewed in Kotiadis et al., 2014). The first line of defense comprises both anti-oxidants enzymes that scavenge ROS produced as by-product of the ETC and molecular chaperones and proteases that insure protein folding or degradation of damaged proteins (reviewed in Bozaykut et al., 2014). The third mechanism of mitochondrial PQC involves the clearance of highly damaged mitochondria through mitophagy (reviewed in Osellame and Duchen, 2014; Scheibye-Knudsen et al., 2015).

Heat shock proteins are molecular chaperones found in all organisms. They are subdivided in distinct families based on their molecular weight and sequence homology: HSP110, HSP90, HSP70, HSP60, HSP40, and sHSP. Each HSP family has specific functions that have been the subject of different reviews (Vos et al., 2008; Priya et al., 2013; Ryabova et al., 2013; Saibil, 2013; Karagoz and Rudiger, 2015). The sHSPs are characterized by the presence of the alpha-crystallin domain and are at the crossroad between two main process namely protein

Abbreviations: ETC, electron transport chain; HSE, heat shock element; HSP, heat shock protein; mtUPR, mitochondrial unfolding protein response; PQC, protein quality control; ROS, reactive oxygen species; sHSP, small heat shock protein.

folding and degradation. Indeed, most sHSPs have the ability to prevent protein aggregation and to maintain their client in a refoldable state hence preventing them to form deleterious interactions (reviewed in Haslbeck et al., 2005; McHaourab et al., 2009; Fu, 2014; Treweek et al., 2015). Additionally, some sHSPs are involved in protein degradation via the proteasome and autophagy (Goldbaum et al., 2009; Bissonnette et al., 2010; Acunzo et al., 2012).

In *Drosophila melanogaster* there are 12 members of the sHSP family that have different chaperone ability, distinctive intracellular localization and cell- and stage-specific pattern of expression (Michaud et al., 2002; Vos, 2009; Morrow and Tanguay, 2015). Almost all of them are stress-inducible, but only seven have been shown to be up-regulated during aging (CG14207, l(2)efl, Hsp67Bc, Hsp22, Hsp23, Hsp26, and Hsp27; King and Tower, 1999; Zou et al., 2000; Landis et al., 2004, 2012; Wang et al., 2005; Girardot et al., 2006; Tanguay and Morrow, 2008; Yang and Tower, 2009). Among these sHSPs, the link between Hsp22 and aging is particularly interesting due to its peculiar mitochondrial matrix localization (Morrow et al., 2000) and given the central role of mitochondria in the aging process (Hill and Van Remmen, 2014; Ziegler et al., 2014). Mitochondria are involved in different metabolic and signaling pathways (ATP production, amino acid catabolism, fatty acid β-oxidation, apoptosis among others) and are in constant communication with the nucleus to adjust to metabolic demand (Haynes et al., 2007; Haynes and Ron, 2010; Runkel et al., 2014). While ROS produced by mitochondria have been at the center of the free radical theory of aging (Harman, 1956), recent reports are now showing that increased ROS production is not always harmful and can even promote longevity (Van Raamsdonk and Hekimi, 2009, 2012; Yee et al., 2014). In recent years, multiple factors have been shown to contribute to aging by favoring accumulation of dysfunctional mitochondria such as impairment of mitochondria-to-nucleus signaling, changes in mitochondrial dynamics (fusion/fission) and clonal amplification of mitochondrial DNA mutations (Bereiter-Hahn, 2014; Hepple, 2014; Ziegler et al., 2014). A failure to maintain mitochondrial homeostasis and integrity is therefore associated with aging (Bratic and Larsson, 2013; Bohovych et al., 2014) and accordingly, the maintenance of mitochondrial stress response has gained recognition as a potential pro-longevity mechanism (Hill and Van Remmen, 2014; Scheibye-Knudsen et al., 2015).

Hsp22 is Preferentially Up-Regulated During Aging

As a member of the sHSP family, Hsp22 is readily up-regulated by a variety of different stresses (Colinet et al., 2010; Hirano et al., 2012; Landis et al., 2012; Morrow and Tanguay, 2015) but its developmental expression pattern is tightly regulated. Indeed, during development its expression is restricted to the metamorphosis of larvae to pupae (Michaud et al., 2002). However, during adulthood Hsp22 is the most up-regulated sHSP, the induction of its mRNA reaching up to 60% in the head of 30 days-old flies comparatively to 6 days-old flies (King and Tower, 1999; Yang and Tower, 2009; Landis et al., 2012). Since *hsp22* mRNA is post-transcriptionally regulated, the protein was only detected starting at 40 days of age in these flies (King and Tower, 1999), and the resulting increase was of $\geq$150%.

Interestingly, fly strains genetically selected for their increased longevity display increased *hsp22* mRNA at the beginning of adulthood comparatively to short-lived strains (Kurapati et al., 2000; Zhao et al., 2005b). These flies were also more resistant to heat-shock and were shown to have a quicker heat-shock response than short-lived flies (Zhao et al., 2005b) suggesting a beneficial role of Hsp22 during aging (Kurapati et al., 2000; Zhao et al., 2005b). This was further confirmed by over-expression and down-regulation studies (see Hsp22 Over-Expression Increases Longevity and Resistance to Stress and Absence of Hsp22 Expression Decreases Lifespan and Resistance to Stress, Morrow et al., 2004a,b). This positive correlation between the *hsp22* mRNA level and lifespan likely indicates a more effective stress response and is consistent with a report showing a positive correlation between the level of induction of a *shsp* reporter in response to stress and the remaining lifespan in *Caenorhabditis elegans* (Rea et al., 2005; Yang and Tower, 2009).

Hsp22 Expression Partially Predicts the Remaining Lifespan of Flies

Due to its stress-inducibility (Colinet et al., 2010; Hirano et al., 2012; Landis et al., 2012; Morrow and Tanguay, 2015) and to the fact that the onset of Hsp22 protein induction is near the beginning of the period of rapid death in the fly population (King and Tower, 1999), the ability of Hsp22 to be an aging biomarker was investigated using transgenic flies expressing the green fluorescent protein (GFP) driven by an *hsp22* promoter (*hsp22*-GFP; Yang and Tower, 2009). It was shown that in a given strain, flies that were robustly expressing the *hsp22*-GFP transgene at younger adult age than their counterpart tended to die sooner. In this case, the abnormal level of *hsp22*-GFP transgene expression would be indicative of the high level of stress experienced by a given individual and would represent that particular individual's susceptibility to stress and failing homeostasis and as such could serve as a stress biomarker announcing imminent mortality (Yang and Tower, 2009). While this may seem contradictory to other studies on the beneficial effects of Hsp22 on longevity (Kurapati et al., 2000; Morrow et al., 2004b; Zhao et al., 2005b), it may only represent the importance to express Hsp22 early in development to observe its effect on lifespan. Unfortunately no data on mRNA/protein level of *hsp22* are available for the long-lived strains harboring increased levels of *hsp22* mRNA early in development (see Hsp22 is Preferentially Up-Regulated During Aging, Kurapati et al., 2000; Zhao et al., 2005b). However, studies using over-expression of Hsp22 have clearly shown that it must be expressed before 4 days of age to confer an increased longevity (Bhole et al., 2004; Morrow et al., 2004b). Moreover, the sensitivity of GFP detection technique may not favor the detection of weak/transient *hsp22*-GFP expression and therefore emphasis on more robust

expression. In the same way, Hsp22 protein expression driven in adult motorneurons was not observable by western blots of whole fly homogenate but was still able to mediate lifespan increase (Morrow et al., 2004b). Together these data suggest that the cell-types in which Hsp22 is expressed and the timing of its expression are important factors in its beneficial effect on aging and that robust expression of Hsp22 at the whole organism level may reflects intensive stress and failing homeostasis.

Hsp22 Expression is Modulated by Factors Influencing Longevity

In the course of understanding the aging process, different proteins, and pathways have been shown to influence lifespan. Interestingly, in some cases the modulation of Hsp22 expression was also reported.

dFoxo as a Regulator of Hsp22 Expression

The up-regulation of Jun-*N*-terminal Kinase pathway and the down-regulation of the insulin/IGF pathway converge to the transcription factor Foxo to increase lifespan and stress tolerance (Tatar et al., 2001; Wang et al., 2003, 2005; Accili and Arden, 2004; Giannakou and Partridge, 2007). In *D. melanogaster*, dFoxo has been shown to regulate the expression of Hsp22 together with Hsp23, CG14207, l(2)efl, Hsp70, Hsp40, Hsp90, and Hop (Wang et al., 2005; Harvey et al., 2008; Hull-Thompson et al., 2009; Demontis and Perrimon, 2010). Hsp22, Hsp23, and l(2)efl increase longevity upon over-expression and it was therefore proposed that they are, at least in part, involved in the lifespan extension mediated by dFoxo (Morrow et al., 2004b; Wang et al., 2005; Tanguay and Morrow, 2008). Accordingly, it was shown that dFoxo null flies have a reduced lifespan as well as a reduced age-induced expression of l(2)efl (Shen and Tower, 2010) and Hsp22 (Morrow and Tanguay, 2015).

Hsp22 Expression is Coordinated with the Life Promoting Protein dDnmt2

In flies, dDnmt2 is the only DNA methyl transferase known up to now. Contradictory with its name dDnmt2 has a relatively poor DNA methyl transferase activity, but it has, however, a rather robust tRNA methyl transferase activity (Schaefer and Lyko, 2010; Schaefer et al., 2010). One of the functions of dDnmt2 is to protect stress-induced cleavage of tRNA in stress granules (Schaefer et al., 2010) and it would also be a life determination gene since it increases lifespan and resistance to oxidative stress upon over-expression and decreases *Drosophila* lifespan when down-regulated (Lin et al., 2005; Schaefer et al., 2010). Interestingly, only Hsp22, Hsp23, and Hsp26 (and no other life promoting genes such as Inr, chico, metuselah, and SOD) were shown to be expressed similarly to dDnmt2 (i.e., up-regulated when dDnmt2 is over-expressed or down-regulated when dDnmt2 expression is decreased) suggesting that the lifespan determination of dDnmt2 is interconnected with sHSP expression (Lin et al., 2005).

Hsp22 Expression is Influenced by Histone Methylation and Acetylation

Histone post-translational modifications are known to control gene transcription. Among the enzymes regulating modifications of histones, the histone demethylase KDM4A has been suggested to regulate longevity gene expression. Indeed, the depletion of KDM4A has been shown to induce cellular senescence in normal fibroblasts (Mallette and Richard, 2012) and to decrease lifespan in flies (Lorbeck et al., 2010). Interestingly, the most down-regulated gene in short-lived KDM4A flies was *hsp22* (Lorbeck et al., 2010). A link between Hsp22 expression and histone acetylation has also been observed in flies. Indeed, inhibition of histone deacetylase by trichostatin and sodium butyrate was shown to increase lifespan and promote *hsp22* and *hsp70* expression (Zhao et al., 2005b). In this case, the binding of hyperacetylated histone H3 at both promoters was shown to increase accessibility of HSEs to the heat shock factor (Zhao et al., 2005a).

Hsp22 has a Protective Role During Lifespan

The beneficial role of Hsp22 during aging was shown by over-expression and down-regulation studies in flies and was also demonstrated in human cells.

Hsp22 Over-Expression Increases Longevity and Resistance to Stress

Using the Gal4-UAS system, it was shown that over-expression of Hsp22 either ubiquitously with the actin driver or in motorneurons with the D42 driver increases resistance to heat and oxidative stresses and longevity by up to 30% (Morrow et al., 2004b). Moreover, flies over-expressing the sHSP maintained their locomotor activity for a longer time suggesting that Hsp22 over-expression increases the health-span (Morrow et al., 2004b). While the beneficial effect of Hsp22 over-expression on lifespan is clear in this system, the timing of its expression is very important. Indeed, over-expressing the sHSP at the beginning of adulthood instead of the beginning of embryogenesis did not result in any increase of longevity (Bhole et al., 2004).

Absence of Hsp22 Expression Decreases Lifespan and Resistance to Stress

The three HSEs of the *hsp22* promoter are required for the age-induced expression of Hsp22 (King and Tower, 1999). Accordingly, flies that carry a p-element insertion in the *hsp22* promoter (in between the HSEs) lack the sHSP expression during aging and consequently have a decreased longevity and resistance to stress (Morrow et al., 2004a).

Hsp22 Increases Population Doubling in Human Fibroblasts

Ten sHSPs are found in humans and up to now none has been found to reside constitutively inside the mitochondria. Interestingly, over-expression of *D. melanogaster* Hsp22 in primary human fibroblasts extended their lifespan from 58 population doublings to 84 population doublings and this was

accompanied by a lower level of the senescence associated β-galactosidase marker (Wadhwa et al., 2010). While it is clear that Hsp22 was functionally active in human cells, its expression was also shown to increase malignant properties of human cancer cell lines (Wadhwa et al., 2010). In mammals, sHSPs are up-regulated in many different cancer cell types and are often linked to bad prognoses (Boncoraglio et al., 2012; Kampinga and Garrido, 2012). The exact mechanisms by which Hsp22 operate in human cells has not been investigated deeply. However, p53 was shown to co-immunoprecipitate with Hsp22 and accordingly, p53 was found in the mitochondria of Hsp22 over-expressing cells (Wadhwa et al., 2010).

Hsp22 Over-Expression Triggers Changes in Gene Transcription

Consistent with the extent of Hsp22 beneficial effect at the organismal level, Hsp22 over-expression was shown to alter gene transcription. Indeed, transcripts from protein involved in multiple functions were shown to be expressed differently in Hsp22 over-expressing flies, notably genes of the ETC, and genes involved in protein translation (Kim et al., 2010). The mechanism by which a mitochondrial sHSP can alter gene transcription is not clear, but is likely to result from an indirect effect and therefore probably involves other proteins and/or messengers. Mitochondria are in constant communication with the nucleus to adjust gene expression as a response to altered metabolic demand and stress (Haynes et al., 2007; Haynes and Ron, 2010; Runkel et al., 2014). Therefore, rather then initiating a signaling cascade between mitochondria and nucleus, over-expression of Hsp22 may simply modify the mitochondrial status by insuring proteostasis hence influencing mitochondrial function and integrity.

Hsp22 Involvement in the Mitochondrial Unfolding Protein Response

Due to its drastic up-regulation upon mitochondrial protein synthesis disruption and following different types of stress, Hsp22 has been proposed to be involved in the mtUPR together with Hsp60 and mitochondrial Hsp70 (Fernandez-Ayala et al., 2010; Tower, 2014; Tower et al., 2014; Morrow and Tanguay, 2015). The mtUPR is a stress response induced by protein misfolding in the mitochondria that involves mitochondria-to-nucleus signaling (Haynes and Ron, 2010). In *Drosophila* Hsp22 has been proposed to work in an amplification loop of mtUPR since it can influence its own level of expression (Shen and Tower, 2013; Tower et al., 2014). Interestingly, the link between mtUPR and longevity is similar to the one between Hsp22 and longevity since both have to be induced before adulthood to have a positive effect (Bhole et al., 2004; Morrow et al., 2004b; Durieux et al., 2011; Houtkooper et al., 2013). Moreover, both of them have been associated with health-span in *Drosophila* (Morrow et al., 2004b; Hill and Van Remmen, 2014).

Hsp22 Expression Reduces Mitochondrial Metabolism

Using a reporter construct consisting in the promoter of *hsp22* fused to GFP, a cell lineage-specific induction of Hsp22 was reported in oenocytes (liver-like cells) during aging (Tower et al., 2014). Interestingly, two genes were found to increase the preferential expression of the *hsp22*-GFP reporter construct during aging, namely MnSOD and Hsp22 itself (Tower et al., 2014). This link between MnSOD and Hsp22 expression was also observed in another study aimed at identifying the changes in gene expression in long-lived flies over-expressing MnSOD (Curtis et al., 2007). Oenocytes that express *hsp22*-GFP reporter were shown to accumulate less age pigment and to have lower levels of oxidative stress suggesting that Hsp22 could prevent age-induced damages by reducing mitochondrial metabolism (Tower et al., 2014). Interestingly, the reduction of the mitochondrial metabolism by Hsp22 is also supported by preliminary data from our lab that show the down-regulation of multiple isoforms of proteins from the ETC and Krebs cycle among others upon Hsp22 over-expression (Morrow et al., submitted).

Concluding Remarks

The suggested effects of Hsp22 on mitochondria are summarized in **Figure 1**. As mentioned above, Hsp22 over-expression has

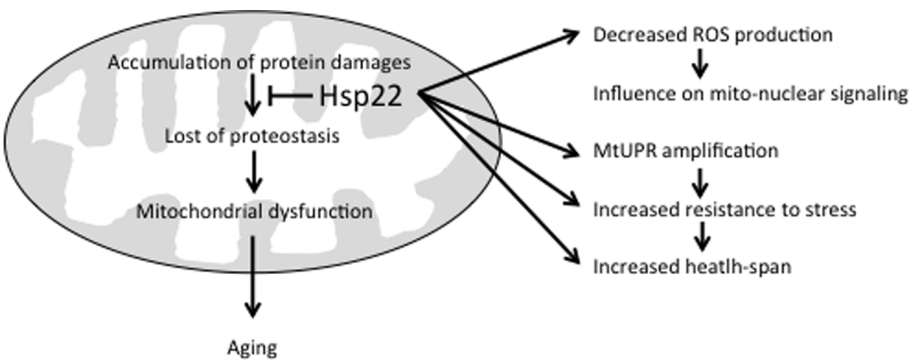

FIGURE 1 | Beneficial effects of Hsp22 on mitochondria. See text for explanation.

been shown to increase resistance to stress and health-span (Morrow et al., 2004a,b). This can be achieved directly by preventing the accumulation of protein damages in mitochondria through its chaperone activity (Morrow et al., 2006) and/or indirectly by amplifying mtUPR signaling and subsequent expression of other chaperones and proteases (Haynes and Ron, 2010; Shen and Tower, 2013; Tower et al., 2014). The fact that a reduced mitochondrial metabolism has been associated with Hsp22 expression suggests that this sHSP may also influence mitochondria-to-nucleus signaling through a decreased ROS production (Tower, 2014; Tower et al., 2014).

Drosophila melanogaster Hsp22 is one of the few sHSPs found inside mitochondria independently of the cellular environment together with plants mitochondrial sHSPs (Waters, 2013) and *C. elegans* Hsp17 (Ezemaduka et al., 2014). The situation in mammals is different as all sHSPs are mainly located in the cytoplasm and shuttles to the different organelles (Nakagawa et al., 2001; van den IJssel et al., 2003; Bryantsev et al., 2007; den Engelsman et al., 2013; Marunouchi et al., 2013). This is notably the case for HSPB1, HSPB2, HSPB5, and HSPB8 that have been shown to shuttle to mitochondria in conditions of oxidative stress (Nakagawa et al., 2001; Jin et al., 2008; Marunouchi et al., 2013). As a general molecular chaperone, Hsp22 may have multiple clients inside the mitochondria and this may account for all the differences observed in flies over-expressing it as well as explain its functionality in orthologous system. Additionally to its role in mitochondrial proteostasis and mtUPR, Hsp22 could help maintain mitochondrial inner membrane integrity in a way similar to what has been observed for the mitochondrial sHSP of *C. elegans*. Indeed, when over-expressed in bacteria, ceHsp17 was shown to maintain cell envelope integrity at lethal temperatures by associating with bacterial inner membrane (Ezemaduka et al., 2014). Other sHSPs such as *Mycobacterium tuberculosis* Hsp16.3, *Synechocystis* Hsp17, and mammalian HSPB5 have been found to be associated with membranes and confer protection (Horvath et al., 1998; Cobb and Petrash, 2000; Torok et al., 2001; Zhang et al., 2005). While we have gained some important clues on the effect of Hsp22 on mitochondrial function, there is still a lot to do to understand exactly how this bona fide chaperone influences longevity and resistance to stress.

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Aging alters circadian regulation of redox in *Drosophila*

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Circadian coordination of metabolism, physiology, and neural functions contributes to healthy aging and disease prevention. Clock genes govern the daily rhythmic expression of target genes whose activities underlie such broad physiological parameters as maintenance of redox homeostasis. Previously, we reported that glutathione (GSH) biosynthesis is controlled by the circadian system via effects of the clock genes on expression of the catalytic (*Gclc*) and modulatory (*Gclm*) subunits comprising the glutamate cysteine ligase (GCL) holoenzyme. The objective of this study was to determine whether and how aging, which leads to weakened circadian oscillations, affects the daily profiles of redox-active biomolecules. We found that fly aging is associated with altered profiles of *Gclc* and *Gclm* expression at both the mRNA and protein levels. Analysis of free aminothiols and GCL activity revealed that aging abolishes daily oscillations in GSH levels and alters the activity of glutathione biosynthetic pathways. Unlike GSH, its precursors and products of catabolism, methionine, cysteine and cysteinyl-glycine, were not rhythmic in young or old flies, while rhythms of the glutathione oxidation product, GSSG, were detectable. We conclude that the temporal regulation of GSH biosynthesis is altered in the aging organism and that age-related loss of circadian modulation of pathways involved in glutathione production is likely to impair temporal redox homeostasis.

Keywords: aging, circadian clocks, redox, glutathione, *Drosophila*

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Introduction

A growing body of evidence suggests that circadian coordination of metabolism, physiology, and neural functions contributes to healthy aging and disease prevention (Reddy and O'Neill, 2010). Indeed, genetic or environmental disruption of circadian rhythms has been shown to lead to premature aging and age-related pathologies (Kondratova and Kondratov, 2012). From a molecular perspective, the clock genes encode a set of transcriptional regulators whose daily fluctuations dictate the rhythmic expression of their target clock-controlled genes (CCGs). Several genome-wide studies performed around the clock suggest that a substantial fraction of genes are expressed rhythmically in different tissues of flies and mice (Hughes et al., 2012; Koike et al., 2012; Rodriguez et al., 2013). CCGs often constitute a rate-limiting step in pathways involved in metabolism, energy balance, DNA-damage repair, and xenobiotic detoxification in both mammals (Claudel et al., 2007; Green et al., 2008; Kang et al., 2009) and *Drosophila* (Hooven et al., 2009; Beaver et al., 2010; Xu et al., 2011). Moreover, clock gene mutations have been shown to have negative consequences in various biological processes and to shorten life span (Kolker et al., 2004; Khapre et al., 2010;

Robertson and Keene, 2013). For example, mice lacking the clock protein BMAL1 (homolog of fly CYC protein) display several symptoms of aging (Kondratov et al., 2006) and show increased neurodegeneration (Musiek et al., 2013). In flies, a null mutation in the clock gene *period* (*per*) leads to shortened life span, accelerated functional decline and increased neuronal degeneration during aging, accompanied by faster and more pronounced accumulation of oxidative damage (Krishnan et al., 2009, 2012). While the connection between the circadian system and aging is relatively well-established, efforts to discern the molecular mechanisms underlying these connections have only recently been initiated.

One of the emerging roles of circadian clocks concerns the regulation of antioxidant defenses and cellular redox (Patel et al., 2014). Studies in *Drosophila* have shown that the levels of reactive oxygen species (ROS) and oxidatively damaged (carbonylated) proteins fluctuate in a daily rhythm in heads of wild type flies and that susceptibility to oxidative challenge is gated by the circadian clock (Krishnan et al., 2008). Recently, a clock-gated response to oxidative injury and regulation of the antioxidant genes was also reported in mice (Pekovic-Vaughan et al., 2014). Circadian regulation was also implicated in oxidative metabolism through rhythmic control of NAD^+ biosynthesis (Peek et al., 2013). Additionally, daily transcriptional rhythms in genes regulating redox and response to oxidative stress have been demonstrated in brain, liver, lungs, and other murine tissues (Wang et al., 2012; Patel et al., 2014). In particular, daily changes in glutathione (GSH) levels, a central player in the antioxidant defense network and redox-sensitive signaling, along with daily changes in the levels of GSH-biosynthetic gene products, were observed in different mammalian organs (Hardeland et al., 2003; Pekovic-Vaughan et al., 2014). These changes were ascribed to clock-gene regulation of GSH synthesis and homeostasis, mediated via the NRF2 signaling pathway (NRF2 signaling; Pekovic-Vaughan et al., 2014) or via the microRNA-controlled rhythms in GSH levels (Kinoshita et al., 2014). We recently reported that the diurnal fluctuations of GSH levels in *Drosophila* were dependent on the rhythmic expression of genes encoding the catalytic (*Gclc*) and modulatory (*Gclm*) subunits of glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH biosynthesis (Beaver et al., 2012). Furthermore, we reported that the expression of *GstD1*, which utilizes GSH in cellular detoxification, is also controlled by the clock, based on the observation that these rhythms were abrogated in arrhythmic clock gene mutants (Beaver et al., 2012).

It is notable that multiple hypotheses of aging postulate an important role for redox state dynamics, and this involvement is buttressed by several lines of evidence. Thus, over-expression of genes, such as GCL, Glucose 6 Phosphate Dehydrogenase, and Peroxiredoxin 5, foster a pro-reducing cellular environment, enhance GSH production and confer strong positive effects on longevity (Orr et al., 2005; Legan et al., 2008; Radyuk et al., 2009). GSH-dependent detoxification responses mediated by glutathione *S*-transferase (GST) activity have also been shown to promote beneficial longevity effects (Sykiotis and Bohmann, 2008). Despite growing evidence that circadian and antioxidant/redox systems both modulate longevity, the possible connections between these systems remain to be fully understood. In order to gain insights into potential links between the clock, redox and aging, we investigated the fate of redox-related rhythms in aging *Drosophila*.

We determined recently that functional clocks are necessary for rhythmic transcription of *Gclc* and *Gclm* and for rhythmicity in GSH levels in young male flies, as the rhythms of these molecules were abolished in the clock mutants cyc^{01} and per^{01} (Beaver et al., 2012). It was also established that clock gene oscillations become significantly reduced in heads of old males (Luo et al., 2012; Rakshit et al., 2012); however, profiles of CCGs have not been studied during aging. To address this question, we investigated how age-related weakening of the clock gene oscillations affects daily profiles of factors involved in glutathione synthesis and metabolism. We report that the rhythms in cellular redox observed in young flies are significantly altered in the heads of old flies, leading to compromised GSH homeostasis.

Materials and Methods

Fly Rearing and Strains

Flies were raised on standard cornmeal-yeast-molasses diet at 25°C under a 12 h light/12 h dark (LD) regimen (where Zeitgeber time (ZT) 0 is time of lights on and ZT12 is time of lights off). Light intensity was kept at ∼1500 lux. Mated males were separated 1–2 days after emergence. Aging males were kept in inverted 8 oz round bottom polypropylene bottles (Genesee Scientific, San Diego, CA, USA) on 35 mm petri dishes (BD Falcon, San Jose, CA, USA) containing 15 ml of diet. Fresh diet was provided three times a week. All experiments were completed using the w^{1118} strain.

Quantitative Real-Time PCR

Fly heads were separated using metal sieves frozen with liquid nitrogen. RNA was extracted from 50 heads, which were homogenized using a Kontes handheld motor in Trizol (Life Technologies, Grand Island, NY, USA) followed by ethanol precipitation. Samples were treated with DNAse (Takara, Mountain View, CA, USA). DNAse was deactivated by phenol/chloroform extraction, and samples were purified with sodium acetate. Synthesis of cDNA was achieved with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Quantitative real-time PCR was performed with Power SYBR Green Master Mix (Life Technologies) on an Applied Biosystems StepOne Plus real-time machine. Primers were obtained from IDT Technology (Coralville, IA, USA). All primers used in this study had efficiencies >96%, and their sequences have been published (Beaver et al., 2012). Data were normalized to *rp49* (Ling and Salvaterra, 2011) as indicated in the results and analyzed using the standard $2^{-\Delta\Delta CT}$ method. Statistics were calculated using GraphPad Prism 6 (San Diego, CA, USA).

Immunoblot Analysis

Samples were collected at 4 h intervals from at least 10 heads obtained from w^{1118} flies separated using sieves frozen with liquid nitrogen, and processed as described Beaver et al. (2012). Briefly, samples in the amount of ∼5 μg of total protein were resolved by PAGE and the immunoblots were developed with antibodies generated against recombinant GCLc and GCLm proteins (Orr et al., 2005) and anti-actin antibodies (MP Biomedicals, Santa Ana, CA, USA) to control for loading. The intensity of signals was analyzed by densitometric scanning, using the digital imaging analysis system with AlphaEase Stand Alone Software (Alpha Innotech Corp., San Leandro, CA, USA).

GCL Enzyme Activity and Free Aminothiols Levels

Glutamate cysteine ligase enzyme activity was measured essentially as described Toroser and Sohal (2005) and Beaver et al. (2012). Linearity of time, protein and substrate concentrations were determined in pilot experiments. GCL activity reaction was performed in duplicate immediately after protein preparation, as suggested (Toroser and Sohal, 2005). The reaction was terminated by adding an equal volume of freshly prepared 10% (w/v) meta-phosphoric acid (MPA). The GCL inhibitor L-buthionine-*S*,*R*-sulfoximine was used to determine specificity of the assay. After GCL reaction, samples were immediately analyzed by HPLC or stored at –80°C for no longer than 24 h before analysis.

Free aminothiol content in fly heads was quantified by HPLC as described Melnyk et al. (1999). Briefly, 50 heads were homogenized in 200 μl of freshly prepared ice-cold 5% MPA, incubated for 30 min on ice and centrifuged at 16000 *g* for 20 min at 4°C. Supernatants were filtered through 0.22 μm PTFE membrane syringe filter and immediately analyzed by HPLC or stored at –80°C. The amount of protein in the precipitate was determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's recommendations (Bulletin 1770 US/EG Rev A, Bio-Rad).

Preparations from GCL assays and free aminothiol extractions were resolved by HPLC under isocratic elution using a reverse-phase C18 Gemini-NX (3 μm, 4.6 × 150 mm) column (Phenomenex, Torrance, CA, USA) with the flow rate of 0.75 ml/min. The mobile phase contains 2% (v/v) acetonitrile, 25 mM monobasic sodium phosphate, 1.5 mM 1-octane sulfonic acid as ion-pairing agent, pH 2.7, adjusted with ortho-phosphoric acid. Aminothiols were detected using the 5600 CoulArray electrochemical detector equipped with four-channel analytical cell (ESA Inc., Chelmsford, MA, USA). Increasing potentials of +100, +200, +750, +850 mV in channels 1–4, respectively, were used for measuring γ-Glu-Cys in GCL activity assay samples. γ-Glu-Cys was detected in channel 3 at +750 mV. Potentials of +550, +600, +750, and +875 mV were used for detection of aminothiols in extracts. Each sample was injected twice. Calibration standards were prepared in 5% (w/v) MPA and injected at regular intervals.

Results

Age-Related Changes in GCLc and GCLm Profiles

To determine how aging affects clock-controlled *Gclc* and *Gclm* transcription, we compared around the clock profiles of these genes in heads of young and old white-eyed flies (*w*, strain w^{1118}). In prior studies involving young Canton S flies we detected strong diurnal patterns in both *Gclc* and *Gclm* mRNA levels and less pronounced, albeit significant rhythmic fluctuations in proteins levels (Beaver et al., 2012). In this study, we confirm that a similar pattern of daily changes in the expression of *timeless* (*tim)*, *Gclc,* and *Gclm* was observed in young w^{1118} flies (**Figure 1**, Beaver et al., 2012). Comparison of young and old ages showed that old flies had a significantly lower peak expression of the clock gene *tim* compared to their young counterparts (**Figure 1A**); this dampening of the amplitude is a typical signature of the aging of the clock mechanism (Rakshit et al., 2012). The young flies had a unimodal rhythm of *Gclc* expression with a distinct peak at ZT20, similar as previously reported in Canton S flies (Beaver et al., 2012). Surprisingly, levels of *Gclc* mRNA were significantly higher in old flies at ZT4, ZT8, and ZT12, thus abolishing the trough that was observed in young flies (**Figure 1B**). The expression of *Gclm* was significantly rhythmic in young flies with elevated expression at ZT 8–16 (**Figure 1C**) similar to that previously reported in Canton S flies (Beaver et al., 2012). In contrast to *Gclc*, expression of *Gclm* did not increase with age, but even slightly decreased reaching significance at ZT16, and the expression remained significantly rhythmic in old flies (**Figure 1C**). Changes in GCLc and GCLm protein expression as well as the GCLc/GCLm ratio in *w* flies paralleled those reported in Canton S flies (**Figures 2A,B** and **3A**, Beaver et al., 2012). Thus, a significant reduction of GCLc was observed at ZT16 in young flies, but old flies showed significantly elevated protein level at this time as well as most other time points (**Figures 2A,C**). In young flies, the levels of GCLm protein were elevated from ZT4 to ZT16, with a significant peak at ZT16, whereas in 50 Da old flies, there was no significant rhythm (**Figures 2B,D**). The relatively weak albeit statistically significant 24-h rhythmic expression patterns of GCLc and GCLm observed in young flies were also reflected in the rhythmic variation in their ratio; likewise absent in old flies (**Figure 3A**).

We next measured the activity of GCL holoenzyme in homogenates of young and old *w* flies collected around the clock. In agreement with GCLc expression patterns (**Figure 2A**) and variations in the GCLc/GCLm ratio (**Figure 3A**), a significant oscillation of GCL enzyme activity was found in young *w* flies (**Figure 3B**) although with a lesser amplitude compared to Canton S flies (Beaver et al., 2012). In old *w* flies, GCL enzyme activity displayed a very different daily pattern than in young flies (**Figure 3B**), and the average enzyme activity was significantly higher (**Figure 3D**). This is consistent with the observation that both GCLc protein levels and the GCLc/GCLm protein ratio exhibited significantly higher levels in the old flies relative to the young ones (**Figures 2A,C** and **3A,C**).

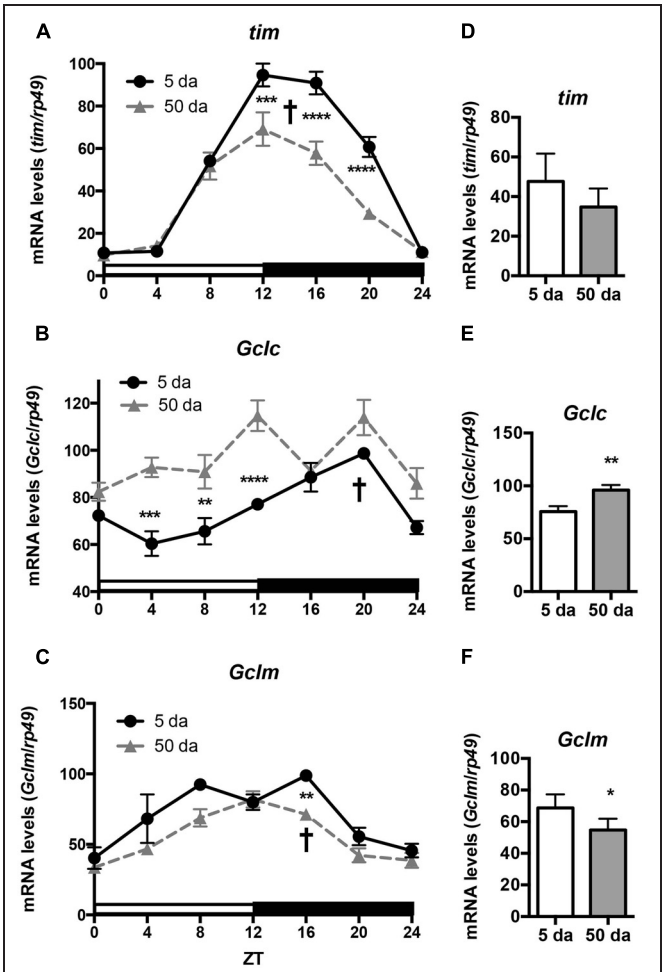

FIGURE 1 | Rhythms in mRNA expression of *Gclc* and *Gclm* show age-related differences. Levels of *tim*, *Gclc*, and *Gclm* mRNA were measured in w^{1118} male heads at ages day 5 and 50 post-eclosion. **(A,D)** Young flies show strong rhythms in transcription of the clock gene *tim*, the rhythm was maintained in old but expression at peak time points was significantly decreased. **(B,E)** Young flies show rhythmic unimodal *Gclc* expression, while in old flies overall mRNA levels are significantly higher at most time points. **(C,F)** Young and old flies show significant rhythms in *Gclm* expression, but overall levels are lower in old. Levels are normalized to the 5 Da peak values set as 100%. Graphics on the right represent average 24-h levels of *tim*, *Gclc,* and *Gclm* mRNA levels in 5 and 50 Da old flies. Significance was analyzed by 2-way ANOVA with Bonferonni post-test; * marks a significant difference between day 5 and day 50 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). † marks a significant difference between minimum and maximum expression in young flies.

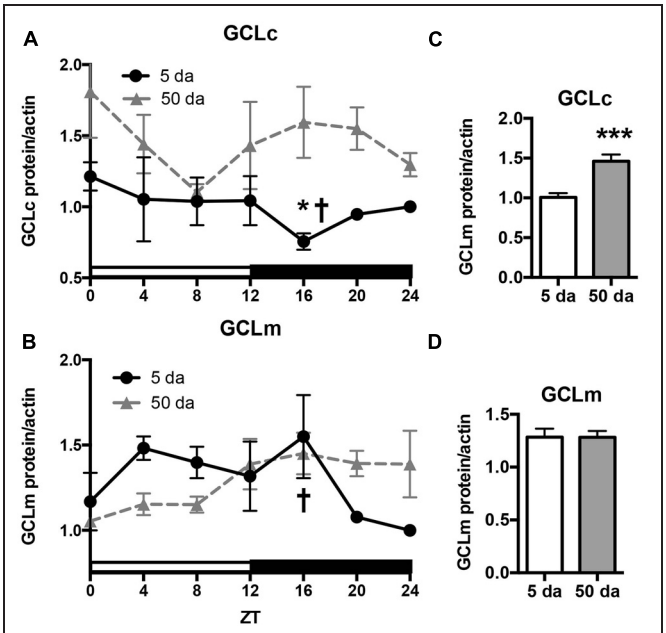

FIGURE 2 | Effects of aging on GCLc (A,C) and GCLm (B,D) protein levels. Protein lysates were prepared from the heads of young (5 Da) and old (50 Da) w^{1118} males and immunoblotted using anti-GCLc and anti-GCLm antibodies followed by re-probing with anti-actin antibodies to control for loading. Each replicate was normalized to the time point with the lowest expression. Data represent average values ± SEM obtained from three independent bio-replicates. Data were analyzed by a 2-way ANOVA and Bonferroni's post-test; * marks a significant difference between day 5 and day 50 (*$p < 0.05$, ***$p < 0.001$). Graphics on the right represent average 24-h levels of GCLc **(C)** and GCLm **(D)** proteins in 5 and 50 Da old flies. † marks a significant difference between minimum and maximum expression in young flies.

Circadian Characteristics of Free Aminothiols Determined in Heads of Young and Old Flies

Previously, we reported that mitochondrial H_2O_2 production and GSH concentrations fluctuate in a clock-dependent manner showing opposite profiles; thus at ZT20, the levels of H_2O_2 in fly heads reached their lowest point and those of GSH were at their highest, defining a pro-reducing peak, while at ZT8 H_2O_2 levels were at their highest and GSH at their lowest (Krishnan et al., 2008; Beaver et al., 2012). Here we show that a significant rhythm in GSH levels is also detected in heads of young *w* flies (**Figure 4A**). In old flies the overall daily levels of GSH were similar to those observed in young flies, although the diurnal oscillations were absent (**Figures 4A,C**). In contrast to GSH, GSSG levels in young flies were relatively low in the morning and continued to decline until ZT8 followed by a significant, approximately twofold increase during the day with a peak at ZT12 (**Figure 4B**). A similar trend was observed in old flies, although the morning GSSG levels did not drop-off as much as they did in young flies, giving rise to a slight albeit non-significant elevation in overall GSSG levels in the heads of aging flies (**Figures 4B,D**, Beaver et al., 2012).

Given that the rhythmicity of GSH levels in young flies was absent in old flies, we also investigated the daily profiles of aminothiols, that are involved in glutathione synthesis and metabolism. Changes in methionine, cysteine, and cysteinylglycine (Cys-Gly) levels were measured in heads of *w* flies collected around the clock. Unlike GSH, the glutathione precursors, cysteine and methionine, as well as Cys-Gly, which is a break-down product of GSH, did not display rhythmic fluctuations in either young or old flies (**Figure 5**). On the other hand, there were statistically significant age-related

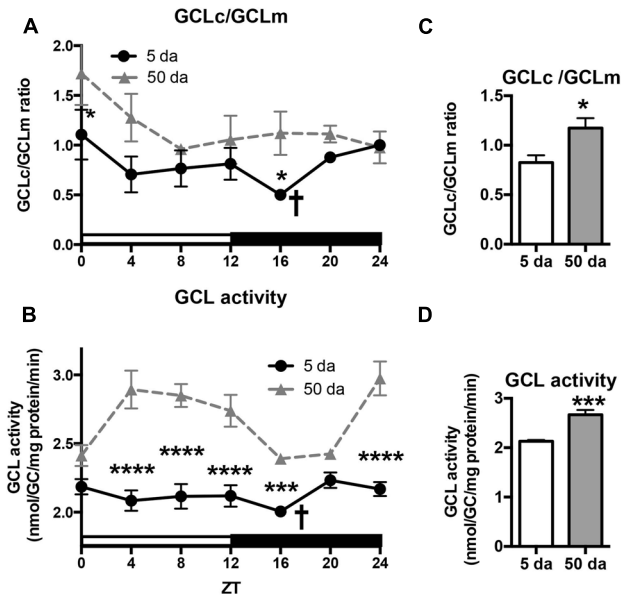

FIGURE 3 | Effects of aging on GCLc to GCLm protein ratio (A,C) and activity of GCL holoenzyme (B,D) in the heads of young (5 Da) and old (50 Da) flies. Samples were prepared from the heads of w^{1118} males. GCL activity was measured by the formation of the GCL product, γ-Glu-Cys (GC). Data represents average values ± SEM obtained from two independent bio-replicates (total $N = 8$). Ratio of GCLc to GCLm proteins was calculated from the results presented in **Figure 2**. Data were analyzed by a 2-way ANOVA and Bonferroni's post-test; * marks a significant difference between day 5 and day 50 (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$). Graphics on the right represent average 24-h levels of GCLc/GCLm protein ratio **(C)** and GCL activity **(D)** in 5 and 50 Da old flies. † marks a significant difference between minimum and maximum expression in young flies.

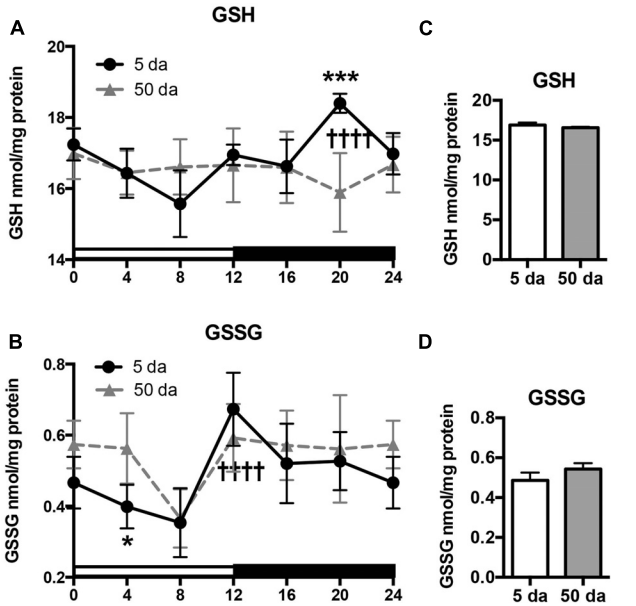

FIGURE 4 | Effects of aging on daily changes in concentrations of GSH (A,C) and GSSG (B,D) in the fly head homogenates. Samples were made from the heads of young (5 Da) and old (50 Da) w^{1118} males. Results are average values ± SEM obtained from three independent bio-replicates (total $N = 6$). Data were analyzed by a 2-way ANOVA and Bonferroni's post-test; * marks a significant difference between day 5 and day 50 (*$p < 0.05$, ***$p < 0.001$). Graphics on the right represent average 24-h levels of GSH **(C)** and GSSG **(D)** in 5 and 50 Da old flies. †††† marks a significant difference ($p < 0.0001$) between minimum and maximum expression in young flies.

differences in these compounds. Levels of free methionine were significantly lower around the clock in old flies (**Figures 5A,D**) while levels of cysteine remained unchanged (**Figures 5B,E**). In contrast to methionine, the levels of Cys-Gly, were significantly higher in the heads of old flies (∼64%, **Figures 5C,F**).

Discussion

In this study we investigated a role for the circadian system in regulating redox in the context of organismal aging. We have determined that the temporal regulation of the redox-active biomolecules, which is modulated by the circadian clocks in young flies, is significantly altered in old flies with dampened clocks. Our results demonstrate that the circadian clocks regulate redox homeostasis via their effects on glutathione biosynthetic pathways, specifically via transcriptional control of the genes involved in *de novo* GSH synthesis, and that age-related reduction of the circadian clock oscillations in old flies is associated with compromised redox rhythms.

Cellular redox homeostasis largely relies on the redox-active compound, glutathione, which is present at concentrations many fold higher compared to concentrations of other redox-active molecules (Rebrin et al., 2004). We had previously shown that the circadian clocks modulate the *de novo* synthesis of GSH via transcriptional control of GCLc and GCLm, the subunits that comprise the GCL holoenzyme (Beaver et al., 2012). In this study we broadened the investigation of the relationship between clock and redox to determine the levels of other redox-active molecules, involved in glutathione synthesis and metabolism. Synthesis of the tri-peptide glutathione, composed of glutamate, cysteine and glycine residues, depends not only on the activity of biosynthetic enzymes, but also on the availability of substrates, where cysteine is a limiting factor. The synthesis of cysteine is mediated by the *trans*-sulfuration pathway, using methionine as the source (Vitvitsky et al., 2003), and this pathway is also active in flies (Kabil et al., 2011). Consequently, we investigated around the clock expression profiles of both cysteine and its precursor methionine. As neither cysteine nor methionine exhibited evidence for diurnal rhythms in either young or old flies, it appears that the contribution of the *trans*-sulfuration pathway to glutathione homeostasis is not regulated by circadian clocks. Consistent with our findings, methionine was found to be arrhythmic in the study of the human metabolome of blood plasma and saliva (Dallmann et al., 2012) although the analyses were performed with healthy but older (57–61 years) males, where age-dependent effects might have influenced the oscillations. In contrast, mouse hepatic metabolome and

transcriptome studies revealed rhythmicity in metabolic subpathways, where oscillations in glutathione were ascertained by oscillations in its precursors, cysteine and methionine, albeit with a lower amplitude for the latter (Eckel-Mahan et al., 2012). In the same study, hepatic rhythmicity was also observed in the concentrations of Cys-Gly, which peaked at 9 h together with cysteine and methionine. In contrast, analysis of the *Drosophila* heads revealed no cycling in the concentrations of Cys-Gly (**Figure 5C**), consistent with the arrhythmic behavior of cysteine and methionine (**Figures 5A,B**). Given that Cys-Gly also serves as a signature of glutathione degradation, interpretation of these results are somewhat tentative. Nevertheless, our results revealed no rhythmicity in cysteine, methionine and Cys-Gly, and suggest that, at least in flies, the pathways responsible for the supply of sulfur-containing precursors for glutathione synthesis are not regulated by the circadian clocks (**Figure 6**). It should be noted that the

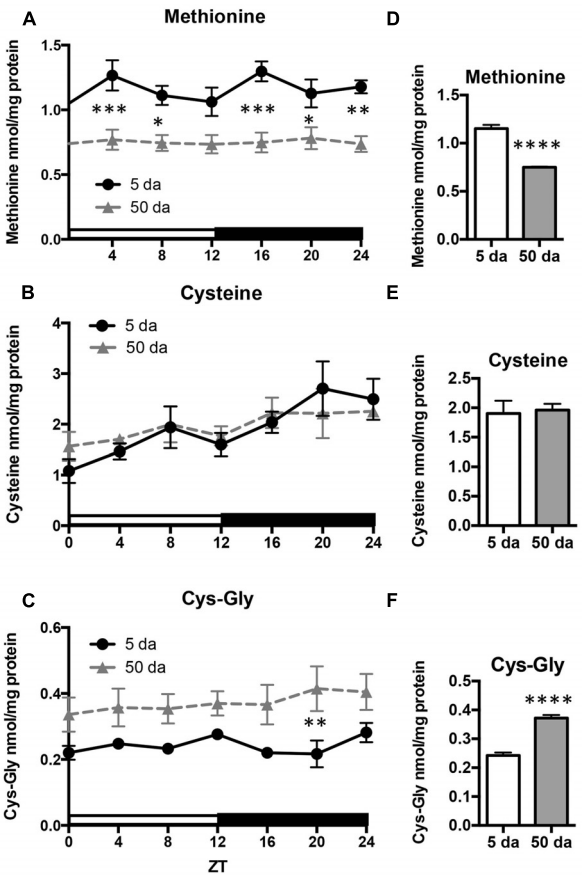

FIGURE 5 | Effects of aging on daily profiles in concentrations of methionine (A,D), cysteine (B,E) and Cys-Gly (C,F) in the fly head homogenates. Samples were made from the heads of young (5 Da) and old (50 Da) w^{1118} males. Results are average values ± SEM obtained from three independent bio-replicates (total $N = 6$). Data were analyzed by a 2-way ANOVA and Bonferroni's post-test; * marks a significant difference between day 5 and day 50 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Graphics on the right represent average 24-h levels of methionine **(D)**, cysteine **(E)**, and Cys-Gly **(F)** in 5 and 50 Da old flies.

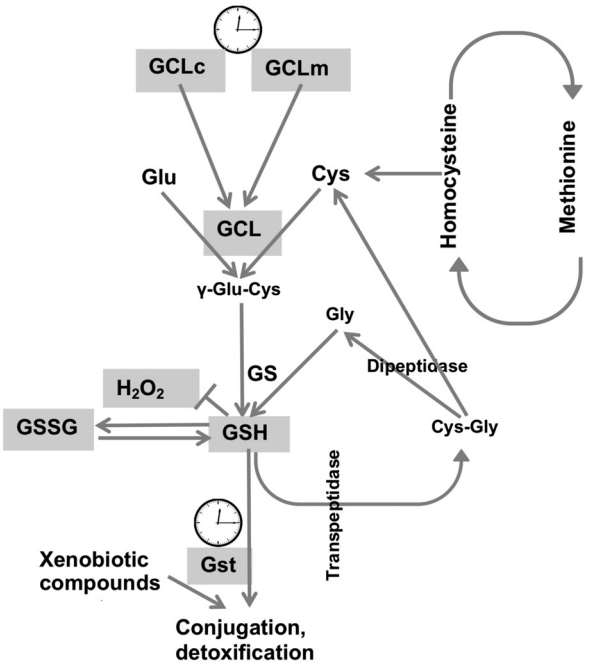

FIGURE 6 | Synthesis and metabolism of glutathione and its regulation by the circadian system. GSH synthesis occurs via two sequential reactions: (i) ligation of L-glutamate and L-cysteine is catalyzed by the activity of GCL, and (ii) the addition of glycine to γ-glutamylcysteine by the activity of glutathione synthetase. GCL is a heterodimer composed of a catalytic subunit (GCLc) and a modulatory subunit (GCLm). Degradation of GSH involves cleavage by *trans*-peptidase, followed by the cleavage of cysteinyl-glycine by a dipeptidase, forming Cys-Gly and cysteine respectively. Cysteine is supplied via *trans*-sulfuration pathway and as a product of GSH degradation. GSH is oxidized by H_2O_2 to its disulphide, GSSG. Methionine is the precursor for cysteine synthesis, in which homocysteine is an intermediate. GSH is also used for detoxification in reactions catalyzed by GSTs, which expression is also rhythmic (Beaver et al., 2012). A clock image denotes genes regulated by the circadian clocks. Cycling components of the glutathione synthesis and utilization systems are shown in gray.

mammalian liver is a homogenous tissue with a strong food-entrained clock mechanism, while fly heads are enriched in nervous tissues with clocks entrained by light-dark cycles. Moreover recent analysis of the circadian transcriptome shows that liver possesses the highest number of rhythmic genes, while brain has the lowest (Zhang et al., 2014).

Another important finding of our study is that the diurnal fluctuations in GSH levels were not followed by similar changes in the products of its degradation (Cys-Gly) and oxidation (GSSG). While Cys-Gly was completely arrhythmic (**Figure 5C**), changes in GSSG profile did not mirror those observed for GSH (**Figures 4A,B**). Even though both shared the same slow drop-off from ZT0 to ZT8 as well as the ZT8 trough, their peaks were quite distinct (ZT12 for GSSG and ZT20 for GSH). Also in old flies, a certain degree of rhythmicity is maintained for GSSG in contrast to the absence of any diurnal GSH patterns.

As mentioned above, circadian oscillations of GSH and GSSG in the mouse liver were recently reported and displayed an opposing pattern at ZT9 where GSH exhibited a trough and

GSSG exhibited a peak (Eckel-Mahan et al., 2012); the decrease in GSH levels was assumed to come largely at the expense of GSSG formation. Our results suggest that this is not the case in *Drosophila* heads, although the observed differences may be related to the specificity of phase of rhythms in different tissues of different species. This differential oscillation of GSH and GSSG also raises some doubt about the functional relevance of the GSH:GSSG ratio and the redox potential in mediating redox-dependent responses, which was recently critically discussed (Flohe, 2013). Indeed, the ratio of GSSG and GSH may reflect little else than the steady state levels of these molecules, which are maintained by independent enzymatic processes of GSH metabolism and synthesis. In this light, concentrations of GSH account for more than 97% of the total non-protein glutathione pool, while GSSG constitutes only ∼3% (**Figure 4**). Consequently, the observed circadian changes in GSSG levels would constitute a trivial change in GSH levels, suggesting that, rather than mediating redox signaling, GSH is predominantly used by antioxidant and detoxification enzymes, e.g., GSTs and peroxidases, as well as in reactions of protein modification (glutathionylation; Manevich et al., 2004; Tew and Townsend, 2012; Zhang and Forman, 2012; Janssen-Heininger et al., 2013).

Another important finding of this study is that the rhythms in glutathione levels observed in young flies were lost in old flies (**Figure 4**), presumably due to the loss of diurnal fluctuations of GCLc, GCLm as well as GCL activity, in response to the weakening of the circadian clocks (**Figures 1–3**). In contrast, GSSG rhythms were largely preserved in older flies, suggesting that the daily changes in glutathione disulfide levels are supported by enzymatic reactions that are not under clock control.

Despite loss of circadian regulation, average daily levels of GSH remained unchanged during aging (**Figure 4C**), while the levels of GSSG were slightly higher, mainly due to lesser drop in the early morning (**Figures 4B,D**). In *Drosophila*, it has been established that whole body GSH levels were either relatively constant (Rebrin et al., 2004) or slightly decreased during aging while GSSG rose 2–3 fold (Rebrin and Sohal, 2006). Similar age-related changes were documented in different mammalian tissues with the most significant reduction in GSH and accumulation of GSSG in the brain, indicative of a more pro-oxidative cellular environment (Suh et al., 2004b; Zhu et al., 2006; Rebrin and Sohal, 2008). As such changes in GSH and GSSG were frequently associated with increases in enzyme activities related to GSH usage, the relatively steady glutathione concentrations observed in the heads of old flies could point to less efficient GSH utilization.

The rather unexpected finding of our study is that the expression of Gclc at both mRNA and protein levels significantly increased in the heads of old flies, and this increase was associated with about 25% higher average daily GCL activity. Despite this increase, the average daily levels of GSH remained unchanged suggesting a loss in GCL catalytic efficiency or an age-related increase in GSH utilization. One possible scenario is that the efficiency of GSH synthesis can be induced by oxidative stress, in part through the well-documented increase in H_2O_2 signaling that accompanies aging (Sohal and Dubey, 1994; Chen et al., 2005; Franklin et al., 2009). For instance, post-translational control of γ-glutamylcysteine (γ-Glu-Cys) synthesis is influenced by oxidative stress, which can dramatically affect formation of GCL holoenzyme and its stabilization (Franklin et al., 2009; Krejsa et al., 2010). Consistent with induction of GCL by stress, we previously reported that *per*-null mutants with disrupted clock displayed arrhythmic as well as elevated GCL activity (Beaver et al., 2012), which was also reflected in arrhythmic and elevated ROS levels relative to the control (Krishnan et al., 2008). It should be noted that previous studies comparing GCL activity and GSH levels in young and old rats showed a decrease of both parameters in liver (Suh et al., 2004b), while in aging brain and heart GSH decreased but GCL activity remained unchanged (Suh et al., 2004a), pointing again to catalytic deficiency of the enzyme.

Other aminothiols that did not show cycling in young flies also remained arrhythmic in old flies, but displayed changes in their steady state levels. Consistent with previous reports, the amounts of Cys-Gly were ∼50% higher in older flies (Rebrin et al., 2004; Rebrin and Sohal, 2006, 2008). Cys-Gly, derived from the breakdown of glutathione, is required for GSH synthesis as a precursor of cysteine, but at the same time it is also a prooxidant generated during the catabolism of glutathione. The requirement of Cys-Gly for GSH synthesis justifies its increase with age, as the tissues require an increased supply of precursors for GSH biosynthesis in older flies. However, we did not observe an increase in cysteine levels during aging. A more plausible explanation is that the increase in Cys-Gly is indicative of an increase in oxidative stress and GSH degradation. In agreement with this view, the average daily levels of methionine were about 35% lower in old flies suggesting the likelihood of an increase in oxidation of methionine to methionine sulfoxide by ROS rather than an increase in methionine consumption for cysteine biosynthesis. Together, these changes indicate a shift in redox homeostasis in the heads of older flies, consistent with the earlier reports in whole flies (Rebrin et al., 2004). Similar alterations in the redox components were also indicative of heightened oxidative stress in pathologies like systemic lupus erythematosus (Wu et al., 2012).

To conclude, this study revealed that the age-related dampening of circadian rhythms in clock genes underlie the age-related loss of rhythmicity of cellular redox.

Author Contributions

Conceived the study and planned experiments: SR, JG, EC. Performed experiments: VK, EC, JK-R SR, JG. Analyzed data and wrote the paper: EC, VK, SR, WO, JG.

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Telomeric aging: mitotic clock or stress indicator?

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Telomeres are regions of tandem arrays of TTAGGG repeats and associated proteins located at chromosomal ends that allow cells to distinguish chromosome ends from double-strand breaks and protect chromosomes from end-to-end fusion, recombination, and degradation (Houben et al., 2008). Telomeres are not linear structures, telomeric DNA is maintained in a loop structure due to many key proteins. This structure serves to protect the ends of chromosomes (Neidle and Parkinson, 2003). Telomeres are subjected to shortening at each cycle of cell division due to incomplete synthesis of the lagging strand during DNA replication owing to the inability of DNA polymerase to completely replicate the ends of chromosome DNA ("end-replication problem") (Muraki et al., 2012). Therefore, they assume to limit the number of cell cycles and act as a "mitotic clock" (Olovnikov, 1996). Shortened telomeres cause decreased proliferative potential, thus triggering senescence (Blackburn et al., 2006).

Telomere length (TL) is highly heterogeneous in somatic cells, but generally decreases with age in proliferating tissues thereby constituting a barrier to tumorigenesis but also contributing to age-related loss of stem cells. Repair of critically short ("uncapped") telomeres by telomerase enzyme, which elongates chromosomal ends by synthesizing new telomeric repeats, is limited in somatic cells, and cellular senescence, apoptosis and/or a permanent cell cycle arrest in G1 phase are triggered by a critical accumulation of uncapped telomeres (D'Souza et al., 2013). Telomerase maintains TL by adding telomeric DNA repeats to chromosome ends in prenatal tissues, gametes, stem cells, and cancerous cells. In proliferative somatic cells, it is usually inactive or expressed at levels that are not high enough to maintain the stable TL (Hiyama and Hiyama, 2007). Alternative lengthening of telomeres (ALT) pathway is a recombination-mediated process, which can increase telomere length by thousands of base-pairs within a few somatic cell cycles (Liu et al., 2007). Telomere-initiated cellular senescence is a mechanism of eliminating cells with damaged DNA and protection against cancerogenesis (through the activation mechanisms of cell cycle arrest or double-strand breaks-induced apoptosis), but it may also impair the cell function contributing to degenerative organ failure and organismal aging (Chen et al., 2007; Campisi, 2013). Cellular senescence (a state of irreversible cell cycle arrest) occurring in response to a telomere shortening may be regulated by immune surveillance. In this process, senescent cells interact with their environment by secreting various growth factors and cytokines, being thereby recognized and eliminated by an antigen-specific immune response (Hoenicke and Zender, 2012; Sagiv and Krizhanovsky, 2013). Shortened telomeres have also been observed in a variety of chronic degenerative diseases, including type 2 diabetes, cardiovascular disease, osteoporosis, and cancer (Armanios, 2013; Babizhayev et al., 2014). The specific molecular mechanism by which short telomeres trigger the development of diseases is, however, not yet determined. It has been proposed that telomere shortening *per-se* might not be a direct signal for cell cycle arrest, but rather the consequence of telomere loss (Vaziri and Benchimol, 1996). It can promote a pro-inflammatory secretory phenotype, in turn contributing to a variety of age-related diseases.

Replicative attrition, however, is not the only explanation for age-dependent telomere shortening. Some studies demonstrate that this process can be non-replicative and significantly stress-dependent because of the deficiency of a telomere-specific damage repair (von Zglinicki, 2003).

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Potential stochastic nature of progressive telomere shortening was shown by Levy et al. (1992). Accumulating research findings suggest that psychophysiological stress may accelerate the telomere attrition from early development, and maybe even prenatally, with potential impact for life-long health (Shalev et al., 2013a; Monaghan, 2014). For example, it has been shown that the more violence children had experienced the shorter were their telomeres from 5 to 10 years of age (Shalev et al., 2013b). In the study by Entringer et al. (2011), young adults whose mothers were exposed to severe stressors during pregnancy (for example, due to the death of a close family member) had more short telomeres than those subjects whose mothers had a relatively stress-free pregnancy. Several studies also indicate that effects of exposure to stress early in life may persist into adulthood. In research by Surtees et al. (2011), it has been demonstrated that the more adversity women had experienced during their childhood the shorter were their telomeres in adult life. By investigating the hypothesis that psychophysiological stress may impact health by modulating the rate of cellular aging, Epel et al. (2004) examined the TLs and telomerase activity levels in mothers of healthy or chronically sick children. The second group was predicted to be more chronically stressed than the first one. In this research, mothers of ill children demonstrated substantially reduced telomerase activity which was equivalent to the 9–17 years of additional cellular aging as compared to those women who had healthy children, and rates of cellular aging were obviously associated with levels of stress exposure. Substantial telomere attrition was also revealed among caregivers of AD (Alzheimer's disease) patients. In this occupational group, telomere shortening was clearly associated with symptoms of depression, in comparison to control subjects matched for gender and age (Damjanovic et al., 2007). The correlation between level of perceived stress and TL was clearly confirmed in a recent meta-analysis conducted by Schutte and Malouff (2014).

Oxidative stress is one of the most important stress factors causing telomere shortening (Houben et al., 2008; Zhang et al., 2014). Telomeric DNA is known to be more susceptible to oxidative damage than non-telomeric DNA, owing to the high content of guanine residues and a reduced DNA repair capacity in telomeric regions (Gomes et al., 2010). Triple guanine repeats of telomeric DNA were shown to be particularly prone to oxidative damage, and they may generate oxidatively modified bases such as 8-oxoguanine which is the main substrate for the base excision repair (BER) pathway (Kawanishi and Oikawa, 2004). Wang et al. (2010) demonstrated that oxidative damage of guanine plays an important role in disruption of telomere integrity and presumes that BER pathway is substantially involved in repair of oxidative base lesions in mammalian telomeres. Moreover, telomeric DNA, in contrast to genomic DNA, is deficient in repair of single strand breaks and double strand breaks, and telomere dysfunction is believed to mediate the effect of oxidative base damage to abnormal nuclear morphology (Coluzzi et al., 2014). In human cell lines, telomeres generally shorten by 30–200 base pairs at each round of DNA replication, but only approximately 10 base pairs of this reduction are a consequence of the end-replication problem; the remaining loss is likely owing to oxidative damage (Monaghan and Haussmann, 2006). Systemic chronic inflammation to accelerate aging via ROS-mediated telomere dysfunction and cell senescence was shown in mice (Jurk et al., 2014).

In several studies, it was found, that DNA damaging agents cause telomere erosion, primarily, by high levels of reactive oxygen species (ROS) regardless of age of the cell donor. For example, in the Richter and von Zglinicki (2007) study, high levels of oxidative stress cause accelerated rates of telomere erosion and, ultimately, arrest of the cell cycle in cell cultures derived from donors of various ages (embryonic to adult). Moreover, it was revealed that over-expression of superoxide dismutase in a cell line with low antioxidant capacity slowed erosion of telomeres (Serra et al., 2003). Further evidence for non-replicative cause of telomere shortening comes from the fact that the telomere erosion rate varies in a stochastic manner among cells in fibroblast clones, derived from single cells, thereby causing sub-clonal heterogeneity in replicative lifespan (Martin-Ruiz et al., 2004). Recently, it was found that such cell-to-cell heterogeneity may come from stochastic heterogeneity in oxidative DNA damage caused, e.g., by variations in the cell's metabolic processes (Snijder and Pelkmans, 2011; Trusina, 2014). Another pathway for non-replicative telomere erosion relates to non-canonical function of telomerase, mainly, protection of mitochondria against oxidative stress (Saretzki, 2014). The fact that TERT is transported out of the nucleus under long-term low level oxidative stress suggests that protection of mitochondrial DNA might have higher priority than protection of telomeres maintenance under stress (Ahmed et al., 2008).

The complexity of processes underlying age-related telomere erosion came from several longitudinal studies of telomere dynamics *in vivo*. Research of TLs in blood cells of snakes, birds, and humans unexpectedly revealed, that no TLs changes with aging were found (for review, see Eisenberg, 2011). Moreover, the faster age-related decline in leukocyte TLs was observed among subjects with longer telomeres at baseline early-adult examinations, suggesting that longer telomeres provide bigger amount of targets for oxidative stress-related telomere erosion (Aviv et al., 2009). Traditionally, it was assumed that telomeres are stable structures, which may be changed only in unidirectional way—shortening over the lifetime. Today, however, it has become increasingly clear that telomeres shortening over time in an oscillatory rather than linear fashion and they may be either shortened or lengthened under certain conditions (Epel, 2012). Several pilot studies indicate that treatment procedures targeting to reduce stress, e.g., meditation, along with the enhanced physical activity and changes in dietary patterns, can slow or even reverse telomere shortening owing to the elevated telomerase activity (Jacobs et al., 2011; Blackburn and Epel, 2012). The elongation of telomeres may be caused by the telomerase-mediated extension or appear due to the "pseudo-telomeric lengthening." The latest is due to the fact that, since TLs are commonly measured in a mixed leukocyte population, mean TL can increase because of a redistribution of cell subpopulations, i.e., change in the percentage of various cell types in the blood samples.

Consistent evidence suggest that telomere erosion may be a common mechanism that triggers all major processes underlying aging, including mutation accumulation, mitochondrial dysfunction and stem cell failure (Armanios, 2013), as well as epigenetic

changes likely through silencing of gene expression by formation of long-range chromatin interactions (Fojtová and Fajkus, 2014; Misteli, 2014). Induction of senescence occurs in reaction to a critically short telomere length and to the triggering of DNA damage response. Therefore, unraveling the factors causing telomere attrition may be important for further progress in understanding the basic mechanisms of aging. In this context, it is particularly important to establish the relative significance of end-replication and oxidative damage events in the age-related telomere erosion.

Given data from recent studies, a concept that replicative senescence is a "clocked" and stepwise process seems doubtful, and repeatedly reported reproducibility of both replicative lifespans and rates of telomere shortening could be the result of stochastic rather than programmed events (von Zglinicki, 2003). In other words, it seems that telomeres can be an indicator of stress-induced damage level rather than a mitosis "counter." Moreover, considering the fact that oxidative stress represents a common causative mechanism for both age-related telomere shortening (Houben et al., 2008) and age-associated disease (Richardson and Schadt, 2014), there are reasons to believe that relationships between TL and morbidity or mortality are non-causal, and TL can be an indicator of previous exposure to oxidative stress that may, in turn, cause both greater telomere shortening and higher risk of chronic disease. Thereby, perceived stressful events, though correlated with TL, may likely have independent effects on health and longevity (Eisenberg, 2011). By summarizing recent research findings, Trusina (2014) conclude that recently obtained knowledge "shift the telomere paradigm from a simple clock counting cell divisions to a more complex process recording the history of stress exposure within a cell lineage." This point of view, based on the accumulated evidence, appears plausible, and requires further investigation.

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Prolactin and growth hormone affect metaphase-II chromosomes in aging oocytes via cumulus cells using similar signaling pathways

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General senescence of the adult organism is closely connected with reproductive one. Meanwhile, the age-related reduction in the female fertility is primarily associated with a decline in the gamete quality. Molecular and cellular changes in oocytes of old mammalian females are very similar to those occurring during aging of matured ova of their young counterparts, suggesting similarities in underlying mechanisms. The aim of the present work was to study actions of two related pituitary hormones, prolactin (PRL) and growth hormone (GH), on age-associated modifications of metaphase-II (M-II) chromosomes in bovine oocytes using a model of the prolonged culture. We analyzed: (1) effects of PRL and GH on abnormal changes in the chromosome morphology in aging matured oocytes and the role of cumulus cells in these effects and (2) signaling pathways involved in the hormone actions. During the prolonged culture of oocytes, a gradual rise in the frequency of destructive modifications of M-II chromosomes was revealed. In the case of cumulus-enclosed oocytes (CEOs), PRL and GH exerted dose-dependent biphasic effects on the frequency of these modifications. Both PRL (50 ng/ml) and GH (10 ng/ml) decelerated the abnormal chromosome changes in CEOs, but did not affect the chromosome configuration in denuded oocytes. Concurrently, the presence of PRL and GH receptors in cumulus cells surrounding matured oocytes was demonstrated. Attenuating effects of both hormones on the chromosome modifications in aging CEOs were abolished by PP2 (an inhibitor of Src-family tyrosine kinases), triciribine (an inhibitor of Akt kinase), and calphostin C (a protein kinase C inhibitor). Our findings indicate that PRL and GH can exert the similar decelerating action on age-associated alterations in the M-II chromosome morphology in bovine ova, which is mediated by cumulus cells and may be related to activation of Src-family tyrosine kinases as well as Akt- and protein kinase C-dependent signal pathways.

Keywords: oocyte aging, chromosome modifications, cumulus cells, prolactin, growth hormone, receptors, signaling pathways

Introduction

General senescence of the adult organism is closely connected with reproductive one. It is well established that the female reproductive performance, primarily the ovarian function, deteriorates with age in many vertebrates including humans, with underlying fundamental mechanisms being conserved among species (Finch and Holmes, 2010; Ottinger, 2010). In mammals, an initial reduction in the female fertility is primarily associated with a decline in the gamete fitness (Tatone et al., 2008). The impairment of the oocyte quality is usually thought to be caused by age-dependent changes in general and specific functions of both oocytes and their nursing somatic follicular cells (Broekmans et al., 2007; Tatone et al., 2008; Tatone and Amicarelli, 2013). Reproductive (or maternal) aging proceeds slowly and involves mainly immature oocytes arrested at the diplotene stage.

Postovulatory aging of mature mammalian oocytes is another biological phenomenon leading to an age-related decline in the quality of female gametes. This type of senescence occurs quickly (within 1–2 days) in ova arrested at the metaphase-II (M-II) stage in the absence of activation stimuli (Miao et al., 2009). Reproductive aging and postovulatory aging are characterized by similar functional changes of oocytes including chromosome abnormalities, spindle defects, disturbances in segregation of homologous chromosomes or sister chromatids, an increased predisposition to apoptosis and parthenogenesis, a decreased fertilizability and developmental capacity (Battaglia et al., 1996; Wu et al., 2000; te Velde and Pearson, 2002; Ma et al., 2005; Tatone et al., 2006; Miao et al., 2009). According to the current state of knowledge, molecular transformations occurring in the oocyte cytoplasm underlie the above listed negative functional changes. These transformations are primarily related to alterations in the pattern of synthesis and post-translational modifications of effector proteins and to disturbances of the mitochondrial function and calcium homeostasis (Eichenlaub-Ritter et al., 2004; Tatone et al., 2006, 2008; Miao et al., 2009; McReynolds et al., 2012). In particular, some functional changes associated with oocyte aging have been shown to be related to the compromised function of intra-oocyte signaling pathways dependent on different protein kinases including MAP kinase, protein kinase C, and Akt (Tatone et al., 2006; Petr et al., 2011; Cecconi et al., 2014; McGinnis et al., 2014). Meanwhile, cellular and molecular changes in postovulatory ova aging *in vivo* are very similar to those occurring during *in vitro* aging of *in vivo* and *in vitro* matured oocytes, suggesting similarities in the underlying mechanisms (Miao et al., 2005, 2009). Thus the prolonged culture of mature mammalian oocytes is a convenient model for the comprehensive study of physiological factors and signal systems involved in regulation of the oocyte senescence.

The available evidence points to participation of two closely-related hormones, prolactin (PRL) and growth hormone (GH), in modulation of the mammalian oocyte maturation and developmental competence (Izadyar et al., 1996; Bole-Feysot et al., 1998; Hull and Harvey, 2002; Lebedeva et al., 2014b). Receptors of PRL and GH or their mRNA have been detected in oocytes and surrounding cumulus cells of different species including cows (Bevers and Izadyar, 2002; Marchal et al., 2003; Picazo et al., 2004; Lebedeva et al., 2014b). Both hormones can modulate the mitochondrial activity and/or calcium homeostasis in bovine oocytes maturing *in vitro* (Kuzmina et al., 1999, 2007), indicating the hormonal implication in processes modified by aging. Furthermore, in various types of mammalian cells, PRL and GH are able to activate signal cascades dependent on MAP kinase, protein kinase C, and Akt (Postel-Vinay and Finidori, 1995; Bole-Feysot et al., 1998; Di Rosa et al., 2009; Devesa et al., 2014), which in turn are involved in regulation of some functional changes in aging oocytes. Mammalian follicular fluid is known to contain both hormones derived from the circulation as well as produced locally by ovarian cells (Borromeo et al., 1998; Mendoza et al., 2002; Modina et al., 2007; Marano and Ben-Jonathan, 2014). Immediately after ovulation, follicular fluid carrying the ovum becomes the major component of tubal fluid (Lyons et al., 2006). Thus, there are reasons to assume that PRL and GH may act at least temporarily within the oviduct and affect aging processes in mature oocytes.

To date, little is known about physiological factors regulating the speed of oocyte senescence. Using the nematode *Caenorhabditis elegans* as the justified model of female reproductive aging, it has been recently demonstrated that two conserved endocrine/growth factor pathways, the insulin/insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β) pathways, act in various somatic tissues to control oocyte aging (Luo et al., 2010). According to the current concept, similar somatic signals might also regulate the oocyte quality in older women (Ellis and Wei, 2010). This concept is supported by data for age-related changes in the expression of some genes associated with the insulin/IGF-1 and TGF-β pathways in human cumulus cells (Al-Edani et al., 2014). Interactions between gametes and somatic cells are of considerable importance in the case of postovulatory aging as well (Miao et al., 2009). During *in vitro* aging of mammalian oocytes, both accelerating and decelerating effects of cumulus cells on different negative functional changes in mature ova have been found (Miao et al., 2005; Takahashi et al., 2009; Wu et al., 2011). Furthermore, the impaired expression of several genes related to the mitochondrial function, metabolism, apoptosis, and the antioxidant defense in cumulus cells surrounding *in vitro* aging goat oocytes has been revealed (Zhang et al., 2013). However, physiological regulators and signal systems involved in cumulus-oocyte interactions determining the matured ovum senescence are still not clearly understood.

We have previously found abnormal modifications of the chromosome morphology in bovine M-II oocytes aging *in vitro*, with the surrounding cumulus investment promoting these negative processes (Lebedeva et al., 2014a). Since PRL and GH at certain concentrations could exert inhibitory effects mediated by cumulus/granulosa cells on destructive changes of chromosomes in bovine oocytes maturing *in vitro* (Kuzmina et al., 1999; Lebedeva et al., 2005), one would expect similar hormonal effects on the senescent oocytes. Therefore, the present study was conducted to test a hypothesis that PRL and GH are able to suppress the M-II chromosome aberrations in aging bovine oocytes by transmitting signals through somatic cumulus cells.

To attain this aim we analyzed: (1) effects of PRL and GH on abnormal changes in the chromosome morphology in aging mature oocytes and the role of cumulus cells in these effects and (2) signaling pathways involved in the hormonal effects. In addition, the presence of PRL and GH receptors in cumulus cells surrounding matured oocytes has been verified, because cumulus expression of different proteins including hormonal receptors is dramatically reduced in the course of oocyte maturation (Devjak et al., 2012). The choice of tested signaling pathways dependent on Akt, protein kinase C, and MAP kinase was due to their implication in both oocyte and somatic aging (Bitar, 2003; Tatone et al., 2006; Salminen and Kaarniranta, 2010; Petr et al., 2011; Cecconi et al., 2014). To assess the participation of the previously mentioned protein kinases in actions of PRL and GH on destructive changes of M-II chromosomes, the influence of triciribine (an inhibitor of Akt kinase), calphostin C (a protein kinase C inhibitor), and U0126 (a MEK 1/2 inhibitor) on these actions was examined. Furthermore, the respective involvement of Src-family tyrosine kinases associated constitutively with PRL and GH receptors (Waters and Brooks, 2011; Martín-Pérez et al., 2015) was tested using genistein (a non-selective inhibitor of tyrosine kinases) and PP2 (an inhibitor of Src-family tyrosine kinases).

Materials and Methods

Unless otherwise stated, all media and chemicals were purchased from Sigma-Aldrich Chemical Corporation (St. Louis, MO, USA).

Oocyte Collection, Handling, and *in Vitro* Maturation

Slaughterhouse-derived bovine ovaries were transported to the laboratory in a thermo box with sterile saline at 30−35°C, and cumulus-enclosed oocytes (CEOs) were obtained by wall dissection of 2−8 mm antral follicles. The oocyte retrieval and handling were performed in a wash medium consisting of HEPES-buffered TCM-199 containing 5% (v/v) fetal calf serum (FCS; Hyclone Laboratories, Logan, UT, USA) and 50 μg/mL of gentamicin sulfate. The CEOs were washed twice in the wash medium and selected under a stereomicroscope. Only oocytes with a complete, compact, multilayer cumulus and finely granulated homogenous ooplasm were used for the study. Groups of CEOs were matured for 20 h in 500 μL of a maturation medium at 38.5°C under 5% CO_2 in humidified air. The following maturation medium was used: HEPES-buffered TCM-199 (with Earle's salts and L-glutamine), containing 0.2 mM sodium pyruvate, 50 μg/mL of gentamicin, and 10% (v/v) FCS, supplemented with 10 μg/mL of porcine follicle-stimulating hormone and 10 μg/mL of ovine luteinizing hormone.

Design of Oocyte Prolonged Culture Experiments

After 20 h of maturation, most of CEOs were immediately used for the prolonged culture. Another portion of the oocytes was denuded of their cumulus cells by incubating the CEOs in the above mentioned wash media containing 0.1% (v/v) hyaluronidase for 1 min at 37°C and subsequent gentle pipetting through a fine needle pipette (with the hole diameter of 130 μm). The denuded oocytes (DOs) were washed twice from hyaluronidase and examined under an inverted light microscope (at magnification × 200) to ensure the complete removal of cumulus cells. Thereafter, CEOs or DOs were transferred to an aging medium consisting of HEPES-buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 50 μg/mL of gentamicin, and 10% (v/v) FCS (Control) and cultured at 38.5°C under 5% CO_2 in humidified air. In experimental groups, either pituitary bovine PRL (20 IU/mg; Research Center for Endocrinology, Moscow, Russia) or recombinant bovine GH (Monsanto, St. Louis, MO, USA) was added to the aging medium. In dose-dependent experiments, two different preparations of bovine PRL (Research Center for Endocrinology, Moscow, Russia and USDA bPRL B-1, Beltsville, MD, USA) were used for comparison. Frozen aliquots of a stock solution (50 μg/mL of PRL or 10 μg/mL of GH in saline) were diluted by the aging medium immediately prior to culture.

In the first experiment, CEOs were cultured for 24 h in the aging medium containing different concentrations of either PRL (0, 20, 50, 150, and 500 ng/mL), or GH (0, 2.5, 5, 10, 20, and 50 ng/mL), or recombinant human epidermal growth factor (EGF; 0, 0.2, 1, 10, 50, and 500 ng/mL; Thermo Fisher Scientific, Waltham, MA, USA). In the second experiment, CEOs and DOs were incubated for 12, 24, 36, or 48 h with and without 50 ng/mL of PRL or 10 ng/mL of GH. In the third experiment, CEOs were cultured for 24 h in the absence and in the presence of either PRL (50 ng/mL) or GH (10 ng/mL) and/or protein kinase inhibitors. The following inhibitors were applied: (1) genistein, the non-selective inhibitor of tyrosine kinases (40 μM; ICN Biomedicals, Aurora, OH, USA), (2) PP2, the inhibitor of Src-family tyrosine kinases (20 μM), (3) triciribine, the inhibitor of Akt kinase (50 μM), (4) calphostin C, the protein kinase C inhibitor (1 μM; Calbiochem, Darmstadt, Germany), and (5) U0126, the MEK 1/2 inhibitor (20 μM; Promega, Madison, WI, USA). At the end of culture, all oocytes were fixed to determine their nuclear status. In all the experiments, the proportion of oocytes at the M-II stage was no less than 72%.

Assessment of Oocyte Nuclear Material

To evaluate nuclear maturation and the M-II chromosome morphology in oocytes, cytogenetic preparations were performed by the method of Tarkowski (1966) with some modifications (Kuzmina et al., 1999). The state of the nuclear material was examined under a light microscope (Opton, Germany) at magnification × 1000 using criteria described earlier (Ernst et al., 1980; Homa, 1988). The following morphological abnormalities were ascribed to destructive changes of M-II chromosomes: (1) decondensation (a loss of clear morphological contours, an increase in the chromosome volume and/or uneven morphological contours), (2) chromosome decondensation and/or partial adherence, (3) chromosome clumping into a single mass, and (4) fragmentation.

Immunocytochemical Analysis

The protein expression of PRL and GH receptors in cumulus cells surrounding *in vitro* matured oocytes was detected by immunocytochemistry as described previously for freshly isolated

bovine CEOs (Lebedeva et al., 2014b). Briefly, following 20 h of maturation, CEOs were washed twice in PBS containing 0.2% (w/v) bovine serum albumin (PBS-BSA) and fixed with 4% (w/v) paraformaldehyde in PBS for 15 min. After washing, the specimens were permeabilized for 30 min with 0.1% (v/v) Triton X-100 in PBS-BSA (in the case of the PRL receptor) or with 0.5% (v/v) Triton X-100 in PBS-BSA (in the case of the GH receptor). Nonspecific binding was blocked by incubating the CEOs with 10% (v/v) goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS containing 1% (w/v) BSA for 1 h at room temperature. For detection of PRL and GH receptors, the specimens were incubated respectively with mouse anti-PRL receptor monoclonal antibody MA1-610 (Thermo Scientific, Rockford, IL, USA; 1:50 dilution) or mouse anti-GH receptor monoclonal antibody MAB 263 (Abcam, Cambridge, MA, USA; 1:50 dilution) overnight at 4°C. The primary antibody MA1-610 reacts both with long and short PRL receptor isoforms and detects the receptor in tissues of different mammalian species including bovine cumulus cells (Lebedeva et al., 2014b). The antibody MAB 263 has been extensively validated for immunohistochemical studies in various types of tissues including bovine ovary (Kölle et al., 1998). Thereafter, the CEOs were incubated with biotinylated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA; 1:500 dilution) for 30 min at room temperature. All antibodies were diluted in PBS containing 1% (w/v) BSA and 3% (v/v) goat serum. For visualization of the specific staining, Vectastain ABC reagent and 3-amino-9-ethylcarbazole (AEC) substrate (all purchased from Vector Laboratories, Burlingame, CA, USA) were applied. The CEOs were counterstained by hematoxylin and mounted in a droplet of the glycerol-PBS mixture (1:3, v/v). All specimens were evaluated for the presence of PRL and GH receptors using the light microscope at magnification × 400.

Specificity of the immunodetection was proved by several negative controls: (1) omission of the first antibody, (2) omission of the secondary antibody, and (3) incubation with AEC alone to show the absence of endogenous peroxidases. Layers of freshly isolated bovine membrana granulosa, expressing PRL and GH receptors (Kölle et al., 1998; Lebedeva et al., 2001), were employed as a positive control. A total of 24 and 41 CEOs were used for immunocytochemical detection of PRL and GH receptors, respectively.

Statistical Analysis

All treatments in culture experiments were repeated 3−6 times. The numbers of oocytes used per each treatment are indicated in figure legends. Results were expressed as means ± SEM. Data were analyzed by one-way or two-way ANOVA followed by the Tukey's HSD test using SigmaStat software package. If the data expressed as percentages did not meet the assumption of normal distribution or homogeneity of variance, they were arcsine transformed before analysis. In the case of two-way ANOVA, the statistical model included the main effects and all interactions. Independent variables were PRL or GH treatments and the aging duration or the inhibitor treatment. A probability of $p < 0.05$ was considered to be statistically significant.

Results

Effects of PRL and GH on M-II Chromosomes During *in Vitro* Aging of Bovine Cumulus-Enclosed and Denuded Oocytes

Immediately after *in vitro* maturation, the proportion of M-II oocytes with signs of chromosome abnormalities did not exceed 20% in all experiments. Following 24 h of the prolonged culture in the control aging medium, the rate of CEOs with destructive modifications of M-II chromosomes increased up to 50−64%. Meanwhile, the chromosome decondensation was the most common abnormal change that was manifested by various morphological signs (**Figure 1**). At the same time the chromosome fragmentation was observed very seldom.

During 24 h aging of CEOs, PRL and GH exerted dose-dependent biphasic effects on the frequency of abnormal chromosome modifications. Since the data obtained for both PRL preparations were identical, they were combined to produce a single dose-dependent curve. The application of PRL at concentrations of 20−50 ng/mL caused a decline in the frequency of M-II chromosome modifications (at least $p < 0.01$), with the maximum reducing effect being observed at a concentration of 50 ng/mL (**Figure 2A**). By contrast, at a concentration of 500 ng/mL, PRL enhanced destructive changes in the M-II chromosome morphology ($p < 0.01$). The pattern of the dose-dependent curve for GH was very similar to that for PRL (**Figure 2B**). As compared to the control medium, the rate of oocytes with abnormal chromosome changes was reduced in the presence of 5−10 ng/mL of GH ($p < 0.05$) and increased in the presence of 50 ng/mL of GH ($p < 0.01$). At the same time EGF, another well-known modulator of oocyte maturation (Conti et al., 2006), did not inhibit destructive modifications of M-II chromosomes at all concentrations tested (**Figure 2C**). So, in further experiments, 50 ng/mL of PRL and 10 ng/mL of GH were used for characterization of the suppressive influence of the hormones on age-associated chromosome changes in bovine oocytes.

In the course of the prolonged culture of CEOs in the control medium, a rise in the rate of the oocytes with destructive changes of the chromosome configuration occurred by 12 h of aging ($p < 0.05$) and persisted up to 36 h ($p < 0.001$; **Figure 3**). The addition of PRL (50 ng/mL) or GH (10 ng/mL) to the aging medium resulted in a decrease of this rate throughout the culture period (at least $p < 0.05$). Thus, the frequency of abnormal modifications of M-II chromosomes in aging oocytes increased more slowly in the hormone-treated groups than in the control group.

Effects of PRL and GH on destructive changes of M-II chromosomes in DOs were explored after 24 and 48 h of aging, since the removal of cumulus cells had been shown to decelerate these changes in bovine oocytes (Lebedeva et al., 2014a). It was found that the gradual rise in the rate of DOs with chromosome abnormalities during the prolonged culture was unaffected by both PRL and GH (**Figure 4**). Therefore, only CEOs were used in the subsequent culture experiments to study signaling pathways involved in the hormone actions.

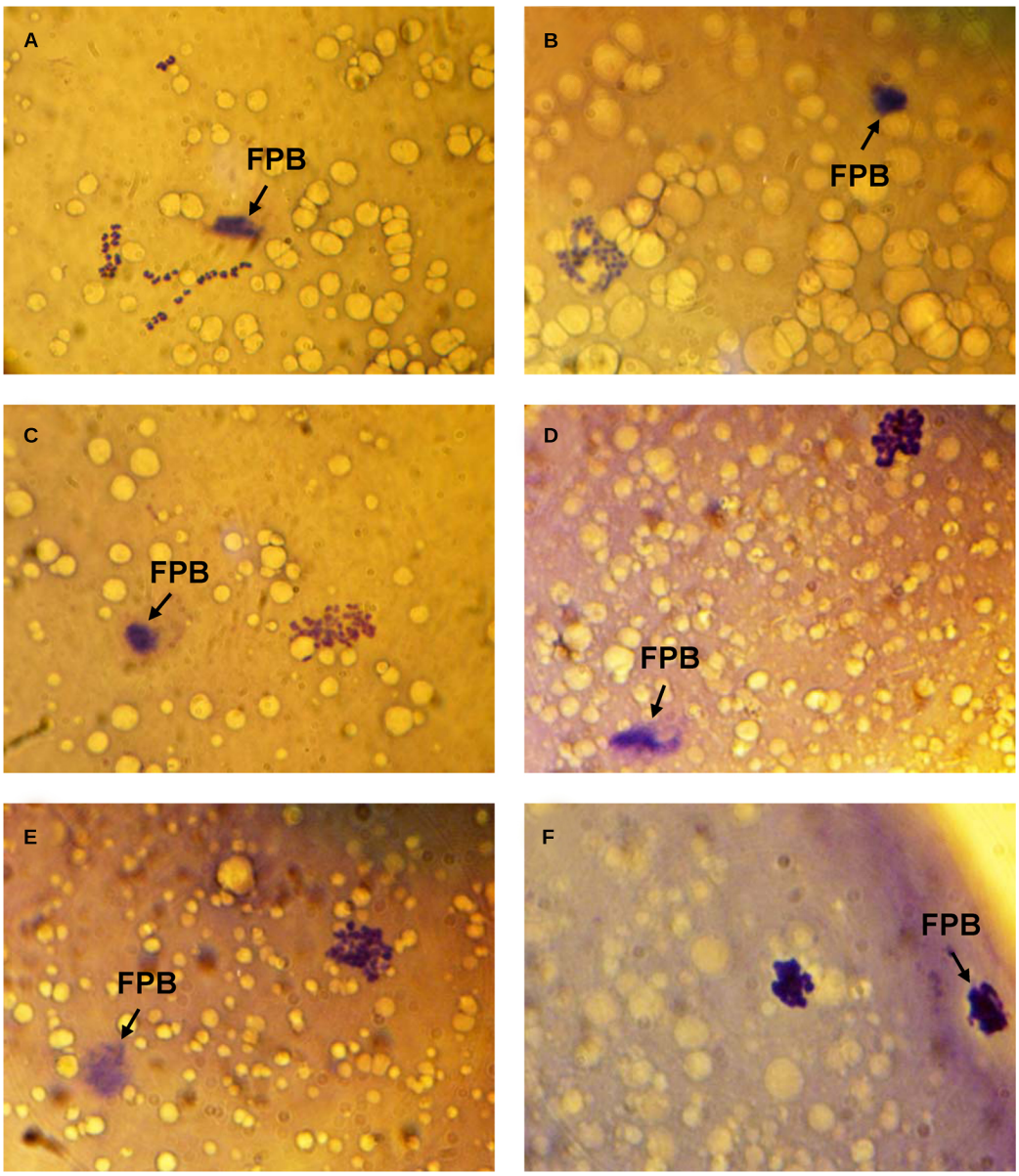

FIGURE 1 | Morphology of M-II chromosomes in aging bovine oocytes. (A) Chromosomes without signs of abnormal changes, **(B)** Chromosome decondensation (a loss of clear morphological contours), **(C)** Chromosome decondensation (unclear and uneven morphological contours), **(D)** Chromosome decondensation (uneven morphological contours and an increase in the chromosome volume), **(E)** Chromosome decondensation (unclear and uneven morphological contours) and partial adherence, **(F)** Chromosome clumping into a single mass. Black arrow indicates the first polar body (FPB). Original magnification: × 1000.

Immunocytochemical Localization of PRL and GH Receptors in Bovine Cumulus Cells Surrounding Matured Oocytes

To confirm the availability of cumulus-mediated pathways of PRL and GH signaling into M-II oocytes, the presence of the respective receptors in cumulus cells following 20 h maturation of CEOs was examined. Receptors of PRL were detected in the cells of all bovine cumulus-oocyte complexes tested using MA1-610 antibody and the red AEC-chromophore (**Figure 5A**). No specific immunoreactivity was found in negative controls performed by omitting the primary antibody (**Figure 5B**). Furthermore, most of cumulus cells surrounding *in vitro* matured oocytes showed the intensive red staining for anti-GH receptor antibody MAB 263 (**Figure 6A**), whereas the specific immunoreactivity was not present in the negative controls (**Figure 6B**). Layers of freshly isolated membrana granulosa (positive control) containing PRL and GH receptors (Kölle et al., 1998; Lebedeva et al., 2001) also demonstrated the red staining (data not shown). Concurrently, CEOs incubated without the secondary antibody or with AEC alone did not show the specific staining.

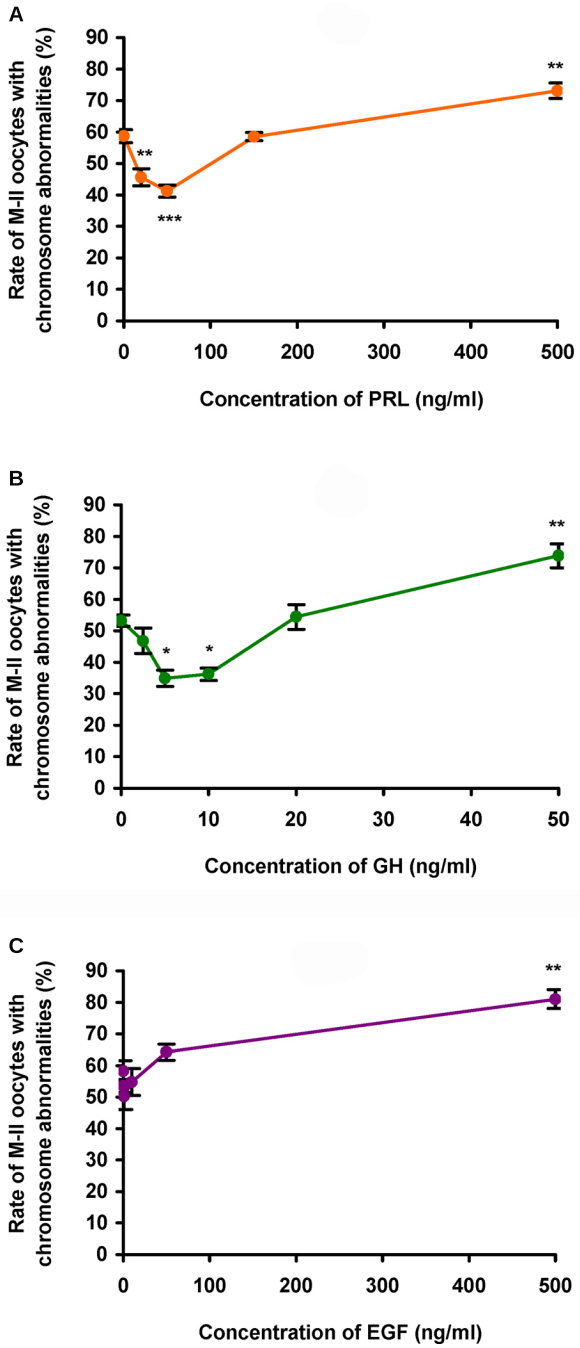

FIGURE 2 | Effects of different concentrations of PRL (A), GH (B), and EGF (C) on destructive changes in the M-II chromosome morphology during 24 h aging of bovine cumulus-enclosed oocytes. Data represent means ± SEM of 3–6 replicates using 51–91 oocytes per treatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the respective groups without PRL, GH, or EGF.

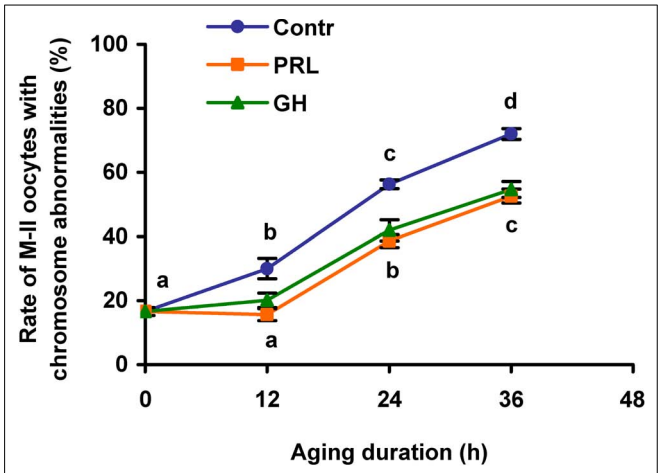

FIGURE 3 | Effects of PRL (50 ng/mL) and GH (10 ng/ml) on destructive changes in the M-II chromosome morphology during aging of bovine cumulus-enclosed oocytes. Data represent means ± SEM of 4–5 replicates using 77–101 oocytes per treatment. Means with different letters differ significantly (at least $p < 0.05$).

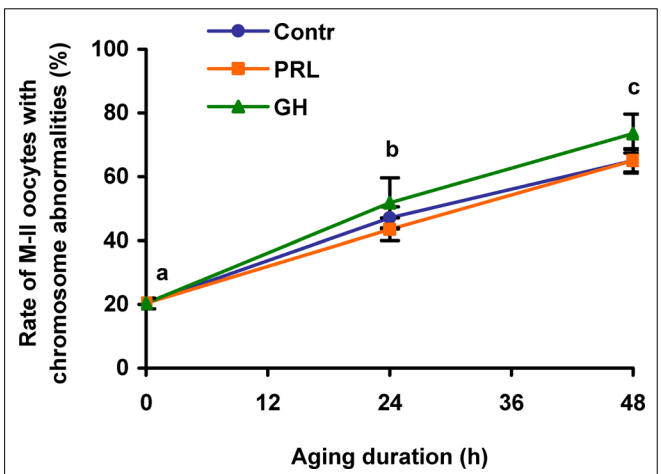

FIGURE 4 | Destructive changes in the M-II chromosome morphology during aging of bovine cumulus-free oocytes in the presence and in the absence of PRL (50 ng/ml) or GH (10 ng/ml). Data represent means ± SEM of 4–5 replicates using 83–104 oocytes per treatment. Means with different letters differ significantly (at least $p < 0.05$).

Signaling Pathways Involved in PRL and GH Modulation Of M-II Chromosome Abnormalities in Aging Bovine Cumulus-Enclosed Oocytes

An involvement of different protein kinases in PRL and GH signaling into aging CEOs was studied by testing effects of inhibitors of tyrosine kinases, Akt kinase, protein kinase C, and MEK 1/2 on the decelerating hormonal influence on the chromosome modifications. The used concentrations of genistein (40 μM), PP2 (20 μM), and U0126 (20 μM) were very close to the respective concentrations, which were effective in suppressing PRL and GH actions on different mammalian cells (Fresno Vara et al., 2001; Huang et al., 2003; Gutzman et al., 2004; Zhang et al., 2004, 2006; Lebedeva et al., 2011). Concentrations of triciribine (50 μM) and calphostin C (1 μM) were chosen on the basis of the published values for the respective IC50 (Tamaoki and Nakano, 1990; Gürsel et al., 2011).

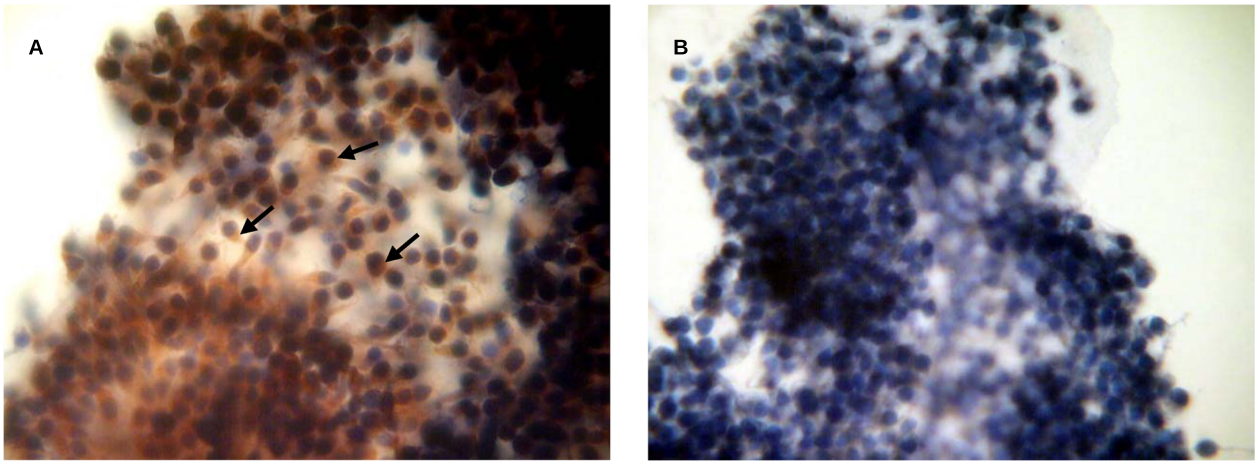

FIGURE 5 | Immunocytochemical detection of PRL receptors in cumulus cells after 20 h maturation of bovine cumulus-enclosed oocytes. Specific localizations were detected by MA1-610 antibody and the red 3-amino-9-ethylcarbazole (AEC) chromophore. Nuclei were counterstained with hematoxylin. **(A)** Positive staining. Black arrows indicate PRL receptor-specific immunoreaction. **(B)** Negative control performed by omitting the primary antibody. Original magnification: × 400.

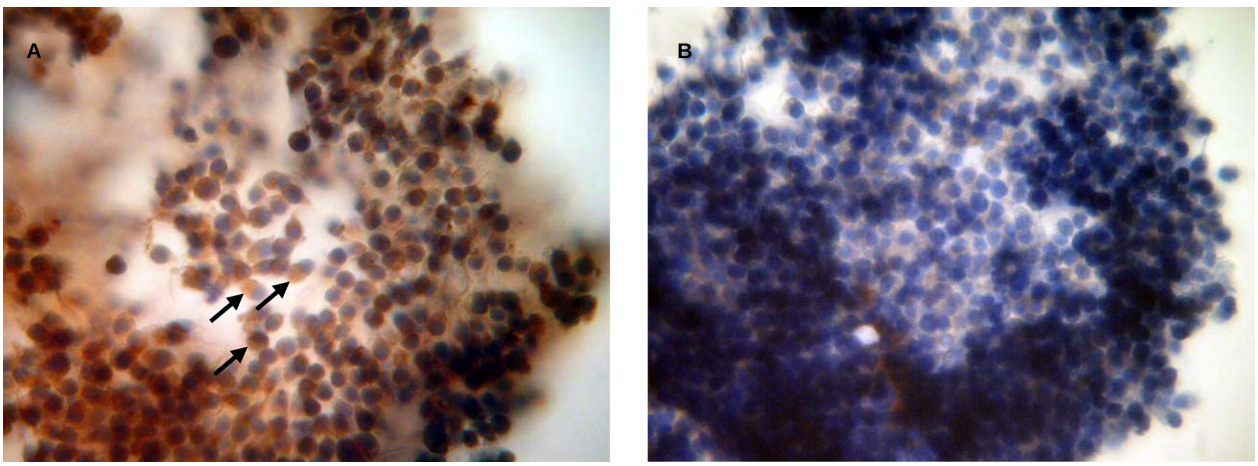

FIGURE 6 | Immunocytochemical detection of GH receptors in cumulus cells after 20 h maturation of bovine cumulus-enclosed oocytes. Specific localizations were detected by MAB 263 antibody and the red 3-amino-9-ethylcarbazole (AEC) chromophore. Nuclei were counterstained with hematoxylin. **(A)** Positive staining. Black arrows indicate GH receptor-specific immunoreaction. **(B)** Negative control performed by omitting the primary antibody. Original magnification: × 400.

When added to the aging medium, genistein, the non-selective inhibitor of tyrosine kinases, and PP2, the inhibitor of Src-family tyrosine kinases, eliminated the revealed effects of PRL and GH on the chromosome destruction (**Figure 7**). Meanwhile, a rise in the rate of CEOs with abnormal modifications of M-II chromosomes was more pronounced in the presence of PP2 ($p < 0.001$) than genistein ($p < 0.05$). Triciribine, the inhibitor of Akt kinase, and calphostin C, the protein kinase C inhibitor, also increased the frequency of chromosome modifications ($p < 0.001$) during the prolonged culture of CEOs in the medium containing PRL or GH (**Figures 8** and **9**). At the same time the hormonal action on the chromosome destruction in the aging oocytes was unchanged in the presence of U0126, the MEK 1/2 inhibitor (**Figure 10**). Concurrently, at concentrations used, all the inhibitors did not affect the frequency of chromosome abnormalities in the respective control groups.

Discussion

The present research has been directed toward elucidating the pattern and mechanisms of action of two closely-related pituitary hormones, PRL and GH, on abnormal modifications of M-II chromosomes in matured bovine oocytes aging *in vitro*. The data obtained indicate for the first time that both hormones are able to eliminate the accelerating effect of surrounding cumulus cells on aging processes associated with chromosome changes in oocytes. In this case PRL and GH can use similar pathways, acting through cumulus cells expressing the respective receptors and activating

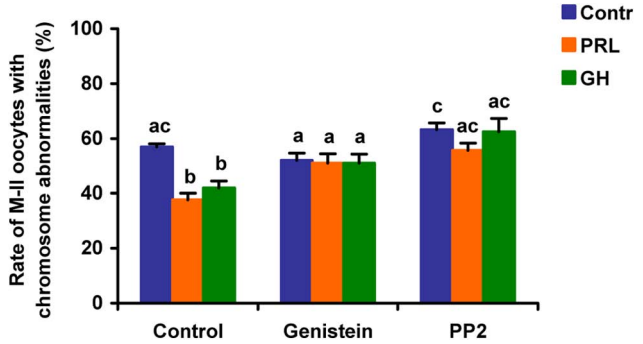

FIGURE 7 | Effects of genistein (40 μM), the non-selective inhibitor of tyrosine kinases, and PP2 (20 μM), the inhibitor of Src-family tyrosine kinases, on destructive changes in the M-II chromosome morphology during 24 h aging of bovine cumulus-enclosed oocytes in the presence and in the absence of PRL (50 ng/ml) or GH (10 ng/ml). Data represent means ± SEM of 4 replicates using 82–96 oocytes per treatment. Means with different letters differ significantly (at least $p < 0.05$).

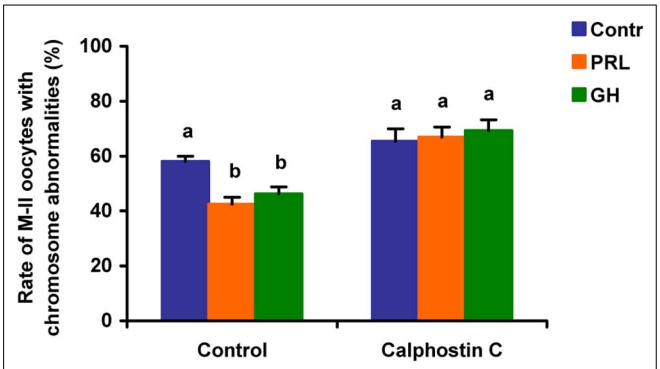

FIGURE 9 | Effects of calphostin C (1 μM), the inhibitor of protein kinase C, on destructive changes in the M-II chromosome morphology during 24 h aging of bovine cumulus-enclosed oocytes in the presence and in the absence of PRL (50 ng/ml) or GH (10 ng/ml). Data represent means ± SEM of 4 replicates using 73–81 oocytes per treatment. Means with different letters differ significantly (at least $p < 0.05$).

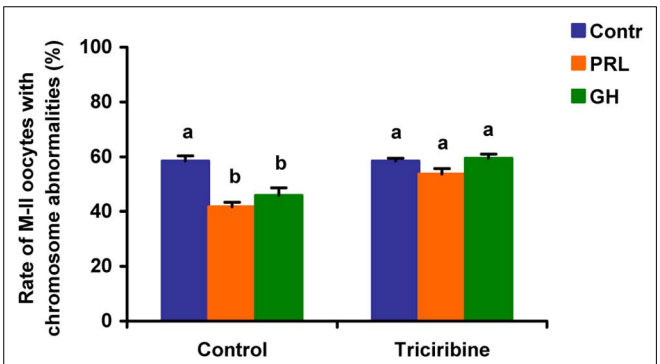

FIGURE 8 | Effects of triciribine (50 μM), the inhibitor of Akt kinase, on destructive changes in the M-II chromosome morphology during 24 h aging of bovine cumulus-enclosed oocytes in the presence and in the absence of PRL (50 ng/ml) or GH (10 ng/ml). Data represent means ± SEM of 4 replicates using 74–92 oocytes per treatment. Means with different letters differ significantly (at least $p < 0.05$).

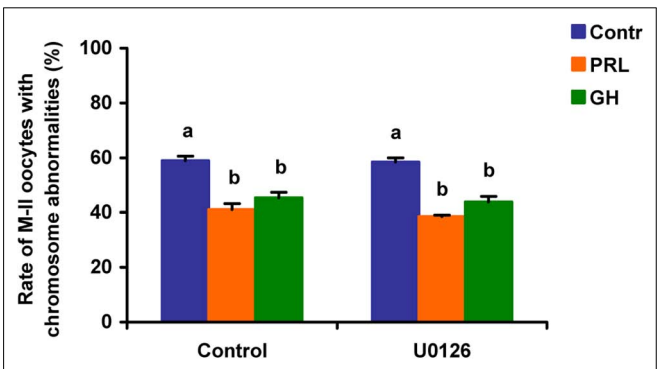

FIGURE 10 | Effects of U0126 (20 μM), the MEK 1/2 inhibitor, on destructive changes in the M-II chromosome morphology during 24 h aging of bovine cumulus-enclosed oocytes in the presence and in the absence of PRL (50 ng/ml) or GH (10 ng/ml). Data represent means ± SEM of 4 replicates using 73–86 oocytes per treatment. Means with different letters differ significantly (at least $p < 0.05$).

signaling cascades dependent on Src-family tyrosine kinases, Akt kinase, and protein kinase C.

In line with our earlier findings (Lebedeva et al., 2014a), in the current study, the prolonged culture of matured bovine oocytes was accompanied by different modifications in the morphology of M-II chromosomes, with the most common abnormal change being their decondensation or decondensation coupled with adherence. Similar chromosome aberrations have been found in human oocytes aged *in vitro* for 3–4 days (Eichenlaub-Ritter et al., 1988). Furthermore, using quantitative 3D analysis of nuclear material in oocytes of aging mice, Tian et al. (2013) have recently demonstrated an enhancement of adhesion between chromosomes in metaphase-I that may be a consequence of their decondensation and the decrease of interchromosomal distance. Thus, a decline in the degree of metaphase chromosome condensation is likely to be one of the functional nuclear alterations associated with M-II oocyte senescence. It might result in the incomplete silencing of gene transcription and subsequent abnormalities in the embryo/fetus development, which are observed following fertilization of aged oocytes (Miao et al., 2009). One can also speculate that destructive changes in the M-II chromosomal morphology are induced by epigenetic modifications driving chromosome transformations during meiosis (Endo et al., 2005; Ivanovska and Orr-Weaver, 2006). To date, various epigenetic changes, mainly a decline in the methylation level of DNA and histones and a rise in the level of histone acetylation, have been revealed in the course of mammalian oocyte aging (Huang et al., 2007; Liang et al., 2008; Manosalva and González, 2010). Furthermore, a decrease in the histone methylation level in oocytes of old female mice has been shown to be associated with different structural chromosomal abnormalities including decondensation (Manosalva and González, 2010).

Our data have demonstrated that both PRL and GH affect the morphology of M-II chromosomes in CEOs in a biphasic dose-dependent manner, which is typical for actions of these

hormones in different cell systems (Ilondo et al., 1994; Hodson et al., 2010; Lebedeva et al., 2014b). A bell-shaped pattern of dose-response curves for PRL and GH is usually attributed to the two-site mechanism of the hormone binding to their receptors involving dimerization of these latter and self-antagonism at high concentrations when monomeric hormone-receptor complexes become predominant (Kelly et al., 1994). However, this theoretical model cannot explain the opposite pattern of effects of low and high hormonal concentrations found in the present study, suggesting an implication of additional factors. Dose-response experiments have revealed inhibitory effects of low concentrations of PRL (20−50 ng/ml) and GH (5−10 ng/ml) but not EGF on abnormal modifications of M-II chromosomes in aging bovine oocytes. These concentrations are the same as concentrations, which have been effective at stimulating *in vitro* the nuclear maturation or developmental capacity of bovine CEOs (Kuzmina et al., 2001, 2007). Moreover, they are very close to the respective hormonal concentrations found in the bovine plasma and follicular fluid (Wise and Maurer, 1994; Borromeo et al., 1998; Modina et al., 2007). Physiological levels of intratubular PRL and GH possibly derived from the circulation are presently unknown; however, follicular fluid containing both hormones flows into the oviduct following ovulation (Lyons et al., 2006), permitting their local effects. The possibility for PRL and GH actions within the oviduct is also supported by evidence for the expression of the respective receptors in mammalian oviductal cells (Shao et al., 2008; Steffl et al., 2009). It should be emphasized that concentrations of PRL and GH enhancing destructive changes of M-II chromosomes are 10 to 20 times higher than physiological ones. This fact gives grounds to expect an inhibitory influence of PRL and GH on the metaphase chromosome modifications during postovulatory aging of matured bovine oocytes.

In mammals, cumulus cells are vitally important for regulating processes of oocyte maturation, ovulation, and fertilization (Tanghe et al., 2002). However, their function in oocyte aging is not evident, since they can play both positive and negative roles in maintaining the quality of the senescent ovum (Miao et al., 2005; Takahashi et al., 2009; Wu et al., 2011). Furthermore, it has been demonstrated that cumulus cells surrounding aging mouse and porcine oocytes are exposed to apoptosis and, in turn, begin to produce soluble substances, which accelerate oocyte aging in a paracrine manner (Wu et al., 2011; Zhu et al., 2015). We have previously shown that bovine cumulus cells surrounding aging oocytes are undergone apoptotic degeneration and their removal lead to delaying the abnormal changes of M-II chromosomes, suggesting an accelerating effect of the somatic investment on these changes (Lebedeva et al., 2014a). In the present work, both PRL and GH at low concentrations decelerated aging processes associated with chromosome modifications in CEOs, exerting an effect much like that of the cumulus removal. Meanwhile, PRL and GH did not affect the chromosomal aberrations in cumulus-free oocytes, implying that the hormonal impacts were achieved through cumulus cells. The availability of cumulus-mediated pathways of the hormone actions was further confirmed by the immunocytochemical localization of PRL and GH receptors in cumulus cells surrounding *in vitro* matured bovine oocytes. Taking into account all these data, one can assume that bovine cumulus cells may produce aging-promoting factor/factors (APF) contributing to abnormal modifications of oocyte chromosomes, while PRL and GH are able to suppress the production or action of APF.

Finally, the implication of the relevant protein kinases into the actions of PRL and GH on aging bovine CEOs was examined using inhibitors of the respective signaling pathways. It has been found that inactivation of Src-family tyrosine kinases, Akt kinase, and protein kinase C results in blocking of the decelerating action of the studied hormones on abnormal modifications of M-II chromosomes. By contrast, the inhibition of the MEK1/2 activity did not abolish the hormonal effects on the chromosome aberrations. Thus, the effects of PRL and GH were attained by activating the similar signaling cascades dependent on Src-family tyrosine kinases, Akt kinase, and protein kinase C. It should be noted that, in the absence of the hormones, inhibitors of these intracellular pathways did not affect the chromosome destruction, suggesting a low basal activity of the respective protein kinases in aging cumulus-oocyte complexes. The reduced activity of the signaling pathways might be a consequence of the impaired functional status of cumulus cells surrounding aging oocytes that had been previously demonstrated (Zhang et al., 2013; Zhu et al., 2015).

The revealed similarity in mechanisms of PRL and GH actions on senescent oocytes is obviously due to the close relationship both between the hormones and between their receptors (Kelly et al., 1994). Although JAK2/STAT (Janus kinase 2/Signal transducers and activators of transcription) is the main signal pathway activated in response to PRL and GH, other pathways are also involved in their effects. To date, it has been well established that Src-family tyrosine kinases are associated constitutively with PRL and GH receptors and can control activation of different signaling cascades including PI3K/Akt and MEK/ERK (Barclay et al., 2010; Waters and Brooks, 2011; Martín-Pérez et al., 2015).

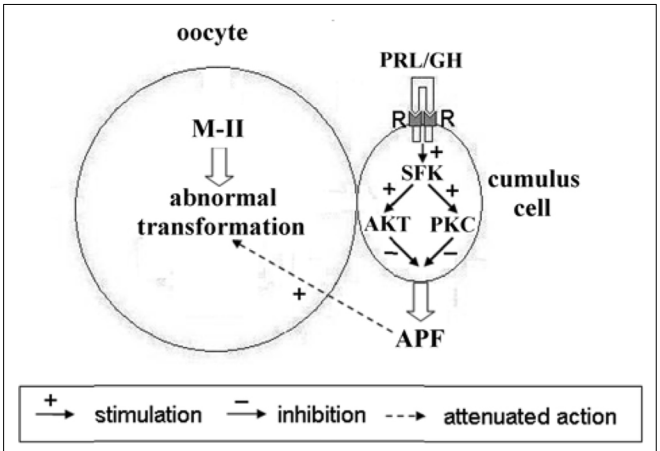

FIGURE 11 | Schematic representation of possible mechanisms of PRL and GH actions on abnormal modifications of M-II chromosomes in aging bovine oocytes. The hormone binding to the respective receptors (R) on cumulus cells causes an activation of receptor-associated Src-family kinases (SFK), which control signaling pathways dependent on Akt and protein kinase C (PKC). These events result in inhibiting the production of the cumulus-derived aging-promoting factor (APF) that accelerates abnormal chromosome modifications.

In its turn, the PI3K/Akt pathway may be connected with protein kinase C dependent pathway through activation of phospholipase C and generation of diacylglycerol (Divecha and Irvine, 1995). Meanwhile, both pathways are involved in regulation of the survival of mammalian cumulus/granulosa cells (Kim et al., 1999; Cecconi et al., 2012). In addition, PRL and GH have been shown to inhibit the apoptotic degeneration of bovine cumulus cells surrounding oocytes maturing *in vitro*, with protein kinase C mediating this effect in the case of PRL (Kölle et al., 2003; Lebedeva et al., 2011). Therefore, it seems probable that PRL and GH may eliminate the accelerating action of cumulus cells on abnormal chromosomal modifications in aging bovine oocytes by decelerating cumulus apoptosis and thereby reducing the production of the putative APF (**Figure 11**). However, other pathways, namely an apoptosis-independent suppression of the APF production or a direct hormonal inhibition of the negative action of APF on bovine oocytes, which can express PRL and GH receptors (Izadyar et al., 2000; Lebedeva et al., 2014b), must not be ruled out.

The findings about activation of the Akt-dependent signaling cascade in bovine aging cumulus-oocyte complexes in response to PRL and GH could explain the opposite pattern of effects of low and high hormonal concentrations on chromosome modifications in oocytes. According to the current knowledge, moderate levels of Akt activity inhibit apoptosis in somatic cells. By contrast, hyperactivation of Akt triggers the cell senescence and apoptotic degeneration by increasing reactive oxygen species (ROS) and suppressing antioxidant enzymes (Los et al., 2009). Thus, PRL and GH at high concentrations might raise the activity of Akt kinase up to critical levels leading to apoptosis and an enhanced production of APF in cumulus cells.

Overall, the findings of the present research point to a possible implication of PRL and GH in regulation of postovulatory aging of bovine oocytes. They suggest that both hormones at physiological concentrations are able to maintain the condensed state of metaphase-II chromosomes by activating intracellular survival pathways in cumulus-oocyte complexes and thereby attenuating negative effects of senescent cumulus cells. Further experiments are needed to clarify interrelations between signal cascades activated by PRL and GH, apoptosis in cumulus cells, epigenetic and chromosome modifications in aging oocytes, and the oocyte competence for the embryonic development. Moreover, an identification of the cumulus-derived aging-promoting factor/factors in the bovine is required.

Acknowledgments

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Pharmacological classes that extend lifespan of *Caenorhabditis elegans*

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Recent progress in the field of aging has resulted in ever increasing numbers of compounds that extend lifespan in *Caenorhabditis elegans*. Lifespan extending compounds include metabolites and synthetic compounds, as well as natural products. For many of these compounds, mammalian pharmacology is known, and for some the actual targets have been experimentally identified. In this review, we explore the data available in *C. elegans* to provide an overview of which pharmacological classes have potential for identification of further compounds that extend lifespan.

Keywords: aging, lifespan, pharmacology, GPCR, drug discovery, *Caenorhabditis elegans*, dietary restriction mimetics

INTRODUCTION

As a consequence of the seminal discoveries demonstrating that lifespan can be modulated by genes, it became clear that lifespan might also be extended using chemicals (Johnson, 1990; Kenyon et al., 1993). This concept has certainly been demonstrated, and today many compounds have been identified that extend lifespan in model organisms such as worms, flies and even mice (Kang et al., 2002; Howitz et al., 2003; Evason et al., 2005; Baur et al., 2006; Wilson et al., 2006; Petrascheck et al., 2007; Benedetti et al., 2008; McColl et al., 2008; Harrison et al., 2009; Pietsch et al., 2009; Alavez et al., 2011; Moskalev and Shaposhnikov, 2011; Oxenkrug et al., 2012; Martin-Montalvo et al., 2013; Priebe et al., 2013; Ye et al., 2014). Among all of these model organisms, *Caenorhabditis elegans* stands out because of the large variety of compounds known to extend lifespan. It is now possible to group these compounds into pharmacological classes, and use these groupings as starting points to search for additional lifespan extending compounds.

In the following paragraphs, we will give a short overview of what is known about the pharmacology of lifespan extension in *C. elegans*. We will summarize the discovery of lifespan extending compounds, comment on problems associated with the interpretation of pharmacological data, and we will end with a discussion of specific pharmacological classes of lifespan extending compounds.

DISCOVERING LIFESPAN EXTENDING COMPOUNDS

There are two fundamentally different approaches to identify compounds that have a desired biological effect. These two approaches are often referred to as forward and reverse pharmacology, analogous to forward and reverse genetics (Bartfai, 2006). Forward pharmacology approaches, also called phenotypic screens, screen for compounds that elicit a desired phenotype, like the extension of lifespan. While forward pharmacology is intuitively appealing, as it searches for the desired effect, it has a number of drawbacks. The first is that screens must generally be conducted *in vivo*. *In vivo* screens are more complex, generally longer, and have higher costs associated than *in vitro* screens. Even if these disadvantages are overcome, elucidating the mechanisms by which a hit-compound achieves the desired effect is difficult. Elucidating drug mechanisms generally requires the identification of the drug target, which even today represents a major challenge (i.e., the binding target of the compound).

Reverse pharmacology circumvents the problem of target identification by screening for compounds that bind to, or inhibit, the function of a specific protein target. Reverse pharmacology screens are largely done *in vitro*, and offer the ability to screen very large chemical libraries (+500,000). Targets are validated based on prior knowledge, such as genetic studies in model organisms or gene association studies in humans affected by the disease.

However, target validation, or choosing the protein target against which to develop a drug, also poses considerable difficulties (Pankevich et al., 2014).

As the process of aging is not easily replicated *in vitro*, most lifespan extending compounds have been identified by simply testing whether or not a given compound extends lifespan in a model organism (forward pharmacology). Thus far, most compounds that have been tested for their ability to extend lifespan had prior known pharmacology. Initially, these compounds were developed to inhibit a specific target, independent of their effect on aging. Only later were they tested for their ability to extend lifespan in *C. elegans* or other organisms. Thus, at its current state, the pharmacology of aging is a hybrid of forward and reverse pharmacology.

Why is the pharmacology of aging a hybrid of these two approaches? The simple answer is that it suffers from the disadvantages of both approaches. Target validation is problematic because most of the genes thus far found to be involved in the determination of lifespan are either essential, or they affect mitochondrial biology, insulin signaling, or general metabolism (Lee et al., 2003; Curran and Ruvkun, 2007; Hansen et al., 2007; Smith et al., 2008). These are difficult targets, as any lifespan extending compound will be given to old and frail people over extended periods of time, and therefore demands an extremely good safety profile. The history of the development of anti-obesity drugs has shown how difficult it is to choose an appropriate target to modulate metabolism in safe ways. While the example of metformin shows that it is possible to safely modulate insulin and/or general metabolism (Onken and Driscoll, 2010; Martin-Montalvo et al., 2013), we should note that the glucose lowering effects of metformin were discovered accidently through malaria research and not by a screen based on a validated target (Bailey and Day, 2004; Madiraju et al., 2014)

The forward pharmacology approach has logistical problems. Screens for lifespan in mammals are prohibitively expensive, and thus screens must be conducted in small model organisms. Even for *C. elegans*, maintaining, treating, and scoring thousands of cohorts of animals to determine their lifespan is inherently difficult, as even small irregularities have considerable effects on lifespan. For these reasons, few medium (>1000) to large (>10,000) screens have been performed using diverse compound libraries (Petrascheck et al., 2007; Ye et al., 2014). Lifespan data in and of themselves are problematic, as they are non-normally distributed, and Z' statistics generally used to evaluate other screens are not appropriate (Zhang et al., 1999). Thus, unless some groundbreaking technical advances are made, the pharmacology of aging is likely to remain a hybrid of forward and reverse pharmacology for the foreseeable future.

Early studies in *C. elegans* used extremely high concentrations of chemicals, creating the impression that worms were especially resistant. However, today many of the lifespan extending compounds are effective at concentrations in the lower micromolar range (Luciani et al., 2011; Ye et al., 2014). When compared to cell culture, these concentrations still seem high, but compared to mouse studies they are not. Drug injections are generally conducted at concentrations of 5–200 mg/kg, resulting in an internal concentration in the lower micromolar range (Hayashi and McMahon, 2002). As concentrations for *C. elegans* are indicated for the external culture medium, the internal concentrations are likely to be lower and thus similar to those in mice.

Interpreting lifespan data obtained from compounds with known pharmacology has its own pitfalls. The pharmacological data available for most lifespan extending compounds are based on human data, while the lifespan data are based on experiments in model organisms (Knox et al., 2011). How well pharmacology between species is conserved is unknown, as we have no method to determine all protein targets of a compound. It may well be that a compound annotated as an inhibitor for a specific kinase extends lifespan by inhibiting an off-target.

Thus, after the identification of a lifespan extending compound, it is important to test multiple, structurally different compounds with the same pharmacology. If several structurally different compounds with the same pharmacology extend lifespan, the lifespan extending effect is likely to come from the annotated target, since off targets tend to be different for different structures. For example, multiple serotonergic antagonists extend *C. elegans* lifespan irrespective of their structure (Ye et al., 2014). Furthermore, combining structural studies with genetic studies, in which the compound is tested on mutants lacking the suspected target, allows the identification of the compound target with a high degree of certainty.

Given these caveats, we will discuss only pharmacological classes for which (i) multiple compounds were identified and (ii) additional genetic data exist that support the notion that targeting this class of proteins will lead to a lifespan extension. The genetic data we considered include mutations in target proteins that either cause a lifespan extension or, as is often the case, abrogate the lifespan extending effect of the compound. As an exception, we include natural compounds because of their general interest and wide use as food-supplements.

PHARMACOLOGICAL CLASSES THAT EXTEND LIFESPAN

ANTIOXIDANTS

Because of Harman's theory of oxidative stress, antioxidants were some of the first compounds to be tested for their ability to extend lifespan and, as a compound class, have received quite a bit of attention (Harman, 1972; Melov et al., 2000; Benedetti et al., 2008). Indeed, antioxidants that extend *C. elegans* lifespan have been identified. These findings initially lent support to the idea that oxidative stress causes aging. However, later experiments guided by the theory of hormesis have challenged this view of aging. The theory of hormesis predicts that low levels of stress extend lifespan by over-activating stress responses, leading to excess stress response capacity and thus stress resistance and long-lived animals. Several reactive oxygen species (ROS) generating compounds such as rotenone, paraquat, arsenite, or naphthoquinone derivatives, were found to extend lifespan of *C. elegans* when used at low concentrations (Schulz et al., 2007; Van Raamsdonk and Hekimi, 2009; Lee et al., 2010; Hunt et al., 2011). Surprisingly, antioxidants blocked the lifespan extension of many hormetic agents, clearly suggesting that oxidative stress was required for the lifespan extension. Therefore, evidence

exists that antioxidants extend lifespan and, paradoxically, also abrogate lifespan extension by hormetic agents. How can these findings be unified? One argument is that, although a compound has antioxidant capabilities, it still may extend lifespan by blocking an enzymatic activity independent of the antioxidant effect. This may indeed be true for some antioxidants. However, given the evidence that oxidative stress increases with age, it is unlikely to be true for all antioxidants. An interesting alternative that could explain how oxidants (hormetic agents) as well as antioxidants extend lifespan is to hypothesize that redox regulation plays an important role in determining lifespan. For example, many proteins known to be involved in aging (e.g., DNA repair enzymes) contain reactive cysteines whose redox status may alter protein activity (Weerapana et al., 2010).

While lifespan extending antioxidants were found based on candidate approaches, unbiased screens testing many pharmacological classes for their ability to extend *C. elegans* lifespan did not result in any lifespan extending antioxidants. This observation suggests that, as a pharmacological class, antioxidants may not be a particularly strong candidate for identification of lifespan extending compounds (Ye et al., 2014).

METABOLITES

The first ever intervention found to verifiably extend lifespan was dietary restriction. Thus, dietary restriction immediately linked the process of aging to metabolism. In recent years, metabolites have received increased interest, due in part to technical advances in metabolomics and the identification of metabolic enzymes important in the determination of lifespan. Today, multiple metabolites are known that play a role in the determination of adult lifespan. These include the nuclear hormone receptor ligand dafachronic acid, ascarosides (ascr#2, ascr#3), *N*-acylethanolamines, and α-ketoglutarate (Motola et al., 2006; Lucanic et al., 2011; von Reuss et al., 2012; Ludewig et al., 2013; Priebe et al., 2013; Chin et al., 2014).

Ascarosides are endogenously produced compounds that are derived from the dideoxy sugar ascarylose, and were discovered as high population density signals. They affect many aspects of *C. elegans* physiology. For example, the lifespan extension of ascr#2 is mediated by the G-protein coupled receptor (GPCR) DAF-37, specifically expressed in the nervous system. This is an excellent example of how environmental signals are able to affect longevity via the nervous system (von Reuss et al., 2012).

Dafachronic acids are bile-acid-like steroids that are ligands for nuclear hormone receptors and are synthesized in starving animals. In adult *C. elegans*, dafachronic acids directly or indirectly activate NHR-8, leading to a reduction in *let-363*/mTOR expression and subsequent longevity (Motola et al., 2006; Thondamal et al., 2014).

Similarly, the tricarboxylic acid cycle intermediate α-ketoglutarate appears to mediate dietary restriction induced lifespan extension. Starvation induces α-ketoglutarate synthesis, which then blocks the ATP synthase, leading to reduced oxygen consumption, reduced ATP synthesis and increased autophagy (Chin et al., 2014).

In contrast, *N*-acylethanolamines, a class of fatty acid amides, are synthesized under favorable conditions. While high levels of *N*-acylethanolamines block lifespan extensions by dietary restriction, low levels of *N*-acylethanolamines are found under conditions of dietary restriction and are sufficient to extend lifespan (Lucanic et al., 2011). There are still few metabolites known to regulate lifespan, but the aforementioned examples show that the metabolome is likely to contain many more interesting compounds involved in the regulation of lifespan.

KINASE INHIBITORS

The first cloned gene found to be important for lifespan determination was the class-I phosphatidylinositol 3-kinase *age-1* (Morris et al., 1996). In addition to *age-1*, numerous mutations in various kinases have been found to extend *C. elegans* lifespan, including the receptor tyrosine kinase *daf-2*, *akt-1*, TOR, and S6 kinase, to name a few. Mutations in kinases like *age-1* and the insulin/IGF receptor *daf-2* cause some of the most dramatic effects on lifespan. As mutations in kinases are also frequently found in cancers and other diseases (Vogt et al., 2010) many kinase inhibitors were found to extend *C. elegans* lifespan (e.g., lithium, GDC-0941, LFM-A13, and rapamycin), with the most promising being rapamycin (Harrison et al., 2009; Ye et al., 2014). However, thus far none of the tested kinase inhibitors has been able to reproduce the spectacular longevity seen in *age-1* or *daf-2* mutants. For example, PI3kinase inhibitors like GDC-0941 do increase *C. elegans* lifespan, but only by about 10%, while the most extreme *age-1* mutant alleles increase lifespan by 700% or more (Ayyadevara et al., 2008; Bharill et al., 2013). Similarly the IIS (*daf-2*) inhibitor NT219 blocks IIS signaling in *C. elegans*, but does not extend lifespan (El-Ami et al., 2014). It is unclear why these kinase inhibitors are unable to mimic the effect of genetic mutations. Possible explanations include: (i) the kinase inhibitors are not specific enough, and thus cause side effects masking longevity, (ii) inhibiting the kinase activity only is insufficient, (iii) longevity in the mutants is partially due to altered development. These three possibilities are testable and, while currently the answer is not known, it should be possible to clarify these issues soon.

NUCLEAR HORMONE RECEPTORS

Nuclear hormone receptors are an important class of regulatory proteins that activate or repress gene expression patterns in response to cellular signals. The fact that these signals generally consist of small molecules, like steroid hormones, makes nuclear hormone receptors important drugs targets. Because of the early discovery of the role of DAF-12 in the determination of lifespan, hormonal regulation of lifespan has been a central theme in aging. Candidate approaches, as well as unbiased screens of synthetic compounds have identified lifespan extending compounds known to bind nuclear hormone receptors. One problem with studying nuclear hormone receptors using *C. elegans* is its vastly expanded repertoire of 284 nuclear hormone receptors, compared to 49 in mammals (Antebi et al., 2000; Robinson-Rechavi et al., 2001; Motola et al., 2006) making it difficult to translate *C.elegans* findings to mammals.

G PROTEIN COUPLED RECEPTOR LIGANDS

Compounds affecting GPCR are among the most important pharmacological classes for drug discovery (Paolini et al., 2006; Yildirim et al., 2007). In medium scale screens for compounds with known pharmacology that extend lifespan, 50% of all hit compounds targeted GPCRs (Ye et al., 2014). This was surprising, as the genome wide RNAi screens only uncovered one GPCR as being involved in the regulation of lifespan (Lee et al., 2003; Hansen et al., 2007). How can these findings be unified? One explanation may be a technical difference. The RNAi screens started to inhibit gene expression early in development, while the chemical screens started inhibition during adulthood. Indeed, the lifespan extending effect of Mianserin was reduced from +33% to less than 10% when added to developing animals rather than adult animals (Petrascheck et al., 2007). This result suggests that GPCRs signal environmental changes, upon which the physiology of the animal adapts. Thus, blocking signaling in adults causes a change that leads to alterations in physiology, while a continuous repression starting in early development does not. While considered "accidental" pharmacology, finding bioactive compounds by chance often results in compounds targeting GPCRs. Examples include Cannabinoid receptors, Opioid receptors, and dopamine receptors to name a few. By contrast, forward genetic screens, which result in the permanent inhibition of a GPCR, have only identified a handful of GPCRs. Out of roughly 6000 *C. elegans* genes cloned, only five were GPCRs, despite the fact that GPCRs represent 5% of the entire genome (Moresco and Koelle, 2004). Thus, it appears that GPCRs exist that must be active during development in order to affect lifespan when blocked in adults, probably because their function is to modulate lifespan in response to environmental change.

NATURAL COMPOUNDS

Many natural compounds or plant extracts such as blueberry polyphenols, curcumin, quercetin, ginkgo extracts, and resveratrol have been identified to extend *C. elegans* lifespan (Wu et al., 2002; Howitz et al., 2003; Wilson et al., 2006; Pietsch et al., 2009). What makes a natural compound approach attractive is that plant extracts are generally regarded as safe, and are often used as food supplements. However, natural compounds are hard to synthesize and modify, and thus target identification is particularly difficult for natural compounds. The ongoing dispute on the mechanism of action of resveratrol certainly gives testimony on such difficulties. The proposed mechanisms range from antioxidant properties, to activation of the histone deacetylase SIRT1, AMPK activation by blocking phosphodiesterase activity, or mimicking tyrosine-activating stress response pathways via TyrRS and PARP1 (Howitz et al., 2003; Park et al., 2012; Sajish and Schimmel, 2014).

SUMMARY

In this short review, we have discussed how lifespan extending compounds are currently identified, what limits progress, and which pharmacological actions seem to extend lifespan. Discussing the different classes above shows that lifespan pharmacology is not inherently different from any other type of drug discovery. It seems that the same pharmacological classes (GPCR's, nuclear hormone receptors, Kinases) that delivered compounds for many other indications are also suitable for lifespan extension. It may, however, be that our conclusions are biased for historical reasons. Most of the compounds tested for lifespan were those that were readily available, which are the same compounds that stand out as promising for other indications. Alternatively, it is also possible that GPCRs, nuclear hormone receptors, kinases, and ion-channels make better drug targets. This may be because all these proteins exert regulatory functions, affect multiple physiological functions, and may also be easier to target with drugs than others. Whatever the reasons, the large overlap between pharmacological classes that extend *C. elegans* lifespan and drugs used in humans is encouraging because it suggests that some degree of conservation can be expected.

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Dose-dependent effects of mTOR inhibition on weight and mitochondrial disease in mice

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Rapamycin extends lifespan and attenuates age-related pathologies in mice when administered through diet at 14 parts per million (PPM). Recently, we reported that daily intraperitoneal injection of rapamycin at 8 mg/kg attenuates mitochondrial disease symptoms and progression in the *Ndufs4* knockout mouse model of Leigh Syndrome. Although rapamycin is a widely used pharmaceutical agent dosage has not been rigorously examined and no dose-response profile has been established. Given these observations we sought to determine if increased doses of oral rapamycin would result in more robust impact on mTOR driven parameters. To test this hypothesis, we compared the effects of dietary rapamycin at doses ranging from 14 to 378 PPM on developmental weight in control and *Ndufs4* knockout mice and on health and survival in the *Ndufs4* knockout model. High dose rapamycin was well tolerated, dramatically reduced weight gain during development, and overcame gender differences. The highest oral dose, approximately 27-times the dose shown to extend murine lifespan, increased survival in *Ndufs4* knockout mice similarly to daily rapamycin injection without observable adverse effects. These findings have broad implications for the effective use of rapamycin in murine studies and for the translational potential of rapamycin in the treatment of mitochondrial disease. This data, further supported by a comparison of available literature, suggests that 14 PPM dietary rapamycin is a sub-optimal dose for targeting mTOR systemically in mice. Our findings suggest that the role of mTOR in mammalian biology may be broadly underestimated when determined through treatment with rapamycin at commonly used doses.

Keywords: aging, rapamycin, mTOR, mitochondrial disease, pharmaceutical intervention

Introduction

Rapamycin, a specific inhibitor of the mechanistic Target Of Rapamycin (mTOR), was the first pharmacological agent reproducibly demonstrated to extend lifespan in multiple organisms including budding yeast, nematodes, flies, and mice (Harrison et al., 2009; Ha and Huh, 2011; Partridge et al., 2011; Choi et al., 2013; Johnson et al., 2013a,b). Rapamycin was first demonstrated to increase murine lifespan by the National Institute on Aging (NIA) Interventions Testing Program (ITP) in a study where genetically heterogeneous UMHET3 mice were fed a diet supplemented with 14 PPM rapamycin in a microencapsulated formula beginning at around 600 days of age (Harrison et al., 2009). This diet was subsequently shown to extend lifespan in UMHET3 mice treated from a young age (Miller et al., 2011) and C57Bl/6 inbred mice when initiated at either 19 months of age (Zhang et al., 2014) or mixed ages (Neff et al., 2013). Surprisingly, dietary treatment with rapamycin at 14 PPM had comparable effects on survival whether treatment was initiated in young or middle-aged animals. Similar to dietary delivery, an intermittent treatment protocol where 1.5 mg/kg rapamycin was injected subcutaneously three times per week, 2 weeks out of every month, was shown to extend lifespan in the 129/Sv background starting in young animals (Anisimov et al., 2011). In addition to increasing lifespan, there is a general consensus that rapamycin attenuates age-associated declines in some measures of cardiac, immune, muscular, and cognitive function, increasing overall healthspan (Spong and Bartke, 2012; Wilkinson et al., 2012; Blagosklonny, 2013; Kaeberlein, 2013; Neff et al., 2013; Zhang et al., 2014). mTOR inhibition by rapamycin has also been shown to provide benefit in models of disease such as the murine model of Leigh Syndrome.

Leigh Syndrome (LS) is a severe mitochondrial disease that occurs in about 1:40,000 newborns and is associated with retarded growth, muscular deficits including myopathy and dyspnea, lactic acidosis, and a characteristic progressive necrotizing encephalopathy of the vestibular nuclei, cerebellum, and olfactory bulb (Budde et al., 2002, 2003; Anderson et al., 2008). *Ndufs4* encodes a subunit of Complex I of the mitochondrial electron transport chain; mutations in the NDUFS4 gene cause LS in humans (Budde et al., 2000, 2003; Darin et al., 2001; Anderson et al., 2008; Quintana et al., 2010), and the *Ndufs4* knockout (KO) mouse is a murine model of LS (Kruse et al., 2008; Quintana et al., 2010). *Ndufs4* KO mice have decreased Complex I levels and activity in multiple tissues and show severe and progressive symptoms of mitochondrial disease that mirror human LS. LS results in death at an average of 6–7 years in humans, and *Ndufs4* KO mice show a similar early-life mortality with an average lifespan of just 50 days.

In a recently published study, we reported a striking suppression of Leigh Syndrome phenotypes by rapamycin in *Ndufs4* KO animals (Johnson et al., 2013c). 8 mg/kg of rapamycin increased survival by roughly 30% when administered every other day via intraperitoneal injection (IP) and more than doubled median survival and tripled maximum survival when administered every day. In addition to the robust effect on survival, daily injection of rapamycin also attenuated multiple aspects of disease including neuroinflammation, weight loss, and behavioral phenotypes associated with neurological decline. Given these findings, we sought to determine whether oral delivery of encapsulated rapamycin could achieve effects similar to daily IP injection in regards to developmental weight gain in wildtype mice and disease in *Ndufs4* KO animals. Here, we report that oral delivery of encapsulated rapamycin has dose-dependent effects on developmental weight in mice and disease onset and progression in the murine model of LS. A dosage of 378 PPM, approximately 27-fold higher than that shown to extend lifespan of WT animals, was necessary to achieve effects on survival and body composition in control and *Ndufs4* KO animals comparable to daily IP injection of 8 mg/kg rapamycin. Importantly, although these high-doses of rapamycin resulted in a dramatic reduction in animal size, reminiscent of pituitary dwarfism, we observed no detrimental effects in treated mice. These studies have major implications for experimental and clinical use of rapamycin.

Materials and Methods

Animals

Heterozygous *Ndufs4* knockout mice on a C57Bl/6NIA background were bred to produce homozygous KO animals. Injectable rapamycin preparation and delivery and animal care and end-of-life criteria were as previously described (Johnson et al., 2013c). All animal use was in accordance with the University of Washington institutional guidelines and experiments were performed as approved by the Institutional Animal Care and Use Committee. *Ndufs4* heterozygous mice and wild-type mice are phenotypically indistinguishable in all studies (both here and previously published) and are pooled as controls for the experiments described. All mice used in these studies were C57Bl/6NIA animals.

Encapsulated rapamycin was obtained from the Barshop Institute at the University of Texas Health Science Center at San Antonio and Rapamycin Holdings, Inc. Standard mouse chow was ground to a powder and mixed with encapsulated rapamycin at 14, 42, 126, or 378 PPM. 300 mL of 1% agar melted in sterile water was added per kilogram of powdered chow and the mixture was pelleted and baked at 55°C for 2–3 h to harden. Pellets were then vacuum-sealed for short-term (4°C) or long-term (−20°C) storage. Control food contained either no drug or encapsulation material (eudragit) alone at a concentration matching that in the rapamycin chow, as indicated.

Mice began receiving assigned diet treatments upon weaning, at 20–21 days of age. Mice assigned to rapamycin (8 mg/kg) or vehicle injection groups began treatments at post-natal day 10 (P10) as previously described (Johnson et al., 2013c).

Rapamycin Blood Level Analysis

Whole-blood samples were collected in EDTA tubes by cardiac puncture immediately following euthanasia. All blood samples were collected at approximately the same time of day, during light cycle, to minimize variation between samples due to feeding. Samples were frozen and shipped on dry ice to the Javors laboratory in the Department of Psychiatry at the University

of Texas Health Science Center San Antonio for analysis by HPLC-MS.

Statistical Analysis

Linear regression and related statistics in **Figure 1A** were generated in GraphPad Prism using data for 14, 42, and 126 PPM rapamycin with y-intercept was set to 0. Survival curves were compared by Log-rank test in GraphPad. Pairwise *t*-tests were performed in Excel. All tests were two-tailed and assumed equality of variance, with a *p*-value less than 0.05 considered statistically significant.

Results

Dietary Rapamycin Reduces Developmental Weight in a Dose-dependent Manner

To assess the efficacy and effects of high-dose dietary rapamycin, control and *Ndfus4* KO animals were fed chow containing 42, 126, or 378 PPM rapamycin beginning at weaning (∼P20, see Materials and Methods). Circulating levels of rapamycin were measured after at least 7 days on the rapamycin diet (**Figure 1**). Levels of circulating rapamycin increased in a linear manner from 14 to 126 PPM at a rate of approximately 3.2 ng/ml serum rapamycin per PPM dietary rapamycin increase, with linearity decaying between 126 and 378 PPM.

Dietary rapamycin reduced developmental weight gain in a dose-dependent manner in control animals, with strength of effect ranging from no observed impact at 14 PPM (data not shown), to a robust and highly significant reduction in body size at 378 PPM that is similar in magnitude to what we previously reported for daily injection of 8 mg/kg (**Figure 2**). Rate of weight gain during 25–30 days of life was decreased in a dose-dependent manner. Mice treated with the highest doses of rapamycin are approximately 50% the size of control littermates at 30 and 60 days of age, reminiscent of genetic interventions in growth signaling such as the long-lived Snell and Ames dwarf mice (Cheng et al., 1983). While gender specific effects of rapamycin at low doses are well established, we found that at 378 PPM rapamycin male and female animals have indistinguishable rates of weight increase. Although a robust decrease in developmental weight gain occurred in both genders by 378 PPM, it should be noted that this may not be the level of maximum possible effect as we did not examine higher doses.

Dietary Rapamycin Increases Ndufs4 Knockout Survival in a Dose-dependent Manner

We next assessed the impact of dietary rapamycin on *Ndufs4* KO animals fed a diet containing 42, 126, or 378 PPM rapamycin beginning at weaning. As previously shown, control treated *Ndufs4* KO mice show a slightly reduced developmental weight gain compared to control animals until 35 days of age where disease symptoms onset. This is followed by a progressive decline in body weight concomitant with the presentation of neurological symptoms and a steady loss of fat mass (Johnson et al., 2013c). We found that dietary rapamycin treatment altered weight profiles of *Ndufs4* KO animals in a dosage dependent manner similar to the effects in wildtype animals (**Figure 3**). 14 PPM dietary rapamycin had no impact on disease onset or progression (data not shown). 42 PPM dietary rapamycin delayed the rapid decline in weight roughly proportionally to the increase in survival for this cohort (**Figures 3C,D**), while mice treated with 126 PPM or 378 PPM showed a reduced rate of weight gain but were largely protected against the rapid weight loss occurring around P35 in control animals, showing similar weight profiles

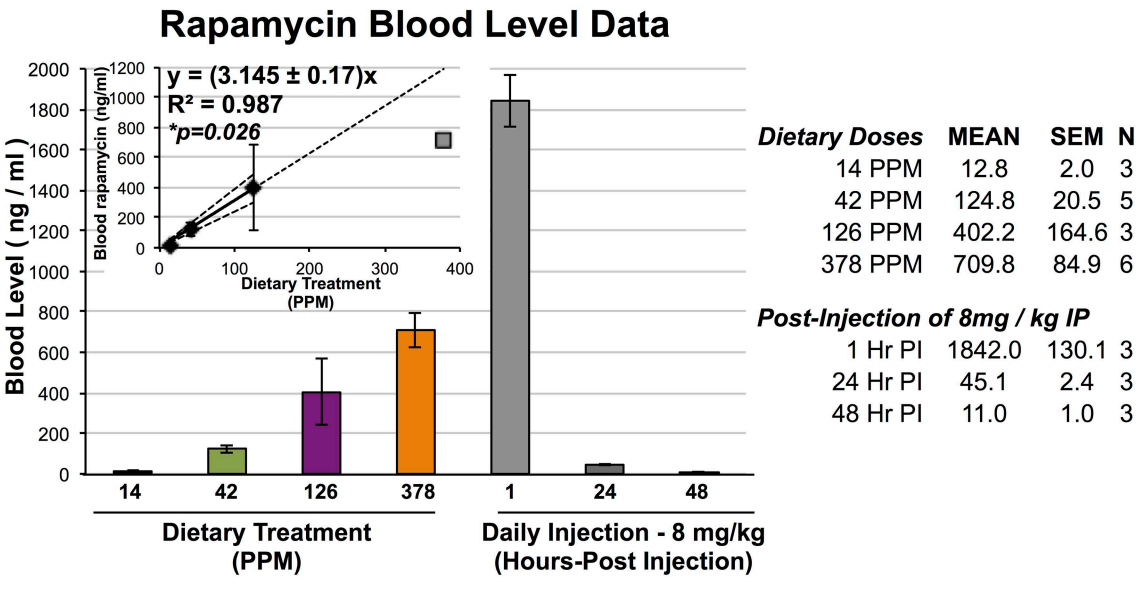

FIGURE 1 | Blood levels of rapamycin resulting from dietary and IP injection delivery. Blood levels of rapamycin in mice treated with dietary rapamycin or intraperitoneal injection as indicated. Dietary concentration to blood level relationship shown in inlayed graph; linear regression generated using 14, 42, and 126 PPM data with 95% confidence intervals, correlation co-efficient, and correlation *p*-value indicated. All error bars represent SEM.

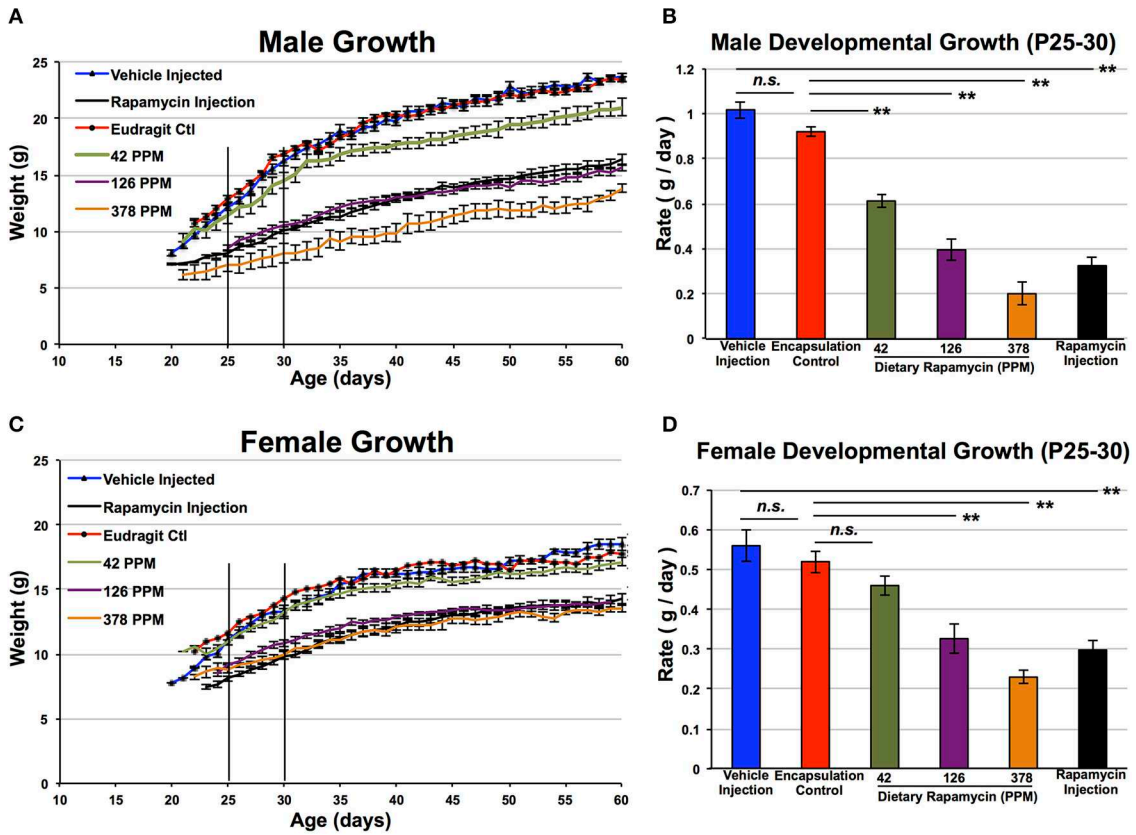

FIGURE 2 | Rapamycin reduces developmental weight gain in a dose-dependent manner. Control male **(A,B)** and female **(C,D)** mice treated with dietary rapamycin from weaning (approximately post-natal day 21). Rate calculated from post-natal day 25–30 was reduced in a dose-dependent manner in both genders. Eudragit (encapsulation material) alone at a quantity equivalent to that in 378 PPM rapamycin chow had no impact on developmental weight gain. Male and female mice treated with 378 PPM dietary rapamycin show similar weight curves and developmental weight gain rates. Rapamycin injection—8 mg/kg/day IP injection. Error bars represent SEM. $^{**}p < 0.005$; n.s., not significant.

to *Ndufs4* KO animals treated with 8 mg/kg/day of rapamycin by IP injection. These observations are consistent with the effects of daily IP injection of 8 mg/kg rapamycin beginning around weaning and demonstrate that high doses of dietary rapamycin are well tolerated and are required in order to achieve robust benefits from the drug in this disease model.

A comparison of the effects of dietary rapamycin and daily rapamycin injection on median lifespan, maximum lifespan, and rate of developmental weight gain reveals that 378 PPM impacts developmental weight gain roughly equivalently to daily IP rapamycin at 8 mg/kg but may appear not to be sufficient to achieve the same benefits in regards to lifespan in the *Ndufs4* KO animals (**Figure 3D**, see discussion).

Rapamycin Treatment Starting at P35 Delays Mortality Without Impacting Developmental Weight Gain in the Ndufs4 $^{-/-}$ Mice

As most cases of severe mitochondrial disease in children are diagnosed after the presentation of symptoms, we sought to determine if rapamycin can have a beneficial impact on mitochondrial disease when initiated after the onset of neurological symptoms. To address this we treated a group of knockout animals with 378 PPM dietary rapamycin starting at P35, the age at which weight peaks in the *Ndufs4* knockout mice and neurological phenotypes become apparent. Animals in this cohort showed a significant delay in mortality relative to controls, although the rescue was not as robust as that of 378 PPM started at weaning (**Figure 4**). These data indicate that the benefits of rapamycin are at least partly independent of any effects the drug may have on very early developmental weight, but that earlier treatment provides the greatest benefit.

New Insight Into the Impact of Rapamycin on Aging and Disease

While the beneficial effects of mTOR inhibition by rapamycin on aging and age-related disease have been reproducibly demonstrated, there has been recent debate over whether the anti-cancer effects of rapamycin may account for the lifespan benefits in the mouse model. Notably, a 2013 paper published by Neff et al. (2013) suggested that the effects of rapamycin on murine lifespan are completely explained by the anti-cancer effects of the drugs, rather than an effect on the aging process. Given our findings that dietary rapamycin at 14 PPM is at the low end of the dose-response curve for each of the

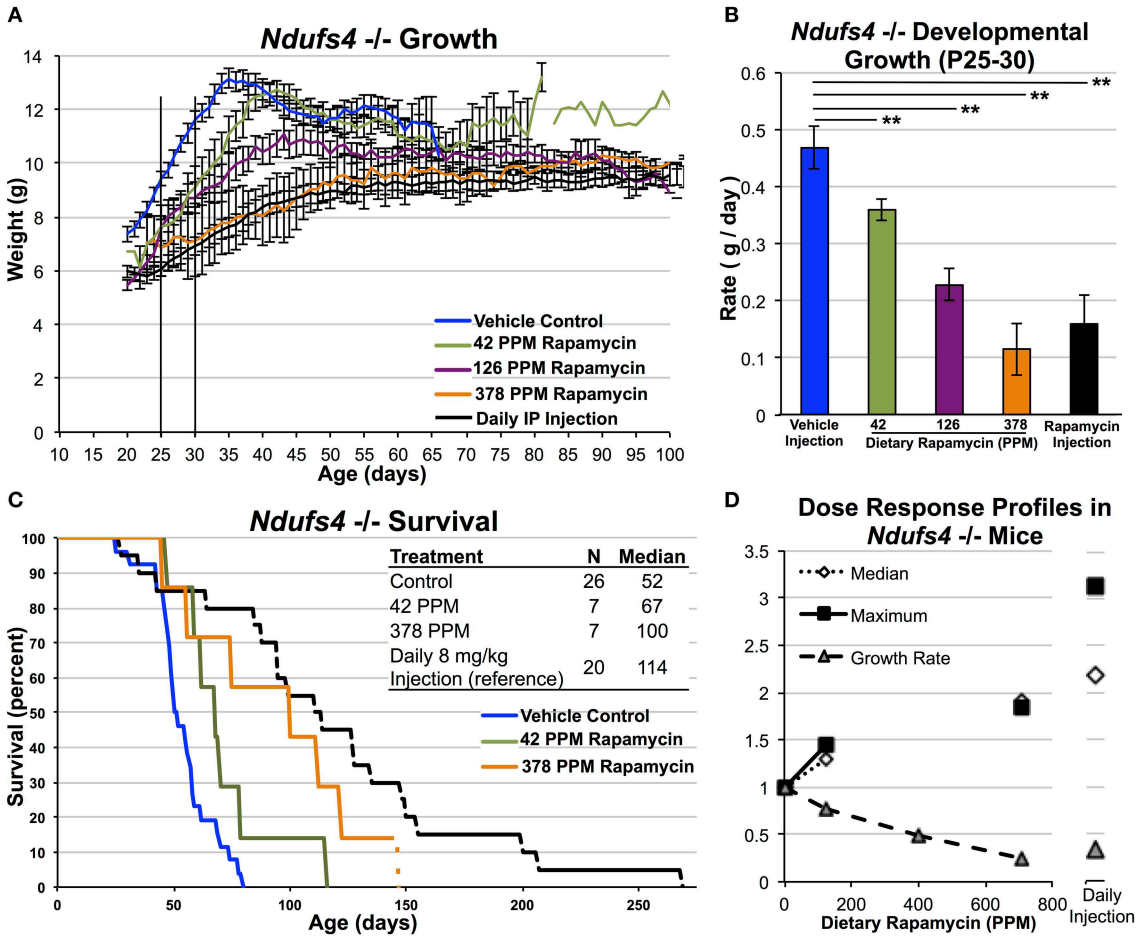

FIGURE 3 | High-dose dietary rapamycin attenuates disease and enhances survival in *Ndufs4* $^{-/-}$ mice. Dietary rapamycin reduces developmental weight gain and attenuates the progressive weight loss phenotype in a dose-dependent manner **(A,B)**. Dietary rapamycin improves survival in a dose-dependent manner **(C)**. The effect of 378 PPM dietary rapamycin on weight is slightly greater than daily IP rapamycin injection while IP injection resulted in a greater increase in median and maximum survival **(D)**. The oldest mouse in the 378 PPM group was euthanized due to lack of chow and was still healthy by gross observation at the time of euthanasia. Rapamycin injection—8 mg/kg/day IP injection. Error bars represent SEM. $^{**}p < 0.005$. 378, but not 42, PPM treatment significantly increased survival by Log-rank test ($p = 0.086$ and 0.006 for 42 and 378 PPM, respectively; IP injection shown for reference, previously reported elsewhere) (Johnson et al., 2013b).

phenotypes described here, we decided to examine relationships between treatment dose, serum levels, and phenotypic outcome in published studies that utilize this drug in an attempt to explain the somewhat discordant results in the rapamycin literature (**Figure 5**, **Table 1**). An examination of studies involving dietary rapamycin use indicates that the blood levels of rapamycin we observed in our mice are consistent with those reported in other studies using 14 and 42 PPM (**Figure 5A**). A closer inspection of the 14 PPM dose data reveals that the initial 2009 ITP publication of lifespan extension using microencapsulated rapamycin resulted in higher than average blood serum levels of the drug, while the 2013 Neff et al. paper fell at the far low end of the distribution by blood level (**Figure 5B**). Critically, there is a greater than 10-fold difference in reported blood levels of rapamycin when comparing the 2009 ITP study and the 2013 Neff et al. study, a difference that may largely account for any differences in outcome reported by these groups.

Discussion

Here we present a dose-response profile for dietary rapamycin in a mouse model. Our observations indicate that the commonly used 14 PPM dose is significantly lower than necessary to observe robust effects on developmental weight gain and attenuate disease in the *Ndufs4* KO mouse model of Leigh Syndrome. This data demonstrate that dietary rapamycin at doses much higher than previously tested in normative aging are not only well tolerated, but are also necessary to significantly impact multiple physiological outcomes, raising the possibility that doses higher than 14 PPM may have a more robust impact on longevity and healthspan in normal aging. The standard dose of 14 PPM is at the threshold of detectable effects, indicating it may be at the border of clinical significance. Suboptimal dosing may explain the results of studies that failed to observe significant effects of 14 PPM rapamycin on some parameters of aging (Neff et al.,

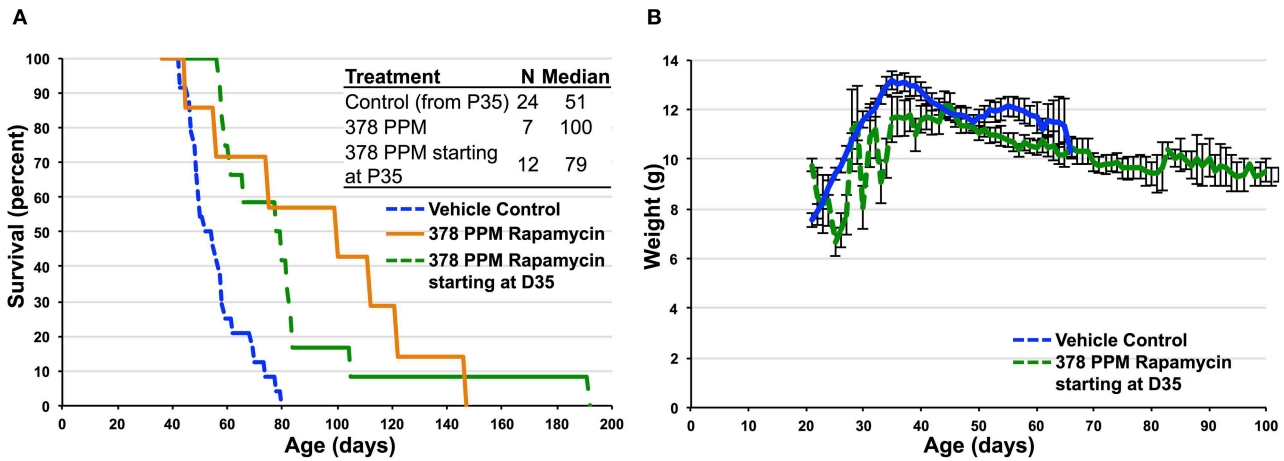

FIGURE 4 | Impact of high-dose dietary rapamycin on *Ndufs4* $^{-/-}$ mice when initiated at P35. (A) High-dose rapamycin provides some benefit to survival when initiated at P35 without impacting developmental weight **(B)**. Control animals in **(A)** only include those surviving past P35. Survival of mice treated starting at P35 is significantly greater than control treated animals ($p = 0.0002$, Log-rank test).

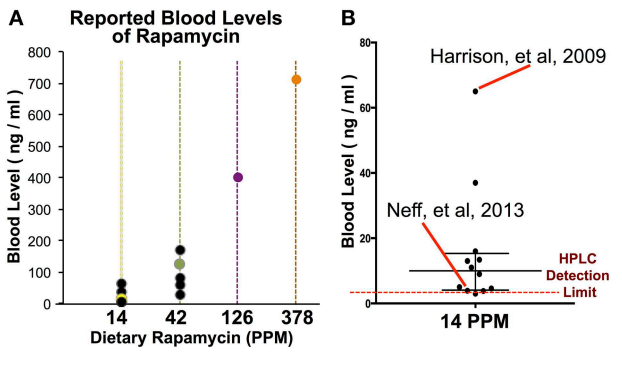

FIGURE 5 | A comparison of rapamycin blood levels in studies using dietary rapamycin treatment. (A) Blood levels of rapamycin reported in studies using dietary treatment (black datapoints) and in our study (colored datapoints). **(B)** A comparison of reported blood levels in studies using 14 PPM dietary rapamycin with the initial ITP report (Harrison et al.) and the 2013 report suggesting few benefits to aging parameters (Neff et al.) indicated. Bar and whiskers represent median and interquartile range of published blood levels for 14 PPM rapamycin. Reported limit of detection by HPLC indicated by dashed line.

2013). Rapamycin may be effective in cancer inhibition at doses lower than that necessary for robust inhibition of normal mTOR signaling, and rapamycin diet at the standard ITP dose of 14 PPM may have robust anti-cancer effects with only a relatively modest influence on other age-related pathologies. We suspect that the high doses tested here are likely to provide more potent longevity and healthspan promoting effect than previously reported.

Maintaining high levels of circulating rapamycin also appears necessary to significantly attenuate mitochondrial dysfunction. We previously found that every-other-day injection of 8 mg/kg rapamycin results in only a modest attenuation of disease in the *Ndufs4* KO model while daily injection provides a strong positive effect. A similar response was also seen in the *Lmna* KO mouse model (Ramos et al., 2012). High-dose injection provided the greatest benefit to survival in the *Ndufs4* KO model while oral rapamycin at 378 PPM had the strongest impact on developmental weight gain (**Figures 3C,D**); the bolus provided by injection provides an enhanced benefit over steady dietary delivery in the mitochondrial disease model, perhaps by overcoming blood-brain barrier. Daily IP injection of rapamycin at 8 mg/kg alters neural activity associated with aging (Yang et al., 2012) and robustly reduces whole brain levels of phospho-s6, an indicator of mTOR activity, in *Ndufs4* KO and WT animals (Johnson et al., 2013c). While the importance of mTOR signaling in brain vs. peripheral tissues in aging and Leigh syndrome is not yet known, these reports demonstrate that circulating levels of rapamycin achieved by daily IP injection are sufficient to reduce mTOR signaling in the central nervous system. Effective inhibition of mTOR in the brain may be necessary for the full benefits of rapamycin in both *Ndufs4* KO mice and in normative aging.

It is worth noting that the use of high dose dietary rapamycin in long-term intervention studies in mice will be restricted by cost, as it is currently only available from a single supplier and this dose is greater than 1000 times more expensive than delivery by injection. 378 PPM dietary rapamycin (378 ug rapamycin per gram-food) is 27 times the dose used by the ITP, while IP injection requires only 8 ug un-encapsulated rapamycin per gram-mouse weight to achieve comparable circulating levels. Alternative methods for delivery of oral rapamycin in mouse chow or the development of stable rapalogs will greatly accelerate efforts to study the effects of high-dose rapamycin in long-term treatment paradigms.

We recognize that the use of high-dose rapamycin raises additional concerns regarding potential side effects. It is important to note that we observed beneficial effects of rapamycin in the mitochondrial disease model when initiated after developmental weight gain—this indicates that the effects of rapamycin treatment on disease in this setting can be at least partially uncoupled from the off-target effects on weight.

TABLE 1 | A comparison of published studies utilizing dietary encapsulated rapamycin.

Dose	Blood levels	Strain	Primary outcome	Study
4.7, 14, 42 PPM	Female: 7, 16, 80 ng/ml Male: 6, 9, 23 ng/ml	UM-HET3	Aging	Miller et al., 2014
14 PPM	Female: ∼5 ng/ml Male: ∼3 ng/ml	C57Bl/6J and C57Bl/6NIA	Aging	Fok et al., 2014
14 PPM	3–5 ng/ml	C57Bl/6NIA	Aging	Zhang et al., 2014
14, 42 PPM	37, 170 ng/ml	Apc^{MIN}	Cancer	Hasty et al., 2014
14 PPM	10–12 ng/ml	TrJ	Nerve mylenation	Nicks et al., 2014
14 PPM	Females: 3.9 ng/ml Males: 3. 8 ng/ml	Rb1+/−	Cancer	Livi et al., 2013
14 PPM	4.57 ng/ml ($n = 3$ mice)	C57Bl/6J	Cancer	Neff et al., 2013
4.7, 14, and 42 PPM	6.5, 13.4, and 57.5 ng/ml	UM-HET3	Aging	Wilkinson et al., 2012
14 PPM	**60–70 ng/ml**	**UM-HET3**	**Aging**	**Harrison et al., 2009**
Oral nanoparticles	>3000 ng/ml, no toxicity observed	CD1	Cancer	Bisht et al., 2008
14, 42, 126, and 378 PPM	13, 125, 402, 710 ng/ml	C57Bl/6NIA	Developmental weight, disease	Data here

The original publication demonstrating murine lifespan extension by microencapsulated rapamycin in bold. Bisht et al., study provided for reference as the highest level of rapamycin reported in mice and no overt toxicity was observed. UM-HET3—genetically heterogenous mice produced from a four-way cross. C57Bl/6 substrain (National Institute on Aging, NIA, vs. Jackson laboratory, J) as indicated.

Conversely, in the setting of a disease with 100% mortality early in life this off-target effect may be seen as tolerable. This will be an important consideration if rapamycin is brought to clinical trials for Leigh Syndrome.

Even at the ITP dosage of 14 PPM, sustained periods of treatment may result in abnormal insulin and glucose responses reminiscent of diabetes (Lamming et al., 2012). However, while mice treated chronically with rapamycin show abnormalities in the context of a glucose tolerance test, this does not equate to a pathogenic state and it remains unclear whether these animals actually have any deficiency in glucose homeostasis under physiologically relevant conditions. Intolerance in this assay may reflect an altered metabolic state wherein metabolism is shifted away from glucose utilization, a state of "starvation diabetes" (Blagosklonny, 2011), where animals are not physiologically primed to deal with a sudden, non-physiological increase in blood glucose levels.

Consistent with this, unpublished data from our laboratory indicates that 90 days of IP injection of 8 mg/kg rapamycin in adult mice does not significantly alter circulating glucose levels in the blood (data not shown). While more work is needed to define the physiological effects of high-dose rapamycin and the true relevance of observed metabolic shifts, our data clearly demonstrate that chronic, very high dose rapamycin has no overt toxicity in mice, and any abnormal physiological findings must be considered in the context of the known benefits of mTOR inhibition in aging or disease paradigms. Furthermore, while it was demonstrated that a modest extension of lifespan in female mice can be achieved in the context of mTORC1 reduction alone, the importance of mTORC2 in the beneficial effects of rapamyicn remains to be determined. The relative roles of mTORC1 and mTORC2 may partially explain the dosage-requirements demonstrated by our work, in addition to the pharmacokinetic properties and tissue specific effects of rapamycin, and will require further study.

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Heating and ultraviolet light activate anti-stress gene functions in humans

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Different environmental factors (i.e., toxins, heavy metals, ultraviolet (UV) rays, and X-radiation) cause damage to DNA, cell membranes and other organelles and induce oxidative stress, which results in the excessive production of reactive oxygen species (ROS) by phagocytes. All types of cell stress are accompanied by the activation of anti-stress genes that can suppress ROS synthesis. We hypothesized that different environmental factors would affect organisms through the activation of anti-stress genes by autologous serum (AS) proteins, followed by the synthesis of molecules that increase cell resistance to oxidative stress. The goal of this work was to study the influence of AS on ROS production by peripheral blood neutrophils isolated from donors in different age groups. Neutrophils were isolated from 59 donors (38–94 years old). AS was heated at 100°C for 30 s. or irradiated by UV light at 200–280 nm and 8 W for 10 min. Neutrophils were exposed to heat shock at 42°C for 1 min. (short-term heating stress) or 43°C for 10 min., followed by the determination of the chemiluminescence reaction induced by zymosan. AS can increase or decrease ROS production by neutrophils depending on the structure of the proteins in the serum; these structures can be changed by heating or UV treatment and the temperature of their interaction (4 or 37°C). We propose that the effect of environmental factors on AS proteins can cause an adverse increase in oxidative stress levels due to the functional reduction of anti-stress genes. We found a negative correlation between the quantity of intracellular Hsp70 and levels of intracellular ROS production following 10 min of heat shock at 43°C. Short-term heating stress (1 min) at 42°C was followed by a prominent reduction in ROS production. This effect may be a result of the impact of the hormone adrenaline on the functions of anti-stress genes. Indeed, the same effect was observed after treatment of the neutrophils with adrenaline at concentrations of 10^{-4} and 10^{-5} M. In contrast, dexamethasone from the other stress hormone group did not evoke the same effect at the same concentrations.

Keywords: reactive oxygen species, autologous proteins, ultraviolet light, heat shock proteins, phagocytosis, short-term heating stress, adrenaline, dexamethasone

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Introduction

Oxidative stress refers to the production of reactive oxygen species (ROS) in excessive concentrations in humans. Cell stress, especially oxidative stress, is one of main causes of many age-related diseases, including coronary heart diseases, cardiosclerosis, arterial hypertension, Alzheimer and Parkinson diseases, and cerebrovascular atherosclerosis (Penke et al., 2011).

Different environmental factors (i.e., toxins, heavy metals, ultraviolet (UV) rays, and X-radiation) cause damage to DNA, cell membranes and other organelles and induce oxidative stress, which results in the production of excessive concentrations of ROS by phagocytes. Phagocytes were discovered by Ilja Mechnikov and are the main cells that produce ROS in organisms. Many studies have used cell heating to produce a stress reaction and activate anti-stress genes. Indeed, all types of cell stresses are accompanied by the activation of anti-stress genes that can suppress ROS synthesis.

The hypothalamus is activated in emergency situations. The hypothalamus stimulates the sympathetic nervous system, adrenal medulla and adrenal cortex. The adrenal medulla releases adrenaline and noradrenaline, while the adrenal cortex releases cortisol. Adrenaline (epinephrine) plays a central role in the stress reaction, and especially in the short-term stress reaction. The stress system relies on two key hormones: adrenaline and cortisol. Adrenaline works over a short time period, while cortisol has a prolonged period of action (Mark, 2007). In the present study, we investigated the influence of adrenaline and dexamethasone (a homolog of hydrocortisone) on ROS production by human neutrophils.

We hypothesized that different environmental factors affected organisms through changes in autologous serum (AS) protein structures. This affect was accomplished by the activation of anti-stress genes, followed by synthesis of Hsp70 molecules that increase cell resistance to oxidative stress. The goal of this work was to study the influence of heat and UV treatments on AS-induced ROS production by neutrophils isolated from the peripheral blood of donors in different age groups.

Our results demonstrate that the adsorption of AS onto neutrophils at 4°C (an unfavorable temperature for phagocytosis) prior to stimulation with opsonized zymosan enhanced ROS production; this was especially true for the adsorption of heated AS. These results coincide with data from other authors, who reported the stimulation of ROS production by human leukocytes independent of phagocytosis using heat-aggregated human IgG or serum-treated zymosan and cytochalasin B-treated neutrophils for the prevention of phagocytosis (Goldstein et al., 1975).

Some authors (Veloso et al., 2008) proposed that the inhibition of ROS production observed following treatment with autologous plasma was due to its antioxidant capacity. Our results did not show a marked antioxidant capacity of AS in patients of different ages following the treatment of neutrophils with AS at 4°C or their stimulation by AS at 37°C.

According to our data the UV irradiation of AS caused changes in the stimulation of ROS production by neutrophils. A possible explanation is that the treatment could have results in changes in the structure of AS proteins. Indeed, UV irradiation has been shown to cause a reduction in human α-lactalbumin by affecting the S–S bonds that form disulfide bridges (Permyakov et al., 2003).

The negative correlation between intracellular ROS production and Hsp70 found in this article may indicate that a higher concentration of Hsp70 in plasma is protective against oxidative stress. This higher concentration may cause lower levels of intracellular ROS production (Njemini et al., 2007).

In our investigations reduction in ROS production was observed after treatment of neutrophils with adrenaline at concentrations 10-4 and 10-5 M. This effect may be associated with both the activation function of the anti-stress genes by adrenaline and its influence as an antioxidant (Shimizy et al., 2010).

Materials and Methods

Participants

A total of 28 donors aged 38–59 years (three men), 17 donors aged 60–75 years (two men) and 14 long-lived donors aged 90 and over (two men) registered as patients in the Moscow Clinical Centre of Gerontology were recruited for the study. These donors suffered from co-morbidities, with coronary heart diseases cardiosclerosis, arterial hypertension, and cerebrovascular atherosclerosis diagnosed as the main pathologies. The inclusion criteria for participation were the absence of active pathologies (history of acute infection, tumors, apoplexy, or myocardial infarction) and treatment with corticosteroids or high doses of non-steroidal anti-inflammatory drugs for all subjects, and independent living for elderly and non-agenarians. The study was approved by Ethics Committee of Russian National Research Medical University named after N.I. Pirogov. All participants gave their informed consent prior to the study.

Neutrophil Isolation

Neutrophils were isolated from the peripheral blood within 2 h after blood sampling. The samples were centrifuged at 500 × *g* for 30 min at room temperature (RT) in a density gradient using PolymorphPrep separation medium (Axis-Shield, Sweden). Fractions containing neutrophils were collected. The cells were washed twice (400 × *g*, 15 min) in Dulbecco's phosphate buffer saline (DPBS), resuspended in RPMI-1640 media (Sigma-Aldrich, USA) supplemented with 2 mM L-glutamine, 15 mM HEPES and 2% fetal calf serum (HyClone, Thermo Scientific, USA; referred to hereafter as assay media) at a concentration of 2×10^6 cells/ml and left for 30 min prior to use in assays. Neutrophil purity was assessed by flow cytometry analysis and was routinely found to be ≥95%. Cell viability determined by trypan blue staining was at least 97%.

ROS Measurement by Luminol-Amplified Chemiluminometry

Reactive oxygen species production was assessed using the luminol-amplified chemiluminometric method (Allen and Loose, 1976). Neutrophils (2×10^5 cells/sample) were stimulated with zymosan A (Sigma-Aldrich, USA) opsonized with a freshly prepared serum pool from 10 donors at a final concentration of 20 mg/ml to induce ROS production. The reaction was performed at 37°C in plastic tubes in colorless Hank's Balanced Salt Solution (HBSS; 200 μl) and 1 μM luminol (Serva, Germany) in a volume of 400 μl. The level of chemiluminescence in the cell samples was

measured using a 3603 chemiluminometer (Dialog Joint Venture, Russia). The number of light pulses per minute (cpm) was registered. The kinetics of the level of chemiluminescence was recorded for 30 min. Spontaneous ROS production was measured before zymosan treatment as the initial count per minute (cpm) level. The maximal cpm level was used for the calculation of zymosan-induced ROS production in a sample. The experiments were performed in duplicate.

Measurement of Intracellular ROS Production

Intracellular ROS generation in neutrophils was determined using 2′-7′-dichlorodihydrofluorescein diacetate (DCFHDA, Invitrogen, USA; Bass et al., 1983). The probe was added to neutrophils resuspended in assay media (500 μl) at a 5 μg/ml final concentration. After incubation for 20 min at 37°C, the cells were washed twice with DPBS at 4°C. Then, fluorescence at 530 nm was measured in the neutrophils by flow cytometry on a BD FACSCalibur flow cytometer (San Jose, CA, USA) with excitation at 488 nm.

Heat Treatment and Intracellular Hsp70 Immunolabeling

Neutrophils in assay media were dispensed into polypropylene tubes (10^6 cells in 500 μl) and heated in a constant-temperature water bath at 43°C for 10 min (heat shock) or at 42°C for 1 min (short-term stress) followed by recovery period of 1 h at 37°C. Intracellular levels of Hsp70 were determined by indirect immunofluorescent staining, followed by flow cytometry analysis. For intracellular labeling, the neutrophils were fixed and permeabilized in DPBS containing 2% paraformaldehyde (Riedel-de Haen, Germany), 0.05% BSA and 0.05% Triton X-100 (Sigma-Aldrich, USA) at 37°C for 15 min. The permeabilized neutrophils were treated in a 100 μl volume with the primary HSP70-specific monoclonal antibody BRM22 (Sigma-Aldrich, USA) or HSP70-specific B-hybridoma supernatants at 1:100 dilutions for 30 min at RT, and then stained with secondary sheep anti-mouse IgG Fab-fragments conjugated with PE (Sigma-Aldrich, USA) for 30 min at RT. Each stage of labeling was followed by two washes with DPBS containing 0.2% BSA and 0.1% Triton X-100. The cells were finally resuspended in DPBS and analyzed by flow cytometry. Intracellular HSP70 levels were determined by means of fluorescence intensity (MFI) corrected for the background fluorescence of the negative controls. The level of Hsp70 production by neutrophils was used as an indicator of anti-stress gene functions. Hsp70 is a very conservative family of cytoprotective proteins that are specifically induced in response to several environmental stresses at the cellular level, including heat shock, cellular energy depletion, oxidative stress or inflammation. Intracellular Hsp70 prevents abnormal folding of newly synthesized polypeptides or assists in the repair of damaged proteins (Ogava et al., 2008).

Variations of AS Interactions with Neutrophils

Autologous serum was prepared from the donor peripheral blood by centrifugation to remove cellular components and fibrinogens. There were two variations of AS interactions with neutrophils. AS In the first variation, prior to the chemiluminescence reaction the neutrophils were treated with different dilutions of serum for 30 min at 4°C, followed by washes. In the second variation, 200 μl of AS (1:10 dilution) was used directly in the chemiluminescence reaction as a stimulator of ROS production by neutrophils at 37°C. This temperature represents a favorable condition for phagocytosis. Prior to the interaction with neutrophils, the AS was heated in a water bath (100°C) for 30 s or irradiated by UV rays of 200–280 nm using a quartz lamp with a power setting of 8 W.

A total of 100 μl of normal or heated AS was added to 100 μl of neutrophils (2×10^5 cells) and incubated for 30 min at 4°C, followed by centrifugation at $400 \times g$ at 4°C for 10 min. The cells were resuspended in 100 μl of colorless Hanks. Control neutrophils suspended in colorless Hanks without AS were centrifuged at 4°C for 10 min and resuspended in 100 μl of colorless Hanks.

The chemiluminescence reactions were performed in plastic tubes in colorless Hanks with Ca^{++} and Mg^{++} using luminol (Sigma) at a concentration of 2.5 μg/ml. A total of 100 μl of neutrophils were added to plastic tubes with 200 μl of Hanks solution and 150 μl of luminol in the revolving drum of the chemiluminometer for 1 h at 37°C; then, the cells were stimulated by the addition of heated or UV irradiated AS or opsonized zymosan. The control tubes were treated with UV irradiated or normal Hanks. AS (1:10 dilution) was used directly in the chemiluminescence reaction as a stimulator of ROS production in a 200 μl volume. Prior to the interaction with neutrophils, the AS was heated in a water bath (100°C) for 30 s or irradiated by UV rays (200–280 nm) using a quartz lamp with a power setting of 8 W for 7 or 14 min.

Treatment of Neutrophils with Hormones

The reaction was performed in plastic tubes in colorless Hanks solution with Ca^{++} and Mg^{++}. The control tubes contained 200 μl of Hanks solution and 150 μl of luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione, Serva, Germany). A total of 100 μl of the neutrophil suspension (2×10^5 cells) was added to the experimental and control tubes. Then, adrenaline or dexamethasone was added to the experimental tubes at a concentration of 10^{-4} or 10^{-5} M for 30 min at 37°C prior to the chemiluminescence reaction.

Flow Cytometry

Flow cytometry analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, USA) equipped with 488 and 640 nm lasers and an appropriate set of detectors and filters. Neutrophils were identified and gated using forward and side light scatter. A minimum of 10,000 gated events was collected for each sample. Data were analyzed using CellQuest ver. 3.4 (BD Biosciences) and FlowJo version 7.6.5.

Statistical Analysis

Statistical analysis was performed using the R 3.0.2 statistical system (The R Foundation for Statistical Computing). The significance of the differences between two groups was attained

using a *t*-test. Correlation analysis was performed using the procedure from the stats package for R. Results were considered statistically significant at $p \leq 0.05$.

Results

Our findings show that AS affects ROS production in a dose dependent manner which is seen from **Figure 1**, presenting influence of different AS dilutions on ROS production. More diluted AS (i.e., 1:40) resulted in reduced enhancement of ROS production.

Centrifugation and resuspension procedures have been reported to reduce the neutrophil response to zymosan and subsequent ROS production (**Figure 1**, control 1 and 2). Adsorption of normal and heated AS onto neutrophils caused an increase in ROS production at AS dilutions of 1:10 and 1:20 in comparison with control 1. All patients were divided into three groups: long-lived (A) – mean age 93 years, senile and elderly patients (B) – mean age 71.4 years, and middle and young patients (C) – mean age 38 years (**Figure 2**). Heated AS evoked more ROS production compared to normal AS ($p < 0.01$) for groups B and C. Interestingly, the stimulation of ROS production by zymosan after treatment of the neutrophils from the long-lived group with normal AS did not differ significantly from the control; however, ROS production in the cell samples from the long-lived group after treatment of the neutrophils with heated AS was higher than the corresponding values in groups B and C. Data presented in **Figure 2** demonstrate that in long-lived group of patients effect of AS on the ROS production is higher than in mean age and young patients groups.

In the second series of experiments, we showed that normal AS stimulated ROS production by neutrophils to a greater

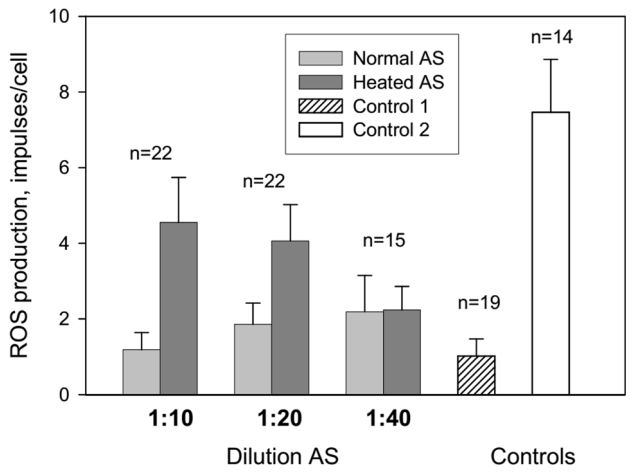

FIGURE 1 | Influence of autologous serum (AS) on reactive oxygen species (ROS) production by the patients' neutrophils measured by luminol-dependent chemiluminescence. Control 1: neutrophils in colorless Hanks without AS were centrifugated at 4°C 10 min and resuspended in 100 ml of colorless Hanks. Control 2: neutrophils in colorless Hanks have not centrifuged.

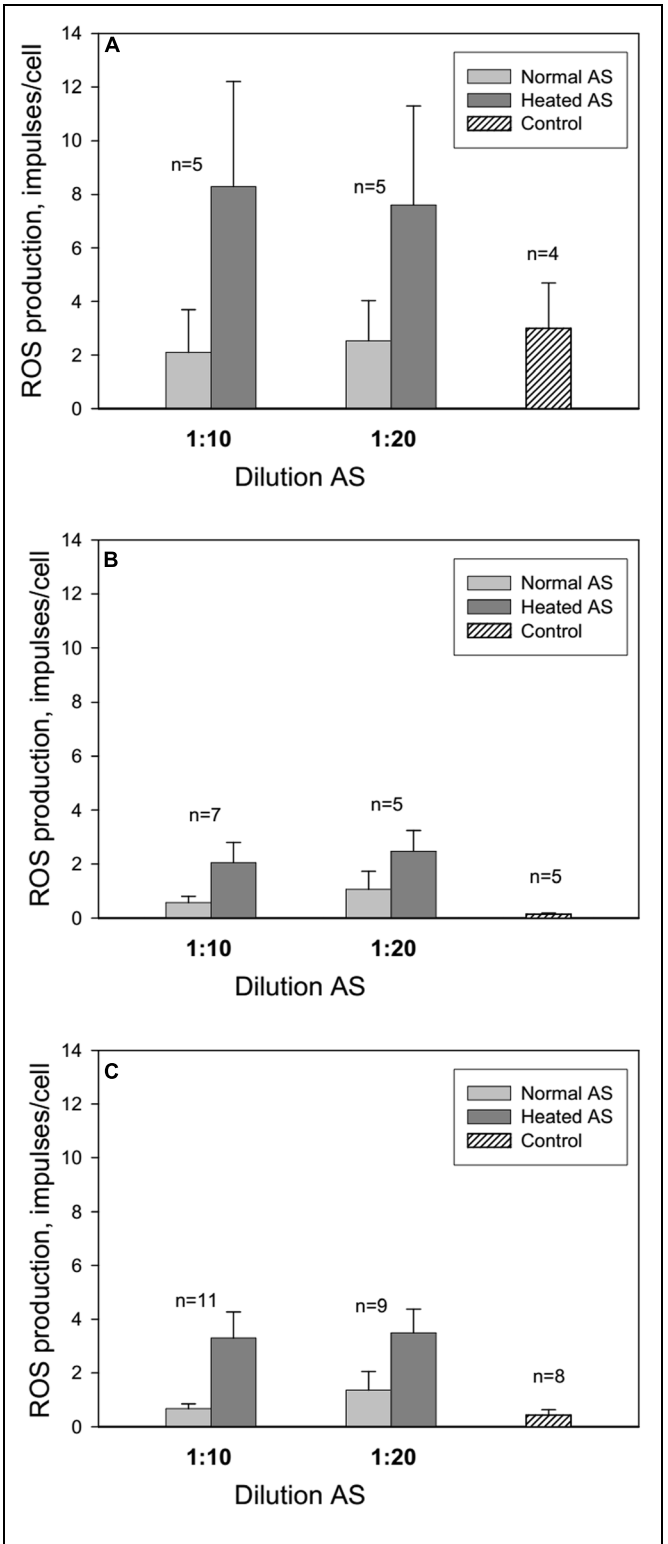

FIGURE 2 | Influence of AS on ROS production by neutrophils from long-lived patients above 90 years of age (A), patients between 60 and 75 years of age (B) and middle aged patients from 40 to 59 years of age (C). Control: neutrophils in colorless Hanks without AS were centrifugated at 4°C 10 min and resuspended in 100 μl of colorless Hanks. The line above the bar indicates the SD; n – number of patients.

extent than heated AS (**Figure 3**). UV irradiation of AS for 7 min induced ROS production by neutrophils to a greater extent than AS that was UV irradiated for 14 min. UV irradiation of Hanks solution for 14 min did not influence ROS production.

Figure 4 shows the dynamics of ROS production from neutrophils collected from an 86 years old patient after stimulation of the cells by normal, heated or UV irradiated AS. The changes in ROS production were similar to the previous data presented in **Figure 3**. Involvement of the heat shock function or anti-stress genes of neutrophils in response to treatment with the different types of AS and zymosan may be determined based on the stress impact on cells prior to their stimulation in the chemiluminescence reaction. Heating stress reduces ROS production as can be seen from **Figure 5** from dynamics of ROS production following the stimulation of heated AS by neutrophils after cells stress. UV irradiation of AS induces ROS production in time which is shown in **Figure 5**.

Figure 5 shows that heat shock (accomplished by heating the neutrophils for 1 min at 42°C) caused a marked reduction of ROS production by neutrophils in response to treatment with normal, heated or UV irradiated AS.

When neutrophils from the long-lived and 60- to 89-years-old patients were exposed to heat shock, the quantity of intracellular Hsp70 was negatively correlated with the level of intracellular ROS (**Figure 6**). Moreover, zymosan-induced extracellular ROS production showed a negative correlation with the level of intracellular ROS in the long-lived group (**Figure 7A**) that was absent in the 60- to 89-years-old patients (**Figure 7B**).

The treatment of neutrophils collected from patients in the different age groups with adrenaline at concentrations of 10^{-4} and 10^{-5} M (**Figures 8–10**) caused a reduction in ROS production. In contrast, treatment with dexamethasone (from the other stress hormone group) at the same concentration did not evoke this effect (**Figures 9** and **10**).

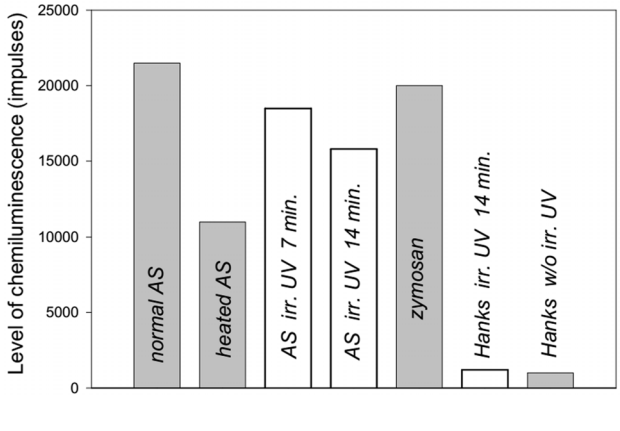

FIGURE 3 | Level of ROS production from human neutrophils stimulated by heated or ultraviolet (UV) irradiated AS (collected from a 38-years-old patient) measured by luminol-dependent chemiluminescence. The maximal levels of induced chemiluminescence of neutrophils are presented.

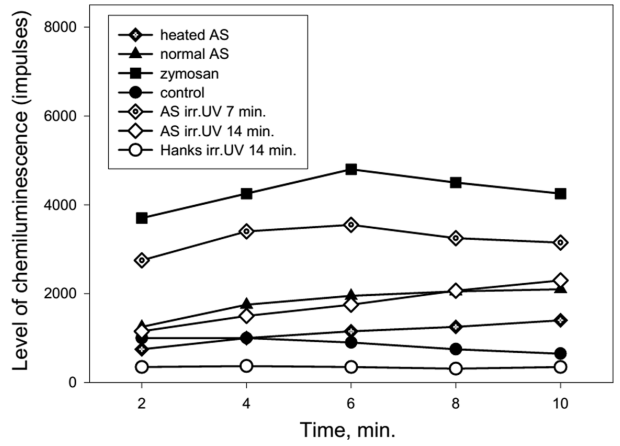

FIGURE 4 | Dynamics of ROS production by human neutrophils stimulated by heated or UV irradiated AS (collected from an 86-years-old patient). AS was heated in a water bath (100°C) for 30 s or irradiated by UV (200–280 nm) using the quartz lamp with a power setting of 8 W for 7 or 14 min prior to neutrophil stimulation.

Discussion

An autologous system is more closely related to physiological processes at the level of a whole organism. It is important to study the concrete mechanisms behind the regulation of ROS production by human phagocytes. Our data demonstrate that the adsorption of AS onto neutrophils at 4°C prior to stimulation with opsonized zymosan enhanced ROS production; this was especially true for the adsorption of heated AS. The adsorption of AS onto neutrophils at 4°C represents an unfavorable condition

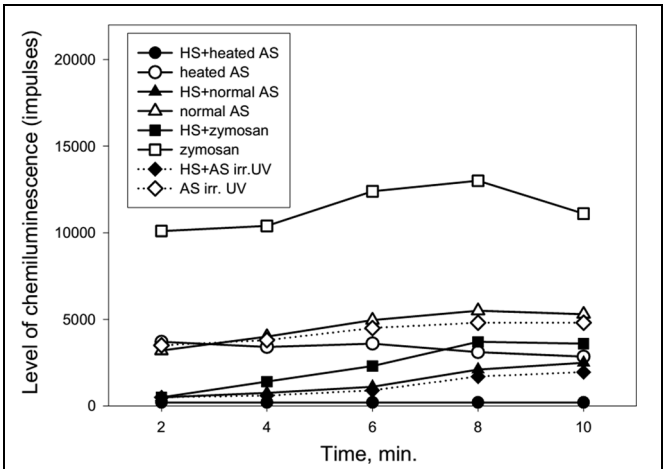

FIGURE 5 | Dynamics of ROS production following the stimulation of heated or UV irradiated AS by neutrophils after cells stress (collected from a 92-years-old patient). AS was heated in a water bath (100°C) for 30 s or irradiated by UV (200–280 nm) for 14 min. The stress impact was evoked by heating the neutrophils at 42°C for 1 min followed by a recovery period of 1 h at 37°C prior to cell stimulation.

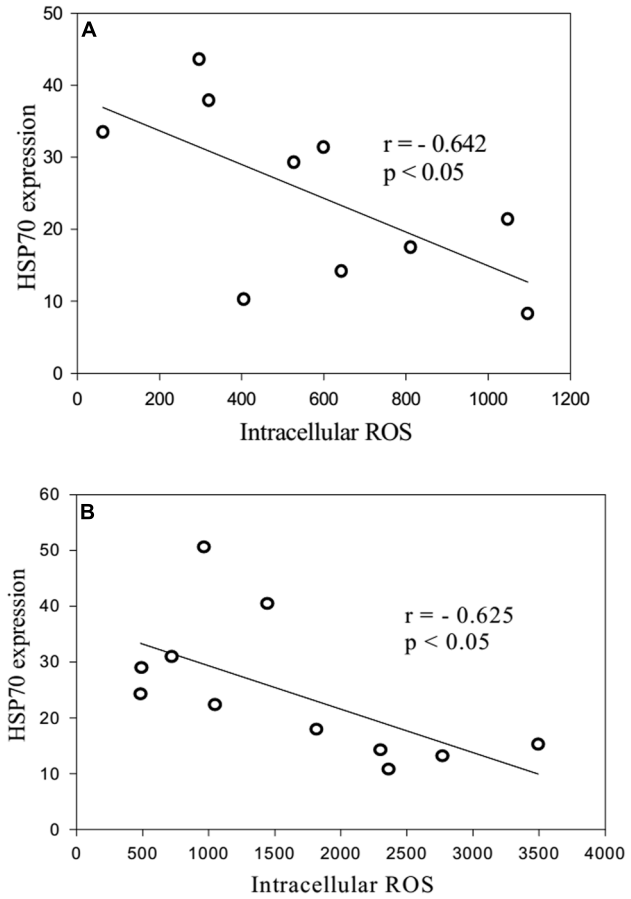

FIGURE 6 | Correlation between the quantity of intracellular HSP70 after stress and the levels of intracellular ROS production from heat shock-exposed neutrophils [(A): long-lived patients; (B) 60- to 89-years-old patients].

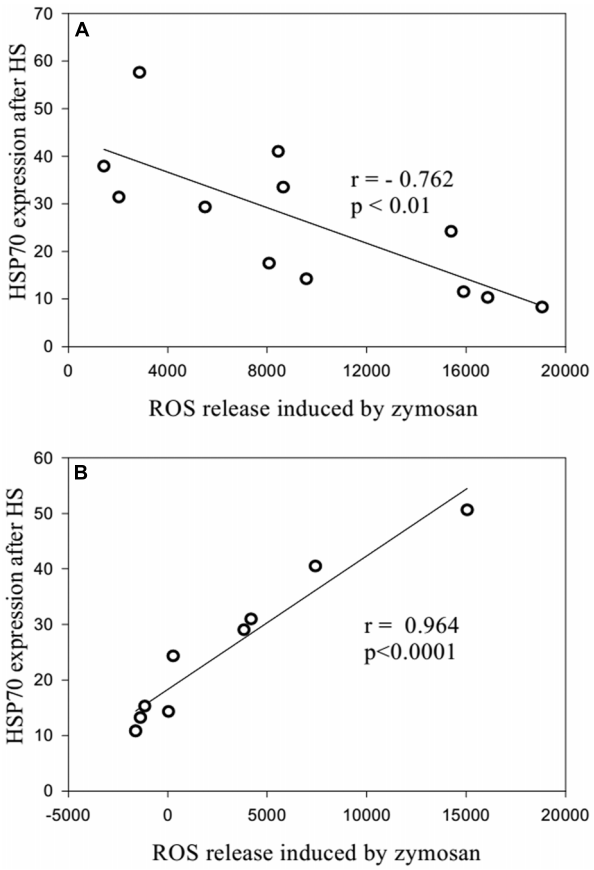

FIGURE 7 | Correlation between the quantity of intracellular HSP70 after stress and the levels of zymosan-stimulated intracellular ROS production [(A): long-lived patients; (B) 60- to 89-years-old patients].

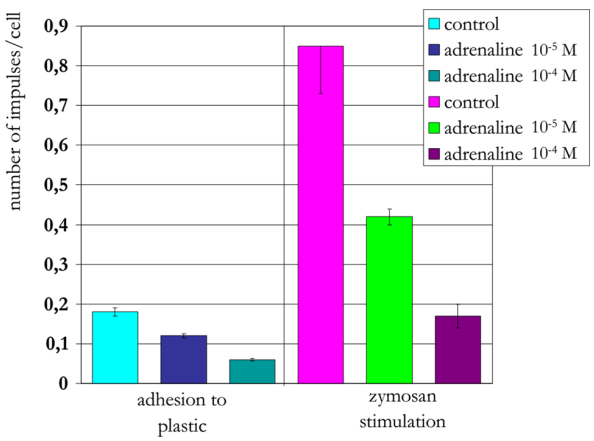

FIGURE 8 | Influence of adrenaline on ROS production by neutrophils from a 22-years-old patient (induced by chemiluminescence).

for phagocytosis. The results of our examination coincide with data from other authors, who reported the stimulation of ROS production by human leukocytes independent of phagocytosis using heat-aggregated human IgG or serum-treated zymosan and cytochalasin B-treated neutrophils for the prevention of phagocytosis (Goldstein et al., 1975). The interaction of aggregated serum proteins with neutrophil Fc receptors may be caused by the increasing ROS production by the cell mitochondria after stimulation with opsonized zymosan through the C3b receptor. Our results attach importance to the quality of serum proteins, because the use of more dilute AS (i.e., 1:40) resulted in a reduced enhancement of ROS production. High concentrations of proteins in AS can reduce the function of anti-stress or heat shock genes and the synthesis of the HSPs that suppress ROS production. HSP70 can inhibit the main producer of ROS synthesis (NADPH oxidase) in human neutrophils, and thereby suppress ROS production (Maridonneau-Parini et al., 1988). The results that in long lived group of patients effect of AS on the ROS production is higher than in mean age and young patients groups can point that with age the conformal changes of proteins increase and anti-stress genes functions decrease. These can be considered as a biological indicator of senescence.

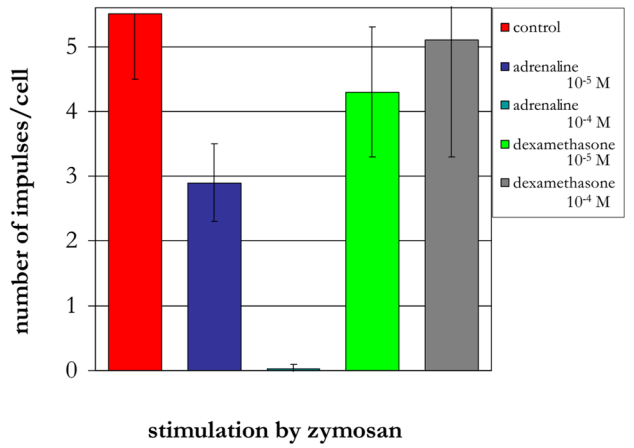

FIGURE 9 | Influence of adrenaline and dexamethasone on ROS production by neutrophils from a 90-years-old patient (induced by chemiluminescence).

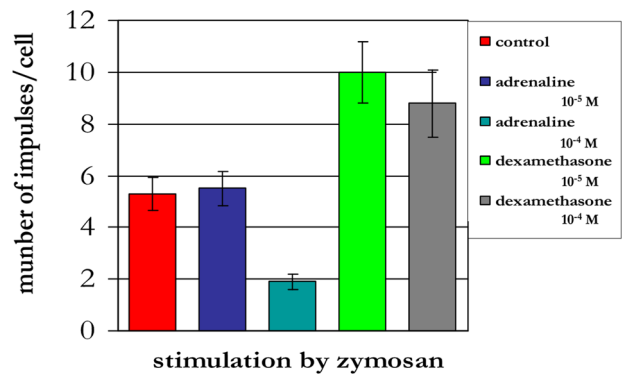

FIGURE 10 | Influence of adrenaline and dexamethasone on ROS production by neutrophils from a 94-years-old patient (induced by chemiluminescence).

In our previous work (Ponomarev et al., 2005), we demonstrated reduced ROS production by human neutrophils by the addition of recombinant HSP70 in a luminol-dependent chemiluminescence assay after stimulation with opsonized zymosan. The stress impact on neutrophils from patients in the different age groups following heating for 1 min at 42°C resulted in the reduction of ROS production in response to opsonized zymosan and normal, heated or UV irradiated AS.

The use of normal or heated AS for the stimulation of ROS production by neutrophils at 37°C (representing the favorable condition for phagocytosis) differed from the results of the treatment of neutrophils with the same types AS at 4°C. The heated AS caused a reduction in ROS production compared with normal AS. This finding may be attributed to the phagocytosis of the aggregated heated AS proteins following the activation of heat shock genes and synthesis of HSPs and the suppression of ROS production. Other authors have demonstrated the inhibition of ROS production in peripheral blood mononuclear cells from healthy and type 2 diabetic patients following treatment with autologous plasma through the Akt/PKB signaling phosphorylation pathway using luminol-dependent chemiluminescence (Veloso et al., 2008). The authors proposed that the inhibition of ROS production observed following treatment with autologous plasma was due to its antioxidant capacity. Our results did not show a marked antioxidant capacity of AS in patients of different ages following the treatment of neutrophils with AS at 4°C or their stimulation by AS at 37°C. ROS production following the treatment of neutrophils with normal AS did not differ from the control in centenarians, probably due to the preservation of some of the antioxidant activity of normal AS. However, ROS production following treatment of neutrophils with normal AS differed significantly from the controls in the senile and elderly and the middle and young patients groups.

The finding that normal AS stimulated ROS production by neutrophils to a greater extent than heated AS may be attributed to the phagocytosis of the aggregated heated AS proteins following the activation of heat shock genes and synthesis of HSPs and the suppression of ROS production. The UV irradiation of AS may have caused changes in the stimulation of ROS production by neutrophils due to its effect on the complement C3 factor and the subsequent enhancement of phagocytosis (Artjuhov et al., 2005). An alternative explanation is that the treatment could have results in changes in the structure of AS proteins. Indeed, UV irradiation has been shown to cause a reduction in human α-lactalbumin by affecting the S–S bonds that form disulfide bridges (Permyakov et al., 2003).

Our data demonstrate the possible influence of the phagocytosis of AS proteins with altered structures by neutrophils on HSG and HSP production, which can suppress synthesis of ROS. Human aging may be associated with a decrease in the functional activity of HSGs and phagocytosis. As a consequence of the decline in the neutrophils HSP response, the suppression of ROS generation is weakened and phagocytes produce high level of ROS within regions of inflammation (Ogava et al., 2008), thereby injuring self tissues. The functional activity of HSGs and the synthesis of HSP70 can affect the level of regulation of gene transcription by heat shock factors (Singh et al., 2007). Cellular senescence may be evoked through enhanced synthesis of ROS by phagocytes as a result of frequent stresses in addition to the prominent reduction in the function of anti-stress genes and the synthesis of HSPs. Damaging environmental factors can induce cell stress in an organism by acting on AS proteins of ("serum" mechanism of aging; Semenkov et al., 2005).

The reduction of the excessive concentration of ROS that results from oxidative stress can be achieved by antioxidant treatment, such as ascorbic acid (vitamin C), glutathione, melatonin, superoxide dismutase, and catalase. The treatment should be focused on the individual sensitivity of the patients, which is reflected in the decrease of ROS levels to normal values and the need of the individual to control the current ROS level (Maridonneau-Parini et al., 1988; Ponomarev et al., 2005; Veloso et al., 2008).

Ultraviolet radiation is an important factor involved in premature aging. The role of sunlight in the process of premature aging is so significant that it has been called "photoaging." In addition to serving as a source for ionizing radiation, UV light may cause the development of oxidative stress in humans. AS protein structures changed by heating or irradiation by UV rays (200–280 nm 8 W) for 10 min resulted in decreased neutrophil ROS production by means of phagocytosis and the activation of anti-stress genes, which control Hsp70 production. We propose that the effect of environment factors on AS proteins can cause an adverse increase in oxidative stress levels due to the functional reduction in anti-stress gene expression.

The negative correlation between intracellular ROS production and Hsp70 may indicate that a higher concentration of Hsp70 in plasma is protective against oxidative stress, which may cause lower levels of intracellular ROS production (Njemini et al., 2007). Our previous results showed the dependence of the correlation on the synthesis of Hsp70 and ROS production on the patient's age (Kovalenko et al., 2014).

There was a negative correlation between zymosan-induced extracellular ROS production and the level of intracellular ROS in the long-lived group that was absent in the 60- to 89-years-old patients. The determination of different types of ROS (i.e., intracellular hydrogen peroxides and extracellular superoxide anions) should be taken into account. These data showed some possible functional defects in the ability of intracellular Hsp70 to suppress extracellular ROS production in elderly and senile patients with polymorbidity.

Short-term heating stress (1 min or less) at 42°C was followed by a prominent reduction in ROS production (Semenkov et al., 2014). Under similar conditions, the same effect was observed after treatment of neutrophils with adrenaline at concentrations 10^{-4} and 10^{-5} M. The reduction in ROS production may be associated with both the activation function of the anti-stress genes by adrenaline and its influence as an antioxidant (Shimizy et al., 2010). In contrast, dexamethasone from the other stress hormone group did not evoke the same type of effect when provided at the same concentration. Dexamethasone is a homolog of hydrocortisone, which can suppress HSG function and HSP production.

Conclusion

In this article, we studied the influence of AS on ROS production in neutrophils isolated from the peripheral blood of donors in different age groups. The presented results show a new mechanism by which some environment factors (e.g., high temperature and UV light) impact oxidative stress inductors by changing the structure of AS proteins. It is known from the literature that after heating at 100°C proteins lose the quaternary structure while UV irradiation may damage disulfide bridge in proteins. These proteins affect the functions of the anti-stress genes that control anti-stress protein synthesis (Hsp70). The interaction of AS treated with heat or UV light with neutrophils evoked a reduction in ROS production at a temperature that was favorable for phagocytosis (37°C).

A negative correlation was found between the quantity of intracellular Hsp70 and the level of intracellular ROS production. This negative correlation was observed following 10 min of heat shock at 43°C. Short-term heating stress (1 min) at 42°C was followed by a prominent reduction in ROS production. The same effect was observed after treatment of neutrophils with adrenaline at concentrations of 10^{-4} and 10^{-5} M. Dexamethasone from the other stress hormone group did not evoke the same effect when provided at the same concentration.

The authors hope that the results of the investigation will be useful for future research into the influence of environment damage on anti-stress gene functions by means of AS proteins.

Funding

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Diagnostics, correction and prophylaxis of prepathological states using the non-invasive cell technology is one of the ways of longevity

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Chronic diseases (cardiovascular, cancer and others) cause the life quality deterioration and early aging. The development of early preclinical human pathology detection methods is necessary for implementation of the modern "4P medicine" paradigm—Predictive, Personalized, Preventive, and Participatory.

A non-invasive multiorgan karyological test for early diagnostics of health state was developed in a laboratory of genetic monitoring of the Research Institute of Human Ecology and Environmental Health named after A. N. Sysin (Moscow). The method involves microscopic studies of buccal, nasal, urothelial and/or bronchial epithelial cells which includes the entire spectrum of cells nuclei states morphological evaluation. In 1992, Tolbert et al. proposed a method of evaluation of nuclear abnormalities in the human buccal cells. The International Project Human Micronucleus Assay in exfoliated cells (HUMNxl) was initiated in 2007 (www.humn.org; Bolognesi et al., 2013). We proposed to increase the number of investigated biomarkers, tissues and integral indicators. We have also determined the indicative guideline values. We propose to consider the following biomarkers: cytogenetic biomarkers (number of micronuclei, nuclear buds and additionally—atypical nuclei); biomarkers of proliferation (binucleated cells and cells with double nuclei) and biomarkers of cell death, which include cells with early destruction of nucleus (with perinuclear vacuoles, nucleus membrane damage, chromatin condensation and vacuolization, early karyolysis) as well as cells with late destruction of nucleus (pycnosis, karyorrhexis, and late karyolysis). The determination and categorization criteria for diagnostic signs of the entire spectrum of karyological biomarkers was described earlier (Sycheva, 2007). Considering the karyological biomarkers categorization, including of cytogenetic parameters (cyt), proliferation (pr) and destruction of nucleus (apop), an accumulative cytogenetic damage index formula [$I_{ac} = (I_{cyt} \times I_{pr}/I_{apop}) \times 100$] as well as three levels of cytogenetic stress (low, acceptable, or high) were proposed (Sycheva, 2012).

Exfoliative cells are renewed during 10–14 days, so cytogenetic status can be monitored and corrected. It is recommended carrying out analysis of cytogenetic status 2 times in year. If the level of cytogenetic stress is high, it is necessary to determine factors inducing the DNA damage by means of interviewing a patient (eating habits, medication, working conditions, living conditions, social habits, solarium usage, etc.). Taking into account the predominating free radical mechanism of cell damage the vitamin-mineral antioxidant complexes administering may be proposed by recommended daily intakes to eliminate or minimize the identified risk factors. The next evaluation of patient's cytogenetic status is necessary to carry out after 2–3 months to determine the direction of changes. We used this approach to evaluate the influence on the population genetic state of

such environmental factors as dioxins, oil-contaminated soils, complex pollution of pulp and paper mill, metallurgical plants, coffee-production enterprise the large office in Moscow etc. Our studies revealed significantly higher numbers of exfoliated cells with cytogenetic damage and, in some cases, the level of apoptosis decreased in the group exposed to environmental pollution, compared to the control group. Decreased apoptosis is very adverse for the population, as it may lead to the accumulation of cytogenetically damaged cells as well as to the cancer induction. Positive results and improvement of human cytogenetic status were obtained after administering of antioxidants. In our study 52% of students have demonstrated decrease frequency of cytogenetically damaged cells after the 1 month of vitamins A and C administration, 38% of students did not have changes, 10% of students have demonstrated higher level of damaged cells within the indicative guideline values range. On the whole the frequency of cells with cytogenetic damage was decreased from 1.93 to 1.17‰ ($P < 0.05$) and apoptotic index was increased from 30.3 to 36.7‰ ($P < 0.001$) (Abasova et al., 2012). Approximately the same results were obtained after the vitamin and mineral complexes ≪ Helvesana ≫, ≪ Lenedex ≫, ≪ Celergen ≫ administering in studies carried out in collaboration with professor Trukhanov et al.

This approach enables the development of "Medicine 4P" paradigm. The "Predictive" medicine concept is based on the analysis of the objective biomarkers of human cytogenetic status and the analysis of epithelial cells which are the most exposed to neoplastic transformation. The "Personalized" medicine means that the evaluation of the cytogenetic status can be carried out for each person and the cytogenetic status monitoring allows determination of deleterious conditions. The "Preventive" medicine concept suggests determination of the earliest deleterious effects before their clinical manifestations. The "Participatory" medicine expects patient participation using non-invasive methods for monitoring their own cytogenetic status at the preclinical stages. The elimination and correction of genotoxic environmental factors may be attempted in cooperation with a patient in order to control and improve their personal cytogenetic status.

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Drosophila nervous system as a target of aging and anti-aging interventions

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Introduction

Nervous system regulates homeostasis and adaptation to environmental changes of a whole organism, thus deregulation of nervous processes accelerates aging (Alcedo et al., 2013a,b). The aging process in different models is associated with progressive degeneration of the nervous system (Lee et al., 2000) and progression of age-related neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Boerrigter et al., 1992; Coppede and Migliore, 2010). The neurodegeneration also characterizes the progeroid syndromes, including Hutchinson-Gilford syndrome and Werner's syndrome (Coppede and Migliore, 2010).

Drosophila melanogaster is a good model organism to study age-related neurodegenerative changes (Lu and Vogel, 2009). Enrichment in mutants with neurodegeneration among flies with shortened lifespan has been reported (Buchanan and Benzer, 1993; Kretzschmar et al., 1997). The brain from old flies demonstrates the ultrastructural neurodegenerative changes such as reduction in the number of synapses, defects in mitochondria, and increase in neuronal apoptosis (Haddadi et al., 2014). However, anti-aging interventions may postpone the neurodegeneration (Bgatova et al., 2015).

Here we consider molecular genetic changes in the *Drosophila* aging brain and the bases for applying the brain as a target for anti-aging intervention.

Aging of the Nervous System

The study of age-related gene transcriptional levels changes in *Drosophila* showed that in different organs (including the brain) there are two critical time points—30 and 60 day of age (Zhan et al., 2007). Comparing those points with *Drosophila* mortality curve it could be mentioned that the 30 day time point can be potentially attributed to the age when almost "linear" part of survival curve is followed by the "exponential" part, reflecting more rapid decrease the amount of live flies. These data are in good agreement with the shape of Gompertz curve, which describes the probability of age-related mortality in *Drosophila*. Gompertz curve has two parameters: R describes background mortality and α—exponential growth of mortality. At the initial 30 day of age Gompertz curve is close to the linear dependence with the R slope, at later 60 day of age the curve decrease exponentially. Our study of normal expression of *D-GADD45* gene during aging showed that *D-GADD45* brain expression is vanishing at critical point of 30 day old (Bgatova et al., 2015).

What are the genes that change the expression level during brain aging? As it is shown in (Girardot et al., 2006) the main effect is down regulation of genes involved in synaptic transmission at different levels divided into three subgroups. The first one includes genes that play a role in neurotransmitter metabolism such as the choline acetyltransferase (*Cha*) and the dopamine N acetyltransferase (*Dat*) genes. In the second subgroup many genes involved in various steps of neurotransmitter secretion: priming for synaptic vesicle fusion (γ-SNAP, unc13, comatose and tomosyn), fusion with presynaptic membrane (*Csp*, *Syx1A* and *rab3-GAP*) and reformation of vesicles through endocytosis (*liquid facets*, *AP-50* and *AP-2σ*). The third subgroup includes several neurotransmitter receptor ion channels. Among these channels, two nicotinic acetylcholine receptors (*nAcRβ96A* and *nAcRα18C*) and three ionotropic glutamate receptors (*Nmdar1*, *GluCla,* and *CG11155*) mediate excitatory synaptic transmission. Moreover, three inhibitory GABAergic channels (*Lcch3*, *GABA-B-R2,* and *Rdl*) are also down regulated in aging *Drosophila* brain. Up regulated genes in aging *Drosophila* brain mostly present signatures similar to those observed in whole flies: genes associated with immune response and amino acid metabolism are over-represented. Based upon those whole genomic data it is possible to develop a set of Gal4 reporters that would permit to determine "biological" brain age markers for a given individual and to understand are there a "schedule" of aging at the gene level or is partially "stochastic" process.

Nervous System as a Target for Anti-Aging Interventions

Genetic manipulations with a single gene expression that extend life span are important tools for discovering mechanisms underlying aging. Mutations in the *Indy* (I'mNotDeadYet) gene dramatically extend the lifespan of the fruit fly, *Drosophila melanogaster* (Rogina et al., 2000). In the past we had identified an allele *Indy-P115*, which shows the same life span extension as the first allele (Bulgakova et al., 2002, 2004). Since we had the *P(lArB)* insertion, we studied the pattern of expression of the gene in the larval tissue. It occurs that the larval brain has clear pattern of expression and we put forward a hypothesis that *Drosophila* brain can be the main target of aging.

Studies on other models confirm our assumption. For example, mutations in *daf-2* disrupting an insulin-like signaling pathway dramatically extend the adult *C. elegans* life span (Guarente and Kenyon, 2000). The study of cell-specificity of *daf-2* action reveals that the neurons are responsible for the effect (Wolkow et al., 2000). The *lit* mutant mouse strain, which has a mutation disrupting the hypothalamic GH releasing hormone (GHRH), lives longer. Homozygous *lit/lit* mice live up to 25% longer than wild-type mice (Flurkey et al., 2001).

The creation of Gene-Switch Gal4 drivers (Osterwalder et al., 2001) now permits to identify the genes, whose ectopic activation/suppression can prolong *Drosophila* life span when overexpressed in adults. In particular *Elav-GS* driver directs conditional RU486 expression in the nervous system. With this approach it was shown, that overexpression of *Cbs*, *Eip71CD*, *G6PD*, *GCLc*, *hep*, *Jafrac1*, *p53*, *Sir2* and the silencing of *CG9172*, *CG18809*, *l(3)neo18*, *Naam* in the adult brain leads to increased life span (**Table 1**). It is also necessary to mention that similar data was published for *D-Gadd45* (Plyusnina et al., 2011). Those data gave the heavy background to consider adult brain as the target of aging. However, the range of the genes tested with the approach is very small, so we like to analyze how large the range of such genes can be. All the genes mentioned above showed not only the life-span extension induced by *Elav-GS* driver, but similar extensions were observed also with one of *Act-GS-Gal4* or *Tub-GS-Gal4* drivers, showing ubiquitous over-expression also results in the life extension. So in **Table 1** we made an attempt to correlate the list of the genes already studied by Gene-Switch approach with the level of their expression in development and tissues (modENCODE Tissue Expression Data). It can be seen that 30 genes studied within the *da-Gal4*, *tub-GS-Gal4*, *Act-GS-Gal4*, *hs-Gal4 UAS-geneX* system are heterogeneous group including high and low expression genes. Among those only *AGBE*, *CalpA*, *Men*, *wdb* demonstrate evident preponderance of head expression level. It is very probable that those genes, preferentially expressed in the head, also affects adult life-span by targeting the brain.

It was discovered cases when ubiquitous drivers: *da-GS-Gal4* and *tub-GS-Gal4* can extend life-span when inducing *RNAi-geneX* constructs (**Table 1**). Among those only *CG17856*, *ms(3)72Dt* have very low level of expression in the head.

Recent investigations shown, that the nervous system may be a target for ant-aging pharmacological interventions also. For example, serotonin antagonists (272N18, mianserin, mirtazapine, methiothepin and cyproheptadine), some of which are used clinically, extend the lifespan of adult *C. elegans* by 20–33% (Petrascheck et al., 2007). Screening of a library of compounds with known mammalian pharmacology revealed 60 compounds that increase longevity in *C. elegans* (Ye et al., 2014). The 33 compounds increased resistance to oxidative stress, and enhanced resistance to oxidative stress was associated primarily with compounds that target receptors for biogenic amines, such as dopamine or serotonin (Ye et al., 2014).

Conclusion

Now the thesis "*Drosophila* nervous system as a target of aging and anti-aging interventions" has been proved for some cases. On the one side of the nervous system is one of the targets of aging process and the state of nervous system may be regarded as a marker of aging. In this context, intervention aimed to combat the aging should lead to postponement of neurodegeneration. On the other hand, many pharmacological and genetic aging-suppressive interventions act through the nervous system. Therefore, it can be considered as one of the targets of anti-aging therapy. However, conditional expression approach reveals also other essential targets. We think that now days, when a large list of longevity genes already become known, it needs to put some efforts for complete longevity targets determination for every case. For example, current studies of the *Indy* mutations

TABLE 1 | Tissue expression data of longevity genes in normal conditions.

GeneX	Expression pattern according to modENCODE Tissue Expression Data	References
da-Gal4, tub-Gal4, Act-Gal4, hs-Gal4 > UAS-GeneX		
Atg8a	Very high expression almost in all organs including nervous system	Simonsen et al., 2008
AGBE	Moderately high, high in the head. Expression is lower in the other organs	Paik et al., 2012
CalpA	Moderate expression in the head. Expression is lower in the other organs	Paik et al., 2012
CG8155	Low expression in the head. Expression in other organs also low	Paik et al., 2012
CG10383	Low expression in the head. Expression in other organs also low	Paik et al., 2012
CG10916	Low expression in the head. Expression in other organs also low	Paik et al., 2012
CG30427	Low expression in the head. Expression in other organs also low	Paik et al., 2012
CG42663	Low expression in the head. Expression in other organs also low	Paik et al., 2012
dFh	Very low and low expression	Runko et al., 2008
Dlc90F	Moderately high, moderate expression in the head. Expression in other organs also high	Paik et al., 2012
dPrx5	Very high in testis, high and moderately high in other organs except salivary gland and fat body	Radyuk et al., 2009
dTsc1	High and moderately high in imaginal discs, ovary, and testis, moderate in almost all other organs	Gao et al., 2002
GCLm	High and very high expression in many organs, moderate expression in nervous system of larvae and pupae	Orr et al., 2005
Hsp22	Low expression in the head. Expression in other organs stronger	Kim et al., 2010
Hsp26	Low expression in the head. Expression in other organs stronger	Wang et al., 2004
Hsp27	Low expression in the head. Expression in other organs stronger	Wang et al., 2004
ImpL2	Moderately high, moderate expression in the head. Expression in other organs also high.	Paik et al., 2012
Men	High expression in the head. High expression in some other organs	Paik et al., 2012
Nlaz	High expression in the head. High expression in some other organs	Hull-Thompson et al., 2009
Pcmt	High expression in imaginal discs and testis, moderate expression in other organs including nervous system	Chavous et al., 2001
PGRP-LF	Weak expression everywhere	Paik et al., 2012
Prx5	High and moderately high in the head. High in other organs	Radyuk et al., 2009
S6k	Moderate, moderately high in the head and other organs	Kapahi et al., 2004
SIFaR	Low expression everywhere except pupae nervous system	Paik et al., 2012
Sin3A	Moderate in the head and some other organs	Paik et al., 2012
sm	Moderately high, moderate in the head. Expression higher in some organs	Paik et al., 2012
Sod2	High and very high in the head and other organs	Curtis et al., 2007
Tor	Low expression everywhere	Kapahi et al., 2004
Trx-2	Moderately high, moderate in the head and other organs	Seong et al., 2001
Tsc1	Moderate in the head and other organs	Kapahi et al., 2004
wdb	Moderately high, moderate in the head and some other organs	Funakoshi et al., 2011
da-Gal4, tub-Gal4 > RNAi-GeneX		
CG17856	Very low everywhere	Copeland et al., 2009
ms(3)72Dt	Very low everywhere	Copeland et al., 2009
Elav-Gal4 > UAS-GeneX		
Cbs	Low everywhere	Kabil et al., 2011
Eip71CD	High, moderately high in the head and some other organs	Chung et al., 2010
G6PD	Moderate expression in testis and head of 20 day male, moderate and low expression in other organs. Expression in nervous system of larvae and pupae—very low	Legan et al., 2008
GCLc	High expression in digestive system and salivary glands	Orr et al., 2005
Hep	Moderate in the head and other organs	Biteau et al., 2010
Jafrac1	Very high, high, and moderately high almost everywhere, exept salivary gland fat body	Lee et al., 2009
p53	Very low everywhere	Shen et al., 2009
Sir2	Moderate in the head and other organs	Whitaker et al., 2013
Elav-Gal4 > RNAi-GeneX		
CG9172	High, moderately high in the head and some other organs	Copeland et al., 2009
CG18809	Moderately high, moderate in the head. Expression lower in other organs	Copeland et al., 2009
l(3)neo18	Very high in the head and some other organs	Copeland et al., 2009
Naam	Very low in the head and other organs	Balan et al., 2008

extending life-span are concentrated on the gene function in the gut (Rogina et al., 2014). However, for the most of the longevity genes the target organs are poorly studied. We suggest that the brain is one of the main aging targets.

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Genetics of aging, health, and survival: dynamic regulation of human longevity related traits

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Background: The roles of genetic factors in human longevity would be better understood if one can use more efficient methods in genetic analyses and investigate pleiotropic effects of genetic variants on aging and health related traits.

Data and methods: We used EMMAX software with modified correction for population stratification to perform genome wide association studies (GWAS) of female lifespan from the original FHS cohort. The male data from the original FHS cohort and male and female data combined from the offspring FHS cohort were used to confirm findings. We evaluated pleiotropic effects of selected genetic variants as well as gene-smoking interactions on health and aging related traits. Then we reviewed current knowledge on functional properties of genes related to detected variants.

Results: The eight SNPs with genome-wide significant variants were negatively associated with lifespan in both males and females. After additional QC, two of these variants were selected for further analyses of their associations with major diseases (cancer and CHD) and physiological aging changes. Gene-smoking interactions contributed to these effects. Genes closest to detected variants appear to be involved in similar biological processes and health disorders, as those found in other studies of aging and longevity e.g., in cancer and neurodegeneration.

Conclusions: The impact of genes on longevity may involve trade-off-like effects on different health traits. Genes that influence lifespan represent various molecular functions but may be involved in similar biological processes and health disorders, which could contribute to genetic heterogeneity of longevity and the lack of replication in genetic association studies.

Keywords: longitudinal data, genetics of longevity, genetics of cancer, CVD, gene-environment interaction, smoking and life span, aging changes

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Introduction

The literature review on the genetic influence on human aging and longevity indicates limited progress in identifying genetic mechanisms regulating human aging, health, and longevity using

genome wide association studies (GWAS). Most associations detected in these studies tend to be weak and have not reached the genome-wide level of statistical significance. They also suffer from the lack of replication in studies of independent populations. What factors and conditions might be responsible for such a situation? What methods and approaches have to be used to properly address these issues?

The literature on genetic studies of human longevity lacks an appropriate biologically-based conceptual framework that would allow one to efficiently address questions about the dynamic roles of genes in forming aging and longevity related traits. Such a framework would help specify necessary steps for efficient comprehensive analyses of corresponding genetic mechanisms using available data. The approaches based on the systems biology of aging, health, and longevity (Kriete, 2013; Yashin and Jazwinski, 2014) merged with biodemography of aging methods (Arbeev et al., 2011; Yashin et al., 2012b) and statistical modeling (Yashin et al., 2012a; Arbeev et al., 2014) have the potential to facilitate progress in this direction.

The literature review also revealed the underutilized research potential of available data that were used in genetic analyses of aging and longevity traits. The possibility of more efficient analyses of genetic associations with such traits was demonstrated in Yashin et al. (1999, 2000) where the joint analyses of genetic and demographic data in centenarians studies have been performed. The methods of such analyses were further developed and adjusted for the biological and biodemographic nature of the traits and successfully used in data analyses (Yashin et al., 2007, 2013; Arbeev et al., 2011). The results of these analyses indicate that the use of biodemographic ideas, models, and methods could shed more light on genetic mechanisms linking human aging, health, and longevity.

In genetic analyses performed in this paper we used a modified statistical procedure of testing genetic associations with longevity related traits. The populations whose data are used in GWAS often have genetic structures called "population stratification" (PS). Such structures are caused by non-random mating between groups of individuals followed by different patterns of genetic drift of allele frequencies in each group. Without proper controlling for PS in the statistical procedures used in GWAS of complex traits the analyses may result in erroneous (false-positive) associations. To avoid such errors the genetic association studies of complex traits include control for potential PS. The method of principal components analyses (PCA) suggested in Price et al. (2006) identifies several top principal components (PCs) in genetic data and uses them as observed covariates in the statistical estimation procedure in GWAS. Although this approach may be efficient in GWAS of many complex traits it may create problems in genetic association studies of human longevity. This is because the process of mortality selection that takes place in genetically heterogeneous cohorts may generate additional genetic structures in the study populations. This structure which involves genetic variants affecting life span may be captured by the selected principal components. As a result, controlling for PS in GWAS of human aging and longevity may reduce the estimates of associations of genetic variants with longevity traits, i.e., it may substantially attenuate the signals from the genetic variants one wants to detect. In this paper we applied the new procedure for controlling for population stratification (see Section S1 in Supplementary Materials) in GWAS of human life span using data on females from the original cohort of the Framingham Heart Study (FHS). This procedure as well as the results of analyses using traditional and modified methods of controlling for potential PS in GWAS of human longevity are described in Yashin et al. (2014). We investigated the associations of the detected genetic variants with life span in males from the original and in males and females from the offspring FHS cohorts as well as with health related traits and age trajectories of biomarkers from the original cohort. We also investigated functional properties of genes related to detected variants.

Data and Methods

In the discovery phase of the analyses we used the life span data on genotyped females collected in the original cohort of the Framingham Heart Study (FHS) (Giroux, 2013) as well as genetic data. The genetic data were represented by 550,000 SNPs. Genotyping was conducted using Affymetrix 500 K and 50 K (non-overlapping) arrays. The lifespan data were available for 1529 participants from the original FHS cohort. The quality control (QC) procedure included 95% call rate for the sample and 95% call rate for SNPs, and HWE p-value $>$ 1E-7. After applying the QC procedure, the data on 1111 individuals with information about lifespan and 429,783 SNPs were available for the analysis. Life spans for 204 study subjects (52 males and 152 females) were censored. We used EMMAX software to perform GWAS of female lifespan data from the original FHS cohort with modified correction for population stratification. In the confirmation phase we used genetic and the life span data on males from the same FHS cohort and the life span data on males and females combined from the offspring FHS cohort. Using available data on cancer (all sites but skin) and CVD as well as longitudinal data on physiological indices we estimated age trajectories of probabilities of staying free of the corresponding diseases for carriers and non-carriers of selected genetic variants as well as associations of these variants with age trajectories of physiological indices. We also investigated how smoking modulates estimated associations. More details about methods of data analyses used in this paper are given in Section S1 of Supplementary Materials.

Results

Genetic Variants Showing Association with Life Span in Females are Replicated in Males

Discovery Phase

The most significant genetic variants (minor alleles) resulted from GWAS on human life span using female data from the original FHS cohort are shown in Table S1 (in Supplementary Materials) together with corresponding estimates of the effect sizes, p-values, genetic frequencies, and other important characteristics of these SNPs.

Confirmation Phase

To test whether the selected genetic variants also show significant associations with lifespan in males we performed genetic analysis of the ten selected SNPs using lifespan data on 432 males from the original FHS cohort. The same (additive) genetic model and the same observed covariates were used in these analyses. The results are summarized in Table S2 together with corresponding estimates of the effect sizes, *p*-values, genetic frequencies, and other important characteristics of these SNPs. This table shows that the eight genetic variants that had negative associations with lifespan in females also showed statistically significant negative associations with lifespan in males.

Statistical estimates in Tables S1, S2 are obtained using mixed effect regression model with imputed life span data on 204 study subjects). It is unclear whether detected associations remain statistically significant when the methods free of life span imputation are used in the association analyses. To address this issue we evaluated genetic associations of selected genetic variants with mortality risk using Cox's regression model that does not require data imputation. The results of these analyses (Table S3) showed highly significant associations between selected genetic variants and mortality risks.

To test whether detected variants are associated with life spans of the members of the offspring FHS cohort we performed genetic analyses of data for this cohort. Since the life span data for some members of this cohort are censored these analyses have been performed using the Cox's regression model using gender as an observed covariate. All selected variants showed significant associations with mortality risk using data from the offspring cohort (Table S7).

Empirical Survival Functions Estimated from Longitudinal Data Confirm Detected Associations

An important advantage of longitudinal data for genetic analyses of lifespan is the opportunity to *verify* the research findings obtaned using regression models in GWAS by constructing and comparing *empirical survival functions* for carriers and non-carriers of selected variants. Note that the case-control analysis, typicaly used in genetic studies of centenarians, does not have data to make such a verification. Since associations of the variants with lifespan in our GWAS have the same direction of the effect in both genders, the Kaplan–Meier estimates of conditional survival functions for the genotyped members of the original FHS cohort were evaluated for males and females combined. In such evaluation we took into account that the life span data on the members of the original FHS cohort are right censored and left truncated. Note that for genetic analyses one should use age at blood collection (not the age at baseline) as the left truncation variable. We calculated these variables using data from the DNA Draw Date file available for FHS data from the dbGaP. Then we calculated ages at death/censoring of those 1111 individuals using age at the first exam and the number of days since the first exam until the death event or censoring which are provided in the Framingham data. Using these data and the R package "survival" we calculated Kaplan–Meier estimates of conditional survival functions for carriers and non-carriers of selected genetic variants who survived until specific ages (e.g., 70 or 80 years).

Figure 1 shows patterns of survival for carriers and non-carriers of the minor allele, for two SNPs: rs7894051 (in ECHS1 gene), and rs4904670 (in NRDE2 gene) that were selected for further analyses after additional QC procedure described in Supplementary Materials.

Figures 1A,B show such functions conditional on survival to age 70 years and the **Figures 1C,D** show these functions for those who survived age 80 years (for males and females combined). For rs7894051, the negative effect of the minor allele on survival became pronounced only after age 80 years. The probabilities of survival for carriers and non-carriers of these variants at the age interval between 70 and 80 years are about the same. For rs4904670, the negative effect on survival starts from the age 80. This SNP manifests a survival trade-off, with minor allele having positive effect on survival at ages before 80 and negative effect afterwards. This illustrates the changing age-specific influence of genes on vulnerability to death. The role of age as a modulator of genetic effects on lifespan was suggested and discussed in our prior works (De Benedictis et al., 1998; Yashin et al., 1999, 2000, 2001; Ukraintseva, 2005). Later, it was also noted in Atzmon et al. (2006). Age-dependence of genetic effects on health and related traits was also extensively discussed and analyzed in the past by different research groups (e.g., Jarvik et al., 1997) including our own (e.g., Kulminski et al., 2013). Note that the harmful influence of the minor alleles on survival after the age 80 was manifested for all eight genetic variants listed in Table S2. The Cox regression analyses applied to the life span data from the original FHS cohort showed that the difference in survival curves among minor allele carriers and non-carriers for each of the eight SNPs, males and females combined, was highly statistically significant (Table S3 Effects of gene-environment interaction on survival: the case of smoking).

Using data on the smoking habit (ever or never) as well as life span data on genotyped members of the original FHS cohort (males and females combined) we evaluated effects of interactions of detected genetic variants with the smoking habit on survival. The results are shown in **Figure 2**.

Figures 2A,B show four survival functions for smoker-carriers, smoker-non-carriers, non-smoker-carriers, and non-smoker-non-carriers of the minor allele, for the two SNPs, rs7894051 and rs4904670, introduced above. These figures show that non-smokers-non-carriers have the best survival, while smokers-carriers have the worst survival among all four groups. This is an important illustration of gene-environment interaction effects on survival, though one can see that the effect of genotype is stronger than the effect of smoking in case of these two SNPs.

How do Detected Genetic Variants Influence Incidence Rates of Cancer and CVD?

Using available data on ages at disease onset, we evaluated conditional probabilities of staying free of cancer (all sites but skin), for males and females combined who survived to 80 years of age. The results for the two SNPs, rs7894051 and rs4904670, are shown on **Figure 3**.

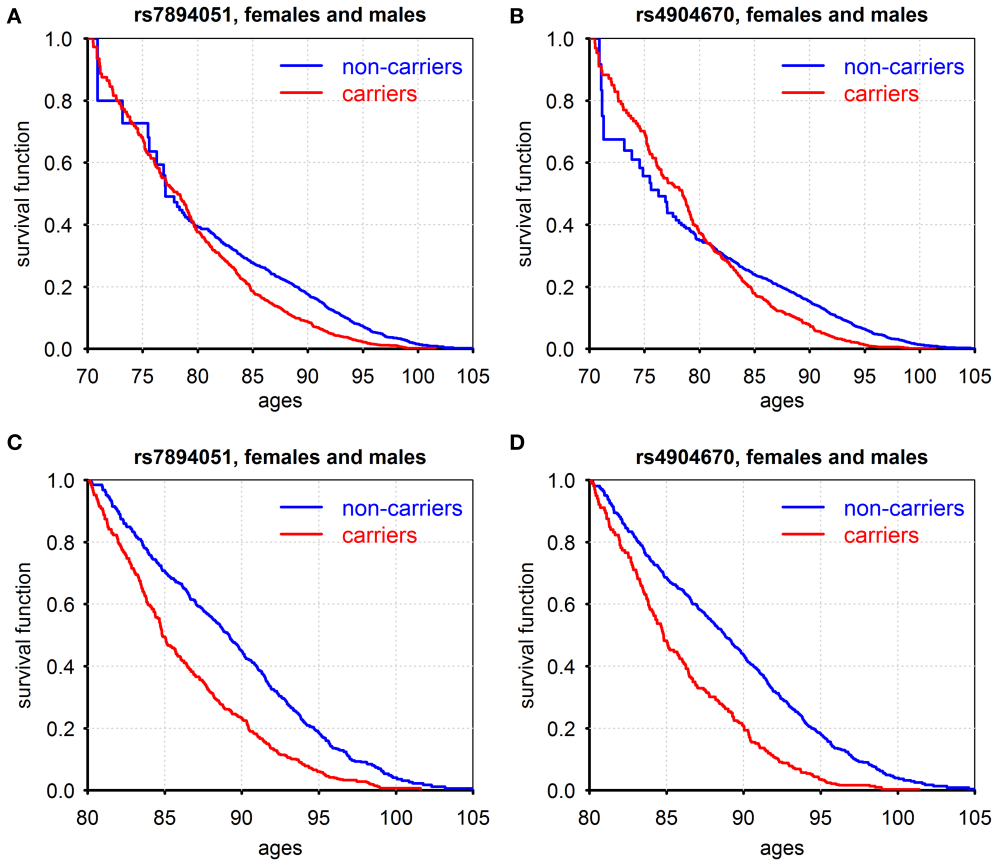

FIGURE 1 | Kaplan–Meier estimates of conditional survival functions for carriers/non-carriers of minor alleles of rs7894051 (A,C) and rs4904670 (B,D) SNPs surviving to age 70 years (A,B) and age 80 years (C,D). Source: Framingham Heart Study, original cohort, genotyped individuals after quality control, females, and males combined.

One can see from this figure that when males and females are combined, the rs7894051 does not influence cancer risk (**Figure 3A**), while carrying the minor allele of rs4904670 (**Figure 3B**) increases that risk. The pattern observed in **Figure 3A** may take place when the effect of this allele on cancer risk is opposite in males and females. To test this possibility, we evaluated the age patterns of probabilities of staying free of cancer for males and females separately. The graphs shown in **Figures 3C,D** provide evidence that such situation does take place: the effects of minor alleles on cancer risk after age 80 were opposite in males and females for both rs7894051 and rs4904670. That is, the presence of the minor allele in person's genome increased the risk of cancer in females and reduced it in males for both the SNPs (**Figures 3C–F**). However, for rs4904670 the reduction of cancer risk in males did not fully compensate for the increase in such risk in females, so that the total effect of the minor allele of this SNP on cancer risk for males and females combined was detrimental. The effects of rs7894051 and rs4904670 on mortality rates from CVD and cancer are shown in Tables S4, S5 in Supplementary Materials. The vulnerability variants of these two SNPs increased the risk of CHD onset after age 80 (Figures S2, S3 and Table S6). Since carrying the vulnerability allele reduced the cancer risk in males at the same ages, these results suggest potentially important role of antagonistic pleiotropic effects of genes in determining their impact on longevity.

Longevity Related Variants can Modulate Patterns of Aging Changes in Biomarkers

Using longitudinal data on body mass index (BMI) for genotyped individuals from the original FHS cohort, we evaluated the average age trajectories of BMI for carriers and non-carriers of the minor allele for the same two SNPs, rs7894051 and rs4904670, in males and females. The results are shown in **Figure 4**.

One can see from **Figures 4A,B** that between ages 40 and 65 years the average values of BMI for carriers of minor alleles exceeded those for non-carriers, in males and females combined. After this age the curves intersected so that the BMI of the carriers became lower than that of the non-carriers. **Figures 4C,D** show the effect of smoking habit on the average age trajectories of BMI for rs7894051 and rs4904670 minor-allele carriers and non-carriers. One can see that the average age trajectories for non-smoker-carriers of the minor allele of any of the two SNPs differed from other groups trajectories. It is also seen that

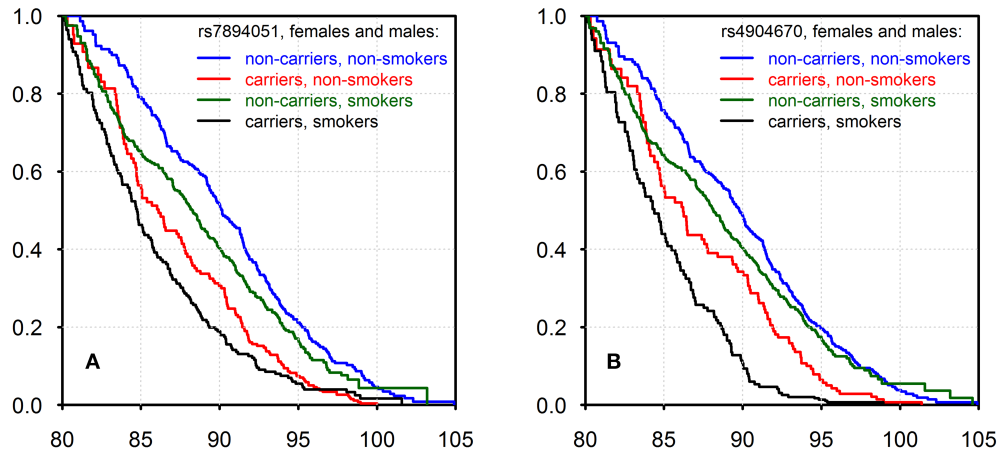

FIGURE 2 | Kaplan–Meier estimates of survival functions for smokers-carriers, non-smokers-carriers, smokers-non-carriers, and non-smokers-non-carriers of the minor alleles of rs7894051 (A) and rs4904670 (B) SNPs. Source: Framingham Heart Study, original cohort, genotyped individuals after quality control, females, and males combined.

smoking did not have a strong influence on the BMI age trajectories for non-carriers, for any of the two SNPs. For rs7894051, values of BMI for non-smoker-carriers were highest at the age interval between 50 and 70 years. For rs4904670 the age trajectory of BMI for the non-smoker-carriers sharply declined after age 70, as for the rs7894051.

Genes Related to the Vulnerability Variants: Biological Meaning

Table 1 shows essential characteristics and biological and health effects of the two SNPs, rs7894051 and rs4904670 that passed additional QC we applied as described in Supplement, as well as related genes. We also included rs1794108 to this table and relevant discussion because it is next top SNP by the size of its effect on lifespan (though not by *P*-value), which could aid biological interpretation of the findings. We conducted review of current knowledge about biological and health effects of these SNPs and related genes using up-to-date publications and reliable online sources such as NCBI's PubMed, Entrez Gene, dbSNP, OMIM, Ensembl, GeneCards, GO, and MetaCore from Thomson Reuters. Genes related to SNPs identified in this study (or to SNPs that are in LD with them) are largely involved in mitochondrial oxidation, apoptosis, and protein degradation in cell. These processes are important part of cell/tissue responses to stress or damage. For example, the NRDE2 (C14orf102) gene is linked to PSMC1, a subunit of 26S proteasome involved in protein degradation, essential for cell damage response. PSMC1 may potentially interact with PSMD13, which is another subunit of the same 26S proteasome involved in protein degradation. ECHS1 is involved in mitochondrial oxidation and apoptosis, also essential for cell/tissue response to damage (**Table 1**).

On the level of health disorders, detected genes were most often involved in cancer and neurological disorders, such as schizophrenia and ASD, which broadly overlaps with biological and health effects of genes found in other studies of aging and longevity (e.g., Walter et al., 2011; Yashin et al., 2012c). The frequent involvement of aging/longevity associated genes in neurological disorders suggests an intriguing possibility that genes predisposing to such disorders may accelerate brain physiological aging, and through this negatively impact longevity (in addition to direct pathological effects). A number of recent studies indicate that this might be the case, especially for schizophrenia (Kochunov et al., 2013; Koutsouleris et al., 2014; Shivakumar et al., 2014; Wright et al., 2014; Silver and Bilker, 2015). Potential mechanisms by which the same genetic factor may influence aging and brain pathology could involve, for example, declines in regenerative response to damage, neural repair, axons outgrowth, and synaptic transmission, typical of both brain aging and brain disorders (Balu and Coyle, 2011; Edwards et al., 2011; Ferguson and Son, 2011; Haroutunian et al., 2014).

As for connection between lifespan related genes and cancer, it appears to be complex and involve trade-offs. Our analyses showed that carrying minor (vulnerability) allele of the two selected SNPs increased cancer risk in females. For males, however, the effect of these variants on cancer was the opposite, and carrying the vulnerability allele reduced the cancer risk in males.

Since most centenarians avoid cancer (Andersen et al., 2005; Joseph et al., 2014), it may seem logical that pro-longevity variants could indeed be among those protecting against cancer. Our results for females are in line with this suggestion. However, vulnerability variants appear to be protective against cancer in males in this study.

This raises another intriguing possibility that pro-longevity variants (in non-carriers of vulnerability alleles) may sometimes do both, promote cancer and at the same time be potentially against certain phenotypes of physical senescence. This

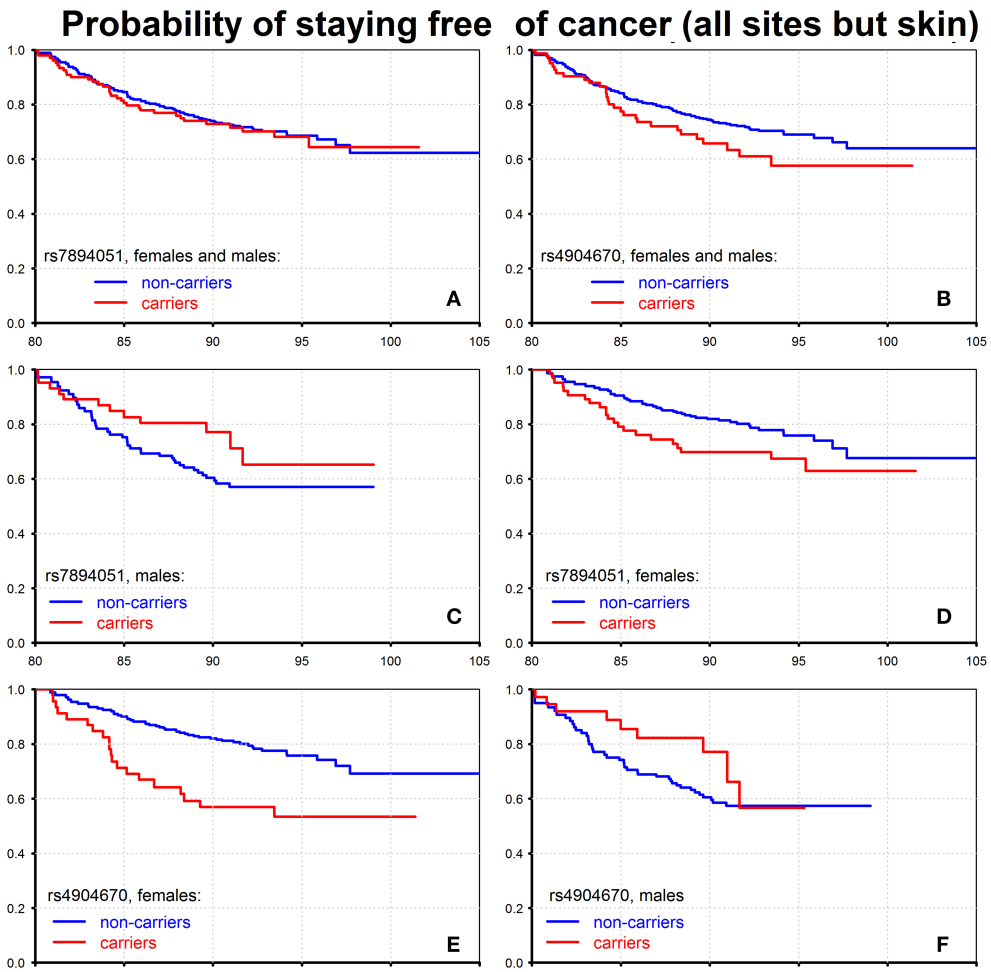

FIGURE 3 | Effect of individual genetic variants on probability of staying free of cancer of all sites but skin. The figure shows the Kaplan–Meier estimates of conditional survival functions for individuals who survived to age 80 years: carriers and non-carriers of the minor allele of rs7894051, females and males **(A)**, males **(C)**, females **(D)** and carriers and non-carriers of the minor allele of rs4904670, females and males **(B)**, females **(E)**, males **(F)**. Source: Framingham Heart Study, original cohort, genotyped individuals after quality control.

could happen because cancer (all sites combined) and senescent phenotypes such as physical frailty, and heart failure due to muscle atrophy, have peak manifestations at different ages: cancer risk reaches its maximum typically before oldest old age (<85), while senescence-related causes become major contributors to mortality risk later (at ages 85+) (Ukraintseva and Yashin, 2003a,b; Ukraintseva et al., 2008; Akushevich et al., 2012). If person who carries such a genetic risk factor for cancer nevertheless survives the period of the highest cancer risk (before age 85), s/he may get an advantage from attenuated physical senescence at older ages, so that such genetic variant may contribute to both longevity and cancer (in case of our study, in males only). Since males and females have markedly different body composition and physical manifestation of senescence, genetic factors could affect this manifestation also differently, and be beneficial or deleterious in only one gender.

Although biological and health effects of identified genes, especially their involvement in damage response, and in cancer, overlap with those earlier found in our and others' studies of aging and lifespan associated genes, the exact replication, such as findings of identical SNPs or genes, is less common across different studies.

Discussion

The process of population aging in the developed part of the world has dramatic consequences for population health and health-care financing, and makes the reduction of the disease burden of the elderly people a high priority research problem. Useful insights into possible strategies of improving the health of the elderly could be produced from studying the roles of genetic factors in connection with aging, health, and longevity. An important implication of the results of such studies could be the possibility of preventing many aging related chronic diseases by slowing down or postponing individual aging processes.

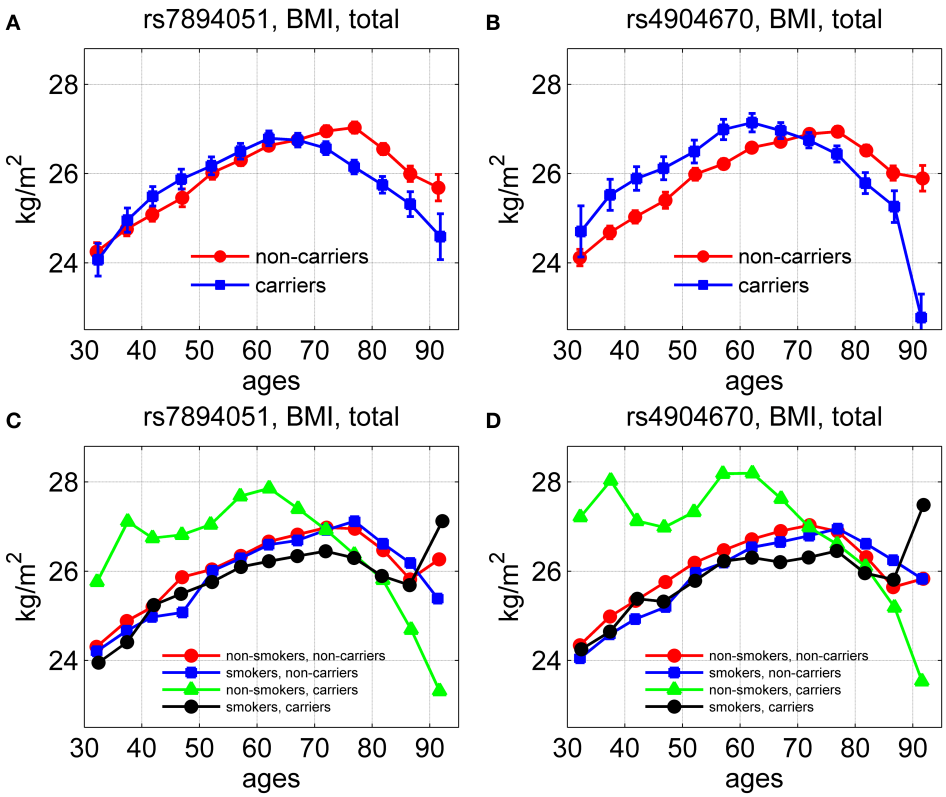

FIGURE 4 | Average age trajectories of BMI for groups stratified by smoking status and genetic background. The figure shows the average age trajectories for carriers and non-carriers of the minor alleles of rs7894051 **(A)** and rs4904670 **(B)** SNPs. **(C,D)** show the average age trajectories of BMI for carriers and non-carriers of the same SNPs with different smoking habits.

Positive Implications from Modest Progress of GWAS of Human Life Span

The genetics of human aging and longevity became the subject of intensive analyses during the last decade ranging from studies of candidate genes (Franceschi et al., 2000; De Benedictis et al., 2001; Tan et al., 2001; Christensen et al., 2006; Christensen, 2008; Willcox et al., 2008) to genome-wide association studies (GWAS) (Lunetta et al., 2007; Newman et al., 2010; Yashin et al., 2010; Deelen et al., 2011, 2013; Nebel et al., 2011) that involved hundreds-of-thousands to millions of SNPs. Although the results of numerous GWAS of human aging and life span have modest success and could not clearly describe factors and mechanisms responsible for exceptional longevity, they have important positive implications. These results helped us identify problems that need to be urgently addressed to have further progress in the research field. Why were most of the detected genetic associations with human life span so weak? Why was it so difficult to replicate the research findings using data on independent studies? What are the mechanisms by which genes influence life span? Can one identify mechanisms that link lifespan-affecting genetic variants with health indicators and aging-related changes in biomarkers? How do detected variants interact with other genetic and non-genetic factors to influence life span? To address these questions, efficient approaches capable of studying the dynamic nature of genetic effects on mortality and morbidity risks, as well as on intermediate variables measured in longitudinal data have to be used. The results obtained with the help of one such approach were described in this paper.

Genetic Estimates in GWAS of Human Life Span Require Proper Control for Population Stratification

We applied a modified procedure for controlling for potential population stratification in GWAS of human life span to detect genetic variants strongly associated with life span in both males and females and used longitudinal data to address questions about the roles of these variants in health and survival outcomes, age trajectories of biomarkers, as well as the effects of interactions with non-genetic factors. Our aim was to make genetic signals stronger, to evaluate genetic connections among aging-related changes, health, and life span, to elucidate possible reasons for the lack of replication, and to develop useful insights into potential mechanisms of life span regulation. The GWAS performed in these analyses used a modified procedure to control for possible population stratification. Such modification was needed because the traditional procedure may interfere with the process of mortality selection. The confirmation of the genome-wide significant findings in a population of opposite sex (males)

TABLE 1 | Essential characteristics of the two "vulnerability" SNPs (and their closest genes) associated with reduced lifespan in both sexes in the FHS Original cohort.

SNP	Ch	Gene region	Closest gene	Gene/protein function in cell and tissue	Physiological processes and health disorders associated with the gene/protein
rs7894051 *This SNP is in LD with rs1049951, exonic non-synonymous SNP in the same gene	10q26.2	Intronic	**ECHS1**—enoyl CoA hydratase, short chain 1, mitoch.	ECHS1 catalyzes the hydration of 2-trans-enoyl-coenzyme A (CoA). Involved in **mitochondrial oxidation** and **apoptosis** suppression (Liu et al., 2010). In LD with 3 intronic SNPs in **PAOX** (oxidase) gene	Overexpressed in different **cancers**; has been implicated in melanoma (Lake et al., 2011), liver and colorectal cancers (Xiao et al., 2013; Zhu et al., 2013; Xie et al., 2014); suggested role in **neurological disorders** (Chiocchetti et al., 2014; Perluigi et al., 2014)
rs4904670	14q32.11	Intronic	**NRDE2** (C14orf102) necessary for RNA interference, domain containing	NRDE2 is highly expressed in the brain (Maiti et al., 2011); linked to **PSMC1**, proteasome 26S subunit, ATPase, 1, involved in **protein degradation.** *The PSMC1 may potentially interact with **PSMD13**, a gene which is in LD with **SIRT3** (see below)	NRDE2 can be associated with **cancer** (melanoma) (Chiu et al., 2014); Suggested role of NRDE2 and PSMC1 in schizophrenia (Maiti et al., 2011); and in **neurodegeneration** (Bedford et al., 2008)
rs1794108	11p15.5	Exon—nonsyn coding	**PSMD13**—proteasome 26S subunit, non-ATPase, 13	PSMD13 is a proteasome regulatory subunit, involved in **protein degradation.** High LD between **PSMD13** and **SIRT3**. *Sirtuins (SIRT1-7) play a central role in epigenetic gene silencing, DNA repair, cell-cycle, microtubule organization, and aging (Giblin et al., 2014)	**Aging, longevity, cancer**. PSMD13-SIRT3 haplotype pools are significantly different between centenarians and younger people (Bellizzi et al., 2007). Plays role in **stem cells aging and stress response** (Brown et al., 2013). SIRT3 can be a tumor promoter or tumor suppressor, depending on context (Alhazzazi et al., 2011)

Other SNPs are not shown because they did not pass additional QC that we applied as described in Supplement. The SNP rs1794108 was added to this table because it is next top SNP, not by P-value but by the size of its effect on LS. It is also in LD with the SIRT3 gene. The minor allele of this SNP had negative effect on lifespan with significance in males ($p < 1.65E-6$) and in females ($p < 1.91E-4$).

in the same (original) cohort and in the offspring generation indicates that these findings are likely to be true-positive. It also indicates that GWAS of human longevity with inappropriate controls for population stratification are likely to suffer from the low level of statistical significance (weak signals) and from the lack of replication.

Additional QC Testing

The genetic data from the original FHS cohort were one of the first prepared for genetic analyses using GWAS, and a substantial portion of genotyped cohort members died before the quality of genotyping was substantially improved. Therefore, these data have higher chances of having genotyping error than more recently produced datasets. Taking this into account we performed testing of the quality of genotyping for eight genetic variants detected in this study (Section S2 in Supplementary Materials). As a result of this testing the two of these SNPs rs7894051 (in ECHS1 gene), and rs4904670 (in NRDE2 gene) were selected for further analyses of their associations with major diseases (cancer and CHD) and physiological aging changes. Thus, although detected genetic associations in eight SNPs were confirmed twice (findings on female data were confirmed using data on males from the original FHS cohort, and then using data from the offspring FHS cohort), additional studies with higher quality genetic data are needed to make final conclusions about the involvement of all eight detected genes in regulation of health, aging, and longevity related traits.

Benefits of Biodemographic Information for Genetic Studies of Human Longevity

Researchers studying the genetics of human aging and longevity tend to underestimate the role of demographic information in genetic analyses of complex traits. Our analyses show that taking this information into account may substantially improve the quality of genetic analyses. Indeed, researchers studying genetics of human life span tacitly assume that population cohorts are genetically heterogeneous with respect to individual susceptibility to death. The aging of individuals in heterogeneous cohorts is accompanied by the process of mortality selection. The genetically vulnerable individuals tend to die first and genetically robust members of the cohort will survive to the old and the oldest-old ages. In Yashin et al. (1999, 2000, 2007, 2013) and Tan et al. (2003), we developed and tested a series of statistical methods for efficient joint analyses of genetic and demographic information

in genetic studies of human longevity. The use of such methods allowed us not only to improve estimates of associations between genetic factors and longevity but also to evaluate and compare age patterns of mortality rates for carriers and non-carriers of candidate alleles and genotypes. Such comparisons are not possible using data on genetic frequencies alone.

Monotonic vs. Non-monotonic Age Patterns of Allele Frequencies

Complex diseases, such as cancer, CVD, diabetes, and AD, are major contributors to mortality in old age. Genetic variants which increase risks of such diseases were shown to negatively affect survival and/or be less common among the long-lived people in many studies (e.g., Benes et al., 2001; De Benedictis et al., 2001; Lescai et al., 2009; Park et al., 2009, 2010; Nebel et al., 2011; Ruiz et al., 2011; Yashin and Jazwinski, 2014). The results of our analyses are in line with negative effects of detected alleles on survival after age 80 years. Note that such connection between genetic variants and survival is manifested by *monotonically* declining age patterns of the allele frequencies.

Previously we found that the *non-monotonic* age patterns of allele frequencies are also possible (Yashin et al., 1999, 2000). These patterns (e.g., when the variant's frequency first declines, then reaches minimum, and then increases) corresponds to a very important property of some genetic variants where their effects on mortality risk change with increasing age from detrimental to beneficial. In this case the mortality curves for carriers and non-carriers of the corresponding genetic variant intersect. Possible reasons for such intersection are discussed in Yashin et al. (2001) and Atzmon et al. (2006). Such a pattern could be responsible for the seemingly paradoxical presence of "risk alleles" for complex diseases in genomes of long-lived people, sometimes reported in literature (e.g., De Benedictis et al., 1998; Bladbjerg et al., 1999; Bonafe et al., 1999; Yashin et al., 1999, 2001b; Beekman et al., 2010; Freudenberg-Hua et al., 2014). Thus, the results of genetic studies of human longevity confirm the existence of two groups of genetic variants which can be characterized by different age patterns of genetic frequencies.

Genetic Heterogeneity of Complex Traits

The results of our analyses show that negative associations of selected genetic variants with human life span in females were confirmed in males; they also influence the incidence rates of cancer of all sites combined (but skin) and CVD. One may argue that the health and longevity traits investigated in this study (i.e., life span and two diseases) are too heterogeneous: individual differences in life spans may depend on susceptibility to different chronic conditions and cause specific mortality risks; cancers of distinct sites are often associated with different genetic and non-genetic factors; the term CVD includes many diseases dealing with cardio-vascular problems, each having its own sets of risk factors; etc. Although these concerns are certainly correct, one often does not have much choice: the selection of a trait for analysis is determined by the available data which has to be enough to get reliable conclusions from the analyses. Studying factors affecting aggregated traits (e.g., cancer of all sites, all-cause, mortality risk, etc.) may help us detect regularities and identify mechanisms that are common for many specific phenotypes (cancers of specific sites, mortality risks by cause, etc.) comprising such traits. An important question is how best to analyze heterogeneous traits when you must? How can an understanding that the trait is genetically heterogeneous affect methods and results of analyses?

Despite the fact that the presence of such heterogeneity is recognized by many researchers in the field, there is no agreement concerning the best strategies for dealing with this issue. The recommendation that follows from our experience would be to investigate genetic heterogeneity by using different statistical models of the connections between genetic factors and phenotypic traits, to consider how the results of analyses (e.g., sets of selected genetic variants) differ for different quality control procedures, to perform careful studies of the non-replicating results, keeping in mind that *different genetic mechanisms may be responsible for the same manifestation of a given trait*. The goal of the analyses thus becomes, not just finding the one or more longevity (or vulnerability) alleles/genotypes responsible for the genetic variation of life span in all individuals, but investigating possible alternative mechanisms responsible for developing this trait. These mechanisms may involve different sets of genes in different individuals or in different groups of individuals. The genetic heterogeneity of longevity could be partly responsible for the lack of replication of respective genetic association studies.

Genetic Mechanisms Linking Aging, Health, and Longevity

Although exact replication, such as findings of identical SNPs or genes, is not common across genetic association studies of aging and longevity, biological and health effects of identified genes, especially their involvement in similar damage responses, and in similar pathology, often overlap between the different studies. In our case, the involvement of selected genes (**Table 1**) in cancer and neurological disorders was reported in both our and others' studies of aging and lifespan associated genes (e.g., Walter et al., 2011).

The results of our review of current knowledge also suggest an intriguing possibility that the vulnerability SNPs in genes involved in neurological disorders might also contribute to accelerated brain aging, and through this negatively impact longevity, which idea deserves further investigation. Connections between lifespan related genes and cancer may involve complex trade-offs. More detail on potential biological mechanisms are provided in section on Biological Meaning above.

In sum, our study demonstrated genetic connections among lifespan, physiological aging changes, and complex diseases. Comparison of functional properties of genes found in this and other genetic studies of aging/longevity indicates that human lifespan may be regulated by different sets of genes; however, these genes tend to be involved in similar biological processes and complex pathology. The fact that the same biological process or health outcome may be realized through the different genes suggests that genes may potentially be interchangeable in their

influence on longevity. This could contribute to both genetic heterogeneity of longevity and to a lack of replication in genetic association studies of lifespan and related phenotypes. Additional studies with a higher quality genetic data are needed to support our conclusions.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2015.00122/abstract

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OPINION
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Anti-aging dilemma: to restore the hardware or to reinstall the software?

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"Metaphors have a way of holding the most truth in the least space."
—Orson Scott Card, *Alvin Journeyman*

Since its early days, the value of deciphering the human DNA has been seen primarily in extracting the set of messages that run the cells that constitute the body. In common understanding, these messages are encoded in DNA and transcribed as cell-specific sets of RNAs, some of which are translated to proteins, then modified with various post-translational add-ons made of sugars, lipids and other moieties. This complex chain of events is further complicated by multi-layered possibilities for the modifications allowed at every step–epigenetics for DNA, editing for RNAs and the recently discovered phenomenon of non-template polypeptide extension allowed by ribosomes (Shen et al., 2015). It seems that when looked at as a whole, the DNA, and all the messages associated with DNA, do not look like a blueprint, or even a clear set of instructions, but rather a messy draft or a stack of notes that are scribbled all over and full of ambiguities.

However, let us hack through the majority of the "omics" and look upon the set of small molecules known as metabolites, and the budding discipline of Metabolomics that researches the true underpinnings of the abundantly complex mechanics of the living cell. It is worthwhile to note that, to a somewhat defined degree, the cell will tolerate the loss of a gene or changes in the levels of RNAs or even the most important of proteins, while even slight deregulation of the levels of some of the smallest metabolites leads to immediate and catastrophic consequences. The potassium ion and ATP may be used as the primary examples of smallest molecules capable of eliciting a systemic response. According to our calculation, a mere 0.5% increase in the total content of potassium chloride, one of the most common electrolytes in the human body, leads to immediate cardiac arrest. The consequences of the depletion of ATP may manifest as a variety of ailments, with their duration inversely proportional to the severity of the defect. Aging, in particular, is associated with a decline in the efficiency of oxidative phosphorylation and an increase in the risk of resulting pathologies. Of course, there are other small molecules, possibly not as well-known as ATP, but still indispensable and irreplaceable. In particular, the metabolites derived from the amino acid tryptophan have the capacity for similar dramatic alteration of system-wide function. Most pertinent to the topic of this discussion, the changes in metabolic profiles are considered as drivers for the pathogenesis of age-associated disorders, including Alzheimer's disease (Tacutu et al., 2010; Demetrius and Driver, 2013; Jia et al., 2014; Obre and Rossignol, 2015). It also is of note that metabolites are not as abundant as commonly studied species of proteins and RNAs. Hence, the world of metabolites is immensely easier to comprehend than the overly complicated world of other famous "omics." The latter point is extremely important, as it provides a possibility for the use of a powerful reductionist approach without falling into ill-fitting or over-fitting of the underlying model, a well-known perpetual source of entrapment.

Let us compare the living (and aging) cell, with its endless "omics"-scale layers of interconnected components, to modern computers. Computer hardware is a collection of interconnected physical devices used in or with your machine. One of these parts may wear itself out and die; however,

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in industrially–built computers, the presence of multiple redundant circuits incorporated in fault-tolerant or multi-modular redundancy designs provides adequate protection against so-called "**soft errors.**" But what are such soft errors? Indeed, these are not synonymous with "software glitches." Soft errors are often defined as "**single-event upsets**" (SEU), the changes of state caused by ions or electro-magnetic radiation striking a sensitive node in a micro-electronic device, usually a unit where the memory is stored. The most common cause of "soft errors" is the direct hit of the circuits by cosmic particles colliding with atoms in the atmosphere, creating cascades or showers of neutrons and protons (Ziegler and Lanford, 1979)–analogous to the occurrence of a mutation. There is even a formula for the calculation of a soft-error rate that is typically expressed as a number of failures-in-time (a.k.a. the mutation rate) (Li et al., 2007). Similarly to living systems, computers could be and are designed to detect SEUs and recover gracefully, either by forward error correction that incorporates redundant error-correcting code into each output, or by roll-back error correction that detects the SEU using "sentinel" (or parity) bits, and, if needed, rewrites the data using a back-up. In the DNA world, both of these functions are executed by the DNA repair machinery. Similarly to redundant routines fixing SEUs, the redundant DNA repair mechanisms are embedded in the original system's design, whether *in vivo* or *in silico*. Hence, both the DNA and the molecules directly encoded by DNA, i.e., the RNA and the proteins, are the components of the hardware of life.

The million dollar question is: "What components make up the living cell's software?" Below we will try to make a case for the community of small molecules extant within the metabolome as the software components that run the living cell. Indeed, metabolites are universal, and also interchangeable between cellular types. In the end, ATP is only ATP; it is difficult to imagine that ATP may be mutated into something else. Hence, the metabolites may be likened to the set of instructions (software), that could be run on one or another type of hardware–i.e., a molecule of ATP extracted from a tapeworm would have addressed cellular needs and functions in the same way as that extracted from a human cell. Within the cell, a set of metabolites, each with its associated local concentrations and, possibly, their ratios may serve as the "net regulator" directing the overall patterns of transcription, translation and further modification of the messages encoded in DNA. Importantly, the concentrations of metabolites may be adjusted externally, either through direct supplementation or the administration of soluble enzyme inhibitors or co-factors. With that, very similarly to computer software, the "net regulation" that is maintained by the cellular metabolome may be restored to the default settings. In the case of aging, the default mode would correspond to one or another earlier timepoints on the living system trajectory, or the "younger" state of the living system. As one of us has previously demonstrated, metabolic profiles are robust, reproducible fingerprints of whole organismal phenotypic states in the nematode, *Caenorhabditis elegans*, accurately reflecting both life stage differentials and environmental modulations (Willett et al., 2010; Sudama et al., 2013). Perhaps it is more than a coincidence that Sydney Brenner, a founding father of the use of *C. elegans* as a transparent model for various scientific inquiries, including aging research, recently pointed at the biological necessity of including the question of information into the eternally studied interplay between matter and energy (Brenner, 2012).

No doubt, all of the above are nothing more than metaphors. However, these analogies may be helpful for understanding the eternal problem of aging as the mundane crackdown of a desktop computer. When the desktop is starting to fail us by slowing down or freezing frames, we either reboot it or, as a last resort, reinstall its operating software. Note that the idea of redesigning or otherwise reinforcing the hardware parts to make them less prone to SEUs, or mutations, in the case of a desktop seems absurd. Similarly, as a countermeasure to aging, we should concentrate on the elements that are easily fixable, or replaceable–the metabolic pattern seems like a suitable candidate for extrinsic or intrinsic modifications (Muradian, 2013). Obviously, this avenue of thinking implies that aging is not a fundamental property of the living system, but rather a time-associated decay, and that one or another routine procedure may be established in order to offset this process, in a way that is quite similar to the treatment of the disease.

In other words, the desktop metaphor provides for a hope that a software ingredient of the living machine, the metabolism, may be amenable to rebooting. Indeed, the analogies between the computer world and the world of living (and aging) things are abundant. Since the beginning of modern science, physics and its extension, chemistry, have been considered to be the foundation of biology. In digital physics, all known laws of physics have consequences that are theoretically computable on a digital computer, and therefore the universe itself must be computable on a classical Turing machine, a hypothetical device that manipulates symbols on a strip of tape according to a table of rules (Turing, 1936). The essential truth postulated above is known as the Strong Church–Turing thesis (Copeland, 1996). Pertinent to biology, living systems are parts of the Turing Universe; hence, all living things are Turing computers and, therefore, are the subjects of biological determinism in the widest sense possible.

While giving both the grounds for an infinite number of scientific papers describing various mechanistic insights into "regulatory" cellular networks and providing hope for the ultimate understanding of living system trajectories, we must admit that digital physics is neither the most modern, nor the most attractive representation of the universe. There are some widely-discussed alternatives, for example, that the Universe is a hypercomputer that is capable of non-recursive calculations (Siegelmann, 1995; Copeland and Proudfoot, 1999).

Importantly, even if the Universe as a whole may be likened to a hypercomputer, it is possible that its parts, i.e., living systems, may firmly remain within the Turing realm. Here we would like to add to a recent argument that makes the case of living systems surpassing the Turing requirements (Maldonado and Gomez Cruz, 2015) by referring to the Turing-unsolvable halting problem. An inability to detect a halt or, in other words, to determine from a description of an arbitrary computer program and an input whether the program will finish running, or continue to run forever, is a feature

embedded in a Turing design (Jack Copeland, 2004). Here we postulate that, for the living system, death is equivalent to a halt. Since in living systems one can both detect and predict death with certainty, we should accept non-recursive hypercomputation as an important underlying principle of biology.

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