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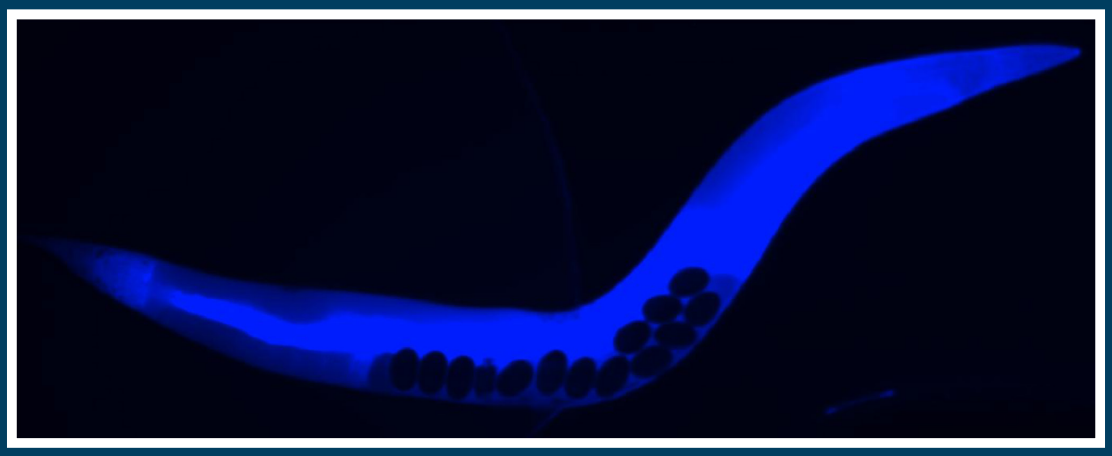

PAPERS OF THE CONFERENCE ON GENETICS OF AGING AND LONGEVITY 2012

Topic Editors
Alexey Moskalev and Elena G. Pasyukova

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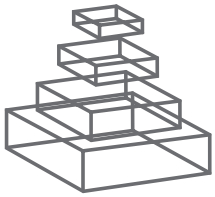

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PAPERS OF THE CONFERENCE ON GENETICS OF AGING AND LONGEVITY 2012

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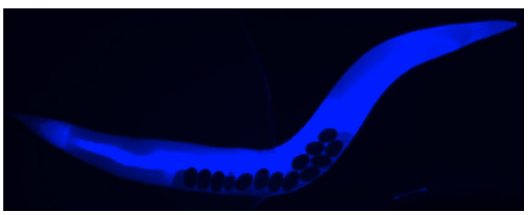

Image of adult *Caenorhabditis elegans* showing intense blue fluorescence, emitted by anthranilic acid glucosyl esters, which is a marker of organismal death. Image courtesy of Cassandra Coburn and David Gems; see also C. Coburn, D. Gems, 'The mysterious case of the C. elegans gut granule: death fluorescence, anthranilic acid and the kynurenine pathway.' Frontiers in Genetics (2013) 4: 151.

The 2nd International Conference «Genetics of aging and longevity» took place 22-25 April, 2012 in the main building of Russian Academy of Sciences, Moscow, Russia. Top gerontologists and geneticists from 25 countries around the world discussed the current problems in many areas related to the genetics of longevity and mechanisms of aging. This Research Topic is aimed to provide a collection of articles based on the talks, reports and experimental outcomes related to the topics of the conference: «Epigenetic Changes Associated with Longevity», «Hormones and Aging», «Proximal and Cellular Mechanisms of Aging», «Nutrient Signaling, Stress Resistance and Longevity», «Identifying Longevity Genes by Mutational, QTL and Association Mapping», «Fundamental Biological Processes Central to Aging», «Interventions to Extend Lifespan and Promote Healthy Aging», «Longevity: Meta-Analysis and Informatics Approaches». Participants of the Conference submitted 20 papers belonging to Original Research Papers, Review Articles (Including Mini Reviews), Opinion and Perspective Papers. All of the submitted manuscripts were peer-reviewed by excellent Frontiers Review Editors and prepared for publication by highly efficient Frontiers team, and it is a pleasure to thank them all for their work and dedication.

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EDITORIAL
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From theories of aging to anti-aging interventions

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Keywords: aging, evolution, genetics, epigenetics, geroprotectors

The 2nd International Conference ≪Genetics of aging and longevity≫ took place 22–25 April, 2012 in Moscow, Russia. Top gerontologists and geneticists from 25 countries around the world discussed the current problems in many areas related to the genetics of longevity and mechanisms of aging. Following this meeting, a collection of articles based on the talks, reports and experimental outcomes related to the topics of the conference was published in Frontiers in Genetics of Aging. This collection represents a comprehensive prospect of recent advances in genetics of aging, ranging from theoretical questions to practical approaches aimed to delay aging and prolong lifespan.

Unraveling the proximal cause of aging and creating a universal aging theory remains a difficult and ambitious task. Complicated mechanisms that preserve homeostasis and prolong lifespan involve interactions of numerous genes, metabolic pathways and environmental cues. Genetic and environmental variations, combinations of programmed and random events underlying the aging process further complicate the objective. Trindade et al. (2013) emphasize that a universal evolutionary aging theory must reconcile "trade-offs (pleiotropy or hitchhiking effect), retrogression (mutational load and drift) and direct adaptation ("program")."

The important notion that aging and lifespan are intimately related to various life history traits is highlighted in several articles from the collection. Novoseltsev and Novoseltseva (2013) used mathematical approaches to assess the origin of stochasticity in Drosophila reproduction, in relation to life expectancy. To add to mathematical predictions, analysis of long-term captive breeding of water voles demonstrated that individuals who began breeding at an older age had a significantly longer lifespan and produced more offspring (Nazarova, 2013).

These results resonate with the basis of the aging theory that set the grounds for a heated debate concerning the proximal cause of aging (Zimniak, 2012). In spite of many new insights into regulatory mechanisms that affect the aging process, the author remains true to the hypothesis of molecular damage and argues that hyper function proposed as a universal cause of aging by Blagosklonny (for references, see Zimniak, 2012) is only one of several sources of molecular damage. It is not proved, however, that a single proximal cause of aging does exist. According to the point of view of Lehmann et al. (2013), telomere length and body temperature are independent drivers of mammalian longevity.

New facts are needed to advance our understanding of aging, and studies of model organisms continue to provide us with valuable information. There are five articles in the collection, which represent new discoveries in genetics of aging of *Caenorhabditis elegans* (Bharill et al., 2013; Coburn and Gems, 2013), *Drosophila melanogaster* (Rogina and Helfand, 2013; Shostal and Moskalev, 2013), and mice (Bartke and Westbrook, 2012). In one of the most intriguing articles, Coburn and Gems (2013) come to the conclusion that the blue fluorescence of gut granules, a marker of death in *C. elegans*, is issued from anthranilic acid glucosyl esters, rather than from lipofuscin. They believe that this removes one reason for believing that worm aging is caused by accumulation of molecular damage, thus entering into the extramural discussion with Zimniak (2012).

In recent years, studies of long-lived wild animals, mainly rodents have complemented studies of model species and open new perspectives in aging research. In particular, the longevity of these species is correlated with cancer resistance, and analysis of molecular mechanisms underlying this property can benefit human health if such mechanisms can be activated in human cells (Azpurua and Seluanov, 2013).

Analysis of human populations represents an invaluable source of information concerning genetic and environmental factors promoting a long and healthy life. The results of several longitudinal studies of centenarians suggest, on the one hand, that factors responsible for exceptional longevity and health are not necessary the same and centenarians often experience chronic age-related diseases, but are able to cope with them. On the other hand, these results suggest that postponing aging changes is associated with extreme longevity (Sebastiani and Perls, 2012; Yashin et al., 2013). To add to the complexity, it is likely that the genetic component of extreme longevity includes many genes with modest effects (Sebastiani and Perls, 2012), which underscores once again that there is no simple universal recipe for a long life.

Unraveling fundamentals of exceptional longevity is essential for the concept of aging; for practical reasons, it may be even more important to get at the mechanisms linking lifespan and health. Theoretical aspects of this problem are reviewed in two articles providing compact but thorough description of the role of mitophagy (Palikaras and Tavernarakis, 2012) and Alu elements (Mustafina, 2013) in aging, while Djansugurova et al. (2013) present new data on genetic markers of cancers.

Understanding the molecular mechanisms underlying aging and age-associated diseases could bring us closer to the development of novel, efficient, anti-aging treatments. In this regard, one of the latest trends, the use of stem cells to increase lifespan, is described by Kovina et al. (2013).

Recently, there is also a great hope for the development of target-specific drugs for age-associated chronic diseases and, possibly, anti-aging drugs. Several articles in this collection are related to this problem. Vaiserman and Pasyukova (2012) present arguments that the development of specific drugs which target epigenetic pathways could be a highly promising anti-aging strategy. Bharill and co-authors demonstrated that commercially available inhibitors of AKT/FOXO signaling are able to enhance longevity and tolerance to oxidative stress in *C. elegans* (Bharill et al., 2013). However, the extreme complexity of the genetic control of homeostasis and aging predetermines caution in the use of new drugs. An example of possible side effects of medical treatments is proposed by Smith et al. (2013): the premature and accelerated aging of HIV-patients can be caused by adverse effects of antiretroviral drugs, specifically those that cause severe mitochondrial damage.

Another problem in drug discovery is the difficulty of extrapolating of the results from model species to humans and the time it takes to evaluate the effects of various interventions on longevity in humans. This problem is addressed in the article of Zhavoronkov et al. (2014) who propose a method for screening and ranking the possible geroprotectors before conducting pre-clinical work and expensive clinical trials.

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How lifespan associated genes modulate aging changes: lessons from analysis of longitudinal data

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Background and Objective: The influence of genes on human lifespan is mediated by biological processes that characterize body's functioning. The age trajectories of these processes contain important information about mechanisms linking aging, health, and lifespan. The objective of this paper is to investigate regularities of aging changes in different groups of individuals, including individuals with different genetic background, as well as their connections with health and lifespan. **Data and Method:** To reach this objective we used longitudinal data on four physiological variables, information about health and lifespan collected in the Framingham Heart Study (FHS), data on longevity alleles detected in earlier study, as well as methods of statistical modeling. **Results:** We found that phenotypes of exceptional longevity and health are linked to distinct types of changes in physiological indices during aging. We also found that components of aging changes differ in groups of individuals with different genetic background. **Conclusions:** These results suggest that factors responsible for exceptional longevity and health are not necessary the same, and that postponing aging changes is associated with extreme longevity. The genetic factors which increase lifespan are associated with physiological changes typical of healthy and long-living individuals, smaller mortality risks from cancer and CVD and better estimates of adaptive capacity in statistical modeling. This indicates that extreme longevity and health related traits are likely to be less heterogeneous phenotypes than lifespan, and studying these phenotypes separately from lifespan may provide additional information about mechanisms of human aging and its relation to chronic diseases and lifespan.

Keywords: age trajectories, physiological variables, longevity genes, genetic dose, integrative genetic mortality model

INTRODUCTION

The influence of genes on lifespan is mediated by biological variables which integrate responses to numerous external and internal challenges to maintain functioning of an organism's life supporting and reproductive machineries. Some of these variables are measured in longitudinal studies of aging, health and longevity. To mediate genetic effects on lifespan, these variables have to be associated with lifespan as well. Such associations have been established in epidemiological studies for a number of physiological variables where their roles as risk factors for all-cause mortality or chronic degenerative diseases have been investigated. Among many other variables, body mass index (BMI), diastolic blood pressure (DBP), serum cholesterol (SCH), and ventricular rate deserve particular attention because their typical average age trajectories are non-monotonic and their associations with all-cause mortality have been widely studied. Specifically, the effect of BMI on risk of diseases and mortality was intensively studied in connection with metabolic syndrome. Freedman et al. (2006) showed that the connection between BMI and mortality risks is generally J-shaped for both genders and different age groups. The authors also found that this risk function changes with age. The relationships between mortality risk and BMI were also assessed (see Zhou, 2002; Gu et al., 2006; Gelber et al., 2007; Klenk et al., 2009, among others).

The connection between DBP and all-cause mortality risk has been investigated to better understand factors and mechanisms of cardiovascular diseases (CVD) (Cruickshank, 1988, 2003; Staessen, 1996). Special attention has been paid to the J-shape of the risk function (see Isles and Hole, 1992; Alderman, 1996; Cruickshank, 2003; Messerli and Panjrath, 2009; Grassi et al., 2010, among others). Franklin et al. (2001) studied changes in this risk function with age. Boshuizen et al. (1998) studied the connection of blood pressure and mortality risk among the elderly. Questions of optimal blood pressure were discussed by Onrot (1993) and Townsend (2005), among others.

Anderson et al. (1987) evaluated the connection between SCH and mortality using 30 years of follow-up data from the Framingham Heart Study (FHS). The authors found that a 1% increase in total cholesterol produced a 2% increase in coronary heart disease incidence among individuals between 60 and 70 years of age. Kronmal et al. (1993) found that the relationship between total cholesterol level and all-cause mortality was positive

at age 40 years, negligible at ages 50–70 years, and negative at age 80 years. Manolio et al. (1992) and Weverling-Rijnsburger et al. (1997, 2003) showed that CVD in old age was independent of total SCH levels. Weverling-Rijnsburger et al. (1997) proposed that this could be a result of selective mortality of those with the highest cholesterol levels in middle age. Weverling-Rijnsburger et al. (1997) and Schatz et al. (2001) showed that low total SCH levels are associated with higher all-cause mortality in the oldest old. The relationship between SCH and all-cause mortality was also studied by Chyou and Eaker (2000) and Li et al. (2004a,b), among others.

The effects of resting heart rate (also called ventricular rate, VR) on cardiovascular mortality have been discussed in Kannel et al. (1987). Mensink and Hoffmeister (1997) and Benetos et al. (1999) investigated the effects of resting heart rate on all-cause mortality. The connection between heart rate and mortality in the elderly has also been investigated by Cacciatore et al. (2007). Kuzuya et al. (2008) found a J-shaped relationship between resting pulse rate and all-cause mortality in community dwelling older people with disabilities. Böhm et al. (2012) showed that resting heart rate in clinical conditions is associated with all-cause mortality, disability, and cognitive decline.

The results described above indicate that studying aging related changes in physiological variables as well as genetic factors involved in their regulation using available longitudinal data could make substantial contributions to clarifying mechanisms linking aging, health, and longevity in humans, and provide useful insights into alternative strategies for improvement of people's health by postponing the aging process (Kristjuhan, 2012). Note that none of the studies mentioned above performed either systematic analyses of age patterns of corresponding variables, or their roles in mediating genetic influences on lifespan. The longitudinal data on aging, health, and lifespan collected in the FHS contain valuable information on biennial measurements of these physiological variables during the life courses of study participants, detailed data on their genetic background, as well as data on health and survival outcomes which can be used for testing the ability of these variables to mediate genetic influences on lifespan.

In this paper we evaluate and discuss the properties of the average age trajectories of the four physiological indices described above, evaluate the connections of the shapes of these trajectories to lifespan, and individuals' health status. We also evaluate how the different doses of pro-survival alleles carried by study participants are associated with the age patterns of their physiological variables. Using a stochastic process model (Yashin et al., 2007a), we evaluate hidden components of the process that drive aging related changes in physiological variables.

DATA AND METHODS

THE FRAMINGHAM HEART STUDY (FHS) DATA

The FHS Original cohort was launched at Exam 1 in 1948 and has continued with biennial examinations to the present (30 exams to date; data from exams 1–28 were available for this study). The FHS Original cohort consists of 5209 respondents (55% females) aged 28–62 years at baseline residing in Framingham, Massachusetts, between 1948 and 1951. Nearly all subjects were Caucasians. The examination included an interview, physical examination, and laboratory tests. Individual information on the SNP genotyping and phenotypic traits collected in the Framingham Study was obtained through the dbGaP website. The data on phenotypic traits collected in the Original FHS cohort over 60 years and relevant to our analyses include: ages at disease onsets for cancer, CVD and diabetes, causes of death, lifespan, and various factors that may affect disease risk and prognosis including BMI (data available at exams 1, 4, 5, and 10–28), DBP (exams 1–28), SCH (exams 1–11, 13–15, 20, and 22–28), VR (exams 4–28), age at exam, sex, birth cohort, and smoking status (exams 1, 4, 5, 7–15, and 17–28).

The occurrence of diseases (CVD and cancer) and death (including information on the cause of death coded as death from cancer, CVD, and all other or unknown causes) has been followed through continuous surveillance of hospital admissions, death registries, clinical exams, and other sources, so that all the respective events are included in the study. We used data on first occurrence of CVD (defined by the FHS investigators as having any of the following: coronary heart disease, intermittent claudication, congestive heart failure, or stroke/transient ischemic attack) and cancer from the follow-up data, and data on current diabetes status (defined by the FHS investigators as a level of blood glucose exceeding 140 mg/dl and/or taking insulin or oral hypoglycemics) in exams in analyses involving the onset of "unhealthy life" (see below).

We also used information about the distribution of 27 "pro-survival" alleles among participants of the FHS Original cohort. **Figure 1** shows distribution of the numbers of these alleles in the sample of genotyped individuals in the FHS Original cohort. These genetic variants showed highly significant joint influence on lifespan in our recent study (Yashin et al., 2012c). They were selected out of 550,000 SNPs in 1471 genotyped participants of the FHS Original cohort as described in Yashin et al. (2012c). **Table 1** below shows essential information about the 27 SNPs and

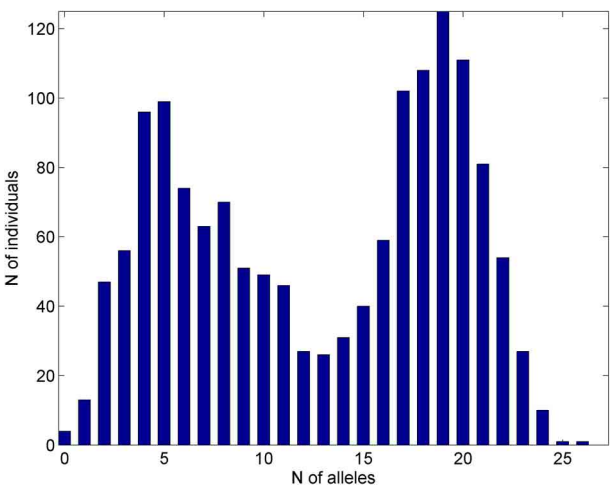

FIGURE 1 | Distribution of the numbers of pro-survival alleles out of 27 of such alleles selected in Yashin et al. (2012c) in the sample of genotyped individuals in the FHS Original cohort.

Table 1 | Essential characteristics of the 27 pro-survival SNPs from Yashin et al. (2012c).

SNP	MAF%	Chr	Relation to gene	Distance to gene	Closest gene	Gene/protein description	Major biological processes or health disorders, in which respective genes were shown/suggested to be involved
rs4648884	27.7	1	INTRONIC	0	RUNX3: runt-related transcription factor 3	Can either activate or suppress transcription; interacts with other transcription factors	Apoptosis; tumor suppression; immune response; asthma
rs3120819	42.1	1	INTERGENIC	−84906	RP11-149P14.1	Pseudogene	
rs1974676	35.4	2	INTRONIC	0	HPCAL1: hippocalcin-like 1	Member of neuron-specific calcium-binding proteins family	Brain information processing; CVD, asthma (?)
rs432203	38.0	2	INTRONIC	0	TGF alpha: transforming growth factor, alpha	A mitogenic polypeptide able to bind to the EGF receptor and act synergistically with TGF beta to promote cell proliferation	Cell proliferation; brain response to damage; cancer; stroke (?)
rs12623542	36.7	2	NON CODING	0	AC131097.3		
rs13008689	23.2	2	INTERGENIC	−153466	AC011747.3		
rs1834497	44.7	3	INTRONIC	0	CLSTN2: calsyntenin 2	Postsynaptic membrane proteins with highest levels in GABAergic neurons; expressed in the medial temporal lobe	Brain information processing; memory formation; AD
rs9876781	34.9	3	NON CODING	0	RP11-24C3.2	Closest gene: ATR interacting protein (ATRIP)	Cell cycle arrest/ replicative senescence
rs10937739	41.9	4	INTRONIC	0	PPP2R2C: protein phosphatase 2, regulatory subunit B, gamma	Serine/threonine phosphatase 2; is abundant in brain; can modify MAPK activity;	Apoptosis; memory; brain disorders; cancer, CVDs (?)
rs1205035	36.2	6	INTERGENIC	−109050	RP1-223B1.1		
rs3800358	25.9	6	3PRIME_UTR	0	BTBD9: BTB domain containing 9	Involved in protein-protein interactions	Sleep disorders
rs10256972	36.5	7	INTRONIC	0	C7orf50: chr7 open reading frame 50		
rs1327533	25.3	9	INTRONIC	0	SVEP1: EGF and pentraxin domain containing 1	A multi-domain protein: Sushi, CCP, vWF-A, EGF/ EGF-like calcium binding, and pentraxin domains	Cell adhesion; muscle growth and regeneration
rs2590504	37.3	9	DOWN-STREAM	−6153	KIAA0649	Is a 1A6/DRIM ("Down-regulated in Metastasis") interacting protein with oncogenic characteristics	Cell proliferation; cancer
rs10819510	26.5	9	INTERGENIC	−37267	RP11-65J3.3		
rs739401	32.4	11	INTRONIC	0	CARS: cysteinyl-tRNA synthetase	Catalyzes tRNA aminoacylation; located near imprinted gene domain in a tumor-suppressor gene region	Cancer; diabetes

(Continued)

Table 1 | Continued

SNP	MAF%	Chr	Relation to gene	Distance to gene	Closest gene	Gene/protein description	Major biological processes or health disorders, in which respective genes were shown/suggested to be involved
rs2370413	35.2	12	INTRONIC	0	CACNA1C: alpha 1C subunit of L-type voltage-gated calcium channel	Mediates the entry of calcium ions into cells; involved in variety of calcium-dependent processes	Calcium-depend. processes; brain volume, memory; brain disorders; CVD
rs9517320	30.8	13	INTRONIC	0	STK24: serine/threonine kinase 24	Participates in the mitogen-activated protein kinase (MAPK) cascade	Brain regeneration; memory; possibly PD and cancer
rs4148544	24.6	13	INTRONIC	0	ABCC4: ATP-binding cassette transporters family	Transport various molecules across extra- and intra-cellular membranes	Transport of xenobiotics, detoxification; cancer
rs41383	25.0	16	INTRONIC	0	NLRC5: NLR family, CARD domain containing 5	IFN-gamma-inducible nuclear transcriptional regulator of the NF-kappaB and type I interferon signaling	Innate immunity; inflammation; viral infection
rs16975963	46.7	19	NON CODING	0	AC016582.2		
rs2024714	26.8	20	INTRONIC	0	CDH4: R-cadherin (retinal)	Participates in calcium-dependent cell-cell adhesion	Cell adhesion; brain volume, brain aging; cancer
rs2826891	36.50	21	INTRONIC	0	NCAM2: neural cell adhesion molecule 2	Brain protein, superfamily of the immunoglobulin; one of plasma membrane-anchored proteins	Neural cell adhesion; cancer, brain disorders (?)
rs139170	34.7	22	INTRONIC	0	PARVG: parvin, gamma	Actin-binding proteins associated with focal adhesion	Cell adhesion; tumor suppression
rs5771675	30.9	22	INTRONIC	0	FAM19A5: member of TAFA family	Related to MIP-1alpha, a member of the CC-chemokine family.	Regulation of brain inflammation and neuronal survival (?)
rs9616906	22.7	22	UP-STREAM	−3552	AC000050.2		
rs13053175	28.0	22	UP-STREAM	−7992	RAC2: ras-related C3 botul. toxin substrate 2	GTPase of the RAS superfamily regulating cell growth, cytoskelet, and the protein kinases activation	Cell growth/ proliferation; cell adhesion; immunity, inflammation

Expanded table with references is provided in Yashin et al. (2012c).

Note: Abbreviations: AD, Alzheimer's disease; CVD, cardiovascular diseases; PD, Parkinson's disease. MAF, minor (pro-survival) allele frequency % at baseline ages (60–75).

their closest genes. More detailed information about the genes closest to the 27 SNPs, including their biological functions and links to aging and disease phenotypes, as found in current literature, is provided in Yashin et al. (2012c). In brief, of the 27 SNPs, 16 were located within functioning genes, and all these SNPs but one were intronic. Overall, these genes have been linked in the literature to multiple phenotypes, although they were more often involved in cancer and brain disorders. While some of the genes, in which the selected SNPs are located, may produce specific physiological effects, none of the 27 SNPs identified in Yashin et al. (2012c) has been found so far to be individually significantly associated with BMI, DBP, SCH, or VR.

EMPIRICAL ANALYSES

First, we calculated empirical estimates of the mean values of four physiological indices (BMI, DBP, SCH, and VR) in age groups <35, 35–39, ..., 85–89, and 90+ years for all participants of the Original FHS cohort (males and females combined) using pooled data on measurements from all exams.

Second, we selected groups of short-lived individuals (those dying at ages 75 or earlier; censored individuals are excluded from this group) and 100 longest lived individuals (which is equivalent to individuals with lifespan exceeding 97.44 years) and evaluated average values of physiological indices in the same age groups for these individuals using pooled data on measurements from all exams.

Third, we used data on the first occurrence of CVD and cancer from the follow-up data, and data on current diabetes status from the exams to calculate ages at onset of "unhealthy life" defined as the minimum of ages of occurrence of these diseases. Then we evaluated average age trajectories of physiological indices in the same age groups described above for "unhealthy" (those with cancer, CVD, or diabetes) and "healthy" (those free of these three diseases) individuals. Measurements of physiological indices before the onset of any of these diseases contributed to the "healthy" trajectory and those after the onset of any of the diseases contributed to the "unhealthy" trajectory. Note that average values based on less than 10 observations are not shown in all figures.

Fourth, we evaluated average age trajectories in the same age groups for carriers of different numbers of alleles among the 27 pro-survival alleles selected as described in Yashin et al. (2012c). Note that the identity of SNPs was not important in this selection procedure. We calculated the total number of pro-survival alleles in the genomes of individuals using data on 27 SNPs selected in Yashin et al. (2012c). For each individual and for each of these 27 SNPs, we created a dichotomous variable equaling 1 if the individual has respective minor allele and 0 if he/she is a carrier of major allele homozygote for respective SNP. Then we created a variable counting the number of 1's for each individual (that is, the number of such minor alleles in these 27 SNPs, which could be any number from 0 to 27). The genotyped sample was divided into two sub-groups, the first containing individuals having less than 14 minor alleles and the second consisting of those having 14 or more minor alleles. For the sake of convenience, we will refer to these sub-groups as the (<14)- and the (≥14) groups, respectively. Then the age trajectories of average values of four physiological indices were calculated for individuals in these two sub-groups. Note that all analyses related to these alleles are based on data from 1471 genotyped participants.

Fifth, we estimated average trajectories among "unhealthy" and "healthy" carriers of different numbers of pro-survival alleles, as described above.

Sixth, we compared average trajectories among carriers and non-carriers of the APOE e4 allele. These calculations are based on data on APOE polymorphisms available for 1258 participants of the original FHS cohort.

Finally, we evaluated associations of the "genetic dose" with mortality rates by cause in the Cox proportional hazards model. "Genetic dose" or "polygenic score" is defined here as a variable calculating the number of pro-survival alleles [out of the 27 alleles from Yashin et al. (2012c)] in the genomes of 1471 genotyped individuals from the Original FHS cohort. The model was adjusted for sex, birth cohort, and smoking status (ever/never smoked). Age at the biospecimen collection available for the genotyped individuals was used as the left truncation in the Cox model. The model was applied to data on total mortality as well as mortality by cause (cancer, CVD, and all other or unknown causes, denoted as "Other" in **Table 3**).

All calculations mentioned above have been performed using SAS 9.3. Graphical output was prepared in MATLAB R2012a.

Table 2 shows subject characteristics in the specific analyses described above.

ADVANCED STATISTICAL ANALYSES USING THE STOCHASTIC PROCESS MODEL

We illustrated how different aging-related characteristics in carriers of different numbers of pro-survival alleles may jointly contribute to the patterns of mortality rates as well as the trajectories of physiological variables applying the stochastic process model of aging (Yashin et al., 2007a) to data on four physiological indices (BMI, DBP, SCH, and VR) and total mortality in the 1471 genotyped individuals from the original FHS cohort. Note that data on genotyped and non-genotyped individuals can be analyzed jointly in the version of the stochastic process model described in Arbeev et al. (2009) but such analyses are beyond the scope of this paper. Some concepts and ideas about the process of aging that are used in the stochastic process model are briefly outlined in section "The need for comprehensive integrative analyses of longitudinal data." Technical details about the specific version of the model used in this paper are given below.

Stochastic dynamics of individual age trajectories of physiological indices

The version of stochastic process model (Yashin et al., 2007a, 2012a), can be used to provide information about adaptive mechanisms forming the age trajectories of average physiological indices. In this model the individual dynamics of one physiological index is described by stochastic differential equation

$$dY_t = a(t)\big(Y_t - f_1(t)\big)dt + B(t)dW_t,\ Y_0 \qquad (1)$$

Here Y_t is the value of a particular physiological index at age t in an arbitrarily chosen individual. In contrast to traditional

Table 2 | Characteristics of study subjects.

Study sample	Baseline age				% Females	Sample size
	Mean	St. Dev.	Min	Max		
TOTAL SAMPLE						
	44.1	8.60	28	62	55.2%	5209
SHORT- VS. LONG-LIVED						
Short-lived	44.0	8.35	28	62	42.4%	1801
Long-lived	47.8	6.58	37	62	85.0%	100
"UNHEALTHY" VS. "HEALTHY"						
"unhealthy"	44.1	8.61	28	62	52.7%	4291
"healthy"	44.2	8.55	29	62	66.4%	788
CARRIERS OF DIFFERENT NUMBER OF PRO-SURVIVAL ALLELES						
<14	42.0	7.32	30	61	54.4%	721
≥ 14	36.0	4.50	29	55	65.7%	750
CARRIERS ("E4") AND NON-CARRIERS ("NOT E4") OF THE APOE E4 ALLELE						
e4	37.9	5.96	28	57	66.1%	277
not e4	37.6	5.66	29	57	63.1%	981
"UNHEALTHY" AMONG CARRIERS OF DIFFERENT NUMBER OF PRO-SURVIVAL ALLELES						
"unhealthy", <14	41.8	7.27	30	61	52.3%	637
"unhealthy", ≥ 14	35.9	4.46	29	54	63.4%	658

Notes: Short-lived are those dying at ages below 75, long-lived are those with 100 longest life spans; see details in the text. "Unhealthy" are those who had onset of at least one of the three diseases (cancer, CVD, diabetes) during the follow-up period; "healthy" are those free of such disease during the follow-up period; see more details on calculations of average trajectories in "unhealthy" and "healthy" individuals in the text. Carriers of different numbers of pro-survival alleles are defined based on the 27 SNPs identified in Yashin et al. (2012c); see details on the selection procedure in the text.

approaches used for analyzing longitudinal data with health or survival outcomes the description of the data in our model includes additional unobserved variables having important biological meaning for the aging process. The coefficient $B(t)$ characterizes the contribution of random external disturbances described by a Wiener process, W_t. Function $f_1(t)$ describes effect of allostatic adaptation, i.e., integrated effect of persistent external or internal disturbances which Y_t is forced to follow by homeostatic forces. Taking this effect into account is especially important in analyses of longitudinal data on aging, health and longevity in which measurements of external disturbances are absent or limited. This adaptation aims at achieving stability of key biological variables (not described here), through physiological or behavioral change. The strength of homeostatic forces is characterized by the negative feedback coefficient, $a(t)$. According to (1) the age trajectory of physiological indices Y_t tends to follow function $f_1(t)$, i.e., adapt to changes in $f_1(t)$. An ability to adapt depends on the absolute values of $a(t)$. Age-related changes in these coefficients characterize changes in adaptive capacity with age. Specifically, $a(t)$ regulates the age trajectory of the physiological index approximated by Y_t, i.e., it characterizes the rate of the adaptive response for any deviation of a physiological index from the state $f_1(t)$ which an organism tends to follow. For example, in a simplified one-dimensional case, when $B(t) = 0$, for all t, in Equation (1), and constant negative $a(t) = a$ for all t, the parameter a is the coefficient of negative feedback in the equation for Y_t, which keeps the trajectory Y_t close to $f_1(t)$. When $f_1(t) = f_1$, constant for all t, the value of Y_t asymptotically approaches f_1. In case of non-zero disturbances, the higher the absolute value of a, the closer Y_t is to f_1, and the faster Y_t tends to f_1. That is why the value $a(t)$ characterizes adaptive capacity. Practical estimation of the changes in adaptive capacity with age involves maximization of the likelihood function of the data in which $a(t)$ is described as parametric functions of age. The random variable Y_0 describes the initial value of physiological index, which is assumed to be independent of W_t for each $t \geq 0$. The introduction of $f_1(t)$ and $a(t)$ into the model facilitates the biological interpretation of the results of statistical analyses of longitudinal data.

Conditional risk function (conditional mortality rate)

Note that individual trajectories of physiological variables must be stopped at random time T describing lifespan of an individual. The probability distribution of this stopping time is characterized by conditional mortality rate given the value of the physiological index. This conditional mortality rate is represented by the quadratic form:

$$\mu\,(t, Y_t) = \mu_0(t) + Q(t)\big(Y_t - f_0(t)\big)^2 \qquad (2)$$

The term $\mu_0(t)$ (the baseline mortality rate) is a function of age. It shows how the total mortality rate would change if a corresponding physiological index Y_t followed the optimal trajectory $f_0(t)$. The function $f_0(t)$ is associated with the notion of the age-dependent "norm" in the model. The positive function $Q(t)$ shows how the steepness of the parabola $Q(t)\left(Y_t - f_0(t)\right)^2$ changes with increasing age.

The model described above takes into account the fact that available longitudinal data do not contain records characterizing when, how, and how long external disturbances affected individuals during their life course. The use of the notions of allostasis

and allostatic adaptation helps us understand how persistent unfavorable conditions get "under the skin" of affected person, increasing his/her susceptibility to diseases and death (McEwen, 2012). Many such conditions affect set-points of physiological homeostasis changing physiological balance from the "normal," $f_0(t)$ to "abnormal," $f_1(t) \neq f_0(t)$ state. These effects, represented in Equation (1) can be estimated from the FHS data thereby providing indirect evaluation of the effects of external disturbances without measuring them.

Version of model used in application to FHS data

We applied a discrete-time version (see Yashin et al., 2007b) of the general model (1)–(2) (with values of a physiological index evaluated at one-year age intervals using respective observations in the adjacent FHS exams) with specification of respective components as follows. We used a constant diffusion $B(t) = \sigma_1$; a linear function for the adaptive capacity $a(t)$: $a(t) = a_Y + b_Y t$; a linear function for the quadratic hazard term $Q(t)$: $Q(t) = a_Q + b_Q t$, and the Gompertz function for the baseline hazard $\mu_0(t)$:$\mu_0(t) = a_{\mu_0}\exp(b_{\mu_0} t)$. Initial values Y_0 are assumed normally distributed, $N(f_1(t_0), \sigma_0)$.

To evaluate the "optimal" trajectories $f_0(t)$, we calculated the average age trajectories of physiological variables for long-lived individuals (those with lifespan exceeding 90 years) in the original FHS cohort and fitted these trajectories by cubic polynomials using the Curve Fitting Toolbox in MATLAB. The fitted curves were used as the estimates of the "optimal" trajectories $f_0(t)$ [see motivation for such specification in Yashin et al. (2012b)]. Note that longitudinal observations in long-lived individuals are available only starting at ages 40 and above. Therefore, we restricted applications of our model to observations at ages 40 and above.

Taking into account the possibility that the homeostatic regulation forces the trajectory of physiological variables to the values different from the optimal values represented by $f_0(t)$ and that this difference can be age-dependent, we specified the function $f_1(t)$ as $f_1(t) = f_0(t) + \Delta f_0(t)$, where $\Delta f_0(t) = a_{f_0} + b_{f_0} t$.

Details of the likelihood maximization procedure can be found in Yashin et al. (2007a,b). The likelihood maximization was performed using the constrained optimization procedure of MATLAB's Optimization Toolbox. The constrained maximization algorithm was used to impose necessary restrictions on parameters of: (1) function $f_1(t)$, to ensure "physiologically reasonable" values at each age; (2) the feedback coefficient $a(t)$, to ensure its negative value at each age so that the trajectories of Y_t tend to $f_1(t)$; (3) the baseline hazard $\mu_0(t)$, to ensure non-negative values for each age; (4) σ_0, and σ_1, to ensure non-negative values; and (5) the quadratic hazard term $Q(t)$, to ensure that the values are non-negative for each age.

The model was applied to data on the two groups of genotyped individuals from the original FHS cohort, those carrying <14 and ≥ 14 alleles out of the 27 pro-survival alleles selected in Yashin et al. (2012c). First, we estimated the unrestricted model that has all different parameters in the two groups and then restricted models imposing respective restrictions on the parameters of the model to test the null hypotheses about the equality of model's characteristics in the two groups. Specifically, we tested four null hypotheses on the equality of (1) baseline hazards, (2) quadratic hazard terms, (3) adaptive capacities, and (4) mean allostatic trajectories in the two groups. The hypotheses were tested using the likelihood ratio test. Respective *p*-values are shown in **Figures 9–11**.

RESULTS

THE NON-MONOTONIC AVERAGE AGE TRAJECTORIES OF PHYSIOLOGICAL VARIABLES

The shapes of the average age trajectories of physiological indices (**Figure 2**) provide useful insights about factors and mechanisms involved in changes developing in aging human body which can be verified using more sophisticated statistical approaches. **Figure 2** displays the age patterns of average values of physiological indices for BMI, DBP, SCH, and VR for males and females combined, evaluated from the data on the Original FHS cohort.

One can see from this figure that all four trajectories are non-monotonic. After a period of increase at ages between 35 and 50–54 years, the values of BMI, DBP, SCH, and VR reached their maximum value and then declined. Note that the shapes of these curves do not necessarily represent the shapes of biological age trajectories in individual organisms. This is because in addition to the contribution of biological aging, these trajectories reflect the effects of compositional changes due to mortality selection in a heterogeneous population.

The average age patterns for males and females look similar to those of the combined estimates, with some differences in details (not shown). Specifically, the average values of BMI for females were lower than those of males at ages between 35 and 74 years. They increased faster and reached their maximum value later than those of males. After age 75 the values of BMI coincided for the two genders. The average values of DBP for females were lower than those of males at ages between 35 and 59. They increased faster and reached their maximum value later than those of males. After age 60, the values of DBP practically coincided with those of males with a tendency to become higher at age 95 years. The average values of SCH for females were lower than those of males at ages between 35 and 44 years. Then they became higher than those of males for the rest of the age domain. The values of SCH for females increased faster and reached their maximum value later than those of males. The values of VR for females were higher than those of males at the entire age domain.

THE AGE TRAJECTORIES OF THE SHORT LIVED (SL) vs. THE LONGEST LIVED (LL) INDIVIDUALS

Figure 3 shows the average age trajectories of DBP, BMI, SCH, and VR, for the short lived (lifespan, LS <75 years) and the 100 longest lived (aged 97+) males and females. One can see that trajectories for the LL individuals were substantially different from those for the SL individuals in all four indices. Specifically, the average values of BMI were higher among the SL persons until age 70 with tendency to intersect trajectory of this index for the LL individuals. The age trajectories of BMI for the SL and LL groups reached their maximum values at ages 60 and 70, respectively. The average values of DBP among the SL people were higher than those of the LL study participants from age 40 until 70 years

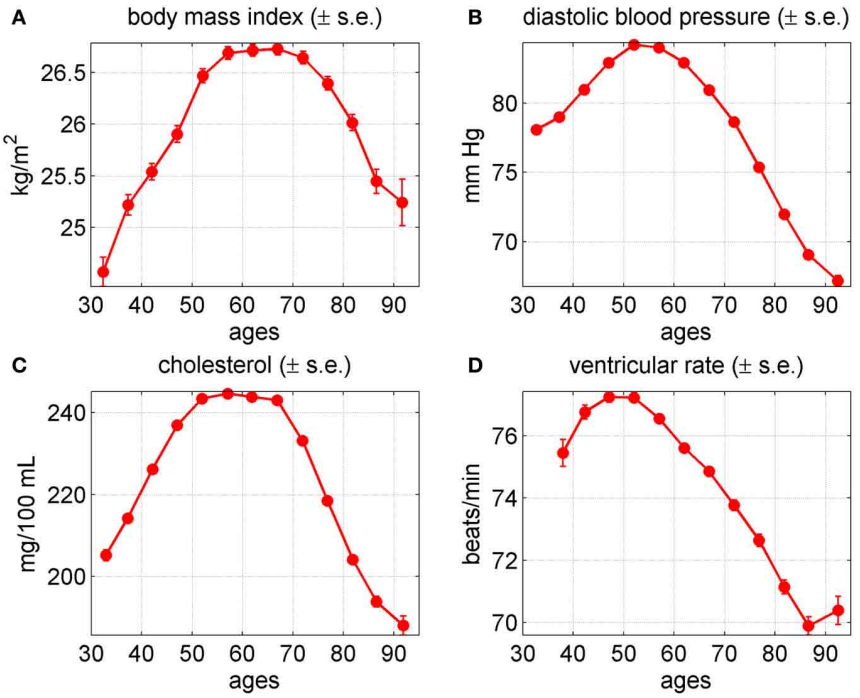

FIGURE 2 | Average trajectories (±standard errors) of four physiological variables in the Framingham Heart Study (original cohort, pooled data from exams 1–28). (A) Body mass index; **(B)** diastolic blood pressure; **(C)** cholesterol; **(D)** ventricular rate.

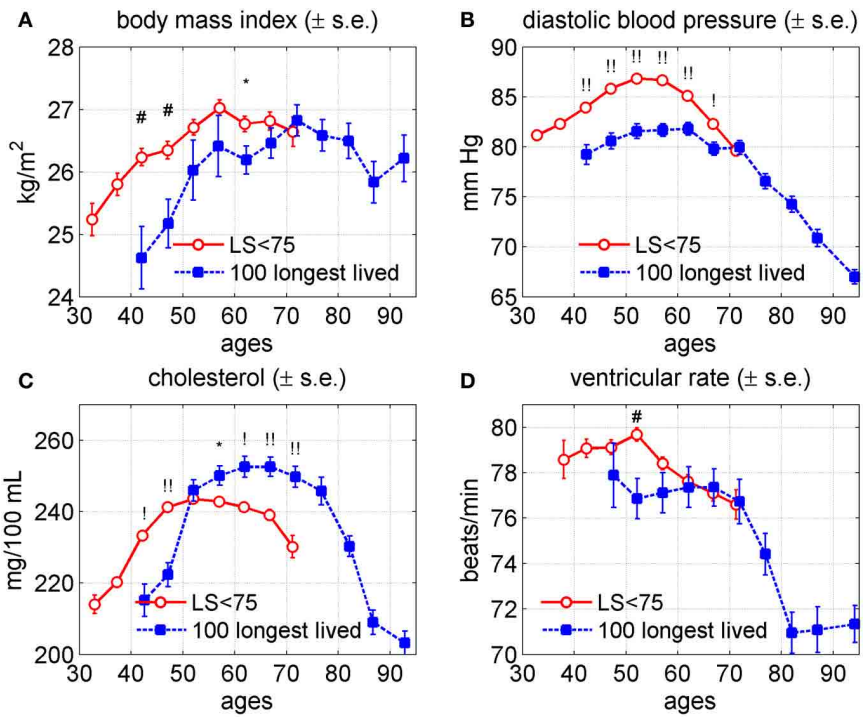

FIGURE 3 | Average trajectories (±standard errors) of four physiological variables in groups of short-lived individuals ("LS < 75," i.e., those dying at ages 75 or earlier; censored individuals are excluded from this group) and 100 longest lived individuals in the Framingham Heart Study (original cohort, pooled data from exams 1–28). (A) Body mass index; **(B)** diastolic blood pressure; **(C)** cholesterol; **(D)** ventricular rate. Symbols above the curves correspond to *p*-values for testing the null hypotheses on equality of means in respective age groups: no symbol: $p \geq 0.05$; *: $0.01 \leq p < 0.05$; #: $0.001 \leq p < 0.01$; !: $0.0001 \leq p < 0.001$; !!: $p < 0.0001$.

of age. At ages 70–74 years the values of DBP were practically indistinguishable between the two groups. The maximum value of the average DBP in the SL group was higher than that of the LL persons and it was reached earlier (50 years for the SL and about 60 years for the LL individuals). The average values of SCH were higher among the SL persons up to age 55, where they reached their maximum value and then declined. At age 55 the average age trajectory of SCH for the SL persons intersected that of the LL persons. The average age trajectory of the SCH for the LL persons reached their maximum value about age 65 and then declined. Note that at average, after age 55 females have higher levels of SCH than males. This fact together with information that females comprise 85% of the LL group and only 57.6% of the SL group (**Table 2**) contributes to difference in magnitudes between SCH trajectories for the SL and LL individuals in **Figure 3**. The values of VR for the SL persons were higher until age 65 and then practically coincided with that of the LL people until age 75 with the tendency to intersect.

Figure 4 displays individual trajectories for the SL and LL groups. It reveals that there is some tendency in trajectories of the LL individuals (to a lesser extent in VR) to avoid extreme values of indices (which, however, may be just an artifact of a smaller number of individuals in the LL group). Nevertheless, **Figure 4** generally shows that, despite the observed differences in average patterns (**Figure 3**), different individuals may have very diverse trajectories and the SL individuals are a more heterogeneous group.

THE AGE TRAJECTORIES OF PHYSIOLOGICAL INDICES FOR HEALTHY AND UNHEALTHY INDIVIDUALS

It is well known from medical practice that health status may influence values of physiological indices, as well as lifespan. In turn, physiological variables associated with lifespan are likely to show associations with some chronic diseases. To elucidate links between health and physiological variables we calculated average age trajectories of BMI, DBP, SCH, and VR for healthy and unhealthy individuals. The unhealthy individuals are defined here as those having at least one of three diseases: cancer, CVD, or diabetes. Since having a disease is likely to increase mortality risk, the unhealthy individuals are likely to be more susceptible to death. **Figure 5** shows average age trajectories for healthy and unhealthy persons (males and females combined).

One can see from this figure that the trajectories differed for healthy and unhealthy individuals. Specifically, the average values of BMI tended to be higher for the unhealthy than for healthy group at ages from 40 to 95 years. The average trajectories of DBP, SCH, and VR for healthy and unhealthy persons intersected at ages between 60 and 70 years. The values of SCH were higher among unhealthy individuals at age between 40 and 60 years. Then the curves intersected, so after age 60 the healthy individuals had higher values of SCH. The values of DBP had a similar pattern with the intersection point around age 65, which was, however, less pronounced. The values of VR were higher among unhealthy people until about age 65, after which they became lower than those among healthy people.

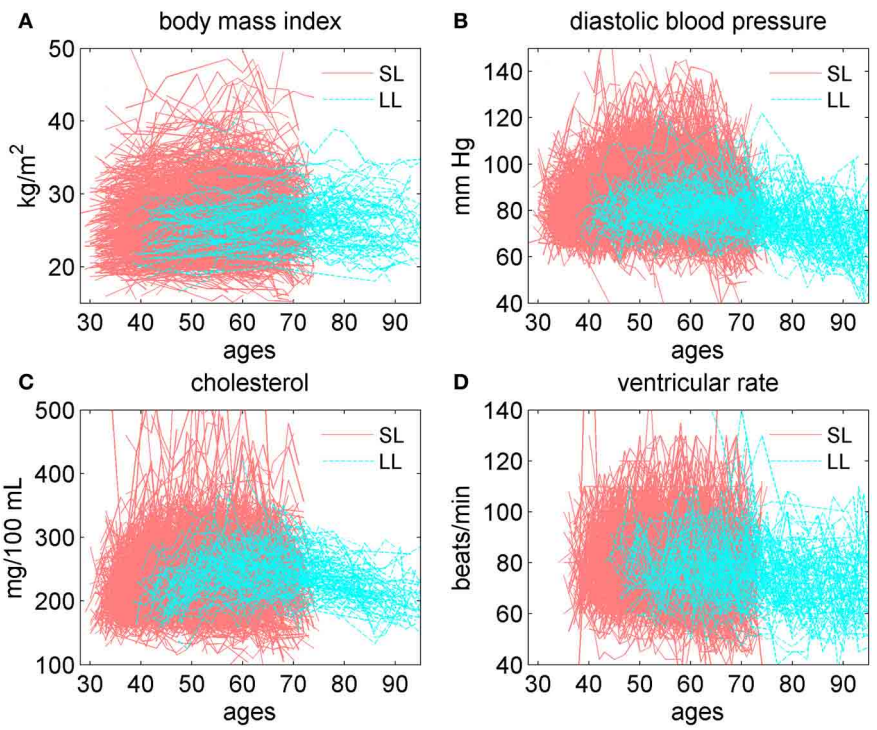

FIGURE 4 | Individual trajectories of four physiological variables in groups of short-lived individuals ("SL," those dying at ages 75 or earlier; censored individuals are excluded from this group) and 100 longest lived ("LL") individuals in the Framingham Heart Study (original cohort, pooled data from exams 1–28). (A) Body mass index; **(B)** diastolic blood pressure; **(C)** cholesterol; **(D)** ventricular rate.

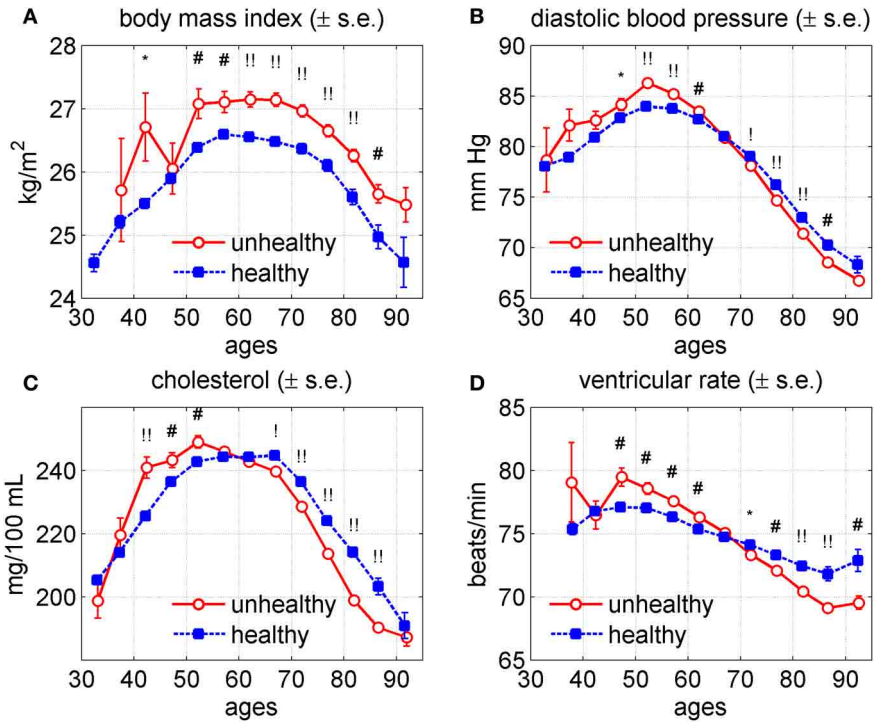

FIGURE 5 | Average trajectories (±standard errors) of four physiological variables for "unhealthy" and "healthy" individuals in the Framingham Heart Study (original cohort, pooled data from exams 1–28). (A) Body mass index; **(B)** diastolic blood pressure; **(C)** cholesterol; **(D)** ventricular rate. Note: "unhealthy" individuals are those with cancer, CVD or diabetes; "healthy" are those free of these three diseases. Measurements of physiological indices before the onset of any of these diseases contribute to the "healthy" trajectory and those after the onset of any of the diseases contribute to the "unhealthy" trajectory. Symbols above the curves correspond to *p*-values for testing the null hypotheses on equality of means in respective age groups: no symbol: $p \geq 0.05$; *: $0.01 \leq p < 0.05$; #: $0.001 \leq p < 0.01$; !: $0.0001 \leq p < 0.001$; !!: $p < 0.0001$.

GENETIC INFLUENCE ON AGE TRAJECTORIES OF PHYSIOLOGICAL INDICES

In Yashin et al. (2012c), we showed that human lifespan in the Original FHS cohort was positively associated with the "dose" of 27 individually selected genetic variants ("longevity" alleles) each having a small positive effect on lifespan. It is clear that genetic effects on lifespan and survival are mediated by a number of intermediate variables whose effects are integrated in the values of physiological variables. Since the genetic dose index was associated with lifespan and the values of physiological variables measured in the Original FHS cohort were also associated with lifespan we expected that the genetic dose index would show an association with the age trajectories of the physiological variables affecting lifespan as well. To illustrate this, we divided the genotyped population of study participants into two sub-cohorts. The first one included individuals carrying up to 13 out of 27 alleles associated with lifespan in our earlier study. Individuals from the second sub-cohort carry 14 and more such alleles in their genomes, referred as the (<14)- and the (≥14)-groups, respectively. **Figure 6** shows how the age trajectories of BMI, DBP, SCH, and VR differed between the (<14)- and the (≥14)-groups of study participants for the two genders combined.

One can see from this figure that the average age trajectories of BMI for the (<14)-group were higher up to age 65 years and then became lower than those in the (≥14)-group. Note that the maximum value of BMI for the members of (<14)-group was reached earlier (around 55 years of age) than for the members of the (≥14)-group (around 75 years). The individuals from the (<14)-group had higher levels of the average values of DBP and VR for the entire age interval 35–95 years shown in the graphs. The average values of the SCH for this group were higher than those for the (≥14)-group between ages 50 and 95 years and were about the same beyond this interval.

The average age trajectories of these physiological indices varied slightly between males and females (figures not shown). Specifically, the average values of BMI among female members of the (<14)-group were higher until age 70, stayed about the same until age 85, and intersected those of the group (≥14) after this age. In males, the values of this variable tended to be higher in the (<14)-group until age 60, became lower than those in the (≥14)-group and then became about the same at age 85 years. For DBP the pattern of differences between the two groups remained the same for each of the two genders with larger differences in DBP trajectories between the groups in females than in males. In males, the values of VR in the two groups were about the same until age 50 years and then diverged. The female levels of SCH in the (<14)-group were higher than in the (≥14)-group until age 85 and were about the same after this age. In males, the values of SCH remained higher in this group until age 50, stayed about the same in both groups until age 75, and then they become lower for the members of the (<14)-group. For comparison we will show age patterns of the same

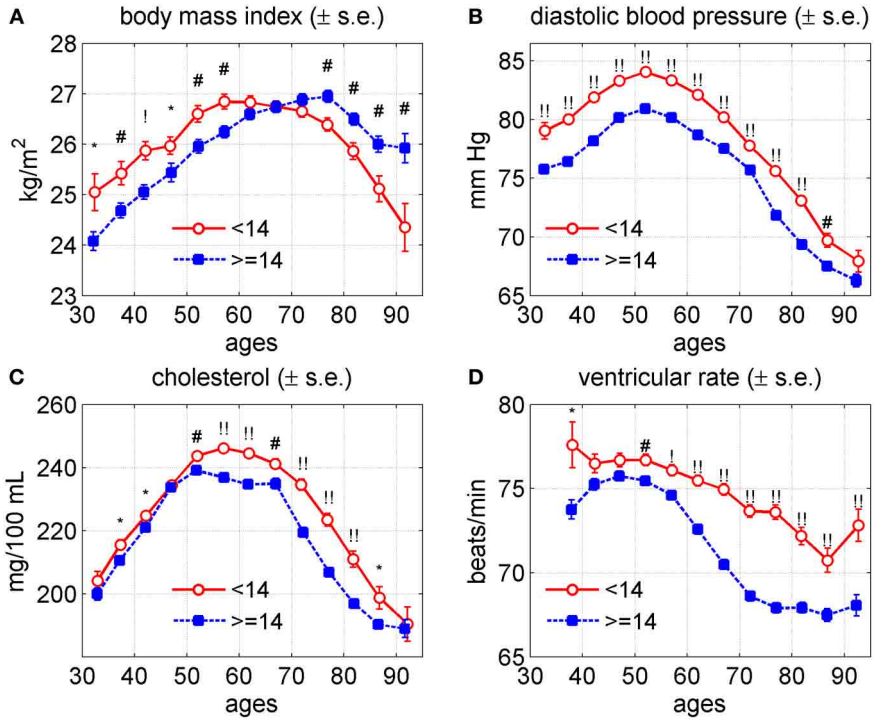

FIGURE 6 | Average trajectories (±standard errors) of four physiological variables in the Framingham Heart Study (original cohort, pooled data from exams 1–28) for individuals carrying different number of pro-survival alleles (<14 and ≥14) out of the 27 such alleles identified in Yashin et al. (2012c). (A) Body mass index; **(B)** diastolic blood pressure; **(C)** cholesterol; **(D)** ventricular rate. Symbols above the curves correspond to *p*-values for testing the null hypotheses on equality of means in respective age groups: no symbol: $p \geq 0.05$; *: $0.01 \leq p < 0.05$; $^{\#}$: $0.001 \leq p < 0.01$; $^{!}$: $0.0001 \leq p < 0.001$; $^{!!}$: $p < 0.0001$.

physiological variables for carriers and non-carriers of the APOE-e4 allele.

THE AGE TRAJECTORIES OF PHYSIOLOGICAL INDICES FOR CARRIERS AND NON-CARRIERS OF THE APOE-e4 ALLELE

The association of the APOE alleles with longevity has been detected in many genetic studies (e.g., Deelen et al., 2011; Nebel et al., 2011). Their influences on age trajectories of physiological indices are less known. In Arbeev et al. (2012) we evaluated effects of the APOE polymorphism on age trajectories of SCH and DBP in the original FHS cohort and found differences in average age trajectories of these indices in long-lived carriers and non-carriers of the e4 allele. The analyses also showed that the average age trajectories in individuals dying at earlier ages markedly deviate from those of the long-lived groups and these patterns differ for carriers and non-carriers of the e4 allele of both sexes. Applying the extended version of the stochastic process model (Arbeev et al., 2009) we found the presence of a genetic component in aging-related mechanisms that is manifested in the observed patterns of the allele-specific age trajectories of physiological indices and mortality rates.

To compare whether the effects of the APOE alleles are similar to those of genetic variants we calculated average age trajectories of the BMI, DBP, SCH, and VR for study participants carrying and not carrying APOE-e4 allele. These trajectories are shown in **Figure 7** for males and females combined.

One can see from this figure that the values of DBP and VR were about the same for carriers and non-carriers of APOE-e4. The most significant difference was in BMI. The curves look about the same until age 65, and then diverge. Among the APOE-e4 carriers, BMI starts to decline. Among non-carriers the values of BMI continued to increase until age 75 and then declined. The rate of decline was higher among carriers of the APOE-e4. The SCH levels were about the same until age 50, after which they became higher in the carriers of the APOE-e4 and remain slightly higher for the rest of the age interval. The figures for males and females are not much different from combined estimates (the graphs are not shown). The figures for females repeat those for the two sexes combined. For males, the values of DBP among APOE-e4 carriers were slightly lower than in non-carriers until age 55, stayed about the same until age 75, and then slightly exceeded those of non-carriers. Comparing **Figure 7** with **Figure 6** indicates that the effect of the APOE-e4 on SCH is similar to that of the (<14)-group at the entire age domain; for BMI the effect is similar after age 65; for DBP the effect is opposite but much less pronounced; and for VR the APOE-e4 effect does not exist.

GENETICS OF AGE TRAJECTORIES FOR HEALTHY AND UNHEALTHY INDIVIDUALS

The graphs of the age trajectories of BMI, DBP, SCH, and VR for unhealthy individuals of the (<14)- and (≥14)-groups are shown in **Figure 8** for the two sexes combined.

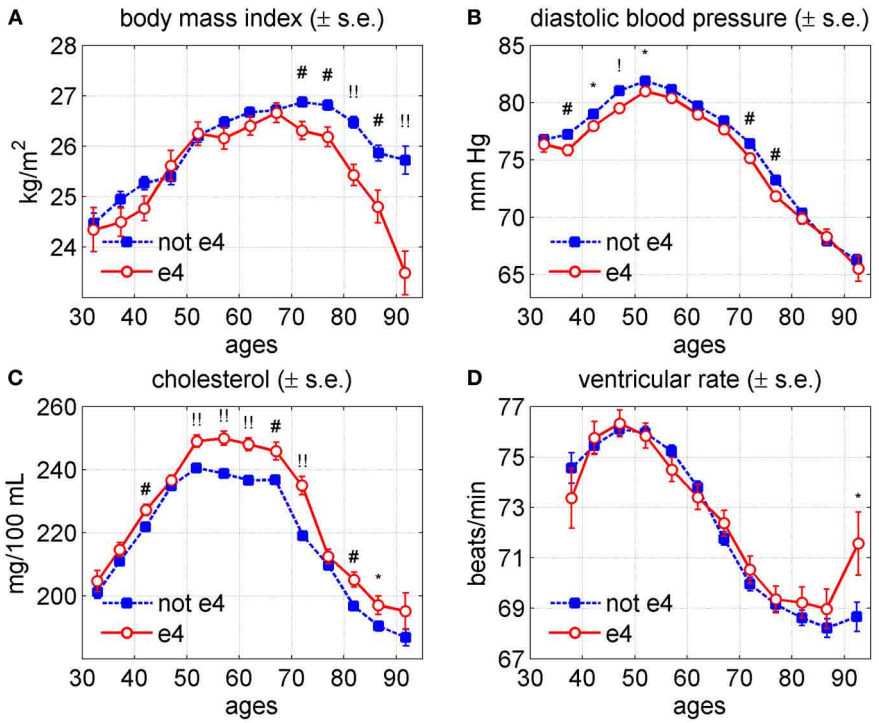

FIGURE 7 | Average trajectories (±standard errors) of four physiological variables for carriers ("e4") and non-carriers ("not e4") of the APOE e4 allele in the Framingham Heart Study (original cohort, pooled data from exams 1–28). (A) Body mass index; **(B)** diastolic blood pressure; **(C)** cholesterol; **(D)** ventricular rate. Symbols above the curves correspond to *p*-values for testing the null hypotheses on equality of means in respective age groups: no symbol: $p \geq 0.05$; *: $0.01 \leq p < 0.05$; $^{\#}$: $0.001 \leq p < 0.01$; $^{!}$: $0.0001 \leq p < 0.001$; $^{!!}$: $p < 0.0001$.

The average values of BMI in the (<14)-group were higher than those in the (≥14)-group until age 65. Then the trajectories intersected so the values of BMI in the (<14)-group became smaller than those in the (≥14)-group for the rest of the age domain. The average values of DBP in both groups were about the same between ages 65 and 75 years. However, beyond this interval the values of this index in the (<14)-group were higher than those in the (≥14)-group. The average levels of SCH were about the same until age 65. Then they become higher in the (<14)-group until age 95 years. The average values of VR were about the same until age 55 and then became higher for individuals from the (<14)-group.

The graphs of the age trajectories of BMI, DBP, SCH, and VR for healthy individuals of the (<14)- and (≥14)-groups for the two sexes combined practically repeat those shown in **Figure 6** for the entire population and therefore are not shown.

THE EFFECTS OF GENETIC DOSE ON MORTALITY BY CAUSE

The results of application of the Cox model to cause-specific mortality data (see details in section "Empirical analyses") are shown in **Table 3**. One can see from this table that the estimates of the effects on all mortality rates are statistically significant.

THE NEED FOR COMPREHENSIVE INTEGRATIVE ANALYSES OF LONGITUDINAL DATA

The values of physiological variables described above are regulated by dynamic biological mechanisms which integrate external influence, internal changes and compensatory activity. An essential part of such mechanism is the negative feedback loop which tends to stabilize the values of regulated variables around certain set points. Many variables involved in regulation of observed physiological indices are not observed, and therefore are not represented in the longitudinal data. To be able to address research questions about fundamental regularities of changes developing in aging human body, the behavior of these additional variables has to be investigated together with the values of physiological variables measured in the study. For these purposes we developed a version of the stochastic process model of human mortality and aging which allows for incorporating established facts, new research findings, and a number of theoretical concepts about aging into the model. Specifically, we used linear stochastic differential equations to describe aging related changes in physiological variables driven by the effect of allostatic adaptation to persistent external disturbances, stochastic components, and subjected to regulation by negative feedback mechanism, which properties may change during the life course. The dynamic effects of physiological variables on mortality risk are described by conditional hazard having quadratic form. This reflects empirical evidence of the U- or J-shapes of risks considered as functions of risk factors (e.g., observed covariates). Variables describing changes in stress resistance and adaptive capacity modulate quadratic hazard and feedback regulation mechanism, respectively. Variables describing physiological norm characterize "optimal" values of conditional risks. Additional variables characterize

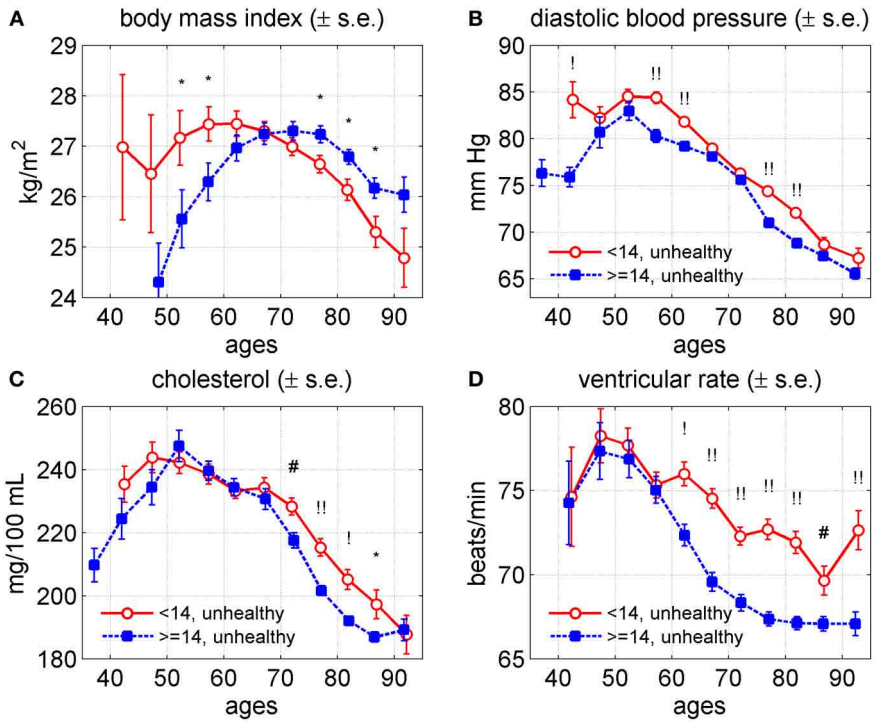

FIGURE 8 | Average trajectories (±standard errors) of four physiological variables in the Framingham Heart Study (original cohort, pooled data from exams 1–28) for "unhealthy" individuals carrying different number of pro-survival alleles (<14 and ≥14) out of the 27 such alleles identified in Yashin et al. (2012c). (A) Body mass index; **(B)** diastolic blood pressure; **(C)** cholesterol; **(D)** ventricular rate. Note: "unhealthy" individuals are those with cancer, CVD, or diabetes; "healthy" are those free of these three diseases. Measurements of physiological indices before the onset of any of these diseases contribute to the "healthy" trajectory and those after the onset of any of the diseases contribute to the "unhealthy" trajectory. Symbols above the curves correspond to *p*-values for testing the null hypotheses on equality of means in respective age groups: no symbol: $p \geq 0.05$; *: $0.01 \leq p < 0.05$; $^{\#}$: $0.001 \leq p < 0.01$; $^{!}$: $0.0001 \leq p < 0.001$; $^{!!}$: $p < 0.0001$.

Table 3 | Effects of polygenic score on total and cause-specific mortality in the Cox model.

Cause	Events #	Cens. #	Beta	SE	*p*-value	HR (95% C.I.)
Total	1267	204	−0.057	0.007	2.7E-18	0.94 (0.93; 0.96)
CVD	368	1103	−0.047	0.012	0.0001	0.95 (0.93; 0.98)
Cancer	243	1228	−0.030	0.015	0.041	0.97 (0.94; 0.99)
Other	656	815	−0.071	0.009	1.9E-15	0.93 (0.92; 0.95)

Notes: "Cause" denotes the cause of death (Total—all causes, CVD, cancer, and Other—all other causes or unknown cause); "Events #" shows the number of deaths from specific cause; "Cens. #" indicates the number of censored cases; "Beta" shows the estimate of the regression coefficient for the polygenic score variable in the Cox model; "SE" is a standard error for Beta; "p-value" characterizes the significance of the estimate; "HR" shows the estimate of hazard ratio per one SNP (pro-survival allele), 95% confidence intervals are shown in parentheses.

levels of stochasticity and effects of allostatic adaptation (Yashin et al., 2007a, 2012a).

The traditional way of describing dynamics of systems with such regulation is the use of ordinary differential equations. To take the random factors that tend to shift the values of corresponding variables from their normal set points into account, the stochastic differential equation with feedback is used (Yashin and Manton, 1997; Yashin et al., 2007a). In the ideal (no stresses) situation the set point of the feedback regulation mechanism for a given variable corresponds to its normal value for a given age, i.e., the value that minimizes mortality risk at this age. In the presence of persistent external disturbances the regulation set point deviates from its normal value by the process of allostatic adaptation. The absolute value of difference between the normal and the realized set points is called allostatic load. The allostatic load may change with age reflecting continuing adjustment of an organism to persistent environmental conditions. Note that neither these conditions nor allostatic load are observed, although a number of studies developed a proxy measure of these characteristics using available measurements (McEwen and Seeman, 1999; McEwen, 2000, 2012). In our analyses the variable representing allostatic load can be introduced into the model and estimated

indirectly from longitudinal data. The quality of feedback regulation is determined by the absolute value of the feedback coefficient. This value characterizes the "adaptive capacity" of a system. Individuals with better capacity are likely to be healthier and have longer life.

Two mechanisms regulating average values of the physiological index in the cohort

It is shown by Yashin et al. (2007a) that average trajectories of any physiological index ($m(t)$) described by Equations (1) and (2) satisfy the following ordinary differential equation:

$$dm(t)/dt = a(t)\big(m(t) - f_1(t)\big) - 2\gamma(t)Q(t)\big(m(t) - f_0(t)\big),\ m(0) = m_0 \quad (3)$$

Here the coefficient $\gamma(t)$ is a positive function of age (Yashin et al., 2007a), and all other coefficients are specified in the descriptions of Equations (1) and (2). The coefficient $a(t)$ is negative by the definition. The coefficient $Q(t)$ is non-negative by definition. Thus equation (3) describes two negative feedback mechanisms regulating age trajectory of $m(t)$. One of them, characterized by the feedback loop coefficient $a(t)$, deals with homeostatic adaptation to the values of slow-time allostatic response $f_1(t)$ to persistent external disturbances (e.g., stresses of life). This mechanism tries to keep the value of $m(t)$ around function $f_1(t)$. The second mechanism is represented by the negative feedback loop describing the effect of mortality selection on $m(t)$ (average age trajectory of physiological index). This mechanism is characterized by the feedback coefficient $-2\gamma(t)Q(t)$. Its task is to keep the value of $m(t)$ around function $f_0(t)$, the optimal age trajectory of physiological index, (i.e., the function of age which minimizes the mortality risk at each given age).

Application to data

The version of this general model has been used in the analyses of data on four physiological indices (BMI, DBP, SCH, and VR) collected in the Original FHS cohort (see section "Advanced statistical analyses using the stochastic process model"). The results of these analyses of longitudinal data are shown in **Figures 9–11**. One can see from these figures that parameters of corresponding models are successfully estimated, and age trajectories of corresponding variables can be well interpreted. Substantially, the analyses revealed significant differences in the parameters of the model associated to the baseline hazard rates (except SCH), the adaptive capacities (except VR) and the average allostatic trajectories between the two sub-groups of individuals carrying <14 and ≥ 14 pro-survival alleles.

Figure 9 shows that the baseline hazard (i.e., the hazard summarizing the effect of all factors except respective physiological variable) is lower in carriers of a larger number of pro-survival alleles (≥ 14), compared to carriers of a smaller number of such alleles (<14) for all variables except SCH.

The lack of significant differences in the baseline hazard rates for SCH indicates that the differences in survival chances in the two groups can be explained by differences in other characteristics such as adaptive capacities and average allostatic trajectories (see **Figures 10C** and **11C**). Note also that for two indices, DBP and VR, the initial value of the baseline hazard is smaller but its slope is larger in carriers of a larger number of alleles (≥ 14) and the curves intersect at some advanced age (around 105 years). This means rectangularization of respective "baseline" survival functions (when going from the (<14)- to the (≥ 14)-group). We note here that the results for BMI should be interpreted with care because the quadratic term for the (<14)-group estimated as zero for this index (data not shown).

Figure 10 displays the estimates of the feedback coefficient representing adaptive capacity in the model.

It reveals that for two physiological variables, DBP and SCH, carriers of a larger number of pro-survival alleles have significantly better adaptive capacity [i.e., larger absolute values of the feedback coefficient in Equation (1)] than individuals with a smaller number of such alleles. That is, in the group carrying a larger number of pro-survival alleles, the trajectories of these physiological variables return faster to the average "allostatic" trajectories that organisms are forced to follow than they do in the individuals carrying a smaller number of pro-survival alleles.

Figure 11 shows these average trajectories that the organisms are forced to follow by the process of allostatic adaptation ("mean allostatic trajectories") in the two groups.

One can see from this figure that the processes of allostatic adaptation in individuals carrying different number of pro-survival alleles act differently in the sense that the resulting trajectories are significantly different in the two groups. The pattern differs by physiological variables. Specifically, the trajectory for the (<14)-group is consistently lower for BMI, consistently higher for VR, and shows similar patterns for the other two variables (DBP and SCH): for the (<14)-group the trajectory is initially higher, reaches the maximum and starts declining at earlier ages, and declines at a faster rate compared to the (≥ 14)-group so that the trajectories in the two groups intersect at ages about 90–95. Finally, we observed that the (≥ 14)-group has a decline in the quadratic hazard term $Q(t)$ with age which means a widening [not narrowing as in the (<14)-group] U-shape of the mortality risk as a function of a physiological variable with age. However, no significant conclusion can be made about the difference in behavior of the quadratic hazard term in these two groups for all physiological variables because of non-significant p-values (about 0.4) and we do not show the results here.

In sum, the evaluated hidden mechanisms of aging-related changes can collectively explain the difference in the mortality risk in the two groups carrying different number of pro-survival alleles. Note that these analyses illustrate the effect of only specific longitudinal variables. As the significant differences between baseline hazard rates in the two groups reveal, there can be many more factors that can explain the difference in the mortality rates in these groups.

DISCUSSION

EXTREME LONGEVITY AND HEALTH ARE LINKED TO DISTINCT TYPES OF CHANGES IN AGE-TRAJECTORIES OF PHYSIOLOGICAL INDICES

Figures 3 and **5** indicate that exceptional longevity and health may be linked to distinct types of changes in the age-trajectories of physiological indices. Indeed, a common point of the differences

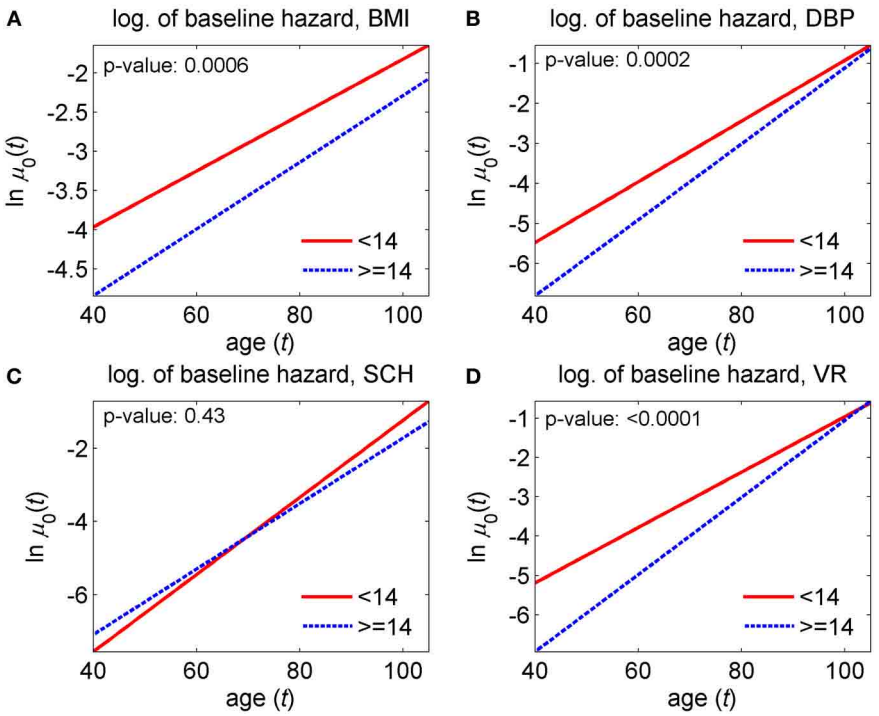

FIGURE 9 | Estimates of the logarithm of the baseline hazard rates in the stochastic process model (Yashin et al., 2007a) applied to data on longitudinal measurements of four physiological indices and total mortality in individuals carrying different number of pro-survival alleles (<14 and ≥14) out of the 27 such alleles identified in Yashin et al. (2012c). (A) Estimates for body mass index (BMI); **(B)** estimates for diastolic blood pressure (DBP); **(C)** estimates for cholesterol (SCH); **(D)** estimates for ventricular rate (VR). *P*-values are for the null hypotheses on the equality of baseline hazards in the two groups. See more details about the model in section "Advanced statistical analyses using the stochastic process model."

between the longest versus shorter living individuals, as seen in **Figure 3**, is that for the LL individuals *the value of an index peaked later in life and started to decline about 10 years later* as compared with the SL individuals. For BMI, in particular, the patterns of its aging changes had similar shape for the LL and SL, as well as had similar peak values of the index. The major difference was that for the LL individuals, the whole age-pattern of the BMI was shifted to the right, and all BMI changes were postponed in time.

This indicates that a postponement of physiological aging changes in time may be essential for achieving extreme longevity. It seems logical, especially if to look at strains of lab animals which significantly differ in longevity and are thought to age at a different pace [such as B6 vs. DBA mice, e.g., Sell and Monnier (1997)]. The longer compared to shorter living strains typically have all the stages of aging changes in physiological indices (such as the rise, peak or decline in BMI) shifted toward an older age (Turturro et al., 1999).

While the typical difference between the long and short living individuals was the shift of the age-trajectory of physiological changes toward older age, the respective trajectories for healthy and unhealthy individuals had similar timing and overall similar age at the peak value of the index, with probable exception for cholesterol (**Figure 5**).

Our results thus suggest that pathways to achieving extreme longevity and health are not necessary the same, or at least do not always overlap and may potentially involve significant trade-offs. For example, consider BMI in **Figures 3** and **5**. Why indeed is the average BMI in healthy old people substantially lower than that BMI in the longest living individuals for similar age intervals 75 + ? One reason could be that at older ages (and especially at the oldest old ages), the level of overall resistance to stresses (such as ability to cope with infections, fractures, bleeding, sarcopenia, atrophy, and generally frailty) starts to play a more important role in person's survival than specific diseases. This is reflected in declining the relative excess of mortality attributed to each specific cause of death with advancing age (Forsen et al., 1999; Horiuchi et al., 2003; Richmond et al., 2003). And as it was discussed at a recent International Conference on Sarcopenia Research, having an excess of fat storage in the body may potentially help to postpone frailty and improve the relative chances of survival in both healthy and unhealthy individuals at older ages (Doehner et al., 2012).

Figure 3 shows that longest lived subjects have significantly higher SCH levels, which seems to contradict with the general knowledge from epidemiological studies that higher SCH levels are associated with a higher mortality. Although the literature analyses show controversial results the evidence is accumulating that the values of total cholesterol have different effects at different age intervals. Specifically, Kronmal et al. (1993) found that the relationship between total cholesterol level and all-cause

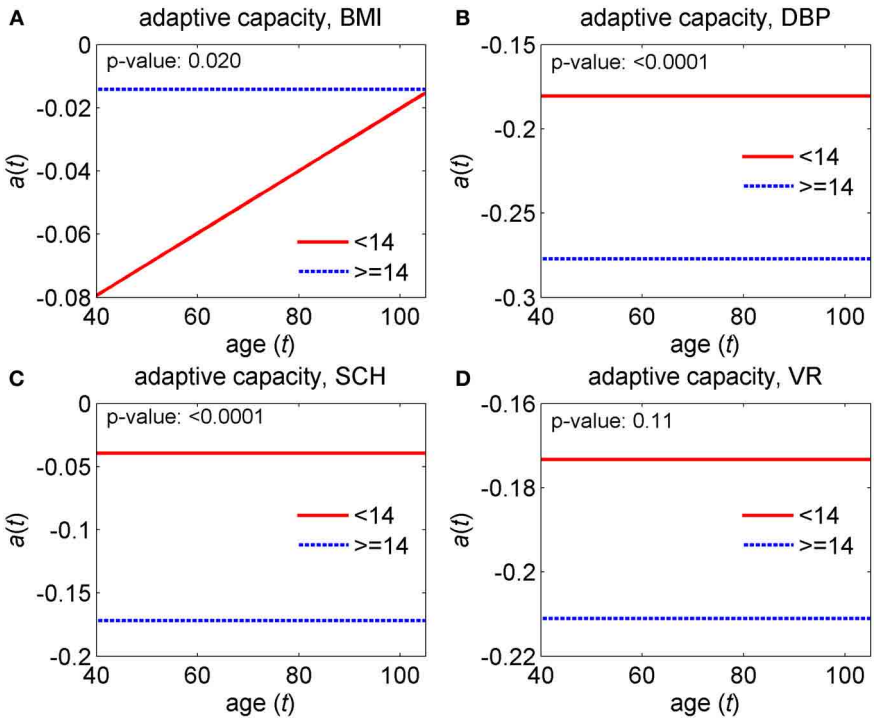

FIGURE 10 | Estimates of adaptive capacity in the stochastic process model (Yashin et al., 2007a) applied to data on longitudinal measurements of four physiological indices and total mortality in individuals carrying different number of pro-survival alleles (<14 and ≥14) out of the 27 such alleles identified in Yashin et al. (2012c).

(A) Estimates for body mass index (BMI); **(B)** estimates for diastolic blood pressure (DBP); **(C)** estimates for cholesterol (SCH); **(D)** estimates for ventricular rate (VR). *P*-values are for the null hypotheses on the equality of adaptive capacities in the two groups. See more details about the model in section "Advanced statistical analyses using the stochastic process model."

mortality was positive at age 40 years, negative at age 80 years, and negligible at ages 50–70 years. Krumholz et al. (1994) concluded that the results of their analyses do not support the hypothesis that hypercholesterolemia or low HDL-C are important risk factors for all-cause mortality, coronary heart disease mortality, or hospitalization for myocardial infarction or unstable angina in this cohort of persons older than 70 years. Weijenberg et al. (1996) reported that total cholesterol seems to be a stronger risk factor for mortality from the disease, whereas HDL cholesterol is more strongly associated with the incidence of a first coronary heart disease event. Weverling-Rijnsburger et al. (1997) concluded that in people older than 85 years, high total cholesterol concentrations are associated with longevity owing to lower mortality from cancer and infection. Chyou and Eaker (2000) reported that an increased ratio of total cholesterol to high-density lipoprotein appears to be associated with an increase in risk for all-cause mortality in men aged 65 and over, while an elevated level of high-density lipoprotein, considered alone, seems to be protective against mortality from all causes in men aged 65–74 years, but this effect diminishes over the age of 75. Karlamangla et al. (2004) reported that increases in cholesterol over time have beneficial associations in some older adults. The authors concluded that the role of cholesterol changes in the health of older individuals needs further exploration. Upmeier et al. (2009) concluded that high levels of serum total cholesterol and particularly low levels of HDL-C seem to be risk factors for cardiovascular mortality even in the elderly population. Newson et al. (2011) found that higher total cholesterol was associated with a lower risk of non-cardiovascular mortality in older adults. This association varied across the late-life span and was stronger in older age groups. The authors concluded that further research is required to examine the mechanisms underlying this association.

Several recent studies in Japan reported that all-cause mortality among individuals with the highest total cholesterol levels was lower than in the other individuals (Ogushi and Kurita, 2008; Noda et al., 2010; Nago et al., 2011; Hamazaki et al., 2012a). Hamazaki et al. (2012b) reported that almost all epidemiological studies in Japan showed that all-cause mortality was lower in subjects with high levels of total cholesterol. Our results in **Figure 3** for age trajectories of SCH levels among long-lived and short lived individuals support Japanese finding. The biological mechanisms responsible for such connection require separate study.

GENES INFLUENCING LIFESPAN MAY BE A MIX OF VARIANTS FAVORING EXTREME LONGEVITY AND HEALTH

A comparative look at **Figures 3**, **5**, and **6** indicates that the 27 SNP alleles associated with lifespan influence the age trajectories of physiological indices in this study in complex ways involving

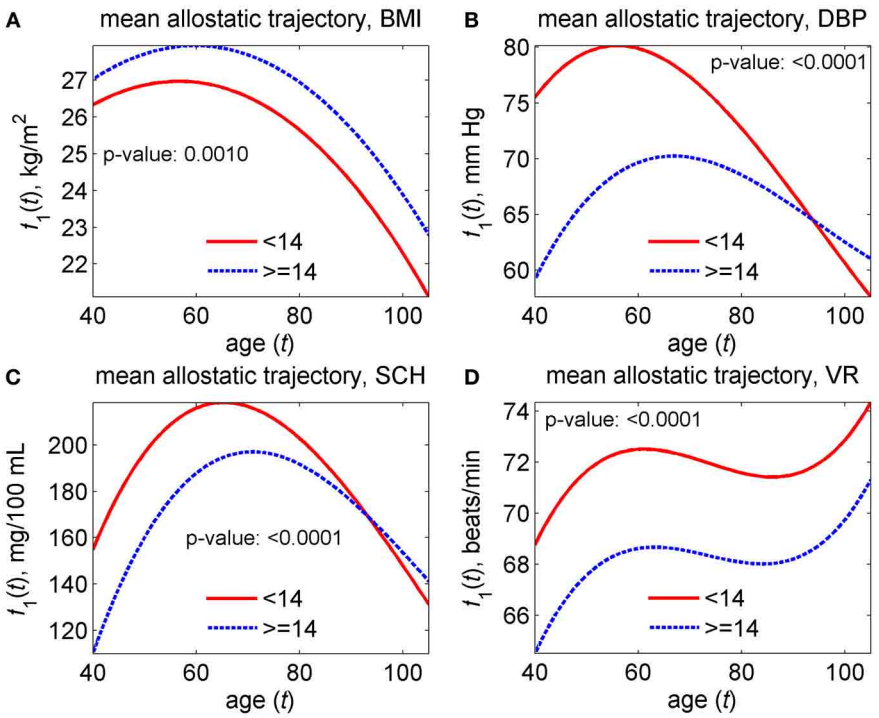

FIGURE 11 | Estimates of mean allostatic trajectories in the stochastic process model (Yashin et al., 2007a) applied to data on longitudinal measurements of four physiological indices and total mortality in individuals carrying different number of pro-survival alleles (<14 and ≥14) out of the 27 such alleles identified in Yashin et al. (2012c). (A) Estimates for body mass index (BMI); **(B)** estimates for diastolic blood pressure (DBP); **(C)** estimates for cholesterol (SCH); **(D)** estimates for ventricular rate (VR). *P*-values are for the null hypotheses on the equality of mean allostatic trajectories in the two groups. See more details about the model in section "Advanced statistical analyses using the stochastic process model."

both health and aging related processes. This is because all types of the effects seen in **Figures 3** and **5** appear in the **Figure 6**, including the parallel shift of the age trajectory of an index to the right (for BMI); the same age at the peak value but different levels of an index at the peak (for DBP and SCH); and different rates of decline in the index value at older ages (for VR). This means that some of the 27 pro-survival alleles may modulate predisposition to particular diseases, some may regulate the rate and onset of physiological aging changes, and some other may have pleiotropic influence on respective traits. These results are in agreement with our earlier analysis of the functional effects of genes linked to the 27 SNPs, where we found that such genes are involved in both physiological aging and common diseases (Yashin et al., 2012c).

Note that we did not match the (<14)- and (≥14)-groups of the study subjects for other confounding factors. This is because we would like to show how age trajectories of physiological indices differ for individuals carrying different numbers of "pro-survival" genetic variants. The age patterns of physiological change for human individuals with different genetic background have never been studied before. These results do not pretend to be interpreted as causal relationships. They, however, help researchers get useful insights and ideas on how such relationship could be evaluated in future studies. The effects of genetic and non-genetic factors on dynamics of each physiological variable deserve separate analyses.

NON-MONOTONIC CHANGES IN PHYSIOLOGICAL INDICES

Our analyses showed that the population average trajectories of BMI, DBP, SCH, and VR, as well as their average biological age trajectories (at least for the LL individuals), are non-monotonic. This property of aging-related changes may reflect the decline in functioning of various tissues and organs involved in the regulation of these variables. This means that the difference in the ages at which a given variable reached its maximum value in different groups of individuals might provide us with useful information about the rates of aging in these groups. The links of this parameter with lifespan and healthy lifespan were established in Yashin et al. (2010a). Several other characteristics of dynamic behavior of age trajectories of biological indices were also associated with lifespan. These include the average values, as well as the slopes and intercepts of physiological indices calculated between ages 40 and 60 years, the value of the maximum, the rate of decline after reaching the maximum value and the individual variability of an index during the life course (Yashin et al., 2006, 2010a).

The non-monotonic age patterns of physiological variables present additional challenges for those who seek biomarkers of aging capable of measuring "biological" age. The indices investigated above are not good for that. This is because of the non-linear changes of their values with age, e.g., they can be about the same at ages 45–50 and 85–90 years. It is important to note that the forces driving the average age trajectories of physiological variables cannot be detected from the data using

standard statistical methods. These mechanisms, however, can be described and their characteristics can be estimated from the data using advanced methods of statistical modeling, see Yashin et al. (2012a) and references therein. Two possible mechanisms contributing to corresponding shapes have been proposed (Yashin et al., 2010b). The first one deals with biological homeostatic regulation of the values of physiological variables in response to aging related changes, and to persistent external challenges. This mechanism is represented by the first term in Equation (3). The second mechanism deals with mortality selection in heterogeneous populations in which individuals with high deviations of physiological variables from the norm have substantially higher mortality risks. The deceased individuals, as well as those who leave the study for other reasons at some age, drop out of the averaging procedure after this age. This part of the mechanism is represented by the second term in the Equation (3).

To evaluate the shape of the biological age trajectories without compositional changes and to compare whether the age trajectories of individuals having long lifespan differ from those who died prematurely, we divided members of the Original FHS cohort into the two sub-cohorts of the SL and LL individuals. The LL individuals included all those whose lifespan exceed 97 years. The results of our analyses indicated that biological age trajectories of the four physiological variables studied in this paper were non-monotonic. Typically the SL persons had initially higher average values of these indices. The average age trajectories for the SL persons reached their maximum values, and started to decline earlier than those of the LL persons. The average age trajectories of the LL persons describe their average biological changes until about age 97, and these changes were non-monotonic. The fact that these trajectories differed substantially for the SL and LL groups of individuals indicates that they contain useful information about remaining lifespan distributions which could be used for predicting this trait.

INTERSECTION OF THE AGE TRAJECTORIES FOR HEALTHY AND UNHEALTHY PERSONS

The fact that average age trajectories of DBP, SCH, and VR for healthy and unhealthy individuals intersect may indicate fundamental aging related changes occurring on the way from the old to the oldest old ages. Such a transition is likely to rearrange factors of susceptibility to diseases and change the role of risk factors from deleterious to neutral or even favorable. These intersections, however, do not explain what kind of forces might be responsible for such behaviors of these curves. One possibility is that the genetic factors could contribute to observed patterns.

The genetic analyses of the age trajectories of the four indices indicate that the genetic influence of pro-survival alleles may differ from one index to the next. For example, the effect of the (<14)- and (≥ 14)-groups on BMI differs from those on DBP and VR, and the effects of these genetic groups on SCH are similar to these two but the effects on SCH become more pronounced only after age 50. This indicates that studying the roles of genes in aging related changes requires more information about biological mechanisms involved in regulation of these characteristics. Analyses of genetic influence on age patterns of average physiological indices for healthy and unhealthy individuals resulted in similar conclusions. They also showed that the selected pro-survival alleles were likely to contribute to mortality from CVD and cancer.

THE USE OF STOCHASTIC PROCESS MODEL IN ANALYSES OF LONGITUDINAL DATA

The statistical analyses of longitudinal data using stochastic process model allowed us to evaluate hidden dimensions of aging related changes which are important for better understanding regulatory mechanisms driving observed aging related changes in physiological variables. The hidden components of aging related changes incorporated into our model include adaptive capacity, resistance to stresses, physiological norm, and the effect of allostatic adaptation [see notations for variables used in Equations (1) and (2)]. All these variables play important role in the aging process but were not directly measured in longitudinal data. The adaptive capacity characterizes the ability of organisms to keep the values of physiological variable around set-point of homeostatic regulation. The higher absolute values of this variable correspond to better organism's capacity to adapt and provide it with better fitness. Physiological norm describes optimal value of physiological variable which minimizes mortality risk. The resistance to stresses characterizes sensitivity of mortality risk to the deviation of physiological variable from the norm. The effect of allostatic adaptation characterizes set-point in mechanism of homeostatic regulation of physiological index. This component of physiological change integrates influence of persistent external disturbances. Note that since external disturbances are not measured in most of longitudinal studies, the estimates of the difference between this variable and physiological norm (allostatic load) may serve as an important indicator of healthy or unhealthy environment individuals under study are exposed to. Our analyses show that these characteristics can be estimated from the data using stochastic process model of human mortality and aging. The use of such model allows for testing statistical hypotheses not only about genetic influence on age trajectories of physiological indices but also about the roles of genes in mechanisms involved in regulation of these trajectories.

CONCLUSIONS

The age trajectories of physiological indices corresponding to individuals with exceptional longevity differ from those of people living without three major human diseases—cancer, CVD, and diabetes. These results may indicate that factors responsible for the long life and good health are not necessary the same. The trajectories for long-living individuals look as if their age related changes were postponed compared to persons died prematurely.

The analyses confirmed that genetic influences on lifespan are realized through dynamic mechanisms regulating changes in physiological variables during the life course as well as through variables describing individual health status. The genetic dose index constructed from genetic variants selected for their individual associations with lifespan showed association with mortality rates by cause. This indicates that these genetic factors influence both health and lifespan.

The average aging related changes in the four selected physiological variables are likely to be driven by hidden components of aging changes and by genetic factors.

The ability of advanced methods of statistical modeling to estimate hidden components of aging changes in humans indicates that the approach can be further extended to perform more comprehensive analyses of available data by incorporating relevant biological knowledge about aging into statistical models. The use of such models in statistical analyses of data will help researchers to untangle complex age-dependent dynamic relationships among biomarkers and elucidate roles of genes and non-genetic factors in aging, health, and lifespan.

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Extreme depletion of PIP_3 accompanies the increased life span and stress tolerance of PI3K-null *C. elegans* mutants

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The regulation of animal longevity shows remarkable plasticity, in that a variety of genetic lesions are able to extend lifespan by as much as 10-fold. Such studies have implicated several key signaling pathways that must normally limit longevity, since their disruption prolongs life. Little is known, however, about the proximal effectors of aging on which these pathways are presumed to converge, and to date, no pharmacologic agents even approach the life-extending effects of genetic mutation. In the present study, we have sought to define the downstream consequences of *age-1* nonsense mutations, which confer 10-fold life extension to the nematode *Caenorhabditis elegans* – the largest effect documented for any single mutation. Such mutations insert a premature stop codon upstream of the catalytic domain of the AGE-1/p110α subunit of class-I PI3K. As expected, we do not detect class-I PI3K (and based on our sensitivity, it constitutes <14% of wild-type levels), nor do we find any PI3K activity as judged by immunodetection of phosphorylated AKT, which strongly requires PIP_3 for activation by upstream kinases, or immunodetection of its product, PIP_3. In the latter case, the upper 95%-confidence limit for PIP_3 is 1.4% of the wild-type level. We tested a variety of commercially available PI3K inhibitors, as well as three phosphatidylinositol analogs (PIAs) that are most active in inhibiting AKT activation, for effects on longevity and survival of oxidative stress. Of these, GDC-0941, PIA6, and PIA24 (each at 1 or 10 μM) extended lifespan by 7–14%, while PIAs 6, 12, and 24 (at 1 or 10 μM) increased survival time in 5 mM peroxide by 12–52%. These effects may have been conferred by insulinlike signaling, since a reporter regulated by the DAF-16/FOXO transcription factor, SOD-3::GFP, was stimulated by these PIAs in the same rank order (PIA24 > PIA6 > PIA12) as lifespan. A second reporter, PEPCK::GFP, was equally activated (∼40%) by all three.

Keywords: insulin signaling, IGF-1, longevity, oxidative stress, PI3K, phosphatidylinositides, *C. elegans*/nematode

INTRODUCTION

Phosphatidylinositides are tightly regulated signaling molecules that participate in a diverse range of cellular events, including cell replication and survival, membrane trafficking, secretion, adhesion, and cell migration (Boss and Im, 2012; Echard, 2012; Mayinger, 2012). Phosphatidylinositol (PI; sometimes abbreviated as "PtdIns") lipid chains are generally integrated into inner cell membranes, while the attached phosphoinositide rings project into the cytoplasm. PI's are formed by additions of phosphate to hydroxyl groups at the 1, 3, 4, and/or 5 position of the inositol ring. Additions at the 3 position are governed by phosphatidylinositol 3-kinases (PI3K's), enzymes with key regulatory roles in cell division and metabolism (Wymann and Schultz, 2012). Class-I PI3K's convert $PI(4,5)P_2$ (often abbreviated as PIP_2) to $PI(3,4,5)P_3$ (or PIP_3), which plays decisive roles in multiple signaling pathways.

Intracellular concentrations of L-α phosphatidylinositol 4,5-bisphosphate (known as $PI(4,5)P_2$, one of several PIP_2 isoforms) lie in the range of 2–30 μM (Gambhir et al., 2004). Class-I phosphatidylinositol 3-kinase (PI3K) can add phosphate to $PI(4,5)P_2$ at the inositol 3 carbon to form phosphatidylinositol 3,4,5-triphosphate[$PI(3,4,5)P_3$ or PIP_3]. This key signaling molecule or "second messenger" is normally present at only ∼0.1% of the levels of its precursor (Weinkove et al., 2006), or <30 nM. In response to stimuli, however, the concentration of PIP_3 can increase up to 100-fold (Pettitt et al., 2006), achieved through activation of PI3K and/or inactivation of the opposing PI 3-phosphatase, PTEN. Many membrane-associated proteins, including a number of kinases involved in signal transduction cascades, have a domain that binds either PIP_3 or a specific PIP_2. The best-studied of these are Pleckstrin Homology (PH) domains, typically ∼120 amino-acid residues long, many of which show quite specific affinity for PIP_3. In the insulin signaling pathway, formation of PIP_3 is required for the downstream activation of AKT/PKB, a protein kinase that promotes cell proliferation and blocks apoptosis in many cell types (Franke et al., 1997). In order to be activated, AKT must bind PIP_3 at its PH domain. This tethers AKT to the inner cell membrane, in relative proximity to its upstream kinase(s) and downstream targets, while also inducing a structural change in AKT to expose a key phosphorylation site to activating kinases such as PDK-1 (Stokoe et al., 1997). Mammalian AKTs are fully active when phosphorylated at residues Thr^{308} and Ser^{473} (Stokoe et al.,

1997); other AKT-activating kinases include DNA-dependent protein kinase (DNA-PK) (Dragoi et al., 2005; Sester et al., 2006) and TOR complex (Hawkins et al., 2006). Following dimerization, AKT1/AKT2 complex phosphorylates dozens of targets, including kinases and transcription factors, leading to their activation or inactivation (Cutillas et al., 2006). FOXO transcription factors (DAF-16 isoforms in *Caenorhabditis elegans*) are among the inactivated targets, as their phosphorylation by the AKT complex prevents their entry into the nucleus (Tissenbaum and Ruvkun, 1998; Berdichevsky et al., 2006). AKT mutations conferring constitutive activation are observed in many cancers (Shtilbans et al., 2008); mutations in the *pten* gene, disrupting the PI 3-phosphatase that opposes PI3K, also produce a high PIP_3/PIP_2 ratio, favoring activated AKT and hence cell proliferation in diverse cancers (Yi et al., 2005). Although direct constitutive activation of PI3K is far less common, the BCR-ABL fusion protein indirectly activates PI3K, thus elevating PIP_3 in chronic myelogenous leukemia (Kharas et al., 2008).

The above findings demonstrate the critical involvement of PIP_3/AKT/FOXO signaling in cell proliferation, and have led to great interest in disruption of such signaling in cancers (Castillo et al., 2004). However, this kinase cascade has also been implicated in other roles beyond cell proliferation. Enhanced PIP_3 signaling in specific hypothalamic neurons is associated with diet-sensitive obesity (Plum et al., 2006). Lithium, commonly used as a mood stabilizer for bipolar disorder, suppresses PIP_3 signaling in *Dictyostelium* and in cultured human cells (King et al., 2009). To date, attention has been largely focused on drugs that target PI3K or AKT. The two PI3K inhibitors in most common use are LY294002 and wortmannin (Vlahos et al., 1994; Schultz et al., 1995; Semba et al., 2002). These drugs bind to the ATP-binding site of PI3K, LY294002 reversibly (IC_{50} 0.5–10 μM) and wortmannin much more avidly (IC_{50} 7 nM)(Wu et al., 2008). Both are known to inhibit other kinases (e.g., PLK1) with similar IC_{50} values; in view of the thousands of proteins with ATP-binding sites, such off-target effects are not surprising. A number of PI3K inhibitors have been developed through small-molecule screens or by synthetic chemistry testing derivatives of partially effective molecules. Among these, ZSTK474 inhibits p110γ somewhat more than α or β (IC_{50}'s of 6, 17, and 53 nM respectively); whereas A66 is rather specific for p110α (IC_{50} of 32 nM), requiring >3 μM to reach the IC_{50} against β or γ. The most avid p110α inhibitor is GDC-0941 with an IC_{50} of 3 nM, but its activity against other PI3K isoforms has not been reported. In another approach, phosphatidylinositol analogs (PIAs) were designed to dock in the PIP_3-binding (PH) domain of AKT and thereby inhibit its activity (Kozikowski et al., 2003). It is not known whether any of these compounds have affinity for the PIP_2-binding catalytic site of PI3K.

The *C. elegans age-1* gene encodes the nematode homolog of p110α, the mammalian class-I or -Iα catalytic subunit of PI3K (Morris et al., 1996) responsible for conversion of PIP_2 to PIP_3. Nonsense mutants of *age-1*, at the second homozygous generation, should lack any active PI3K. These worms are extremely long-lived and resistant to multiple stresses; they develop very slowly and are completely infertile, reflecting severely impaired cell division (Ayyadevara et al., 2008). Those phenotypes were largely blunted in the first homozygous generation, presumably due to carry-over of oocyte PI3K, *age-1* mRNA, or PIP_3 from their *age-1*-heterozygous parent.

Because PIP_3 can allosterically alter the conformation of a PH-domain protein, permitting its activation by one or more kinases, PIP_3 may serve as a catalyst for that activation and be required only in minute amounts. In contrast, it is required continuously (and hence stoichiometrically) for its membrane-tethering role. We therefore predict that, over most of its physiological range of concentration, PIP_3 will have an essentially linear dose-response curve with respect to activity of any individual PIP_3-binding protein (although it would become non-linear for any process that depends on several such proteins). However, for binding proteins that also depend on PIP_3 "catalytically," such as AKT, a far more dramatic effect might be expected on removal of the last few PIP_3 molecules per cell – perhaps accounting for the marked phenotypic differences between first- and second-generation *age-1*-null homozygotes (Ayyadevara et al., 2008).

We have now examined PIP_3 levels by quantitative immunofluorescence as well as functional assays, in first and second-generation *age-1*(*mg44*) homozygotes, comparing them to worms that bear wild-type or weaker *age-1* mutant alleles. We also have asked whether PI3K inhibitors can partially "phenocopy" *age-1* mutation in wild-type animals, to enhance longevity and stress tolerance.

RESULTS

PI3K IS WIDELY DISTRIBUTED IN WILD-TYPE *C. ELEGANS*, BUT IS NOT DETECTED OVER BACKGROUND IN *age*-1-NULL MUTANT WORMS

The *age-1*(*mg44*) allele encodes a truncated PI3K p110α protein, due to replacement of the Trp codon at position 387 (of 1146) by an amber stop codon (Ayyadevara et al., 2008). Antibody raised to the helical and kinase domains of AGE-1, lying downstream of (C-terminal to) the mutation, registered full-length enzyme as a diffuse cytoplasmic signal in virtually all cell types of wild-type *C. elegans* adults (**Figures 1A,C**). The same antibody, however, failed to detect AGE-1 protein in second-generation *age-1*(*mg44*) homozygotes, over the background seen in wild-type worms exposed only to the fluorescent secondary antibody (**Figures 1B,C**).

PIP_3 IS SIGNIFICANTLY REDUCED IN FIRST-GENERATION *age*-1(*mg*44) HOMOZYGOTES, AND IS BELOW DETECTABLE LIMITS IN THEIR SECOND-GENERATION PROGENY

Despite the absence of full-length, catalytically active AGE-1 protein, it is possible that PIP_3 might be generated by a PI3K p110 of a different class, or *via* an alternative biosynthetic pathway. We therefore assessed PIP_3 levels, initially and most sensitively by *in situ* immunofluorescence but with confirmation by activity-based assays. Using a highly specific antibody against PIP_3 (Chen et al., 2002; Kharas et al., 2008), we found that *age-1*(*mg44*) second-generation homozygotes have no PIP_3-specific immunofluorescence above background, i.e., their level is indistinguishable from negative control samples from which the primary antibody was omitted (**Figure 2**). In several replicate experiments comparing groups of day-8 adults (of which **Figure 2** is typical), we observed 15–25% reductions in PIP_3 signal for worms carrying the weaker *hx546* allele of *age-1* (each $P < 0.001$ compared to wild-type), 60–75% reductions for first-generation *mg44* homozygotes

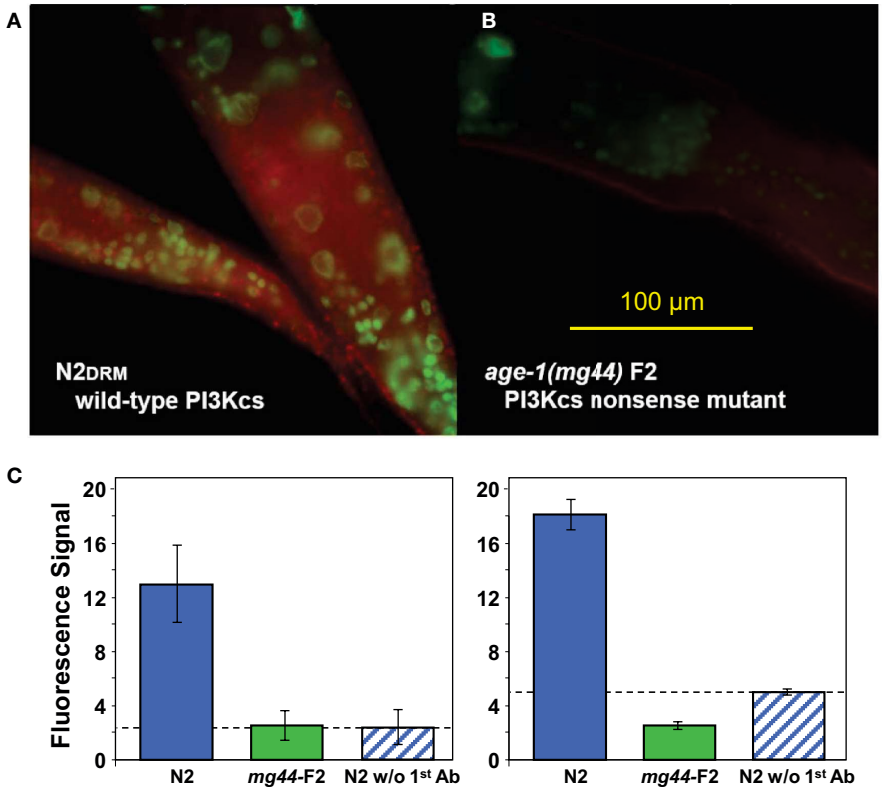

FIGURE 1 | Immunodetection of AGE-1 protein (class-I PI3K catalytic subunit) in adult *C. elegans* of wild-type strain N2DRM (A) or second-generation *age-1*(*mg44*) homozygotes at adult age 3 days (B). Synchronized worms, fixed 14 h in 1% formaldehyde at 4°C, were permeabilized by successive exposures to 1% β-mercaptoethanol, 10-mM DTT, and 0.3% hydrogen peroxide. Primary antibody was goat anti-AGE-1 at1:50 (Santa Cruz Biotech.), followed by rabbit anti-goat ALEXA680-tagged IgG at 1:200 (Invitrogen), imaged on an Olympus BX51 fluorescence microscope at 10×. The histograms **(C)** show mean fluorescence intensity, ±SEM, in two independent experiments. Each includes a negative control, staining of wild-type worms without primary antibody (rightmost bar).

(each $P < 10^{-12}$), and essentially no signal above background in second-generation *mg44*/*mg44* adults ($P < 10^{-12}$ relative to any group except negative controls). Because the upper bound of the 95% confidence interval for these "F2" *mg44*$^{-/-}$ worms is ∼1.4% of the wild-type level, we infer that their PIP$_3$ level is reduced at least 70-fold relative to wild-type adults. PIP$_3$ levels were also assessed at several ages. Wild-type N2 worms showed maximal signal at 3 days of adult age (coinciding with peak fecundity) and fell to 35–40% of maximum at 6–10 days, whereas second-generation *mg44* homozygotes never differed significantly from background at adult ages 3, 5, or 10 days (data not shown).

Activity-based assays were used to confirm immunofluorescence quantitation of class-Iα PI3K, and of PIP$_3$. PI3K activity is difficult to quantify in unstimulated cells, in which it is below the limits of detection, but it can be measured after induction by oxidative stress (Weinkove et al., 2006). We induced oxidative stress by exposing adult worms to 4 mM H_2O_2 for 40 min at 20°C, in the presence of $^{32}PO_4^{=}$. Worms were then lysed and their PIP's isolated and resolved by thin-layer chromatography. PI3K activity was calculated as the ratio of ^{32}P incorporation coinciding with the position of a PIP$_3$ standard, to ^{32}P signal migrating with PIP$_2$. As expected, no PIP$_3$ signal was detected in the absence of peroxide stress. After H_2O_2 exposure, it reached a measurable level (a ratio of 0.07) only for wild-type worms, but remained near-zero for worms carrying either *age-1* allele (**Figure 3**).

Similarly, we were able to confirm PIP$_3$ depletion in *age-1*(*mg44*) second-generation homozygotes, using a functional assay based on the requirement for PIP$_3$-binding to bioactivate AKT *via* phosphorylation at Thr^{308} (Stokoe et al., 1997). Antibodies recognizing unphosphorylated human AKT, or specific for AKT phosphorylated at Thr^{308}, were used to evaluate the products of incubating bacterially synthesized human AKT with *C. elegans* lysates (**Figure 4**). The results support our immunofluorescence data, indicating the virtual absence of any PIP$_3$ in the very long-lived *mg44*-mutant worms, relative to wild-type. Dependence of the assay on endogenous PIP$_3$ was demonstrated by adding synthetic PIP$_3$ (**Figure 4**, rightmost bar).

NEMATODE LIFESPAN AND PEROXIDE RESISTANCE ARE MODESTLY ENHANCED BY SEVERAL PI3K INHIBITORS, IN PARTICULAR PHOSPHATIDYLINOSITOL ANALOGS

We tested a variety of PI3K inhibitors for the ability to extend nematode lifespan, in effect seeking a partial "pharmacopy" of the *age-1* phenotype. Neither wortmannin, LY294002, A66, nor

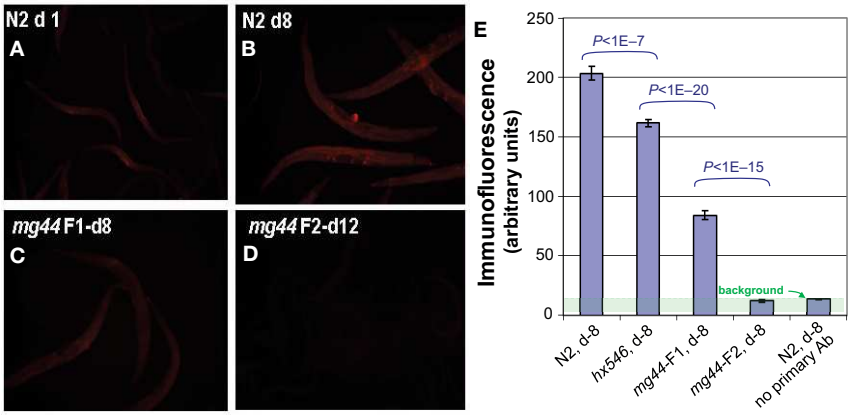

FIGURE 2 | Immunofluorescence quantitation of PIP$_3$ in *C. elegans*. Worms were permeabilized as for **Figure 1** and incubated with mouse antibody to PIP$_3$ (Echelon), followed by a 1:200 dilution of secondary antibody, ALEXA594-labeled goat anti-mouse IgG (Invitrogen). Images **(A–D)**, acquired on an Olympus BX51 microscope, were quantified with ImageJ and summarized in **(E)** for a typical experiment. Histogram bars show means ± SEMs for 20–50 worms per group. Background, assessed without primary antibody, is shown (rightmost bar) but has not been subtracted. Worm ages are given in days (d) as adult.

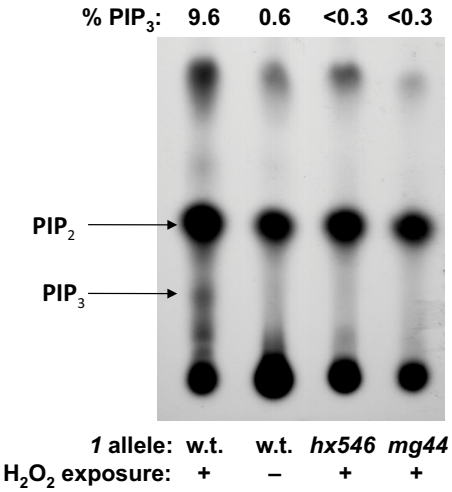

FIGURE 3 | *In vivo* ^{32}P-labeling of PIP$_2$ and PIP$_3$. Young adult worms were washed and incubated 16 h in phosphate-free medium to which 0.6 mCi $^{32}PO_4$ were added. Where indicated, worms were exposed 45 min to 16-mM H_2O_2; lipids were extracted, chromatographed, and autoradiographed. Quantitative results (PIP$_3$ as a percent of the sum of PIP$_2$ and PIP$_3$), determined by scintillation counting of excised spots, are given above the TLC image.

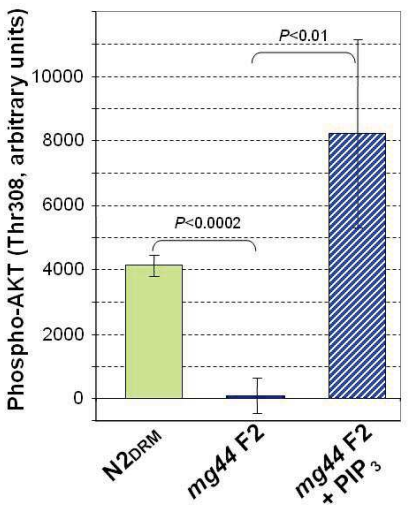

FIGURE 4 | Functional assay of PIP$_3$ based on phosphorylation of AKT(Thr^{308}). Bacterially synthesized AKT, with an N-terminal His$_6$ tag, was added to cleared *C. elegans* lysates, and after 1 h at 20°C, was bound to AKT monoclonal antibody on AlphaBeads (PerkinElmer) in an AlphaScreen PI3K Assay (Echelon), and scored for also binding a tagged antibody to AKT(P-Thr^{308}). Results were read on an Envision 2104 Microplate Reader (PerkinElmer). Error bars are standard deviations ($N = 3$).

ZSTK474 (each tested at 1–10 μM) increased the lifespan of wild-type worms (**Table 1**), However, GDC-0941 – reportedly the most avid p110α inhibitor – at 1 μM extended lifespan by 10% (nominally significant, Gehans–Wilcoxon $P < 0.03$). We then tested four phosphatidylinositol analogs (PIAs) previously shown to be potent inhibitors of signal transduction *via* AKT activation (Gills et al., 2006, 2007; Memmott et al., 2008). PIA6 and PIA24 increased adult life span in two of three repeats, relative to PIA7 (inactive control) or DMSO vehicle-treatment. In the experiment shown (**Figure 5**), mean longevity was extended 10% ($P < 0.05$) by PIA6, and nearly 14% by PIA24 ($P < 0.015$, Gehans–Wilcoxon log-rank test; **Table 2**).

We then compared PIAs 6, 12, and 24 to LY294002 or an inactive PIA, for their ability to extend survival of *C. elegans* exposed to a lethal oxidative stress, 5 mM hydrogen peroxide. PIAs 6 and 24 again conferred the greatest protection to wild-type worms, extending survival by 18 and 19%, respectively (each $P < 10^{-6}$), while PIA12 initially increased peroxide survival 12% ($P < 0.003$) and LY294002 by <1% (NS) relative to controls (**Figure 6A**;

Table 1 | Effects of PI3K inhibitors on wild-type lifespan at 25°C.

	DMSO	LY294002	Wortmannin	A66	GDC-0941	ZSTK474
		1 μM	1 μM	1 μM	1 μM	1 μM
Mean survival (days)	10.72	10.51	10.28	9.78	11.64	10.96
SD	2.31	2.66	2.91	2.26	2.32	2.24
SEM	0.31	0.55	0.69	0.49	0.42	0.41
N	54	23	17	22	30	29
% Of DMSO control		98.0	95.9	91.2	109.6	102.2
P, Gehans–Wilcoxon	–	–	–	–	<0.03	–
		10 μM	10 μM	10 μM	10 μM	10 μM
Mean survival (days)		9.80	10.83	9.97	10.97	9.60
SD		2.62	2.34	2.20	2.03	1.97
SEM		0.57	0.52	0.49	0.45	0.40
N		21	20	21	20	24
% Of DMSO control		91.4	101.0	93.0	102.3	89.6

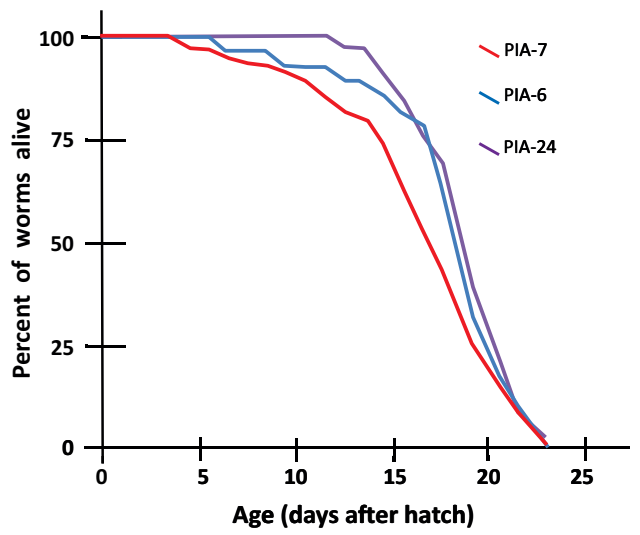

FIGURE 5 | Longevity survivals of wild-type (N2DRM) *C. elegans* maintained at 20°C on plates with phosphatidylinositol analogs (PIAs). Worms from synchronous cultures were selected as L4 larvae, and placed on 10-cm agar plates containing 1 μM of either PIA6, PIA24, or an inactive control, PIA7. They were transferred daily to identical plates on days 1–7 of adulthood to isolate them from their progeny, and transferred every 2–3 days thereafter. At each transfer, worms were scored as dead if they failed to move either spontaneously or in response to gentle prodding. Worms dying from desiccation due to stranding on the side walls of dishes, or from internal hatching of progeny, were censored at the midpoint of transfers between which death was discovered.

Table 2 | Effects of PIAs on wild-type (N2) lifespan at 20°C.

	DMSO	PIA7 1 μM	PIA6 1 μM	PIA24 1 μM
EXPERIMENT 1				
Mean survival (days)	18.8	19.2	20.3	20.5
SD	2.8	2.7	1.8	2.3
SEM	0.63	0.56	0.36	0.47
N	19	24	26	24
% (DMSO + PIA7)/2	–	–	106.8	108.0
P, Gehans–Wilcoxon	–	–	<0.10	<0.03
EXPERIMENT 2				
Mean survival (days)	17.3	16.6	18.2	18.8
SD	3.2	4.6	3.8	2.5
SEM	0.40	0.63	0.72	0.45
N	64	52	28	32
% (DMSO + PIA7)/2	–	–	109.9	113.5
P, Gehans–Wilcoxon	–	–	<0.06	<0.015
Combined Signif.:			<0.006	<0.0004

Tables 3A,B). In these experiments, PIAs generally conferred less (and less significant) protection to *age-1*(*hx546*) worms, although PIA12 appeared equally effective for either strain (**Figure 6B**; **Tables 3A,B**). When newly synthesized batches of PIAs 6 and 12 were subsequently tested (**Tables 3C,D**), PIA12 was more protective than PIA6, indicating that differences in these analogs may in large measure reflect purity and freshness of the compounds. LY294002 was only moderately effective at doses ranging from 1 to 20 μM, whereas the tested PIAs remained equally protective from 1 to 1000 μM (**Table 3** and additional data not shown).

PIP$_3$ ANALOGS INDUCE GENES THAT ARE POSITIVELY REGULATED BY INSULIN/IGF-1 SIGNALING

DAF-16 target genes such as those encoding SOD-3 and PEPCK are strikingly upregulated in *age-1*(*mg44*) worms, and somewhat less so in *age-1*(*hx546*) mutants – increases that are blocked when *daf-16* is deleted (Shmookler Reis et al., 2009; Tazearslan et al., 2009). The DAF-16 transcription factor was shown to mediate strong modulation of expression (by factors of 2 to >1000) by the *age-1* allele for a total of 64 genes (Ayyadevara et al., 2009; Tazearslan et al., 2009). SOD-3 is an Fe^{++}/Mn^{++} superoxide dismutase, believed to be mitochondrial and to protect against superoxide produced *via* the electron-transport chain; its mRNA is upregulated >9-fold in *age-1*(*mg44*)-F2 worms (Tazearslan et al., 2009). If PIA treatment, like *age-1* mutations, disrupts insulinlike signaling through DAF-16 activation, it should enhance transcription of *sod-3*. We treated young adult worms expressing a SOD-3::GFP fusion protein with several PIAs, each at 1 μM concentration. PIA24

Table 3 | Protection against 5-mM peroxide by PI3K inhibitors at 20°C.

A. EXPERIMENT 1						
	DMSO	**PIA7 1 μM**	**LY294002 1 μM**	**PIA6 1 μM**	**PIA12 1 μM**	**PIA24 1 μM**
N2, wild-type						
Mean survival (h)	6.8	6.6	6.7	7.9	7.5	8.0
SD	0.7	0.7	0.6	0.7	1.0	2.2
SEM	0.15	0.16	0.13	0.16	0.23	0.41
N	20	20	20	20	20	22
% of PIA7 control	–	–	101	118	112	119
P, Gehans–Wilcoxon	–	–	N.S.	<1E–6	0.002	<1E–6
Age-1(hx546)						
Mean survival (h)		9.8	10.8	10.0	11.0	9.6
SD		2.6	2.3	2.2	2.0	2.0
SEM		0.57	0.52	0.49	0.45	0.40
N		21	20	21	20	24
% of PIA7 control		–	96.5	116	112	112
P, Gehans–Wilcoxon		–	N.S.	0.0008	0.005	0.009

B. EXPERIMENT 2				
	PIA7 10 μM	**LY294002 10 μM**	**PIA6 10 μM**	**PIA6 (10 μM) + LY294002 (10 μM)**
N2, wild-type				
Mean survival (h)	6.0	7.1	7.1	7.5
SD	1.0	1.5	1.1	1.4
SEM	0.15	0.24	0.17	0.23
N	40	40	40	40
% of PIA7 control	–	118	118	125
P, Gehans–Wilcoxon	–	<1E–3	<1E–5	<1E–6
Age-1(hx546)				
Mean survival (h)	8.3	8.6	9.0	9.0
SD	1.5	1.2	1.0	1.1
SEM	0.23	0.19	0.16	0.17
N	40	40	40	40
% of PIA7 control	–	103	107	107
P, Gehans–Wilcoxon	–	NS	<0.04	<0.05

C. EXPERIMENT 3					
	PIA7 1 μM	**PIA6 1 μM**	**PIA12 1 μM**	**PIA6 + LY 1 μM, 10 μM**	**PIA12 + LY 1 μM, 10 μM**
N2, wild-type					
Mean survival (h)	6.9	8.6	10.0	8.6	8.5
SD	1.6	1.6	1.1	1.7	1.7
SEM	0.25	0.25	0.17	0.26	0.27
N	40	40	40	40	40
% of PIA7 control	–	124	144	124	122
P, Gehans–Wilcoxon	–	<1E–5	<1E–12	<1E–4	<1E–4

D. EXPERIMENT 4						
	PIA7 1 μM	**PIA6 1 μM**	**PIA12 1 μM**	**PIA7 + LY 1 μM, 10 μM**	**PIA6 + LY 1 μM, 10 μM**	**PIA12 + LY 1 μM, 10 μM**
N2, wild-type						
Mean survival (h)	6.2	7.3	9.4	6.4	7.4	7.4
SD	1.32	1.5	1.2	1.4	1.8	1.6
SEM	0.30	0.33	0.28	0.32	0.40	0.35
N	20	20	20	20	20	20
% of PIA7 control	–	118	152	103	119	118
P, Gehans–Wilcoxon	–	<0.02	<1E–8	0.65	<0.04	<0.02

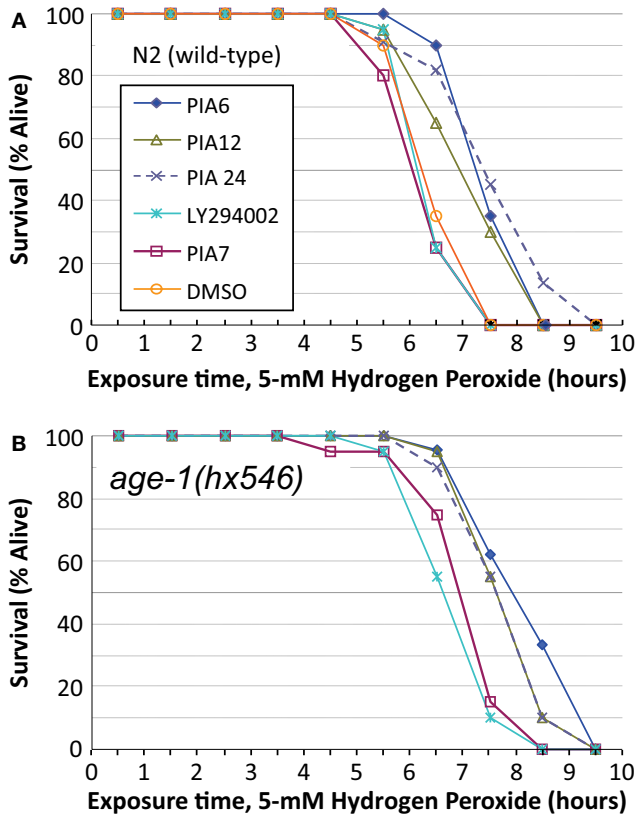

FIGURE 6 | Survival time during exposure to a toxic level of hydrogen peroxide, after development and growth in the presence of PIAs or LY294002. Adult worms (day 2–3 after the L4/adult molt) of strain N2DRM [**(A)**, wild-type] or *age-1*(*hx546*) [**(B)**, a moderately long-lived *age-1* mutant] were maintained from hatching on 1 μM of LY294002 (a widely used inhibitor of class-I PI3K); PIA6, PIA12, or PIA24 (active PIP$_3$ analogs); PIA7 (inactive control analog); or their common solvent, DMSO (added in the amount transferred with the inhibitors). Worms at adult day 3 were transferred to liquid medium without bacteria, containing 5 mM hydrogen peroxide, and were then monitored hourly for survival as described in the **Figure 5** legend.

increased SOD-3::GFP levels by 26% after 48 h ($P < 0.0002$), chiefly affecting diffuse global expression, whereas PIA6 and PIA12 produced increases of only 9% ($P < 0.05$) and 6% (not significant) respectively, while LY294002 slightly reduced fluorescence (**Figure 7, panels A–E,K**). We tested putative PI3K inhibitors for effects on *pck-2*, the *daf-16* target gene encoding phosphoenolpyruvate carboxykinase (PEPCK), which is upregulated >8.5-fold in *age-1*(*mg44*)-F2 worms (Tazearslan et al., 2009). PEPCK is a key enzyme of metabolic regulation which extends lifespan and increases physical activity levels as well as metabolic rate when overexpressed in mice (Hakimi et al., 2007). Adult worms expressing a PEPCK::GFP fusion protein were treated with 1 μM LY294002 or PIAs for 48 h. LY294002 increased PEPCK::GFP fluorescence by 25% ($P < 0.01$ relative to PIA7 controls), while PIAs 6, 12, and 24 elicited increases of 39–41% (each $P < 0.002$; **Figure 7, panels F–J,L**).

DISCUSSION

Since *age-1*(*mg44*) F2-homozygous worms show no full-length PI3K catalytic subunit, it does not appear to be synthesized by either alternative-splicing or read-through routes that avoid the mutationally introduced stop codon. We note that the expression of other components of insulin/IGF-1 signaling are also suppressed at the transcript level in this strain, as are catalytic subunits for all three classes of PI3K (Tazearslan et al., 2009). This underscores the point that the unique survival phenotypes associated with this mutant may not derive entirely from direct effects of the *age-1* nonsense mutation, but may instead be indirect consequences.

These worms evidence no measurable PI3K activity, even when strongly induced by peroxide stress in a transiently starved state. Although measures of PI3K activity averaged zero, such negative findings (no detectable class-I PI3K enzyme or activity) are constrained by the limited sensitivity of the assays, to the rather weak conclusion that F2 *mg44*$^{-/-}$ worms have significantly less than half or one-fifth as much activity as wild-type (**Figure 1C**, left and right panels, respectively). In the PIP$_3$ immunoassay, however, the F2 mutant level had such a small variance that it was significantly below 1.4% of the wild-type value. In contrast, their "F1" homozygous parents had 40% of wild-type PIP$_3$ levels, which was far more than F2 progeny possessed ($P < 10^{-15}$). These results are consistent with the hypothesis of maternal protection, wherein F1-homozygous worms acquire significant amounts of *age-1* mRNA, AGE-1 protein, and/or PIP$_3$, from the oocytes formed in their heterozygous mothers. The *hx546* worms show higher levels of PIP$_3$ than *age-1* F1 or F2 worms, suggesting that they retain substantial kinase activity despite our failure to detect any by PI3K activity assay (**Figure 3**). Given that strong *age-1* mutation indirectly suppresses transcription of *pten*, encoding the PTEN phosphatase that opposes AGE-1/PI3K (Tazearslan et al., 2009), it is possible for steady-state PIP$_3$ levels to be reduced far less, in either *age-1* mutant, than PI3K itself.

We next determined the downstream consequences of chemical treatments thought to disrupt insulin/IGF-1 signaling, by monitoring global expression of SOD-3::GFP and PEPCK::GFP transgenic fusion proteins. The three active PIAs were of similar efficacy in stimulating PEPCK::GFP expression (**Figure 7**), and each was more effective than LY294002. When SOD-3::GFP fluorescence was assessed, however, LY294002 was actually inhibitory, and PIA24 was substantially more effective than either of the other PIAs. Since both *sod-3* and *pepck* are targets of the same FOXO transcription factor, DAF-16, it appears incongruous that the spectrum of drug activities should differ for these two endpoints. However, the tissue distribution differs between SOD-3 (globally expressed, with highest expression at the anterior tip, followed by the nerve ring just posterior to that, very similar to WormBase Expr8145 and Expr3925) and PEPCK (PCK-2, Expr6502 in WormBase: seen in adult intestine, reproductive organs, and vulval muscle, with lower expression in body-wall muscle); see **Figure 7**. Perhaps of even greater importance is the likelihood of multiple drug targets which differ among the PIAs (Gills et al., 2007). Differences between PIAs and more conventional PI3K inhibitors such as LY294002 are also expected, since PIAs

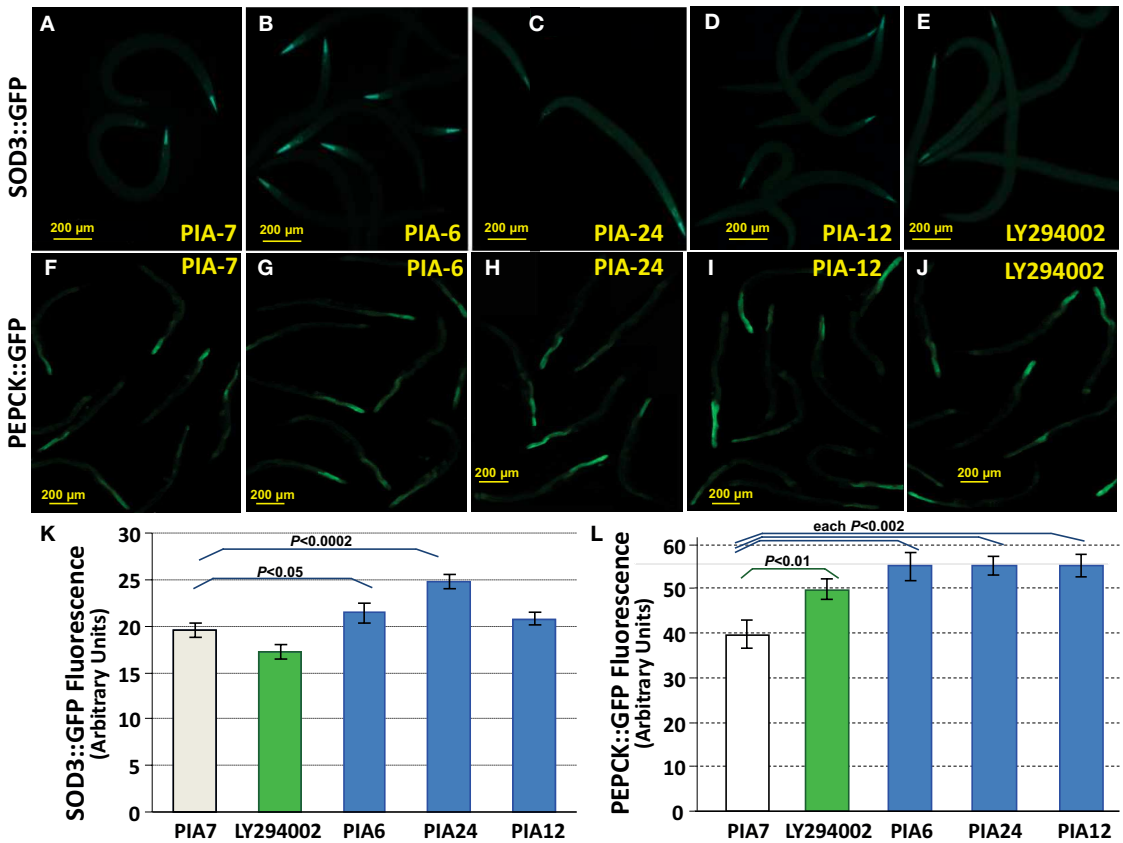

FIGURE 7 | PIAs induce elevated expression of known insulinlike signaling reporters, SOD-3:: GFP, and PEPCK::GFP. Worms with integrated *sod-3::gfp* and *pepck::gfp* reporter constructs were grown 3 days in medium containing 1 µM PIA6, PIA12, or PIA24 (active PIP$_3$ analogs); PIA7 (inactive control analog); or LY294002 (an inhibitor of class-I PI3K). Reporter fluorescence was then imaged with a 10× objective and quantified using ImageJ. **(A–E)**, Fluorescence images of worms at 3 days of adult age, expressing SOD-3::GFP, after exposures as indicated. **(F–J)**, Fluorescence images of worms at 3 days of adult age, expressing PEPCK::GFP, after exposures as indicated. Bars in images indicate 200 µm. Note that the SOD-3::GFP images are shown at slightly greater magnification than the PEPCK::GFP images. **(K)**, Histogram of mean ± SEM fluorescence for 10–20 worms per group, expressing SOD-3::GFP. **(L)**, Histogram of mean ± SEM fluorescence for 10–20 worms per group, expressing PEPCK::GFP. Statistical significances indicated for specific inter-group comparisons were calculated by two-tailed *t*-tests without adjustment for multiple comparisons.

were designed to target the PIP$_3$-binding site of AKT [and may also target other PIP$_3$-binding sites (Gills et al., 2007) including that of PI3K], whereas LY294002, wortmannin, and many other PI3K inhibitors, instead target the ATP-binding pocket of the PI3K catalytic subunit (Walker et al., 2000). It is thus quite possible that "collateral targets" of these drugs differ among tissues, and some of these targets may interact differentially with SOD-3 vs. PEPCK reporters. We note that such unintended drug targets might include effects on translation and protein turnover components, which could then influence expression of reporters.

These same drugs also varied with respect to protection of N2 adults from peroxide stress (**Figure 6**). For a given synthesis, they followed the same rank order (PIA24 > PIA6 > PIA12) as lifespan and the induction of SOD-3, which contributes to oxidant protection. The *age-1*(*hx546*) mutation only blunted the protection by PIA24 to approximately the same level as PIA12 (**Figure 6B**), which may be explicable if PIA24 confers part of its oxidative stress resistance through interaction with AGE-1, whereas the other PIAs act entirely *via* AGE-1-independent mechanisms (e.g., interaction with AKTs). LY294002 provided little or no benefit to either lifespan or peroxide survival; indeed, of all the drugs (other than PIAs) previously reported to inhibit PI3K, only GDC-0941 produced a nominally significant increase in *C. elegans* longevity, i.e., $P < 0.03$ without adjustment for multiple endpoints (**Table 1**). The absence of any short-term toxicity (or increased susceptibility to peroxide stress), for PIAs across a 1000-fold dose range, argues against a mechanism involving hormesis *via* deleterious drug effects. It remains to be seen whether drugs can be developed with greater specificity for class-I PI3K catalytic subunit, and whether those drugs will better recapitulate the extreme longevity and oxidative stress resistance of strong *age-1*-null mutations.

MATERIALS AND METHODS

STRAINS

Nematode strains were supplied by the *Caenorhabditis* Genetics Center (CGC, Minneapolis) and were maintained at 20°C

on 0.6% peptone NGM-agar plates seeded with *E. coli* strain OP50, as described (Ebert et al., 1993; Ayyadevara et al., 2001; Shmookler Reis et al., 2007). Cohorts were synchronized by alkaline hypochlorite lysis of parents (sparing eggs/larvae), and propagated on fresh nutrient-agar plates (Sulston and Hodgkin, 1988).

DETERMINATION OF LIFE SPAN

Nematodes, grown on NGM-agar plates containing 0.6% peptone, were harvested by rinsing off each plate with S buffer (0.1 M NaCl, 0.05 M potassium phosphate, pH 6.0) (Sulston and Hodgkin, 1988). Adults were allowed to settle, and then resuspended in alkaline hypochlorite (0.5 N NaOH, 1.05% hypochlorite; 5 min at 20˚C). The recovered eggs (containing unenclosed larvae) were rinsed in S buffer and placed on fresh agar plates seeded with *E. coli* strain OP50. Survival cultures were established on 60-mm NGM-agar plates (Sulston and Hodgkin, 1988; Ebert et al., 1993; Ayyadevara et al., 2003) seeded with OP50. PI3K inhibitors, including LY294002, wortmannin, A66, ZSTK474, and GDC-0941 (Selleckchem, Houston, TX, USA) or phosphatidylinositol analogs (PIAs, from A. Kozikowski, University of Illinois, Chicago) dissolved in DMSO, were overlaid on plates to achieve final concentrations of 1, 2, or 10 μM. Worms were added 6 h later, 1 day after the L4/adult molt; 30–50 adults were transferred to each 60-mm dish. Worms were maintained at 20˚C, and scored as alive, dead, or lost during daily transfer to fresh dishes. Worms were considered dead if they did not move either spontaneously or in response to touch; those lost (stranded on dish walls or beneath the agar) or killed by internal hatching of progeny ("bagging") were censored at the midpoint of the time interval in which this occurred; worms inadvertently killed were censored at the time of the event.

HYDROGEN PEROXIDE STRESS TOLERANCE AFTER PIA TREATMENT

Adult worms [N2DRM or *age-1*(*hx546*), as indicated] were synchronized by alkaline hypochlorite lysis of hermaphrodites; surviving embryos were transferred to fresh NGM-agar plates and allowed to mature. On reaching the L4 stage, worms were placed on fresh NGM-agar plates seeded with OP50 and overlaid with PIAs to achieve 1 μM. After 48 h, they were transferred to 24-well plates (20–25 worms per well) containing S medium (S buffer plus 0.5% cholesterol) and 5- or 7-mM hydrogen peroxide (Sigma) at 20˚C, as previously described (Ayyadevara et al., 2005, 2008). Survival was scored as above, initially at 1-h intervals, until no worms remained alive. Assays, each comprising 20 or 40 worms, were performed two to six times per strain.

PIP_3 STAINING AND IMMUNOFLUORESCENCE

N2DRM, *age-1*(*hx546*), and *age-1*(*mg44*) worms were fixed at indicated ages by a modified Finney–Ruvkun protocol (Finney and Ruvkun, 1990). Briefly, worms were rinsed from plates in S buffer, rinsed again, and fixed at room temperature in S buffer containing 1% formaldehyde. The solution was frozen on dry ice and thawed overnight at 4˚C with slow agitation. After centrifugation, 30 s at 2000 rpm, supernatant was aspirated and worms were washed twice with Tris-Triton buffer ("TTB," comprising 0.1 M Tris-Cl, pH 7.4; 10 mM EDTA; and 1% v/v Triton X-100), then incubated 2.5 h at 37˚C in TTB plus 1% w/v β-mercaptoethanol to reduce disulfide bonds. Worms were washed twice in 25-mM borate buffer, pH 9.2, incubated 15 min at 37˚C with borate plus 10- mM DTT, and exposed 15 min at 20˚C to 0.3% H_2O_2 in borate buffer. After rinsing in antibody B buffer [0.14 M NaCl; 50-mM sodium phosphate buffer, pH 7.9; 0.1% w/v bovine serum albumin (BSA); 0.1% v/v Triton X-100; 10-mM EDTA; and 0.05% w/v NaN_3], they were incubated at 4˚C overnight with mouse IgM antibody to $PI_{(3,4,5)}P_3$ (Echelon Biosciences Inc.) diluted 1:50 in antibody A buffer (identical to B buffer but with 1% w/v BSA). Worms were washed in antibody B buffer (3 min × 30 min), then incubated 1.5 h at room temperature with ALEXA594-labeled donkey anti-mouse IgG (Molecular Probes) diluted 1:200 in antibody A buffer. After three 30-min washes in antibody B buffer, worms were counterstained with DAPI (Invitrogen) and mounted on slides with Prolong Gold Antifade Reagent (Invitrogen); images were captured with an Olympus BX51 fluorescence microscope.

EXPRESSION OF SOD-3::GFP AND PEPCK::GFP FOLLOWED BY PIA TREATMENT

PIAs were dissolved in DMSO, creating 20× stocks that produce final PIA concentrations of 1 or 10 μM in the agar plates. These solutions were overlaid on 60-mm dishes previously seeded with *E. coli* strain OP50. Adult worms were of strain TJ374 (zEx374) expressing SOD-3::GFP fusion protein, or strain BC10543 (sEx10543) expressing a PCK-2(R11A5.4)::GFP fusion protein. They were synchronized by alkaline hypochlorite lysis of hermaphrodites, and surviving embryos were transferred to fresh NGM-agar plates. Young adults (post-L4 molt) were transferred to NGM-agar plates with PIAs. After 48 h, adult worms were collected, washed three times in S buffer, and fixed in 1% formaldehyde. Worms were mounted on slides under a coverslip, and fluorescence images were taken using an Olympus BX51 microscope.

IN VIVO PIP_3 ASSAY

Wild-type and *age-1* mutant worms were collected and washed in phosphate-free RPMI-1640 medium, then labeled at 20˚C with 1 mCi/ml [^{32}P]-orthophosphate for 16 h in phosphate-free RPMI-1640 medium. ^{32}P-labeled worms were then washed once in phosphate-free RPMI-1640 medium, and lipids were extracted and separated by thin-layer chromatography according to a published procedure (Weinkove et al., 2006). After autoradiography, spots were scraped from the chromatograph and β emissions quantified in a scintillation counter (Beckman).

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Indy mutations and *Drosophila* longevity

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Decreased expression of the fly and worm *Indy* genes extends longevity. The fly *Indy* gene and its mammalian homolog are transporters of Krebs cycle intermediates, with the highest rate of uptake for citrate. Cytosolic citrate has a role in energy regulation by affecting fatty acid synthesis and glycolysis. Fly, worm, and mice Indy gene homologs are predominantly expressed in places important for intermediary metabolism. Consequently, decreased expression of *Indy* in fly and worm, and the removal of *mIndy* in mice exhibit changes associated with calorie restriction, such as decreased levels of lipids, changes in carbohydrate metabolism and increased mitochondrial biogenesis. Here we report that several *Indy* alleles in a diverse array of genetic backgrounds confer increased longevity.

Keywords: *Indy*, *Drosophila melanogaster*, aging and longevity, fruit flies, single gene mutation

INTRODUCTION

Aging is a complex process that can be modulated by environment and affected by genetic manipulations, such as single gene mutations. Understanding the underlying mechanisms by which single gene mutations extend life span can contribute to our understanding of the process of aging, and allow us to design therapeutic interventions that could postpone age-related decline and extend healthy aging. For example, based on the genetic data shown that down-regulation of the TOR signaling pathway extends longevity of yeast, worms, and fruit flies, experiments were performed that show that rapamycin, a drug that down-regulates the TOR signaling pathway, extends mice and fruit flies longevity (Vellai et al., 2003; Jia et al., 2004; Kapahi et al., 2004; Kaeberlein et al., 2005; Harrison et al., 2009; Bjedov et al., 2010).

Mutations in the *Indy* (*I'm Not Dead Yet*) gene extend life span of the fruit fly, *Drosophila melanogaster* (Rogina et al., 2000; Wang et al., 2009). Similarly, decreased expression of two of the worm *Indy* homologs extend worm longevity (Fei et al., 2003, 2004). *Indy* encodes the fly homolog of a mammalian di and tricarboxylate transporter involved in reabsorbing Krebs cycle intermediates, such as citrate, pyruvate, and α-ketoglutarate (Knauf et al., 2002, 2006; Pajor, 2006). Functional characterization of the transporter encoded by the *Indy* structural gene confirmed that it is a transporter of Krebs cycle intermediates (Inoue et al., 2002; Knauf et al., 2002). Studies in frog oocytes and mammalian cells showed that INDY mediates Na^+, K^+, and Cl^- independent high-affinity flux of dicarboxylates and citrate across the plasma membrane (Inoue et al., 2002; Knauf et al., 2002). Further studies have shown that INDY functions as an anion exchanger of dicarboxylate and tricarboxylate Krebs cycle intermediates (Knauf et al., 2006). Crystal structure of a bacterial INDY homolog from *Vibrio cholera* (VcINDY) reveals that one citrate and one sodium molecule is bound per protein but the mature transporter is likely found in the form of a dimer (Mancusso et al., 2012).

The fly INDY is most highly expressed in the gut, fat bodies, and oenocytes, all places where intermediary metabolism takes place, suggesting its role in metabolism (Knauf et al., 2002). Similarly, worm homologs (ceNaDC1 and ceNaDC2) are expressed in the intestinal tract (Fei et al., 2003), and the mouse gene *mIndy* (mINDY; SLC13A5) is predominantly expressed in liver (Birkenfeld et al., 2011). Based on INDY expression and a role in transporting Krebs cycle intermediates it has been hypothesized that decreased INDY activity creates a state similar to calorie restriction (CR). Studies in flies and mice support this hypothesis mainly by showing similarities between the physiology of *Indy* mutant flies and *mIndy* knockout mice on high calorie food and control flies and mice on CR (Wang et al., 2009; Birkenfeld et al., 2011).

It has recently been reported that longevity was not extended in worms with decreased levels of the *Indy* or in fruit flies with one of the alleles utilized by Rogina et al. (2000) and Toivonen et al. (2007). Toivonen et al. (2007), attributed the life span extension in *Indy* to the genetic background and bacterial infection (Toivonen et al., 2007). Subsequently, it was confirmed that the original $Indy^{206}$ mutation extends longevity after backcrossing into the *yw* background but not after backcrossing into the w^{1118} genetic background (Wang et al., 2009; reviewed in Frankel and Rogina, 2012). Furthermore, it was demonstrated that the results published in Toivonen et al., are most likely due to differences in the caloric content of the food (Toivonen et al., 2007; Wang et al., 2009).

Here we report that the presence of one copy of an $Indy^{206}$ mutant chromosome extends longevity in several genetic backgrounds when compared to genetically matched controls. In order to further address the issues of *Wolbachia* contamination we treated the previously reported $Indy^{159}$ allele, and several new

alleles, with tetracycline and backcrossed all of these *Indy* alleles into a *yw* genetic background for 10 generations. We determined survivorships of all *Indy* alleles on standard laboratory diet and found that several new *Indy* mutant alleles can also extend the longevity of male and female *Drosophila*. The data presented here further confirm the role of the *Indy* gene in *Drosophila* longevity and show the relationship between life span extension and reduction in *Indy* mRNA.

RESULTS

MUTATION IN *Indy^{206}* EXTENDS LIFE SPAN IN DIFFERENT GENETIC BACKGROUNDS

In order to further examine if genetic background may contribute to the life span extension of heterozygous *Indy* mutant flies, we determined the survivorship of *Indy* heterozygous mutant flies in *Hyperkinetic^{1} (Hk^{1})* and long- and short-lived selected Luckinbill lines (**Figures 1A-E**) (Kaplan and Trout, 1969; Luckinbill and Clare, 1985). *Hk^{1}* is a recessive mutation characterized by hyperactivity and shorter life span of *Drosophila*. Hyperactivity is due to mutation of the beta (*Hk^{1}*) subunit of the potassium channel, which causes increased neuronal excitability (Trout and Kaplan, 1970). *Hk^{1}* is an X-linked recessive mutation, thus only male flies in those background live shorter (Trout and Kaplan, 1970; Rogina and Helfand, 1995). We used the *Hk^{1}* line since it was isolated by an EMS mutagenesis of *Canton-S* (*CS*) stock in 1969 and therefore had many years of divergence from the *CS* background of the original *Indy* lines. We determined the survivorship of flies heterozygous for *Hk^{1}* and either *Indy^{206}* or control-*2216*. The 2216 and 1085 lines that were derived from the same mutagenesis as *Indy^{206}*, but do not have a P-element insertions in the *Indy* region were used as control in Rogina et al., 2000. Survivorship analysis revealed that the median life span of male flies with one copy of the *Indy* mutation in *Hk* background is 52.0% increased as compared to the control *Hk;2216*. A similar increase in survivorship of 57.0% was observed in *Hk;Indy^{206}* female flies when compared to the control females, **Figures 1A,B**; **Table 1**. (Median life span: *Hk;Indy^{206}* males = 38.0 days, females = 68.0; *Hk;2216* males = 25.0, females = 43.3).

Indy^{206} MUTANT HETEROZYGOUS FLIES LIVE LONGER IN LUCKINBILL SHORT- AND LONG-LIVED LINES COMPARED TO CONTROL LINES

Luckinbill short- and long-lived lines were selected based on reproduction of a population of outbreed *Drosophila* early or late in life (Luckinbill and Clare, 1985). Selective breeding was carried out for 21 or 29 generations and resulted in a large difference in median longevity between short- and long-lived lines. For instance, median life span of males for the short-lived *1S9* line was 33.9 day, while median longevity for the long-lived line *1L6* = 65.3, *2L9* was 93.0 and *2L18* was 93.0 days. Similar differences in median longevity between short and long-lived line can be seen in females (Median longevity *1S9* = 32.0, *1L6* = 61.4, *2L9* = 80.0, and *2L18* = 81.0 days.) We examined if *Indy* mutant flies can affect longevity of Luckinbill short- and long-lived line differently as compared to controls and further extend the life of long-lived lines beyond that expected from hybrid vigor. Our data show that the *Indy^{206}* mutation increases longevity of both short- and long-lived lines in all conditions, with one exception, the female *1S9;Indy^{206}* flies have a similar median longevity compared to the controls. While, F1 heterozygous males flies from a cross between the control 1085 and the *1S9* short Luckinbill line show the expected life span extension due to hybrid vigor and have a 77% increase in median longevity as compared to the homozygous *1S9* line, F1 heterozygous *Indy^{206};1S9* male flies have much higher increase in median longevity of 98.8% compared to *1S9* homozygous flies, **Figure 1C**; **Table 1**. *Indy^{206}* mutation further increased longevity of all long-lived Luckbill lines. F1 heterozygote animals from a cross between the *Indy^{206}* enhancer-trap line and the laboratory selected long-lived line *1L6* of Luckinbill (*Indy^{206};1L6)* live 20.7% longer compared to the homozygous *1L6*. In contrast, heterozygous control *1085;1L6*, live only 2.5% longer then homozygous *1L6* flies. *2L9* homozygous long-lived Luckinbill line live much longer compared to *1L6* **Figure 1D**. However, *Indy* mutant heterozygous flies in *2L9* (*Indy^{206};2L9*) still live 7.2% longer compared to the *2L9* homozygous flies **Figure 1E**. In contrast, F1 heterozygous control males, *1085;2L9* have 4.4% shorter median life span compared to the homozygous *2L9* flies. Median male life span in days: *Indy^{206};2L9* males = 99.7, *1085;2L9* = 88.9, **Figure 1E**; **Table 1**. Thus, heterozygous *Indy^{206};2L9* male flies have an increase in life span of 12% over matched controls (*2L9;1085*), and 7.2% over the homozygote Luckinbill long-lived *2L9* line itself (**Table 1**). Median life span of female *1085;2L9* is decreased by 21.4% compared to median longevity of homozygous *2L9* female flies, while longevity of *Indy^{206};2L9* females is only 3.2% shorter compared to homozygous flies. (Median female longevity in days: *1085;2L9* = 62.9, *Indy^{206};2L9* = 77.4, **Table 1**). Heterozygous *Indy^{206}* flies in the background of the *2L18* long-lived line do not live significantly longer compared to homozygous *2L18* flies; however, they live significantly longer compared to control *1085;2L18* heterozygous male flies, which live 9.5% shorter compared to *2L18* homozygous male flies. (Median male life span in days: *2L18* = 93.0, *Indy^{206};2L18* = 94.1, *1085;2L18* = 84.2, **Figure 1F**; **Table 1**). Similarly, *Indy^{206};2L18* heterozygous females live longer in *2L18* background compared to the controls.

LIFE SPAN EXTENSIONS IN DIFFERENT *Indy* ALLELES

We have previously reported that five independent *Indy* mutant alleles extend the life span of male and female *Drosophila* in wild type *CS* and *yw* genetic backgrounds (Rogina et al., 2000; Wang et al., 2009). We have now tested an additional six *Indy* alleles for their effect on fly longevity (*Indy^{EP3044}*, *Indy^{EP3366}*, *Indy^{EY01442}*, *Indy^{EY01458}*, *Indy^{EY013297}*, *Indy^{KG07717}*). Genomic organization of the *Indy* locus and position of P-elements insertion in different *Indy* mutant alleles used in this manuscript is shown in **Figure 2A**. These six new alleles and three previously tested *Indy* alleles (*Indy^{206}*, *Indy^{302}*, *Indy^{159}*) and *yw* control flies, were all treated with tetracycline to eliminate any possible bacterial contamination by *Wolbachia*. Although the absence of *Wolbachia* contamination after tetracycline treatment was not confirmed by PCR, we have previously confirmed the absence of Wolbachia after identical treatment (Wang et al., 2009). All of the *Indy* alleles and one of the control stocks 1085, which has the same genetic background as *Indy^{206}*and *Indy^{302}*, were backcrossed into the *yw* genetic background for 10 generations. We have determined longevity of all *Indy* alleles as heterozygotes in

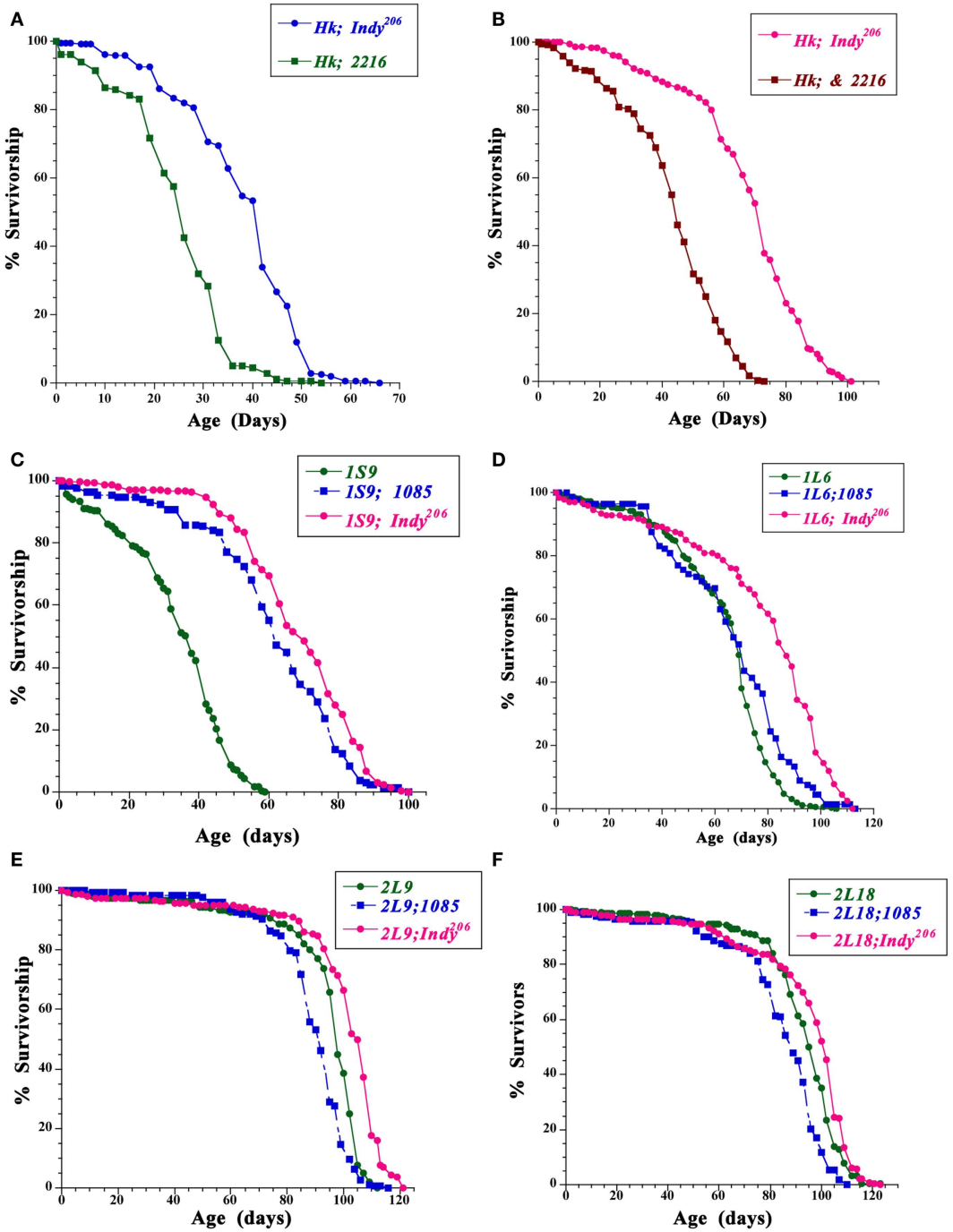

FIGURE 1 | Mutation in the *Indy* gene extends the life span of male and female *Drosophila* in different genetic backgrounds. (A,B) Survivorships for male **(A)** and female **(B)** heterozygous for the *Indy*^{206} (*Hk;Indy*^{206}), and control (*Hk;2216*) enhancer-trap line in *Hyperkinetic* background. **(C–F)** Survivorship for male homozygous flies for the Luckinbill short live line 1S9 **(C)**, the Luckinbill long-lived line *1L6* **(D)**, *2L9* **(E)**, or *2L18* **(F)** and heterozygous for the *Indy*^{206}, or 1085 and the Luckinbill *1S9*, *1L6*, *2L9*, or *2L18* lines at 25˚C. Between 135–537 flies were used for each life span.

yw background and calculated median longevity for males and females, **Table 2**. Representative survivorships of two new *Indy* alleles are plotted in **Figures 2B,C**. Heterozygous *yw;Indy*^{206}/+, *yw;Indy*^{302}/+, *yw;Indy*^{159}/+, *yw;Indy*^{EY01442}/+ *yw;Indy*^{EY01458}/+, and *yw;Indy*^{EY013297}/+ male and female flies have a significantly longer life compared to control *yw* flies. Longevity extension in males with one copy of *Indy* mutant allele varies from 34.4 to 14.0%, and in females *Indy* mutant extension range from 29.4 to

Table 1 | Life span of *Indy*^{206} heterozygous flies is longer compared to the control flies in different genetic backgrounds.

Gender	Genotype	N	Median life span (% change)	X^2	p	Maximal life span (% change)
M	*Hk;Indy*^{206}/+	210	38.0 (52.0)	146	$p < 0.0001$	53.6 (35.7)
M	*Hk;2216*	184	25.0			39.5
F	*Hk;Indy*^{206}/+	294	68.0 (57.0)	286	$p < 0.0001$	91.0 (41.9)
F	*Hk;2216*	344	43.3			64.2
M	*1S9*	301	33.9			51.7
M	*1S9-Indy*^{206}	284	67.4 (98.8)	526	$p < 0.0001$	89.5 (73.1)
M	*1S9-1085*	170	60.0 (77.0)	278	$p < 0.0001$	86.2 (66.7)
F	*1S9*	360	32.0			52.2
F	*1S9-Indy*^{206}	245	52.6 (64.4)	252	$p < 0.0001$	83.5 (60.0)
F	*1S9-1085*	255	54.2 (69.5)	210	$p < 0.0001$	88.7 (69.9)
M	*1L6*	323	65.3			90.7
M	*1L6-Indy*^{206}	240	78.8 (20.7)	169	$p = 0$	108.1 (19.2)
M	*1L69-1085*	135	66.9 (2.5)	7	$p = 0.008$	98.2 (8.3)
F	*1L6*	317	61.4			87.6
F	*1L6-Indy*^{206}	258	75.4 (22.7)	155	$p = 0$	101.4 (15.8)
F	*1L69-1085*	154	48.7 (−20.7)	6.3	$p = 0.01$	90.5 (3.3)
M	*2L9*	291	93.0			106.3
M	*2L9-Indy*^{206}	158	99.7 (7.2)	81.6	$p < 0.0001$	115.8 (8.9)
M	*2L9-1085*	177	88.9 (−4.4)	42.2	$p = 8.3e{-}11$	105.1 (−1.0)
F	*2L9*	340	80.0			99.6
F	*2L9-Indy*^{206}	236	77.4 (−3.2)	17.9	$p = 2.28e{-}05$	104.5 (4.9)
F	*2L9-1085*	228	62.9 (−21.4)	140	$p < 0.0001$	87.9 (−12.0)
M	*2L18*	312	93.0			112.1
M	*2L18-Indy*^{206}	457	94.1 (1.2)	21.7	$p = 3.15e{-}6$	114.1 (1.8)
M	*2L18-1085*	169	84.2 (−9.5)	42.9	$p = 5.8e{-}1$	105.3 (−6.0)
F	*2L18*	286	81.0			99.4
F	*2L18-Indy*^{206}	537	73.2 (−9.6)	0.1	$p = 0.764$	100.7 (1.3)
F	*2L18-1085*	199	64.6 (−20.2)	112	$p < 0.0001$	85.6 (−14.0)

M, males; F, females; N, number of flies in the experiment, Hk, Hyperkinetic; 1S9, Luckinbill short 1S9 line; 1L6, 2L9, or 2L18 Luckinbill long-lived 1L6, 2L9, or 2L18 lines.

10.7%, **Table 2**. In addition, female, but not male heterozygous *yw;Indy*^{EP3044} flies live 9.2% longer compared to the controls. No effect on longevity was observed in male and female heterozygous *yw;Indy*^{KG07717}/+, *yw;Indy*^{3366}/+, and male heterozygous *yw;Indy*^{3044}/+ mutant flies. We determined the levels of *Indy* mRNA isolated from Head& Thorax of male heterozygous for two of the new *Indy* alleles (*yw;Indy*^{EY01442}/+, *yw; Indy*^{EP3366}/+), one old (*yw;Indy*^{206}/+), and their genetic control (*yw*). The levels of *Indy* mRNA in heterozygous *yw;Indy*^{206}/+ allele are 51.1% and in heterozygous *yw;Indy*^{EY01442}/+ allele are 60.6% of the levels of *Indy* mRNA found in *yw* flies, **Figure 2D**. A similar decrease in the levels of *Indy* mRNA in *yw;Indy*^{206}/+ was previously reported (Wang et al., 2009). We found only a minor, non-significant decrease in the levels of *Indy* mRNA in heterozygous *yw;Indy*^{EP3366}/+ mutant flies. Lack of longevity effect in *yw;Indy*^{EP3366}/+ allele is most likely due to only a small effect of the P-element insertion on the *Indy* mRNA levels in *yw;Indy*^{EP3366}/+mutant flies. Our data show a strong correlation between the level of *Indy* mRNA and longevity extension.

DISCUSSION

We have previously identified and characterized five independent mutations in the *Indy* gene in *Drosophila* that cause an increase in average and maximal life span for both male and female fruit flies (Rogina et al., 2000). The original five alleles were derived from three different mutageneses (Boynton and Tully, 1992; Rogina et al., 2000). Life spans of flies carrying one copy of P-element in the *Indy* gene were compared with their close genetically matched controls, flies from the same mutagenesis without a P-element insertion in the *Indy* gene. Here we show that *Indy*^{206} heterozygous mutant flies also live longer when crossed into three different genetic backgrounds, *Hk*, short, and long-lived Luckinbill lines as compared to control flies from the same genetic background as *Indy* also crossed to these three different genetic backgrounds. Luckinbill short and long-lived lines have been generated by selective breeding for early and late female fecundity (Luckinbill and Clare, 1985). Presence of the *yw;Indy*^{206} mutant chromosome significantly extends longevity in the background of the Luckinbill short *1S9* line compared to the control line 1085. Moreover, the

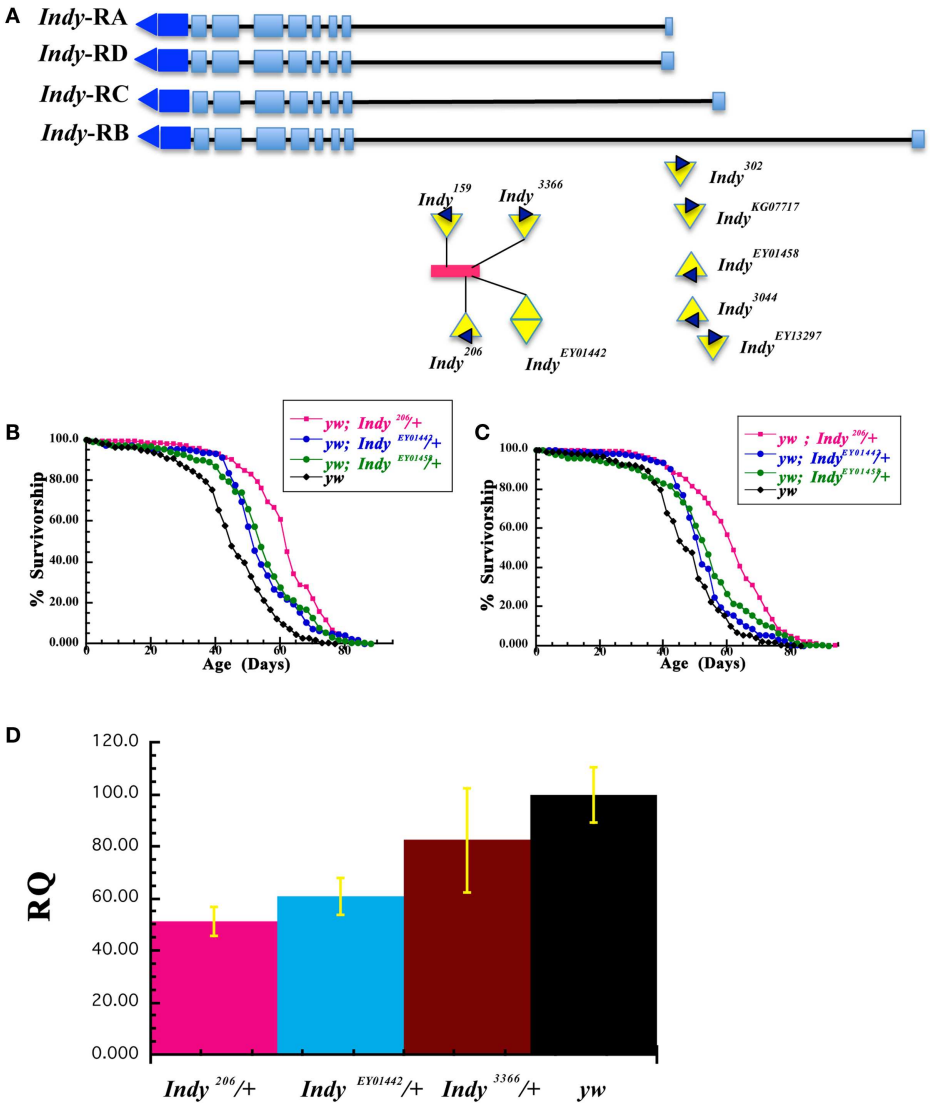

FIGURE 2 | Survivorships of different *Indy* mutant alleles in *yw* background. (A) Genomic organization of the *Indy* locus with insertion sites and orientation of P-element in $Indy^{206}$, $Indy^{159}$, $Indy^{EY01442}$, $Indy^{3366}$, $Indy^{302}$, $Indy^{KG07717}$, $Indy^{EY01458}$, $Indy^{EP3044}$, and $Indy^{EY013297}$ alleles used in this manuscript. Orientation of P-element in $Indy^{EY01442}$ allele is not known. The red rectangle represents the conserved Hoppel transposable element. *Indy* encodes four putative transcripts (RA, RB, RD, and RC), which have different 5′ exon. **(B,C)** Life span of males **(B)** and females **(C)** heterozygous for $Indy^{206}$, $Indy^{EY01442}$, $Indy^{EY01458}$, and *yw* on standard laboratory corn diet after 10× backcrossing into the *yw*. **(D)** *Indy* mutants have decreased levels of *Indy* mRNA. Q-PCR determination of *Indy* mRNA expression levels in Heads and Thorax of $Indy^{206}$/+, $Indy^{EY01442}$/+, $Indy^{33662}$/+, and *yw* 20 days old male flies. Experiments were done in two ($Indy^{206}$/+) or three ($Indy^{EY01442}$/+, $Indy^{3366}$/+, and *yw)* replicates with 2 × 15($Indy^{206}$/+), 3 × 40 ($Indy^{3366}$/+), or 3 × 50 ($Indy^{EY01442}$/+ and *yw)* flies in each group.

$Indy^{206}$ mutation further extends longevity of two long-lived Luckinbill lines and does not cause shortening of life span of *2L18* long-lived line. At the same time, median longevity of control lines when crossed to Luckinbill long-lived lines are significantly shorter compared to homozygous Luckinbill lines. These data show that extension of life span by this *Indy* allele is not limited to the background of the short-lived lines, but further extends lines already selected for long life span.

We also report extension of longevity by additional *Indy* mutant alleles. All *Indy* mutant alleles were treated by tetracycline to prevent any effects of *Wolbachia* and backcrossed to *yw* background. *Wolbachia* infection was proposed as a contributing factor to *Indy* longevity by Toivonen et al. (2007). $Indy^{EY01442}$, $Indy^{EY01458}$, $Indy^{EY013297}$, $Indy^{KG07717}$ were generated by the Berkeley *Drosophila* Genome Project (BDGP) gene disruption project (Bellen et al., 2004). The *Indy* gene region appears to be a "hot spot" for P-element insertions illustrated by isolation of 5 KG, 28 EY, and 10 EP element insertions in the *Indy* region (Bellen et al., 2004). P-element insertion in $Indy^{206}$, $Indy^{159}$, $Indy^{EY01442}$, and $Indy^{EP3366}$ are within the Hoppel element in the first intron

Table 2 | Life span of several different *Indy* mutant alleles as heterozygous is longer compared to the control flies in *yw* genetic background.

Gender	Genotype	N	Median life span (% change to *yw*)	X^2	p	Maximal life span (% change)
M	*yw;Indy^{206}*	224	61.3 (34.4)	146	$p < 0.0001$	78.9 (19.7)
M	*yw;Indy^{302}*	161	52.0 (14.0)	14.1	$p = 0.000164$	69.0 (4.7)
M	*yw;Indy^{159}*	169	54.5 (19.5)	35.8	$p = 2.16e{-}09$	69.2 (5.0)
M	*yw;Indy^{EY01442}*	179	53.5 (17.3)	30.5	$p = 3.37e{-}08$	76.8 (16.7)
M	*yw;Indy^{EY01458}*	151	53.8 (18.0)	33.5	$p = 6.95e{-}09$	75.5 (14.6)
M	*yw;Indy^{EY13297}*	178	53.8 (18.0)	31.4	$p = 2.15e{-}08$	73.0 (10.8)
M	*yw;Indy^{KG07717}*	178	45.9 (0.6)	0.9	$p = 0.339$	62.5 (−5.0)
M	*yw;Indy^{EP3044}*	168	48.5 (6.3)	1.6	$p = 0.207$	75.3 (14.3)
M	*yw;Indy^{EP3366}*	181	42.8 (−6.0)	12.1	$p = 0.000499$	57.2 (−13.0)
M	*yw;1085*	175	43.6 (−4.0)	5.5	$p = 0.0191$	62.3 (−5.0)
M	*yw*	169	45.6			65.9
F	*yw;Indy^{206}*	316	61.7 (29.3)	149	$p < 0.0001$	80.7 (21.9)
F	*yw;Indy^{302}*	192	52.8 (10.7)	10.8	$p = 0.00102$	71.9 (8.7)
F	*yw;Indy^{159}*	212	56.5 (18.4)	51.5	$p = 6.15e{-}13$	76.9 (16.3)
F	*yw;Indy^{EY01442}*	186	52.8 (10.7)	11.4	$p = 0.000731$	74.1 (12.0)
F	*yw;Indy^{EY01458}*	206	53.0 (11.1)	24.5	$p = 8.97e{-}07$	79.4 (20.0)
F	*yw;Indy^{EY13297}*	190	54.7 (14.7)	30.5	$p = 3.36e{-}08$	76.1 (15.0)
F	*yw;Indy^{KG07717}*	181	48.6 (1.9)	0.1	$p = 0.795$	70.3 (6.3)
F	*yw;Indy^{EP3044}*	186	52.1 (9.2)	7.8	$p = 0.00522$	76.9 (16.2)
F	*yw;Indy^{EP3366}*	189	48.9 (2.5)	2.4	$p = 0.119$	57.3 (−13.0)
F	*yw;1085*	187	48.4 (1.4)	2.2	$p = 0.141$	59.2 (−11.0)
F	*yw*	200	47.7			66.2

M, males; F, females; N, number of flies in the experiment.

of the *Indy* gene, upstream of the putative translational start site, **Figure 2A**. The conserved Hoppel element is present in the same position in wild type flies (Rogina et al., 2000). The insertion in *Indy^{302}*, *Indy^{EY013297}*, *Indy^{EY01458}*, *Indy^{KG07717}*, and *Indy^{EP3044}* lines is upstream from putative transcriptional start sites. *Indy* encodes four putative transcripts, which have different 5′-exons. The positions of P-elements in *Indy^{302}*, *Indy^{EP3044}*, *Indy^{EY01458}*, *Indy^{EY013297}*, and *Indy^{KG07717}* are located close to the three putative transcriptional start sites for three putative *Indy* transcripts (*Indy-RA, Indy-RD,* and *Indy-RC*) and about 5,000 bp upstream from the putative transcriptional start site in *Indy-RB*. Genomic organization of the *Indy* locus and positions of P-element insertion in different *Indy* alleles used in this manuscript are shown in **Figure 2A**. Positions of additional P-elements insertion can be seen in FlyBase: http://flybase.org/reports/FBgn0036816.html. It was previously shown that the presence of the P-element in *Indy^{206}* and *Indy^{302}* mutant alleles decreases the levels of *Indy* mRNA most likely by affecting transcription (Knauf et al., 2006; Wang et al., 2009). The levels of *Indy* mRNA are decreased about 95% in homozygous *Indy^{206}* and about 40% in homozygous *Indy^{302}* alleles (Wang et al., 2009). The levels of INDY protein are also dramatically decreased in *Indy^{206}* homozygous mutant flies (Knauf et al., 2002). Similarly, here we show that the levels of *Indy* mRNA are decreased about 39% in the heterozygous *Indy^{EY01442}*/+ allele and about 49% in the heterozygous *Indy^{206}*/+ allele compared to the levels of *Indy* mRNA found in *yw* flies. No significant decrease in the levels of *Indy* mRNA were observed in heterozygous *Indy^{3366}*/+ flies, which correlates with the absence of longevity extension. It is likely that variation in longevity effects of different *Indy* alleles correlates to actual *Indy* mRNA levels and differential effects of P-elements on transcription. We found that male flies heterozygous for six *Indy* alleles have longevity extension ranging from 14.0 to 34.4%. Females heterozygous for seven *Indy* alleles show similar result having longevity extension ranging from 9.2 to 29.3%. Our data further confirm our hypothesis that the level of *Indy* expression is central for longevity extension. When the levels of *Indy* mRNA are decreased approximately 49%, as in *Indy^{206}*/+ heterozygous mutant flies, there is dramatic longevity extension of 34%. We have previously reported that when the levels of *Indy* mRNA are radically reduced, as in *Indy^{206}* homozygous flies, longevity extension is less than extension of the *Indy^{206}*/+ heterozygous flies (Wang et al., 2009). A smaller longevity effect of 17% was observed when *Indy* mRNA levels are moderately reduced, as in *Indy^{EY01442}*/+. Insignificant reduction of *Indy* mRNA levels, as in *Indy^{EP3366}*/+ mutant flies, resulted in no longevity effect. Besides *Indy^{EP3366}*/+, no longevity extension was found in another one of the new alleles, *Indy^{KG07717}*. In summary, maximal longevity in *Indy* mutant flies is associated with optimal reduction of *Indy* mRNA levels. When *Indy* levels are too low or close to normal, longevity effects are diminished. Although a recent report attributed life span extension in *Indy* to hybrid vigor, due to life span evaluation in an incorrect genetic background, and bacterial infection, our data presented here corroborate a link

between the *Indy* mutations and longevity in flies (Toivonen et al., 2007; Wang et al., 2009). The effect of the *Indy* mutation on longevity was supported by findings that decreased activity of NaDC2, a *C. elegans* homolog of the *Indy* gene, extends the life span of worms (Fei et al., 2003, 2004). Similar effects of increased longevity associated with mutations in the fly and the worm *Indy* gene suggests a possibility of evolutionary conservation and a universal role of INDY in longevity (Fei et al., 2003, 2004).

Several studies have investigated the molecular mechanisms underlying the effects of the *Indy* mutation on longevity and health span of worms, flies, and mice (Fei et al., 2003; Marden et al., 2003; Neretti et al., 2009; Wang et al., 2009; Birkenfeld et al., 2011). INDY is a plasma membrane transporter that may mediate the movement of dicarboxylic acids through the epithelium of the gut and into organs important in intermediary metabolism and storage (Knauf et al., 2002, 2006). Location of the INDY transporter in the fat body and oenocytes suggest a role in intermediary metabolism and expression in the gut suggests a role in uptake of nutrients. Reductions in INDY activity may alter uptake, utilization, or storage of important nutrients and affect normal metabolism. It has been hypothesized that reductions in *Indy* activity seen in *Indy* mutations might be altering the normal energy supply in flies resulting in life span extension through a mechanism similar to CR. CR has been shown to increase life span and delay the onset of age-related symptoms in a broad range of organisms (McCay et al., 1935; Weindruch and Walford, 1988). Consistent with the hypothesis that *Indy* is important in metabolism is the finding that *Indy* mutant worms, flies, and mice have disrupted lipid metabolism (Fei et al., 2003; Wang et al., 2009; Birkenfeld et al., 2011). Similarly to CR animals, *Indy* mutant flies have increased spontaneous physical activity, decreased starvation resistance, weight, egg production, and insulin signaling. Furthermore, wild type flies on CR have significantly decreased levels of *Indy* mRNA (Wang et al., 2009). *Indy* homozygous mutant flies live shorter on low calorie foods compared to controls, which is consistent with our hypothesis that *Indy* mutant flies are already in a state of reduced nutrition on normal food and when food is further reduced, life span is shortened due to starvation (Wang et al., 2009). In addition, *Indy* mutant flies have increased mitochondrial biogenesis in heads and thoraces similar to CR animals (Neretti et al., 2009). Similarly, *mIndy* knockout mice have increased mitochondrial biogenesis in the liver. The mechanism of the effect of a decrease in INDY on metabolism is likely from its physiological function as a citrate transporter. Cytosolic citrate is the main precursor for the synthesis of fatty acid, cholesterol, triacylglycerols, and low-density lipoproteins. In addition, cytosolic citrate inhibits glycolysis and fatty acid β-oxidation. Therefore, INDY by affecting the levels of cytosolic citrate may alter glucose and lipid metabolism in a manner that favors longevity. Additional support that *Indy* mutation mimics CR comes from the findings that *mIndy* knockout mice are protected against adiposity and insulin resistance when kept on high fat diet (Birkenfeld et al., 2011). The data from worm, fly, and mice studies highlight the importance of INDY in health span and longevity. New *Indy* alleles described here should provide additional tools to further explore the role of INDY in metabolism and its connection to extended longevity and health.

MATERIALS AND METHODS

FLY STRAINS

1S9 a short-lived and *1L6, 2L9,* and *2L18* long-lived lines were a kind gift from James W. Curtsinger and originally described in Luckinbill and Clare (1985). $Indy^{206}$, $Indy^{302}$, 1085, and 2216 were obtained from Tim Tully (Boynton and Tully, 1992). $Indy^{159}$ was kind gift from the Bier lab (Bier et al., 1989). $Indy^{EP3044}$, $Indy^{EP3366}$, $Indy^{EY01442}$, $Indy^{EY01458}$, $Indy^{EY013297}$, $Indy^{KG07717}$alleles, and Hk^{1} were obtained from the Bloomington Stock Center or Exelexis. Heterozygous flies used in survivorship analysis are F1 generations from crosses in which virgin females homozygous for Hk^{1}, short-lived, long-lived Luckinbill lines, or *yw* were mated to males homozygous for different *Indy* alleles, or the control lines 1085 or 2216.

BACKCROSSING SCHEME

$Indy^{206}$, $Indy^{302}$, $Indy^{159}$, $Indy^{EP3044}$, $Indy^{EP3366}$, $Indy^{EY01442}$, $Indy^{EY01458}$, $Indy^{EY013297}$, $Indy^{KG07717}$, and 1085 were backcrossed into the *yw* background. Female virgins from *yw* were mated with males of different *Indy* alleles or *1085.* Heterozygous females were then backcrossed to *yw* males for 10 generations.

FOOD RECIPE

We used standard yeast, corn, sucrose food in our experiments: 113 g Sucrose (MP Biomedicals, Fischer Scientific) and 28 g Brewers yeast (MP Biomedicals, Fischer Scientific) was mixed with 643 ml water and autoclaved for 20 min. 49 g corn (MP Biomedicals, Fischer Scientific) and 8.1 g Agar (SciMart) were mixed in 268 ml water and added to the food mixture and autoclaved for 20 min. The food was cooled down with constant mixing. 2.4 g tegosept (Fischer Scientific) dissolved in 10.7 ml 100% EtOH was added when the food temperature was 65°C. Approximately 10 ml food was poured to plastic vials using Fly food dispenser (Fischer Scientific), and vials were covered with Kimwipes and cheese cloth. Once the food was cooled down it was stored at 4°C. Before use the food was warmed up to room temperature.

LIFE SPAN

Vials were cleared of adult flies in the morning and the collection of newly eclosed flies occurred in the afternoon. Approximately 20 male and 20 female flies were kept together in a plastic vials with approximately 5–10 ml of a standard cornmeal media (Rogina et al., 2000). Flies were housed in humidity-controlled incubators, maintained at 25°C on a 12 h light: dark cycle. Vials of fresh food were supplied three times weekly (Monday, Wednesday, and Friday) and the number of dead flies was recorded during each passage from old to new vials.

mRNA ISOLATION Q-PCR ANALYSIS

The standard Chomczynski protocol and Trizol reagent (Gibco BRL) were used to isolate mRNA (Chomczynski and Sacchi, 1987). Male flies at age 20 were placed on a cold block and Head with Thorax were dissected. Three biological replicates of 50 males were used in each isolations of $Indy^{EY01442}$/+ and *yw* flies, three

biological replicates of 40 *Indy*^{3366}/+ males and two biological replicates of 15 *Indy*^{206}/+ males. Q-PCR was performed with *Indy* and Ankyrin specific primers obtained from Applied Biosystems according to the manufacturers protocol. Ankyrin was used as an endogenous control. The samples were run on the AB 7500 System.

STATISTICAL ANALYSIS

Life span data were analyzed by long-rank tests (http://bioinf.wehi.edu.au/software/russell/logrank/). Maximum life span was calculated as the median life span of the longest surviving 10% of the population.

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The determination of genetic markers of age-related cancer pathologies in populations from Kazakhstan

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Aging associates with a variety of pathological conditions such as cancer, cardiovascular, neurodegenerative, autoimmune diseases, and metabolic disorders. The oncogenic alterations overlap frequently with the genes linked to aging. Here, we show that several aging related genes may serve as the genetic risk factors for cervical and esophagus cancers. In our study, we analyzed samples obtained from 115 patients with esophageal and 207 patients with cervical cancer. The control groups were selected to match the ethnicity and age of cancer patients. We examined the genes involved in the processes of xenobiotics detoxification (*GSTM1* and *GSTT1*), DNA repair (*XRCC1* and *XRCC3*), and cell cycle regulation and apoptosis (*CCND1* and *TP53*). Our study revealed that deletions of *GSTT1* and *GSTM1* genes or the distinct point mutations of *XRCC1* gene are associated with cervical and esophageal cancers. These results will lead to development of screening for detection of individuals susceptible to esophageal and cervical cancers. Introduction of the screening programs will allow the early and effective preventive measures that will reduce cancer incidence and mortality in Kazakhstan.

Keywords: age-related disease, cervical cancer, esophageal cancer, genetic susceptibility, single nucleotide polymorphism

INTRODUCTION

The aging of human populations is the most significant demographic change of the twentieth century. Despite the fact, that the population aging is particularly noticeable in the industrialized countries, a rapid increase of elder people is expected in populations of developing countries. Kazakhstan is one of the states with accelerated speed of aging. According to the United Nations data, every fourth person in Kazakhstan will represent the older population by 2050.

Aging is a complex biological process determined by genetic and environmental factors. Aging processes are associated with the accumulation of toxic metabolites, damages of biologically important molecules, and increased predisposition to the development of a number of pathological conditions. Age-related pathologies include cancer, cardiovascular, neurodegenerative, autoimmune diseases, diabetes, obesity, and other. It is known that the mode and speed of aging vary among different people due to individual genetic characteristics (Wheeler and Kim, 2011). The important areas of aging medicine are the elucidation of genetic and molecular mechanisms of aging including the role of genetic and epigenetic factors in the etiology and pathogenesis of various age-related pathologies. The development of age-related diseases or the ability to take an active longevity is greatly influenced by ethnicity (Gavrilov and Heuveline, 2003). A large number of centenarians are known for some ethnic groups, for example, Caucasian (Caucasian is also used for white-skinned people) people. The determination of genetic features of centenarians and genetic status of key genes involved in the pathogenesis of age-related pathologies represent the main approaches to define the key components of active aging and longevity.

Candidate genes participating in aging can be classified as following: (1) genes involved in tissue homeostasis (apoptosis and telomerase); (2) genes controlling integrity of genome and DNA repair; (3) genes involved in stress resistance (heat shock and oxidation); (4) genes contributing epigenetic changes (methylation, carbonylation, and nitrosylation).

Researchers are mainly focused on the genes whose orthologs determine longevity in other species and also on the genes responsible for development of the main age-related disorders.

Cancer is primarily a disease of older people where incidence rates increased substantially with age for most cancers. The genetic components of cancer overlays the range of candidate genes controlling aging. During the multistage carcinogenesis process, the cells predisposed to cancer accumulate mutations of proto-oncogenes, tumor suppressor genes, and other genes that are directly or indirectly involved in regulation of cell proliferation, survival, and migration.

Mutations of the same gene may cause the several cancer types. Thus, mutations of tumor suppressor gene *TP53* were detected in the tumors of all tissues and organs. The spectrum of mutations in key genes involved in the control of genome instability, DNA repair, cell cycle, apoptosis, and such processes as xenobiotics detoxification may vary for different cancer types.

As a result of natural selection the genetic polymorphism spectrum depends on the geographical conditions, diet, and ethnicity. In certain circumstances, genetic polymorphisms can predispose to the development of specific diseases, or to protect organism. Analysis of genomic polymorphism, which forms the basis of predictive medicine, helps to identify the individual genotypes

that predispose to the development of diseases (Baranov, 2009; Yuzhalin and Kutikhin, 2012).

Here, we present the results of molecular epidemiological study of populations from Kazakhstan representing healthy individuals and patients with esophageal and cervical cancers. These cancer types have been selected in our study because of their high morbidity and mortality in Kazakhstan. Esophageal cancer is one of the most aggressive forms of cancer. It is ranked on the ninth place by malignancy and on the seventh place by mortality. Esophageal cancer often diagnosed at an advanced stage, and therefore the 5-year survival rate for this type of cancer is only in a range of 5–10%. The incidence of esophageal cancer in males reaches 25.7 cases in population of 100,000.

Cervical cancer in women is diagnosed in the reproductive age. Kazakhstan is among the countries with high levels of cervical cancer and its incidence is on the second place following the breast cancer.

Identification of genetic markers for these types of cancer will help to determine strategies of prevention, early diagnosis, and personalized treatment.

The choice of candidate genes for our study has been selected based on the previous studies (Tan et al., 2000; Gao et al., 2002; Dumont et al., 2003; Zhang et al., 2003, 2012; Abbas et al., 2004; Lu et al., 2006; Cescon et al., 2009; Francisco et al., 2010; Barbisan et al., 2011; Liu and Xu, 2012 and others) showing the strong association with development of different cancer types including esophageal and cervical cancers.

We studied the following genetic markers: (1) deletion polymorphism of genes participating in second phase of xenobiotic detoxification – glutathione-*S*-transferases – *GSTM1* and *GSTT1*; (2) two types of single nucleotide polymorphism (SNP) of *XRCC1* (Arg194Trp and Arg399Gln), responsible for the repair of double strand DNA breaks; (3) SNP of *XRCC3* (Thr241Met), responsible for the repair of single strand DNA breaks; (4) SNP of gene regulating cell cycle and apoptosis – *TP53* (Arg72Pro); (5) SNP of cell cycle regulating gene cyclin D1 – *CCND1* (A870G).

MATERIALS AND METHODS

SAMPLING

This "case-control" study was approved by the Ethics Committee of the Asfendiyarov Kazakh National Medical University (Almaty, Kazakhstan). The material was collected in the Kazakh Research Institute of Oncology and Radiology (Almaty, Kazakhstan) by approbation of the patients. We examined clinical material (blood, buccal smears, biopsy materials, cervical smears) obtained from 115 esophageal cancer patients and 207 cervical cancer patients. The control groups of healthy individuals (100 and 160 respectively) were selected according to the ethnic background and age of the esophagus and cervical cancer patients. Detailed questionnaires and informed consents were filled prior collection of samples. The clinical diagnosis of cancer patients was verified by the cytological or histological methods using biopsy materials.

DNA ISOLATION

DNA samples were extracted by standard phenol-chloroform method with modifications in lysis buffer composition (for blood samples: 0.2 M sodium acetate and 1% sodium dodecyl sulfate, pH 8.0; for tissue: 0.02 M ethylenediaminetetraacetic acid (EDTA); 0.02 M Tris-HCl, pH = 8.0; 0.16 M NaCl; 0.3% sodium dodecyl sulfate, 1 U of protease E). Water diluted DNA samples were used for all type of polymerase chain reaction (PCR).

GENOTYPING BY SITE-SPECIFIC PCR AMPLIFICATION

The genotyping of *GSTM1* and *GSTT1* deletion polymorphisms was carried out by multiplex PCR amplification. The method of site-specific PCR amplification followed by restriction of amplified fragments was used for the genotyping of *XRCC1* Arg194Trp; *XRCC1* Arg399Gln, *XRCC3* Thr241Met, and *TP53* Arg72Pro SNPs. Twenty to one hundred nanogram of target DNA was amplified in total volume of 20 μl of PCR mixtures using amplifier "Mastercycler" (Eppendorf). PCR mixture contains 15 pM of each specific primer, 10 mM of each dNTP, 2 μl of 10× PCR buffer (10 mM KCl, 100 mM Tris-HCl, pH 9.0), and 0.5 U of Taq-polymerase (Sigma-Aldrich). The 1.4% agarose gel electrophoresis and Lambda/*Hin*dIII DNA marker (Sigma-Aldrich) were used for detection of the amplified DNA fragments length. The PCR products were digested at 37°C for 8–16 h with 1–3 U corresponding restriction enzymes (Fermentas, Lithuania). Restriction products were analyzed using 3% agarose MetaPhor (Lonza) gel. The PCR details and corresponding references are represented in **Table 1**.

GENOTYPING BY DIRECT SEQUENCING

The method of direct sequencing was applied for genotyping of *CCND1* A870G polymorphism for all DNA samples. This method also was used for some DNA samples representing esophageal cancer in the case of determination of *TP53* Arg72Pro polymorphism. Previously we performed the PCR amplification of the gene fragments which contain the studied polymorphic sites: for *CCND1* (281 bp) and for *TP53* gene (141 bp). The PCR was carried out in a total volume of 25 μl reaction mixture containing 50 ng of target DNA, 0.625 U of ExTaq™ HS enzyme (TaKaRa Biotechnology, Japan), 0.2 mM of each dNTP, 2.5 μl of 10× PCR buffer (100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Non-idet P-40, 50% glycerol, 20 mM Tris-HCl, pH 8.0), and 200 nM of each primer (for the *CCND1* gene – s 5′-CGG GCC GCT TGC TCA GAG-3′ and as 5′-AAG GCT GCC TGG GAC ATC ACC-3′, for *TP53*: gene – s 5′-CGT CCC AAG CAA TGG ATG ATT-3′ and as 5′-CCG GTG TAG GAG CTG CTG G-3′). The PCR amplification conditions consisted of initial denaturation step at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s (for the *CCND1* gene), or 61°C for 30 s (for the *TP53* gene), 72°C for 30 s, and final elongation step at 72°C for 10 min. The 1.5% agarose gel electrophoresis and Lambda/*Hin*dIII DNA marker (Sigma-Aldrich) were used for detection of the amplified DNA fragments length. Amplified DNA fragments was purified from residues of PCR reaction mixture using ExoSAP-IT® (GE Healthcare, USA). To 5 μl of PCR product we added 1 μl of ExoSAP-IT® and incubated at 37°C for 40 min, at 80°C for 20 min, and at 4°C for 10 min. Sequencing of PCR products was carried out using the BigDye® Terminator v3.1 kit (Applied Biosystems) in accordance with standard protocol[1]. Sequence-amplification was carried out in the total volume

[1]http://www.appliedbiosystems.com

Table 1 | The site-specific PCR amplification protocols.

Genes	Primers for PCR	PCR conditions	The length of amplified fragments (bp)	Restriction enzyme	Restricted products length and corresponding genotype	Reference
GSTM1	s 5′-GAACTCCCTGAA AAGCTAAAG C-3′, as 5′-GTTGGGCTCAAATATACGGTGG-3′	Initiation denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 2 min, 59°C for 1 min, 72°C for 1 min and final elongation step at 72°C for 10 min.	215	Not used	–	Abbas et al. (2004)
GSTT1	s 5′-TTCCTTACTGGTCC TCACATCTC-3′, as 5′-TCACCGGATCATGGCCAGCA-3′		480	Not used	–	
β-глобин (as a internal control)	s 5′-CCACTTCATCCACGTTCACC-3′, as 5′-GAAGAGCCTAGGACAGGTAC-3′		268	Not used	–	
XRCC1 Arg194Trp	s 5′-GCCCCGTCCCAGGTA-3′, as 5′-AGCCCCAAGACCCTTT-3′	Initiation denaturation step at 95°C for 2 min, followed by 40 cycles of 94°C for 15 s, 57°C for 45 s, 72°C for 45 s and final elongation step at 72°C for 5 min	490	*Pvu*II, 10 × Tango buffer	Arg/Arg – 490 bp; Arg/Trp – 490, 294, and 196 bp; Trp/Trp – 294 and 196 bp	Au et al. (2003)
XRCC1 Arg399Gln	s 5′-CAAGTACAGCCAGGTCCTAG-3′, as 5′-CCTTCCCTCATCTGGAGTAC-3′	Initiation denaturation step at 95°C for 2 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, 72°C for 45 s and final elongation step at 72°C for 5 min	248	*Bcn1*, 10 × Tango buffer	Arg/Arg – 159 and 89 bp; Arg/Gln – 248, 159, and 89 bp; Gln/Gln – 248 bp	Au et al. (2003)
XRCC3 Trp241Met	s 5′-GCCTGGTGGTCATCGACTC-3′, as 5′-ACAGGGCTCTGGAAGGCACTGCTCAGC TCACGCACC-3′	Initiation denaturation step at 94°C for 3 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°Cfor 1 min and final elongation step at 72°C for 5 min	136	*Nco1*, 10 × Tango buffer	Trp/Trp – 136 bp, Trp/Met – 136, 97, and 39 bp; Met/Met – 97 and 39 bp	Au et al. (2003)
TP53 Arg72Pro	s 5′-TGAGGACCTGGTCCTCTGAC-3′, as 5′-AGAGGAATCCCAAAGTTCCA-3′	Initiation denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 30 s and final elongation step at 72°C for 5 min	412	*Bsh*1236I *(Acc II)*, 10 × r buffer	Arg/Arg – 252 and 160 bp; Arg/Pro – 412, 252 and 160 bp; Pro/Pro – 412 bp	Lu et al. (2004)

20 μl reaction mixture containing 3 μl of PCR product, 4 μl of 5 × BigDye® buffer, 0.5 μl ready mix BigDye® Terminator v3.1, 3.2 pM of specific forward primer (for gene *CCND1*: s 5′-CGG GCC GCT TGC TCA GAG-3′and for the gene *TP53*: s 5′-CGT CCC AAG CAA TGG ATG ATT-3′).

Sequence-PCR conditions were the same for *CCND1* and *TP53* genes: initial step at 94°C for 2 min, followed by 30 cycles of 96°C for 30 s, 60°C for 4 min, and final elongation step at 4°C for 10 min. Sequence-PCR products were filtered using Sephadex™ G-50 (Amersham Biosciences) by centrifugation at 1000 × *g* for 3 min. Then 20 μl of formamide was added to the purified sequence-PCR product and denatured at 95°C for 2–3 min followed by cooling on ice. Genotyping of the products obtained sequencing was performed using capillary analyzer ABI PRISM® 3130 (Applied Biosystems). Data analysis was carried out using the program FinchTV (Geospiza, USA).

GENOTYPING BY THE TaqMAN ALLELIC DISCRIMINATION METHOD

Genotyping of *TP53* Arg72Pro polymorphism of some esophageal cancer samples was also carried out by the TaqMan allelic discrimination method. We used the specially synthesized probes (Applied Biosystems) containing the fluorescent reporter dye on the 5′-end and the quencher dye on the 3′-end. The probe complementary to the Pro72 allele was labeled by FAM™ dye, the probe complementary to the Arg72 allele – by VIC™ dye. Amplification of *TP53* gene fragment (141 bp) containing the polymorphic site Arg72Pro was performed by real-time PCR using a thermocycler iCycler iQ5 (Bio-Rad). The amplification was carried out in a total volume of 25 μl reaction mixture containing 50 ng of target DNA, 12.5 μl of universal PCR-mix TaqMan® (Applied Biosystems), 10 μM of each primers (s 5′-CGT CCC AAG CAA TGG ATG ATT-3′ and as 5′-CCG GTG TAG GAG CTG CTG G-3′), and 14 μM of each probe [for the Pro72 (FAM) allele – 5′-CTC CCC GCG TGG CCC C-3′ and for the Arg72 (VIC) allele – 5′-CTC CCC CCG TGG CCC C-3′]. The real-time PCR conditions consisted of initiation denaturation step at 50°C for 2 min and 95°C for 10 min followed by 35 cycles of 95°C for 15 s and 61°C for 1 min, and final step at 4°C for 10 min. Fluorescence end point detection and analysis of data were performed using ABI PRISM® 7700 Sequence Detection System (Applied Biosystems) and supporting Software.

STATISTICAL ANALYSIS

The allele frequencies was calculated in accordance with standard Hardy–Weinberg equilibrium: $p^2 + 2pq + q^2 = 1$, where p – the frequency of allele 1 and q – the frequency of allele 2.

To estimate the relative risk of cancer development we use the method of odd ratio (OR) calculation for case-control epidemiologic study taking into account the dominant and recessive models (Jedrychowski and Maugeri, 2000). In general model OR is calculated for each genotype (11, 12, 22) separately. According to the dominant model OR is calculated comparing the normal homozygous (11) versus combination of mutant homozygous (22) with heterozygous (12). The recessive model: combination of normal homozygous (11) with heterozygous (12) versus mutant homozygous (22). An approximate index of relative risk, or OR, is the ratio of disease development chances among persons, exposed and did not exposed to some factor (in our case this is genotype). OR, close to 1, indicates the absence of the genotype effect on disease development. More than 1 OR value indicates the influence of genotype on development of disease, and less than 1 OR value indicates a positive effect of genotype on health. OR may be calculated by the following formula:

$$\mathrm{OR} = ad/bc, \tag{1}$$

where

a – The number of persons in case group (patients with disease) having genotype 1;
b – the number of persons in case group having genotype 2;
c – the number of persons in control group (healthy persons) having genotype 1;
d – the number of persons in control group having genotype 2.

The estimates of 95% confidence intervals (CI) can be computed from the following formula:

$$\text{Lower 95\% CI} = \exp\left(\ln \mathrm{OR} - 1.96\sqrt{\left(1/a + 1/b + 1/c + 1/d\right)}\right)$$

$$\text{Upper 95\% CI} = \exp\left(\ln \mathrm{OR} + 1.96\sqrt{\left(1/a + 1/b + 1/c + 1/d\right)}\right)$$

To verify the significance (p-values) of the observed differences between case and control groups, we performed the standard χ^2 test. An alpha error (P) of less than 0.05 was used as the criterion of significance.

All statistical analysis of the obtained data was performed using GraphPad InStat™ Software (V. 2.04. Ralf Stahlman, Purdue University) and "Case-Control Study Estimating Calculator" from TAPOTILI company (Laboratory of Molecular Diagnostics and Genomic Dactiloscopy of "GosNII Genetika" State Scientific Centre of Russian Federation[2]).

RESULTS

A DISTINCT POLYMORPHISM ASSOCIATES WITH ESOPHAGEAL CANCER

All 115 patients representing the esophageal cancer group were diagnosed as the squamous-cell carcinoma cancer type. In this group, the high differentiated carcinoma was detected in 8 patients, the moderately differentiated carcinoma in 48 patients, and the low-grade differentiated carcinoma in 59 patients. The control cohort represented the healthy people without any noticeable pathologies was matched to case cohort by the age, sex, and ethnicity, and smoking habit (**Table 2**).

Genotyping of the candidate genes (deletions *GSTM1* and *GSTT1*, *XRCC1* Arg194Trp and Arg399Gln, *XRCC3* Thr241Met, *TP53* Arg72Pro, and *CCND1* A870G) was performed for the case and control cohorts. Frequencies of the allele variants are shown in **Table 3**.

[2]http://www.tapotili.ru

Table 2 | The correspondence of the esophageal cancer case and control cohorts by age, ethnicity, sex, and smoking habit.

Cohort	Years of birth	Nationality, persons (%)		Sex, persons (%)		Smoking habit, persons (%)	Total, persons
		Kazakh	Russian	Male	Female		
Case	1920–1977	102 (88.69)	13 (11.31)	62 (53.91)	53 (46.09)	30 (26.08)	115
Control	1921–1976	89 (89.00)	11 (11.00)	54 (54.00)	46 (46.00)	26 (26.00)	100

Table 3 | The frequencies of alleles of candidate genes in control and case cohorts for esophageal cancer.

Polymorphism	The allele variant	The frequency of allele	
		Control cohort	Case cohort
GSTT1	Functional (+)	0.365	0.178
	Deletion (−)	0.635	0.822
GSTM1	Functional (+)	0.510	0.204
	Deletion (−)	0.490	0.796
XRCC1 Arg194Trp	194Arg	0.855	0.852
	194Trp	0.145	0.148
XRCC1 Arg399Gln	399Arg	0.725	0.657
	399Gln	0.275	0.343
XRCC3 Trp241Met	241Trp	0.815	0.822
	241Met	0.185	0.178
TP53 Arg72Pro	72Arg	0.760	0.657
	72Pro	0.240	0.343
CCND1 A870G	870A	0.450	0.404
	870G	0.550	0.596

Genotyping of the candidate genes shows no contradictions with the Hardy–Weinberg equilibrium. However, the frequencies of allele variants differ between the control group and the group representing esophageal cancer patients. We also performed the statistical analysis of association between genetic polymorphism and development of esophageal cancer. The statistical powers of the general, dominant, and recessive models were evaluated for each type of polymorphism. **Table 4** shows the adjusted association of candidate gene polymorphisms, which calculated by general model of inheritance for each genotype separately.

The prevalence of *GST*-deletions (−/−, "null" – genotype) was observed in esophageal cancer cases cohort. Our data show significant association of "null" *GST*-genotypes (−/−) with susceptibility to esophageal cancer for *GSTM1* (OR = 5.30) and *GSTT1* (OR = 3.29) genes. These findings are also confirmed by the dominant (+/+ versus combination of ±and −/− genotypes) and recessive models (−/− versus combination of +/+ and ±genotypes) of OR calculation. Thus, according to the dominant model the risk of esophageal cancer development was significantly higher for the following combinations of genotypes: *GSTM1* (± and −/−) (OR = 9.75, $p < 0.0001$); and *GSTT1* (± and −/−) (OR = 3.29, $p = 0.02$). The recessive model corresponds to the results of general model of inheritance: for the *GSTM1* −/− (OR = 5.30, $p < 0.0001$) and *GSTT1* −/− (OR = 3.29, $p < 0.0001$). The presence of the functional allele variants of *GSTM1* and *GSTT1* genes in homozygous states shows a strong protective effect (for *GSTM1* +/+ genotype – OR = 0.10 and for *GSTT1* +/+ genotype – OR = 0.30).

The difference of *XRCC1* Arg194Trp polymorphism genotypes distribution between control and esophageal case cohorts was not significant. The *XRCC1* Trp194Trp homozygote did not show a statistically reliable association with esophageal cancer (OR = 3.57, $p = 0.39$). Application of the dominant and recessive model of OR calculation did not reveal any significant risk. We have noticed a similar relationship following analysis of the *XRCC1* polymorphism – Arg399Gln that indicated OR values [OR = 2.54, $p = 0.2$ (general model of inheritance); OR = 1.39, $p = 0.23$; dominant model] were not statistically significant. On the contrary, analysis of the homozygote genotype *XRCC3* Met241Met (OR = 7.40, $p = 0.02$) detected a strong linkage to the esophageal cancer progression. This finding has been supported by the dominant model. Instead, the protective effect (OR = 0.52, $p = 0.02$) was observed with the heterozygous genotype *XRCC3* Trp241Met.

Comparison of the *TP53* Arg72Pro genotypes distribution between control and case cohorts shows the prevalence of Pro/Pro homozygous (13 versus 5%) and Arg/Pro heterozygous (43 versus 38%) among esophageal cancer patients. The association of *TP53* Pro72Pro genotype with susceptibility to esophageal cancer has been analyzed by the total model of inheritance – OR = 2.85, $p = 0.06$. The presence of Arg in 72 codon of *TP53* gene reduced the risk: OR = 1.66, $p = 0.06$ (dominant model – Pro72Pro in combination with Arg72Pro); for Arg72Pro genotype – OR = 1.21, $p = 0.06$. Whereas, a strong protective effect has been observed for the Arg72Arg genotype – OR = 0.60, $p = 0.06$.

Substantial prevalence of *CCND1* A870A homozygous has been detected in the esophageal cancer case cohort (38 versus 18% in control). The *CCND1* A870A genotype is statistically reliable to determine its susceptibility to esophageal cancer (OR = 2.82, $p = 0.004$). Combination with *CCND1* G870A genotype (dominant model) reduces the risk: OR = 1.64, $p = 0.12$. The G870G genotype demonstrates strong protective effect: OR = 0.61, $p = 0.004$.

ASSOCIATION OF GENOMIC POLYMORPHISM WITH DEVELOPMENT OF CERVICAL CANCER

All 217 women representing cervical cancer case cohort were cytologically or histologically examined for cancer type. Squamous-cell carcinoma (cancer *in situ*) is the predominant histotype in selected cohort. Within this cohort, 15 patients were at the stage I, 167 patients were at the stage II, 26 patients were at the stage III (invasive), and 9 patients were at the stage IV (invasive, metastatic).

The control cohort of healthy women was selected taking into account the personal data of patients suffering from cervical

Table 4 | Association between genetic polymorphism and development of esophageal cancer.

Type of polymorphism	Genotype	Esophageal cancer, persons (%)	Control, persons (%)	Odds ratio (OR)	Confidence interval (CI), (95%)	$\chi 2$	*p*
GSTT1	+/+	5 (4.35)	13 (13.00)	0.30	0.10–0.89	18.66	<0.0001
	±	31 (26.96)	47 (47.00)	0.42	0.24–0.74		
	−/−	79 (68.69)	40 (40.00)	3.29	1.88–5.77		
GSTM1	+/+	4 (3.48)	26 (26.00)	0.10	0.03–0.31	40.64	<0.0001
	±	39 (33.91)	50 (50.00)	0.51	0.30–0.89		
	−/−	72 (62.61)	24 (24.00)	5.30	2.93–9.61		
XRCC1 Arg194Trp	Arg/Arg	85 (73.91)	72 (72.00)	1.11	0.60–2.01	1.86	0.39
	Arg/Trp	26 (22.61)	27 (27.00)	0.79	0.42–1.47		
	Trp/Trp	4 (3.48)	1 (1.00)	3.57	0.39–32.46		
XRCC1 Arg399Gln	Arg/Arg	47 (40.87)	49 (49.00)	0.72	0.42–1.23	3.24	0.2
	Arg/Gln	57 (49.57)	47 (47.00)	1.11	0.65–1.90		
	Gln/Gln	11 (9.56)	4 (4.00)	2.54	0.78–8.24		
XRCC3 Thr 241Met	Trp/Trp	82 (71.30)	64 (64.00)	1.40	0.79–2.48	8.32	0.02
	Trp/Met	25 (21.74)	35 (35.00)	0.52	0.28–0.94		
	Met/Met	8 (6.96)	1 (1.0)	7.40	0.91–60.25		
TP53 Arg72Pro	Arg/Arg	51 (44.38)	57 (57.00)	0.60	0.35–1.03	5.71	0.06
	Arg/Pro	49 (42.61)	38 (38.00)	1.21	0.70–2.09		
	Pro/Pro	15 (13.04)	5 (5.00)	2.85	1.00–8.15		
CCND1 A870G	G/G	22 (19.13)	28 (28.00)	0.61	0.32–1.15	10.87	0.004
	G/A	49 (42.61)	54 (54.00)	0.63	0.37–1.08		
	A/A	44 (38.26)	18 (18.00)	2.82	1.50–5.32		

Table 5 | The correspondence of the cervical cancer case and control cohorts by age, ethnicity, and smoking habit.

Cohort	Years of birth	Nationality, persons (%)		Smoking habit, persons (%)	Total, persons
		Kazakh	Russian		
Case	1945–1990	176 (81.11)	41 (18.89)	10 (4.61)	217
Control	1942–1987	128 (80.00)	32 (20.00)	8 (5.00)	160

cancer. The age, ethnicity, and smoking habit data of cervical cancer case and control groups are represented in **Table 5**.

DNA samples representing the cervical cancer case and control cohorts were genotyped for detection of different types of gene polymorphisms: deletions *GSTM1* and *GSTT1*, *XRCC1* Arg194Trp and Arg399Gln, *XRCC3* Thr241Met, *TP53* Arg72Pro, and *CCND1* A870G.

The genotyping results revealed that the distribution of genotypes in the control and case cohorts follows to Hardy–Weinberg equilibrium. Frequencies of allele variants are summarized in **Table 6**.

Comparison of the candidate genes allele frequencies in cohorts of healthy women and women suffering from cervical cancer shows the differences including a prevalence of the *GST*-deletions and rare allele variant *XRCC3* 241Met in case cohort. The data of statistical analysis of associations between the studied gene polymorphisms and susceptibility to cervical cancer, which calculated for each genotype separately by general genetic model, are presented in **Table 7**.

Deletion of *GSTT1* in homozygous state (−/−) shows the significant association with susceptibility to cervical cancer (OR = 3.99, $p = 0.0$). *GSTT1* "null" genotype in combination with heterozygous genotype (−/− and ±) significantly increases the risk: in accordance with dominant model of OR calculation – OR = 9.45, $p = 0.0$. The *GSTM1* "null" genotype also shows strong association with development of cervical cancer (OR = 6.50, $p < 0.0001$). But the *GSTM1* functional allele presence in genotype reduces the risk. In accordance with dominant model for the combination of *GSTM1* genotypes (± and −/−) – OR = 2.66, $p < 0.0001$. The presence in genotype of the functional allele variants of *GSTM1* and *GSTT1* genes in homozygous states shows strong protective effect (for *GSTM1* +/+ genotype – OR = 0.38 and for *GSTT1* +/+ genotype – OR = 0.11).

The *XRCC1* 194Arg allele variant shows association with susceptibility to cervical cancer in homozygous (Arg194Arg) and heterozygous (Arg194Trp) states. According to general model of inheritance for Arg194Arg genotype – OR = 1.58, $p = 0.01$. According to the dominant model for combinations of genotypes (Arg194Arg and Arg194Trp) – OR = 3.64, $p = 0.006$.

Table 6 | The frequencies of alleles of candidate genes in control and case cohorts for cervical cancer.

Polymorphism	The allele variant	The frequency of allele	
		Control cohort	Case cohort
GSTT1	Functional (+)	0.544	0.230
	Deletion (−)	0.456	0.770
GSTM1	Functional (+)	0.850	0.677
	Deletion (−)	0.150	0.323
XRCC1 Arg194Trp	194Arg	0.781	0.862
	194Trp	0.219	0.138
XRCC1 Arg399Gln	399Arg	0.694	0.634
	399Gln	0.306	0.366
XRCC3 Trp241Met	241Trp	0.875	0.776
	241Met	0.125	0.224
TP53 Arg72Pro	72Arg	0.572	0.647
	72Pro	0.428	0.353
CCND1 A870G	870A	0.500	0.491
	870G	0.500	0.509

Polymorphism of 399 codon of *XRCC1* gene also shows association with susceptibility to cervical cancer. For the homozygous genotype Gln399Gln – OR = 3.96, $p = 0.03$. The presence of 399Arg allele variant in genotype reduces the risk, but not statistically reliably: OR = 1.25, $p = 0.90$ (dominant model – Gln399Gln in combination with Arg399Gln). The protective effect of *XRCC1* Arg399Arg genotype is weakly expressed (OR = 0.80, $p = 0.03$).

Another DNA repair gene *XRCC3* demonstrates the strong association between Trp241Met polymorphism and susceptibility to cervical cancer. The risk is expressed for homozygotes Met241Met: OR = 3.96, $p = 0.006$. In combination with heterozygous genotypes (dominant model – Met241Met and Trp241Met versus Trp241Trp) the risk is significantly reduced: OR = 1.89, $p = 0.007$. The Trp241Trp genotype shows the statistically reliable protective effect: OR = 0.53, $p = 0.006$.

Analysis of *TP53* Arg72Pro polymorphism genotypes distribution in control and cervical cancer case cohorts shows that Arg72Arg genotype can increase risk of cervical cancer development (OR = 1.46, $p = 0.08$), but not significantly. The risk is increased (OR = 1.89, $p = 0.06$) in combination with heterozygous genotype (dominant model of inheritance – Arg72Arg and Arg72Pro versus Pro72Pro). *TP53* Pro72Pro genotype demonstrates the protective effect (OR = 0.55, $p = 0.08$).

The *CCND1* G870A polymorphism does not show significant differences in genotype distribution among healthy women and the cervical cancer patients. We also did not observe association of this variation with susceptibility to cervical cancer.

Table 7 | Association between genetic polymorphism and development of cervical cancer.

Type of polymorphism	Genotype	Cervical cancer, persons (%)	Control, persons (%)	Odds ratio (OR)	Confidence interval (CI), (95%)	χ2	*p*
GSTT1	+/+	12 (5.53)	57 (35.62)	0.11	0.05–0.21	67.15	0.00
	±	76 (35.02)	60 (37.50)	0.90	0.59–1.37		
	−/−	129 (59.45)	43 (26.88)	3.99	2.56–6.21		
GSTM1	+/+	108 (49.77)	116 (72.50)	0.38	0.24–0.58	25.31	<0.0001
	±	78 (35.94)	40 (25.00)	1.68	1.07–2.65		
	−/−	31 (14.29)	4 (2.5)	6.5	2.25–18.81		
XRCC1 Arg194Trp	Arg/Arg	163 (75.12)	105 (65.63)	1.58	1.01–2.48	8.72	0.01
	Arg/Trp	48 (22.12)	40 (25.00)	0.85	0.53–1.38		
	Trp/Trp	6 (2.76)	15 (9.37)	0.27	0.10–0.73		
XRCC1 Arg399Gln	Arg/Arg	78 (35.94)	66 (41.25)	0.80	0.53–1.22	7.24	0.03
	Arg/Gln	119 (54.84)	90 (56.25)	0.94	0.63–1.42		
	Gln/Gln	20 (9.22)	4 (2.50)	3.96	1.33–11.82		
XRCC3 Trp241Met	Trp/Trp	140 (64.51)	124 (77.50)	0.53	0.33–0.84	10.28	0.006
	Trp/Met	57 (26.27)	32 (20.00)	1.43	0.87–2.33		
	Met/Met	20 (9.22)	4 (2.50)	3.96	1.33–11.82		
TP53 Arg72Pro	Arg/Arg	85 (39.17)	49 (30.63)	1.46	0.95–2.25	5.15	0.08
	Arg/Pro	111 (51.15)	85 (53.12)	0.92	0.61–1.39		
	Pro/Pro	21 (9.68)	26 (16.25)	0.55	0.30–1.02		
CCND1 A870G	G/G	54 (25.12)	41 (25.62)	0.97	0.61–1.56	0.09	0.96
	G/A	103 (47.91)	78 (48.75)	0.97	0.64–1.46		
	A/A	58 (26.97)	41 (25.63)	1.07	0.67–1.71		

DISCUSSION

Aging is a complex process of functional decline and increased disease risk that has been resulted from accumulation of DNA mutations. Series of mutations in key regulatory genes are the main reason of cancer induction. Genes involved into the control of genome instability, DNA repair, cell cycle regulation, apoptosis, and such processes as xenobiotics detoxification, are the main candidate for aging and carcinogenesis. Mutations affecting the functions of these genes cause the range of abnormalities. Polymorphic variants of their DNA sequences can modify the functions of genes and predispose to the disease development in combination with other genetic and environmental factors.

The frequency of alleles of polymorphic genes is determined by natural selection. Many factors affect the genomic polymorphism spectrum in populations, such as geographical location, ethnicity, type of diet, habits, etc. The ethno-genetic status, age, the radiation background, and bad habits strongly influence on mutagenic processes.

Numerous of molecular epidemiological studies have been devoted to finding biomarkers of age-related diseases. However, the revealing of reliable association between polymorphic allele variant and susceptibility to disease depends on allele frequency in population. The high frequency of allele facilitates the identification of the association with high probability. In the case of rare allele the detection of association is more complicated, and it requires an increase of sample size.

Epidemiological studies require a careful selection of the control group for the research, especially for small sample sizes. The control cohort should correspond to case cohort on many parameters. Matching control can help to identify the reliable association between genetic polymorphism and risk of disease in the cases of small sample sizes or rare allele frequency.

The studied cohorts represent inhabitants of Almaty city (Kazakhstan). Radiation background in Almaty is not significant and this factor has not been considered in our study. However, smoking and age are known risk factors for many cancer types. Also, the case cohorts representing patients suffering from esophageal and cervical cancer are mixed by ethnicity. The majority of both case cohorts are Kazakhs (about 80%), but there are Russians too (about 20%). To minimize the effects of ethnicity, age, and smoking influence on the susceptibility to studied cancer types, we have selected the healthy control groups matched to the corresponding case groups (**Tables 2** and **5**).

The genotyping on candidate gene polymorphisms allowed us to determine all possible genotypes in the studied control and case cohorts in accordance with Hardy–Weinberg equilibrium.

Because these types of genetic polymorphisms were first studied for Kazakh populations, we compared the obtained frequency of allele variants in control cohorts with data presented in NCBI SNP database and literature (d'Errico et al., 1999; Ketterer et al., 2007; Gao et al., 2011). For the increasing of sample size we have combined both control cohorts because all investigated persons were healthy inhabitants of Almaty city. The integrated data presented in **Table 8**.

The frequencies of *GSTT1* deletions in healthy residents of Almaty city (0.525) are more similar to Asians (0.80–0.540). The *GSTM1* deletions are widely distributed among Asian (0.490–0.540) and European (0.420–0.540) peoples with similar rates. But in our study the frequency of *GSTT1* deletions was low: 0.281. The low frequencies of *GSTT1* deletions have been suggested for African populations (0.160–0.360) (d'Errico et al., 1999; Ketterer et al., 2007; Gao et al., 2011).

The data on frequencies of rare *XRCC1* 399Gln, *XRCC3* 241Met, and *CCND1* 870G alleles in healthy residents of Almaty city do not contradict to experimental data obtained from the analysis of most Asian and European populations (**Table 8**).

The rates of *XRCC1* 194Trp (0.190) and *TP53* 72Pro (0.356) alleles do not correspond to the populations from Europe and Asia (**Table 8**). One of the possible explanations is the mixed ethnic composition of Almaty city residents (**Tables 2** and **5**): 80% Kazakh (Asians) and 20% Russian (Europeans). Also it should be noted that most of studied populations from Asia, represented in NCBI SNP database and other sources (Chinese, Japanese, Malaysian, etc.), were distinct from Kazakh population.

Identified associations between candidate genes polymorphism and esophageal and cervical cancer are not surprising. Glutathione *S*-transferases (*GST*s), a multigene family of phase II metabolic enzymes, are active in the detoxification of a wide variety of potentially toxic and carcinogenic substances by conjugating them to glutathione. Deletions of *GST*-genes

Table 8 | The comparison of rare allele frequencies of healthy inhabitants of Almaty city with earlier studied populations.

Polymorphism	The rare allele variant	The frequency of allele		
		Healthy residents of Almaty city (260 persons)	Integrated data from different sources	
			Asian populations	European populations
GSTT1	Deletion	0.525	0.480–0.540	0.160–0.385
GSTM1	Deletion	0.281	0.490–0.540	0.420–0.540
XRCC1 Arg194Trp	194Trp	0.190	0.239–0.289	0.092–0.093
XRCC1 Arg399Gln	399Gln	0.294	0.274–0.279	0.303
XRCC3 Trp241Met	241Met	0.150	0.000–0.148	0.000–0.417
TP53 Arg72Pro	72Pro	0.356	0.409–0.511	0.233
CCND1 A870G	870G	0.481	0.456–0.656	0.475–0.483

are associated with susceptibility to many cancer types. The previous study (Tan et al., 2000; Gao et al., 2002; Lu et al., 2006; Liu and Xu, 2012) reported that deletions of *GSTT1* and *GSTM1* genes play a significant role in development of esophageal or cervical cancers. Most of these studies were carried on Chinese and Caucasian populations. Association of *GSTT1* and *GSTM1* deletions with esophageal and cervical cancer susceptibility is supported by data obtained by studying populations from India, Korea, Turkey, Great Britain, Italy, USA, and other countries (Ketterer et al., 2007; Gao et al., 2011; Zhang et al., 2012). Our results demonstrate that *GSTT* and *GSTM* "null" genotypes are strongly associated with susceptibility to esophageal and cervical cancers in population from Kazakhstan (Almaty city).

But some data obtained on different populations from Japan, Brazil, Thailand, and Greece were distinct from our results (Morita et al., 1997; Rossini et al., 2007; Gao et al., 2011; Zhang et al., 2012). This fact can be explained by the insufficient knowledge about influence of *GST*-deletions on development of esophageal and cervical cancer or the distinct ethnic backgrounds or accounting the risk related factors, such as smoking, chemotherapy, or radiation therapy.

There are many opinions about influence of *XRCC1* (X-ray repair complementing defective repair in Chinese hamster cells 1) and *XRCC3* (X-ray repair complementing defective repair in Chinese hamster cells 3) genes on different cancer types. These genes participate in excision repair of bases and repair of single and double strand breaks. The previous studies also point out to the relation of *XRCC1* (Arg399Gln, Arg194Trp) and *XRCC3* Trp241Met polymorphisms with colorectal cancer, skin cancer, lung cancer (Cui et al., 2012; Zhang et al., 2012), and others.

There are data confirming the participation of *XRCC1*-genes polymorphism to cervical cancer (Li et al., 2012). Interestingly, that Barbisan et al. (2011) have made a conclusion, that Arg194Trp polymorphism may be associated with cervical cancer risk, Arg399Gln polymorphism might be a low-penetrant risk factor for cervical cancer only at Asians. The meta-analysis of 16 studies (Li et al., 2012) found out that there were no obvious associations of *XRCC1* Arg399Gln polymorphism with cervical cancer risk. But in the subgroup analyses by ethnicity/country, a significantly increased risk was observed among Asian, especially among Chinese. The study of one Chinese population (Yu et al., 2004) shows the strong association between *XRCC1* Gln399Gln genotype and squamous-cell carcinoma of esophagus, and the smoking people have 4.2-fold increased risk in comparison with not smoking persons.

We found out that *XRCC1* Arg194Trp polymorphism had been associated with esophageal and cervical cancer in Kazakhstan population, but in different manner. *XRCC1* Trp194Trp genotype was associated with susceptibility to esophageal cancer, and *XRCC1* Arg194Arg genotype – with cervical cancer. Interestingly, the data of meta-analysis revealed the protective effect of the *XRCC1* 194Trp allele for tobacco-related types of cancer, which was compatible with the evidence of lower mutagen sensitivity for this allele (Rayjean et al., 2005). Possibly this protective effect of 194Trp allele is related not only tobacco, but also other toxic influences, such as drug and contraceptive treatment. Our research revealed the strong protective effect of *XRCC1* 194Trp allele in cervical cancer patients.

Regarding the *XRCC1* Arg399Gln polymorphism our results show the evidence of associations between *XRCC1* Gln399Gln genotype carriers and increased risk of cervical and esophageal cancer development, which is confirmed by other studies (Yu et al., 2004).

Published data on the relationship of *XRCC3* Trp241Met polymorphism with cancer risk are inconsistent (Au et al., 2003; Konstantinos and Theodoros, 2010; Settheetham-Ishida et al., 2011). However, the most studies show the association of *XRCC3* 241Met allele. But *XRCC3* 241Met allele did not increase the risk of cervical cancer development in the Chinese population (He et al., 2008) and among Thai women (Settheetham-Ishida et al., 2011). Our data demonstrate the strong association between *XRCC3* Met241Met genotype and expressed risk of susceptibility to both cervical and esophageal cancer in Kazakhstan populations.

Mutations and polymorphisms of cell cycle regulating genes (*CCND1* and *TP53*) can play the main role in many types of cancer. Proto-oncogene cyclin D1 is an activator of CDK kinases, whose activity is required for cell cycle G1/S transition. This protein has been shown to interact with tumor suppressor protein Rb and the expression of this gene is regulated positively by Rb. One meta-analysis (Chen et al., 2012) exhibited the statistically significant association between *CCND1* G870A polymorphism and a risk for cancers of the digestive tract, including esophageal cancer (Zhang et al., 2003; Cescon et al., 2009). Another meta-analysis suggested that this polymorphism has no significant association with esophageal cancer risk in the Caucasian or the Asian populations (He et al., 2013). There are no substantial data confirming the correlation of this type of polymorphism with cervical cancer. In our study we have shown that *CCND1* A870A genotype associates with susceptibility to esophageal cancer, but not to cervical cancer.

Polymorphism of *TP53* Arg72Pro can play dual role in cancer development (Francisco et al., 2010). On the one side, protein product of 72Arg allele more effectively induces apoptosis (Dumont et al., 2003). On the other side, 72Pro allele variant provide longevity of being in cell cycle G1-phase in which DNA repair processes are active (Sullivan et al., 2004). Also it was established, that oncoprotein E6 coding by viruses HPV-18 and HPV-16, can interact with p53 protein inducing its degradation. And 72Arg allele faster degradates E6 than 72 Pro (Storey et al., 1998; Tada et al., 2001). Further investigations show contradictive results. Thus, women from Taiwan, Thailand, Korea, Japan, China, and Hong-Kong show no association between *TP53* 72Arg/Pro polymorphism and HPV-associated and HPV-non-associated cervical cancer (Nishikawa et al., 2000; Settheetham-Ishida et al., 2004; Wu et al., 2004 and others). The study of women from India, Brazil, Chili, Peru, and women from Africa show this association (de Araujo and Villa, 2003; Ojeda et al., 2003; Mitra et al., 2005). Study of women in Greece, Holland, and Hungary revealed this positive association (Madeleine et al., 2000; Habbous et al., 2012 and others). And also there are evidences of influence of *TP53* Arg72Pro on development of esophageal cancer (Cescon et al., 2009; Ma et al., 2012). We find out that *TP53* 72Pro allele associates with susceptibility to cervical cancer and 72Arg allele shows strong association with esophageal cancer development.

A large number of molecular epidemiologic studies have been performed to evaluate the role of polymorphisms of *GST*-, *XRCC*-, *TP53*, and *CCND1* genes in various neoplasms. The results of these studies obtained on different populations can be contradictory. The evidence of an association between some rare allele variant and risk of disease can be achieved by the quantitative analysis of available publications – meta-analysis (Yin et al., 2009; Gao et al., 2011; Jiang et al., 2011; Chen et al., 2012; Cui et al., 2012; Li et al., 2012; Zhang et al., 2012; He et al., 2013).

Studies investigating the combined effect of *GST*-deletions, *XRCC1* (Arg194Trp and Arg399Gln), *XRCC3* (Thr241Met), *TP53* (Arg72Pro), and *CCND1* (A870G) will be very important for further evaluate the role of these polymorphism in different cancers. Data of association between seven genetic polymorphism types and two types of age-related cancers obtained on unstudied populations from Kazakhstan can be substantial input for meta-analysis. It is required for understanding the role of studied polymorphisms in the development of age-related pathologies in populations from Eurasia. And also the research results have a high practical significance.

Conducted research allowed to determine the panels of genetic markers of predisposition to the development:

1. Esophageal cancer – deletions of *GSTT1* (OR = 3.29) and *GSTM1* (OR = 5.30) genes; *XRCC3* Met241Met (OR = 7.40); *TP53* Pro72Pro (OR = 2.85), *CCND1* A870A (OR = 2.82).
2. Cervical cancer – deletions of *GSTT1* (OR = 3.99) and *GSTM1* (OR = 6.50) genes; *XRCC1* Arg194Arg (OR = 1.58); *XRCC1* Gln399Gln (OR = 3.83), *XRCC3* Met241Met (OR = 2.84), and *TP53* Arg72Arg (OR = 3.96).

These results are statistically reliable and will be used for developing of test-kits for the defining susceptibility to esophageal and cervical cancers. The introduction of these tests to the screening programs will allow to develop the large-scale preventive measures and will have an impact on reducing cancer incidence and mortality in Kazakhstan, helping to extend the qualitative longevity.

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Effects of seasonal, ontogenetic, and genetic factors on lifespan of male and female progeny of *Arvicola amphibius*

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The water vole (*Arvicola amphibius*) in the forest-steppe of West Siberia is known to have wide fluctuations in abundance. These fluctuations are accompanied by changes in birth and death rates, sex-age structure of the population, and individual morphophysiological and behavioral characteristics of the animals. Survival of the animals depends on season, phase of population cycle, and sex. Based on the data of long-term captive breeding of water voles, the maximal lifespan of males was found to be 1188 days and that of females, 1108 days. There were no differences between the sexes in mean lifespan. The probability of living 2 years or longer was 0.21. Individuals who began breeding at an older age had a significantly longer lifespan and produced more offspring. The survival curves of the spring-born animals were steeper than of those summer-/autumn-born. Maternal factors had a differential effect on males and females with respect to lifespan. Male lifespan correlated negatively with maternal age, parity, and litter size, whereas female lifespan did not correlate with these characteristics. To estimate heritability, parent-offspring correlations of lifespan were calculated, as well as full-sib intraclass correlations. No statistically significant correlation was found for lifespan between sons and mothers, sons and fathers, and daughters and fathers. Daughters' lifespan correlated positively with maternal lifespan ($r = 0.21$, $p < 0.001$). Female full-sibs and male full-sibs had the same intraclass correlations, 0.22, $p < 0.001$.

Keywords: lifespan, heritability, maternal environment, age of sexual maturity, seasonal cohorts, sex

INTRODUCTION

Population cycles in voles and lemmings are found mainly in northern latitudes (Hansson and Henttonen, 1985; Norrdahl, 1995). In spite of long history studies of this phenomenon, demographic mechanisms of population cyclicity remain insufficiently understood. It is assumed that there are "extrinsic" causes of cyclic fluctuations in animal numbers (Elton and Nicholson, 1942; Erlinge et al., 1983; Sinclair et al., 1993; Krebs et al., 1995; Potapov et al., 2004), as well as "intrinsic" causes, which may act synergistically (Sinclair et al., 2003). The latter are related to behavioral or physiological traits of the animals that can be passed through generations either by genotypic or by maternal processes (Chitty, 1960, 1967; Inchausti and Ginzburg, 2009).

Maternal effects mediated by the age, hormonal, or nutritional state of mothers are considered to be of great significance in inducing a delayed density-dependent feedback on population growth rate (Bernardo, 1996; Rossiter, 1996; Inchausti and Ginzburg, 2009). According to the results of several long-term population studies, mothers from decline phases have lower nutritional conditions, fecundity and quality offspring than those from increase or peak phase of the population cycle (Norrdahl and Korpimäki, 2002; Evsikov et al., 2008; Nazarova and Evsikov, 2012a).

Life history traits, especially age at first reproduction and longevity have a major impact on population growth rate (Krebs and Myers, 1974; Evsikov et al., 1997; Oli and Dobson, 1999, 2001; Erlinge et al., 2000). There are composite, quantitative, polygenic traits whose expression is highly contingent upon plasticity, pleiotropy, and epistasis (Braendle et al., 2011). In rodents, life history traits are characterized by high flexibility and show tremendous temporal and spatial variation (Stearns, 2000; Millar and McAdam, 2001). To understand the peculiarities of self-regulatory mechanisms in population dynamics, it is important to evaluate the heritability of life history traits as well as their dependence on seasonal and maternal environment.

Age at first reproduction and longevity are especially sensitive to factors of population density or seasonal environment (Tkadlec and Zejda, 1998; Erlinge et al., 2000). Tkadlec and Zejda suggested that seasonal environmental variation is from causal factors of bimodality of age at first reproduction and, as a consequence, population cyclicity (Tkadlec and Zejda, 1998). The results of population studies have demonstrated that animals belonging to spring or summer-autumn cohorts differ in growth trajectory, age at first reproduction, hormonal status and rate of ageing (Shvarts et al., 1964; Zejda, 1971; Millar, 1980; Malzahn, 1985; Shintaku et al., 2010). Individuals born in spring months breed in the current reproductive season, whereas those born in summer or autumn months delay maturation until the next year. The proportion of matured young individuals has been found to closely correlate with cyclic fluctuations in abundance (Gliwicz, 1996; Evsikov et al., 1997, 1999; Erlinge et al., 2000).

Some authors have found that in decline phases the average age of the wintering population shift toward older animals due to delayed sexual maturation of young animals and shortening of the breeding season (Zejda, 1967; Wiger, 1979). Boonstra assumed the cause of the declines to be senescence and associated deterioration of physiological functions and fecundity (Boonstra, 1994). Indeed, females trapped during a decline phase and kept in the benign laboratory environment, show poorer reproductive performance, growth, and survival than those trapped during other phases of the population cycle (Mihok and Boonstra, 1992; Boonstra et al., 1998; Nazarova and Evsikov, 2010). However, age-related pattern of reproductive performance in rodents is still poorly understood.

The water vole (*Arvicola amphibius*) provides a good model for the study of internal and external causes of variation of life history traits and their interrelationships. The water vole in the forest-steppe of West Siberia is known to have large-scale fluctuations in abundance. These fluctuations are accompanied by changes in birth and death rates, sex-age structure of the population, individuals' physiological state, and reproductive and behavioral characteristics of the animals (Evsikov et al., 1997; Rogov et al., 1999). As in other cyclic species (Krebs and Myers, 1974), length of the breeding season and the age at sexual maturity change markedly during the population cycle. In decline, breeding season is 2 months shorter than in the increase phase, and therefore, overwintering females make a major contribution to reproduction. Due to extremely low reproductive output (about 1 young caught per 10 reproductively active females) (Rogov, 1999), population existence after population crash is highly dependent on the persistence of individuals capable of surviving adverse conditions of a decline phase.

In wild populations, the survival of water voles depends on season, phase of population cycle, and sex of animals (Rogov et al., 1999). In captivity, seasonal patterns of growth, maturation and reproductive activity are similar to that observed in the wild, providing an opportunity to evaluate the impact of season of birth and several characteristics of maternal environment on variability of life history traits.

Longevity is a critical parameter of fitness, determining population growth rates, abundance, and sex structure of the population. Because studies dealing with variability of lifespan in myomorphic rodents associated with individual morphophysiological and genetic traits and sex are scare, this long-term multigenerational study conducted on captive-bred water voles aimed (1) to clarify the effects of season of birth, age of sexual maturity, and some characteristics of family environment on lifespan and (2) to evaluate heritability of lifespan. The results help understand the internal mechanisms underlying the dynamics of a cyclic rodent population and evolution of lifespan.

MATERIALS AND METHODS

The study was conducted on an outbred colony of water voles (*Arvicola amphibius*), established in 1984 in the vivarium of the Institute of Systematics and Ecology of Animals. Their ancestors were taken from the cyclic population near Lis'yi Norki village (55° 50′N, 80° 00′E), Novosibirsk oblast. Each 1 to 3 years, new animals from the source population were added to the established colony to limit inbreeding.

The animals were kept in separate 48 by 25 by 25 cm hay-bedded cages under natural photoperiod (55° 1′N, 82° 55′E), with *ad-libitum* access to food (stewed grains, carrots, and cereal germs) and water.

During the breeding season (March–October), females and males were paired, for which their separate cages were connected with 8 by 6 cm tubular passages which were removed 10–18 days after mating. To verify copulation by the presence of sperm, vaginal smears were taken daily for 2 weeks of pairing. The young were weaned when they were 20 or 21 days old and were placed in separate cages. Females unmated for two weeks were caged with a different male. During breeding season, each individual was paired with 2–3 mates on average. Coefficient of relationship of mates was not greater than 0.125.

Survival and lifespan were estimated only in ≥20-day-old animals. Mean, median, and maximal lifespan were estimated from life table data composed of a total of 2016 observations (1424 uncensored and 592 censored).

The data on 1013 males (74.3% of the uncensored observations) and 1003 females (66.9% of the uncensored observations) were used for survival analyses of spring- and summer-/autumn-born individuals. The former were the individuals born in March–May (386 males and 380 females). The latter were those born in June–October (627 males and 623 females). Survival curves were constructed using the Kaplan-Meier method, and intergroup differences were evaluated using Cox's *F*-test.

The narrow-sense heritability of lifespan was estimated by doing lifespan regressions of an average offspring on each parent or by calculating intraclass correlations of lifespans of full sibs (for males and females separately) and multiplying the values obtained by 2 (Falconer, 1989). Only uncensored data were used for this purpose. A total of 355 mother–son and 365 father–son pairs, 354 mother–daughter and 335 father–daughter pairs, 202 sibs male groups (2 to 6 individuals each), and 166 female sibs groups (2 to 5 individuals each) were studied. Standard errors of the intraclass correlation were determined using the formula in Swiger et al. (1965).

The data were analyzed using one-way analysis of variance, survival analysis, and Spearman rank correlations. In the text and tables, means were given with their standard errors. Probabilities of less than 0.05 were accepted as significant. Statistical differences between the means were estimated by the Mann–Whitney *U*-test and Student *t*-test. The statistical package Statistica 6.0 was used for all computations.

RESULTS

MEAN AND MAXIMAL LIFESPAN OF MALES AND FEMALES

The analysis of life tables showed that the median lifespan was 421.8 days, with the 25th and 75th percentiles being 273.0 and 679.0 days, respectively. The probability of living 2 years or longer was 0.21 ± 0.01.

Males and females showed no statistically significant differences in mean lifespan calculated for uncensored data:

393.2 ± 9.6, $n = 753$ and 367.6 ± 9.1, $n = 671$, respectively ($U = 241742$, $z = 1.40$, $p > 0.05$), and had a similar median and maximal lifespan (**Table 1**). However, according to the Cox test, a difference in survival curves was found between males and females, [$T_1 = 699.8$, $T_2 = 725.2$, $F_{(1342,\ 1506)} = 1.16$, $p = 0.002$], especially in older ages. Kaplan–Meier survival curves for males and females are shown in **Figure 1**.

REPRODUCTIVE CHARACTERISTICS OF 1- AND 2-YEAR-OLD INDIVIDUALS

Female reproductive capacity did not deteriorate with age. One- and two-year-old females did not significantly differ in the percentage of mated females that delivered litters and average litter size at birth. Two-year-old males had lower percentage of sires than one-year-old ones (**Table 2**).

Table 1 | Mean, median, and maximum life span.

Sex	No. of individuals	25%	50%	75%	Maximum
Males	1013	264.9	419.5	681.2	1188
Females	1003	286.5	423.8	678.7	1108

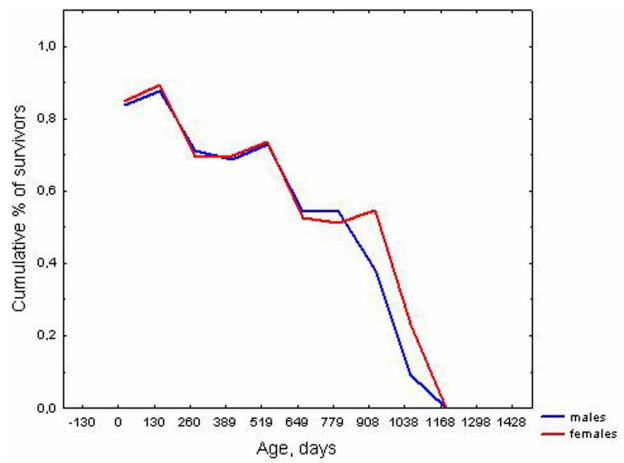

FIGURE 1 | Survival curves for males and females.

Correlation between lifespan and reproductive characteristics

The correlation between lifespan and reproductive characteristics (age at first mating, number of pups born throughout life, and offspring sex ratio (% male pups) in all litters are shown in **Figure 2**. Spearman rank correlation was performed on uncensored data only. The results indicated that individuals who began breeding at an older age had a significantly longer lifespan. Lifespan of both males and females correlated positively with the number of pups born throughout life. However, females who had more sons in progeny had significantly shorter lifespan.

Effects of maternal environment on lifespan

Male lifespan correlated negatively with maternal age, parity, and litter size. These characteristics accounted for about 1% of variance of male lifespan. Female lifespan did not correlate with these maternal characteristics (**Table 3**).

SURVIVAL OF SPRING- AND SUMMER/AUTUMN-BORN INDIVIDUALS

According to Cox's *F*-test, males and females showed significant differences between survival curves for spring- and summer-/autumn-born individuals [males—$F_{(846,\ 660)} = 1.53$, $p < 0.001$; females—$F_{(780,\ 562)} = 1.27$, $p = 0.001$]. The survival curves of spring-born animals were steeper than of those of summer-/autumn-born (**Figure 3**).

HERITABILITY OF LIFESPAN

Parent–offspring correlation

Parent-offspring correlations of lifespan are presented in **Figure 4**. Lifespan of daughters correlated positively with maternal lifespan ($r = 0.21$, $n = 354$, $p < 0.001$) and did not correlate with paternal lifespan ($r = -0.04$, $n = 335$, $p < 0.455$). Heritability, calculated as the mother–average daughter regression multiplied by 2, was 0.46 ± 0.14 ($p < 0.001$).

No significant correlation was found between sons' and parental lifespans (mother–son: $r = 0.04$, $n = 355$; father–son: $r = 0.02$, $n = 365$).

Sib lifespan correlations

One-Way ANOVA revealed a significant sibship effect on lifespan of female [$F_{(166,\ 252)} = 1.72$, $p < 0.001$] and male [$F_{(201,\ 312)} = 1.71$, $p < 0.001$] progeny. Female and male sibs had the same intraclass correlations, 0.22 ± 0.003. Heritability was 0.44 ± 0.006.

Table 2 | Reproductive characteristics of females and males aged one and two years.

Sex	Females		Males	
Age, years	1	2	1	2
No. of animals	63	63	68	68
No. of mated animals (% ±*SE*)	49 (77.8 ± 5.2)	55 (87.3 ± 4.2)	58 (85.3 ± 4.3)	50 (73.5 ± 5.4)
No. of sires or dams (% ±*SE*)	42 (66.7 ± 5.9)	41 (65.1 ± 6.0)	51 (75.0 ± 5.2)*	36 (52.9 ± 6.0)
Total no. of litters	79	70	95	50
Litter size at birth	4.4 ± 0.2	4.2 ± 0.2	4.3 ± 0.2	4.5 ± 0.3

*$p < 0.05$.

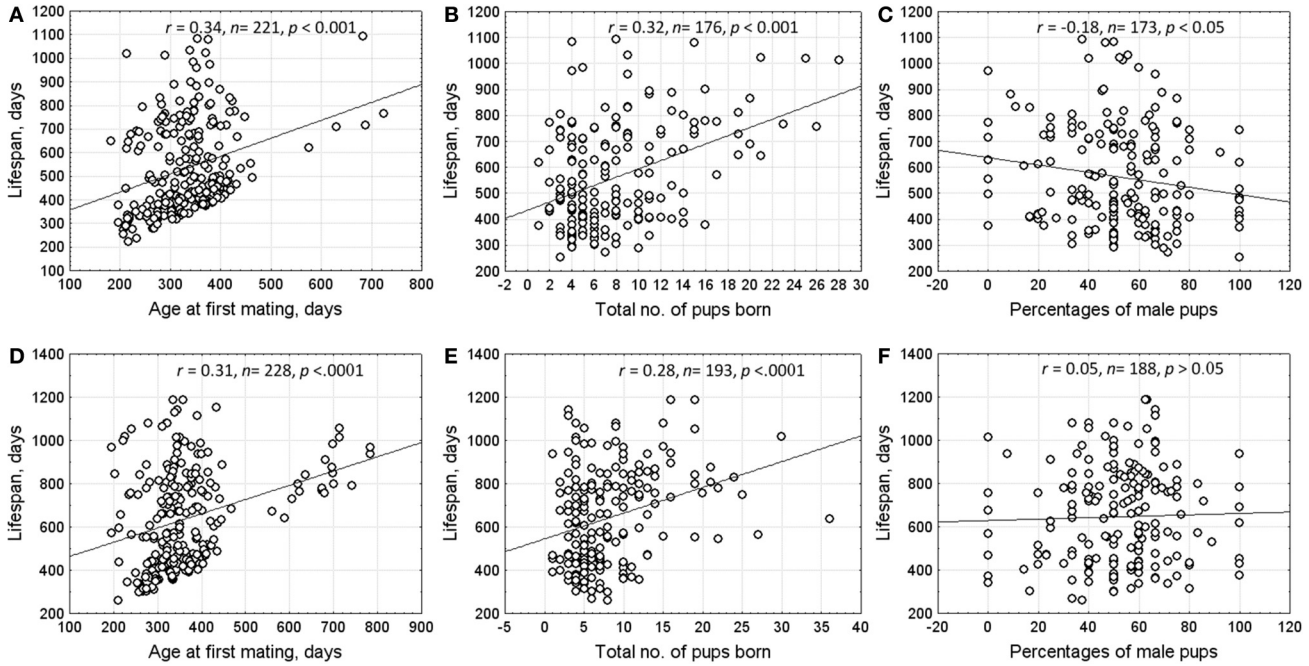

FIGURE 2 | Correlations between lifespan and reproductive characteristics of males and females. Upper row—females **(A)** age at first mating; **(B)** total no. of pups born; **(C)** percentage of male pups). Lower row—males **(D)** age at first mating; **(E)** total no. of pups born; **(F)** percentage of male pups.

Table 3 | Spearman rank correlations between lifespan and maternal environment.

Sex	Maternal age	Parity	Litter size
Males	−0.11*** (672)	−0.14*** (667)	−0.10* (674)
Females	Ns (596)	Ns (587)	Ns (596)

*$p < 0.05$; ***$p < 0.001$. Ns, non-significant.

Heritability of lifespan for sons, assessed from full-sib intraclass correlation, significantly exceeded that determined by parent–offspring regression ($p < 0.001$).

DISCUSSION

Differential survival of males and females is a major factor influencing the sex ratio of adult animals in wild populations of the water vole (Rogov et al., 1999). The main purpose of this study was to examine gender differences in lifespan and their dependence on season of birth and factors of maternal environment.

LIFESPAN AND SURVIVAL CURVES OF MALES AND FEMALES

In captivity, maximal lifespan of males was 1188 days and that of females, 1108 days. Mean lifespan did not differ between the sexes. The lack of differences in mean lifespan between the sexes has been noted in *Microtus ochrogaster, M. pennsylvanicus,* and *M. townsendii* (Boonstra, 1994; Getz et al., 1997).

However, there was a statistically significant difference in the survival curves of males and females, with males having a lower survival probability in older ages, than females. The observed gender difference in survival curves can be accounted by the more rapid progression of senescence in males than females.

AGE-RELATED REPRODUCTIVE PATTERNS IN MALES AND FEMALES

Lifespan is an important component of individual fitness. In water voles, 20% of individuals lived longer than 2 years and most of them maintained reproductive ability. As a result, long-lived animals produced more offspring. The observed negative correlation between lifespan of females and the proportion of males in their progeny can be attributed to a higher physiological cost of rearing sons than daughters (Evsikov et al., 1994). Reproductive investment to predominantly male progeny shortens the lifespan of mothers.

The results of analysis of age-related variation of reproductive characteristics indicated that there was no evidence of reproductive senescence in females. One- and two-year-old females did not significantly differ in the percentage of mated females that delivered litters and average litter size at birth. Similar results were obtained in Richardson's ground squirrel and Siberian lemming females (Erlinge et al., 2000; Broussard et al., 2005). However, in deer mice and white-footed mice (Millar, 1994; Morris, 1996), older females have decreased reproductive success.

As for water vole males, their reproductive capacity decreased with advancing age. It is a common feature of most polygynous vertebrates (Clutton-Brock and Isvaran, 2007).

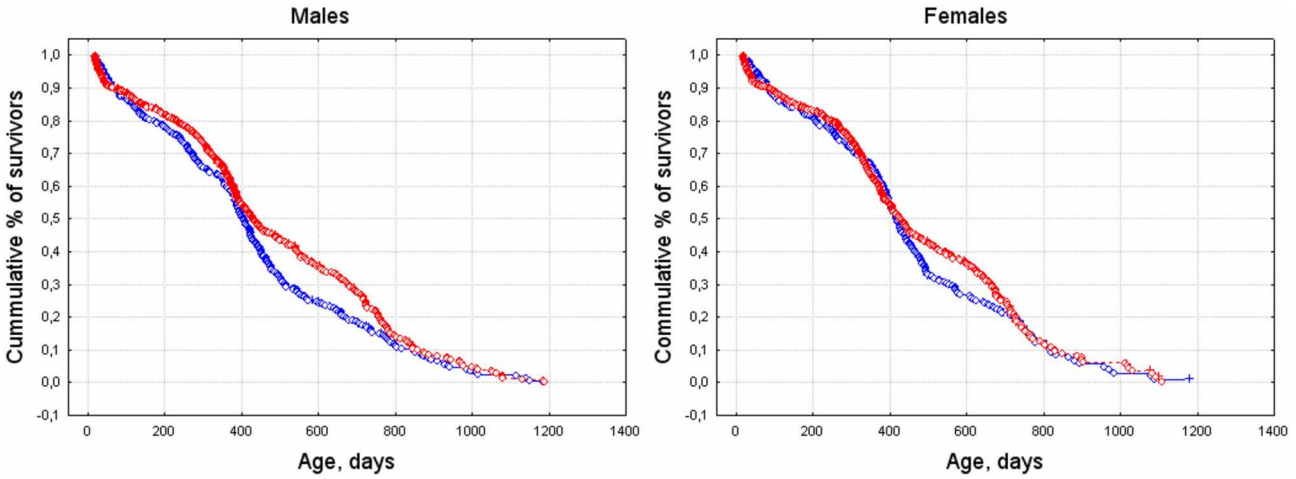

FIGURE 3 | Survival curves for spring- and summer-/autumn-born males (left) and females (right). Blue line: born March–May, red line: born June–October.

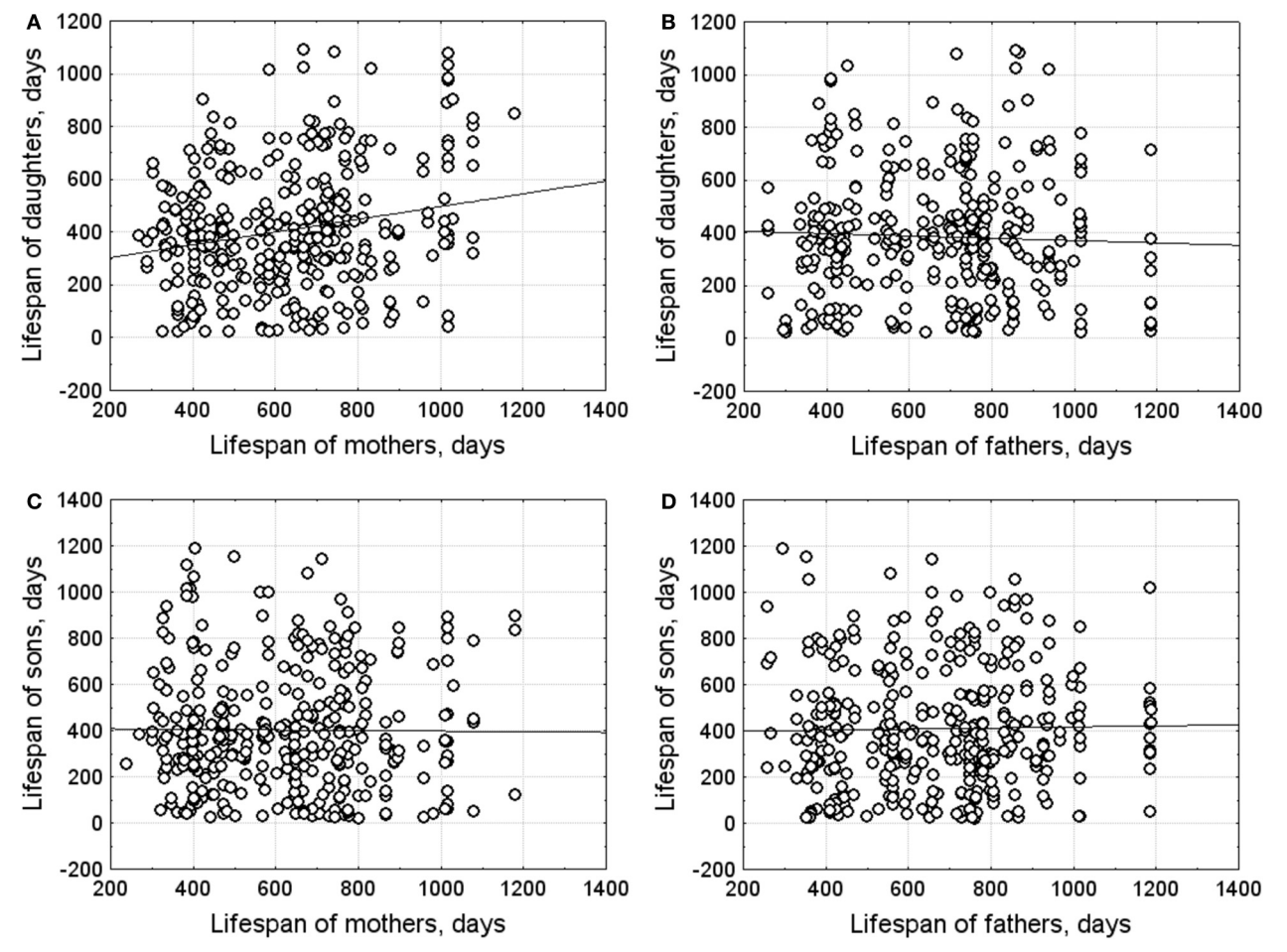

FIGURE 4 | Parent-offspring correlations of lifespan. (A) mother-daughter; **(B)** father-daughter; **(C)** mother-son; **(D)** father-son.

EFFECT OF SEASON OF BIRTH ON LIFESPAN

Arvicoline rodents in high-latitude environments are known to undergo pronounced seasonal changes in their physiology (Bronson, 1985; Ebling and Barrett, 2008). Spring- and summer-/autumn-born individuals are known to exhibit considerable biological differences, as in natural populations, as in captivity (Shvarts et al., 1964; Panteleev, 1968; Getz et al., 1997).

In this study, statistically significant differences in survival curves between the spring-born and summer-/autumn-born cohorts were observed in males and females. The curves of spring-born individuals showed a steeper decline than those of summer-/autumn-born ones. This appears to be determined by physiological response of an organism to yearly changes of day length, because other environmental conditions were under control.

The potential role of day length in the regulation of lifespan was experimentally shown in mice by Blom et al. (1994). The authors found that the immune status of offspring is affected by prenatal photoperiod. It is lower in mice carried under long prenatal photoperiod than in those carried under a short one. Other studies in *Microtus montanus* and *Microtus pennsylvanicus* showed that some characteristics of life history, correlating with lifespan, such as growth and sexual maturation, are also influenced by the prenatal and postnatal photoperiod (Horton, 1984; Lee et al., 1987).

AGE AT FIRST REPRODUCTION AND LIFESPAN

Costs and benefits of early and delayed maturation play an important role in the evolution of life history. The cost of reproduction usually increases with decreasing age at first reproduction (Adams, 1985). The extensive comparative studies conducted in mammals support that species-specific lifespan is inversely related with age of sexual maturity and fecundity (Severtsov, 1930; Clutton-Brock and Isvaran, 2007; De Magalhães et al., 2007; Jones et al., 2008). Kirkwood (1977) hypothesized that lifespan correlated negatively with ratio of expenditures devoted to growth and reproduction on the one hand and maintenance of body integrity and organism viability on the other hand. The results obtained in this study revealed that early reproduction impaired organism viability and shortened lifespan. A similar phenomenon was found for females from the cyclic population of water voles: the younger the age of a female at first reproduction, the fewer such females survived winter (Rogov, 1999).

EFFECTS OF MATERNAL CHARACTERISTICS ON LIFESPAN

The analysis of the effects of maternal environment on lifespan revealed a weak negative correlation between male lifespan and maternal age, parity, and litter size. Female lifespan did not correlate with these characteristics. Therefore, maternal environment has a different effect on males and females in terms of survival and lifespan. However, several recent studies on mice have demonstrated an opposite tendency: lifespan of females is more affected by maternal age (Carnes et al., 2012).

Negative correlation between male lifespan and litter size at birth would imply the existence of a trade-off between offspring number and offspring quality (Smith and Fretwell, 1974).

HERITABILITY OF LIFESPAN

Lifespan heritability, evaluated from the mother–average daughter regression and showing the percentage of additive genetic variance in the total phenotypic variance, was 0.46. Lifespan heritability, estimated from intraclass correlation of full-sib females, was 0.44. These values are similar to those obtained in mice (Klebanov et al., 2000). The correlation between daughters' and paternal lifespan was nearly zero. The difference between "daughter-mother" and "daughter-father" correlation coefficients indicates that female longevity could depend on family environment or inheritable maternal physiological qualities that affect both reproductive success and probability of death associated with reproduction, for example, the ability to accumulate body reserves during pregnancy for lactation needs (Nazarova and Evsikov, 2012b). It is known, that reproduction has considerable energy demands in mammals and is risky for females (Gittleman and Thompson, 1988).

The ecological factors of local environment and individual maternal characteristics play an important role in fulfillment of reproductive potential and demographic dynamics of the water vole (Nazarova and Evsikov, 2000, 2004, 2007, 2008, 2012a; Muzyka et al., 2010). During breeding season, reproductive groups composed of males and females of various ages form the basic unit of the spatial organization of a water vole population (Evsikov et al., 2001; Muzyka et al., 2010). These groups may have different reproductive success and duration. Breeding females have non-overlapping home ranges and produce several litters per breeding season. Before birth, pregnant voles move to a new territory, leaving their older offspring in their natal territory (Waser and Jones, 1983; Jeppson, 1986). Daughters of many rodent species are more likely to remain within the maternal home range than sons (Solomon and Keane, 2007). The present results showed that water vole females inherited not only the home range but also qualities that determine longevity.

Sons' lifespan heritability estimated from the parent–offspring regressions did not differ significantly from zero. Lifespan heritability, calculated from intraclass correlations between full-sib males, was 0.44, the same for males and females. The higher sib-sib than parent-offspring heritability coefficients may be accounted by effects of the common rearing environment.

In conclusion, developmental factors have substantial influence on water vole longevity. Potentially, phase-related changes in characteristics of maternal environment or mean age at first reproduction of females could be primary factors influencing the sexual structure of populations.

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Effect on lifespan of high yield non-myeloablating transplantation of bone marrow from young to old mice

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Tissue renewal is a well-known phenomenon by which old and dying-off cells of various tissues of the body are replaced by progeny of local or circulating stem cells (SCs). An interesting question is whether donor SCs are capable to prolong the lifespan of an aging organism by tissue renewal. In this work, we investigated the possible use of bone marrow (BM) SC for lifespan extension. To this purpose, chimeric C57BL/6 mice were created by transplanting BM from young 1.5-month-old donors to 21.5-month-old recipients. Transplantation was carried out by means of a recently developed method which allowed to transplant without myeloablation up to 1.5×10^8 cells, that is, about 25% of the total BM cells of the mouse. As a result, the mean survival time, counting from the age of 21.5 months, the start of the experiment, was +3.6 and +5.0 (±0.1) months for the control and experimental groups, respectively, corresponding to a 39 ± 4% increase in the experimental group over the control. In earlier studies on BM transplantation, a considerably smaller quantity of donor cells (5×10^6) was used, about 1% of the total own BM cells. The recipients before transplantation were exposed to a lethal (for control animals) X-ray dose which eliminated the possibility of studying the lifespan extension by this method.

Keywords: bone marrow transplantation, stem cells, longevity, life extension

INTRODUCTION

It is presently held that many tissues of an adult organism are capable of regeneration (self-renewal) by means of resident or circulating stem cells (SCs). The self-renewal of tissues occurs continuously. In the heart of rats, for example, about 7% of the cells are replaced every month (Kajstura et al., 1996), whereas the renewal of blood and epithelial tissues proceeds much faster. The participation of not only local but also circulating SC in the renewal of tissues has been shown by numerous studies on sex-mismatched transplantation which was accompanied by significant y/x-chimerism (Körbling et al., 2002; Herzog et al., 2003; Thiele et al., 2004). The significance of these studies is especially increased now since it has become possible to create cellular material for transplantation that is genetically identical to cells of the patient (Grewal et al., 2004; Takahashi and Yamanaka, 2006; Wang et al., 2011).

Our previous *in vitro* studies have demonstrated that non-differentiated SC can indeed, under certain conditions, be effectively differentiated into the cell type corresponding to their cellular microenvironment (Kovina and Khodarovich, 2011). These findings may explain the previously found, and not enough accounted for, effectiveness of bone marrow transplantation (BMT) in treating not only hematology diseases (Fanconi anemia; Gluckman et al., 1995) but also such systemic diseases as mucopolysaccharidosis and senile hearing impairment (Birkenmeier et al., 1991; Iwai et al., 2001; Corti et al., 2004; Willenbring et al., 2004). Mucopolysaccharidosis is caused by a deficiency in enzymes required for degradation of glucosamines that are accumulated in lysosomes of many organs, thereby leading to dysfunction and reduction of lifespan. Transplantation of syngenic bone marrow (BM) cells from healthy mice resulted in an increase of lifespan from 6 months to the control value of 2 years. The lysosomal activity was recovered in full or in part in all studied tissues. For the thymus gland, spleen, and BM the recovery was complete, for the lungs it was 50%, for kidney and liver, 20%, and for the brain, 7% (Birkenmeier et al., 1991). This result can be logically accounted for by tissue replacement with progeny of donor BM; however, the authors did not determine the degree of chimerism. In the next work of this series, the curing of a hereditary skin disease by BMT was shown; the chimerism of skin was determined to be between 10 and 30%, and the chimerism of the mucous epithelium of the gastrointestinal tract was 50% (Wagner et al., 2010). These facts led to the assumption that the BM, besides hematopoietic and stromal cells, contains cells capable to be differentiated into mature cells of many other tissues (Herzog et al., 2003).

Nevertheless, it is still unclear whether such regeneration can retard the normal process of aging. The BMT methods employed before demanded a strong X-ray irradiation which negatively affected the lifespan of control animals (Birkenmeier et al., 1991). Though there are some life extension reports about myeloablating SC transplantation on mice (Shen et al., 2011), the usage of

lethal irradiation cannot be recommended for human anti-aging therapy. In the present work, we report the influence upon lifespan of high yield non-myeloablating transplantation of BM from young mice to old mice.

MATERIALS AND METHODS

ISOLATION OF BONE MARROW

All animal experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology (http://www.toxicology.org/ai/air/air6.asp) and were approved by Institutional Animal Care and Use Committee (IACUC). The donors of BM were C57BL/6 mice aged 6 weeks. The donors were sacrificed by cervical dislocation and sanitized with 70% ethanol. Isolation of BM was carried out as described (Colvin et al., 2004) with small variations. Firstly, the spine and skull were cut out and freed of surrounding tissues using sterile scalpel, scissors, and forceps. The caudal part of the spine was removed. The spine and skull bones were then minced with sterile pistil and mortar in 1–2 ml of cooled sterile Hanks' balanced salt solution (HBSS) buffer of the following composition: 0.44 mM potassium phosphate, 5.37 mM potassium chloride, 0.34 mM dibasic sodium phosphate, 136.89 mM sodium chloride, 5.55 mM D-glucose. The mixture was filtrated through four layers of a fine-mesh nylon-6 tissue, washed in fresh HBSS buffer and then centrifuged under mild conditions (50 *g*). The deposit was resuspended in 1 ml HBSS, the cells were counted in a Goryaev chamber and HBSS was added to a final concentration of 5×10^7 cells per ml. The overall number of isolated cells of various sizes and morphology was $3.5–4.5 \times 10^8$.

TRANSPLANTATION OF BONE MARROW

Bone marrow was transplanted to C57BL/6 mice aged 21.5 months. Pre-treatment of suspension of the cells included filtration through four layers of a fine-mesh nylon-6 tissue and addition of 5 U of heparin (Synthesis, Russia) per 2.5×10^7 cells in 0.5 ml HBSS to prevent occlusions of vessels with cellular material. The tail of a mouse was warmed in water at 50–55°C until the twin caudal veins were clearly seen, and then the cell suspension was slowly (during 20 s) injected into one of the veins by means of an insulin syringe.

Transplantations were carried out twice a day on three subsequent days with an interval of 8–16 h, which brought the amount of transplanted cells up to 1.5×10^8 per recipient.

The control animals did not receive any treatment.

STATISTICAL TREATMENT OF DATA

Approximation of experimental results was done using the Origin software (OriginLab, USA) on the basis of the Gompertz function, traditionally used for survival description (Anisimov et al., 2003; Gavrilov and Gavrilova, 2006):

$$F(t) = 100 \times \exp[-\mathrm{P1} \times \exp(\mathrm{P2} \times t - \mathrm{P3})], \quad (1)$$

where $F(t)$ is the percentage of animals that attained age t, 100% is the number of animals at the age 21.5 month (the age-point when the transplantation was performed), and P1, P2, and P3 are parameters to adjust. To find the best fit, we conducted a three-parametric fit for each experimental curve and obtained three pairs of parameters. Then the least sensitive parameter (P1) was averaged and fixed for a two-parametric fit (P2 and P3). Then again the least sensitive (P3) was averaged and fixed for a one-parametric fit (P2).

To find the 50%-survival time ($t_{1/2}$) for each curve, we used the following equation, derived from Eq. 1:

$$50 = 100 \times \exp[-\mathrm{P1} \times \exp(\mathrm{P2} \times t_{1/2} - \mathrm{P3})],$$
$$t_{1/2} = [\mathrm{P3} + \mathrm{In}((\mathrm{In}(2))/\mathrm{P1})]/\mathrm{P2} \quad (2)$$

RESULTS AND DISCUSSION

In the beginning, we selected conditions for massive transplantation of BM cells to mice. According to the idea of the experiment, we needed to inject about 150 million cells (1.5×10^8) to each animal, which amounts to nearly one-fourth of overall BM cells of an adult mouse. It turned out that after injection of a large volume (over 1 ml) or of a highly concentrated cell suspension ($1–1.5 \times 10^8$/ml) most mice died within 2–10 min. Therefore, the following conditions of transplantation were chosen: the cells were injected in six portions of 2.5×10^7 cells in 0.5 ml at intervals of 8–16 h. An important factor for successful transplantation was the addition of 5 U of heparin per cell dose (2.5×10^7 cells in 0.5 ml) and a thorough filtration of the cells before injection; combining these two measures greatly reduced post-transplantation death of the animals.

The recipients of BM cells were C57BL/6 mice at the age of 21.5 months. Each of the 10 females received 1.5×10^8 cells obtained from 6-week-old males of the same line. In each case the quantity of live BM cells exceeded 90%, which was ascertained by counting cells stained with trypan blue in a Goryaev chamber. Two recipients were lost during transplantations, presumably from formation of thrombi in large vessels; they were excluded from the statistical analysis. The remaining eight mice were observed until their natural death in parallel to nine mice of the same age from a control group not exposed to transplantation. **Figure 1** depicts the effect of BM transplantation on lifespan. The gray line corresponding to the group of recipients is always above the black line of the control group.

Statistical analysis of the results was performed as described in Section "Materials and Methods." The Gompertz equation describes the experimental data sufficiently good, with the correlation coefficient 0.9. The best fit gave the following values of the mean total lifespan:

$$t_{1/2\mathrm{cntrl}} = 25.1 \pm 0.1 \text{ months and } t_{1/2\mathrm{trnspl}} = 26.5 \pm 0.1 \text{ months.}$$

Then, the mean residual lifespan, calculated from the beginning of the observations (age = 21.5 months), was $25.1–21.5 = 3.6 \pm 0.1$ months and $26.5–21.5 = 5.0 \pm 0.1$ months for the control and experimental groups, respectively, corresponding to a gain of 5.0–3.6 = 1.4 months (**Figure 1**). Hence, the BMT carried out using the described procedure resulted in a $(1.4/3.6) \times 100\% = 39 \pm 4\%$ increase of the mean survival time counting from the moment of transplantation. While the overall lifespan extension is modest ($100 \times 1.4/25.1 = 6\%$) we discuss here only the post-transplantation extension (39%), since it properly reflects the

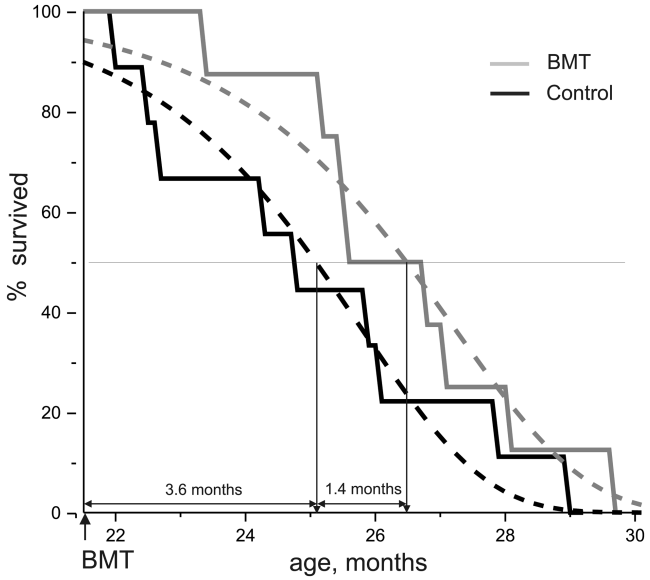

FIGURE 1 | Effect of massive non-myeloablating syngenic transplantation of bone marrow on the lifespan of C57BL/6 mice. Black line – Kaplan–Meier graph for animals from the control group (*solid line*) and its Gompertz approximation (*dashed line*); gray line – Kaplan–Meier graph for animals from the experimental group which underwent bone marrow transplantation (*solid line*) and its Gompertz approximation (*dashed line*). BMT, bone marrow transplantation.

effect of the treatment while the overall ratio includes the untreated time. This is a common practice (Dhahbi et al., 2004). The untreated time is several fold greater than post-treatment time and, therefore, cannot be ignored as might be done when the treatment starts soon after weaning. There is no known method to extend the mean wt-mouse life for more than 40% after the start of the treatment, even with dietary restriction, the most powerful method of life extension.

The oldest transplanted animal lived 3 weeks longer than the oldest control animal., However we cannot calculate the maximal lifespan here, since it is, by definition, the mean lifespan of the most long-lived 10% of each group. In our small group, 10% would be less than one mouse. So, the investigation of an influence of BMT on maximal lifespan is the task for future work.

The obtained positive influence of BMT on the mean lifespan in our work is underestimated because of transplantation complications (including the occlusion of vessels) from which, obviously, suffered not only the two mice that died during transplantation and were excluded from the statistics, but also those that survived, though to a lesser degree. We expect a greater difference in lifespan between control and experimental groups by (i) the use of high-quality commercial filters for purification of transplanted material from cell aggregates and (ii) the use of more accurate controls injected with old BM (in this work the control animals did not get the parallel invasive treatment because of the absence of additional 20 months old animals to produce old BM for control transplantation).

Data from the literature also suggest an effect of BMT on the lifespan of mice. For instance, an attempt was made in 2004 to extend the lifespan with syngenic radiation-free transplantation of 4–10 $\times$ 10^6 BM cells from young donors (Kamminga et al., 2005). The effect was not large though (less than 10% of the survival increase), presumably because a relatively small number of cells were transplanted; the resulting chimerism of the BM was also quite modest (1–10%). However, very soon another research team managed to achieve 30–37% chimerism of young recipients after transplantation of 2 $\times$ 10^8 BM cells without irradiation (Colvin et al., 2004), which is in a perfect agreement with the SC competition hypothesis (an adult mouse has a total of 6 $\times$ 10^8 BM cells). The SC competition hypothesis predicts a still greater effectiveness of replacement of the aging recipient's BM with young donor's SCs, as the amount of SCs decreases sharply with age.

In perfect agreement with this prediction, the chimerism of old recipients in the above-mentioned study (Kamminga et al., 2005) was 10- to 20-fold greater than chimerism found in younger recipients (5–10 vs. 0.5–1%). Therefore, when the amount of donor cells is increased 10–20 times (from 5–10 $\times$ 10^6 to 1–2 $\times$ 10^8) one may expect an almost complete replacement of the aged BM with that from the donor. Thus, our results which demonstrate a significant effect of BMT on mice survival may be explained as solely by a blood renewal effect (for example, immune boosting effect or increased blood oxygenic capacity), as well as by large number of transplanted cells incorporated in the solid organs and differentiated into the surrounding tissue, thus renewing it. The last conclusion is supported by the results of our previous work on contact differentiation *in vitro*, where we achieved nearly 100% differentiation of SCs into endotheliocytes after seeding them into a primary endothelial culture (Kovina and Khodarovich, 2011).

In our work, we used a slightly different source of BM as it is obtained traditionally – spine and scull instead of femurs. Spine and scull together contain the majority (63%) of total body BM cells, according to Colvin et al. (2004). Besides, BM from spine and scull has a twice higher percentage of high proliferative potential colony-forming cells, than femurs (Colvin et al., 2004). So, it must have a higher potential to renew old tissues than femur's BM. Finely, spine and scull are easily cleaned from surrounded tissues. Thus, in order to recover the maximal amount of active BM within minimal time, we decided to use only spine and scull as BM sources. A quantitative assessment of the degree of post-transplantation chimerism and its correlations with lifespan on a larger quantity of animals with variations in both: the sources of circulating SC and the lines of recipients will be the next stage of our work. A success along these lines will allow developing approaches for therapy for not only a number of hereditary diseases but also for retardation of the aging process in general.

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The genetics of extreme longevity: lessons from the New England Centenarian study

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The New England Centenarian Study (NECS) was founded in 1994 as a longitudinal study of centenarians to determine if centenarians could be a model of healthy human aging. Over time, the NECS along with other centenarian studies have demonstrated that the majority of centenarians markedly delay high mortality risk-associated diseases toward the ends of their lives, but many centenarians have a history of enduring more chronic age-related diseases for many years, women more so than men. However, the majority of centenarians seem to deal with these chronic diseases more effectively, not experiencing disability until well into their nineties. Unlike most centenarians who are less than 101 years old, people who live to the most extreme ages, e.g., 107+ years, are generally living proof of the compression of morbidity hypothesis. That is, they compress morbidity and disability to the very ends of their lives. Various studies have also demonstrated a strong familial component to extreme longevity and now evidence particularly from the NECS is revealing an increasingly important genetic component to survival to older and older ages beyond 100 years. It appears to us that this genetic component consists of many genetic modifiers each with modest effects, but as a group they can have a strong influence.

Keywords: centenarians, genetic of longevity, heritability of longevity, compression of morbidity, genetic variation

THE NEW ENGLAND CENTENARIAN STUDY AND EXCEPTIONAL LONGEVITY

The New England Centenarian Study (NECS)[1] was founded in 1994 as a population-based study of all centenarians living within eight towns in the Boston area with the goal of better understanding the bio-psycho-social characteristics of centenarians and their family members and discovering determinants of exceptional longevity and healthy aging (Perls et al., 1999). The study soon expanded enrollment to include siblings of centenarians and their offspring from throughout North America, and since 2008 there has been a particular effort to locate and recruit subjects 105 years old and older (what Nobu Hirose of the Japanese Centenarian Study has termed "semi-supercentenarians"). In addition to recruiting long lived individuals, the NECS has also recruited younger referent subjects from families lacking longevity as well as spouses of centenarians' offspring. Between 1994 and 2012, the study has enrolled more than 1,800 centenarians and 123 supercentenarians (age 110+ years), more than 600 centenarian offspring and 437 controls. The majority of the NECS centenarians were born between 1880 and 1910 and reached a median survival of 103 years, thus surviving 30–40 years past the median survival of their birth year cohort. Birth certificates were available for only about 30% of the centenarians and therefore US census data from the early 1900s and other techniques were used for validating date of birth (Young et al., 2010; Andersen et al., 2012). Typically, 99% of age claims 115 years and older are false, and therefore in the case of supercentenarians, the NECS takes extra steps to prove a person's age including family reconstitution and collecting multiple forms of proof that all must be consistent with one another (Young et al., 2010). Demographic, health, and family history data, as well as physical and cognitive function data are collected at least once for the majority of study subjects and are updated annually for living subjects. DNA samples have also been collected on the majority of subjects.

WHAT IS THE CUT OFF AGE FOR EXCEPTIONAL SURVIVAL?

Birth cohort life tables provide an indication of the exceptional survival of centenarians (Bell and Miller, 2005). Based on the U.S. Social Security Administration birth cohort life table,[2] the median survival for males born between 1896 and 1905 was 63 and 72 years for females and 1% of males and 5% of females lived past the age of 95 years. 0.1% of males and 1% of females lived past the age of 100 (Bell and Miller, 2005). The frequencies of survivors past the age of 100 decrease by a factor of approximately one half for each additional year of life after 100. Their

[1] http://www.bumc.bu.edu/centenarian

[2] http://www.ssa.gov/oact/NOTES/as120/LifeTables_Tbl_7_1900.html

life span represents a phenomenon of extreme survival that is very rare in the population, and much more extreme than the human longevity examined for example in the Leiden Study of Longevity (Deelen et al., 2011), in which subjects reached an average survival of 94 years, or the CHARGE consortium (mean age 81 years; Walter et al., 2011).

Though still rare, the prevalence of people at age 100 years is growing. When the NECS began in 1994, the estimated prevalence in the US and other developed nations was one centenarian per 10,000. Now, in 2012, the prevalence has doubled to 1 per 5,000. One explanation for such growth is that at the turn of the last century, marked improvements were taking place in public health, particularly ones which impacted upon neonatal and maternal mortality. In 1900, infant mortality was 10–30% for the first year of life depending upon the area of the country and 6–9% of women died due to complications of childbirth.[3] Cleaner water supplies, plumbing, milk pasteurization, marked socioeconomic improvements, vaccines, and a major increase in the mean years of education led to major improvements in survival. Then in the 1930s and 1940s, the introduction of antibiotics (sulfa and penicillin), additional vaccines, safe blood transfusion, and of course many other medical and non-medical advancements led to marked further improvements in infant survival and also survival in adulthood. Figure 2 in Armstrong et al. (1999), illustrates the dramatic decline in mortality due to infectious diseases in the United States from 1900 to 1960. Now, in the last 20 or so years, there has also been a marked effect upon survival to 100 due to reduced mortality rates amongst the geriatrics population (Vaupel et al., 1998).

The result is that with these improvements in our environment, many more people that might have otherwise died during infancy have the opportunity to take advantage of their longevity potential and live to much older age. For example, according to James Evans of the UK's Department of Work and Pensions, a 20-year-old today has a three times greater chance of living to age 100 than when their grandparents were 20 (Evans, 2011). That work produced **Table 1** indicating the probabilities of men and women from different birth cohorts living to 100.

Kaare Christensen and coauthors provide an even more optimistic picture predicting that 50% of French girls born in 2010 will live to 100 (Christensen et al., 2009). Certainly, a phenotype achieved by 50% of the population can not be considered exceptional. However, such projections assume that these large proportions of the population have the biological wherewithal to survive to 100+ years if given the appropriately facilitative environment and this really is not known.

As discussed below there is a growing body of evidence indicating that an increasingly greater positive genetic influence is necessary for survival to age 100 and older ages. Presumably, the vanishing small proportion of people that is able to reach the ages in the extreme tail of the population is due to the increasing rarity of genetic and environmental factor combinations that improve the odds of such rare survival. Supporting this hypothesis is the observation that while the percentage of the population made up of 100 year olds over the past 10 years might still be climbing, the rate of people living to ages 112+ has remained flat.[4] The reason for this could be that specific and very rare genetic signatures are necessary to achieve these ages. For younger ages, for example 105–109 years, the necessary genetic/environmental signatures may be less rare and not all people who have the potential to achieve these ages have yet done so, thus we see a continued growth in their prevalence rate. Once we have a better idea of who is predisposed and by how much, we will be better able to understand what is exceptional longevity versus average and perhaps even below average longevity. Complicating matters however is that signatures are likely different according to some ethnicities and some specific environmental exposures and therefore definitions of exceptionality will vary within these contexts. This complex model of many common and rare genetic variants with modest effects collectively having an increasingly strong effect on survival to older and older ages speaks to the importance of centenarian studies from around the world working closely with one another to effectively compare and contrast their findings.

EXCEPTIONAL HEALTH SPAN AND THE COMPRESSION OF MORBIDITY HYPOTHESIS

Ever since Jim Fries proposed his compression of morbidity hypothesis, published in the New England Journal of Medicine in 1980, most researchers in Gerontology believed that in order to survive to 100 years, one necessarily had to markedly delay both morbidity and disability toward the end of their life. Then, in 2002 Jesse Evert, collected the data and published findings that less than 20% of centenarians had escaped major age-related diseases by the time they reached the age of 100 and approximately 45% developed at least one of these diseases before the age of 65 (Evert et al., 2003). However, consistent with at least part of Fries' hypothesis, we also previously noted that despite the presence of diseases, approximately 90% of centenarians delayed disability until the mean age of 93 years indicating perhaps greater functional reserve that enabled these individuals to remain independent for a long time despite the presence of diseases associated with high risks of disability and mortality (Hitt et al., 1999).

In the past 7 years, as the NECS emphasized the enrollment of even older subjects, those 105+ years, we noted much later ages of onset of age-related diseases. At this point we surmised that Dr. Fries had underestimated the practical limit of human life span (e.g., 100 years) and we simply had not looked at old enough ages to test his hypothesis that age-related diseases and disability

[3] http://www.cdc.gov/mmwr/preview/mmwrhtml/mm4838a2.htm

Table 1 | Chance of living to age 100 according to birth year and sex (directly from with permission).

Year of birth	Male (%)	Female (%)
1931	2.5	5.1
1961	10.5	16.2
1991	19.2	26.4

[4] http://www.mapfre.com/fundacion/html/revistas/gerencia/n111/docs/Estudio1En.pdf

were compressed to shorter and shorter periods of time as subjects approached the limit of life span. Once 100 of our subjects had reached 110+ years, we analyzed our longitudinal data again and indeed found that at progressively older and older ages of survival beyond 100 years, there is a progressive compression of disability and morbidity such that by the survival age of 110 years, subjects had compressed age-related diseases into the last 5.2% of their extremely long lives (compared to 17.9% for controls, 9.4% for subjects age 100–104 years, and 8.9% for those age 105–109 years; Andersen et al., 2012). Furthermore, at these most extreme ages, subjects became much more alike in terms of the types and ages of onset of age-related diseases. This increased homogeneity at oldest ages and our genetic findings to-date hold great promise for many more findings regarding factors that facilitate slower aging and the delay or escape of age-related diseases.

EVIDENCE FOR A STRONG FAMILIALITY OF EXCEPTIONAL LONGEVITY

Twin studies have shown that only 20–30% of the overall variability of living to the mid 80s is attributable to genetic variation (Herskind et al., 1996), and this result is unfortunately and erroneously used to indicate the heritability of exceptional longevity. Work by the Adventist Health Study indicates that with optimal health related behaviors (e.g., no tobacco or alcohol use, regular exercise, vegetarianism, effective management of stress) people should generally be able to achieve an average life expectancy of about 86 years (Fraser and Shavlik, 2001). This suggests that the average genome, in combination with optimal health behaviors, facilitates an average life span of the late eighties and it would make sense that the vast majority of why one lives to their sixties or seventies versus these later octogenarian years would be explained by health habit choices. As indicated above though, the odds of living to the mid-eighties are many folds more common than living to 100 years or older and it is likely that these twin studies cannot inform us about the heritability of living to 100.

The NECS has enrolled several 100 families with centenarian siblings and these data have provided increasingly stronger evidence that exceptional longevity clusters in families (Perls et al., 1998, 2000). As an example, **Figure 1** shows two centenarians and their siblings enrolled in the NECS who reached remarkable ages. The rarity of such families with clusters of exceptional longevity was discussed in (Perls et al., 2000). The exceptionality of familial longevity in a larger number of NECS sibships relative to Framingham Heart Study sibships was also described in (Sebastiani et al., 2009) using an objective measure of familial longevity. Less than 1% of all the families in the FHS would meet the exceptionality of the sibships enrolled in the NECS. Additionally, the NECS has also shown that siblings of centenarians had between 8 and 17 times greater chances of living past 100 years compared to individuals from the same birth year cohort (Perls et al., 2002).

The sex-specific sibling relative risk and the prevalence of centenarians estimated as 1 centenarian every about 5,000 individuals in the US population can be used to estimate the heritability of the liability of living past 100 using formulas in (Wray et al., 2010). Using the online calculator http://gump.qimr.edu.au/genroc/ the heritability of liability to longevity ranges between 0.33 (females) and 0.48 (males). Estimates of relative risk of exceptional longevity for siblings of semi-supercentenarians and supercentenarians are needed to have a better understanding of the heritability of living to extreme ages even greater than 100.

While variability in average life span can be explained by environmental factors and genetics, exceptional longevity seems to be more the resultant of genetics rather than environment. Recently, the Ashkenazi Jewish Centenarian Study has shown that centenarians in their study do not differ from population controls in major risk factors such as increased BMI, drinking, or smoking (Rajpathak et al., 2011). Although lifetime exposures in centenarians are difficult to measure in a reliable way, this analysis suggests that environmental factors have little contribution to extreme longevity, so that most of the heritability of the trait is likely to have a genetic basis.

GENETIC INFLUENCE UPON SURVIVAL TO VERY OLD AGES

Per the phenomenon of demographic selection (Vaupel et al., 1979; Carey and Judge, 2001), we initially hypothesized that centenarians necessarily lack genetic variants associated with premature mortality and perhaps additionally also have genetic variants associated with slower aging and reduced risk for age-related diseases and subsequent mortality, so-called "longevity enabling genes" (see **Figure 2**).

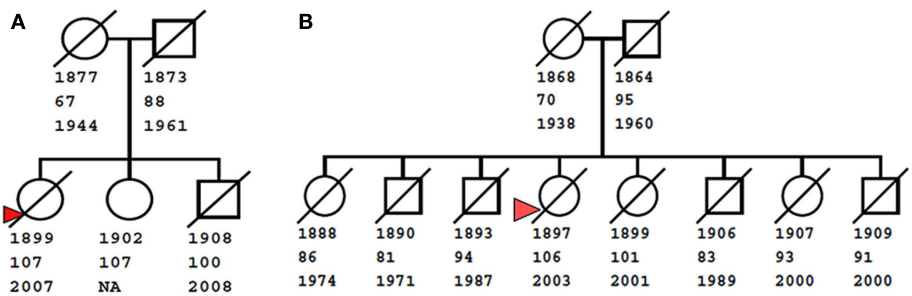

FIGURE 1 | Examples of familial clusters of exceptional longevity. The small pedigree on the left **(A)** shows a sibship of three centenarians, with youngest age at death of 100 years. The pedigree on the right **(B)** shows a larger sibship of eight, with two centenarians (ages at death 101 and 106 years), three nonagenarians (ages 91, 93, and 94), and three siblings who live past the age of 80. Squares and circles represent males and females, diagonal bars represent deceased subjects. Numbers below nodes are birth years, last age at contact, and death year. For living subjects, the death year is not available (NA). Red triangles denote probands enrolled in the New England Centenarian Study.

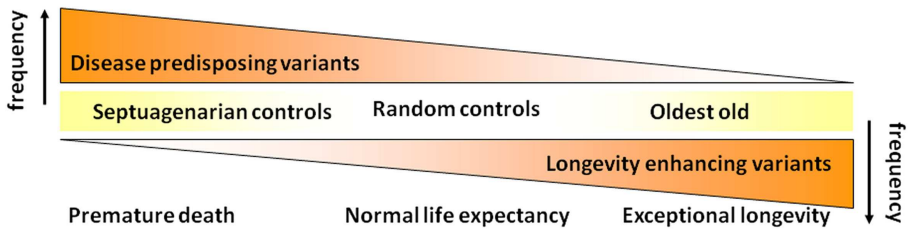

FIGURE 2 | Initial hypothesis on the genetic basis of exceptional longevity.

Since there are many different phenotypic presentations of survival to 100 years we also expected that many genetic variants contributed to a complex genetic model for exceptional longevity. Complicating matters, environmental factors which can be deleterious (e.g., tobacco use is associated with specific cancers in some people and not others) and others which can be health-promoting (e.g., daily aspirin use), will effect survival risk-associated with a person's genetic profile.

To be able to discover the complex genetic basis of exceptional longevity and dissect the trait into sub-phenotypes that represent different patterns of exceptional survival, we conducted a genome-wide association study of exceptional longevity with 801 unrelated centenarians from the NECS (median age at death 104 years) and 914 genetically matched controls from the Illumina control repository and the NECS. The initial version of the results in Science Express in July 2010 contained errors and we retracted it (Sebastiani et al., 2011). We published the corrected version in (Sebastiani et al., 2012b) after extensive cleaning of the data and independent validation of the genotype data. Analysis of approximately 240,000 single nucleotide polymorphisms (SNP) showed that only one SNP in TOMM40/APOE (rs2075650) reached irrefutable genome-wide significance, while a large number of SNPs were significantly associated with exceptional longevity but did not pass corrections for multiple comparisons (p-value for association between 10^{-2} and 10^{-7}). This result was consistent with other studies of longevity that failed to identify genome-wide significant associations beyond APOE but detected many associated SNPs with more moderate levels of significance (Newman et al., 2010; Deelen et al., 2011; Walter et al., 2011). In addition to traditional one-SNP-at-a-time analysis we introduced a new method to capture the simultaneous effect of many genetic variants that individually have minor to modest effects, but as a group assert a substantial influence upon survival to extreme old age. The method comprised essentially three steps:

1. The first step builds a set of mathematical models that can be used together to distinguish centenarians from individuals selected from the general population using only genetic data.
2. The second step uses the set of models to generate a genetic risk profile of exceptional longevity for each study subject. These genetic risk profiles of centenarians can be analyzed using cluster analysis that essentially groups centenarians based on different patterns of genetic risk. We termed the average profiles associated with these clusters as "genetic signatures" of exceptional longevity and they represent combinations of genetic variants that produce a similar chance or probability for exceptional longevity.
3. The third step correlates these genetic signatures with different phenotypic paths to exceptional longevity, to begin to understand what genetic variants are associated with different patterns of exceptional longevity.

For step 1, we used a class of Bayesian classification models that are suitable to analyze case-control studies and developed a forward search algorithm to build nested models with increasing numbers of SNPs and a stopping rule based on sensitivity and specificity of the classification models. To derive the models, all approximately 240,000 SNPs were ranked according to their posterior probability of association with exceptional longevity, and SNPs in strong linkage disequilibrium were pruned out (Sebastiani et al., 2012b). Then beginning with the SNP demonstrating the strongest probability, a set of nested Bayesian classification models was built by adding one SNP at a time from the sorted list of SNPs, and the sensitivity (centenarians predicted as centenarians) and specificity (controls predicted as controls) was assessed. SNPs were no longer added when both the specificity and sensitivity did not increase significantly. These types of Bayesian models can be mathematically related to logistic models with a genetic risk score (Sebastiani et al., 2012c), but they can be adapted more easily to include multiple traits and additional covariates (Hartley et al., 2012).

This algorithm identified 281 SNPs in 130 genes and intragenic regions that include well known aging and age-related disease genes, but also novel genes. The set of models was used together to distinguish between centenarians and controls using an average (ensemble) of the predictions of each single model. It is well known that an ensemble of prediction models usually outperform single "best" models because it is more robust to inclusion of false positive SNPs (Rokach, 2010).

To validate this set of models, we tested their joint accuracy to distinguish between centenarians and controls in independent sets of centenarians and controls reaching 60% specificity and a sensitivity between 58 and 85% (the older the centenarians the greater the sensitivity). The sensitivity of the set of models to distinguish controls from nonagenarians and older was low (58%), but was higher to distinguish controls from older centenarians. This result is consistent with an increasing genetic contribution to reach older and older ages beyond 100 years. The lower specificity could be due to the fact that longevity variants are also present in

the controls and that other non-genetic factors may be needed to for individuals to survive to very extreme ages.

In step 2, the set of 281 SNPs and models were used to first summarize the prediction of the set of nested models in the form of genetic risk profiles. The display of genetic risk profiles provides information about the enrichment of longevity variants of an individual because subjects with similar genetic profiles share most of the longevity SNPs and therefore a similar risk for longevity (See **Figure 3**). By cluster analysis of the genetic risk profiles, centenarians with similar genetic risk profiles can then be grouped to generate what we called genetic signatures of exceptional longevity. We used a Bayesian model-based cluster analysis that is described in (Ramoni et al., 2002) to group centenarians with similar genetic risk profiles. This approach essentially clusters centenarians with genetic risk profiles that follow the same probability distribution. Full details are in (Sebastiani et al., 2012b). We further found that different genetic signatures correlate with significantly different life spans and significantly different ages of onset of major age-related diseases such as dementia, cancer, and cardiovascular disease. Note that while a genetic risk profile informs about the enrichment of risk variants that an individual carries, genetic signatures represent the prevalent genetic risk profiles that lead to different risks. Therefore, genetic signatures can be used for dissection of a complex trait, patient stratification, experimental design, and understanding the mechanism that links genotype to phenotype.

THE ROLE OF DISEASE ALLELES AND LONGEVITY

One of our hypotheses about the genetic make-up of exceptional longevity was that a relative lack of disease-associated variants could in part explain centenarians' survival advantage. To test this hypothesis we computed the rate of SNP alleles that were associated with a variety of diseases and traits in several GWASs from the catalog of published genome-wide association studies[5] (Hindorff et al., 2009) and the Human Gene Mutation Database (HGMD; Stenson et al., 2009). We found 1,214 of the 62,339 disease-associated SNPs in the GWAS of exceptional longevity and we did not observe a significant difference in the rate of disease-associated variants carried by centenarians and population controls. The analysis was conducted by stratifying the rates by disease type and in none of the 14 groups of disease that we analyzed did we note a difference between centenarians and a large number of controls from the NECS and other GWAS. This result agreed with recent findings from the Leiden Longevity and Leiden 85+ Studies that in their nonagenarian sample, the rate of disease-associated variants for a select group of age-related diseases was the same as in the general population (Beekman et al., 2010).

Although this analysis is incomplete because the genotype data did not include most of the known disease variants and the Illumina controls are likely healthy, we noted an equivalent result in the whole genomes of two supercentenarians (Sebastiani et al., 2012a). These two genomes included only 1% of mutations from the HGMD and approximately 50% of the mutations that were linked to common diseases in genome-wide association studies. These disease mutations included known SNPs linked to age-related diseases such as Alzheimer's and ALS, cancer and cardiovascular disease. Compared to 11 other whole genome sequences, the

[5]http://www.genome.gov/26525384

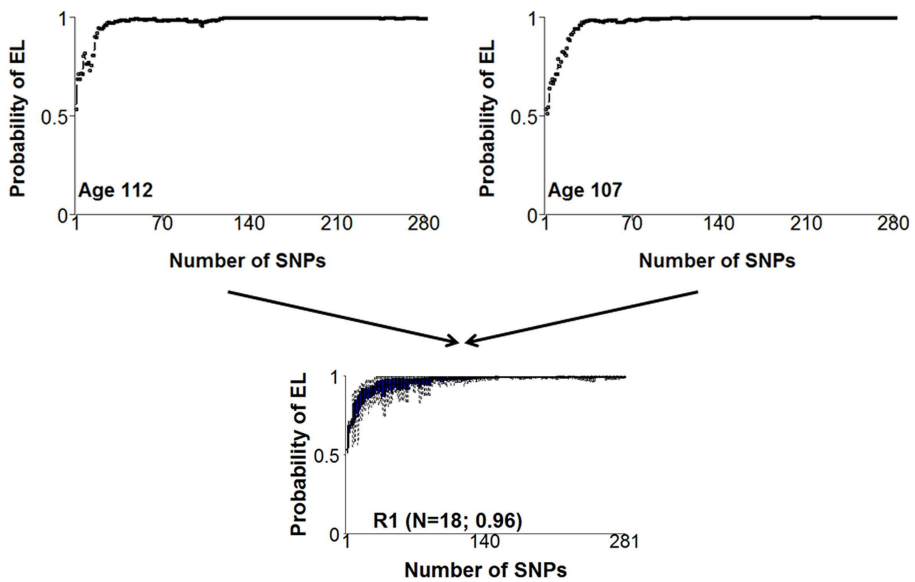

FIGURE 3 | Examples of two similar genetic risk profiles that are highly predictive of exceptional longevity and a cluster of profiles that include the two profiles. The *x*-axis displays the 281 SNPs, sorted by the significance of the association with exceptional longevity. The *y*-axis represents the posterior probability of exceptional longevity given the nested sets of SNPs. The two genetic risk profiles in the top panel represent the pattern of risk for varying combinations of genotypes of the 281 SNPs that we found associated with exceptional longevity. The two plots are not exactly the same, meaning that the two subjects have some different alleles of the 281 SNPs. However, the similar pattern of risk means that they essentially have the same genetic basis for exceptional longevity and were assigned to the same cluster displayed in the bottom panel.

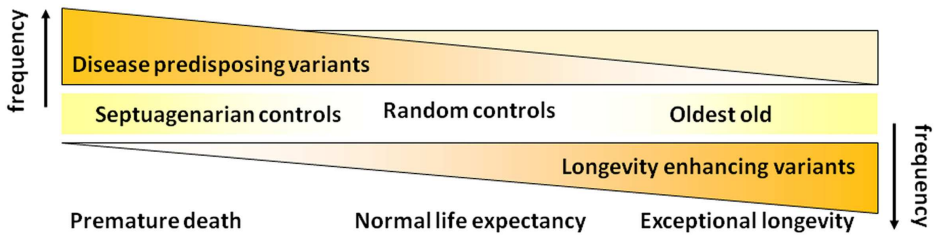

FIGURE 4 | Revised hypothesis for the prevalences of disease and longevity (or protective) associated variants with increasing age at very old ages.

two supercentenarian genomes carried a rate of disease variants comparable to the Venter and Watson genomes and even higher rates compared to Caucasians from the 1,000 Genomes project.

Realizing that disease-associated variants are not necessarily weeded out of the population as people survive to the most extreme ages, we have subsequently revised the hypothesis that we began our studies with (**Figure 2**). As shown in **Figure 4**, we now hypothesize that with increasing age there is not so much a decline in the prevalence of disease-associated genetic variants but rather there is a selection for longevity-associated variants which not only can counter the deleterious effects of genetic and environmental factors but also afford protection against basic mechanisms of aging, slow the rate of aging and delay the onset of age-related diseases, and syndromes.

NOTICEABLE GENES LINKED TO EXCEPTIONAL LONGEVITY AND FOLLOW UPS

The 281 SNPs that we found predictive of exceptional longevity include SNPs in genes associated with age-related diseases such as Alzheimer's, dementia, and cardiovascular disease. Although the SNP rs2075650 in TOMM40/APOE reached genome-wide significance and the AA genotype is associated with increased odds for exceptional longevity, its effect was limited and we showed that this SNP explains only 1% of the predictive accuracy of the joint set of 281 SNPs (Sebastiani et al., 2012b). The GG genotype of this SNP is linked to the ε4 allele of the *APOE* gene and while carriers of this genotype are clearly predisposed to early mortality, carriers of the AA genotype seem to have a small survival advantage compared to AG carriers (Schupf et al., 2012). We are currently conducting replication studies of these 281 SNPs to identify the most robust longevity variants to be carried forward in functional studies. The centenarians' genomes that we published in (Sebastiani et al., 2012a) suggest that most of the SNPs in the list of 281 are located closer to coding SNPs compared to randomly chosen SNPs. For example, the two genomes included more than 50% of the longevity variants in genes and approximately 20% of these mutations were within 10 kb from coding mutations. These data helped us identify new coding mutations for example in the progeria genes LMNA and WRN which is particularly interesting since these findings suggest that different variations of these genes may lead to either extreme premature aging or extreme longevity. Details of the specific mutations are in (Sebastiani et al., 2012a).

CONCLUSION

People surviving to 100 years and older generally delay the onset of disability well into their nineties. For those who survive to the most extreme ages, for example beyond 105 years, we have observed a progressive compression of morbidity as well. These oldest of the old individuals also appear to be more phenotypically homogeneous compared to people surviving to just 100 years. Numerous genome-wide association studies of centenarians have yielded only very few statistically significant findings. The most notable and consistent of these is a variant of apolipoprotein E which has been shown in candidate gene studies to be the variant APOE ε4 which is both rare and deleterious in centenarian studies of various ethnicities. Interestingly though, for some ethnicities, the frequency of this allele can be substantially greater. Analyses of sibships that cluster for exceptional longevity and the increasing but rare phenotypic homogeneity of the most extreme old still suggest a strong genetic component. Thus, the NECS and other studies hypothesize that exceptional longevity is a complex trait influenced by multiple genetic variants that individually have modest effects, but as a group can exert a strong effect. Using a Bayesian analytic approach to construct a genetic model that predicts exceptional longevity, we have found that with older and older age beyond 100 years, this genetic influence appears to get stronger and stronger. While the APOE ε4 allele has garnered much attention for its negative association with exceptional longevity, we found that its contribution to distinguishing between centenarians and healthy controls is minimal (Sebastiani et al., 2012b). Also counter to the conventional wisdom, now several studies have shown that centenarians have many of the disease-associated variants found in the general population thus suggesting an important role for protective variants that counter the effects of these deleterious variants as well as possibly slow the rate of aging and the decrease the risk for age-related diseases that contribute to premature mortality. Genetic models such as these may be used to discover new target genes and pathways related to aging and longevity.

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Metabolic characteristics of long-lived mice

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Genetic suppression of insulin/insulin-like growth factor signaling (IIS) can extend longevity in worms, insects, and mammals. In laboratory mice, mutations with the greatest, most consistent, and best documented positive impact on lifespan are those that disrupt growth hormone (GH) release or actions. These mutations lead to major alterations in IIS but also have a variety of effects that are not directly related to the actions of insulin or insulin-like growth factor I. Long-lived GH-resistant GHR-KO mice with targeted disruption of the GH receptor gene, as well as Ames dwarf ($Prop1^{df}$) and Snell dwarf ($Pit1^{dw}$) mice lacking GH (along with prolactin and TSH), are diminutive in size and have major alterations in body composition and metabolic parameters including increased subcutaneous adiposity, increased relative brain weight, small liver, hypoinsulinemia, mild hypoglycemia, increased adiponectin levels and insulin sensitivity, and reduced serum lipids. Body temperature is reduced in Ames, Snell, and female GHR-KO mice. Indirect calorimetry revealed that both Ames dwarf and GHR-KO mice utilize more oxygen per gram (g) of body weight than sex- and age-matched normal animals from the same strain. They also have reduced respiratory quotient, implying greater reliance on fats, as opposed to carbohydrates, as an energy source. Differences in oxygen consumption (VO_2) were seen in animals fed or fasted during the measurements as well as in animals that had been exposed to 30% calorie restriction or every-other-day feeding. However, at the thermoneutral temperature of 30°C, VO_2 did not differ between GHR-KO and normal mice. Thus, the increased metabolic rate of the GHR-KO mice, at a standard animal room temperature of 23°C, is apparently related to increased energy demands for thermoregulation in these diminutive animals. We suspect that increased oxidative metabolism combined with enhanced fatty acid oxidation contribute to the extended longevity of GHR-KO mice.

Keywords: growth hormone, aging, calorie restriction, dwarf mice, metabolism

INTRODUCTION: GROWTH HORMONE-RELATED MOUSE MUTANTS

Studies of hypopituitary, growth hormone (GH) deficient, and GH-resistant mice provided evidence that deletion of GH signals can produce an impressive extension of longevity (Brown-Borg et al., 1996; Flurkey et al., 2001; Coschigano et al., 2003). Mice lacking GH or GH receptors show numerous symptoms of delayed aging, are partially protected from age-related diseases, and outlive their normal siblings by 30–65% depending on genetic background, sex, and diet composition (reviewed in Bartke, 2011; Bartke, 2012; Brown-Borg and Bartke, 2012). Candidate mechanisms linking the absence of GH signals with extension of longevity include altered expression of numerous genes related to glucose homeostasis, protein synthesis, lipogenesis, lipolysis, and energy metabolism (Tsuchiya et al., 2004; Al-Regaiey et al., 2005; Papaconstantinou et al., 2005; Masternak and Bartke, 2007). Apparently, anti-aging effects of reduced GH signaling involve metabolic adjustments of which some resemble those that mediate the effects of calorie restriction (CR) on aging and longevity (Tsuchiya et al., 2004; Al-Regaiey et al., 2005; Bonkowski et al., 2009).

In this brief review, we will discuss metabolic characteristics of GH-deficient and GH-resistant mice which are likely to represent mechanisms of their extended longevity. Metabolic characteristics of other long-lived mutants, gene knockouts and transgenics as well as phenotypes of mice from strains with different longevity are outside the scope of this article, and the reader is referred to other reviews (Brown-Borg, 2006; Chen et al., 2010; Yuan et al., 2011).

ROLE OF IMPROVED INSULIN SIGNALING

Improved action of insulin on carbohydrate homeostasis is among the key metabolic alterations in long-lived GH-related mutants. GH receptor disrupted GHR-KO mice with profound GH resistance (Zhou et al., 1997), GH releasing hormone disrupted (GHRH-KO) mice with isolated GH deficiency (Alba and Salvatori, 2004), and hypopituitary Ames ($Prop1^{df}$) and Snell ($Pit1^{dw}$) dwarf mice with deficiency of GH, along with prolactin and thyrotropin (Bartke, 2011; Brown-Borg and Bartke, 2012; Bartke et al., in press), have reduced insulin levels and enhanced insulin sensitivity (Zhou et al., 1997; Bonkowski et al., 2006; Bartke, 2011; Bartke, 2012; Brown-Borg and Bartke, 2012; Spong and Bartke, unpublished). Since hypoinsulinemia promotes insulin sensitivity and *vice versa*, it could be debated which of these characteristics is primary and which is secondary. However, available evidence suggests that reduction in GH signals affects both the secretion and actions of insulin. GH and insulin-like growth factor I (IGF-I), a key mediator of GH action, promote development and secretory function of insulin-producing beta cells in the islets of Langerhans

in the pancreas. Islet volume is reduced in GHR-KO mice (Guo et al., 2005), and the number of large islets is reduced in Ames dwarf mice (Parsons et al., 1995). Insulin sensitivity is negatively regulated by GH by a variety of mechanisms including reduced adiponectin levels, enhanced mammalian target of rapamycin (mTOR) signaling, and alterations in serum lipid profiles as well as ectopic fat accumulation. Many of these effects of GH on insulin signaling are mediated by enhanced inhibitory (serine) phosphorylation of insulin receptor substrate 1 (IRS-1; Aguirre et al., 2002; Ishizuka et al., 2004; Adochio et al., 2009). All of these mechanisms appear to be involved in improving insulin sensitivity in GH-related mouse mutants (Al-Regaiey et al., 2005; Wang et al., 2006; Bonkowski et al., 2009; List et al., 2011).

ROLE OF ADIPOSE TISSUE AND ITS PRODUCTS IN THE METABOLIC PROFILE OF GH-RELATED MUTANTS

We have recently obtained evidence that enhanced insulin sensitivity of long-lived GHR-KO mice is due to the altered secretory profile of intra-abdominal ("visceral") adipose tissue and, in particular, to enhanced adiponectin secretion by these fat depots. It is well documented that adiponectin is an important insulin sensitizer. In comparison to normal mice, GHR-KO mutants have increased levels of adiponectin in the epididymal fat and in peripheral circulation (Al-Regaiey et al., 2005; List et al., 2011). To assess the impact of altered secretory activity of visceral fat on insulin signaling, we have compared the impact of removing most of this tissue on insulin and glucose tolerance in these mutants versus normal mice. We removed as much of the epididymal and perinephric (retroperitoneal) fat pads as was possible without endangering blood supply to the testes and the adrenals. In normal mice this resulted in significant improvements in insulin and glucose tolerance (Masternak et al., 2012) as expected from previous studies in this and other species (Shi et al., 2007; Muzumdar et al., 2008). Plasma adiponectin levels were not altered, indicating that in these animals circulating adiponectin is derived primarily from subcutaneous fat, or that other fat depots readily compensate for the consequences of removing visceral fat. In sharp contrast to these findings, visceral fat removal in GHR-KO mice reduced circulating adiponectin levels and reduced, rather than enhanced, tolerance to injected insulin or glucose (Masternak et al., 2012). Apparently, visceral fat is a major source of adiponectin in these animals and visceral fat-derived adiponectin importantly contributes to or perhaps accounts for enhanced insulin sensitivity of GHR-KO mice. In addition to differences in the levels of adiponectin, the levels of interleukin 6 (IL-6), which promotes insulin resistance, are reduced in both epididymal and perinephric fat of GHR-KO as compared to normal mice (Masternak et al., 2012). Altered IL-6 levels may have also contributed to the differential impact of visceral fat removal on insulin sensitivity in GHR-KO versus normal mice.

INTERACTIONS OF CALORIE RESTRICTION AND GH-RELATED MUTATIONS

Association of reduced insulin levels and enhanced insulin sensitivity with extension of longevity was shown in a comparison of GH-related mutants (GHR-KO, GHRH-KO, Prop1^{df}, Pit1^{dw}) with their normal siblings and in studies of the interaction of some of these "longevity genes" with CR (Masternak et al., 2009). Strikingly, CR improves insulin signaling in Ames dwarf mice, in which it also extends longevity (Bartke et al., 2001; Masternak et al., 2009), but has no such effect in GHR-KO mice or in GHRH-KO males in which effects of CR on longevity are absent or minimal (Bonkowski et al., 2006, 2009; Spong, Salvatori, and Bartke, unpublished). Moreover, longevity is not enhanced in transgenic mice overexpressing a GH antagonist in which insulin levels are not suppressed (Coschigano et al., 2003). It deserves emphasis that a reduction in insulin levels and enhancement of insulin sensitivity are among the most consistently observed responses to CR in different mammalian species ranging from mice and rats to nonhuman primates and humans (Fontana et al., 2004; Anderson and Weindruch, 2012).

In contrast to the strong association of improved insulin signaling with extended longevity in GH-related mutants, several mutations affecting events "downstream" from GH and/or IGF-I receptors are long-lived and insulin resistant (Kurosu et al., 2005; Selman et al., 2009). Further work, including examination of insulin signaling at different stages of life history will be needed to reconcile these findings but possible explanations include the well-documented opposite effects of GH and IGF-I on insulin signaling, as well as a possibility that insulin resistance may mimic some of the effects of hypoinsulinemia by protecting the cells from excessive insulin stimulation (Taguchi et al., 2007; Selman et al., 2009).

INFLAMMATION MARKERS AND METABOLIC ADJUSTMENTS

In addition to influencing glucose homeostasis, suppression of GH signaling promotes β oxidation of fatty acids. Fatty acid oxidation is promoted by the direct or indirect actions of peroxisome proliferator activator receptor α (PPARα), PPARγ coactivator 1α (PGC1α), fibroblast growth factor 21 (FGF-21), adiponectin, and AMP-activated protein kinase (AMPK) – and GH negatively regulates the expression or activation of each of these factors (Al-Regaiey et al., 2005; Masternak and Bartke, 2007; Bonkowski et al., 2009; Louis et al., 2010). Increases in the levels of adiponectin and activation of AMPK in GH-resistant and GH-deficient animals also reduce pro-inflammatory signals by inhibiting nuclear factor kappa B (NFκB) signaling (Salminen et al., 2011; Masternak and Bartke, 2012). The resulting shift in the balance of pro- and anti-inflammatory cytokines constitutes yet another potential mechanism of enhancing insulin sensitivity (Salminen et al., 2011). Association of an altered balance of pro- and anti-inflammatory markers with shifts in carbohydrate and lipid homeostasis in long-lived GH-related mutants can thus be related to the involvement of the same mediators of GH action in the control of inflammation and metabolism.

MITOCHONDRIAL FUNCTION AND OXIDATIVE METABOLISM

Enhanced hepatic expression of PGC1α and reduced serum lipid levels in GH-resistant mice (Al-Regaiey et al., 2005; List et al., 2011) suggest alterations in the number and function of mitochondria. PGC1α is a key regulator of mitochondrial biogenesis, and mitochondrial utilization of fatty acids as a metabolic fuel has a major impact on lipid homeostasis and circulating lipid levels.

There is little information on the number or morphology of mitochondria in long-lived GH-related mutants, while available data suggest lack of major changes in mitochondrial density in the liver or muscle of GHR-KO mice (Westbrook et al., unpublished). In Ames dwarf mice, generation of reactive oxygen species (ROS) by the skeletal muscle mitochondria is reduced, suggesting improved mitochondrial efficiency (Brown-Borg, 2006).

We are using indirect calorimetry to study the impact of GH signaling on energy metabolism. Twenty-four hour recordings of oxygen consumption and carbon dioxide output revealed that oxygen consumption (VO_2) per gram of body weight is significantly increased and respiratory quotient (RQ) significantly reduced in Ames dwarf and GHR-KO mice (Westbrook et al., 2009).

These differences were present whether the animals were fed *ad libitum* or fasted during the recording (Westbrook et al., 2009). Moreover, similar differences between GHR-KO and normal mice were detected after exposing the animals to a prolonged period of caloric restriction or every-other-day-feeding (Westbrook et al., unpublished). Interestingly, opposite changes (reduced VO_2 and increased RQ) were seen in giant PEPCK-bGH transgenic mice which are hypersomatotropic, hyperinsulinemic, insulin resistant, and short-lived (Bartke, 2003; Westbrook et al., 2009). The increase of VO_2 in GHR-KO and Ames dwarf mice was apparently not due to expressing the data per unit of body mass, because differences between mutant and normal animals were, if anything, magnified when the data were recalculated per unit of lean body mass (as determined by DEXA in age- and sex-matched mice; Westbrook, 2012).

Detecting this increase in VO_2 was not anticipated particularly in Ames dwarf mice which are hypothyroid and hypothermic and have reduced spontaneous locomotor activity (Bartke, 2011; Bartke, 2012; Brown-Borg and Bartke, 2012). Moreover, VO_2 was reported to be reduced in Snell dwarf mice which phenotypically resemble the Ames dwarfs (Benedict and Lee, 1936). We suspected that the increase of VO_2 in GH-related mutants could reflect increased energy expenditure for thermogenesis needed to compensate for increased heat loss. Increased radiation of heat would be expected in these diminutive animals because of the increased body surface to mass ratio. To test the validity of this explanation, we have compared VO_2 in GHR-KO and normal mice at a thermoneutral ambient temperature of 30°C. Under these conditions, VO_2 of the mutants greatly declined from the values measured at lower temperature and no longer differed from the normal animals (Westbrook et al., unpublished). We conclude that increased VO_2 in long-lived dwarf mice reflects increased energy demand for thermogenesis under conditions imposed by housing at the standard animal room ambient temperature (approximately 22°C). It is an intriguing possibility that this increase in energy expenditure might contribute to slow aging and extended longevity of these mutants. Koizumi et al. (1996) reported that the beneficial impact of CR on cancer incidence and longevity in mice can be reduced or eliminated by housing the animals at a thermoneutral temperature. However, these authors suggested that the effects of thermoneutral temperature in their study were due to eliminating torpor which was a common (daily) occurrence under the conditions of fairly severe CR they employed (Koizumi et al., 1996). We very rarely observe torpor in our animals.

Since metabolic rate declines during aging, an increase in VO_2 in long-lived mutant mice could be viewed either as a potential mechanism of extended longevity or as a "biomarker" of delayed and/or slower aging. Association of increased metabolic rate with improved life expectancy might be due to the benefits of increased uncoupling of mitochondrial electron transport from ATP production (Brand, 2000) and activation of AMPK. Reduced mTOR signaling and S6K activity in Ames dwarf and GHRKO mice (reviewed in Bartke, 2011) may provide yet another link between the regulation of aging, oxidative metabolism, and energy substrate utilization. It was recently reported that a leucine-deficient diet which suppresses hypothalamic S6KI activity produces an increase in VO_2 per unit of body mass and a reduction in RQ; these are alterations similar to those we detected in long-lived dwarf mice (Xia et al., 2012). Examples of the association of increased VO_2 and reduced RQ with resistance to detrimental effects of high fat diet are provided in the next section of this article.

ALTERED USAGE OF ENERGY SUBSTRATES

In addition to demonstrating an increase in VO_2, indirect calorimetry studies of Ames dwarf and GHR-KO mice revealed another metabolic characteristic of these long-lived animals, namely a reduction of RQ. As was the case with VO_2, these differences were detected during both dark (active) and light (resting) parts of the 24-h period, were present in both fully fed and fasted animals, and were opposite to changes measured in short-lived giant PEPCK-GH transgenics (Westbrook et al., 2009). Reduced RQ values indicate increased reliance on fat, as opposed to carbohydrate, as a metabolic fuel and thus denote an important shift in mitochondrial function. Increased "fat burning" by mitochondria is believed to be associated with improved metabolic efficiency and reduced production of potentially harmful ROS (Lopez-Lluch et al., 2006; Ukropcova et al., 2007; Anderson and Weindruch, 2010). Similar metabolic adjustments are associated with extension of longevity in animals exposed to CR (Anderson and Weindruch, 2010). Moreover, reduced RQ and enhanced VO_2 were associated with protection from high fat diet-induced obesity, glucose intolerance and diabetes in mice with ablated agouti-related protein (AgRP) producing neurons and in retinaldehyde dehydrogenase 1a1 knock-out mice (Joly-Amado et al., 2012; Kiefer et al., 2012). Likely mechanisms of increased β oxidation of fatty acids in GHRKO and Ames dwarf mice include increases in adiponectin levels (Al-Regaiey et al., 2005; List et al., 2011), activation of AMPK (Al-Regaiey et al., 2005), and expression of hepatic PPARα (Masternak and Bartke, 2007).

In contrast, to findings in Ames dwarf and GHR-KO mice, extended longevity in mice with fat-specific deletion of insulin receptors, as well as improvement of the metabolic profile of obese mice after gastric bypass, are associated with increases in both VO_2 and RQ (Katic et al., 2007; Nestoridi et al., 2012). From the data that are currently available, it is difficult to determine whether the association of increased VO_2 and reduced RQ in long-lived GH-related mutants is in any way related to the uncommon association of increased obesity with reduced insulin and increased adiponectin levels in these animals.

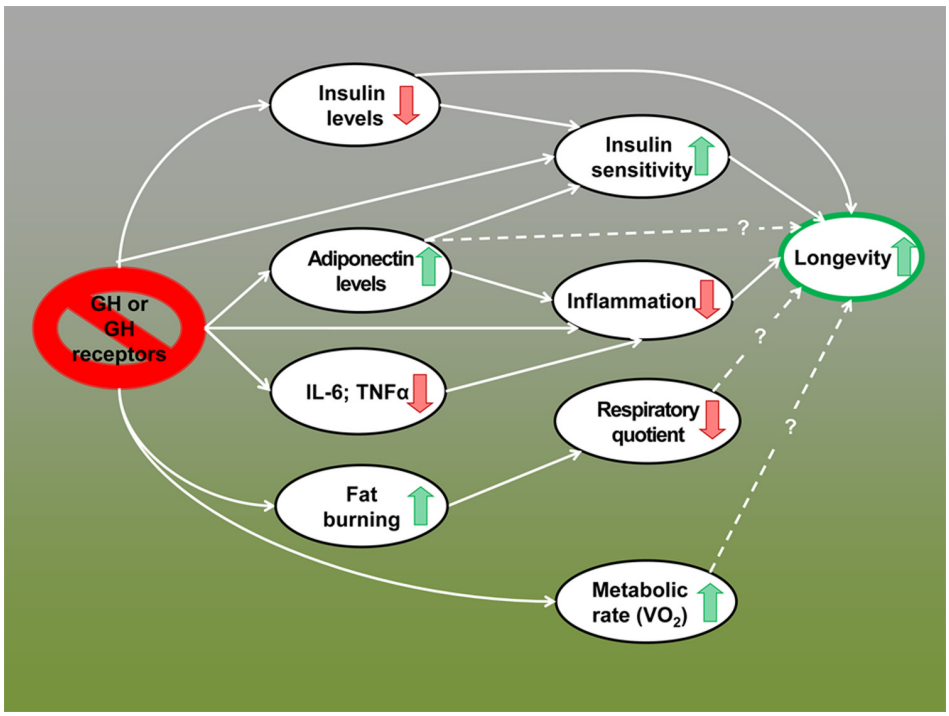

FIGURE 1 | Metabolic alterations in GH-deficient and GH-resistant mice; possible mechanisms of extended longevity.

SUMMARY AND RELATIONSHIP TO REGULATION OF HUMAN AGING

The remarkable extension of longevity in mice lacking GH or GH receptors appears to be due to multiple interacting mechanisms including reduced activation of growth-promoting pathways, greater stress resistance, reduced inflammation, increased reservoir of pluripotent stem cells, and improved genome maintenance (Flurkey et al., 2001; Coschigano et al., 2003; Murakami et al., 2003; Garcia et al., 2008; Bokov et al., 2009; Bartke, 2011; Ratajczak et al., 2011; Bartke, 2012; Brown-Borg and Bartke, 2012). Data summarized in this article indicate that alterations in energy metabolism and improved insulin control of carbohydrate homeostasis have to be added to this list. In fact, these metabolic adaptations may represent key features of the "longevous" phenotype of these animals and important mechanisms of the extension of both healthspan and lifespan in GH-related mutants (**Figure 1**).

Importantly, many of the metabolic features of long-lived mutant mice described in this article have been associated with extended human longevity. Comparisons between centenarians and elderly individuals from the same population and between the offspring of exceptionally long-lived people and their partners indicate that reduced insulin, improved insulin sensitivity, increased adiponectin, and reduced pro-inflammatory markers consistently correlate with improved life expectancy (Kojima et al., 2004; Atzmon et al., 2006; Baranowska et al., 2006; Bonafè and Olivieri, 2009; Rozing et al., 2011; Wijsman et al., 2011).

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Mitophagy in neurodegeneration and aging

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Macroautophagy is a cellular catabolic process that involves the sequestration of cytoplasmic constituents into double-membrane vesicles known as autophagosomes, which subsequently fuse with lysosomes, where they deliver their cargo for degradation. The main physiological role of autophagy is to recycle intracellular components, under conditions of nutrient deprivation, so as to supply cells with vital materials and energy. Selective autophagy also takes place in nutrient-rich conditions to rid the cell of damaged organelles or protein aggregates that would otherwise compromise cell viability. Mitophagy is a selective type of autophagy, whereby damaged or superfluous mitochondria are eliminated to maintain proper mitochondrial numbers and quality control. While mitophagy shares key regulatory factors with the general macroautophagy pathway, it also involves distinct steps, specific for mitochondrial elimination. Recent findings indicate that parkin and the phosphatase and tensin homolog-induced putative kinase protein 1 (PINK1), which have been implicated in the pathogenesis of neurodegenerative diseases such as Parkinson's disease, also regulate mitophagy and function to maintain mitochondrial homeostasis. Here, we survey the molecular mechanisms that govern the process of mitophagy and discuss its involvement in the onset and progression of neurodegenerative diseases during aging.

Keywords: aging, autophagy, neuron, mitochondria, mitophagy, neurodegeneration, parkin, PINK1

INTRODUCTION

Macroautophagy (henceforth referred to as autophagy) is a high-regulated catabolic process responsible for the lysosomal degradation of cytoplasmic constituents. The main characteristic of the autophagic pathway is the formation of a double-membrane structure known as autophagosome, which engulfs cytoplasmic cargo and delivers it to lysosomes for degradation (Klionsky, 2007). In direct correlation with the large variety of autophagy substrates, including cytoplasmic proteins, ribosomes, organelles, bacteria and viruses, autophagy defects have been associated with a wide range of human disorders, such as cancer, autoimmune and neurodegenerative diseases (Mizushima et al., 2008). The main physiological role of autophagy is to supply the cell with essential materials and energy by recycling intracellular components, under conditions of nutrient deprivation when nutrients cannot be obtained from the extracellular environment. Selective types of autophagy, including pexophagy (Sakai et al., 2006), ribophagy (Kraft et al., 2008), ER-phagy (Bernales et al., 2007), protein selective chaperone-mediated autophagy (Cuervo et al., 2004), nucleophagy (Mijaljica et al., 2010), mitochondrial autophagy (mitophagy; Lemasters, 2005) take place under nutrient-rich conditions to rid the cell of damaged organelles or protein aggregates that would otherwise compromise cell viability.

Mitochondria are double-membrane-bound organelles, essential for energy production and cellular homeostasis in eukaryotic cells. In addition, mitochondria have vital roles in calcium signaling and storage, metabolite synthesis, and apoptosis (Kroemer et al., 2007). Thus, mitochondrial biogenesis, as well as, elimination of damaged and superfluous mitochondria are highly regulated processes. Mitophagy is a selective type of autophagy that mediates the removal of mitochondria. Through mitophagy cells regulate mitochondrial number in response to their metabolic state and also implement a quality control system for proper elimination of damaged mitochondria. The process of mitophagy is highly regulated and conserved from yeast to mammals (**Table 1**). While mitophagy shares key regulatory factors with the general autophagy pathway, it also involves distinct steps, specific for mitochondrial elimination. Studies in yeast identified specific genes that are required for mitophagy, but not for other types of autophagy (Kanki et al., 2009a; Kanki and Klionsky, 2010), demonstrating the selective regulation of this process. Despite the fact that the actual selection of mitochondria for degradation is a still obscure part of the process, recent studies shed light on the mechanisms that govern mitophagy and regulate removal of mitochondria during developmental processes or upon mitochondrial damage. In this review, we survey the molecular mechanisms that mediate mitophagy and also highlight how defects in this process may contribute to the onset and progression of neurodegenerative diseases during aging.

MOLECULAR MECHANISMS OF MITOPHAGY

The molecular mechanisms of mitophagy were studied in the yeast *Saccharomyces cerevisiae*. The yeast *uth1* gene encodes a Sad1p/UNC-84 (SUN)-domain protein that is located in the outer mitochondrial membrane and is essential for the specific autophagic elimination of mitochondria upon nitrogen starvation or rapamycin treatment, without influencing general autophagy (Kissova et al., 2004). The protein Aup1, a member of protein phosphatase 2C (PP2C) superfamily that is located in the

Table 1 | Mitophagy-specific factors are highly conserved form yeast to mammals.

Organism				Function	Role
Saccharomyces cerevisiae	***Caenorhabditis elegans***	***Drosophila melanogaster***	***Mus musculus***		
Atg32	–	–	–	Mitophagy receptor	Interaction with Atg8 recruits the autophagic machinery
–	DCT-1	–	NIX/BNIP3	Mitophagy receptor	Interaction with LC3/GABARAP recruits the autophagic machinery
–	PINK-1	Pink1	PINK1	Ser/Thr protein kinase	Phosphorylates and recruits Parkin to mitochondria
–	PDR-1	Parkin	PARKIN	E3 ubiquitin ligase	Ubiquitinates outer membrane mitochondrial proteins such as Mfn1/2, VDAC, MIRO1/2
–	SQST-1 (T12G3.1)	Ref(2)P	SQST-1/p62	Adaptor protein	Interacts with ubiquitinated proteins to recruit the autophagic machinery
Fzo1	FZO-1	Fzo, Dmfn	MFN-1/2	Outer membrane fusion	Ubiquitinated by Parkin; their degradation precedes mitophagy induction
Vdac1	VDAC-1 (R05G6.7)	DmVDAC	VDAC1	Voltage-dependent anion channel; outer mitochondrial membrane	Upon ubiquitination by Parkin induces the recruitment of the autophagic machinery

mitochondrial intermembrane space, is essential for efficient mitophagy at the stationary phase (Tal et al., 2007). Aup1 may regulate mitophagy by also controlling the retrograde response pathway (Journo et al., 2009).

Another factor required for mitophagy is Atg32, a 59 kDa protein, located in the outer mitochondrial membrane (Kanki et al., 2009b; Okamoto et al., 2009a). The amino- and carboxy-terminal domains of Atg32 are oriented toward the cytoplasm and intermembrane space, respectively. Atg32 is thought to act as a mitochondrial receptor that binds the adaptor protein Atg11, to sequester mitochondria to the phagophore assembly site (PAS), during mitophagy (Okamoto et al., 2009b). The cytosolic domain of Atg32 contains an evolutionary conserved WXXL-like motif, which is critical for the interaction with Atg8 (the yeast homolog of the mammalian autophagosome protein LC3; Okamoto et al., 2009b). Thus, Atg32 can interact with Atg8 directly through the WXXL-like motif or indirectly through Atg11. This association is thought to recruit autophagosomes to mitochondria (**Figure 1A**). Atg32 is the first protein shown to interact with the core autophagic machinery, and be required specifically for mitophagy. Interestingly, loss of Atg32 does not alter cellular reactive oxygen species (ROS) levels or growth on non-fermentable carbon sources (Kanki et al., 2009b). This suggests the existence of additional Atg32-independent mitophagy pathways. Recent studies identified two mitogen-activated protein kinases (MAPKs), Stl2 and Hog1, also required for the specific elimination of mitochondria via autophagy in *S. cerevisiae* (Mao et al., 2011). These two positive regulators establish an additional regulatory step in the process of mitophagy, underlining the complexity of this organelle quality control system.

THE PINK1/PARKIN PATHWAY IN MITOPHAGY REGULATION

Mutations in the genes encoding the cytosolic E3 ubiquitin ligase Parkin and the mitochondrial phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) have been shown to cause a recessive form of parkinsonism (Kitada et al., 1998; Valente et al., 2004). However, the involvement of these proteins in the pathogenesis of Parkinson's disease remained obscure. Studies in *Drosophila melanogaster* indicate that PINK1 and Parkin act in the same genetic pathway to regulate mitochondrial network integrity (Greene et al., 2003; Park et al., 2006). In healthy mitochondria, PINK1 is probably imported through the translocase complexes of the outer and inner mitochondrial membrane (TOM and TIM, respectively). PINK1 is subsequently cleaved by several proteases such as the mitochondrial-processing protease (MPP), the inner membrane presenilin-associated rhomboid-like protease (PARL; Meissner et al., 2011; Greene et al., 2012). Upon mitochondrial depolarization, import of PINK1 to the inner mitochondrial membrane is blocked and PINK1 is stabilized on outer mitochondrial membrane (Lazarou et al., 2012). Accumulation of PINK1 on the mitochondrial surface induces mitophagy by recruiting Parkin to damaged mitochondria through a mechanism that is not well-understood. Thus, PINK1 likely functions as a sensor for damaged mitochondria. Recent studies have demonstrated that translocation of Parkin to impaired mitochondria requires PINK1 activity (Narendra et al., 2008; Geisler et al.,

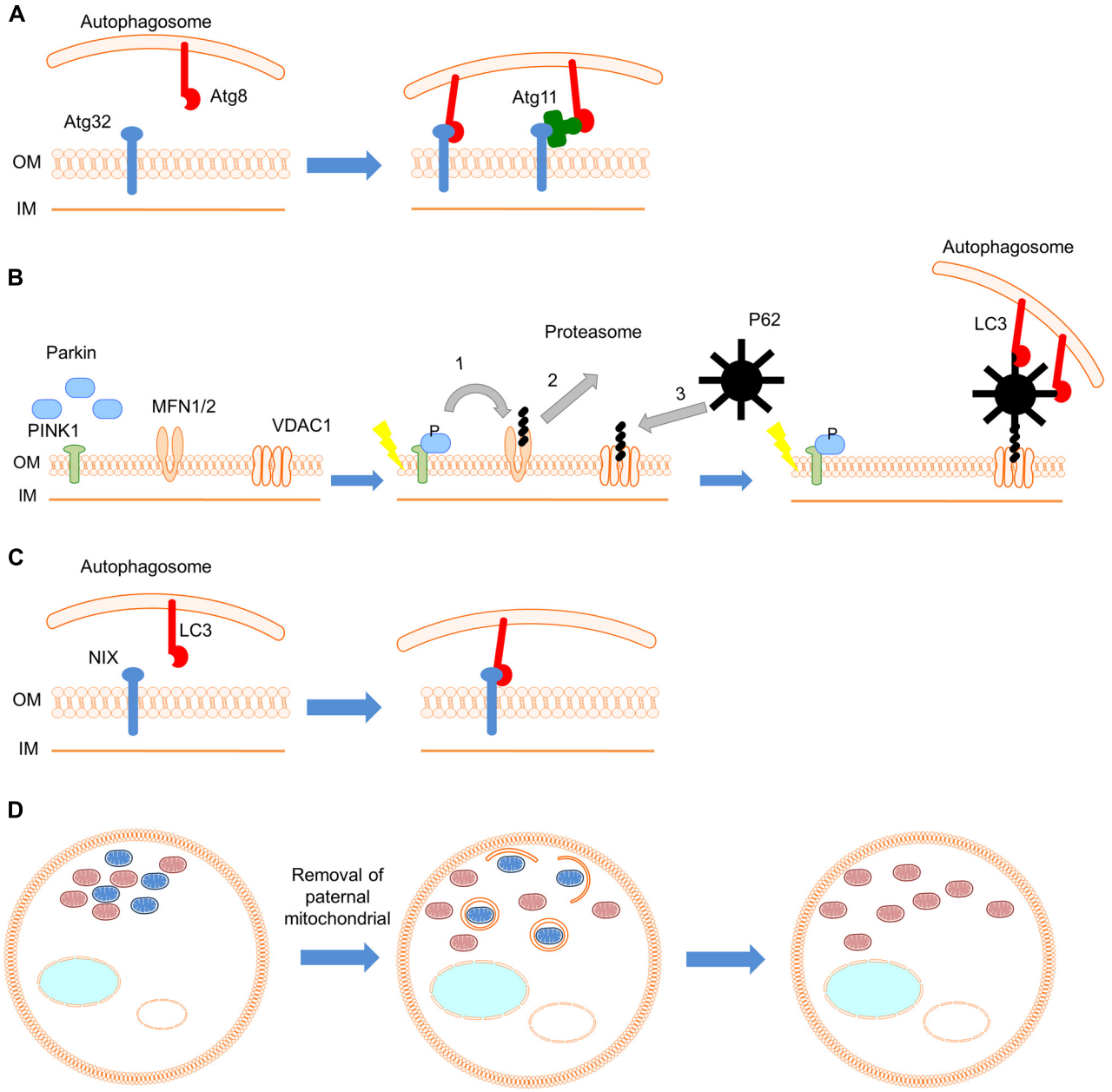

FIGURE 1 | Mechanisms and roles of mitophagy. (A) In yeast Atg32 (blue), a mitochondrial outer membrane protein, interacts with Atg8 (red) directly or indirectly through the adaptor protein Atg11 (green), and links mitochondria to autophagic machinery. **(B)** During red blood cell development the mitochondrial population is eliminated by mitophagy. Nix (blue), an outer mitochondrial membrane protein, serves as a receptor for targeting mitochondria to autophagosomes through its interaction with the autophagosomal protein LC3 (red). **(C)** In the fertilized *C. elegans* embryo, the autophagic pathway selectively degrades sperm-derived mitochondria (blue; oocyte-derived mitochondria are shown in pink). **(D)** Upon mitochondrial depolarization, PINK1 (green) is stabilized on the outer mitochondrial membrane. Subsequently, Parkin (blue) is recruited and ubiquitylates outer mitochondrial membrane proteins such as MFN1/2 and VDAC1. (1) Ubiquitinated MFN1/2 is degraded by the proteasome system. Damaged mitochondria are isolated and cannot fuse with the healthy mitochondrial population. (2) Next, ubiquitin-binding adaptor molecules, such as p62 (black), are recruited to mitochondria to initiate mitophagy through their interaction with LC3 (red).

2010; Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010). Following translocation, Parkin ubiquitylates outer mitochondrial membrane proteins. Subsequently other adaptor molecules, such as p62, are recruited to mitochondria to initiate mitophagy (**Figure 1D**). The mitochondrial fusion proteins mitofusin 1 and 2 have been identified as substrates of Parkin (Gegg et al., 2010; Poole et al., 2010; Tanaka et al., 2010; Rakovic et al., 2011). Parkin prevents mitochondrial fusion through degradation of mitofusins, thereby isolating impaired mitochondria from the healthy mitochondrial population. Apart from

mitofusins, overexpression of Parkin also mediates the ubiquitination of other outer mitochondrial membrane proteins, such as the voltage-dependent anion channel (VDAC), the mitochondrial Rho GTPases (MIRO) 1 and 2, as well as components of mitochondrial translocase complex (TOM70, TOM40, and TOM20; Chan et al., 2011; Yoshii et al., 2011). However, the relevance of these substrates to the induction of mitophagy *in vivo* remains to be investigated.

THE ROLE OF MITOPHAGY IN DEVELOPMENT

Certain developmental processes entail removal of non-damaged mitochondria, a process that is essential for successful organ and tissue development. During erythrocyte differentiation, mitophagy eliminates healthy mitochondria in programmed fashion. Erythrocytes transfer oxygen form the lungs to peripheral tissues and are characterized by lack of internal organelles, including mitochondria, an adaptation that perhaps serves to increase their oxygen carrying capacity. Recently, Nix was identified as a protein that mediates elimination of mitochondria in reticulocytes (immature red blood cells; Schweers et al., 2007; Sandoval et al., 2008). Nix is a Bcl2-related protein with an atypical BH3 domain that is localized to outer mitochondrial membrane and is required for the elimination of reticulocyte mitochondria. $Nix^{-/-}$ mice retain mitochondria in erythrocytes and develop anemia because of decreased survival of these cells (Schweers et al., 2007; Sandoval et al., 2008). Studies of erythrocyte differentiation suggest that Nix is not required for induction of mitophagy *per se*, but for the engulfment of mitochondria by autophagosomes. Nix contains a cytoplasmic WXXL-like motif, which interacts with LC3 (the mammalian homolog of the yeast Atg8) and the GABA receptor-associated protein (GABARAP) *in vivo* and *in vitro* (Schwarten et al., 2009; Novak et al., 2010). Therefore, Nix appears to act as a receptor for targeting autophagosomes to mitochondria in a manner similar to the yeast Atg32 (**Figure 1B**). Nevertheless, despite the requirement of Nix in erythrocyte differentiation, treatment of reticulocytes with uncoupling agents induces mitophagy upon mitochondrial depolarization in a Nix-independent manner (Sandoval et al., 2008). The mechanisms mediating Nix-independent mitophagy in reticulocytes remain unclear.

An additional important developmental role for mitophagy is the removal of paternal mitochondria in fertilized oocytes (Al Rawi et al., 2011; Sato and Sato, 2011). Although, sperm contains mitochondria, which are transferred to the oocyte upon fertilization, only maternal mitochondrial DNA (mtDNA) is ultimately inherited. Two studies in *Caenorhabditis elegans* revealed that the autophagic pathway selectively degrades sperm mitochondria during the early stages of embryogenesis (**Figure 1C**). However, the signal that activates mitophagy, to selectively eliminate sperm-derived mitochondria remains unknown.

MITOPHAGY IN NEURODEGENERATION

Neuronal cells typically require increased numbers of mitochondria, since most neuronal ATP is generated through oxidative phosphorylation. This high-energy demand is dictated by numerous neuronal processes, such as axonal transport of macromolecules and organelles, maintenance of membrane potential, loading and releasing neurotransmitters, and buffering cytosolic calcium. Therefore, neuronal survival and activity are critically dependent on mitochondrial integrity and functionality (Rugarli and Langer, 2012). Mitochondria are highly dynamic organelles that constantly move and undergo frequent fission and fusion events. Several components of the fission/fusion machinery have been linked to various neurological diseases, underlying the significance of mitochondrial dynamics in neuronal homeostasis (Alexander et al., 2000; Zuchner et al., 2004; Waterham et al., 2007). Recent studies have shown that fission/fusion dynamics not only sort out damaged mitochondrial components by distributing them throughout the mitochondrial network, but also fragment and isolate defective mitochondria prior to mitophagy (Twig et al., 2008a,b). The interplay between mitochondrial dynamics and mitophagy is further underscored by the fact that excessive fusion prevents autophagic mitochondrial degradation (Twig and Shirihai, 2011). Indeed, increased fusion protects mitochondria from massive degradation by starvation-induced autophagy (Rambold et al., 2011). Therefore, modulation of mitochondrial dynamics, to increase fission or decrease fusion, facilitates isolation of damaged mitochondria and their subsequent elimination by mitophagy. Hence, mitochondrial damage and deregulation of mitophagy has been implicated in the onset and progression of several age-associated neurodegenerative diseases, such as Parkinson's (Schapira, 2011), Alzheimer's, and Huntington disease (Batlevi and La Spada, 2011).

PARKINSON'S DISEASE

Parkinson's disease is caused by loss of dopaminergic neurons in the substantia nigra, a region important for motor control and coordination. Loss-of-function mutations in PINK1 and/or PARK2 genes have been linked with the early onset of hereditary forms of Parkinson's disease. The PINK1/Parkin pathway has been shown to regulate the elimination of damaged mitochondria through mitophagy (Narendra et al., 2008, 2010). In addition, mtDNA mutations and/or deletions are more frequent in patients with Parkinson's disease compared to age-matched individuals in the population (Bender et al., 2006). Such mutations and/or deletions commonly appear and accumulate during aging in mitochondria of the substantia nigra neurons (Kraytsberg et al., 2006). Consistently, loss of dopaminergic neurons in the substantia nigra that leads to the development of Parkinson's disease correlates with mitochondrial damage accumulation in these neurons. Thus, excessive mitochondrial stress upon exposure to environmental toxins or defects in mtDNA, and the inability of the cell to eliminate damaged mitochondria through mitophagy, may contribute to Parkinson's disease pathogenesis (Ethell and Fei, 2009). However, mitophagy pathways have been characterized in non-neuronal cells, with neuronal mitophagy remaining a relatively obscure process. Some reports suggest that mitochondrial depolarization and respiratory deficiency do not induce Parkin recruitment in neurons (Sterky et al., 2011; Van Laar et al., 2011). Other studies in neuronal cells indicate that Parkin is recruited to depolarized mitochondria and mediates mitochondrial elimination by mitophagy in a Parkin-dependent manner (Wang et al., 2011; Cai et al., 2012). Thus, although mutations in PINK1 and Parkin have been associated with neurodegeneration in Parkinson's disease, further work is needed to clarify if the

PINK1/Parkin pathway regulates damage-induced mitophagy in neurons.

ALZHEIMER'S DISEASE

Alzheimer's disease is the most common age-associated neurodegenerative disorder, characterized by cognitive dysfunction and loss of memory, caused by neuronal cell death in cerebral cortex. Tissue sections from Alzheimer's disease patient brains show distinctive intracellular neurofibrillary tangles and extracellular amyloid plaques composed of beta-amyloid derived from amyloid precursor protein (APP). While, the predominant hypothesis is that excess beta-amyloid leads to neuronal death, the mechanism that underlies pathogenesis is still unclear. Mitochondrial damage has been implicated in the development and progression of Alzheimer's disease, since abnormalities in mitochondrial structure have been observed in afflicted individuals (Baloyannis, 2006). Moreover, beta-amyloid fragments have been found to localize and accumulate within mitochondria (Casley et al., 2002; Lustbader et al., 2004). In addition, the presence of autophagic vacuoles in neurons of Alzheimer's disease patients further implicates cytoplasmic and organelle-specific degradation in disease progression (Boland et al., 2008). In this context, mitophagy may have pivotal role in ameliorating, or defending against the development of Alzheimer's disease through elimination of defective mitochondria, carrying cytotoxic beta-amyloid fragments.

HUNTINGTON'S DISEASE

Huntington's disease is an autosomal dominant neurodegenerative disease caused by the abnormal expansion of the cytosine, adenine, and guanine (CAG) repeats within huntingtin (Htt) gene. The severity of pathology correlates with the number of CAG repeats, the length of expansion (Costa and Scorrano, 2012). Huntington's disease is characterized by progressive motor dysfunction, as well as psychiatric and cognitive abnormalities caused by loss of cortical and striatal neurons (Purdon et al., 1994). Expression of mutant Htt is associated with mitochondrial dysfunction both in patients and mouse models of Huntington's disease. Decreased mitochondrial membrane potential, defects in mitochondrial calcium uptake, decreased respiratory function, reduced mitochondrial mobility and changes in mitochondrial structure are some of the observed mitochondrial defects in Huntington's disease patients (Bossy-Wetzel et al., 2008). Additionally, the peroxisome proliferator-activated receptor gamma coactivator-1a (PGC-1a), the master regulator of mitochondrial biogenesis, has been linked to metabolic and transcriptional defects in Huntington's disease (Weydt et al., 2006). Mitophagy may serve a protective function against neuronal loss in Huntington's disease by eliminating damaged mitochondria. Consistent with this notion, recent findings indicate that Huntington's disease pathology is associated with autophagic cargo recognition defects that lead to accumulation of damaged mitochondria in cytoplasm (Martinez-Vicente et al., 2010).

MITOPHAGY IN AGING

Mitochondrial dysfunction has already been correlated with aging. Mitochondria are the primary source of ROS, such as nitroxides, hydrogen peroxide, and superoxide anions (Hekimi et al., 2011; Sena and Chandel, 2012). Aging particularly affects mitochondrial homeostasis, as ROS generation in mitochondria leads to mitochondrial protein and mtDNA damage. mtDNA is more sensitive and susceptible to oxidative damage due to lack of histones. mtDNA repair mechanisms are also less efficient or robust, compared to nuclear DNA. Accordingly, inhibition or abnormal synthesis of mitochondrial proteins exacerbates mitochondrial dysfunction. mtDNA mutations accumulate during aging, an event that has been correlated with age-related decreased autophagic activity (Cuervo, 2008; Hubbard et al., 2012). In mammals, morphological and enzymatic mitochondrial defects occur during aging (Navarro and Boveris, 2004). Therefore, it is possible that accumulation of damaged mitochondria could induce mitophagy to preserve cellular homeostasis. Studies in yeast have shown that deletion of the mitochondrial membrane protein Uth1 results in a selective defect in mitophagy and decreased lifespan upon nutrient deprivation (Kissova et al., 2004). Caloric restriction is known to promote longevity from yeast to mammals. Given that caloric restriction induces autophagy, increased longevity may in part originate from enhanced elimination of dysfunctional mitochondria (Yen and Klionsky, 2008). Further studies should clarify whether mitophagy is indeed involved in mediating part of the effects of caloric restriction on lifespan.

CONCLUDING REMARKS

Although findings in diverse organisms indicate that the process of mitophagy requires the core autophagic machinery of cell, the initial signals that trigger and activate this selective type of autophagy, remain obscure. These signals appear to differ according to nutrient conditions, developmental processes, and damage-induced mitochondrial loss. Despite the fact that several proteins, such as ATG32, Nix, PINK1, Parkin have been identified as being critical for targeting mitochondria to autophagosomes, important information about the recruitment of these proteins to mitochondria and their interaction with the core autophagic machinery is lacking. Further investigation of the mechanisms mediating mitophagy will elucidate these key steps and shed light onto the link between mitophagy and aging. As a corollary, these studies are also likely to provide novel potential targets for therapeutic interventions against age-associated pathologies such as neurodegenerative disorders.

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Long-lived cancer-resistant rodents as new model species for cancer research

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Most rodents are small and short-lived, but several lineages have independently evolved long lifespans without a concomitant increase in body-mass. Most notable are the two subterranean species naked mole rat (NMR) and blind mole rat (BMR) which have maximum lifespans of 32 and 21 years, respectively. The longevity of these species has sparked interest in the tumor suppression strategies that may have also evolved, because for many rodent species (including mice, rats, guinea pigs, gerbils, and hamsters) tumors are a major source of late-life mortality. Here, we review the recent literature on anti-cancer mechanisms in long-lived rodents. Both NMR and BMR seem to have developed tumor defenses that rely on extra-cellular signals. However, while the NMR relies on a form of contact inhibition to suppress growth, the BMR evolved a mechanism mediated by the release of interferon, and rapid necrotic cell death. Although both organisms ultimately rely on canonical downstream tumor suppressors (pRB and p53) the studies reveal species can evolve different strategies to achieve tumor-resistance. Importantly, studies of these cancer-resistant rodents may benefit human health if such mechanisms can be activated in human cells.

Keywords: aging, cancer, naked mole rat, blind mole rat, long-lived rodents

Mice have become the preferred model for cancer research due to the presence of a powerful arsenal of molecular and genetic tools, their short generation time, strain variety, and propensity for neoplasia. A major goal of cancer research is to understand the genetic and molecular changes that underlie transformation and what defense mechanisms fail as people grow older or are exposed to oncogenic insults. Due to the extreme tumor propensity of mice, focusing research solely in this species may miss some important tumor suppression mechanisms used by longer-lived animals.

Some important differences at the molecular level between mouse and human cells have already been identified. The tumor suppression profile of mice is markedly different from that of human cells, with the most immediate distinction being the presence of telomerase activity in somatic tissue (Gorbunova and Seluanov, 2009). Mouse fibroblasts require fewer mutations to transform than human cells; elimination of pRB and p53 signaling coupled with constitutive Ras signaling is sufficient for mice whereas humans require the activation of telomerase as well as mutations that prevent dampening of the AKT signaling pathway (such as PP2A or PTEN) (Hahn and Weinberg, 2002; Rangarajan and Weinberg, 2003). The three products of the *INK4* tumor suppression locus, $p15^{INK4b}$, $p16^{INK4a}$, and ARF, contribute differently to the tumor-resistance of human and mice, with ARF loss being much more deleterious to mice than humans (Kim and Sharpless, 2006).

To find novel tumor suppressive mechanisms that could potentially be applied to human treatments, we chose to study organisms that have a tumor-resistance profile similar to that of humans, but are tractable to research and investigation in ways similar to mice. Furthermore, by investigating animals that are phylogenetically related to Murinae, we could ask questions about the evolution of tumor suppression mechanisms (and longevity in general). New results from non-model rodents show that there are still gaps in our understanding of anti-cancer mechanisms. Currently, we are investigating two such non-standard model rodent species: the naked mole rat (*Heterocephalus glaber*, hereafter referred to as "NMR") and the blind mole rat (*Spalax sp*. or *Nannospalax sp*., hereafter referred to as "BMR") (**Figure 1**).

H. glaber has been of interest to a wide range of biologists due to its unusual life history traits and adaptations for a subterranean ecological niche. It is considered to be a eusocial rodent, with only one breeding female per colony (and is consequently a naturally highly inbred animal), and has a maximum lifespan of more than 30 years (Buffenstein and Jarvis, 2002; Buffenstein, 2005, 2008) making it an outlier on the body-mass/longevity plot at the same order as *H. sapiens*. Furthermore, it has a much lower incidence of neoplasia than traditional inbred laboratory mice, with no observed tumors from various laboratories housing thousands of the rodents (Edrey et al., 2011).

The NMR has already started yielding interesting observations regarding its tumor-resistance, despite being a relatively new experimental animal. In a 2009 paper by Seluanov et al., we showed that NMR fibroblasts grow much more slowly in tissue culture than other rodent cells, and halt their cell cycle at much lower cell densities than other rodents (a phenomenon termed early contact inhibition, or ECI) (Seluanov et al., 2009). NMRs were also shown to be highly resistant to induced tumorigenesis: primary fibroblasts could not be transformed (induced into anchorage independent growth) solely by disrupting p53 and

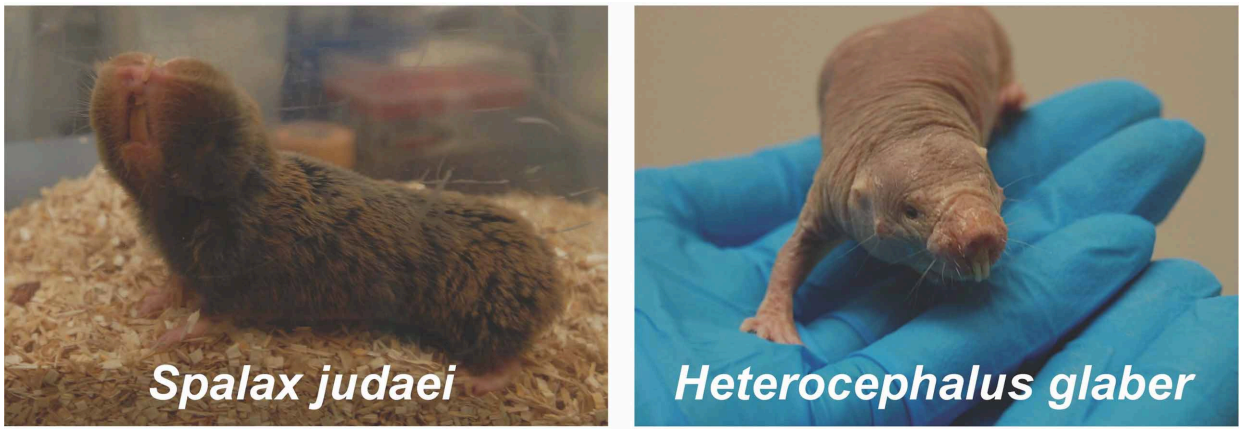

FIGURE 1 | Images of naked mole rat (right) and blind mole rat (left).

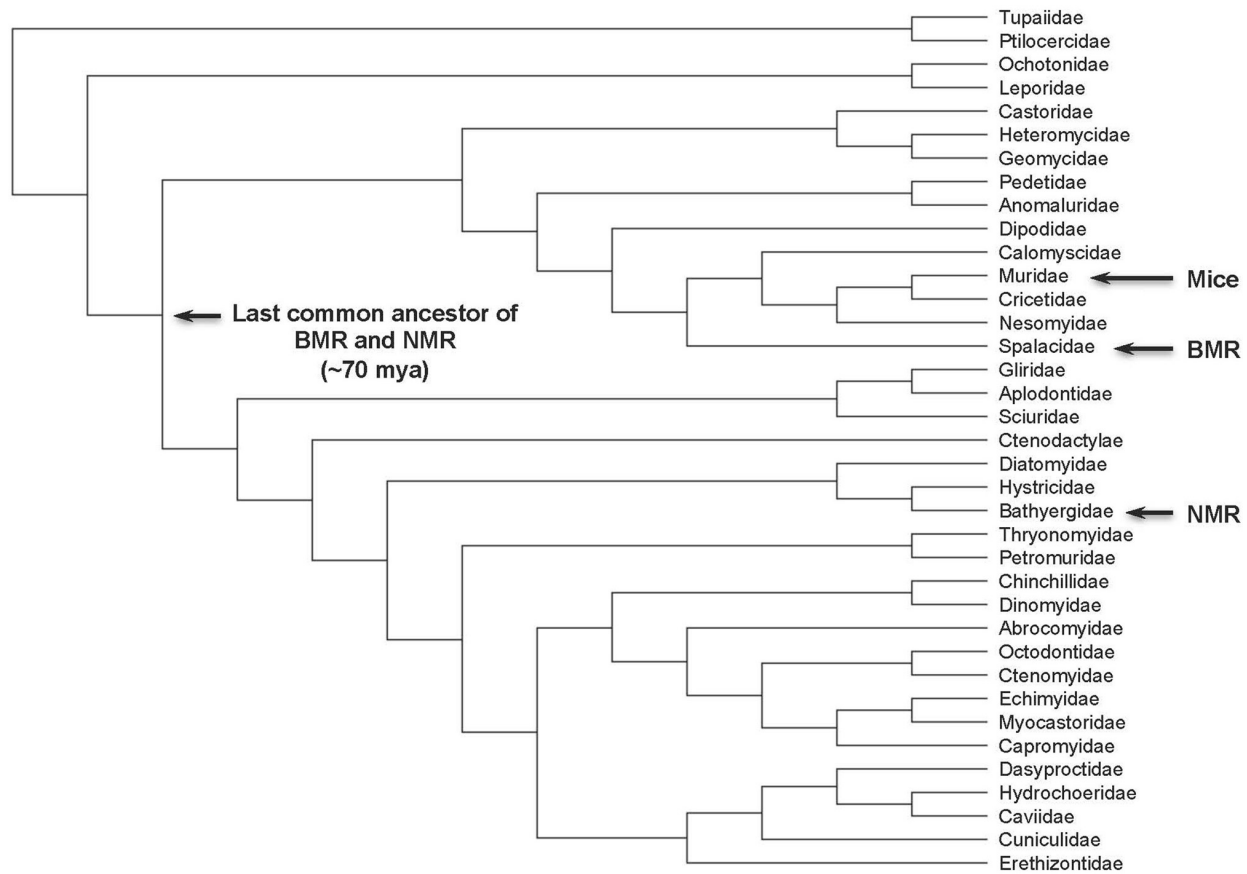

FIGURE 2 | Rodent phylogeny. The positions of naked mole rat, blind mole rat, and mouse are indicated. The tree topology is based on molecular phylogenies inferred from Meredith et al. (2011).

pRB in the presence of oncogenic Ras signaling, the cells had to undergo additional mutations during passaging in tissue culture to allow transformation. The nature of the mutations was not established, but disruption of $p16^{INK4a}$ was observed, as well as a loss of the ECI phenotype. Therefore, although loss of ECI in tissue culture was not sufficient for transformation, it was a necessary step before the cells could undergo anchorage independent growth in soft agar.

The tumor-resistance of NMR cells has also been investigated *in vivo* by Liang et al. by injection of NMR cells with various

combinations of transforming factors into immunocompromised mice (Liang et al., 2010). Again, knockout of the p53 and pRB pathways by LargeT antigen was not sufficient to induce tumorigenesis, even in the presence of a constitutive Ras oncogenic protein, while these mutations were sufficient to allow mouse cells to form large tumor masses in the immunocompromised mice. Tumor formation was observed, however, by addition of hTERT in addition to these other factors. While NMR fibroblasts express their own telomerase (Seluanov et al., 2007; Gomes et al., 2011) and can be passaged indefinitely in tissue culture, the additional pro-growth targets of hTERT (Rahman et al., 2005; Lee et al., 2008) may be sufficient to induce tumor formation when present with other transforming factors.

The BMR shows a similar longevity and tumor-resistance (de Magalhaes and Costa, 2009; Nasser et al., 2009) to the NMR despite being phylogenetically more related to mice. They are also long-lived and subterranean, although they are solitary and genetically heterogeneous. In our recent study Gorbunova et al. showed that in tissue culture, BMR fibroblasts displayed a novel phenotype which we named concerted cell death (CCD) (Gorbunova et al., 2012). Here, fibroblasts grow normally for several population doublings before undergoing synchronized rapid cell death. Like the NMR, the BMR also has somatic telomerase expression, which eliminated a telomere attrition-based response as the culprit. We showed that in both growing and dying cells, the telomeres were still long and telomerase was still active.

Because the death of cells was synchronized, we hypothesized that during growth, a signaling factor was being secreted that would kill cells upon reaching a threshold concentration. The authors identified interferon beta (IFN-β) as the secreted factor that was increasingly released by the cells into the media during passage in tissue culture. Freshly isolated primary BMR cells treated with media conditioned by BMR cells that were near CCD were rapidly induced to undergo CCD themselves. It was also possible to induce very high levels of apoptosis in mouse lines (but not human lines, possibly due to divergence in the structure of the IFN-β receptor). We interpreted the secretion of IFN-β as a response to rapid growth in tissue culture, reflecting the sensitivity of the cells to over-proliferation or abnormalities in the local microenvironment. Intriguingly, the BMR evolved this p53-dependent mechanism despite undergoing a mutation in its p53 gene as an adaptation to hypoxia that renders it less capable of directly promoting apoptosis (Ashur-Fabian et al., 2004). Presumably, the other targets of p53 are sufficient to kill the cell in the presence of IFN-β.

The BMR CCD and the NMR ECI responses are markedly different in their phenotype (cell death vs. growth arrest) and show how convergent evolution toward tumor-resistance can take different paths. Nonetheless, there are some important similarities. In both cases, the authors show that elimination of both the pRB and the p53 pathways is important. If either tumor suppression pathway is left intact, the cells will still undergo arrest or death. This is one trait which these long-lived rodents share more with humans than mice. Additionally, in both NMR and BMR an extra-cellular signal is mediating some aspect of the tumor-resistance (cell density and interferon response, respectively), suggesting that in long-lived species with somatic telomerase activity, selection favors increased cellular sensitivity to the external environment.

During the evolution of extreme longevity in rodents, selection for tumor-resistance seems to be tremendously important (**Figure 2**). Comparative studies in rodents are yielding novel insights into the evolution of tumor suppressor mechanisms, which seem to arise sporadically rather than being a conserved basal trait of the rodent lineage. Interestingly, each lineage evolves its own tumor suppression mechanism, which means there may more to learn from other small, long-lived rodents, such as the Eastern gray squirrel, chinchilla, or muskrat. By learning about alternative tumor suppression mechanisms evolving in different lineages, novel targets for anti-cancer therapy may be revealed or important cell cycle regulatory circuits hitherto ignored may be discovered.

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Premature and accelerated aging: HIV or HAART?

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Highly active antiretroviral therapy (HAART) has significantly increased life expectancy of the human immunodeficiency virus (HIV)-positive population. Nevertheless, the average lifespan of HIV-patients remains shorter compared to uninfected individuals. Immunosenescence, a current explanation for this difference invokes heavily on viral stimulus despite HAART efficiency in viral suppression. We propose here that the premature and accelerated aging of HIV-patients can also be caused by adverse effects of antiretroviral drugs, specifically those that affect the mitochondria. The nucleoside reverse transcriptase inhibitor (NRTI) antiretroviral drug class for instance, is known to cause depletion of mitochondrial DNA via inhibition of the mitochondrial specific DNA polymerase-γ. Besides NRTIs, other antiretroviral drug classes such as protease inhibitors also cause severe mitochondrial damage by increasing oxidative stress and diminishing mitochondrial function. We also discuss important areas for future research and argue in favor of the use of *Caenorhabditis elegans* as a novel model system for studying these effects.

Keywords: mitochondria, HIV, HAART, antiretroviral, *C. elegans*, immunosenescence, premature and accelerated aging, NRTI

HIV-INFECTION

The human immunodeficiency virus (HIV-1) is a *Retrovirus* of the *Lentivirus* genus that primarily infects cells of the host immune system. Once an individual is infected, HIV-1 replication takes place in several steps. In the first step, the virion attaches itself to the host cell with the help of co-receptors, whereupon it fuses with the host cell membrane and the two single-stranded RNA molecules and three different viral enzymes are released into the host cell cytoplasm. The viral reverse transcriptase transcribes the viral RNA into DNA, at which point the viral DNA is transported into the nucleus. With the aid of the viral integrase the viral DNA is processed and incorporated into the host genome. The integrated viral DNA, now known as a provirus, is transcribed and translated by the host machinery to synthesize viral proteins and single-stranded RNA for new virions. After assembly of these components at the plasma membrane, the new virions bud off and mature using the viral protease, completing the HIV-1 life cycle (**Figure 1;** Teixeira et al., 2011). HIV infection of host immune cells causes them to die and thus drastically deplete in number. As immune cell counts decline, the host gradually becomes immune-incompetent and more susceptible to opportunistic infections. If untreated, this leads to acquired immune deficiency syndrome (AIDS) and eventually death.

ANTIRETROVIRAL THERAPY

For the treatment of HIV-1 infection there are currently six different classes of anti-HIV drugs. Each class of drug acts on a particular aspect of the viral life cycle (**Figure 1**), and are used in unison to increase therapy efficacy, overcome problems of tolerance, and decrease emergence of viral resistance. The major classes include the entry inhibitors (EIs), the nucleoside reverse transcriptase inhibitors (NRTIs), the non-nucleoside reverse transcriptase inhibitors (NNRTIs), and the protease inhibitors (PIs). The additional two anti-HIV drug classes are the maturation inhibitors (MIs) and integrase inhibitors (IIs), of which most compounds are still in clinical development.

Since 1996 the combination of at least three antiviral drugs, preferably from at least two different classes, has become standard practice and is known as highly active antiretroviral therapy (HAART). Due to the large variety in drug combinations, standard HAART has been defined as one or more NRTIs combined with a PI (**Table 1**) and often supplemented with one drug from another class (Dybul et al., 2002). Due to the replicative speed of HIV-1 and the inability of antiretroviral drugs to eradicate infection, patients need to medicate daily for the rest of their lives. Nonetheless, the therapeutic use of a combination of drugs was a major advance in HIV therapy and has significantly improved the quality and length of patient lives.

HAART TREATED HIV-PATIENTS AGE PREMATURELY

Without antiretroviral therapy HIV-infected patients usually die within years because of immune system failure. Due to HAART however, early death is prevented, allowing HIV-patients to live decades as long medication is continued (May and Ingle, 2011). It was recently estimated that more than 50% of HIV-infected patients in the United States will be over the age of 50 in 2015 (Effros et al., 2011). Even though this gain in lifespan is celebrated as a success, data show that the life expectancy of treated patients remains shorter than that of the normal population (The Antiretroviral Therapy Cohort Collaboration, 2008). Life expectancy for treated HIV-patients is dependent on the age at which antiretroviral therapy is started and is estimated to be 10–30 years less than that of the uninfected (Lohse et al., 2007). Several studies have also observed that co- and multi-morbidities, like cardiovascular disease, diabetes, and osteoporosis, which are normally witnessed later on in

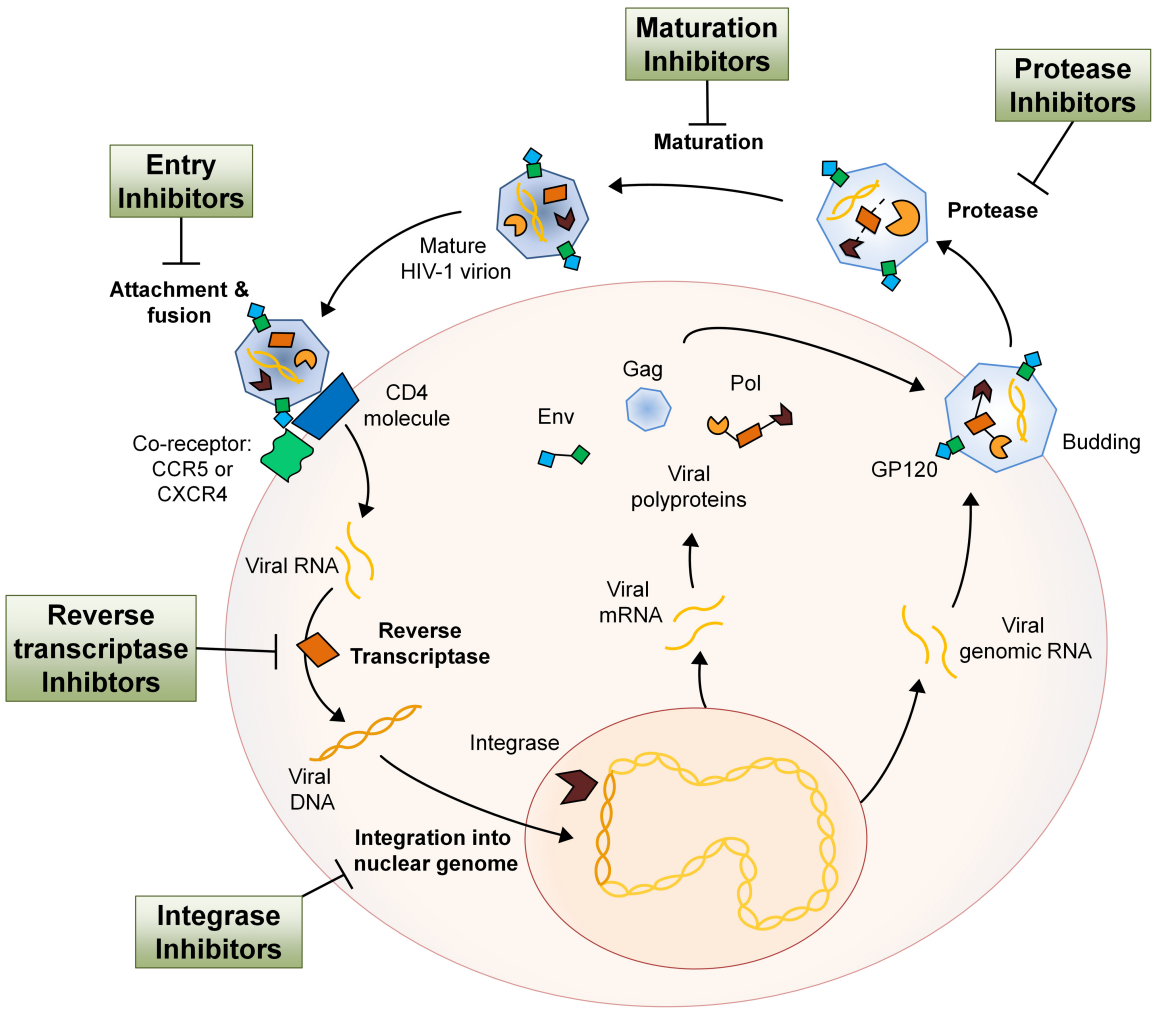

FIGURE 1 | The HIV-1 life cycle and the antiretroviral drug class intervention points. Entry inhibitors interfere with viral entry into the host cell and are comprised of a complex group of drugs with multiple mechanisms of action. By inhibiting several key proteins that mediate the process of virion attachment, co-receptor binding and fusion, virus spreading can be mitigated (Tilton and Doms, 2010). NRTIs imitate endogenous deoxyribonucleotides and have a high affinity for the viral reverse transcriptase, thus facilitating incorporation into the viral DNA strand during synthesis. NRTI incorporation results in transcription termination as they all lack the 3′-OH group necessary for phosphodiester bond formation in DNA strand elongation (Cihlar and Ray, 2010). NNRTIs are compounds that fit into the allosteric "pocket" site of the HIV-1 reverse transcriptase and disrupt its enzymatic activity, selectively blocking HIV-1 transcription (De Clercq, 2004). Integrase inhibitors bind cofactors of the viral integrase that are essential in host DNA interaction and therefore block insertion of proviral DNA into the host genome (Schafer and Squires, 2010). Protease inhibitors bind the viral protease active site with high affinity and therefore inhibit cleavage of viral polypeptides and subsequent maturation of the virion after budding from the host cell (Adamson, 2012). HIV-1 maturation inhibitors act much like protease inhibitors in that they inhibit the processing of the HIV-1 polypeptides. However, maturation inhibitors do not bind the protease but rather the polypeptide itself, rendering it uncleavable (Richards and McCallister, 2008). The relative size of different components has been altered for pictorial clarity.

life as a result of natural aging, were increasingly prominent among the HIV-infected population (Deeks and Phillips, 2009; Guaraldi et al., 2011). These observations led to the hypothesis that the HAART treated HIV-infected population is aging more rapidly, a phenomenon now known as premature and accelerated aging.

THEORIES FOR PREMATURE AND ACCELERATED AGING IN HAART TREATED PATIENTS

There are several factors that influence lifespan of the HIV-infected, but have limited effects on progression of premature and accelerated aging phenotypes. These include lifestyle risk factors such as smoking, drinking, and illicit drug use, which are prevalent across the HIV-infected population (Shurtleff and Lawrence, 2012). Illicit drug use for example, is associated with poorer medication adherence and lesser immunological and virological control (Lucas et al., 2001). Additionally, co-infection, such as with viral hepatitis, is common among the HIV-infected population and is known to decrease life expectancy (Sulkowski, 2008). HIV-1 patients also run a greater risk for adverse drug interactions due to the increase in "pill-burden" to combat co-morbidities (Marzolini et al., 2011). Moreover, both natural aging or HIV-1 infection

Table 1 | Antiretroviral drugs discussed in this review.

Antiretroviral drug class	Drug name	Other names/abbreviations
Nucleoside reverse transcriptase inhibitor (NRTI)	Alovudine	FLT (3′-deoxy-3′-fluorothymidine)
	Didanosine	ddI (2′,3′-dideoxyinosine)
	Stavudine	D4T (2′,3′-didehydro-2′,3′-deoxythymidine)
	Zalcitabine	ddC (2′,3′-dideoxycytidine)
	Zidovudine	AZT (3′-azido-3′-deoxythymidine)
Protease inhibitor (PI)	Indinavir	IDV
	Lopinavir	LPV
	Nelfinavir	NFV
	Ritonavir	RTV
	Saquinavir	SQV

cause changes in gastrointestinal tract, liver, and kidney function that collectively affect the pharmacology of administered drugs (McLean and Le Couteur, 2004). None of these factors however can directly be related to causing the premature and accelerated aging phenotype witnessed in treated HIV-patients (Martin and Volberding, 2010).

Most research in this relatively new field focuses on how HIV-1 infection depletes $CD4^+$ cell counts and exhausts the patient's immune system (Appay and Sauce, 2008; Desai and Landay, 2010). In this way, HIV-infection itself if left untreated has been shown to convert the immune system of a young individual into one similar to someone 40 years older (Ferrando-Martínez et al., 2011). This theory of an accelerated aging process of the immune system is called immunosenescence and is characterized by continuous immune provocation and systemic low-grade inflammation, which predisposes patients to co-morbidities and natural aging symptoms more frequently seen in the elderly (Dock and Effros, 2011; Deeks et al., 2012).

The immunosenescence theory of aging has substance when considering untreated patients, as it principally focuses on viral effects. However, this theory is less plausible for treated patients as HAART has proven highly successful in swiftly replenishing $CD4^+$ cell counts and reducing viral-load to barely detectable limits (Camacho and Teófilo, 2011). Additionally, various antivirals have been shown to induce inflammatory signals and it is therefore plausible that if an altered immune-organization is seen in HAART treated patients it is due to antiretroviral therapy (Mondal et al., 2004; Lagathu et al., 2007; Lefèvre et al., 2010). The influence HAART has warrants thorough investigation as HIV-patients take HAART daily and for the rest of their lives. Very few premature and accelerated aging studies in the HIV-infected population however, focus upon the influence that antiretroviral drugs have on aging and age-related co-morbidities. Accordingly, no consensus has arisen as to why the successfully treated HIV-infected population shows signs of premature and accelerated aging.

IS HAART THE PREDOMINANT CAUSE OF PREMATURE AND ACCELERATED AGING?

Antiretroviral therapy as an explanation for premature and accelerated aging was first mentioned in studies wherein clinical symptoms of aging were shown to correlate with adverse side effects of antiretroviral therapy (Onen et al., 2010). For example, cardiovascular disease, diabetes, kidney and liver disease, metabolic disorders, osteoporosis, and lipodystrophy have all been associated with HAART (Effros et al., 2011; Klein, 2011). Accelerated Tau deposition, a marker for neurodegenerative diseases such as Alzheimer's and Parkinson's, has also been shown to be elevated in patients receiving HAART compared to HIV-infected non-treated patients (Anthony et al., 2006). These symptoms collectively seem to be related to tissues with high-energy demand and show a strong similarity to hereditary mitochondrial diseases (Schapira, 2012). Indeed, after introduction of HAART to treat HIV-1 infection, it quickly became apparent that mitochondrial toxicity is a major reason for antiretroviral-related adverse events (Brinkman et al., 1998). HAART-induced mitochondrial dysfunction therefore likely plays a role in most, if not all complications associated with premature and accelerated aging (White, 2001; Hulgan and Gerschenson, 2012). The specific influence of HAART upon mitochondria and aging however, is often not addressed.

HAART-RELATED MITOCHONDRIAL TOXICITY IN AGING

Mitochondria are essential organelles in the life cycle and fitness of the cell. They are principal regulators of apoptosis and ATP production. Mitochondria are also involved in calcium and reactive oxygen species (ROS) homeostasis. Therefore, a perturbation of any of these functions impairs cellular life-expectancy and has been shown to have tissue and systemic repercussions including accelerated aging (Trifunovic and Larsson, 2008). In consensus, an accumulation of mitochondrial DNA (mtDNA) mutations, increased mitochondrial oxidative stress and a decrease in mitochondrial energy metabolism are all important contributors to aging (Lee and Wei, 2012). Mitochondria therefore play dominant roles in aging and marked effects of HAART upon mitochondria likely accelerate these effects. In this review we discuss how HAART is known to influence mtDNA integrity, alter mitochondrial morphology and function, induce oxidative stress, inflammation, and cell senescence, and how it is directly connected to aging symptoms and co-morbidities.

DRUG INDUCED ACCUMULATION OF mtDNA DAMAGE

Because mitochondria contain their own DNA, mitochondrial genome integrity is essential for organelle function. The mtDNA encodes vital components of the mitochondrial respiratory chain and therefore damage to mtDNA is directly detrimental to energy metabolism and organelle fitness. Not surprisingly, cell senescence and aging are associated with an increase in the amount of damaged mtDNA. Additionally, accumulation of mutations in mtDNA is known to increase with age, and aberrant mtDNA replication contributes to premature-and-accelerated-aging phenotypes (Park and Larsson, 2011; Cline, 2012).

DNA damage and unreliable replication can be induced by the backbone of antiretroviral therapy, namely NRTIs (Sundseth et al.,

1996; Payne et al., 2011). NRTIs have been shown to inhibit the mitochondrial specific DNA polymerase-γ causing a decrease in mtDNA amount and quality. This discovery led to the theory of NRTI-induced toxicity commonly known as the "polymerase-γ theory" (**Figure 2;** Lewis and Dalakas, 1995). In short, a NRTI-induced decrease in mtDNA leads to malfunctioning of mitochondrial protein complexes and changes in respiration rate, decreased ATP production, a diminished mitochondrial membrane potential, and an escalation in ROS production (Lewis et al., 2006; Maagaard and Kvale, 2009). Besides direct inhibition of mtDNA replication, NRTIs also obstruct base excision repair and proofreading capabilities of polymerase-γ (Lim and Copeland, 2001; Lewis et al., 2003). Mice with impaired polymerase-γ proofreading ability show rapid accumulation of mtDNA mutations leading to disrupted mitochondrial function, a variety of aging phenotypes and early death (Trifunovic et al., 2004). Additionally, antiretroviral therapy likely hastens the expansion of pre-existing mutations in mtDNA as depleted mtDNA pools display accelerated digression from their original genetic content (Khrapko, 2011; Payne et al., 2011). The NRTI 3′-deoxy-3′-fluorothymidine (*alovudine* or FLT), known for its high toxicity, can cause DNA fragmentation and induce apoptosis (Sundseth et al., 1996). Interestingly, the NRTIs 3′-azido-3′-deoxythymidine (*zidovudine* or AZT) and 2′,3′-didehydro-2′,3′-deoxythymidine (*stavudine* or d4T) also disrupt telomerase maintenance and have telomere shortening effects, properties often related to cell senescence and aging (Strahl and Blackburn, 1994; Blasco, 2007).

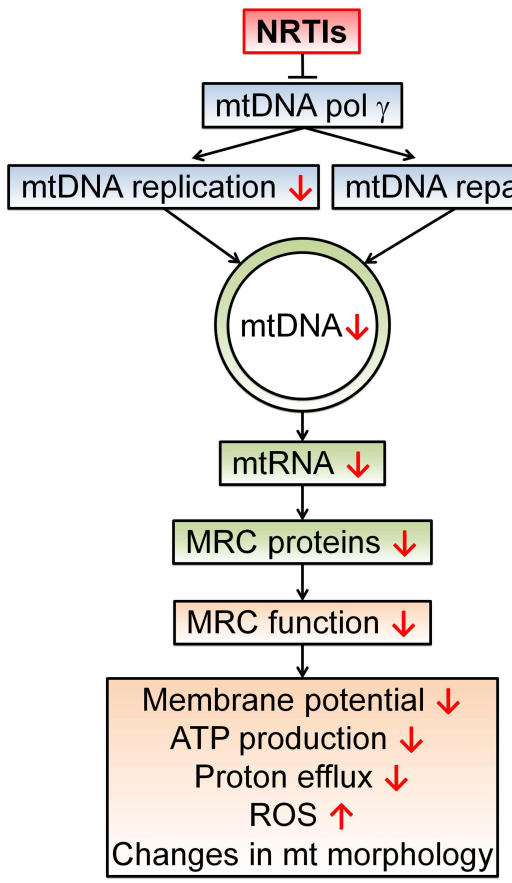

FIGURE 2 | The polymerase-γ theory. NRTIs compete with endogenous nucleotides and nucleosides for transcriptase binding. Due to the surplus and high affinity of NRTIs for polymerase-γ, NRTIs are frequently incorporated into the new DNA strand which results in chain termination as they all lack the 3′-OH group necessary for phosphodiester bond formation in DNA strand elongation. This results in a reduced number of mtDNA molecules and possibly a reduction in mtDNA encoded proteins, essential components of the mitochondrial respiratory chain (MRC) complexes. In turn, this leads to disrupted electron transport through the MRC and a concomitant reduction in proton efflux, reducing the membrane potential and ATP production by the mitochondrion. This disturbed mitochondrial function can result in augmented ROS production and morphological changes. Disturbed mitochondrial function due to polymerase-γ inhibition has been proposed as a central mechanism for NRTI-induced adverse events (Lewis and Dalakas, 1995; Lewis et al., 2006).

During HAART it is very likely that NRTIs and PIs augment each other's ability to steer the cell into premature senescence. This is especially the case when the "booster" PI *ritonavir* (RTV) is used in the HAART cocktail. RTV impedes the enzyme cytochrome P450-CYP3A4, which is responsible for the metabolism of xenobiotics, and therefore RTV induces an increase in intracellular drug concentrations in the patient (Zeldin and Petruschke, 2004). Interestingly, mtDNA damage has also been found to correlate with PI RTV use in human endothelial cell cultures in a dose-dependent manner (Zhong et al., 2002). Although mitochondria are the most important players in antiretroviral toxicities, outside these organelles PIs can cause accumulation of the farnesylated pro-senescence protein prelamin A. Prelamin A accumulation has been shown to cause genomic instability (Worman et al., 2009; Reddy and Comai, 2012). Furthermore, PI-induced prelamin A build-up is directly linked to increased oxidative stress and lipodystrophy-associated symptoms (Caron et al., 2007).

Mitochondrial DNA quantity and quality are important factors in mitochondrial functionality, and therefore cellular fitness, as mtDNA encode for vital components of the organelle's respiratory chain complexes. Mitochondrial toxicity caused by NRTIs, however, does not necessarily follow the chronological steps of the polymerase-γ theory. Not every case of mtDNA depletion leads to changed expression levels or activity of mitochondrial respiratory chain proteins (Stankov et al., 2010). In addition, altered mitochondrial gene expression and impaired respiratory chain activity have been observed without mtDNA depletion (Mallon et al., 2005; Viengchareun et al., 2007). Expression profiles of mitochondrial mRNA possibly explain these occurrences as they have been shown to adjust, both in a peripheral blood mononuclear cell line and mice upon exposure to NRTIs. These adjustments likely reflect cellular adaptation to pressure on the mitochondrial transcriptional machinery (d'Amati and Lewis, 1994; Papp et al., 2008). In an elegant review, Apostolova et al. (2011a) show that mitochondrial toxicity of antiretroviral drugs goes beyond the polymerase-γ theory as disruption of many other mitochondrial mechanisms is also involved.

OXIDATIVE STRESS

Reactive oxygen species, especially superoxide and hydrogen peroxide, are habitually produced in small quantities by mitochondria during oxidative phosphorylation. However, a decrease in, or malfunction of, mitochondrial proteins, due to diminished mtDNA

for instance, can disrupt electron flow through the electron transport chain and cause increased ROS formation (Brand, 2010). Consequently, this increase in ROS can damage mitochondrial components, such as the electron transport complexes, and hence induce even more ROS production (Sastre et al., 2000). A fundamental feature of aging is a decline in mtDNA transcription and repair capacity which can lead to mitochondrial malfunction and set in motion a vicious cycle of enhanced ROS production (Desler et al., 2011). Interestingly, polymerase-γ is highly sensitive to oxidative damage and modification of its amino acid residues by oxidation brings about a decline in DNA-binding ability and polymerase activity (Graziewicz et al., 2002).

An increase in oxidative stress, observed as increased oxidant and reduced antioxidant levels in serum, has frequently been associated with HAART in patients (Mandas et al., 2009). Several studies conclude that symptoms of aging such as cardiovascular disease, lipodystrophy, and insulin resistance are all influenced by antiretrovirally induced ROS production (Day and Lewis, 2004; Caron-Debarle et al., 2010). A common side effect of AZT, namely cardiomyopathy, is likely caused by stimulation of ROS production in heart and endothelial mitochondria (Sutliff et al., 2002; Valenti et al., 2002). Prompt heart injury has even been ascribed largely to 2′,3′-dideoxycytidine (*zalcitabine* or ddC) induced ROS production, independent of mtDNA depletion or damage, a finding that emphasizes the impact of antiretroviral-induced ROS toxicity (Skuta et al., 1999). Increased oxidation of lipids, mtDNA and the major antioxidant glutathione (GSH), further relate AZT to skeletal muscle myopathy (de la Asunción et al., 1998). d4T is known to cause oxidative stress in human hepatoma cells and may underlie hepatic steatosis and lactic acidosis, which are often experienced by patients on HAART (Velsor et al., 2004). Thymidine analogs have additionally been shown to cause cell senescence through an increase in oxidative stress and induction of mitochondrial dysfunction in human fibroblast cell lines and in subcutaneous adipose tissue from HAART patients (Caron et al., 2008).

Protease inhibitors also have the potential to induce oxidative stress, although it is not always clear whether PI induced elevated ROS is produced at the mitochondrial level. The most clearly PI affected cell type is endothelial cells, although other cell types are also afflicted, and strong connections exist between drug toxicity and ROS production (Wang et al., 2007). RTV and *lopinavir* (LPV), two frequently prescribed PIs, can increase ROS production in human arterial endothelial cells (Lefèvre et al., 2010) and are known to induce ROS through a perturbed mitochondrial function in cardiomyocytes (Deng et al., 2010). *Indinavir* (IDV) and *nelfinavir* (NFV) have been shown to elicit ROS production in skin fibroblast cultures *in vitro* and in patients' adipose tissue *in vivo* (Viengchareun et al., 2007). IDV and NFV have furthermore been shown to cause ROS production in human aortic endothelium and are thus involved in recruitment of mononuclear cells and exacerbation of inflammation, prerequisites for vascular complications (Mondal et al., 2004). Additionally, treatment with IDV or NFV was shown to cause increased mitochondrial ROS production and premature senescence in skin fibroblasts (Caron et al., 2007), and an IDV and AZT combination induces ROS mediated apoptosis in human brain microvascular endothelial cells (Manda et al., 2011). Short-term treatment of NFV increases ROS generation and diminishes levels of GSH and the detoxification enzyme superoxide dismutase in a pancreatic insulinoma cell line (Chandra et al., 2009). Moreover, NFV has been linked to adipocyte insulin resistance through oxidative stress induced apoptosis and necrosis (Vincent et al., 2004; Ben-Romano et al., 2006), which is noteworthy as the anti-apoptotic properties of PIs in a low-dose have been documented (Badley, 2005). *Saquinavir* (SQV) however, was shown to cause apoptosis in human umbilical vein endothelial cells via higher levels of ROS production (Baliga et al., 2004). SQV, IDV, NFV, and RTV also elevate ROS in cerebral endothelial cells and interfere with proper blood brain barrier maintenance. Therefore, these PIs conceivably play a significant role in antiretroviral-induced neurological symptoms and could also increase viral entry into the central nervous system (Grigorian et al., 2008). Collectively, these results indicate that oxidative stress is a powerful driving force behind antiretroviral-induced toxicity and has important roles in premature-and-accelerated-aging symptoms (Blas-Garcia et al., 2011).

ALTERED MITOCHONDRIAL MORPHOLOGY AND FUNCTION

Mitochondria are no longer considered as static spherical bodies, but highly dynamic organelles that readily fuse, divide, propagate, and diminish according to cellular requirements. Mitochondrial morphology plays an essential role in mtDNA rescue, protein quality control, and cell survival (Bess et al., 2012; Shutt and McBride, 2012). Certain distinct morphological changes in mitochondrial structure and organization are therefore considered indicators of aging in worms, mice, and humans (Jendrach et al., 2005; Yasuda et al., 2006). Specifically, mitochondria of aged individuals are often swollen and their structures contain less villous cristae, while the mitochondrial network is frequently disrupted (Sastre et al., 2000). Mitochondrial function, especially respiration and ATP production, has been demonstrated to decline with age and even be an important mediator of senescence (Desler et al., 2012). Energy deficiency can cause a broad range of metabolic and degenerative diseases including aging (Wallace et al., 2010). Mitochondrial processes for example play important roles in adipocyte differentiation and function, which in turn influence a wide array of homeostatic processes including insulin sensitivity and lipid accumulation (Caron-Debarle et al., 2010). Changes in mitochondrial structure and function are known to occur in age-associated disorders such as Parkinson's disease, sarcopenia and metabolic diseases, including heart-disease and diabetes mellitus (Desler et al., 2012; Galloway and Yoon, 2012).

Not surprisingly then, antiretroviral drugs are found to alter mitochondrial morphology and function, although specific mechanisms and the chronology of these events remain to be fully unraveled. Electron microscopy of AZT-treated striated skeletal muscle from rats, and AZT-, ddC-, and 2′,3′-dideoxyinosine (*didanosine* or ddI)-treated human hepatocytes show widespread mitochondrial swelling with poorly organized cristae (Lewis et al., 1992; Pan-Zhou et al., 2000). Muscle biopsies from AZT-treated patients give similar results with striking variations in mitochondrial size, shape, and network organization (Pezeshkpour et al., 1991). AZT and d4T induce a rapid increase in mitochondrial proliferation in human fibroblasts (Caron et al., 2008), and their combination with or without IDV increase mitochondrial mass

in both white and brown murine adipocytes (Viengchareun et al., 2007). Individual exposure of HeLa cells to NFV, RTV, and SQV caused fragmentation of the mitochondrial network and decreased mitochondrial number and volume (Roumier et al., 2006).

Mitochondrial fusion, fission, and autophagy have important roles in mitochondrial maintenance, specifically in protection against persistent mtDNA damage (Chen et al., 2010; Bess et al., 2012). Therefore, altered mitochondrial morphology might be considered a compensatory mechanism to help preserve mitochondrial functions. Increased proliferation for example, may be an attempt of mitochondria to recover mtDNA and increase functional capacity under pressure (Lee and Wei, 2005). However, evidence exists that the newly formed mitochondria could be non-functional (Caron et al., 2008). Mitochondrial autophagy on the other hand, has been interpreted as a protective mechanism against NNRTI *efavirenz*-induced respiratory chain malfunction (Apostolova et al., 2011b).

Murine adipocytes exposed to AZT, d4T, and/or IDV displayed impaired mitochondrial function as measured by lower respiration rate and decreased ATP production (Jiang et al., 2007; Viengchareun et al., 2007). AZT is also known to competitively inhibit the ADP/ATP antiporter in rat heart mitochondria and thus could contribute to the ATP deficiency syndrome witnessed in patients (Valenti et al., 2000). Cells with diminished oxidative phosphorylation shift to glycolysis for their energy demands which results in accumulation of lactate and, if left untreated, can cause lactic acidosis. AZT-, d4T-, or ddC-treated human hepatoma cells show increased lactate concentrations and, in some cases, decreased activity of mitochondrial respiratory chain complexes (Velsor et al., 2004). An analysis of mitochondrial genes in adipose tissue and monocytes from HIV-negative subjects receiving dual NRTI therapy revealed a significant decrease in mitochondrial respiratory chain component expression (Mallon et al., 2005). AZT and IDV have additionally been found to suppress membrane potential and cause apoptosis in blood–brain barrier endothelial cells (Manda et al., 2011). Moreover, PI-induced mitochondrial effects are typically related to an altered membrane potential (Apostolova et al., 2011a). A randomized, double-blind, placebo-controlled study found that short-term AZT exposure reduced mitochondrial function and insulin sensitivity in non-infected participants (Fleischman et al., 2007). Additionally, a randomized clinical trial in non-symptomatic antiretroviral-naïve patients showed that long-term exposure to PIs or NNRTIs is associated with disrupted glucose transport as well as disrupted lipid metabolism with increased insulin resistance (Shlay et al., 2007). In conclusion, antiretroviral therapy has frequently been implicated in metabolic diseases as a result of mitochondrial dysfunction (Caron-Debarle et al., 2010) and mitochondrial impairment is found in the absence of HIV infection.

IS HAART INVOLVED IN IMMUNOSENESCENCE?

With the success of HAART in viral suppression the question arises whether HIV-1 is the sole plausible cause for immunosenescence in HIV-treated patients. HAART, which is taken daily for lifelong periods, is probably also responsible for immune system malfunction. Besides that various antivirals have been shown to induce inflammatory signals (Mondal et al., 2004; Lagathu et al., 2007; Lefèvre et al., 2010), senescent cells have also been shown to change their phenotype, secreting proinflammatory cytokines and contributing to systemic low-grade inflammation (Freund et al., 2010). The systemic exposure and relatively high concentration of antiretrovirals undoubtedly affects all cell types, immune system cells included. The direct relationship between antiretroviral drugs and inflammation needs to be addressed further.

Hematopoietic progenitor and lymphoblastoid cell toxicity of NRTIs may explain immune cell depletion independent of inflammation (Faraj et al., 1994; Sundseth et al., 1996; Sharma, 2010). Moreover, a decline in mitochondrial genetic integrity in hematopoietic progenitor cells could also explain continued immune dysfunction upon cessation of therapy. mtDNA levels do recover in patients after discontinuation of HAART (Côté et al., 2002), but due to generation of somatic mutations by antiretrovirals and ROS, it is likely that replenished mtDNA harbors mutations predisposing the recuperating mitochondria to continued dysfunction.

A SUITABLE MODEL SYSTEM TO STUDY PREMATURE AND ACCELERATED AGING

Many questions remain unanswered in the antiretroviral drug field. Most HIV-1-infected individuals use a numerous combination of antiretroviral drugs from two or more different classes, making singular drug impacts difficult to assess. Furthermore, patient populations are diverse and administered drug cocktails as well as research methods are often dissimilar (Fisher and Cooper, 2012). There are multiple *in vivo* and *in vitro* model systems in use to study drug toxicity, however complex systems are time consuming and expensive and they do not permit straightforward analysis. Undeniably, the lack of a good model system has hampered consistent and coherent research into specific effects of antiretroviral therapy.

The nematode *Caenorhabditis elegans* has proven itself to be one of the most versatile model organisms for the elucidation of molecular pathways implicated in many human diseases, including those of mitochondria and aging (Culetto and Sattelle, 2000; Markaki and Tavernarakis, 2010). Aging in *C. elegans* is entirely post-mitotic, reflecting the gradual loss of function in somatic cells as they grow old. Although limited, this model system can also help researchers dissect tissue- and compartment-specific effects. *C. elegans* normally has a relatively short lifespan of two weeks, enabling researchers to rapidly assess the effects of different mutations or treatments on lifespan. Mitochondrial research in *C. elegans* has given us many insights into the genetic regulation of aging and mitochondrial function, and it has provided us with a vast array of mutants to study these effects (Tsang and Lemire, 2003; Addo et al., 2010; Bratic et al., 2010). Not only is *C. elegans* a very practical system, this nematode has also been used to study drug-specific impact on mitochondria (Zubovych et al., 2010). With this knowledge we can use *C. elegans* to quickly evaluate the effects of individual antiretroviral drugs, not only on mitochondrial function directly, but in relation to organism genetics, physiology, and longevity.

Caenorhabditis elegans has successfully been used to elucidate specific effects of NRTIs on physiology and longevity

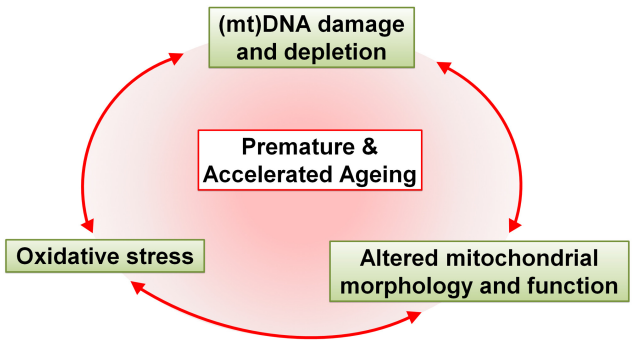

FIGURE 3 | Schematic representation of the major effects of antiretroviral drugs that drive premature and accelerated aging. Antiretroviral drugs cause mtDNA damage and depletion, oxidative stress and altered mitochondrial morphology and function. These alterations in the mitochondria contribute, either alone or in unison, to premature and accelerated aging in HAART-treated patients.

(R. de Boer and H. van der Spek, personal communication). *C. elegans* demonstrated that NRTIs decrease mtDNA copy number, disrupt both structure and function of mitochondria and shorten the average lifespan. Using oxygen consumption as a measure of mitochondrial function, NRTIs were shown to induce a rapid decrease in mitochondrial fitness. These findings compare well to earlier studies wherein abnormal mitochondrial respiratory activity was correlated with altered expression or deficiency in various respiratory chain complexes (Pan-Zhou et al., 2000; Caron et al., 2008). Additionally, *C. elegans* mitochondria showed signs of increased mass, fragmentation, and disrupted organization, as is typically found in HAART treated patients (R. de Boer and H. van der Spek, personal communication).

CONCLUSION

With the increase in life expectancy it has only recently become clear that HIV-1 patients are suffering from symptoms of aging ahead of time. In this review we postulate that strong correlations exist between antiretroviral drug-induced mitochondrial toxicity and premature and accelerated aging (**Figure 3**). However, there are a number of questions that remain unanswered, simply because we do not fully understand the effects of antiretroviral drugs individually, let alone in combination.

For example, questions remain about mitochondrial toxicity of NRTIs beyond the polymerase-γ theory. Various cellular transport systems interact with NRTIs and once inside the cell NRTIs are actively phosphorylated from their pro-drug form (Lewis et al., 2003). Changes in cellular thymidine kinase kinetics by interaction with NRTI thymidine analogs have been linked to cardiomyopathy and lipodystrophy (Apostolova et al., 2011a). Additionally, the occasionally divergent relationship between mtDNA copy number and respiratory chain protein levels needs to be explained. The influence NRTIs have on gene expression could give us insight into cellular adaptation to antiretroviral drugs. Alleviation of oxidative stress could prove an easy way to improve the well-being of patients and delay the detrimental effects of antiretroviral drugs. Interestingly, most of the above mentioned ROS complications have experimentally been found to lessen upon co-administration of antioxidant compounds. Antioxidant- or mitochondria-directed supplementation may therefore benefit HAART patients, although thorough research remains to be done before any definitive advice can be given to patients (Neustadt and Pieczenik, 2008).

We propose the use of *C. elegans* as a model system to study the effects of antiretroviral therapy on premature and accelerated aging. Using *C. elegans* we can begin to study the effects of specific genetic backgrounds on HAART toxicities. Effects of HAART seen in the nematode can direct more specific research into human conditions. In addition, *C. elegans* could provide an easy platform, not just for toxicity studies of various antiretroviral drugs, but also to screen for suitable compounds that neutralize toxic effects of HAART, which remains crucial as long as total HIV eradication is not possible. Combining these genetic and toxicology approaches we can initiate research leading to efficient, personalized, anti-HIV treatment in humans.

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A novel classification system for evolutionary aging theories

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Theories of lifespan evolution are a source of confusion amongst aging researchers. After a century of aging research the dispute over whether the aging process is active or passive persists and a comprehensive and universally accepted theoretical model remains elusive. Evolutionary aging theories primarily dispute whether the aging process is exclusively adapted to favor the kin or exclusively non-adapted to favor the individual. Interestingly, contradictory data and theories supporting both exclusively programmed and exclusively non-programmed theories continue to grow. However, this is a false dichotomy; natural selection favors traits resulting in efficient reproduction whether they benefit the individual or the kin. Thus, to understand the evolution of aging, first we must understand the environment-dependent balance between the advantages and disadvantages of extended lifespan in the process of spreading genes. As described by distinct theories, different niches and environmental conditions confer on extended lifespan a range of fitness values varying from highly beneficial to highly detrimental. Here, we considered the range of fitness values for extended lifespan and develop a fitness-based framework for categorizing existing theories. We show that all theories can be classified into four basic types: secondary (beneficial), maladaptive (neutral), assisted death (detrimental), and senemorphic aging (varying between beneficial to detrimental). We anticipate that this classification system will assist with understanding and interpreting aging/death by providing a way of considering theories as members of one of these classes rather than consideration of their individual details.

Keywords: senemorphism, caloric restriction, longevity, altruism, senescence, evolution

INTRODUCTION

After a century of aging research, the dispute over whether the aging process is active or passive persists and a comprehensive and universally accepted theoretical model remains elusive (Jin, 2010). Contradictory data and theories supporting both exclusively programmed and exclusively non-programmed theories continue to grow (Jin, 2010; Mitteldorf, 2010c; Goldsmith, 2011; Martins, 2011). The idea that aging must be either active or passive is fundamentally incorrect because it is surely the case that aging could in principle be active in some species and passive in others. Moreover, some, or possibly all species could have evolved plasticity of lifespan within both programmed and non-programmed aging phenotypes in order to cope with environmental changes; occasionally favoring the kin, occasionally favoring the individual. Clearly different gerontologists have different points of view, and to understand the evolution of aging/lifespan, all data, theories and arguments must be considered and reconciled. To do this, all hypotheses must be classified into a small number of well-understood categories. Here, we offer a fitness-based framework for categorizing existing evolutionary aging theories. Firstly we describe causality theories of death, which are concerned simply with the process of dying (**Figure 1**). They are subdivided into "entropy-based" and "sudden death" mechanisms. Secondly we characterize evolutionary theories of aging, which are concerned with both the selective pressures and the evolutionary processes that could inhibit the evolution of longer lifespan (**Figure 1**). Evolutionary theories consist of "maladaptive aging," "secondary aging," "assisted death," and "senemorphic aging." This approach should reveal common themes that will prove helpful to researchers. Below, we explain this system of classifying aging/death theories in detail.

CAUSALITY THEORIES OF DEATH

We make the important distinction between causes of death theories and evolutionary theories of aging (**Figure 1**). Causality theories are solely concerned with the main cause of intrinsic death. Consistently, it has been shown that the proximal causes of aging and death differ depending on species and environmental conditions (see for example: Andrade, 1996; Demetrius, 2005; Rattan, 2006; Greer and Brunet, 2011). We divide these causes into

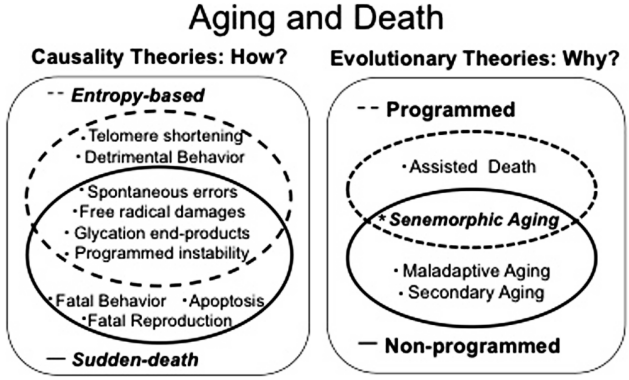

FIGURE 1 | Aging and death theories can be classified into two groups: (1) causality theories which address questions of how aging and death occur and can be subdivided into entropy-based processes and "sudden death." (2) Evolutionary theories which try to explain why species age and die in the way they do. They consist of programmed aging, non-programmed aging and senemorphic aging which is a special case where parallel evolution of "senemorphisms" (independent aging phenotypes encoded by the genome) are related to both a genetic profile to accelerate aging and a genetic profile to maximize lifespan.

two broad groups: (a) entropy-based: when death follows a relatively long period of degeneration (senescence); (b) sudden-death: when death follows a relatively short period of degeneration or is an almost instantaneous process.

ENTROPY-BASED THEORIES

Senescence is clearly a deteriorative process featuring increasing disorder. In the course of senescence, intrinsic death ultimately occurs as a result of physical deterioration due to this increasing disorder, e.g., from the accumulation of molecular-level damage. In these situations it is reasonable to say that senescence is the cause of death. Entropy-based theories of aging have advanced extraordinarily in the past three decades, revealing possible causes of aging and death for most species and include: spontaneous errors (e.g., DNA mutations, protein misfolding), free radical damage, advanced glycation end-products, gerontogenes, etc. (Rattan, 2006). More recently it was proposed that a combination of factors rather than a single mechanism is responsible for age-related death (Rattan, 2006). This is an important area of research as an understanding of the processes involved may lead to the design of treatments to inhibit or reverse age-related diseases.

SUDDEN DEATH

Entropy-based death applies when senescing individuals gradually deteriorate until a tipping point is reached. Sudden death on the other hand is death which occurs in non-senescent individuals over a short time scale or even instantaneously. The classic examples include: (1) fatal reproduction: semelparous species that die rapidly after reproduction (Robertson, 1961; Wodinsky, 1977; Bradley, 2003) or males of some social insect species, which expel their penis in order to enhance fecundity; bringing together the internal organs and automatically killing the animal (Gary and Marston, 1971); (2) cannibalism: in some cases, this is thought to be a result of sexual competition such as in the golden orb-web spider (Schneider et al., 2001). In other cases it is thought to be an important form of death in order to recycle energy in several species (Andrade, 1996; Foellmer and Fairbairn, 2003; Prenter et al., 2006); (3) kin protection: as seen in the female honey bees upon stinging: where the stinger and part of the abdomen remain in the skin of the potential aggressor and releases pheromones to attract more bees (Hunt et al., 2003); (4) apoptosis: proposed as a population survival strategy in unicellular organisms (Lane, 2008).

CAUSALITY VERSUS EVOLUTIONARY AGING THEORIES

The existence of senescence in no way suggests that biological systems cannot act as islands of reverse entropy to avoid death indefinitely. Indeed the order inherent in living systems is one of their defining features. The Second Law of Thermodynamics asserts that closed systems will become disordered. However, living organisms are open self-organizing systems and thus in principle potentially able to maintain a high level of order. In brief, life exists by using energy to maintain order in the face of entropic pressure (Mitteldorf, 2010a). Some individuals can carry out this process for hundreds of years or more (Medawar, 1952; Abele et al., 2008). Causality theories of aging consider the immediate causes of aging and death. It is self-evident that entropy increases as individuals senesce while enough order is maintained to avoid death. It is unclear why living systems do not continue maintaining order to the same level of stringency indefinitely: we are left searching for evolutionary explanations to understand why at some point in time body maintenance decreases; and why this point is distinct in different species.

The central idea for understanding the evolution of aging/lifespan is straightforward: natural selection favors traits related with efficient reproduction whether they benefit the individual or the kin and much evidence has accumulated in support of this idea (for example: Sundström et al., 1996; Bourke, 2011). As predicted mathematically by Hamilton (1964), interesting recent data using model robotic systems also suggests that altruism will always evolve when the benefits to the kin overwhelm the detrimental effects for the individual (Waibel and Keller, 2011). Consequently, the same principle of a balance between individual and kin benefit could allow aging/death to evolve as an adaptation, an idea supported by several authors (see for example: Andrade, 1996; Crespi and Teo, 2002; Mitteldorf, 2010c; Martins, 2011; Fukuyo et al., 2012).

The segregation of causality from evolutionary theories of aging is thus crucial to avoid any "non-sequitur" fallacy. For instance, it is often suggested that senescence, being a degenerative and detrimental process, cannot be adapted by natural selection. This is logically incorrect – altruistic behaviors are by definition detrimental to individuals yet are believed to have evolved by natural selection. Furthermore, several forms of death (e.g., by self-starvation, submissive cannibalism) have been shown to be beneficial for the kin and are supposed to have evolved for this reason (see examples: Andrade, 1996; Larkin and Slaney, 1997). Workers from social species have the same genetic background as queens and yet do not reproduce and have significantly shorter lifespans. Thus, we must not overlook the fact that some of these

altruistic adaptations and senemorphism (i.e. the worker-specific age-related phenotype) are even more detrimental than merely senescence itself.

In conclusion: to understand the evolutionary reasons for species-specific lifespan, we must first understand the balance between the advantages and disadvantages of extended lifespan in the process of spreading genes. This balance is likely to be niche and environment-dependent and thus cannot be understood by metabolism and physiology alone.

EVOLUTIONARY AGING THEORIES

A central concern of evolutionary aging theories is to track down the population genetic processes restricting the evolution of lifespan. In other words, why species have the lifespan they have rather than a longer or shorter one. As discussed above, whether faster or slower aging will evolve depends on whether or not the chance of spreading genes is increased and so can only be understood by consideration of life-history and environmental conditions. From an evolutionary perspective, every characteristic can be classified for its fitness value, which means a specific trait can be considered neutral, beneficial, or detrimental for the individual or kin under specific environmental conditions. We suggest that the central conflict among evolutionary aging theories is that each theory only attributes one fitness value for longevity (e.g., extended lifespan being exclusively beneficial, neutral, or detrimental). The apparent conflict arises from the fact that each theory describes distinct scenarios that apply different selective pressures on longevity (and reproduction) and so cannot be compared. These different selective pressures determine the type of the evolutionary process affecting the evolution of lifespan potential. Therefore, we classify actual evolutionary aging theories according to a range of fitness values assumed for extended lifespan; highlighting the possible corresponding evolutive processes (**Table 1**). We sub-divide the evolutionary theories of aging into four sub-groups depending on specific selective pressure as follows: (a) maladaptive aging: when fitness associated with extended lifespan is neutral, thus longevity could be arrested or lost by retrogression (mutational load and drift); (b) secondary aging: when fitness associated with extended lifespan is beneficial, yet lifespan potential could be lost by a trade-off (pleiotropy/hitchhiking effect); (c) assisted death: when fitness associated with extended lifespan is significantly detrimental for the kin, thus senescence could evolve as a direct adaption to enhance reproduction; (d) senemorphic aging: when fitness associated with extended lifespan historically varied between beneficial and detrimental depending on changes in environmental conditions, thus parallel senemorphoses (distinct senescence patterns encoded by the genome) could have evolved within the same species (**Table 1**).

According to this view, distinct programs resulting in specific lifespan potential could have evolved to enforce optimal adaptation under different environmental conditions (as seen in social species). To our knowledge this is the first time a classification system has listed all fitness values related with longevity to explain the evolution of lifespan.

Table 1 | Fitness-based classification system for the evolutionary aging theories.

Fitness value/specific environmental conditions	Theoretical group	Processes inhibiting the evolution of lifespan	Theories examples
The fitness associated with extended lifespan is neutral when the force of natural selection decreases with aging. Longer lifespan cannot evolve and even could be lost by retrogression.	Maladaptive aging	Mutational load + genetic drift (retrogression) Genetic linkage	Mutation accumulation Somatic damage Infectious diseases
The fitness associated with extended lifespan is beneficial but secondary, when it is overwhelmed by another trait. Lifespan could be exchanged for such more beneficial trait.	Secondary aging	Trade-offs (e.g., reproduction) Pleiotropy Hitchhiking effect	Antagonistic Pleiotropy Disposable Soma
The fitness associated with extended lifespan is detrimental when longer lifespan of parents negatively affects the kin fitness. An intrinsic program could have evolve to inhibit extended lifespan (direct adaptation).	Assisted death	Down-regulation of protection Down-regulation of repair Programmed instability Programmed death	Release resources Demographic control Increase variability
The fitness associated with extended lifespan most likely varies between beneficial and detrimental depending on environmental conditions. Distinct adaptations could emerge: one to maximize lifespan and one to inhibit extended lifespan.	Senemorphic aging	*Combination of mechanisms*	Germ-soma conflict Senemorphic aging

Unifying evolutionary aging theories. The table is based on all possible outcomes for extended lifespan (beneficial, detrimental, neutral, or variable). Column 1 highlights the environmental condition restricting the evolution of longer lifespan. Column 2 shows the respective class of evolutionary aging theory. Column 3 shows the corresponding evolutionary processes restricting the evolution of longer lifespan. Column 4 gives examples of existing theories that fall under these headings.

MALADAPTIVE AGING THEORIES

An attractive theory for the existence of death in some species is the declining force of natural selection with age: the lower reproductive efficiency of long-lived individuals will eventually and indirectly lead a species to adapt a shorter lifespan. That the force of natural selection declines with age would be ensured by environmental factors such as: (1) somatic damage (e.g., limb trauma) which could accumulate even in an "immortal" individual (Weismann, 1891); (2) eventual death through extrinsic forces (e.g., predation, accidents; Medawar, 1952); (3) population-wide infectious diseases that cause sterility but not death (Ricklefs, 1998; Kirchner and Roy, 1999). In these environmental conditions the evolution of a longer lifespan is inhibited as a consequence of harsh extrinsic forces (which cause pro-longevity mutations to be ineffective). A well-accepted example of this class of theory is the mutation accumulation theory of Medawar (1952). In this theory, once strong extrinsic mortality imposes a limit on lifespan, then lifespan is supposed to be arrested or even lost by retrogression (mutational load and drift; **Figure 2**). If true, it may be thought that conditions of low extrinsic mortality would lead to evolution of a longer lifespan, independent of an individual's rate of reproduction. Interestingly, evidence that this is possible can be found in nature and also was demonstrated in laboratory conditions (Rose, 1984; Keller and Genoud, 1997; Møller, 2006). In actual fact, it is now appreciated that changes in longevity via changes in mortality can only occur if the extrinsic mortality is not only strong but also age-dependent (Caswell, 2007). However, in reality as discussed above it is quite reasonable that older individuals could be more vulnerable to extrinsic challenges due to inevitable accumulation of damage over time. In summary, in a maladaptive aging theory, lifespan could in principle always be lengthened, if not for the assumption that extrinsic forces are always extremely harsh. The robust impact of extrinsic forces would invariably impose a longevity maladaptation.

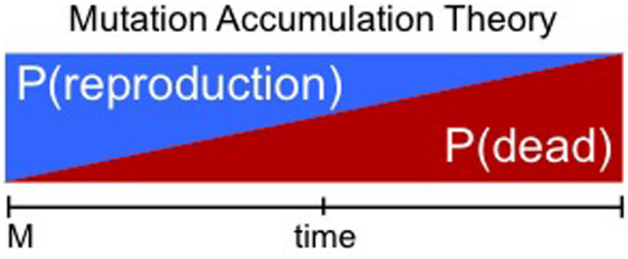

FIGURE 2 | Maladaptive aging theories. Illustration of "Mutation Accumulation Theory." Here the probability of an individual being dead (P(dead), red) increases over time solely due to harsh extrinsic mortality. Consequently the likelihood of successful reproduction (P(reproduction)) at any given time decreases over time (blue). The increasing probability of being dead acts as the main force restricting the selection for longer lifespan. It is reasonable to assume that there is no selective pressure for longevity after a certain threshold at which the likelihood of reproducing is very low. In this case the fitness associated with extended lifespan is neutral. Here death is supposed to occur before senescence has an effect, thus senescence is not necessary to affect the probability of death. M, maturity.

MALADAPTIVE AGING CANNOT BE UNIVERSAL

According to the predictions of maladaptive aging theories a species lifespan depends upon lifespan being limited by random and harsh extrinsic mortality, which assures the probability of reproduction decreases with age (**Figure 2**). In such conditions, individuals would not have the chance to senesce and thus further increases of lifespan potential would be irrelevant (neutral). However, as demonstrated by Caswell (2007) and mentioned above, extrinsic mortality *per se* could not have an effect on lifespan; effects are only seen if the mortality is age-specific. In support of this, there is strong evidence that slight decreases in fitness due to senescence (an intrinsic process) are enough to negatively affect fecundity and survival of older individuals from some species (Ricklefs, 1998, 2008). This means that we cannot generally assume that it is extrinsic mortality alone which restrains the evolution of lifespan in all species. The works of Ricklefs (1998, 2008) suggest not only that senescence can be seen in nature, but also senescence itself decreases fecundity and survival of individuals from several species. If senescence increases vulnerability to extrinsic forces (predation, infection, etc.) then senescence itself determines lifespan (Mitteldorf, 2010c). In addition, lengthening of lifespan could be extremely detrimental for the kin in several situations (see assisted death section below). In this case, senescence could be selected for as an altruistic trait. Furthermore, it seems to be the case that inter-species competition significantly favors reproduction over longevity under several conditions (see next section). In this case a longevity–reproduction trade-off could restrict the evolution of longer lifespan regardless of extrinsic mortality.

In summary, low extrinsic mortality could allow species to evolve longer lifespan but it seems to be only in cases accompanied by a compensatory effect on overall fecundity (see next section). Interestingly, however, senescence itself seems to determine lifespan in the wild, i.e., extrinsic mortality only exerts an effect on lifespan if senescence already exists. This phenomenon cannot be explained by a maladaptive aging theory, which explains senescence as being the result of extrinsic mortality.

SECONDARY AGING THEORIES

Here, senescence is a result of selection for a trait more useful than maintenance of body fitness after reproduction. In this case, beneficial alleles associated with later life could be exchanged for a more useful trait due to pleiotropy or a hitchhiking effect. By definition, a longevity trade-off can only be justified when the fitness associated with extended lifespan is higher than zero (non-neutral). Antagonistic Pleiotropy, proposed by Williams (1957) is an important example of longevity being restricted by a trade-off for faster reproduction. This theory elegantly suggests how numerous adverse side effects in later life could be maintained by being linked to beneficial effects at younger ages: assuming that faster reproduction is an advantage in the competition among and within species, it will be selected for regardless of accompanying side effects that shorten lifespan (**Figure 3A**; Williams, 1957).

Disposable Soma, proposed by Kirkwood and Holliday (1979) is another important theory of this kind and it is widely appreciated by gerontologists. This theory assumes that both maintenance

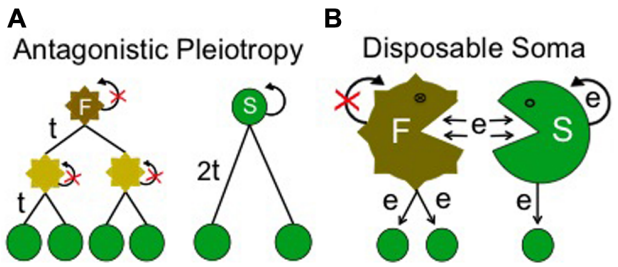

FIGURE 3 | Secondary aging theories. This class of evolutionary aging theory assumes that a continuous trade-off restricts selection for longer lifespan. **(A)** Example representing "Antagonistic Pleiotropy" where selection for longevity is assumed to be restricted due to the pressure for faster reproduction. Here a fitter individual (F) is able to produce two offspring with generation time, t. After 2t, individual F has four descendents. In this case, fast reproduction is supposed to cause a side effect on body homeostasis (shown by jagged edges and non-green color) and to minimize regeneration (curly arrow with red cross). A more slowly reproducing individual (S), although more able to regenerate and limit deterioration, is unable to compete under these conditions, as it produces only two descendents in 2t. **(B)** Example representing "Disposable Soma" where selection for longevity is assumed to be restricted due to optimized, efficient utilization of energy (e) for reproduction. Here the fitter individual (F) uses more of the energy consumed to produce offspring rather than regenerate its own body. As a result it deteriorates faster but produces more offspring than an individual (S) which uses more energy for regeneration.

of soma and reproduction are processes requiring significant amounts of energy. It suggests that organisms in general will adapt to expend resources on optimizing reproduction, trading this off at the expense of soma maintenance. Even if extra energy becomes available, it will be utilized to further optimize reproduction rather than increase lifespan. This is because the theory hypothesizes that optimized reproduction will be always favored over lifespan (**Figure 3B**; Kirkwood and Holliday, 1979). In summary, in a secondary aging theory, the benefits associated with extended lifespan are always considered secondary (non-essential) due to the assumption that a trade-off for faster or increased reproduction would invariably favor the individual.

SECONDARY AGING CANNOT BE UNIVERSAL

Certainly some niches and conditions favor faster development, growth, and maturation over extended lifespan [e.g., ad libitum (AL) food conditions]. However, secondary aging theories are unlikely to be universally applicable. It must be the case that some environmental conditions favor longevity over fecundity. For example, longevity is positively associated with lower fecundity among species (Holliday, 1995). Indeed, the theory seems not to hold in various examples: Reproduction for females is more costly than for males, yet females of several species live longer than males (Mitteldorf, 2010b). In addition, if reproduction significantly impairs body maintenance in all species, all iteroparous individuals should show a decrease in lifespan with each round of reproduction. This is not the case (Ricklefs and Cadena, 2007). Furthermore, some conditions, i.e., famine, have been shown to in fact favor longevity over reproduction (Holliday, 1989). Most importantly, even given an energetic cost associated with reproduction, there are clearly other environmental conditions in which the selective pressure seems to favor both reproduction and lifespan simultaneously: Solitary insects live for days or weeks, but queens from social species are able to live remarkably longer (reaching almost 30 years) and show significantly higher reproductive capacity (Keller and Genoud, 1997). Naked mole-rats (a species of rodent) are similar in size to mice, but can live up to 30 years and are able to give birth to up to 28 pups at once (Sherman et al., 1999). Finally, Rose (1984), using artificial evolution in *Drosophila melanogaster* demonstrated the selected lines exhibited increased longevity but roughly preserved fecundity through decreased early fecundity and increased later fecundity. After further continued selection, these lines in fact increased early fecundity also (Leroi et al., 1994). The results of Rose prove that it is possible for evolution to increase both lifespan and reproduction at the same time. Yet this does not seem to occur often in nature and when it does it seems to be only in special cases accompanied by a compensatory effect on overall fecundity (e.g., social species where only queens can reproduce). It could be the case that slight increases of longevity are detrimental to kin fitness in nature (e.g., result in parent-offspring conflict, overpopulation, decreased variation).

In conclusion: strong evidence suggests that under several conditions, longevity is in fact favored over reproduction (Holliday, 1995; Mitteldorf, 2010b). However, the most important point to bear in mind here is that it is possible to select increased reproduction and longevity at the same time (Leroi et al., 1994; Keller and Genoud, 1997; Sherman et al., 1999). However, in nature, longer lifespan is always accompanied by a compensatory effect on overall fecundity (either through low numbers of offspring or zero/low potential fecundity of offspring produced). Could even a slight increase in lifespan be detrimental for the spreading of genes?

ASSISTED DEATH THEORIES

Faster reproduction and genetic variability of a species are crucial for adaptability within a population (Angelo and Van Gilst, 2009). It has been suggested that overpopulation is detrimental to the kin, enforcing suppression of reproduction (Bourke, 2007; Mitteldorf, 2010c; Ronce and Promislow, 2010). Thus, it could be the case that even a small degree of superfluous longevity is enough to cause a detrimental effect on fitness during harsh competition in the wild. Martins (2011), using computational simulations demonstrated that reproduction is crucial for adaptability, while longevity is detrimental. These results raise the distinct possibility that evolution may adapt a genetic pathway to inhibit useless and otherwise harmful increases in longevity. As discussed above, even if senescence is detrimental to the individual, natural selection could still favor faster death if it enhances the fitness of the kin. If one accepts that longevity could be traded-off to enhance individual reproduction (Kirkwood and Holliday, 1979), it also must be acceptable that longevity could be traded-off to enhance kin reproduction (exactly the same effect and in agreement with natural selection). This suggests the possibility of the existence of a "senescence program" to ensure death in order to inhibit or delay the evolution of longer lifespan. Note, however, that such a "program" need not be a direct set of genetic instructions

to die: Remembering that rigorous repair and maintenance is constantly required to preserve an organism as an island of negative entropy then we see that the senescence "program" could simply be the adaptation of "master controls" to down-regulate these maintenance processes. In this sense and due to the lack of evidence for a genetic program for death, we can consider senescence as adapted/programmed through selection for mechanisms of decreased protection and repair. In this case the term "assisted death" is more appropriate than "programmed death" in the sense that death is "passively" encoded by the genome. Thus, it would be difficult or impossible to differentiate the molecular mechanisms involved in programmed versus non-programmed senescence.

Acknowledging that evolution could favor reproduction and longevity at the same time (see secondary aging), it is difficult to determine if any down-regulation of body maintenance is a side-effect or an adaptation. For instance, the specific age-related changes during AL conditions clearly are not adapted to optimize body fitness (Trindade et al., 2012). In any case, the significant age-related decrease in body maintenance during AL conditions cannot be uncritically assumed to be a side effect of metabolism and growth. Could for ecological reasons the AL genetic profile be in fact an adaptation?

Several reasons have been hypothesized to favor the adaptation of assisted death: (1) release of resources for offspring; (2) for demographic control; (3) to speed up adaptation (Weismann, 1891; Woolhouse, 1967; Wodinsky, 1977; Kirkwood and Holliday, 1979; Bradley, 2003; Mitteldorf, 2004; Lane, 2008). These ideas are strongly supported by the discovery of genes and mechanisms whose sole role appears to be to decrease lifespan (Kenyon, 2005; Lane, 2008).

Semelparous strategies are the most commonly suggested examples of assisted aging/death. For example, in a range of different phyla, including mollusks, fish, reptiles, and mammals an apparently unnecessary "self-starvation" of parents during and after the breeding season is observed (Woolley, 1966; Wodinsky, 1977; Larkin and Slaney, 1997; Bradley, 2003). These species often live in hash environments where it is plausible to think that abstention from food releases resources for the offspring, increasing their chances of survival. In the ultimate example, in some species the parents' dead bodies themselves provide, directly or indirectly, a crucial source of food for the young (Andrade, 1996; Watkinson, 2000). Indeed simple mathematical models of semelparity in animals and plants predict that it will be favored when a small number of simple criteria are met such as increased juvenile survivorship and population growth (benefiting the kin; Young, 1981).

Examples supporting the idea of an assisted death adaptation such as mentioned above are generally of the "sudden death" variety where death occurs quickly and it is easy to quantify the benefits to the kin. However, if one accepts this evidence on the basis that parents' death is beneficial or even crucial for the kin in some niches and environmental conditions, then it may be possible that more gradual death (entropy-based aging) is also a viable means to achieve the same goal. Ultimately, both entropy-based and sudden death could be direct adaptations, particularly if a slight decrease in body maintenance is enough to significantly increase the chance of extrinsic mortality as proposed by Ricklefs (1998, 2008). In summary, in an assisted death theory, lifespan could always be lengthened, if not for the assumption that longer lifespan would invariably cause a detrimental effect on spreading genes. In this case it is postulated that an assisted death program would evolve to benefit the kin.

ASSISTED DEATH CANNOT BE UNIVERSAL

The benefits of assisted death (programmed aging) have been discussed exhaustively in the literature and briefly above. Nevertheless it seems unlikely that assisted death is universally and irreversibly applicable. In many cases a persistent, "strict" assisted death program would be detrimental for an individual that is unable to reproduce. For example, a starved individual incapable of reproducing, must in fact not die and must survive until food returns and successful reproduction becomes possible (Holliday, 1989). Also a semelparous individual which fails to breed in a particular breeding season must in fact not die and must survive until the subsequent breeding season (Bradley, 2003). Therefore, even if an assisted death program has been shown to significantly favor the kin, in several situations its irreversible activation would be detrimental for the individual and for the kin.

The main challenges for an assisted death theory be accepted are: (1) to propose a genetic pathway leading to death; (2) to suggest how this pathway was maintained during the course of evolution by natural selection. Nevertheless, we do not need to understand how death could evolve and be maintained by natural selection to assume it could be possible. It is still somewhat unclear how sexual reproduction, sociality, and altruism had evolved, but it is clear they did. Faster death of parents by an assisted death program, mainly by self-starvation would likely release food for the offspring, decelerate population growth, increase species genetic variability, and thus the adaptability rate. Thus, some environments may directly favor the adaptation of assisted death. On the other hand, some environments surely favor longevity. This conclusion leads us to a possible unification of aging theories, discussed in the next section.

SENEMORPHIC AGING THEORIES

Recently, we have highlighted the existence of environment-dependent senemorphic strategies (independent aging patterns encoded by the genome). The evolution of independent genetic pathways to enforce distinct lifespan potentials can be easily identified for example in social species (caste-related senemorphism) where workers and queens have the same genetic background, but show distinct aging patterns modulated by differential gene expression (Trindade et al., 2012). However, the most common senemorphic adaptation among species is the distinct and independent aging patterns of individuals undergoing AL versus caloric restriction (CR) feeding (Trindade et al., 2012). This diet-related "plasticity" in lifespan we termed "diet-related senemorphism." In brief, there is ample evidence that the response to AL and CR conditions are independent adaptations, as we previously stated: "(1) comparing the two dietary groups, several age-related changes run in the opposite direction over time; (2) switching from an AL to a CR diet clearly reverts (not only delays) several "normal" accumulated changes; (3) major causes

of death are as different between both groups as they are between species." These observations strongly support the idea that independent genetic pathways evolved to modulate distinct lifespan potential during different food conditions. Such an ability to activate a particular genetic program when it is advantageous to do so has been, in the case of social insects, referred to as "parallel evolution of phenotypes" (Rajakumar et al., 2012). In the case of the parallel evolution of aging patterns, we use the term "senemorphic aging" (Trindade et al., 2012). The environment-dependent regulation of lifespan potential offers a good example of how it is of benefit to switch longevity strategies as an adaptation. Surely, the efficient spreading of genes may be favored by either an extended lifespan or a shortened one (**Table 1**). Therefore, here we propose the possibility that the evolution of these distinct genetic pathways are in fact related to the adaptation of both a genetic profile to accelerate aging (altruism, AL, assisted death) and a genetic profile to maximize lifespan (selfishness, CR, maladaptive, and secondary aging). Such distinct aging patterns allow individuals to cope with environmental changes by optimizing indirectly both short and long-term reproduction (**Table 1**).

In summary, senemorphic theory suggests the possibility that the observed diet-related "plasticity" of lifespan potential is a result of direct adaptation for different environmental conditions with long-term activation or deactivation of energy sensing pathways selecting a different downstream cascade. In this case, AL cascade activation could be related to an altruistic program for faster death while the CR cascade could be related to an "individual selection" program to increase lifespan.

DISCUSSION OF SENEMORPHIC AGING THEORIES

Some authors strongly support the evolution of universal active aging, while others strongly support universal passive aging. The genetic mechanisms associated with diet-related lifespan potential have been evolving conservatively since unicellular life (Flatt and Schmidt, 2009). Therefore, the evolution of pluricellularity (including sexual reproduction) only appeared after the adaptation of diet-related senemorphism (energy sense pathways controlling antagonistically longevity and reproduction). Consequently, a universal evolutionary theory of aging must consider the drastic fluctuations of food availability that species have experienced since LUCA (last universal common ancestor). Since different food conditions determine the fitness associated with extended lifespan, the hypothesis that the AL genetic profile is in fact a direct pro-senescence adaptation and the CR genetic profile is related to the adaptation for an optimized extended homeostasis gives the best explanation for the evolution of aging/lifespan. In this section we offered a general aging theory in which the evolution of appropriate response to available energy results in a strategy that is able to explain all current ideas and evidence discussed above.

In this work, we have not attempted to explain how senemorphic aging evolved, merely to show that it could have been advantageous to have done so. Senemorphic aging offers a useful perspective as it potentially unifies evolutionary aging theories enabling a new perspective in gerontology (**Table 1**).

CONCLUSION

Currently, evolutionary aging theories are unable to explain convincingly how and why species have a limited lifespan. Each aging hypothesis has significant flaws that we have discussed briefly and which were elegantly described by Mitteldorf (2010a,b,c). It remains to be seen if a single hypothesis can be developed which is able to unify all of these often contradictory ideas into a single aging theory. The major challenge of a universal evolutionary aging theory is to reconcile the possible existence of trade-offs (pleiotropy or hitchhiking effect), retrogression (mutational load and drift) and direct adaptation ("program"). From this perspective, we have described a universal classification system for aging theories (**Table 1**). Our novel framework based on discriminatory selective pressures categorizes aging/death theories as secondary aging, maladaptive aging, assisted death, or senemorphic aging. Considering an individual theory at the level of the category to which it belongs will assist in judging its merits and should help to bring clarity to the field.

Each category is supported by theoretical and experimental data and are not necessarily mutually exclusive: while increase of biological entropy is an important contribution to a limit for lifespans, maladaptive and secondary aging theories suggest that extrinsic forces restrict the evolution of lifespan (through extrinsic mortality and inter-species competition, respectively). Thus, in beneficial environmental conditions lifespan could be lengthened. Is it always beneficial to increase lifespan if the environmental conditions allow it? Increases of longevity are related to decreases of fecundity (Holliday, 1995). Therefore, an altruistic behavior as described by assisted death could accelerate the turnover of generations maintaining the reproductive rate and at the same time releasing resources for offspring. This would clearly be beneficial for the spread of genes leading to the adaptation of assisted death being favored by some environments. In contrast, some environmental conditions (e.g., starvation, non-breeding semelparous animals) surely favor longevity as far as possible.

This conclusion leads us to a possible unification of aging theories: the existence of environment-dependent lifespan programs encoded by the genome could account for both active and passive aging programs (Trindade et al., 2012). Senemorphic aging could be an adaptation for variable environmental conditions, sometimes favoring the kin (e.g., as an assisted death program under AL conditions and in breeding semelparous individuals), sometimes favoring the individual (e.g., as non-programmed aging under CR conditions and in non-breeding semelparous individuals; **Table 1**).

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The mysterious case of the *C. elegans* gut granule: death fluorescence, anthranilic acid and the kynurenine pathway

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Gut granules are lysosome-like organelles with acidic interiors that are found in large numbers within the intestine of the nematode *Caenorhabditis elegans*. They are particularly prominent when viewed under ultraviolet light, which causes them to emit intense blue fluorescence. Yet the function of these large and abundant organelles in this heavily-studied model organism remains unclear. One possibility is that they serve as storage organelles, for example of zinc. A new clue to gut granule function is the identification of the blue fluorescent material that they contain as a glycosylated form of anthranilic acid, which is derived from tryptophan by action of the kynurenine pathway. This compound can also serve a surprising role as a natural, endogenous marker of organismal death.

Keywords: aging, *C. elegans*, death fluorescence, gut granule, kynurenine, lipofuscin, organismal death, tryptophan

THE GUT GRANULE: AN ENIGMATIC NEMATODE ORGANELLE

Despite decades of research on the nematode *Caenorhabditis elegans*, it still contains many hidden secrets. One such is the function of the prominent organelles known as gut granules, which are numerous in the intestinal cells of nematodes throughout the suborder Rhabditina (Chitwood and Chitwood, 1950). A striking feature of gut granules is the blue fluorescence that they emit under ultraviolet light (Klass, 1977; Gerstbrein et al., 2005). Clues to gut granule function include their acidic interior and capacity for endocytosis (Clokey and Jacobson, 1986; Hermann et al., 2005), both lysosome-like features (though gut granules are much bigger than normal lysosomes). This and the fluorescent material within identify gut granules as lysosome-like organelles (LROs; Hermann et al., 2005; Bernabucci et al., 2012), akin to pigment-containing melanosomes in mammals and eye pigment granules in *Drosophila* (Raposo and Marks, 2007). Thus, the identity of the blue fluorescent substance could provide a key to understanding gut granule function.

One suggestion is that the source of gut granule fluorescence is lipofuscin, a complex molecular waste production that accumulates within lysosomes in aging mammalian cells (Jung et al., 2007). Lipofuscin can contain Schiff bases, which have similar spectral similarities to the worm blue fluorescence (Fletcher et al., 1973; Klass, 1977). Consistent with this, blue fluorescence levels increase in aging worm populations (Klass, 1977; Davis et al., 1982; Gerstbrein et al., 2005). Another idea, derived from studies of *C. elegans* Flu mutants with altered fluorescence color and intensity, is that the blue fluorescence emanates from L-tryptophan-derived metabolites called kynurenines (Babu, 1974).

Over the years the lipofuscin interpretation has been favored (see e.g., Gill, 2006; Masse et al., 2008; Fujii et al., 2009; Jain et al., 2009; Minniti et al., 2009), perhaps because of the good fit with the theory that aging is caused by accumulation of molecular damage. Unfortunately, this interpretation (i.e., that the blue fluorescent substance is lipofuscin) is not the correct one. According to recent chemical analysis, the fluorescent substance within gut granules is a kynurenine pathway product, anthranilic acid (AA) glucosyl ester (Coburn et al., 2013), consistent with the proposal of P. Babu and S. S. Siddiqui so many years ago (Babu, 1974; Bhat and Babu, 1980; Siddiqui and Babu, 1980). This chemical identification was effected by comparing wild-type worms with *glo-1* mutants, which lack gut granules (Hermann et al., 2005). Whether or not lipofuscin exists in *C. elegans* remains an open question. Thus, *C. elegans* gut granules contain large quantities of AA. But what it is there for? Here, one may seek clues from kynurenine pathway action in mammals.

THE KYNURENINE PATHWAY AND NEURODEGENERATION

In mammals, the kynurenine pathway generates a variety of important molecules, including the co-factor nicotine adenine dinucleotide (NAD) and the neurotransmitter serotonin. Around 95% of tryptophan (the rarest essential amino acid) is consumed by this pathway (Vecsei et al., 2013). Although discovered over 150 years ago, the action of the kynurenine pathway's intermediate metabolites, known as kynurenines, has until recently been relatively little studied (Schwarcz et al., 2012). One role of kynurenines is in modulating CNS excitability (Perkins and Stone, 1982; Hilmas et al., 2001; Vecsei et al., 2013). For example, the kynurenine quinolinic acid stimulates *N*-methyl-D-aspartate (NMDA) receptors (Stone and Perkins, 1981; Schwarcz et al., 2012), while kynurenic acid antagonizes all excitatory amino acid receptors.

Kynurenine pathway dysregulation has been implicated in neurological disorders, including Huntington's, Alzheimer's, and

Parkinson's disease, multiple sclerosis, and epilepsy (Vecsei et al., 2013) as well as in neurodegeneration caused by acute insults, such as ischemia and excitotoxicity (Stone et al., 2012). Excitotoxic neurodegeneration is caused by release of high levels of excitatory neurotransmitters, which trigger an influx of calcium ions after depolarization (Rothman and Olney, 1987). Thus, calcium can act as a second messenger, triggering the initiation of necrotic cell death (Rothman and Olney, 1995). The kynurenine quinolinic acid can act as an excitotoxin: levels increase following ischemia, and correlate with increased neurodegeneration (Saito et al., 1993). Thus, one of the ways in which kynurenines may contribute to neurodegenerative disease is by inducing excitotoxic neurodegeneration.

THE KYNURENINE PATHWAY IN *C. elegans*

Is there a link between kynurenines and aging, particularly neurodegeneration, in *C. elegans*? Very little is known about the biology of kynurenines in nematodes. One exception relates back to gut granules: among the Flu mutants alluded to previously, altered intestinal fluorescence (Flu) phenotypes can arise from mutations affecting kynurenine pathway enzymes. For example, *flu-1* mutants, which show an altered, bluish-purple gut granule fluorescence, have reduced kynurenine-3-hydroxylase activity (Siddiqui and Babu, 1980), and *flu-2* mutants, which show a dull green fluorescence, have reduced kynureninase (Bhat and Babu, 1980; **Figure 1A**). The *C. elegans* genome contains homologs of genes encoding these two enzymes in the vicinity of the *flu-1* and *flu-2* loci: a kynurenine hydroxylase, R07B7.5, and a kynureninase C15H9.7, respectively (Altschul et al., 1990; Kanehisa, 2012). Other predicted kynurenine pathway genes are also present in *C. elegans* (van der Goot and Nollen, 2013).

In *Drosophila* genetic and pharmacological inhibition of the kynurenine pathway enzyme tryptophan 2,3-dioxygenase (TDO) extends longevity (Oxenkrug, 2010; Oxenkrug et al., 2011). This suggests that kynurenines may contribute to pathologies of aging; however, whether this is true in *C. elegans* remains uncertain. Here RNAi knock-down of *tdo-2* reduced the toxicity of α-synuclein aggregation in a Parkinson's disease model, and increased lifespan (van der Goot et al., 2012). However, these effects proved to be caused by increased levels of tryptophan rather than altered levels of kynurenines (van der Goot et al., 2012; for a detailed review of the kynurenine pathway and aging

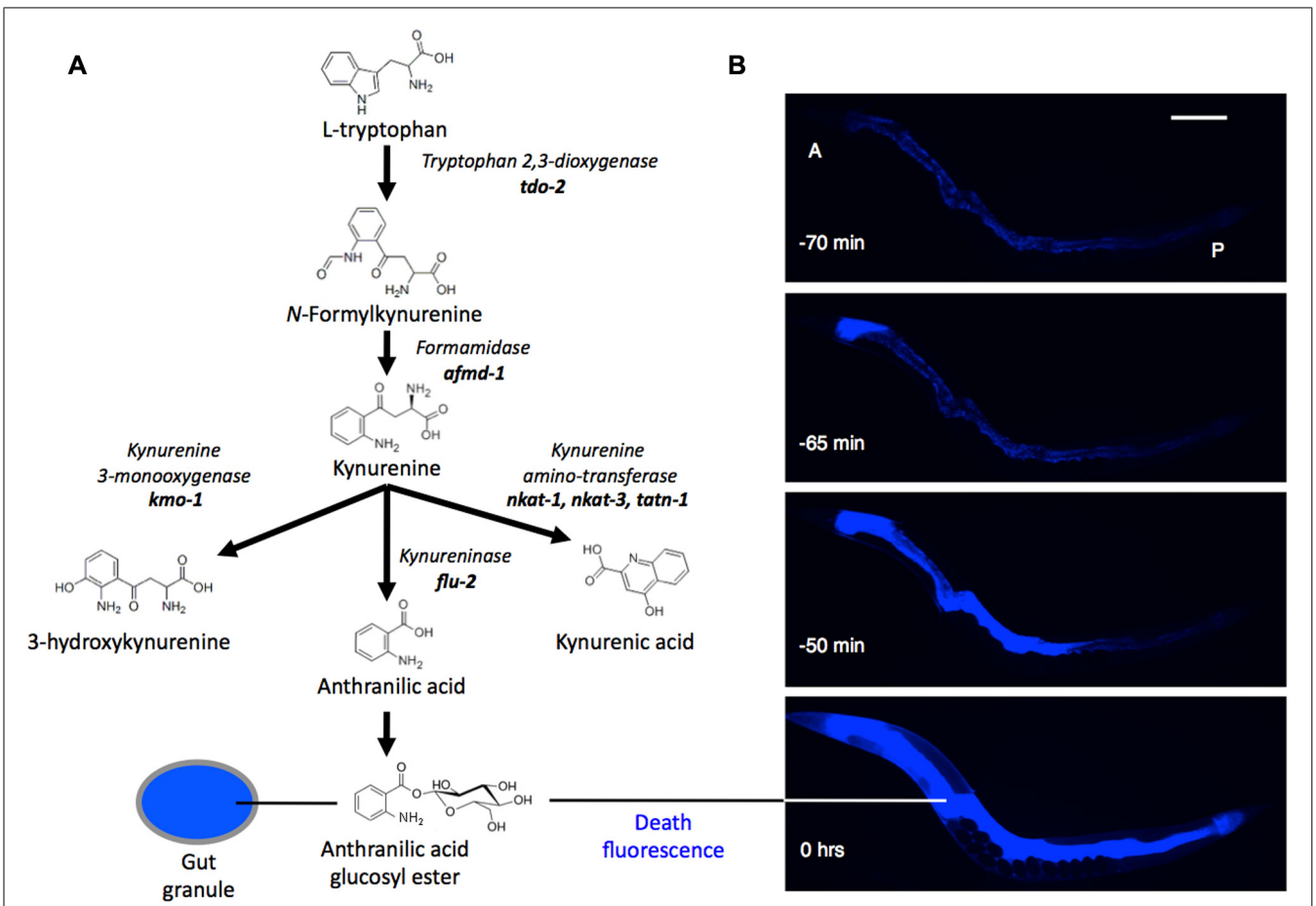

FIGURE 1 | (A) Synthesis of anthranilic acid by the kynurenine pathway. **(B)** Death fluorescence in young adult *C. elegans* killed with a heated wire (DAPI filter). During death fluorescence the pattern of fluorescence changes from punctate (issuing from gut granules) to diffuse, and much brighter. A, P, anterior and posterior ends of intestine. Time is relative to peak fluorescence. Scale bar, 200 μm.

see van der Goot and Nollen, 2013). *tdo-2* RNAi also abrogates gut granule fluorescence in the worm (Coburn et al., 2013).

Kynurenines also play a startling role in the biology of death in *C. elegans*. As they die, worms emit a dramatic burst of blue AA fluorescence (Coburn et al., 2013; **Figure 1B**). This *death fluorescence* typically occurs in an anterior to posterior wave that courses along the intestine, and is seen in both young worms subjected to lethal injury, and worms dying peacefully of old age. Death fluorescence is a somewhat eerie phenomenon in that it renders visible the passage of death through the semi-transparent body of the worm as a spectral blue glow.

Death fluorescence is promoted by the calpain–cathepsin necrotic cell death cascade. In this cascade, intracellular Ca^{2+} levels rise, activating Ca^{2+}-dependent calpains (cysteine proteases; Yamashima et al., 1996). These cause lysosomal lysis, leading to cytosolic acidosis and the destructive release of lysosomal cathepsin proteases (Yamashima and Oikawa, 2009). Mutational attenuation of this cascade often reduces death fluorescence (Coburn et al., 2013). Moreover, the intercellular propagation of death fluorescence (and, probably, necrosis) is dependent upon the innexin gap junction INX-16, reminiscent of the spread of excitotoxic neuronal death from one cell to another in mammals. How exactly the necrotic cascade leads to increased AA fluorescence remains unclear, but one possibility is that it reflects AA fluorescence dequenching as it is released from the gut granules upon organellar lysis.

POSSIBLE FUNCTIONS OF ANTHRANILATES AND GUT GRANULES IN *C. elegans*

The significance of AA concentrated within gut granules remains unclear. One possibility is that glycosylation of AA contributes to its accumulation; in *Arabidopsis*, glycosylation by UDP-glucosyltransferases promotes AA accumulation by increasing compound stability (Quiel and Bender, 2003). Regarding function, one possibility is that AA serves a protective role. In mammals kynurenines can contribute to immune function (Munn et al., 1998; Fallarino et al., 2002; Piscianz et al., 2011). Moreover, AA can inhibit growth of bacterial pathogens, e.g., *Legionella pneumophila* (Sasaki et al., 2012). Thus, AA might have antibiotic properties in *C. elegans*, in which case gut granules could serve as a store of anti-bacterial agents in the event of pathogen attack. This suggests a broader role for gut granules: that of chemical weapons depots for *C. elegans* in their war against the diverse pathogens that beset them in their natural environment (Felix and Braendle, 2010). This could also explain the presence of gut granules in the intestine, the site most likely to experience pathogenic invasion in *C. elegans* (Hodgkin and Partridge, 2008). Another possibility, suggested by similarities between gut granules and melanosomes, is that they are photoprotective. AA fluorescence (peak $\lambda_{ex}/\lambda_{em}$ 340 nm/430 nm) entails the conversion of damaging UV light to relatively harmless visible light, and so may protect against UV damage.

The large size of gut granules relative to ordinary lysosomes is consistent with function as a storage organelle. Moreover, gut granules are the major site of storage of zinc in the worm (Roh et al., 2012). Interestingly, when zinc levels are high, gut granule morphology changes, becoming bilobed, including an apparently non-acidic compartment in which zinc is concentrated. How distribution of zinc and AA compares in such bilobed gut granules remains to be established. It is also notable that both metal toxicity and kynurenines are determinants of neurodegenerative disease. Gut granules also stain with the lipid staining vital dye Nile red; however, results of careful analysis imply that this does not reflect the presence of lipid within gut granules (O'Rourke et al., 2009).

Ultimately, the role in *C. elegans* biology of gut granules and the AAs they contain remains obscure and a topic for future investigation. But we now know at least that the fluorescence of these prominent organelles issues from AA glucosyl esters, rather than lipofuscin – removing one reason for believing that worm aging is caused by accumulation of molecular damage, and opening the way for alternatively theories (Gems and de la Guardia, 2012). And we know that gut granule decay contributes to a wave of intestinal necrosis accompanied by a burst of blue anthranilate fluorescence, which serves as a useful marker for organismal death in *C. elegans*.

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What is the proximal cause of aging?

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In spite of exciting new insights into regulatory mechanisms that modulate the aging process, the proximal cause of aging remains one of the unsolved big problems in biology. An evolutionary analysis of aging provides a helpful theoretical framework by establishing boundary conditions on possible mechanisms of aging. The fundamental insight is that the force of natural selection diminishes with age (Medawar, 1952; Comfort, 1956; Williams, 1957). This does not preclude senescence (age-related decrease in individual fitness) from occurring in natural populations (Nussey et al., 2012). Senescence can develop because some genes have non-separable, but typically different or opposite, functions in reproductive-age and in old individuals (antagonistic pleiotropy; Williams, 1957). Such genes, selected according to their "youthful" function, may thus impose a distinct senescent phenotype in old age. In general, however, unless a controversial formulation of group selection (Nowak et al., 2010; Wilson, 2012) is invoked, traits that would become manifest only in old age cannot evolve. This precludes the evolutionary emergence of aging programs, which have been sometimes postulated to exist (Goldsmith, 2012; Mitteldorf, 2012) in analogy to developmental and other biological programs. (By the same token, selective pressure that diminishes with age would also prevent extreme longevity from evolving, if "extreme" denotes a potential life span much longer than that imposed by extrinsic mortality in a given environment.) This and other arguments against the existence of an aging program have been discussed previously (e.g., Zimniak, 2008; Kirkwood and Melov, 2011).

The evolutionary perspective sketched out above does not specify the mechanisms that underlie aging, but it helps to narrow down the possibilities. As already discussed, an evolved deterministic aging program can be ruled out, perhaps with the exception of specific niche situations. In the absence of adaptive life-curtailing processes driven by a putative aging program, we are left with untargeted pro-aging, destabilizing phenomena which, in principle, may range from purely stochastic to side-effects of "legitimate" biochemical pathways. These destabilizing forces are counteracted by evolved, and genetically controlled, longevity assurance (or repair/maintenance) processes. The interplay of these countervailing forces determines the life span. While I have previously presented my detailed interpretation of this model (Zimniak, 2008, 2011), its central tenets bear repeating: (a) the destabilizing processes that drive aging are neither evolved nor adaptive; (b) in contrast, longevity assurance mechanisms are under genetic control; (c) together, these two opposing forces determine life span; (d) the average life span of a species is set by evolving longevity assurance mechanisms so as to optimize reproductive success under environmental conditions typical for that species.

It is important to stress that the above model allows for longevity assurance, and thus life span, being acutely regulated at the level of an organism via sensory pathways such as insulin or mTOR signaling, as long as the resulting life expectancy optimizes reproductive success under particular environmental conditions. In other words, reproductively optimal life spans evolved for different environmental situations via adaptive selection of distinct set points of anti-aging repair and/or maintenance processes. Thus, the model is fully consistent with the disposable soma theory (Kirkwood, 2005 and references therein).

What exactly, in molecular terms, are the maintenance mechanisms able to extend life? In addition to its intellectual interest, this question has considerable practical ramifications, including the holy grail of prolonging human life. A good way to approach this question is to identify first the life-curtailing destabilizing factors that are the proximal cause of aging. A focus on destabilizing factors does not imply that longevity assurance is somehow less important. As already discussed, both parts of the equation are equally significant in determining life span. However, longevity assurance mechanisms evolved in response to destabilizing factors, so defining the latter is a good point to start.

Destabilization is often thought of as a purely physical or chemical phenomenon, epitomized by the infamous (because of its incompleteness) comparison of an aging organism to a rusting car. Most emphatically, a biological system is subject to all laws of physics and will deteriorate just as a car, but this is only one of several processes relevant to a living organism. This, however, is a topic for another discussion. In the context of the present article it is important to note that destabilizing factors include, in addition to physico-chemical, also biological processes. These processes did not evolve to drive aging, at least in general. However, some side-effects of otherwise homeostatic biological reactions clearly contribute to aging (Zimniak, 2011).

Historically, the first types of reactions proposed to destabilize biological systems and to cause aging were free radical and oxidative processes (Pearl, 1928; Harman, 1956). As a consequence, even in today's literature, molecular damage is often assumed to be limited to oxidative damage, and the two terms are used interchangeably. This is unfortunate because a wide variety of

errors, on scales ranging from molecular through microscopic to macroscopic, is likely to be relevant to aging.

In addition to the already mentioned oxidative and free radical damage, possible destabilizing factors are thought to include entropy-driven loss of organization inevitable in any system that is far from thermodynamic equilibrium, stochastic events inherent in biological processes which often involve relatively small numbers of molecules, modifications of essential macromolecules by reactive xenobiotics as well as by intermediary metabolites, including electrophiles derived mostly from lipid peroxidation, and protein misfolding and aggregation. Because of space limitations, I must refer the reader to my previous reviews of these topics (Zimniak, 2008, 2011) for additional details and references, as well as for a discussion of longevity assurance mechanisms able to offset the various types of damage. Here, I would like to focus on a new and radical development in the aging field, namely an attempt to falsify the above model of aging and to replace it by a new paradigm.

In a series of papers (e.g., Blagosklonny, 2006, 2007a,b, 2008, 2009, 2010a,b, 2011a,b, 2012; Blagosklonny and Hall, 2009), Mikhail Blagosklonny proposed that a novel conceptual framework is necessary to understand aging. According to the new theory, which is gaining acceptance of leading researchers in the field (Gems and de la Guardia, 2012), aging is driven not by untargeted molecular damage, but by hyperfunction and hypertrophy secondary to an inappropriate continuation into adulthood of developmental programs, in particular mTOR signaling. In this theory, mTOR, which is adaptive during growth, would become a quasi-program with detrimental consequences during adulthood, turning the model into an example of antagonistic pleiotropy (Blagosklonny, 2010b). The failure to terminate the quasi-program in adulthood could be attributed to the impossibility of evolving an off-switch in the face of a selective pressure that diminishes with age. It should be noted that, independently of hyperfunction, accumulation of molecular damage would still occur, as required by laws of physics and chemistry, but such damage would be irrelevant to aging because death triggered by hyperfunction-related pathologies would precede any life-curtailing effects of molecular damage (Blagosklonny, 2012, and other works by this author). A schematic depiction of the hyperfunction theory is shown in **Figure 1A**, in comparison with the molecular damage theory of aging (**Figure 1B**). A hypertrophy-based hypothesis has been also proposed to explain the replicative life span of yeast (Bilinski and Bartosz, 2006; Bilinski et al., 2012).

The new perspective provided by the hyperfunction theory of aging is attractive because of recently identified deficiencies of more conventional models, especially of the oxidative stress theory. Specifically, it has been pointed out that the expected correlation between antioxidant status and longevity is not consistently observed in many experimental settings (e.g., Gems and Doonan, 2009; Perez et al., 2009; Pun et al., 2009). This may merely reflect the need for a more nuanced understanding of the chemistry and biology of oxidative stress (Gutteridge and Halliwell, 2010; Murphy et al., 2011; Halliwell, 2012). In addition, the oxidative damage theory may require modifications or refinement. For example, it has been proposed that oxidative damage may limit life span in the wild but not under protected laboratory conditions, or that oxidative stress is relevant to health span but not to life span (Salmon et al., 2010). The hyperfunction theory sidesteps these questions by declaring all molecular damage to be irrelevant to aging. The claim of hyperfunction to exclusivity is, however, worrisome for several reasons, elaborated below.

As illustrated in **Figure 1A**, the hyperfunction model postulates a causal chain leading from hypertrophy to macroscopic pathologies (organ damage) and to death (Blagosklonny, 2012). However, I would hesitate to accept that catastrophic events, such as a stroke in a middle-aged person or sepsis in an otherwise healthy individual, are aging. Rather, loss of homeostasis, i.e., aging, can lower cell/tissue robustness and precipitate catastrophic events (**Figure 1B**). If so, the hyperfunction model may be better at explaining mortality than aging. This may be considered an artificial distinction; however, it would be difficult to identify catastrophic death events in, for example, bacteria, organisms that also age (Rang et al., 2011).

Another criticism of the hyperfunction model may appear trivial. It has been claimed that atrophy, a classical sign of aging-related decline, can be in fact secondary to an initial hyperfunction and hypertrophy (Blagosklonny, 2012). At the

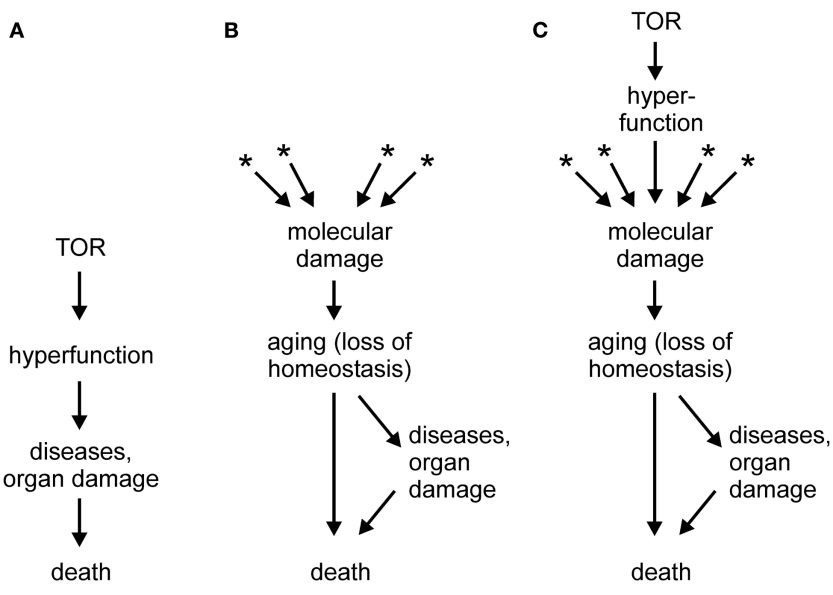

FIGURE 1 | (A) Scheme of the hyperactivity theory of aging (based on Blagosklonny, 2011a; Gems and de la Guardia, 2012). **(B)** Scheme of the molecular damage accumulation theory of aging; asterisks denote multiple sources of molecular damage, such as electrophilic stress, oxidative stress, protein misfolding, stochastic events, and others. **(C)** Scheme of a generalized molecular damage accumulation theory of aging which includes hyperfunction/hypertrophy as a source of destabilizing molecular damage which acts in addition to other sources of damage. See text for more details.

risk of sounding petty, I would counter that with a sufficiently broad definition, almost any abnormality could be subsumed under the term hyperfunction. In fact, increased ROS production is an example of hyperfunction. However, the problem goes beyond semantics and touches on mechanism. According to the paper quoted above (Blagosklonny, 2012), hyperfunction results in hypertrophy and, eventually, in cell failure or death, i.e., atrophy. But, what is the mechanism of this chain of events? Cells and organisms are ultimately chemical systems; therefore, they are susceptible to chemical (or physical) interference. In itself, a mere increase in the abundance of an overproduced component should not matter. However, if that component interacts with normal cell constituents and interferes with their function, it causes damage – molecular damage – which may kill the cell. For example, an overproduced ligand may over stimulate or desensitize a receptor, and an overabundant protein may aggregate and interfere with intracellular trafficking, or co-precipitate with and thus withdraw essential cell constituents. Macroscopic hypertrophy can have molecular sequelae as well; for example, obesity results in a pro-inflammatory and pro-oxidant state (Grimsrud et al., 2007; Holguin and Fitzpatrick, 2010). From this point of view, hyperfunction is one of several sources of molecular damage, on equal footing with reactive metabolites, toxicants, ROS, electrophiles, stochastic events, and many others (**Figure 1C**).

Whereas aging is likely to have multiple contributing causes (Zimniak, 2008; Gladyshev, 2012), one of the looming questions in gerontology is whether any one type of damage predominates, and if so, which. This question is as important as it is difficult to answer, in part because many seemingly distinct experimental interventions lead to overlapping or identical molecular perturbations of a biological system. Among the contenders, oxidative damage has lost much of its appeal, perhaps prematurely, whereas protein misfolding/aggregation is gaining support (Morimoto and Cuervo, 2009; Morimoto et al., 2011). Even if hyperfunction turns out to be the predominant driver of aging, I propose that it does so by causing molecular damage, rather than by killing organisms through triggering catastrophic organ failures. Thus, regardless of its nature, molecular damage remains the proximal cause of aging (**Figure 1C**).

The advent of the hyperfunction theory of aging has been compared to the replacement of the geocentric with the heliocentric worldview (Gems and de la Guardia, 2012). Within this rather grand conceptual framework, I may be seen as an old-timer who desperately tries to salvage a doomed theory by piling up epicycles. Perhaps so – time will tell. Meanwhile, I would like to invoke another old-timer, William of Ockham. Wielding his razor, I propose that, if hyperfunction is treated as a destabilizing process that generates molecular damage, all experimental evidence can be accommodated by the generalized molecular damage theory of aging, without the need to establish a new paradigm.

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Epigenetic drugs: a novel anti-aging strategy?

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Aging is a natural phenomenon which is peculiar to all living organisms. However, it is believed by some scientists that senescence could be postponed or prevented by certain approaches. Some dietary ingredients and supplements have been suggested to have anti-aging and life-extending effects. Natural and synthetic dietary supplements including anti-oxidants, vitamins, and hormones are among the most popular products on the market, even without solid scientific evidence supporting their efficacy (Olshansky et al., 2002). Recently, a number of synthetic drugs used for various therapeutic applications have also been assumed to have anti-aging potential (Kapoor et al., 2009). In most cases, however, the high expectations for these drugs were not fulfilled. The effects of several substances, e.g., anti-oxidants, have been supported by data obtained in animal models, but when carefully controlled human trials have been conducted, questions about the efficacy and safety of these substances have emerged (Jerome-Morais et al., 2011). Excessive intake of anti-oxidants, vitamins, or hormones is known to destroy delicate control mechanisms of homeostatic balance, and it is not yet clear under which conditions, if any, they may have a long-term beneficial impact on life expectancy in humans (Cochemé and Murphy, 2010). Given this situation, it is necessary to increase the choice of chemical compounds which have the potential to positively affect longevity. In this Opinion, we present arguments that the development of specific drugs which target epigenetic pathways could be a highly promising anti-aging strategy.

Epigenetic factors including DNA methylation, histone modifications, and alteration in microRNA expression play key roles in controlling changes in gene expression and genomic instability throughout the human lifespan. Epigenetic modifications are finely balanced and highly reversible in normal tissues. However, they may be imbalanced and heritable in tumor and other abnormal cells. Epigenetic dysregulation has a causal effect on age-associated disorders including cancer, atherosclerosis, type 2 diabetes, neurodegenerative and psychiatric diseases, and the decline in immune response (Berdasco and Esteller, 2012). There is increasing evidence to indicate that epigenetic mechanisms are intimately involved in synaptic plasticity and are essential for learning and memory. Dysfunction of epigenetic gene expression in the brain may be involved in neurodegenerative and psychiatric diseases (Sananbenesi and Fischer, 2009; Berdasco and Esteller, 2012).

Therefore, there is nothing surprising in the fact that, at present, great expectations in the treatment of diseases are associated with the use of so-called "epigenetic drugs" that can modulate the activity of enzymes capable of causing epigenetic changes. In this context, members of the superfamily of histone deacetylases (HDACs) comprising HDAC 1–11 and sirtuins (SIRT) 1–7 are currently the focus of significant interest (de Oliveira et al., 2012). The potential reversibility of epigenetic aberrations has made them attractive targets for therapeutic intervention. Several drugs which target the epigenetic machinery, such as HDAC modulators, mainly inhibitors, have recently been used in human clinical trials, and some have been recommended for the treatment of age-associated diseases (Gryder et al., 2012; Price et al., 2012; Sato, 2012).

Above all, HDAC inhibitors are considered promising anti-cancer therapeutics (Karagiannis and Maulik, 2012). Recently, HDAC inhibitors have shown anti-tumor activity against certain hematological malignancies; their therapeutic potential in solid tumors remains more uncertain (Gryder et al., 2012). Vorinostat and romidepsin have recently been approved for the treatment of relapsed or refractory T-cell lymphoma in the USA and Japan (Price et al., 2012; Sato, 2012). Numerous studies have identified HDAC inhibitors as candidate drugs for the treatment of neurodegenerative disorders. These agents can ameliorate deficits in synaptic plasticity, cognition, and stress-related behaviors in a wide range of neurologic and psychiatric disorders including Huntington's disease, Parkinson's disease, Alzheimer disease, anxiety and mood disorders, Rubinstein–Taybi syndrome, and Rett syndrome (Abel and Zukin, 2008; Xu et al., 2011). HDAC inhibitors are also a new class of immunomodulatory and anti-inflammatory therapeutics (Akimova et al., 2012; Cantley et al., 2012; Licciardi and Karagiannis, 2012). Recent data identified an essential role for HDAC inhibitors in regulation of the expression of innate immune genes and host defenses against microbial pathogens (Roger et al., 2011). The anti-cancer effects of HDAC inhibitors may also be linked to long-term stimulation of the immune response (Leggatt and Gabrielli, 2012).

In recent years, high hopes have been placed on the therapeutic potential of modulators of NAD-dependent class III histone deacetylases (sirtuins). Sirtuins are becoming increasingly recognized as attractive novel therapeutic targets for metabolic, cardiovascular and neurodegenerative diseases, and cancer (Huber and Superti-Furga, 2011; Carafa et al., 2012). Both activation and inhibition of sirtuins may be useful for preventing and treating age-related diseases, depending on the pathological condition, and the target

tissue (Mahajan et al., 2011). A number of sirtuin inhibitors demonstrated anti-proliferative effects in cell assays as well as in mouse tumor models, thus suggesting a possible role in cancer therapy. The selective inhibitor of SIRT2, AGK-2, has been reported to have protective effects against Parkinson's disease, and resveratrol and other sirtuin activators can be useful in the treatment of Alzheimer's disease (Mai, 2010).

HDACs are global transcriptional regulators; they affect gene expression by deacetylation of not only histones but also non-histone proteins, including transcription factors, and are involved in the regulation of signal transduction, cell cycle and cell growth, DNA damage response, apoptosis, and differentiation. Sirtuins have been implicated in determining the balance between apoptosis, cell survival, and cell proliferation, and are also involved in the regulation of metabolism and stress, two key factors that affect the process of aging (Satoh et al., 2011). The therapeutic effects of HDAC inhibitors are based on their ability to affect the transcription of various genes; in particular, anti-tumor effects can be attributed to the transcriptional reactivation of silent tumor suppressor genes and the transcriptional repression of proto-oncogenes (Boumber and Issa, 2011). Overall, as a major mechanism of transcriptional regulation, protein acetylation is a key controller of many physiological processes essential for the maintenance of homeostasis and a healthy lifespan. Consequently, it is believed that the development of specific drugs which target HDAC activity could be a highly promising anti-aging strategy.

Indeed, the efficiency of promising anti-aging dietary compounds, such as resveratrol and curcumin, can by explained, at least partly, by their ability to modulate gene expression via interaction with HDACs. For example, resveratrol has been shown to inhibit HDAC activity in a concentration-dependent manner (Dayangaç-Erden et al., 2009). Albeit the action of resveratrol is complex, the resveratrol-induced SIRT1 activation is believed to be the main reason for its anti-aging effect (Price et al., 2012). Some of the biological activities of curcumin, such as its anti-oxidative, anti-inflammatory, anti-cancer, chemopreventive, and anti-neurodegenerative effects, may be due to its capability to modulate gene expression through its interaction with HDACs, histone acetyltransferases, DNA methyltransferases, and microRNAs (Reuter et al., 2011). For example, in the study by Lee et al. (2011) curcumin-induced apoptosis and cell cycle arrest at the G2/M phase in medulloblastoma cells were accompanied by reduced HDAC4 expression.

Among the chemicals affecting HDAC activity, HDAC inhibitors are obvious candidates for the role of anti-aging agents. Indeed, a decrease in HDAC activity would lead to increased transcription of many genes. With age, the transcription profiles of different genes change in different ways. However, for the majority of genes, primarily metabolic and biosynthetic genes, a decline in transcription is observed in old age (Lee et al., 1999; Seroude et al., 2002). There is hope that HDAC inhibition will promote temporal homeostasis and delay aging due to preservation of the level of transcription, which is characteristic of the young, in aging organisms. In addition, HDAC inhibition may result in up-regulation of inflammatory response and stress response genes—changes that are usually associated with increased longevity (Vermeulen and Loeschcke, 2007; Kourtis and Tavernarakis, 2011).

It still remains an enigma how fine-tuning of the expression of different genes necessary for normal functioning of an organism could be provided in this case. An imbalance in HDAC activity, similar to an imbalance in the supply of anti-oxidants, vitamins, or hormones, can destroy delicate control mechanisms providing homeostasis. However, it is worth mentioning that epigenetic regulation of gene transcription is a highly coordinated process mediated by central regulatory mechanisms, which can take over functions necessary for a proper orchestration of epigenetic interventions. It makes epigenetics an attractive candidate molecular mechanism suited for the control of highly integrated biological process such as aging (Vaiserman, 2011). Alternatively, one can expect that stage-, tissue-, and HDAC-specific inhibitors will be developed. A similar approach is now being implemented with respect to protein kinase inhibitors, aimed at treating various diseases, especially cancer (Lamba and Ghosh, 2012). Certainly, use of epigenetic drugs can have multiple and complex effects on different systems and tissues. For example, one side effect of modulation of epigenetic processes aimed at human life extension could be reduced reproductive activity. However, the reproductive capabilities of modern people can in fact be realized only very partially due to social (but not biological!) reasons. Therefore, such price for long life span does not seem too high.

Notwithstanding all doubts, in recent years, experimental research has emerged on the life-extending potential of synthetic HDAC inhibitors. A substantial increase in both mean and maximum survival by up to 30–50% without diminution of locomotor activity, resistance to stress, or reproductive ability was observed by feeding *Drosophila melanogaster* the HDAC inhibitor, PBA (4-phenylbutyrate), throughout adulthood (Kang et al., 2002). Flies fed PBA showed a global increase in histone acetylation accompanied by a dramatically altered pattern of gene expression of numerous genes, including genes putatively involved in enhancing longevity: superoxide dismutase, elongation factor 1, glutathione S-transferase, cytochrome P450, and three chaperones. All these genes were induced by PBA. Tao et al. (2004) found that the HDAC inhibitor, trichostatin A (TSA), significantly extended the lifespan of flies. Furthermore, TSA promoted *hsp22* gene transcription. In another study conducted by Zhao et al. (2005), the HDAC inhibitors, TSA, and sodium butyrate, were also shown to significantly extend the lifespan and promote *hsp22* and *hsp70* expression in *Drosophila*. In our recent study, flies fed sodium butyrate at concentrations of 10 and 20 mmol/l throughout both pre-adult and adult stages demonstrated significant increases in mean lifespan in both males and females compared with controls; moreover, treatment with 20 and 40 mmol/l sodium butyrate during the adult stage only resulted in a statistically significant increase in male (but not female) lifespan (Vaiserman et al., 2012). The exact molecular mechanisms of these positive effects remain to be elucidated. In particular, it would be good

to know whether anti-aging effects of epigenetic drugs in model animals have the same molecular basis as their therapeutic effects in humans.

In conclusion, understanding the molecular mechanisms underlying the protective role of HDAC inhibitors and other modulators of epigenetic processes could bring us closer to the development of novel drug targets for age-associated chronic diseases. In our opinion, this approach may also provide a new way for the development of efficient anti-aging treatments.

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The genetic mechanisms of the influence of the light regime on the lifespan of *Drosophila melanogaster*

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Light is a crucial environmental factor influencing living organisms during their whole lives. It contributes to the regulation of circadian rhythms, affects growth, metabolic rate, locomotor activity and reproduction. The mechanisms of the influence of light on longevity are poorly understood.

We have suggested that there are two relatively independent genetic mechanisms of the influence of light on lifespan (Moskalev and Malysheva, 2010). The first mechanism is related to the damaging effects of light, leading to a reduced lifespan (**Figure 1A**), the second mechanism is related to the influence of the dark as a mild stressor, which can stimulate the body's defense system and lead to an increased lifespan (**Figure 1B**).

It is known that an increase in the photoperiod usually decreases the lifespan of experimental animals (Massie and Whitney, 1991; Massie et al., 1993; Sheeba et al., 2000; Majercak, 2002; Anisimov et al., 2004; Vinogradova et al., 2009). An increase in day length promotes a higher level of metabolism due to the intensification of locomotor activity and changes in body temperature of *Drosophila* (Sheeba et al., 2000, 2002). An increase in metabolic rate, in turn, leads to the additional formation of toxic by-products—free radicals (Massie and Whitney, 1991; Helfand and Rogina, 2003), damaging the cell's mitochondrial and nuclear DNA, membranes and proteins (Le Bourg, 2001), and as a result this can lead to accelerated aging and a reduced lifespan.

In our works (Moskalev et al., 2006, 2008) we investigated the strain *Drosophila melanogaster* with the defective cytoplasmic superoxide dismutase gene (*Sod*) which has only 36.7% of the normal activity of the *Cu/Zn Sod* enzyme (Phillips et al., 1995) and the strain with the defective *mutagen-sensitive 210* gene (*mus210*), protein-coding, involved in nucleotide-excision repair (homolog of the XPC protein in mammals) (Isaenko et al., 1994). This gene group contributes directly to the elimination of oxidative damage—through free radical detoxication (gene *Sod*) and DNA repair (gene *mus210*). It has been shown that mutations in genes responsible for the removal of oxidative damage can alter the lifespan of animal models. In particular, in *Drosophila* with zero Cu/Zn-superoxide dismutase activity, the lifespan is reduced by 80% (Staveley et al., 1990), and injecting into the short-lived strain *Drosophila* genome additional superoxide dismutase and catalase genes, basic antiradical protection enzymes, resulted in a lifespan increase (Orr and Sohal, 1994, 2003). *Sod* overexpression in *Drosophila's* motor neurons only prolonged the lifespan by 40% and made *Drosophila* more resistant to agents stimulating active oxygen formation such as ionizing radiation and paraquat (Mattson et al., 2002). We also know that the ability to repair oxidized DNA bases decreases in XPC-deficient cells (D'Errico et al., 2006). In our experiments, the median lifespan of flies with an impaired *Sod* gene function also decreased compared with the lifespan of *Canton-S* wild-type flies by 41% for males and 38% for females (Moskalev et al., 2006). In the strain with the defective *mus210* gene the median lifespan was reduced by 48% in males and 21% females compared with wild-type flies (Moskalev et al., 2006). We hypothesized that in strains with a dysfunction of *Sod* and *mus210* genes there will be a significant reduction in lifespan if subject to lighting round the clock, as compared to the wild-type strain. According to our results, in strains with *Sod* and *mus210* gene mutations, there was a significant increase in the difference between the median and maximum lifespan in the dark and in the light compared with the *Canton-S* wild-type strain (Moskalev et al., 2006). In the strain with a free radical detoxication defect, the gap in the median lifespan in the dark and in the light was 36% for males and 14% for females; the maximum lifespan was 11% for males and 24% for females. At the same time, the addition of the antioxidant melatonin into the food for *Drosophila* with this defect reduced this variation (Moskalev et al., 2008). In the strain with the DNA repair defect, the gap in the median lifespan in the dark and in the light was 11% for males and 23% for females; and the maximum lifespan was 21% for males and 9% for females. The lifespan parameters of wild-type flies varied insignificantly (within 0–7%). Thus, our results confirm our hypothesis about the importance of detoxification and DNA repair genes in the regulation of lifespan under a changing day length (Moskalev et al., 2006, 2008).

It is known that in the regulation of the oxidative stress response and lifespan a key role is played by sirtuin family proteins (Guarente and Kenyon, 2000; Balaban et al., 2005). In response to stress sirtuins deacylate histones and various transcription factors (including p53, FOXO, HSPs), activating the expression of the stress response genes and inactivating and inhibiting apoptosis, thus contributing to the cell survival rate and lifespan increase (Tanno et al., 2007; Niedernhofer and Robbins, 2008). It is known that sirtuins play a key role in the regulation of the aging rate and longevity. In particular,

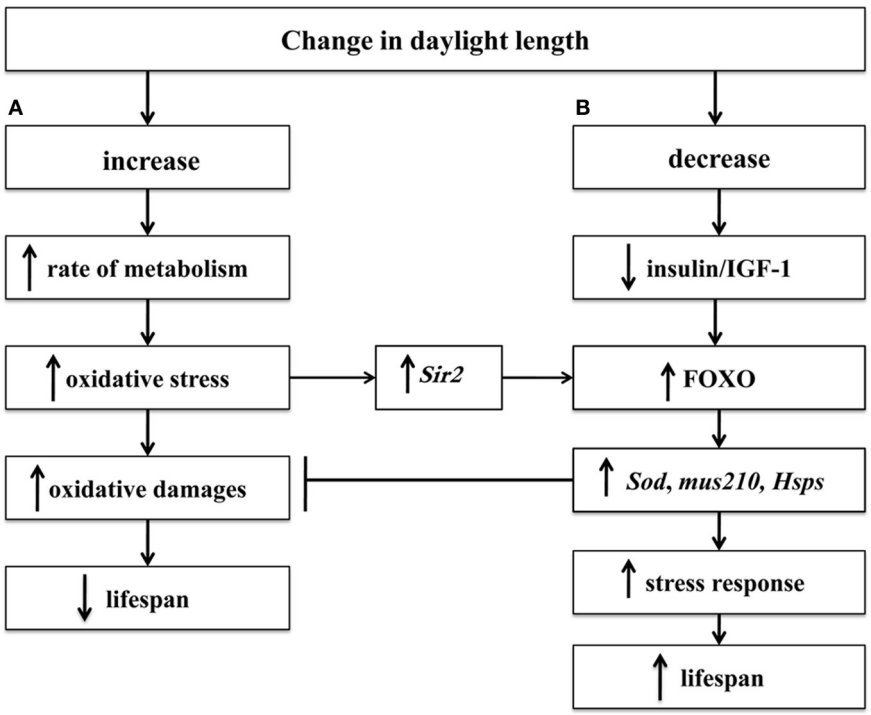

FIGURE 1 | The mechanism of the influence of the light regime on lifespan: metabolism intensification in light conditions (A) and FOXO-dependent increase of lifespan in dark conditions (B). Symbol: →, induction; ⊣, inhibition; ↑, increase; ↓, decrease.

the ubiquitous suppression of expression of the *dSir2* and two *dSir2*-like genes (*CG5085* и *CG6284*) by means of RNA interference is lethal, and the suppression of gene expression in *Drosophila* neurons only reduces the lifespan, while at the same time, increasing the activity of sirtuins in yeast, worms and flies prolongs their lifespan (Kusama et al., 2006; Russell and Kahn, 2007; Niedernhofer and Robbins, 2008). For example, transgenic expression of *sir-2.1* prolongs the lifespan of nematodes by 50%, and overexpression of the *dsir2* gene in the nerve tissue of *Drosophila* at the larval stage prolongs the median lifespan of males and females by 20% and 52%, respectively (Rogina and Helfand, 2004). In our studies *dSir2* gene mutation led to a significant reduction in lifespan compared with the wild-type strain. The median lifespan of males and females of the strain with a deletion of the *Sir2* gene was lower than the median lifespan of the control strain by 19% in males and 33% in females (Moskalev and Malysheva, 2010). The role of sirtuins in the change of lifespan under the influence of different lighting regimes has not previously been studied. We investigated the *Drosophila* strain homozygous with deletion of the *dSir2* gene. It has been shown that the difference in the median lifespan in the dark and light was 24–33%, while in the control wild-type strain the difference in lifespan was 3–17%. Thus, our data point to the important role of the *Sir2* gene in the regulation of lifespan under a changing day length (Moskalev and Malysheva, 2010).

Equally important in the cellular stress response are heat shock proteins (Hsps) involved in the process of repair and proteolysis of damaged proteins (Hunt et al., 2004; Arya et al., 2007). Besides, higher Hsps activity is associated with the longer life in various model animals (Morrow et al., 2004; Kimura et al., 2006). The *Hsp70* gene expression intensifies after oxidative damage (Guo et al., 2007; Soti and Csermely, 2007), which contributes to the improvement of the redox status of cells and increased activity of antioxidative enzymes (Guo et al., 2007). Moreover, in *Drosophila* with mutations in genes encoding catalase and Cu, Zn-Sod, a decrease of the *Hsp70* induction period is observed with age, which provides evidence of *Hsp70* involvement in the response to oxidative stress (Landis et al., 2004). We have suggested that the *Hsp70* gene deletions in *Drosophila* can reduce lifespan in light conditions compared to dark conditions (Moskalev and Malysheva, 2010). Notwithstanding the fact that the median lifespan in the light and in the dark of the strains with *Hsp70* gene mutations changed slightly, under the exposure to light, the maximum lifespan dropped significantly (by 7–29%) (Moskalev and Malysheva, 2010).

Thus, the above evidence supports the fact that the lifespan control mechanism in varying daylight conditions is related to the damaging effects of the additional lighting.

Recently, we proposed a hypothesis which suggests that light affects the animal lifespan via neuroendocrine regulatory networks (Moskalev and Malysheva, 2009). According to this hypothesis, in response to the shortening of the photoperiod the activity of the insulin/IGF-1 signaling is decreased, and stress response machinery, including FOXO transcription factor is activated. FOXO plays a crucial

role in maintaining the balance between growth and reproduction, on the one hand, and stress resistance and lifespan, on the other hand (Calnan and Brunet, 2008). It is known that FOXO mediates the response to oxidative and other stresses, which is often connected with a prolonged lifespan (Giannakou and Partridge, 2004; Vogt et al., 2005; Lam et al., 2006; Honda et al., 2010). It was shown that permanent overexpression of *dFOXO* in the fat body of an adult *Drosophila* reduces the mortality rate, stimulates the resistance to the free radical inducing factor—paraquat and prolongs the lifespan of flies (Giannakou and Partridge, 2004; Giannakou et al., 2007), whereas any defects in the functioning of *dFOXO* raises sensitivity to oxidative stress and decreases the lifespan (Junger et al., 2003). We have suggested that FOXO plays the key role in the increased lifespan when in the dark. In this case, the experimental reduction in the activity of this gene should eliminate the difference between the lifespan in the dark and in the light. Flies with reduced *dFOXO* gene function were obtained by mating flies of two strains containing the $dFOXO^{21}$ and $dFOXO^{25}$ alleles. In $dFOXO^{21}/dFOXO^{25}$ transheterozygotes the survival curves in the dark and under normal lighting conditions did not differ significantly in three out of four cases (Moskalev and Malysheva, 2010). This result points to the connection between the longevity of fruit flies in the dark and the activity of the FOXO transcription factor. Apparently, the minimum differences in survival of flies with reduced FOXO function in the dark and under normal lighting conditions remained because the induction of FOXO-dependent stress resistance mechanisms can occur not only in response to the suppression of insulin-like peptides in the dark, but also in response to oxidative stress in the light, adding to the reduction in lifespan in the light (Moskalev and Malysheva, 2010).

Thus, we obtained the first evidence of the role of FOXO transcription factor in the control of lifespan in varying light regimes. This role is expected to be determined by its involvement in the response to the mild stress effect of darkness.

Overall, the results of our research substantiate our hypothesis regarding the existence of two relatively independent regulatory pathways that respond to changes in lighting regimes (**Figure 1**). On the one hand, the increased photoperiod resulting in the intensified metabolism leads to the decreased lifespan of *Drosophila*. Actually, the strains with *Sod*, *mus210,* and *dSir2* gene mutations demonstrate a significantly higher difference in the lifespan in the dark and in the light with regard to the wild-type strains, and for the strains with mutations in *Hsp70* gene such regularity is a trend. On the other hand, the decreased photoperiod, although it does not cause damage, stimulates the stress response and prolongs the lifespan. In the case of reduced activity of FOXO transcription factor the increase in the *Drosophila* lifespan in the dark is poorly pronounced or absent, which substantiates our hypothesis that *Drosophila* have a FOXO-dependent mechanism of increasing lifespan in the dark (Moskalev and Malysheva, 2010).

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Why is individual reproduction in *Drosophila* flies stochastic?

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Reproduction is essential in studying of aging and senescence in fruit flies as for the resources devoted to egg-laying are subtracted from the total resources of the organism and thus intensive reproduction shorten the life span of an individual. According to prevailing opinion, the stochastic nature of spatial and temporal distribution of external factors leads to randomness of reproductive patterns in fly populations in the wild. Environments are often not constant, but can vary stochastically. The amplitude of variability in environmental conditions influences organismal responses (Boyce et al., 2006; Boggs, 2009). It was believed that the random character of egg-laying can be studied with stochasticity of reproductive behavior (Markow, 1996). Nonetheless, the question of why real egg-laying patterns are stochastic was never asked until 2003 (Novoseltsev et al., 2003b).

To analyze random individual reproductive patterns for the first time it was proposed that *Drosophila* females should be studied by a three-stage non-random approximation embracing the time interval from hatching to death. The analysis included maturation (when eggs are ripened in ovarioles), adult stage (when eggs are laid in a maximal rate), and the senescence stage, when energy shortages produce an exponential decrease of this rate.

This means that the total "stochasticity" in reproductive process was reflected by a set of random parameters adjusted individually for each pattern. Five parameters were used—the moment of the first egg laid was t_0, duration of constancy interval of egg-laying T, time constant of exponential tail τ, reproductive capacity RC, and life span LS (Novoseltsev et al., 2004).

This three-stage approximation technique is the only one which produces the whole pattern description starting with an individual's hatching and ending at death. In this, it is different from the approximations related to various segments of the reproductive process. For example, in Muller et al. (2001) it is shown that individual reproduction experiences exponentially decline in advanced ages, and in Rauser et al. (2003) the assertion is expressed that eggs laying is not reduced to zero in the end of life, but stabilizes at some low level.

Still, the description of individual reproductive patterns contains two types of stochasticity. Slow changes in reproduction, connected with age alterations, are reflected in the "skeleton" of the pattern, but also there exist fast, day-to-day variations in egg-laying, reflected in the tooth-like graph imposed on the three-stage skeleton.

Here, we discuss a hypothesis about the mechanisms that lead to the emergence of the second type of the randomness in the pattern, and on this basis we construct the model of egg production *in silico*. Then this model is used to study heterogeneity in resource allocation and causes of death in fruit flies.

The death of a fly usually occurs at advanced ages when reproductive process has been completed. This is a death caused by senescence. However, in the case of a large share of resource for reproduction and a lack of resources for somatic maintenance, death may occur earlier. In this case, the fly is still in the full swing of egg-laying and death from reproductive overload occurs.

INDIVIDUAL REPRODUCTION PATTERN

Individual reproduction pattern represents the graphical picture of a sequence of numbers showing the quantity of eggs laid day-to-day by a fly, from hatching until death. Three individual reproductive patterns, which correspond to three various phenotypes in resource allocation (*s*-, *m*-, and *l*-patterns), are shown in **Figures 1A–C**. Letters *s*-, *m*-, and *l*- (from *shortened*, *medium*, and *long*) define the life span as related to the reproduction period. *S*-flies have a large energy resource devoted to reproduction but a small resource assigned for somatic maintenance. Their lives are interrupted when egg production continues at a high rate and hasn't diminished yet. Death caused by reproductive overloading arises so that flies die prematurely, not achieving the senescence stage (panel **A**). *M*-flies have a balanced resource allocation and their reproductive potential is realized practically entirely (**B**). In *l*-flies, balance is displaced to a large somatic resource; thus they continue to live after completing reproduction (**C**). Females of *m*- and *l*-types die from senescence whereas *s*-flies die from reproductive overload (Novoseltsev et al., 2005).

Individual reproductive pattern is a random process in the formation of which at least three body systems are involved: (1) the energy balance system, (2) the system of dietary protein processing in the yolk-protein, and (3) the system of ovarioles in which eggs mature before laying.

The first two systems define regular changes in reproduction (the structure of

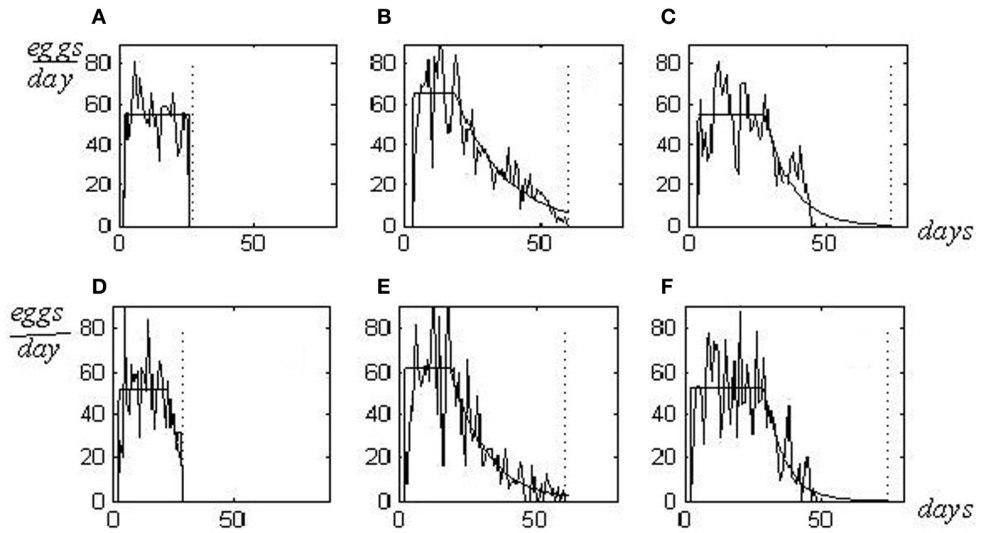

FIGURE 1 | Experimental and *in silico* reproductive patterns in *D. melanlgaster*. Patterns for *s*-, *m*-, and *l*-flies are shown as shortened, medium and long lives as related to the reproduction periods [**(A–C)**—experimental patterns for flies 226, 29, and 2; Arking (1987) population database; **(D–F)**—the correspondent *in silico* patterns]. Death caused by reproductive overload is shown (*s*-flies, **A** and **D** plates), as well as deaths from senescence [*m*- and *l*- flies, plates correspondingly **(B,C** and **E,F**)]. Parameters of regularly approximated patterns for to, *T, RC, LS* and ½ are as follows: 2, 24, 55, 27, 0.02 in *s*-flies; 3, 16, 66, 60, 18 in *m*-flies; 3, 16, 25, 55, 74, 10 in *l*-flies. The *in silico* values of the parameters *a, T* and *LS* are 50, 19, 29 in *s*-flies; 65, 20, 60 in *m*-flies, and 55, 26, 75 in *l*-flies. Vertical dotted lines—the life spans.

the pattern). Energy relations define the "skeleton," determining moments of the transition from stage to stage—the beginning of the period of maturity (egg-laying) and the beginning of the senescence period. The ovarioles system provides fast (day-to-day) fluctuations, reflecting the number of eggs laid per day.

The energy source of the organism $E(t)$ is defined as:

$$E(t) = \int_0^t [P_1(\tau) - P_2(\tau)]d\tau,$$

where P_1 stands for the daily energy intake, and P_2 is the total daily energy expenditure: $P_2(t) = P_s + P_r(t)$. Here P_s = const—daily somatic energy consumption, and $P_r(t)$—daily consumption of energy for reproduction (proportional to the per day number of eggs laid).

Daily intake of energy $P_1(t)$ is subject to the aging process. After a period of constancy its quasi-exponential decline starts with maximum daily energy intake P_{1MAX} and oxidative vulnerability β_1.

The energy balance is responsible for the slow alterations in the oviposition and creates a "skeleton" pattern. While the possibility of the energy system is such that the allowable production of energy P_{1MAX} exceeds operating costs, processes in the body flow constantly. But at a time $t = T$, when the daily consumption of energy $P_s + P_r(t)$ becomes greater than its arrival, production and egg-laying process begin to decline. Death occurs when energy resource is exhausted, $E(LS) = 0$.

A simplified model of the system which processes protein into yolk-protein is described as follows. Let a = const stands for the intake of protein from outside (a unit of measurement being the amount of protein required for the formation of a single egg), and $x(k)$—the amount of protein in the body at the k-th day. Further, let $n(k)$—protein intake in the system, $x(k)$—the amount of protein in it, and L—its capacity (the maximum amount of protein, which can be processed simultaneously). The whole protein $n(k)$, received by the system at the k-th day, turns into a yolk-protein and the next day goes into ovarioles. All ovarioles are identical and each of them can mature to M eggs simultaneously.

If the quantity of yolk-protein in the body denoted as $y(k) = n(k-1)$, the balance of protein can be determined by the equation $x(k) = x(k-1) + a - n(k)$, and the processing of the protein in yolk-protein is described as $x_y(k) = x_y(k-1) + n(k-1) - n(k-2)$.

The resulting yolk-protein enters the ovarioles and fills them in turn. Admission of new eggs in the ovarioles is determined by the condition $x_{ov}(j, k) \leq M$, where $x_{ov}(j, k)$ is a number of eggs in the j-th ovariole at the k-th day. This condition, once satisfied, begins as soon as the process of laying eggs forms "free place" in the ovariole. Immediately after that, a new portion of the eggs is loaded. The quantity of yolk-protein $y(k)$, entering the ovarioles, depends on the values of a, L, and the initial amount of protein in the body $x(0)$.

The rate of the yolk-protein admission to the system of ovarioles, $y(k)$, determines the rate of egg production. If a lot of protein comes with the food ($a > L/2$), the plateau level RC is determines by the possibilities of the processing system. Otherwise ($a \leq L/2$), RC depends on the rate of protein income. Thus, $RC = \min(L/2, a)$. This model considers variant $a \leq L/2$ to avoid a necessity of analysis of forced oscillations.

These relations are valid only until the beginning of the aging process: a decline in egg production starts after time T. According to the model (Novoseltsev et al., 2003b), which reflects the oxidative

damage theory, the aging of the system of the protein into the yolk-protein processing occurs in the same way as that of the energy system. Thus, these processes take place under the condition $x_y(k) \leq N(k)$, where: $N(k) = L$, if $0 \leq k \leq T$. Otherwise, $N(k) = L.\exp[-\beta_2 \int_{k-T}^{k} P_2(\tau)\, d\tau]$, if $k > T$.

Here β_2 is the coefficient of oxidative vulnerability in the protein processing system. It was previously shown that in the *Drosophila* organism the protein processing system ages faster than the power system (Novoseltsev et al., 2003a). Thus, $\beta_2 > \beta_1$ in the model.

STOCHASTIC NATURE OF EGGS LAYING

It can be assumed that the randomness of the process of oviposition in nature is primarily concerned with the uneven provision of energy to numerous ovarioles. At early stages of development, when the future ovarioles are only starting to form, genetic noise plays an important role. Thus, ovarioles receive a variety of power equipment, and eggs in different ovarioles mature at different times ξ.

To form a random reproductive pattern, we define the duration of the maturation process at random: ξ is the integer uniformly distributed in the interval $[p + 1, p + q]$. The value of p determines the day of the first egg laid, and q, the time delay from the first mature egg to the last one. Maturation itself lasts for several days. Thus, for $p = 0, q = 4$ the maturation of eggs in the ovarioles takes from 1 to 4 days.

Let us denote the total number of ovarioles in the body with J, and yolk-protein intake in the j-th ovaryole at the k-th day with $m(j, k)$. If there is a lot of yolk-protein in the body, first ovarioles are filled completely, i.e., each of them receives a portion of exactly M oocytes: $m(j, k) = M$. The number of eggs laid from the j-th ovarioles for the k-th day, is denoted as $s(j, k)$. Then the number of eggs in the ovarioles

$$x_{ov}(j, k) = x_{ov}(j, k - 1) + m(j, k - 1) - s(j, k - 1).$$

Ripe eggs are laid from the ovariole: $y_2(j, k) = s(j, k)$. The total number of eggs laid on the k-th day, $s(k)$, obtained by summing the eggs laid from all J ovarioles. Daily energy consumption devoted to reproduction on the k-th day, $P_r(k)$, is defined by the number of eggs laid on this day: $P_r(k) = \alpha \cdot s(k)$. Here a is the energy cost of laying of a single egg. The aging of ovarioles is not provided in the model.

Changing the setting P_{1MAX} allows the reproduction of patterns of *s*-, *m*-, and *l*-flies shown above in **Figures 1A–C**. These patterns *in silico* are presented in **Figures 1D–F**.

DISCUSSION

The assumption is made above that the stochastic nature of oviposition in fruit flies is completely determined by internal properties of the organism, although environmental factors might have a certain impact.

In formalizing the random nature of eggs maturation in ovarioles the following approach is possible. It assumes all ovarioles the same, and the number of days required for each portion of maturing oocytes to turn into ripened eggs is random. In this case, each portion of eggs laid from the ovariole needs a different amount of time to mature. The process of laying eggs is random due to the fact that every day the eggs are laid from different ovarioles having different maturation time. This assumption allows the construction a model which reproduces *in silico* the entire cycle of egg production and laying. The resulting pattern is virtually indistinguishable from the ones in real experiments, and the model allows confirmation of the early results of the death of a fly caused by reproductive overload.

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The possible roles of human *Alu* elements in aging

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The huge amount of knowledge about the organization and functions of genome structural units accumulated through genome research allows us to examine cell aging processes in detail and to test existing hypotheses of aging. One of the hypothesis currently of interest is that instability of the cell genome is one of the main causes of aging (Vijg and Suh, 2013). Putative causes of genome instability during aging include breakage of double-stranded DNA, telomere shortening, activation of mobile elements, and decreased efficiency of repair systems (Chen et al., 2007; Aubert and Lansdorp, 2008; Maxwell et al., 2011). It is suggested that genome instability of somatic cells has an impact on gene expression and results in disturbance of cell processes, cessation of cellular growth, degeneration and atrophy of cells and tissues as well as aging of the whole organism. The role of mobile elements, in particular *Alu* retrotransposons, in genome instability and aging deserves special attention. Important questions include whether *Alu* elements in the human genome are an endogenous source of DNA damage and genome instability and whether they can promote aging of an organism and make a significant contribution to lifespan variation. *Alu* elements are characterized by considerable polymorphism in human populations. We asked, therefore, whether polymorphism of *Alu* elements can influence the human lifespan.

Opinions about mobile genetic elements have changed radically over the last two decades. Originally, they were characterized as selfish DNA but today we recognize their role in the organization of a genome and the regulation of gene expression (Deininger, 2011). *Alu* elements are classified as short interspersed elements (SINEs). The human genome contains about $\sim 10^6$ copies of *Alu* retrotransposons and they represent ~10.6% of nuclear DNA. The distributions of different *Alu* elements within a one chromosome and between different chromosomes are uneven but are not random. *Alu* elements in human chromosomes 14, 16, and 21 are concentrated in centromeric areas, but clusters of *Alu* elements are not found in chromosomes 4, 19, 20, X or Y. The distribution of *Alu* elements is correlated positively with the presence of GpC-containing genome sequences and the distribution of protein-coding genes. These repeating elements are clustered near the genes controlling metabolic, transport, and signaling processes (Grover et al., 2003).

Genome instability has been found for all sites containing *Alu* elements, which serve as "substrates" for homologous recombination owing to their high frequency of occurrence in the eukaryotic genome and the identity of their sequences. Deletions and duplications can appear as the result of crossing-over between similarly oriented elements; e.g., between inversions of opposite orientation (Kolomietz et al., 2002). The existence of an inserted *Alu* element (*Alu*Y) is a predictor of increased recombination variability within 2 kb of the *Alu* element (Witherspoon et al., 2009). As a result of the analysis of human DNA sequences adjoining the site of recombination, the 26 nt sequence of an *Alu* element was found within the site or at a distance of 20–50 bp from it. This sequence is similar to that of a χ site, which stimulates recombination in *Escherichia coli*. Further, a sequence with homology to a translysine-binding site was detected within the *Alu* element. This protein is involved in partial untwisting of the DNA helix and its linkage with DNA results in increased sensitivity to the action of nucleases and greater probability of recombination (Martinelli et al., 2000). Thus, a large number of *Alu* elements in the genome and the existence of protein-binding sites in sequences involved in recombination lead to their functioning as potential sites for recombination and, perhaps, promotion of this process.

Alu elements are 7SL RNA-like SINEs (Deininger, 2011). Owing to structural features and various functions, *Alu* elements can participate in the regulation of gene expression and likely influence the expression of many genes by insertion into or close by gene promoter regions. *Alu* elements contain binding sites for nuclear hormone receptor complexes and a large number of functionally active transcription factors (Polak and Domany, 2006; Deininger, 2011). These sites can compete for linkage of transcription factors with gene promoters or act as promoters for nearby genes. For example, ~90% of sites responsible for the linkage of retinoic acid are located in *Alu* elements (Laperriere et al., 2007). Human aging is characterized by dysregulation of alternative splicing (Harries et al., 2011) and *Alu* elements can interfere with the mechanism underlying gene splicing. The presence of *Alu* elements in non-translation sites of a gene can result in alternative or aberrant splice sites. About 5% of all human alternative exons contain *Alu* sequences (Sorek et al., 2002). One of the consequences of the insertion of *Alu* elements into protein-coding sequences is the occurrence of an additional stop codon and a premature stop of translation resulting in the development of different diseases (Hancks and Kazazian, 2012). For example, the insertion of an *Alu* element into intron 18 of the human factor VIII gene leads to the absence of exon 19 during the splicing

process, which results in development of the severe form of hemophilia (Ganguly et al., 2003). *Alu* elements can act as anti-sense regulators of transcription. *Alu* elements in gene introns might be located in anti-sense orientation regarding the direction of gene transcription; therefore, anti-sense RNA complementary to mRNA can be synthesized, and this is able to suppress splicing and mRNA translation. Anti-sense interactions of *Alu* transcripts with mRNA likely have a major role in the regulation of translation, degradation of mRNA, and change of gene transcription (Häsler and Strub, 2006). The insertion of *Alu* elements into genes creates alternative sites of polyadenylation, which is one of the important stages of mRNA maturation before translation. The human genome contains ∼10,000 *Alu* elements located in the 3′-untranslated region of coding genes, and 1% of them are active as polyadenylation sites (Chen et al., 2009). The vast majority of transcribed human pre-mRNA contains surprisingly high numbers of *Alu* elements, which likely have an essential role in adenosine-to-inosine (A-to-I) pre-mRNA editing (DeCerbo and Carmichael, 2005). Targets for editing are partially double-stranded RNA that is formed from the inverted repeats of conservative *Alu* sequences (IRAlu) localized in introns and untranslated regions, but not in coding regions. mRNAs without, or containing only low levels of inosine residues move into the cytoplasm and highly edited mRNA molecules are localized in the nucleus. As a result of editing, the expression of mRNA containing IR*Alu* can be modulated by regulating the quantity of mRNA arriving in the cytoplasm (Athanasiadis et al., 2004). Hyper-expression of *Alu* elements as a result of the suppression of the translation process by inhibitors (e.g., cycloheximide and pyromicin) reveals the close connection between the expression of *Alu* elements and the translation state within a cell (Liu et al., 1995). The expression of an *Alu* element is stimulated in response to various factors (e.g., viral infections, translation inhibitors, and factors of cellular stress) and it is believed they can participate in the regulation of translation during stress reactions (Rudin and Thompson, 2001). Up to 33% of all CpG sites in the human genome are located in *Alu* elements and their methylation is a primary mechanism of transposon activity suppression (Schmid, 1991; Slotkin and Martienssen, 2007). It is supposed that changes in the methylation status of *Alu* elements can act as global modifiers of gene expression and it is worth noting the inter-individual variability of the methylation profile of *Alu* elements; indeed, their epigenetic changes throughout life have been observed in monozygotic twins (Fraga et al., 2005; Sandovici et al., 2005). It was suggested that demethylation of *Alu* elements makes a significant contribution to global hypomethylation of the genome in aging (Bollati et al., 2009; Jintaridth and Mutirangura, 2010; Gentilini et al., 2012). Some research data reveal regulatory interactions between *Alu* elements and microRNAs (miRNAs) (Smalheiser and Torvik, 2006; Lehnert et al., 2009). The *Alu* elements localized in 3′-untranslated regions can serve as donors of miRNA target sites to various genes. Many of these genes are involved in regulation of transcription, cell cycle, cell proliferation, apoptosis, cell–cell contact, and signal transduction (Daskalova et al., 2006). miRNAs have recently emerged as important regulators of cellular senescence and aging (Smith-Vikos and Slack, 2012). As a result of genome-wide miRNA study, changes in miRNA expression with human aging were revealed (Elsharawy et al., 2012). Thus, it is clear the mobile elements connect different systems of regulation of gene expression, and it is important for understanding their role in aging.

The insertion/deletion polymorphism of *Alu* elements is widespread in human populations. Hypothetically, *Alu* element polymorphisms contribute to the variability of the human lifespan. Insertion of *Alu* elements into genes could cause epigenetic alterations and altered levels of gene expression, which is in accord with the results of some studies. For example, experiments with a cell line mono-allelic for *Alu* insertion/deletion have shown that the insertion of *Alu*Ya5 into the progesterone receptor gene *PGR* mediates the increased level of DNA methylation in the surrounding area of the genome, causes the inactivation of histone tail modifications and results in inactivation of the expression of neighboring genes (Byun et al., 2012).

In conclusion, *Alu* elements, which are components of the complex network of interrelated molecular and genetic changes; can have a role in structural and functional damage of the human genome during aging. Activation of *Alu* elements in response to environmental factors could be one of the triggers that mediate genome instability, alter the expression levels of genes, and lead to the gradual diminution of cell functions and organism functionality as a whole.

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Telomere length and body temperature—independent determinants of mammalian longevity?

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WHY DO SPECIES DIFFER IN LIFESPAN AND WHAT ARE THE DETERMINANTS OF THEIR LONGEVITY?

Understanding the main factors that determine variation in species longevity may provide a clue into the leading mechanisms of aging and—what is even more important—outline the key targets for longevity-promoting interventions. Comparative studies on longevity in mammals revealed numerous variables that correlate with maximum lifespan (MLS). However, because of the intangibly intertwined biological relationships, only a limited number of the variables could be considered independent determinants of longevity. Most other correlations reflect intermediated (formal) co-variations rather than the "cause-and-effect" links. It is obvious that manipulations of the formal correlates have little chances to effect the longevity-ensuring systems, and thus be helpful for developing experimental strategies of lifespan extension. Therefore, in comparative longevity studies, it is important to discriminate the independent determinants from the formal correlates.

One of the simple criteria to distinguish determinants of longevity is consistently high correlation of a given variable with MLS observed in various model systems. The other approach is based on more ambiguous statistical methods, multivariate analyses included (see, for example, Lehmann et al., 2008a). In particular, we have previously shown that body mass (BM) or resting metabolic rate alone explain around 40–50% of the variation in mammalian longevity, whereas their combination with mitochondrial DNA (mtDNA) GC content could explain over 70% of the MLS variation (Lehmann et al., 2008a,b). Consequently, we hypothesized that other putative players in MLS determination should have relatively small contribution or their effects should be mediated by the above factors.

Recent finding by Gomes et al. (2011) demonstrating a strong negative correlation ($p = 0.0032$) between telomere length and MLS in 59 mammalian species calls for re-evaluation of this hypothesis. Indeed, the coefficient of MLS determination (R^2) calculated using the data in their paper indicates that the telomere length could alone explain more than 1/3 of the variation in the lifespan of mammals. Here, we explore whether the telomere length has an independent impact on mammalian longevity or its effect is attributed to co-variation with other determinants of MLS, such as BM and mtDNA GC content. Our analysis was based on the set of mammalian species with the telomere length records presented in Gomes et al. (2011) ($n = 55$; four species from the orders Monotremata and Diprotodontia, with unusually short telomeres of 1 kb, were not included in the analysis). The MLS, BM, and body temperature (T_b) records were retrieved from the AnAge database (Tacutu et al., 2013; http://genomics.senescence.info/species/). Calculation of the mtDNA GC content was described elsewhere (Lehmann et al., 2008a). To ensure linear relationships, MLS and BM values were ln-transformed, i.e., MLS was presented as a natural logarithm of MLS (lnMLS) and BM was presented as a natural logarithm of BM (lnBM).

Re-evaluation of combined effect of lnBM and mtDNA GC on lnMLS in the set of species analyzed by Gomes et al. (2011) gave an extremely significant coefficient of MLS determination ($R^2 = 0.713$, $P = 6.2\text{E-}10$, $n = 37$), which is very close to that obtained on much bigger dataset of mammals ($R^2 = 0.703$, $P < \text{E} - 25$, $n = 215$; unpublished data). The telomere length significantly correlates with both lnBM ($R = -0.479$, $P = 0.00025$, $n = 55$) and mtDNA GC content ($R = -0.44$, $P = 0.006$, $n = 37$), suggesting that the relationship between lnMLS and telomere length ($R = -0.609$, $P = 1.2\text{E-}10$, $n = 54$) could, at least in part, be due to the telomere length association with lnBM and/or mtDNA GC. Nevertheless, partial correlation and multivariate analyses showed that the telomere length has an independent impact on longevity determination.

The partial correlation analysis allows eliminating the co-variation effects. We found that the correlative links between telomere length and BM or mtDNA GC do not significantly alter its association with longevity. Indeed, removing the effects of lnBM or GC or both did not affect the correlation between lnMLS and telomere length (coefficients of partial correlations are -0.421, -0.608, and -0.442, respectively; $P < 0.01$). Moreover, the links between telomere length and lnBM or mtDNA GC are apparently mediated via longevity-associated factors because removing the effect of MLS resulted in an insignificant correlation of telomere length with lnBM ($P > 0.5$) or GC content ($P > 0.4$).

As further demonstrated in **Figure 1A**, after extraction of lnBM and mtDNA GC input, the lifespan residuals significantly correlate with telomere length ($R = 0.351$, $P = 0.033$). The multivariate analysis showed that the telomere length together with lnBM and mtDNA GC determine 76.9% ($P = 1.3E\text{-}10$) of lnMLS variation, thus increasing the lnMLS R^2-value (71.3%) of two variables, lnBM and mtDNA GC, by 5.6%. That is, the telomere length could explain part of the variation in the mammalian longevity, which is not explained by the lnBM and mtDNA GC. However, there is still a place for unaccounted factors as the lnMLS residuals of lnBM, mtDNA GC, and telomere length significantly correlated with lnMLS ($R = 0.481$, $P = 0.003$). In attempt to discover these still unaccounted factors, we further included in the analysis an additional variable closely related but not identical to the metabolic rate—body temperature (T_b).

Gomes et al. (2011) hypothesized that the evolution from exothermic to homeothermic organisms was accompanied by telomere shortening as a tumor protective adaptation to an enhanced mutation load caused by high T_b. Yet, within mammalian species we did not observe any significant correlation between the telomere length and typical T_b ($P > 0.8$, $n = 37$). There was also no significant correlation ($P > 0.3$) between T_b and lnMLS.

Unexpectedly, we found that T_b significantly correlates with the residual lifespan of lnBM, mtDNA GC and telomere length ($R = 0.597$, $P = 0.002$; **Figure 1B**). This may explain some cases of considerable deviations from the lnMLS predicted by lnBM, mtDNA GC, and telomere length. For example, the naked mole-rat (*Heterocephalus glaber*) and North American pika (*Ochotona princeps*) have similar values of lnBM, mtDNA GC content and telomere length, yet the naked mole-rat lives 4.4 times longer. This apparent "discrepancy" could be largely attributed to the difference in T_b which, in the sample analyzed, is the lowest for the naked mole-rat (32.1°C) and the highest for the North American pika (40.1°C). Of note, the lnMLS residuals of all four variables (lnBM, mtDNA GC, telomere length, and T_b) did not correlate significantly with lnMLS ($P = 0.112$). As a result, **Figure 1C** demonstrates extremely high fitting between predicted and observed lnMLS values ($R^2 = 0.889$, $R = 0.943$, $P = 8.0E\text{-}9$). Thus, in the analyzed set of mammalian species, the combination of lnBM, mtDNA GC, telomere length, and T_b explains the vast majority of lnMLS variation. The remaining variation of about 11.1% is most likely attributed to the "noise" in measuring of the above variables. Yet, an input of still unaccounted factors or cross-talk effects cannot be dismissed and continued study is warranted. For example, the interactions within the telomeres—p53—mitochondria axis (Sahin and DePinho, 2012), could be an important area for future search.

The results obtained gained further support from the multivariate analysis with standardized coefficients showing a significant impact of each of the variables under analysis, i.e., lnBM ($P = 2.6E\text{-}6$), mtDNA GC ($P = 0.0007$), telomere length (Tel; $P = 0.032$) and T_b ($P = 0.0015$), on lnMLS determination:

$$\text{lnMLS} = 0.586\text{lnBM} + 0.356\text{mtDNA GC} - 0.230\text{Tel} - 0.294T_b$$

As could be expected, lnBM and mtDNA GC content display positive coefficients of regression while telomere length and T_b display negative coefficients, highlighting the role of potentially damaging and protective (stabilizing) factors in lifespan determination (**Figure 2**). The higher BM is associated with a lower metabolic rate and lesser generation of damaging substances (e.g., ROS). The higher GC content may ensure the higher thermodynamic stability of mtDNA against denaturizing factors such as high T_b. While short telomeres have less probability to be damaged than longer ones, their maintenance and efficient repair are of crucial importance for chromosome and genome

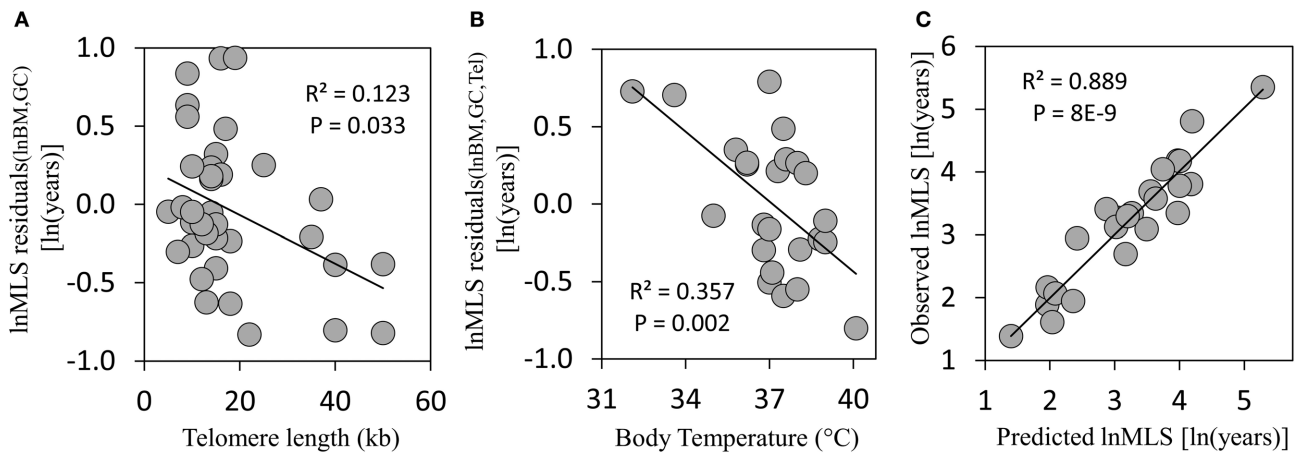

FIGURE 1 | (A) The lnMLS residuals of lnBM and mtDNA GC correlate with telomere length (Tel). **(B)** The lnMLS residuals of lnBM, mtDNA GC, and Tel correlate with T_b. **(C)** Observed lnMLS plotted against predicted lnMLS. The predicted lnMLS was calculated from the multivariate linear regression: $\text{lnMLS} = 0.14\text{lnBM} + 0.015\text{mtDNA GC} - 0.017\text{Tel} - 0.17\ T_b$, where MLS is in years, BM in g, mtDNA GC content in b/kb, Tel in bp, and T_b in °C. Statistical calculations were performed using the statistical package for the social sciences (SPSS, Inc., Chicago, IL) software.

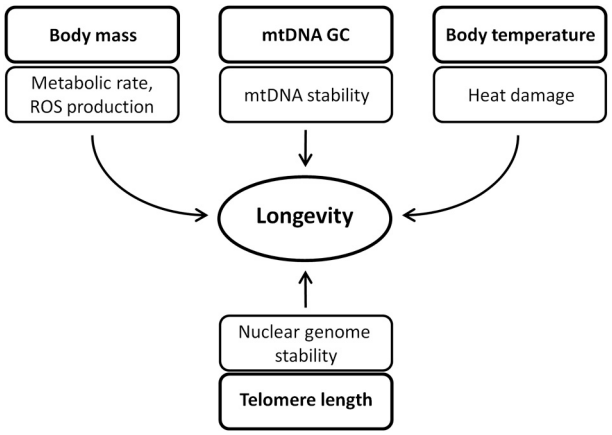

FIGURE 2 | Determinants of mammalian longevity. In the examined set of species, the relative contribution to MLS determination decreases in a descending order: Body mass > mtDNA GC content > Body temperature > Telomere length. Of note, 3 of 4 determinants (upper panel) are directly associated with mitochondria. For explanations, see the text.

stability. It seems plausible that the age-related reduction in telomere length is more pronounced in short-lived than in long-lived species. This notion gained experimental support from a recent observation of Vera et al. (2012), who showed that the mouse telomeres shortened 100 times faster than human telomeres. The shorter (but more stable) telomeres in long-lived/large mammals evolved apparently due to more efficient DNA repair [reviewed by Moskalev et al. (2012)], along with a prominent reduction in telomerase activity with an increase in BM (Seluanov et al., 2007; Gomes et al., 2011). An important observation is that mammalian species (large rodents, humans) that use replicative senescence, a potential tumor suppression mechanism, have also relatively short telomeres (Seluanov et al., 2008). Altogether, the increase in BM and mtDNA GC content, reduction in telomere length and lower T_b could result in a higher genomic and metabolic stability, more efficient cellular homeostasis, and ultimately in increased longevity (**Figure 2**).

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Signaling pathway cloud regulation for *in silico* screening and ranking of the potential geroprotective drugs

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The major challenges of aging research include absence of the comprehensive set of aging biomarkers, the time it takes to evaluate the effects of various interventions on longevity in humans and the difficulty extrapolating the results from model organisms to humans. To address these challenges we propose the *in silico* method for screening and ranking the possible geroprotectors followed by the high-throughput *in vivo* and *in vitro* validation. The proposed method evaluates the changes in the collection of activated or suppressed signaling pathways involved in aging and longevity, termed signaling pathway cloud, constructed using the gene expression data and epigenetic profiles of young and old patients' tissues. The possible interventions are selected and rated according to their ability to regulate age-related changes and minimize differences in the signaling pathway cloud. While many algorithmic solutions to simulating the induction of the old into young metabolic profiles *in silico* are possible, this flexible and scalable approach may potentially be used to predict the efficacy of the many drugs that may extend human longevity before conducting pre-clinical work and expensive clinical trials.

Keywords: geroprotector, aging-suppressive drug, signaling pathway cloud, validation of drugs, transcriptomics

The increasing burden of the aging on the economies of the developed countries is turning the quest to increase healthy life spans from an altruistic cause into a pressing economic priority required to maintain the current standards of living and facilitate economic growth (Zhavoronkov and Litovchenko, 2013). There is an urgent need to develop and validate interventions with geroprotective properties to increase the productive health spans of the working population and maintaining performance and avoiding loss of function (Kennedy, 2012).

While no doubt exists that aging is a complex multifactorial process with no single cause or treatment (Zhavoronkov and Cantor, 2011; Trindade et al., 2013), the issue whether aging can be classified as the disease is widely debated (Rattan, 2013). However, many strategies for extending organismal life spans have been proposed including replacing cells (Rodgerson and Harris, 2011) and organs, comprehensive strategies for repairing the accumulated damage, using hormetins to activate endogenous repair processes (Gems and Partridge, 2008; Gaman et al., 2011), modulating the aging processes through specific mutations, gene therapy (Bernardes De Jesus et al., 2012) and small molecule drugs (Kennedy and Pennypacker, 2013). An animal's survival strongly depends on its ability to maintain homeostasis and achieved through intracellular and intercellular communication within and among different tissues (Alcedo et al., 2013). Many strategies for the development and validation of drugs with geroprotective properties have been proposed to help maintain the homeostasis including drugs that act on specific targets or combinations of molecular pathways (Moskalev and Shaposhnikov, 2010, 2011; Zhavoronkov et al., 2012; Danilov et al., 2013) and epigenetic drugs (Vaiserman and Pasyukova, 2012). However, none of the proposed strategies for aging-suppressive drug development provide a roadmap for rapid screening, validation, and clinical deployment. No methods currently exist to predict the effects of currently available drugs on human longevity and health span in a timely manner. This is partly due to the absence of the clear panel of human processes involved in aging to effectively run clinical trials.

Many processes are involved in the aging of cells and organisms including telomere length (Lehmann et al., 2013), intracellular and extracellular aggregates, racemization of the amino acids and genetic instability. Both gene expression (Wolters and Schumacher, 2013) and DNA methylation profiles (Horvath et al., 2012; Horvath, 2013; Mendelsohn and Larrick, 2013) change during aging and may be used as biomarkers of aging. Many studies

analyzing transcriptomes of biopsies in a variety of diseases indicated that age and sex of the patient had significant effects on gene expression (Chowers et al., 2003) and that there are noticeable changes in gene expression with age in mice (Weindruch et al., 2002; Park et al., 2009) resulting in development of mouse aging gene expression databases (Zahn et al., 2007) and in humans (Blalock et al., 2003; Welle et al., 2003; Park and Prolla, 2005; Hong et al., 2008; De Magalhaes et al., 2009).

Combination of protein-protein interaction and gene expression in both flies and humans demonstrated that aging is mainly associated with a small number of biological processes, might preferentially attack key regulatory nodes that are important for network stability (Xue et al., 2007).

Our prior work with gene expression and epigenetics of various solid tumors (Kuzmin et al., 2010; Mityaev et al., 2010; Zabolotneva et al., 2012a,b) using the OncoFinder system (www.oncofinder.com), provided clues that transcription profiles of cancer cells mapped onto the signaling pathways may be used to screen for and rate the targeted drugs that regulate pathways directly and indirectly related to aging and longevity. Instead of focusing on individual network elements, this approach involves creating the signaling pathway cloud, a collection of signaling pathways involved in aging and longevity each comprised of multiple network elements and evaluating the individual pathway activation strength. Despite significant advances in aging research, the knowledge of the aging processes is still poor, and combining all available factors involved in cellular aging, aging of the organisms, age-related diseases, stress-resistance, and stress-response along the many other factors into a comprehensive signaling pathway cloud may be more beneficial than focusing on the narrow collection of elements. The creation of the pathway cloud may allow for the annotated databases of molecules and other factors to be screened for effectiveness of individual compounds in replicating the "young" signaling activation profiles *in silico*.

Several new methods evaluating the robustness and response ability of the gene regulatory network have been developed and applied to gene expression data sets from young and old patients (Tu and Chen, 2013). Prior studies suggested that a combination of pathways, termed pathway cloud, instead of one element of the pathway or the whole pathway might be responsible for pathological changes in the cell (Voronkov and Krauss, 2013). Long-lived species like the sea urchin (*Strongylocentrotus franciscanus*) and naked mole rat (*Heterocephalus glaber*) that senesce at a slower rate than members of the same order show less transcriptome changes with age (Kim et al., 2011; Loram and Bodnar, 2012). Gene network analysis using gene expression data was effective in identifying the possible drug targets (Imoto et al., 2003; Savoie et al., 2003). *In silico* drug discovery algorithms that attempt to transform the metabolism to the healthy state have been proposed and validated (Yizhak et al., 2013). We theorize that in order to be effective, the geroprotector or a combination of aging-suppressive drugs must regulate the pathway cloud in a way that minimizes the difference in the net differences in pathway cloud activation or downregulation between samples of young and old patients. Small molecules and other factors that may influence gene expression may be ranked by their ability to minimize the net difference between the pathway activation profiles of young and old cells. The algorithms for calculating the ability of the potential geroprotector to minimize signaling disturbance may be parametric and account for the effects on specific targets within signaling pathways or machine learned.

Despite the differences in life span and aging phenotypes, many molecular mechanisms of aging are common in all eukaryotes. Pathway analysis revealed that there are many common age-related transcriptomic changes between different species, including yeasts, worms, flies, rodents, and human (Murphy et al., 2006). Hypothetically, the human orthologs of aging-related genes of model organisms are also involved in aging process. To select longevity-associated pathways for future analysis we perform the following procedure. Using (Tacutu et al., 2013) database we selected genes, where knockout, loss-of-function mutation, deletion or RNA interference significantly extended lifespan in several model organisms (yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, nematode *Caenorhabditis elegans*, fruitfly *Drosophila melanogaster* and mouse *Mus musculus*) from 10 to 200%. We converted the obtained gene lists from different models to the general list of human orthologs where it is possible. 226 genes of 315 from our set were subjected to over-representation pathway analysis in (Kamburov et al., 2011). *P*-value and corrected by FDR *P*-value are calculated according to the hypergeometric test based on the number of physical entities present in both the predefined set of genes to each (Kanehisa et al., 2004) pathway and our list of aging-associated genes (**Table 1**). We established limits of 2 genes of minimum overlap with input list and 0.01 *p*-value cut off threshold.

As a result we revealed overrepresented cell signaling pathways (mTOR, insulin/IGF-1, PI3K-Akt, PPAR, HIF-1, TGF-beta, chemokines, adipocytokine, prolactin, estrogen), general metabolism (TCA cycle, ribosome, oxidative phosphorylation), RNA transport, cell cycle and meiosis, gap junction, peroxisome, cyrcadian rhythm, different synapse types (dopaminergic, glutamatergic, cholinergic, serotonergic, GABAergic), gastric acid secretion as well as age-related diseases pathways (Parkinson's disease, type II diabetes mellitus, Huntington's disease, long-term depression, amyotrophic lateral sclerosis, Alzheimer's disease), Hepatitis B, HTLV-I infection and cancer pathways (prostate cancer, colorectal cancer, glioma, pancreatic cancer, chronic myeloid leukemia, proteoglycans in cancer). We considered obtained such a way pathways as probably associated with the human longevity. Human genes known as key activators/repressors of these pathways may be used in provided further mathematical model.

The methods that may be applied for the possible analysis of geroprotector efficiency by pathways regulation have been arisen from our research experience of cell signaling pathways. As far as we have seen before (Kiyatkin et al., 2006; Borisov et al., 2009; Kuzmina and Borisov, 2011), most signal transduction proteins are essentially far from saturation even at the peak concentrations of the activated form in comparison with the total protein abundances. Thus, we can consider that all activator/repressor genes/proteins have equal importance for the pathway activation/downregulation, and then arrive at the following assessment function for the overall signal pathway cloud disturbance outcome (*SPCD*) is proportional to the following estimator function,

Table 1 | KEGG pathways and list of aging-associated genes.

KEGG pathway name	Background quantity of genes in the pathway	Overlap with candidates list	%	*p*-Value	FDR
Citrate cycle (TCA cycle)	30	5	16.7	0.000401	0.0037
Ribosome	136	21	15.7	7.52E-13	6.21E-11
Parkinson's disease	131	20	15.4	3.87E-12	2.02E-10
Aldosterone-regulated sodium reabsorption	39	6	15.4	0.000165	0.00199
Type II diabetes mellitus	48	7	14.6	6.66E-05	0.00105
Oxidative phosphorylation	133	19	14.4	4.55E-11	1.78E-09
mTOR signaling pathway	60	8	13.3	3.89E-05	0.000764
Huntington's disease	183	24	13.2	7.92E-13	6.21E-11
Progesterone-mediated oocyte maturation	86	11	12.8	2.08E-06	4.66E-05
Insulin signaling pathway	142	17	12.1	8.01E-09	2.10E-07
Ovarian steroidogenesis	51	6	11.8	0.000735	0.00525
Long-term depression	60	7	11.7	0.000281	0.00294
Amyotrophic lateral sclerosis (ALS)	53	6	11.3	0.000905	0.00592
Alzheimer's disease	170	19	11.2	3.36E-09	1.05E-07
Cardiac muscle contraction	77	8	10.5	0.000214	0.0024
Gap junction	89	9	10.1	0.000118	0.00155
Prostate cancer	89	9	10.1	0.000118	0.00155
Estrogen signaling pathway	100	10	10.0	5.44E-05	0.00095
Colorectal cancer	62	6	9.7	0.00206	0.0101
Glioma	65	6	9.2	0.00263	0.012
Pancreatic cancer	66	6	9.1	0.00284	0.0124
GABAergic synapse	90	8	9.0	0.000631	0.00524
Adipocytokine signaling pathway	71	6	8.6	0.00382	0.0154
PPAR signaling pathway	71	6	8.5	0.0041	0.0157
Circadian entrainment	97	8	8.3	0.00104	0.00629
Prolactin signaling pathway	72	6	8.3	0.0044	0.0157
Chronic myeloid leukemia	73	6	8.2	0.0047	0.0164
Cholinergic synapse	113	9	8.0	0.000667	0.00524
Oocyte meiosis	112	9	8.0	0.000667	0.00524
Serotonergic synapse	114	9	8.0	0.000711	0.00525
Insulin secretion	87	7	8.0	0.00261	0.012
Gastric acid secretion	75	6	8.0	0.00537	0.0183
HIF-1 signaling pathway	106	8	7.5	0.00198	0.01
Peroxisome	81	6	7.5	0.00734	0.0226
TGF-beta signaling pathway	80	6	7.5	0.00734	0.0226
Cell cycle	124	9	7.3	0.00138	0.00803
Dopaminergic synapse	131	9	6.9	0.00192	0.01
Glutamatergic synapse	118	8	6.8	0.00366	0.0151
Proteoglycans in cancer	225	14	6.2	0.000348	0.00342
RNA transport	165	10	6.1	0.00268	0.012
Hepatitis B	147	9	6.1	0.00439	0.0157
HTLV-I infection	267	14	5.3	0.00161	0.00869
Chemokine signaling pathway	192	10	5.3	0.00732	0.0226
PI3K-Akt signaling pathway	347	16	4.6	0.00312	0.0133

$$SPCD = \frac{\prod_{i=1}^{N} [AGEL]_i}{\prod_{j=1}^{M} [RGEL]_j}$$

Here the multiplication is done over all possible activator and repressor proteins in the pathway, and $[AGEL]_i$ and $[RGEL]_j$ are gene expression levels of an activator i and repressor j, respectively. To obtain an additive rather than multiplicative value, it is enough just turn from the absolute values of the expression levels to their logarithms, arriving at the *pathway activation strength* (*PAS*) value for each pathway (see **Figure 1**). To obtain the values of *Old (case)-to-Young ratio*, YOR_n, one just has to divide the expression levels for a gene n in the sample taken for the senescent person by the same average value for the normalized young group. The discrete value of *ARR* (*activator/repressor role*) equals to the following numbers:

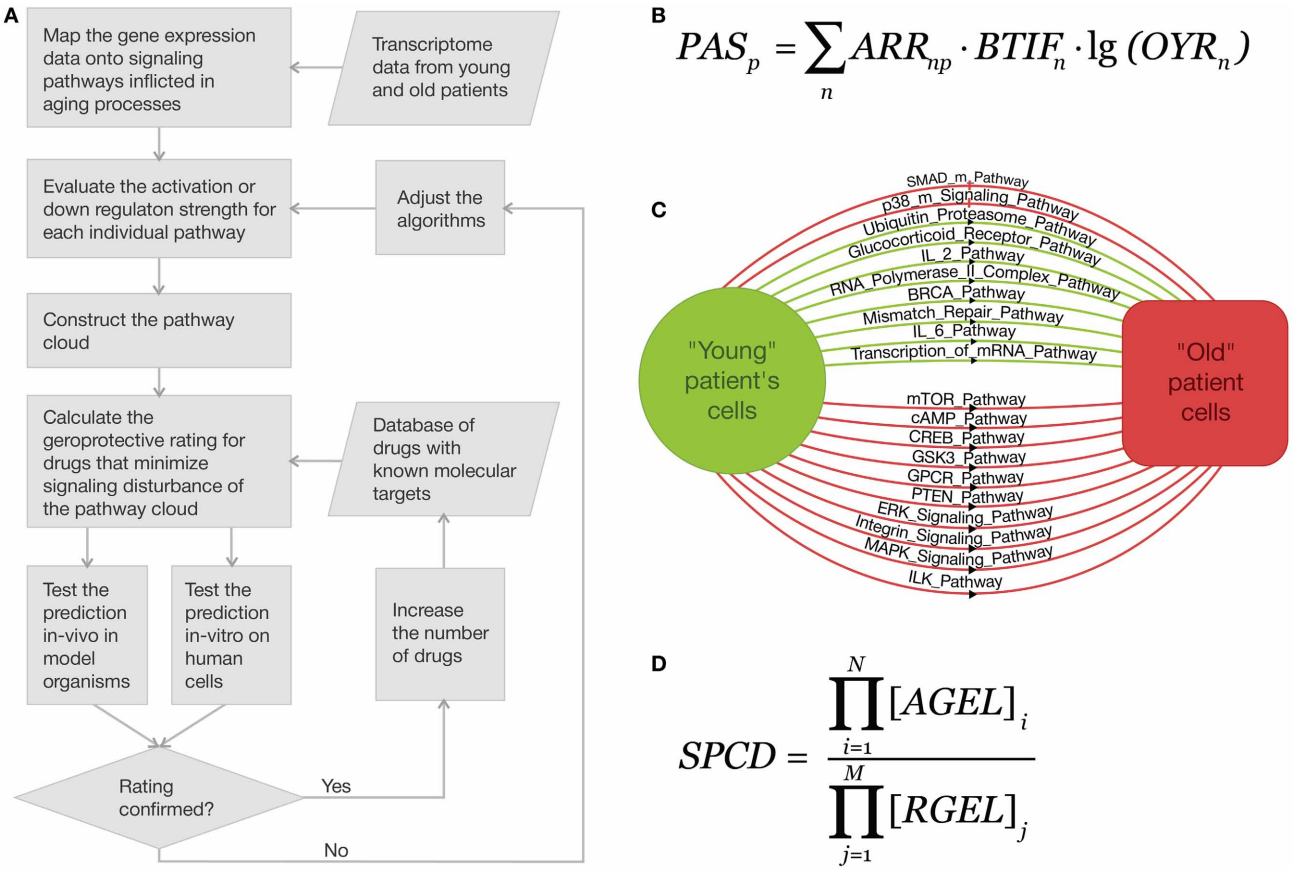

FIGURE 1 | Gene expression-based approach to *in silico* screening for drugs with geroprotective properties and estimating the predicted efficacy. (A) Using signaling pathway cloud regulation for theoretical *in silico* aging-suppressive drug identification and ranking. The proposed method for identifying and ranking of geroprotective drugs by evaluating the net effect on the many elements of signaling pathway cloud that brings the "old" metabolic state closer to the "young." **(B)** An example of how multiple pathways are activated and down-regulated during aging. **(C)** Pathway Activation Strength (PAS) is the logarithmic additive value that characterizes the up-/downregulation of signaling pathways. **(D)** Function for the overall signal pathway cloud disturbance outcome (SPCD).

−1, when the gene/protein *n* is a repressor of pathway excitation;

1, if the gene/protein *n* is an activator of pathway excitation;

0, when the gene/protein *n* can be both an activator and a repressor of signal transduction;

0.5 and −0.5, respectively, if the gene/protein *n* is more an activator or repressor of the signaling pathway *p*.

The information about the activator/repressor role of a particular gene/protein may be obtained from the analysis of open-access or customized pathway databases and from the literature.

The Boolean flag of *BTIF* (*beyond tolerance interval flag*) equals to zero when the *OYR* value lies within the tolerance limit, and to one when otherwise. During the current study, we have admitted that the *OYR* lies beyond the tolerance limit if it satisfies simultaneously the two criteria. First, it either higher than 3/2 or lower than 2/3, and, second, the expression level for a corresponding gene from an old patient of an individual patient differs by more than two standard deviations from the average expression level for the same gene from a set of analogous young tissue/organ samples.

We propose a new computational approach for identifying and rating the variety of factors including small molecules, peptides, stress factors and conditions with the known effects on the transcriptomes at different ages of one or more cell or tissue types or known targets (**Figure 1A**). The approach may be used for general geroprotector screening, but after the validation of the algorithms *in vivo* and *in vitro* may be expanded to identify and predict the efficacy of personalized aging-suppressive intervention regimens for individual patients based on the transcriptome information from various tissue biopsies and blood samples.

The generic geroprotector rating approach involves collecting the transcriptome data sets from young and old patients and normalizing the data for each cell and tissue type, evaluating the pathway activation strength (PAS) for each individual pathway (**Figure 1B**) and constructing the pathway cloud (PC, **Figure 1C**) and screen for drugs or combinations that minimize the signaling pathway cloud disturbance (SPCD, **Figure 1D**) by acting on one or multiple elements of the pathway cloud. Drugs and combinations may be rated by their ability to compensate the changes in signaling pathway activation patterns that are related to aging,

thus bringing all the *PAS* values for the general set of the pathways as close to zero as possible. Since many of the drugs approved for use in humans have known molecular targets and some have been screened for the impact on longevity in model organisms (Ye et al., 2013), the predictions may be then tested both *in vitro* and *in vivo* on human cells and on model organisms such as rodents, nematodes and flies to validate the screening and rating algorithms.

CONCLUSION

Longevity studies of aging-suppressive drug efficiency in higher mammals take several years and decades and may cost millions of dollars. An intelligent process for predicting the activity and ranking the geroprotective activity of various factors and strengthening the prediction in rapid and cost-effective studies on cell cultures and model organisms may help increase the longevity dividend of these studies. In this paper we propose a method for *in silico* screening and ranking of drugs and other factors that act on many signaling pathways implicated in aging processes by calculating their ability to minimize the difference between signaling pathway activation patterns in cells of young and old patients and confirming the results using *in vivo* and *in vitro* studies.

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