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Using DNA Methylation Data to Understand the Evolutionary Basis of Human Aging

By
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Doctor of Philosophy

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Using DNA Methylation Data to Understand the Evolutionary Basis of Human Aging

By

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B.A., Northwestern University, 2011

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Abstract

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The evolutionary basis of human biological aging is not yet well understood. The evolutionary theories of 1) mutation accumulation, 2) antagonistic pleiotropy, and 3) disposable soma provide possible explanations for existence and evolution of aging. These three theories are not mutually exclusive, and it is likely that all three play some role in explaining how and why humans age. My dissertation work increases our understanding of human aging by testing predictions of evolutionary models in new ways. After reviewing the assumptions, predictions, and past empirical tests of each theory, I suggested the novel use of DNA methylation data to test previously unexplored theory predictions related to aging as a lifelong process. DNA methylation patterns are known to be highly dynamic throughout life and have recently been proposed as a biomarker of aging. Using DNA methylation data, I specifically tested: 1) whether the heritability of DNA methylation is consistent with disposable soma or mutation accumulation models; and 2) whether DNA methylation data support a stochastic aging process implied by the disposable soma model. The results of both tests suggest that most age-related DNA methylation changes are consistent with the disposable soma model of aging and may result from random environmental insults and methylation maintenance and repair errors, while a small number of aging-related changes are consistent with the mutation accumulation model and may be targeted to mediate the deleterious age-specific effects of aging genes. This indicates that both the mutation accumulation and disposable soma models play a role in explaining aging and aging-related changes, but that disposable soma is more important in understanding the age-related changes of DNA methylation.

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Chapter 1: Introduction

The existence of aging remains a fundamental problem in evolutionary biology. Biological aging, or senescence, is the functional decline of an organism with age [1]. This familiar process results in decreased fertility and an increased risk of death, and it is associated with major decreases in Darwinian fitness [1-3]. Basic evolutionary theory suggests that such a trait should be opposed by natural selection. Yet, aging is universal within many species including humans. Aging, therefore, poses a paradox. Given the associated decreases in fitness, why has aging not been effectively selected against? Why is it that all humans age?

Evolutionary theories have proposed both adaptive and non-adaptive explanations for the existence of human aging. Major theories include: 1) the theory of mutation accumulation [1], and 2) the theory of antagonistic pleiotropy [4], with the theory of disposable soma as a special case [5]. These theories differ in the modes of selection and the types of genes proposed to underlie aging, but they are not mutually exclusive. Past empirical tests using lifespan as the measure of senescence have provided support for portions of each theory, suggesting that all three may play some role in explaining the features and existence of aging. However, the relative importance of each theory is not yet known. Alternative measures of senescence that are more reflective of aging as a process may provide additional information for such investigations.

DNA methylation is an epigenetic modification has been recently suggested as a biomarker of aging [6-8]. Epigenetic modifications are chemical additions to DNA that can alter gene expression without changing DNA sequence. DNA methylation involves the addition of a methyl group to a single nucleotide base. In humans, this typically occurs at CpG sites, where cytosine bases sit directly next to guanine bases [9, 10]. Microarrays allow DNA methylation to

be easily measured at thousands of CpG sites across the genome [11]. Using this technology, the methylome has been found to be dynamic throughout life, with many studies reporting thousands of age-associated methylation changes across the genome [12-14]. I hypothesize these changes to be reflective of the aging process, and suggest DNA methylation data as an innovative measure against which evolutionary theories of aging can be tested.

My dissertation work aims to increase our understanding of the evolutionary basis of human aging by testing predictions of mutation accumulation and disposable soma using DNA methylation data. I use longitudinal and familial DNA methylation data to test theory predictions at sites across the genome. In Chapter 2, I review the assumptions and predictions of evolutionary theories of aging, and determine which theory predictions are testable with DNA methylation data. In Chapter 3, I test whether age-related changes in the heritability of DNA methylation are consistent with the disposable soma or mutation accumulation models. If DNA methylation helps mediate the aging process, the theory of mutation accumulation suggests that the heritability of DNA methylation will increase with age, while the theory of disposable soma suggests that the heritability of DNA methylation will decrease with age. In Chapter 4, I test whether DNA methylation data support a stochastic aging process implied by the disposable soma model. The theory of disposable soma predicts aging to result from stochastic damages. If age-related changes in DNA methylation are consistent with this prediction, DNA methylation data will fit a stochastic model where the gains and losses of methyl groups occur at random throughout the genome rather than at targeted genes or CpG sites. These analyses categorize CpG sites across the genome as consistent or not consistent with the tested evolutionary theories. In Chapter 5, I discuss the conclusions drawn from this work, including the relative importance of the theories of mutation accumulation and disposable soma in explaining the aging process.

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Chapter 2: Testing evolutionary models of senescence: traditional approaches and future directions

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Introduction

Senescence, or commonly "aging," is the progressive and general deterioration of an organism, resulting in decreased fertility and an increased risk of death (Kirkwood 2005; Medawar 1952; Partridge and Barton 1993). From an evolutionary perspective, it is a challenge to understand how senescence has evolved despite its associated decreases in Darwinian fitness. According to basic evolutionary theory, selection acts to maximize Darwinian fitness, and evolution favors those better able to survive and reproduce. Since the process of aging causes reductions in Darwinian fitness, or the ability to survive and reproduce, why hasn't it been more effectively selected against?

The purpose of this paper is three-fold: 1) review prominent evolutionary explanations for the existence of aging; 2) discuss the assumptions and predictions of these evolutionary explanations and the methods and results of previous empirical tests; 3) present new measurements of senescence that could be used to investigate previously under-explored predictions.

Throughout this text we use senescence interchangeably with the more common term of "aging." Both refer to the biological process of age-dependent decline, and stand in contrast to chronological aging, or the passage of time.

Evolutionary theories of senescence

Mutation Accumulation

P.B. Medawar developed one of the first evolutionary models of senescence in the 1950s (Charlesworth 2000; Medawar 1952). His model, now termed the theory of mutation accumulation (MA), was inspired by observations relating mortality and the strength of selection.

Medawar noticed that in most natural populations, the major cause of death is extrinsic mortality, or death due to external factors such as starvation, accident and predation (Hughes and Reynolds 2005; Kirkwood 2005; Medawar 1952; Zwaan 1999). As a result of extrinsic mortality, the probability of survival decreases with time, and fewer individuals survive to higher ages. This decline in survival causes older ages to matter increasingly less to lifetime reproductive success, and makes selection at older ages increasingly powerless (Baudisch 2008a). Put more simply, Medawar observed that the strength of selection declines with age (Medawar 1952).

To better understand this notion, consider two deleterious mutations with age-specific effects. Assume that one mutation has a negative effect on fitness early in life, while the other mutation has a negative effect on fitness late in life. The early-acting mutation will greatly reduce lifetime reproductive success, and will be strongly selected against. The late-acting mutation, however, will have a much smaller effect on lifetime reproductive success, as carriers of this mutation will have already reproduced and transmitted their genes to the next generation. As a result, the late-acting deleterious mutation will experience weaker selection, and will be less effectively removed from the population (Gavrilov and Gavrilova 2002). Huntington's disease can be seen as an example of such a phenomenon (Medawar 1952). Huntington's disease is a dominant lethal mutation with late onset at approximately 30 to 40 years of age. This late-onset allows carriers to reproduce before any effects are seen. As a result, Huntington's disease evades

the full force of selection and remains in the population (Haldane 1941; Medawar 1952; Weinert and Timiras 2003).

According to Medawar, because the effectiveness of selection diminishes with age, any mutations that arise in the germline with deleterious late-life effects will only be weakly selected against. These mutations will remain in the gene pool under mutation-selection balance and accumulate over many successive generations (Figure 1A). It is this load of deleterious late-acting mutations that Medawar suggested to cause senescence.

Antagonistic Pleiotropy

In 1957 G.C. Williams built upon Medawar's MA theory (Williams 1957). He suggested that deleterious late-acting mutations could be favored by selection and actively kept in the population if they have beneficial pleiotropic effects. His theory, called the theory of antagonistic pleiotropy (AP), assumes the existence of a special type of pleiotropic gene with opposite, or antagonistic, effects at different points in life. That is, a single gene may affect traits that are both beneficial to fitness early in life and detrimental to fitness late in life, or vice versa. For instance, a mutation that causes the overproduction of sex hormones may beneficially increase reproductive success early in life, but also cause cancer and decrease survival late in life (Gavrilov and Gavrilova 2002). Such genes represent a selective trade-off between their early life benefits and their late life costs. Williams points out that as a result of the declining strength of selection with age, the early-life advantages conferred by antagonistically pleiotropic genes can selectively outweigh accompanying late-life costs (Figure 1B). As a consequence, the active selection of early life benefits of antagonistically pleiotropic genes is suggested to cause the accumulation of associated harmful late-life effects and lead to senescence.

Disposable Soma

The AP theory put forth by Williams rests on the idea of an evolutionarily optimized trade-off between early and late life fitness, or more generally, between longevity and reproduction. In the 1970s T.B.L. Kirkwood further developed this theory by proposing an alternative trade-off mechanism for senescence, now termed the disposable soma (DS) theory (Kirkwood 1977). Kirkwood suggested that because resources are finite, organisms face trade-offs in the allocation of energy between the biological functions of growth, maintenance, and reproduction. Any energy that is put towards one function becomes unavailable for another. Given these trade-offs, selection optimizes energy allocation between functions to maximize fitness. An evolved limit on the energy allocated towards maintenance is suggested to cause somatic maintenance and repair mechanisms to be imperfect, and cellular and molecular damage to accumulate over time (Figure 1C). Accumulation of somatic damage over the lifespan of an individual is what Kirkwood suggests ultimately causes senescence (Kirkwood 2005).

Many different types of molecular and cellular damage are expected to contribute to senescence. This is because many different somatic maintenance and repair functions are thought to require significant amounts of energy and to be affected by energy allocation limits. That is, in addition to genetic damage, other damage such as telomere erosion, mitochondrial dysfunction, and epigenetic damage are also expected to contribute to senescence (Campisi and Vijg 2009; Kirkwood 2005).

Like AP, DS considers aging to be the consequence of the evolutionary optimization of a general trade-off between longevity and reproduction. For this reason, many regard DS to be a special case of AP. However, here we will treat DS and AP as related but separate theories, and

will discuss the differences in the proposed trade-off mechanisms and underlying causes of aging.

Testing evolutionary alternatives

The theories of MA, AP, and DS form the foundation of current thinking on the evolution of senescence (for further review of these theories see Gavrilov and Gavrilova 2002; Kirkwood 2005; Partridge and Barton 1993). Since these theories are not mutually exclusive, it is possible that all three play a role in the senescent process. Below we summarize the assumptions and predictions of the three evolutionary models and the results of previous empirical tests. Table 1 provides a summary of the partially contrasting assumptions and predictions discussed.

Heritability and genetic variation

A major assumption underlying all evolutionary theories of aging is that features of the aging process, such as age-specific fecundity and lifespan, are both heritable and variable, and can therefore be modified by natural selection. All three theories discussed here subscribe to this view, but differ in the manner by which genes control the process of aging and the predicted patterns of genetic variation in aging-related traits.

Under MA, the heritability of lifespan is predicted to be nonlinear, with increased lifespan heritability for offspring with longer-lived parents (Figure 2A) (Charlesworth 1994; Gavrilov and Gavrilova 2002). The equilibrium frequency of deleterious mutations is expected to increase with the age of mutation action due to a declining strength of selection. As a result of this increase in mutation frequency, genetic variation and heritability of lifespan are also predicted to increase with age (Figure 2B) (Charlesworth 1994; Gavrilov and Gavrilova 2002; Medawar 1952; Partridge and Barton 1993). Evidence for a nonlinear heritability of lifespan has

been found in humans using longevity data from European aristocratic families (Gavrilov and Gavrilova 2001a; Gavrilov and Gavrilova 2001b; Gavrilova et al. 1998). These studies found that the correlation between offspring and parental lifespan increased with increasing parental longevity, as predicted by MA. Data from aristocratic families was chosen to minimize social and environmental heterogeneity, as the same pattern of increasing heritability could be seen if those with longer-living parents simply live in more similar environments (Gavrilova et al. 1998). Under this alternative environmental hypothesis, the observed phenotypic variance for longevity is expected to be smaller for those born to longer-lived parents than those born to shorter-lived parents. No difference was found in longevity variance for offspring born to longer-lived parents compared to shorter-lived parents, suggesting that the observed patterns for lifespan heritability cannot be simply explained by differences in environmental variance (Gavrilova et al. 1998).

An additional prediction of MA is an age-dependent increase in inbreeding depression and genetic variance of fitness traits (Charlesworth and Hughes 1996; Hughes et al. 2002; Moorad and Promislow 2009). Since these measures are proportional to equilibrium allele frequencies, they are similarly expected to increase with age under MA due to a declining strength of selection (Charlesworth and Hughes 1996). Quantitative genetic experiments measuring age-specific fecundity in populations of *D. melanogaster* have found significant age-dependent increases in inbreeding depression, as well as additive and dominance genetic variances, as predicted under MA (Charlesworth and Hughes 1996; Hughes et al. 2002). These results, while initially thought to uniquely distinguish the role of MA, have also been suggested to be consistent with AP, confounding study conclusions (Moorad and Promislow 2009).

Unlike MA, AP makes no straightforward predictions about genetic variance (Zwaan 1999). Under different assumptions, AP may produce a wide range of patterns of genetic variation, from virtually non-existent to a pattern of variation resembling that expected under MA (Moorad and Promislow 2009). For instance, if the inheritance of a single allele with AP effects is advantageous to lifetime fitness, selection will drive the allele towards fixation, and extant genetic variation will be maintained only on the basis of mutational pressure. Alternatively, AP loci could instead maintain genetic variation through balancing selection if two alleles with opposite AP effects generate marginal overdominance (i.e. a pattern of heterozygote advantage when fitness is averaged over lifespan). In this case, because the age-related allelic effects are not necessarily equal, the dominance effects may change with age. An increase in dominance effects with age will cause inbreeding depression and additive and dominance genetic variances to increase. These effects will produce a pattern of genetic variation similar to MA (Moorad and Promislow 2009). Because a range of allelic effects may exist under AP, it is difficult to distinguish between the role of MA and AP on the basis of quantitative genetic experiments. Any result consistent with MA may also be consistent with AP.

All three of the evolutionary theories discussed here suggest senescence to be a polygenic trait (Kirkwood 1996). Genetic components of senescence have been investigated most frequently through genetic analyses of longevity. Many of these analyses have taken the form of genome-wide association studies (GWAS) using case-control methodology, where long-lived individuals are compared to younger individuals. Surprisingly, these studies have consistently revealed apolipoprotein E, or APOE, as the only genetic association observed at genome-wide significance (Deelen et al. 2013; Deelen et al. 2011; Nebel et al. 2011; Sebastiani et al. 2012); (for a detailed review of this topic see Brooks-Wilson 2013). It has been suggested that

additional genes of smaller effect may be found to achieve significance with larger sample sizes than have previously been used for GWAS of longevity.

While a relatively large body of literature has explored the heritability and genetic variation expected under MA and AP, the corresponding predictions under the special case of DS have been largely undefined and represent an area where further work is needed. Stearns suggests that genes affecting maintenance and repair processes that are implicated in aging based on DS may have antagonistically pleiotropic effects (Stearns 1992). Additionally, in a review of general evolutionary trade-offs, Roff and Fairbairn suggest both mutation-selection balance and antagonistically pleiotropic genes may play a role in the maintenance of variation in trade-offs (Roff and Fairbairn 2007).

Trade-offs

AP and DS differ from MA in their shared consideration of the role of trade-offs in evolution of senescence. Both suggest senescence to be the consequence of the evolutionary optimization of a general trade-off between longevity and reproduction, but differ in the proposed trade-off mechanisms. In the case of AP the trade-off is suggested to result from single genes with opposite effects on early and late life fitness, while with DS the trade-off is suggested to result from the allocation of finite energy resources between different life-history traits.

Under both AP and DS, the trade-off between longevity and reproduction is predicted to result in a negative correlation between early and late life fitness traits. Selection for late life fitness is expected to result in decreased early life fitness and vice versa. These specific trade-off predictions have been extensively investigated via laboratory selection experiments. Rose and Charlesworth demonstrated that selection for increased late-life reproduction resulted in decreased early-life fertility and increased longevity in *Drosophila melanogaster,* as predicted by

AP/DS (Rose 1984; Rose and Charlesworth 1980; Rose and Charlesworth 1981a; Rose and Charlesworth 1981b). These results were corroborated by a similar study examining the lifespan effects of selection for late vs. early reproduction (Luckinbill et al. 1984) and another study applying selection directly to longevity (Zwaan et al. 1995).

Other selection experiments on *D. melanogaster*, however, have been unable to find evidence supporting the existence of a trade-off. Partridge and Fowler, for instance, found selection for late life reproduction to result in increased longevity with no associated decrease in early life fecundity (Partridge and Fowler 1992). This result may be consistent with MA but does not refute the role of AP. Selection for late-life reproduction may act against late-acting deleterious mutations that affect both survival and reproduction, and uncover the effects of MA. However, an AP/DS trade-off may still be present. The negative result simply suggests that if present, the trade-off does not involve the measured trait of early-life fecundity or cannot be measured in the laboratory environment.

Other more recent studies have suggested a diminished importance for AP in explaining the observed life history trade-off between longevity and reproduction (Khazaeli and Curtsinger 2010; Khazaeli and Curtsinger 2013). These studies, conducted by Khazaeli and Curtsinger, used recombinant inbred lines of *D. melanogaster* to show lifespan and early life fecundity to be genetically separable. Recombinant genomes were created from parental lines selected for longevity via late reproduction and unselected controls. Since pleiotropic effects are unaffected by recombination, this experimental method allowed the pleiotropic nature of the genes underlying the trade-off between longevity and reproduction to be tested. If AP genes significantly mediate the trade-off, a negative correlation between early and late life fitness traits is expected in both parental and recombinant lines. If, however, the trade-off is dominated by

non-pleiotropic genes, recombination is expected to create new phenotypes that exhibit a positive correlation between the early and late life fitness traits that were negatively correlated in parental lines. Khazaeli and Curtsinger found flies in the recombinant inbred lines that demonstrated both long lifespan and high early life fecundity. This result indicates that reproduction and longevity can be genetically separated, and suggests that AP does not fully explain the observed tradeoff between longevity and reproduction.

The inconclusive results of the discussed selection experiments regarding support for a specific evolutionary model exemplify the difficulties of devising differentiating tests due to non-contrasting theory predictions. In the case of the general trade-off between reproduction and longevity, results that are found to be consistent with AP/DS do not simultaneously discredit the role of MA in the evolution of senescence and vice versa. MA suggests genes with late-acting detrimental effects to be neutral in early life, with no implied trade-off or correlation between early and late life fitness. This indicates that even if MA plays a prominent role in the evolution of senescence, its role may not be observable through these methods. Furthermore, experimental results that do not provide support for a trade-off do not dismiss the role of AP/DS in favor of MA. Rather, such results may suggest that the experimental environment may be inadequate for the measurement of a particular trade-off or that the measured fitness traits are uninvolved, but do not disprove the existence of the trade-off (Charlesworth 1990; Stearns 1989).

Demographic studies have shown similarly mixed evidence for the trade-off between longevity and reproduction in humans. Some have reported significant negative correlations between number of offspring and postmenopausal longevity (Doblhammer and Oeppen 2003; Westendorp and Kirkwood 1998), while others have reported positive correlations (Gogele et al. 2011; Le Bourg et al. 1993; Muller et al. 2002). The validity of the results supporting an AP/DS

trade-off between longevity and reproduction in humans, however, has been criticized due to data and methodological issues. The data used by Westendorp and Kirkwood, for instance, was not cross-checked for accuracy, resulting in the analysis of a dataset that contained an unrealistically high percentage of childless women (Gavrilova and Gavrilov 2005). Additionally, Doblhammer and Oeppen were unable to find evidence of a trade-off through standard data analysis. Only after childless women and women with one child were removed from the analysis was a trade-off between longevity and reproduction observed (Gavrilova and Gavrilov 2005). Furthermore, examination of the relationship between longevity and infertility, rather than number of offspring, has been suggested to be less susceptible to confounding by social factors and more informative for tests of evolutionary theories (Gavrilova and Gavrilov 2005). Studies comparing the longevity of parents versus non-parents have found either no significant difference (Gavrilova and Gavrilov 2005; Gavrilova et al. 2004) or increased longevity for parents over those that are childless (Chereji et al. 2013). These results better support alternative hypotheses, such as the reproduction potential hypothesis where the presence of a young child has a life-lengthening effect on a mother (Muller et al. 2002), rather than an AP/DS trade-off between longevity and reproduction. These results also align with classic Darwinian theory such that fitter individuals are more likely to contribute more offspring to the next generation.

In addition to empirical testing, a large body of theoretical work has investigated the role of trade-offs in senescence. In particular, mathematical optimization models have been extensively used to understand the conditions under which senescence exists as an evolutionarily optimal life history trait given particular trade-off constraints. The trade-offs investigated using these models are generally of the type suggested by DS – an energy allocation trade-off between the life-history traits of growth, reproduction, and maintenance. Numerous optimization models

have been used to confirm and clarify predictions and assumptions of DS. Many have demonstrated that senescence, often defined as an age-dependent increase in mortality and decrease in fertility, is indeed a possible byproduct of the evolutionary optimization of the DS suggested trade-off between maintenance and reproduction (Abrams and Ludwig 1995; Baudisch 2008b; Cichon 1997; Cichon 2001; Cichon and Kozlowski 2000; Kozlowski 1996). In general, optimization approaches to test theories of senescence create functions to relate energy allocation trade-offs and extrinsic mortality levels to fertility and mortality rates. These functions are then optimized to maximize lifetime reproductive success, akin to the action of selection, and to find optimal resource allocation schemes and resulting age-trajectories of mortality and fertility. This general method has been used to demonstrate that the evolutionary optimization of trade-offs between maintenance, growth, and reproduction can, under some conditions, result in senescent mortality and fertility age-trajectories that are consistent with data. Under alternative conditions, many of these models have also shown optimization to result in sustenance and negative senescence, where mortality and fertility rates remain constant or increase with age respectively (Baudisch 2012; Baudisch and Vaupel 2010; Vaupel et al. 2004). Such results demonstrate the trade-off assumption of DS to be robust enough to describe a range of age-dependent fertility and mortality patterns that exist across the tree of life (Jones et al. 2013).

The results of optimization models depend on assumed conditions, such as level of extrinsic mortality and available resources, as well as the specific functional formulation of the trade-off. Models with different trade-off assumptions have been shown to result in different conclusions about the qualitative patterns of aging (Baudisch 2009; Baudisch 2012). Key assumptions include linear vs. nonlinear trade-offs and constraints on the shape of mortality and fertility curves (Baudisch 2012). Little is known about the exact biological nature of these trade-

offs, and, as a result, many functions are created on the basis of mathematical convenience rather than biological realism. Conclusions of optimization models should therefore be carefully examined with respect to the specific trade-off functions and biological assumptions.

The concept of a resource allocation trade-off between longevity and reproduction, as proposed by DS, has been challenged by the results of calorie restriction experiments. As discussed, DS assumes that senescence results from the allocation of finite energy resources between the biological functions of growth, reproduction, and maintenance and repair. Logically, if the total amount of energy available for allocation decreases, the amount of energy allocated towards somatic maintenance and repair should too, and senescence should be more rapid. However, calorie restriction experiments have shown the opposite to be true in a number of species, including rats, mice, flies and worms (Merry 2002). A large body of data now shows that a reduction in calories leads to an increase in longevity (Heilbronn and Ravussin 2003). Some theorists have explained this result as a shift in the relative fitness values of immediate reproduction and long-term survival. Limited food may signify a decreased probability for offspring survival, and a fitness advantage to delayed reproduction. This may translate to an increased investment in maintenance and repair when resources are scarce, compared to the optimal investment during times of plenty (Mitteldorf 2001; Shanley and Kirkwood 2000).

Extrinsic mortality

All three models suggest the evolution of aging to be driven by extrinsic mortality (Kirkwood 1977; Kirkwood 2005; Medawar 1952; Williams 1957). Populations facing high extrinsic mortality are predicted to age more rapidly than those experiencing low levels of extrinsic mortality. In the case of MA and AP, increased extrinsic mortality means fewer individuals survive to reproduce at later ages, resulting in a faster declining strength of selection,

accumulation of earlier-acting deleterious age-specific mutations, and ultimately more rapid senescence within the species. In the case of DS, high extrinsic mortality shifts the optimal energy allocation scheme. In an environment with high extrinsic mortality it becomes optimal to invest in reproduction over maintenance to maximize reproductive success during the organism's expectedly short lifespan. This decreased investment in somatic maintenance is predicted to cause somatic damage to accumulate at a faster rate and to translate to more rapid aging. Stearns and colleagues tested this prediction in populations of *D. melanogaster* (Stearns et al. 2000). Replicate populations subjected to different adult extrinsic mortality rates evolved lifespan differences in line with predictions. Populations with high adult mortality evolved shorter lifespans than populations with low adult mortality. Additionally, optimization models of Cichon and Kozlowski have shown levels of extrinsic mortality to directly affect the optimal energy allocation strategy (Cichon 1997; Cichon 2001; Cichon and Kozlowski 2000). When extrinsic mortality is low, increased allocation toward maintenance repair is optimal, slowing aging. However, Reznick and colleagues tested this prediction on wild populations of Trinidadian guppies, and found results contrary to prediction (Reznick et al. 2004). Populations of guppies experiencing high extrinsic mortality rates were found to exhibit longer lifespans in the laboratory than populations from low mortality environments. These unexpected results have been suggested to be artifacts of unanticipated selection for increased vitality due to extrinsic mortality caused by predation (Bronikowski and Promislow 2005). This exemplifies that both the existence of extrinsic mortality and the actual sources of extrinsic mortality may influence the evolution of senescence.

Heterogeneity in aging

Under DS, the diverse cellular and molecular damage that contributes to senescence is predicted to be stochastic, as it results from random failure events of somatic maintenance and repair mechanisms (Kirkwood 2005). This DS prediction of an inherently stochastic process of aging differentiates it from both AP and MA. Unrepaired damage due to both extrinsic and intrinsic factors is expected to accumulate stochastically within an individual, and to contribute to inter-individual variation, or heterogeneity, in aging. Here, we use the term heterogeneity in aging to refer to the idea that individuals in a population differ with respect to exactly how they age (e.g. pattern, rate, etc.), in addition to when they die.

Many of the investigations into heterogeneity and stochasticity in human aging have come from the field of demography (Steinsaltz et al. 2012; Yashin et al. 2000). Several analyses of demographic mortality data have incorporated notions of heterogeneity in aging, and have provided a framework for the consideration of mechanisms controlling inter-individual differences in aging and longevity (Li and Anderson 2009; Steinsaltz et al. 2012; Vaupel et al. 1979; Yashin et al. 2000). These models have been built on two different concepts of individual heterogeneity within a population. The first considers heterogeneity in lifespan to be caused by innate differences between individuals (Vaupel et al. 1979). In contrast, the second considers heterogeneity in lifespan to be caused by stochastically acquired differences in mortality risk (Gavrilov and Gavrilova 1990). These concepts have not yet been explicitly considered with respect to evolutionary models, but may provide a framework for the investigation of the evolution of heterogeneity in aging in the future. The first concept of heterogeneity in lifespan is potentially consistent with all three evolutionary models, while the second concept is uniquely consistent with DS.

DS predicts the pattern of inter-individual heterogeneity in aging to result from the stochastic accumulation of both genetic and non-genetic damage over the lifespans of individuals. Heterogeneity in rate of damage accumulation, and consequently lifespan, is likely dependent on both genetic and environmental factors, but the relative impact of each is unknown. DS may therefore be consistent with the first concept of heterogeneity if genetic differences between individuals impact mechanisms of maintenance and repair and lead to differences in the rate of damage accumulation and ultimately lifespan. Additionally, DS is uniquely consistent with the second concept of heterogeneity. This concept assumes differences in longevity to be mainly caused by the stochastic acquisition of different environmental or internal damage between individuals.

In general, evolutionary investigations into the stochastic nature of aging have been limited. A recent paper by Le Cunff et al. investigates the evolution of inter-individual differences in aging under the influence of a DS-type trade-off between longevity and reproduction (Le Cunff et al. 2013). This work relies on assumptions of the DS evolutionary model of senescence, but does not explicitly test its biological predictions. To our knowledge, the DS prediction of stochastic individual age-dependent accumulation of cellular and molecular damage has not yet been explicitly tested and provides a line of inquiry for future research. Statistical models combined with stochastic processes may provide a relatively straightforward method of testing this prediction if longitudinal data on damage contributing to senescence are available. Familial data may also provide additional insight into genetic vs. environmental sources of heterogeneity.

Species variation in aging

Wide variations in patterns of aging have been observed across the tree of life (Jones et al. 2013). Humans, for instance, experience age-dependent declines in fertility and increases in mortality with age (senescence), but species such as hydra experience constant mortality and fertility over all ages (sustenance) (Martinez 1998). Furthermore, other species, like mangrove trees, exhibit increasing fertility and declining mortality with age (negative senescence) (Jones et al. 2013). This variation is not directly predicted by the evolutionary theories of aging, as they were originally formulated to explain the anthropocentric pattern of senescence. MA and AP both assume a declining strength of selection with age. Under MA this causes the accumulation of a deleterious mutational load and inevitable senescence. Under AP, this causes antagonistically pleiotropic genes with early-life benefits and late-life costs to be selected for and the genes with the opposite effects to be selected against. Genes with AP effects will therefore only able to explain senescence and not other life history patterns. Of the three theories described here, DS is the most able to explain great variation in life histories. Under DS, different species may experience different trade-off functions and constraints, which may result in different optimal energy allocation strategies and different age-dependent patterns of fertility and mortality. At this point, little is known to explain why some species have evolved senescent life histories while others have not. In the future, comparative studies concentrating on the relationships between differences in the environmental pressures experienced by different species and differences in species fertility and mortality curves for a wide range of taxa may help to unravel what conditions lead to the evolution of senescence (Jones et al. 2013).

Methods of testing evolutionary alternatives: past approaches and future directions

A variety of methods have been used to test between the MA, AP, and DS models of senescence. Many of the previous attempts to distinguish between these alternatives have been largely inconclusive due to various non-contrasting model predictions. Here we summarize the conclusions drawn in the previous section in terms of the methods used to distinguish between evolutionary models of senescence.

Analysis of genetic variance: The measurement of age-specific genetic variance in aging-related fitness traits, such as age-specific fecundity and lifespan, has been proposed as a method for differentiating between MA and AP evolutionary models of senescence. MA clearly predicts an increase in inbreeding depression and additive and dominance genetic variances as a result of increasing equilibrium allele frequencies with age. AP, however, does not make clear predictions about genetic variation, and can been seen to be consistent with a wide range of patterns. For this reason, it is difficult to differentiate between the theories of MA and AP on the basis of analysis of genetic variance. Experimental analyses observing variation in fitness traits consistent with MA may also be consistent with AP (Moorad and Promislow 2009).

Selection experiments: Laboratory selection experiments have demonstrated responses to selection and the existence of trade-offs between early and late life fitness traits in accordance with predictions of MA, AP, and DS evolutionary models of senescence. As discussed above, these experiments have been shown to be capable of testing specific evolutionary predictions and providing support for individual models, but they are unable to exclude or distinguish between the alternatives. Taken together, the body of literature provides evidence in support of both MA and AP/DS models of senescence. These experiments have exclusively relied on observations of fecundity and lifespan to quantify and measure a manipulation's effect on senescence. In the

future it may be beneficial to complement selection experiments with other genetic analyses to better understand the genetic basis of evolutionary change related to senescence (Flatt and Schmidt 2009).

Mathematical models: Mathematical optimization models have been developed to demonstrate how MA and DS trade-offs can shape fertility and mortality curves and result in senescence. Typically, these models define and quantify senescence as an age-associated decline in fertility and increase in mortality. Assumptions of MA and DS are often analyzed using quantitative genetics and optimization models, respectively. To our knowledge, there have only been two cases where these adaptive and non-adaptive processes have been placed together into a single model (Charlesworth 1990; Danko et al. 2012). Charlesworth incorporated trade-offs into a quantitative genetics model, while Danko and colleagues added MA into a trade-off framework. Charlesworth's model demonstrated genetic correlations, or trade-offs, to be largely unaffected by MA, while the model of Danko and colleagues showed MA to play only a minor role in shaping life histories. Further extension of models combining assumptions of alternative evolutionary theories may help to understand the relative importance of each process in explaining senescence.

Statistical models and demographic data: Statistical models using demographic mortality and reproduction data have been used to test and provide support to predictions of heritability under MA and to test for trade-offs between longevity and reproduction under AP/DS. Additionally, statistical models have been used to assess heterogeneity in aging and investigate the importance of genetic and environmental factors in determining mortality (Yashin et al. 1994). These models have not yet been used to investigate evolutionary theories of aging, but may offer a framework for testing differing predictions surrounding patterns of phenotypic

heterogeneity in aging in the future. DS, for instance, may be differentiated from MA and AP if heterogeneity in aging can be better described by stochastic processes of damage accumulation over the lifetimes of individuals than by innate genetic differences between individuals. Several statistical models have been extended to include longitudinal data of many types in addition to survival data (Tan et al. 2013; Yashin et al. 2007). Mortality data describes only the ultimate outcome of aging, and does not adequately describe the process of aging occurring within individuals. The inclusion of longitudinal data on other measures of senescence would help to better describe aging as a process, and the addition of familial data could help elucidate the relative importance of the different heterogeneity-producing mechanisms proposed by evolutionary theory.

Beyond mortality: other useful measures of senescence for demographic studies

The methods for testing evolutionary theories of aging in humans have generally relied heavily on lifespan and mortality data (Nusbaum et al. 1996). Death is the ultimate result of aging, and lifespan is often assumed to represent a good quantitative estimate of senescence (Baudisch 2008a; Tartar 2001). However mortality data provides an incomplete measurement of senescence that may not be ideal to investigate all aspects and predictions of evolutionary models.

Lifespan acts as a single proxy measurement for an individual's rate of senescence, but provides no information on an individual's senescent state or "biological age" throughout life. Without such measures, investigations into intra- and inter-individual heterogeneity in aging become difficult. For instance, the specific DS prediction of stochastic damage accumulation over an individual's lifespan is not directly testable with only mortality data, but stochastic

models of change and other predictions involving individual rates of senescence can be tested if repeated measures of senescence are available. Furthermore, while familial lifespan data can be used to deduce the heritability of lifespan and test predictions under MA, without longitudinal measures of senescence the heritability of rate of aging, as suggested by DS, can only be inferred. Moreover data for contemporary populations may be incomplete since lifespan can only be observed for deceased members of a pedigree. Alternative measures of senescence and "biological age" may offer the necessary data to test these and other predictions of the proposed evolutionary models.

Biomarkers of aging are quantifiable parameters thought to be reflective of "biological age", or an individual's rate of functional decline (Baker and Sprott 1998; Johnson 2006). The American Federation for Aging Research suggests that a well-defined biomarker of aging should do the following: 1) predict the rate of aging and lifespan better than chronological age; 2) monitor a basic process underlying aging; 3) be repeatedly measureable in humans without causing harm; 4) work in both humans and laboratory animals for testing and validation (Baker and Sprott 1998; Johnson 2006). Previously proposed biomarkers of aging have included telomere length, mtDNA mutations, and levels of oxidative stress and inflammation (Johnson 2006; Simm et al. 2008). These quantitative measures have been suggested as biomarkers of aging due to their correlation with age, high inter-individual variation, and associations with age-related disease and mortality (Bekaert et al. 2005; Cawthon et al. 2003; Eshaghian et al. 2006; Harley et al. 1990; Mather et al. 2011; Trifunovic and Larsson 2008).

To our knowledge, biomarkers of aging have only been loosely connected to evolutionary models. Kirkwood and Kowald created a network model of cellular aging based on the DS prediction of multiple mechanistic causes, and they described processes underlying proposed

biomarkers (Kirkwood and Kowald 1997). Their model included mitochondrial damage, aberrant proteins, free radicals, and antioxidants. Simulations of this model suggested that it is plausible that multiple processes interact to collectively cause the overall process of cellular aging.

In future studies, it may be valuable to use data on biomarkers of aging as alternative or supplementary measures of senescence. This will allow investigations to focus on senescence as an evolved longitudinal process, rather than as a single mortality event. The use of biomarkers will facilitate tests of individual-level predictions of evolutionary models that are not feasible with the population-level measure of death rate. For example, longitudinal data on the senescent state of individuals will allow for direct modeling of the DS prediction of stochastic senescence-causing damage accumulation throughout life. Many of the previously used methods for testing evolutionary models of senescence (e.g., heritability estimates, selection experiments, and statistical models) can be altered to include biomarkers of aging as alternative or supplementary measures of senescence. For instance, selection experiments including additional longitudinal analysis of biomarkers of aging may help elucidate the relationship between AP/DS proposed trade-offs and the rate of senescent decline or damage accumulation. Such a relationship is suggested by DS, but its functional form has yet to be unraveled. Furthermore, analysis of longitudinal data on biomarkers of aging with statistical models can explicitly test the DS prediction that stochastic damage accumulation underlies intra- and inter-individual heterogeneity in the process of aging.

A new biomarker of aging: DNA methylation

Recent research on the epigenetics of aging in humans has led to the proposal of DNA methylation as a new biomarker of senescence (Brocklant et al. 2011; Hannum et al. 2013; Horvath 2013; Koch and Wagner 2011; Teschendorff et al. 2010; Teschendorff et al. 2013).

Epigenetics is the study of heritable DNA alterations that do not change DNA sequence. The most well-known epigenetic modification is DNA methylation, which involves the addition or removal of a methyl group from the 5-position of a cytosine base. DNA methylation is often associated with gene expression silencing, and typically occurs at CpG sites, where cytosine bases sit immediately adjacent to guanine bases (Jaenisch and Bird 2003; Razin and Riggs 1980).

Changes in DNA methylation and chronological age were first reported decades ago (Wilson and Jones 1983) and due to the recent availability of DNA methylation microarrays, a large body of literature now describes patterns of both hyper- and hypo-methylation with age across the human genome (Alisch et al. 2012; Bell et al. 2012; Bjornsson et al. 2008; Bollati et al. 2009; Christensen et al. 2009; Fraga and Esteller 2007; Hannum et al. 2013; Numata et al. 2012; Rakyan et al. 2010; Teschendorff et al. 2010; Xu and Taylor 2014). Nearly indistinguishable epigenetic markings of young identical twins have been observed to be increasingly different as a function of age, based on a cohort study of older vs. younger twins (Fraga et al. 2005; Martin 2005). The exact mechanisms driving these changes are not yet understood, but the influence of external environmental factors and internal stochastic events have been hypothesized (Fraga and Esteller 2007; Hannum et al. 2013). These observations have led researchers to investigate if DNA methylation patterns can be used as a biologically meaningful measure of senescence. Recently, two studies have demonstrated that age can be predicted from genome-wide DNA methylation data with extraordinary accuracy compared to previous biomarkers (Hannum et al. 2013; Horvath 2013). When tested in independent datasets, correlation between chronological age and predicted "methylation age" ranged from 0.91 – 0.96, compared to correlations of -0.52 and -0.74 between chronological age and telomere length in granulocytes and lymphocytes, respectively (Rufer et al. 1999). Measurements of methylation

age were strikingly consistent across individual cell and tissue types (Horvath 2013), suggesting that this predictor does not simply reflect changing cell type proportions with age, and can be generalized to many tissues. Methylation-based estimates of biological age can be used to estimate an individual's rate of aging (i.e. fast or slow) based on the ratio of observed to expected methylation given chronological age.

DNA methylation may be an appropriate biomarker of aging, as it meets three of the four published criteria established by The American Federation for Aging Research. Hannum and collaborators demonstrated that differences in methylation age calculated from whole blood samples are significantly associated with factors known to be relevant to senescence, such as gender and genetic variation, satisfying criteria two and three. Horvath similarly demonstrated observable age-associated methylation changes in human blood samples, and that age-related methylation changes in chimpanzees are highly correlated with those in humans, satisfying criterion four. Further work is needed to establish whether the remaining criterion holds: That is, whether measures of methylation age can predict lifespan and other measures of senescence better than chronological age (Horvath 2013). Early results suggest that this may be the case, as mortality risk in 2,100 individuals aged 40 to 92 was estimated to increase by 15% for each five-year increase in age acceleration (the difference between methylation age and chronological age), adjusting for chronological age (Chen et al. 2014; Gibbs 2014).

DNA methylation as a quantitative measure of senescence can be usefully applied to many evolutionary investigations, either through calculation of predicted methylation age or through site-specific analysis. Since methylation can be readily measured at thousands of CpG sites across the genome, the use of DNA methylation data uniquely enables evolutionary theories to be tested at numerous genomic locations. As the theories discussed are not mutually exclusive,

it is possible that different combinations explain the evolution of senescence in different portions of the genome. Analysis incorporating DNA methylation data will uniquely be able to assess the relative contribution of each theory across the whole genome and within specific sections.

Longitudinal DNA methylation data lends itself to the investigation of stochastic accumulation of damage proposed by DS. The possible stochastic nature of methylation and aging has never been explicitly modeled in a longitudinal framework in the same individuals. Such an analysis could help to elucidate the dynamic process of aging, and could be used to understand the relative importance of DS in evolution of senescence. Statistical analysis of mortality data alone has been shown to be inadequate to understand underlying factors causing observable heterogeneity in aging (Yashin et al. 1994). Age-associated methylation changes have been found to be widespread and reproducible across studies (Alisch et al. 2012; Bollati et al. 2009; Horvath 2013; Teschendorff et al. 2010), suggesting that longitudinal measurements of both methylation age and methylation at individual CpG sites could provide information useful for investigating stochastic intra- and inter-individual variation in aging.

Furthermore, familial DNA methylation data could be used to investigate the heritability of rate of aging. For example treatment of the deviation of methylation age from chronological age as a measure of biological fitness could be useful in testing the prediction of increasing heritability of fitness-related traits with age implied by the MA model. Similarly, heritability studies could be useful for distinguishing between genetic vs. stochastic sources of heterogeneity in somatic maintenance implied by DS. DS assumes the evolution and therefore heritability of energy allocation schemes, which generally dictate the level of somatic maintenance. As a result, the rate of damage accumulation, and therefore the rate of aging, can be expected to be at least somewhat heritable under DS. However, DS also suggests a potentially large environmental

component to aging, as environmental factors may also directly impact the type and quality of damage accumulation. Low heritability or decreasing heritability of fitness-related traits with age may therefore be characteristic of DS, and may differentiate it from MA and AP, which explicitly suggest a large genetic basis for senescence. This prediction and the corresponding predictions under MA and AP have not been formally defined or investigated and offer an interesting line of inquiry for future research.

Conclusions

Strides have been made in understanding the contributions MA, AP, and DS models make in explaining the paradox of senescence, but a consensus has not yet been reached. Clear and non-contrasting predictions are necessary to elucidate the relative importance of each model. However, because these models are not mutually exclusive, tests capable of simultaneously distinguishing between the actions and contributions of all these models are difficult to devise. As a result, empirical tests of these theories have focused on testing underlying assumptions and partially contrasting model-specific predictions. A variety of methods and empirical tests have provided support for portions of each individual model. There are, however, some predictions and assumptions that have received little or no attention. Such predictions may have been overlooked in the past due to limitations of defined measurements of senescence. Thus, the incorporation of longitudinal and/or familial biomarker data into future research as alternative or supplementary measurements of senescence may facilitate the investigation of previously neglected aspects of evolutionary models. Specifically, DNA methylation, a newly proposed measure of biological aging, may be useful in investigating intra- and inter-individual heterogeneity in the longitudinal process of aging as related to evolutionary models.

TABLES

Table 1. Assumptions and predictions of the MA, AP, and DS evolutionary models of senescence.

	Mutation Accumulation	Antagonistic pleiotropy	Disposable Soma
Basic assumptions of each model			
Adaptive vs. non-adaptive	Non-adaptive: senescence exists because selection is not strong enough to eradicate it	Adaptive: senescence is the byproduct of evolutionary optimization	Adaptive: senescence is the byproduct of evolutionary optimization
What causes senescence at the individual level?	Numerous late-acting deleterious alleles	The deleterious late-life effects of antagonistically pleiotropic genes	The progressive accumulation of multiple types of somatic damage
What is inherited?	Deleterious germline mutations with late-life effects	Deleterious germline mutations with antagonistically pleiotropic effects	An energy allocation strategy and a level of somatic maintenance
Mutations/ damage causing senescence are accumulated over…	Many generations	Many generations	An individual's lifespan
Predictions: Heritability and genetic variation			
Genetic variance	Inbreeding depression and genetic variances of fitness traits will increase with age due to an increase in equilibrium frequencies of deleterious alleles with age	AP genes may produce different patterns of genetic variance under different assumptions	Genes affecting maintenance and repair processes may have antagonistically pleiotropic effects and show similar genetic variation
Predictions: Trade-offs			
What trade-off is assumed?	None	Longevity vs. reproduction	Longevity vs. reproduction
Specific predictions based on trade-off	No trade-off predicted	Late-life fitness vs. early-life fitness, resulting from single genes that have opposite effects on early and late life fitness	Somatic maintenance vs. reproduction, resulting from finite energy resources. Energy allocation to one life-history trait necessarily detracts from another
Correlation between early- and late-life fitness	None	Negative correlation	Negative correlation
Selection for longevity causes…	Slowed or delayed senescence with no accompanying decrease in fitness in youth	Slowed or delayed senescence with decreased fitness in youth	Slowed or delayed senescence with decreased fitness in youth
Predictions: Extrinsic mortality			
High rates of extrinsic mortality are predicted to cause high rates of aging because…	Fewer individuals survive to reproduce at later ages, resulting in an faster declining force of selection	Fewer individuals survive to reproduce at later ages, resulting in an faster declining force of selection	Early reproduction becomes the optimal strategy. It is beneficial to invest in reproduction over maintenance due to decreased chances of survival

FIGURES

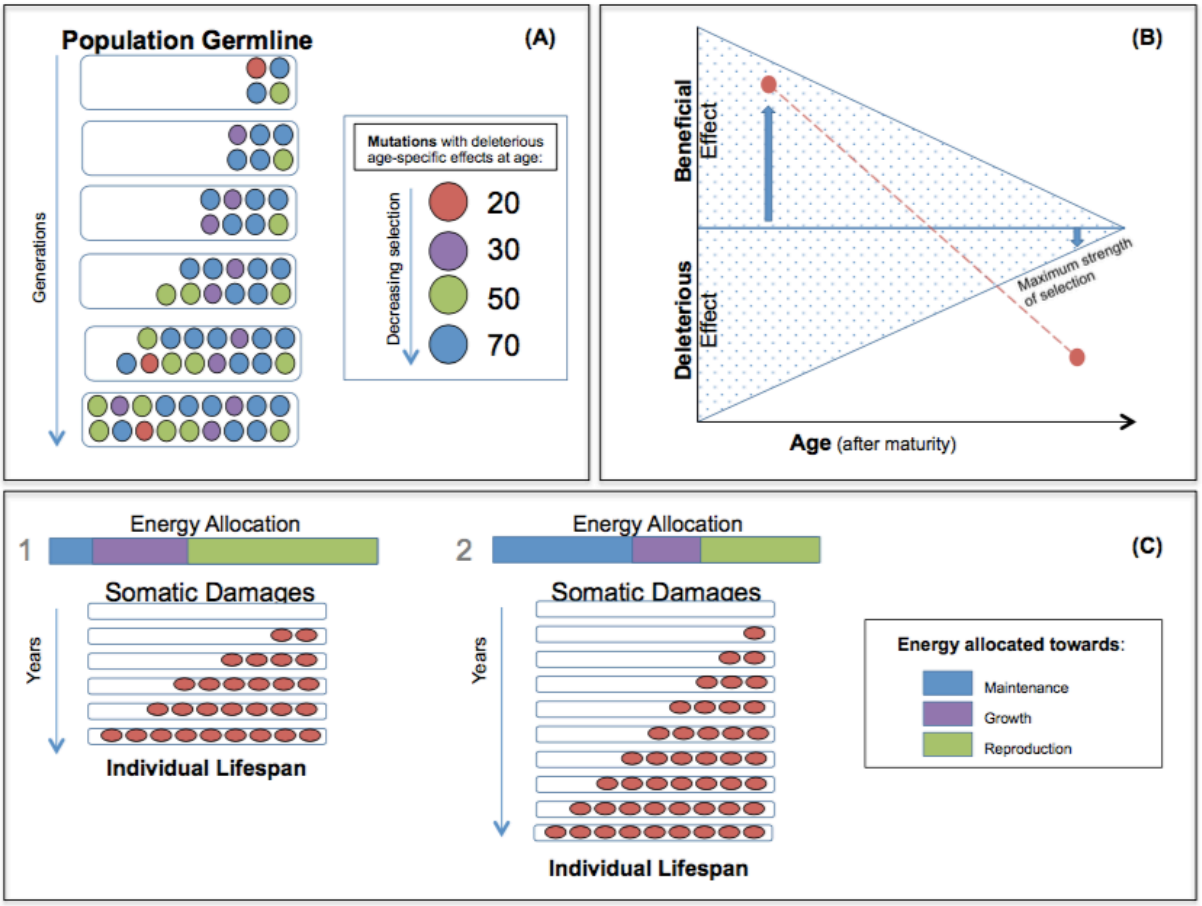

Figure 1. Evolutionary Theories of Aging. (**A**) Under the Mutation Accumulation theory of aging, mutations with later ages of action accumulate in the gene pool over many generations and grow to higher equilibrium frequencies due to declining strength of selection and a constant mutation rate. The deleterious mutation with age-specific effects at age 20 in the first generation is lost due to selection. A load of deleterious late-acting mutations collected throughout a population's history, shown in the last generation, is suggested to cause senescence. (**B**) Under the Antagonistic Pleiotropy theory of aging, the decline in the strength of selection with age (shown in blue) causes early-life benefits of antagonistically pleiotropic genes (shown in red) to selectively outweigh associated late-life costs. Active selection for the early-life benefits of antagonistically pleiotropic genes causes corresponding late-life costs to accumulate within the

germline, which leads to senescence. (**C**) Under the Disposable Soma theory of aging, trade-offs in the allocation of energy between biological functions causes maintenance and repair mechanisms to be imperfect. As a result, somatic damages accumulate over an individual's lifespan and cause senescence. Two energy allocation schemes are shown: (1) and (2). Allocation scheme (1) gives less energy to maintenance than allocation scheme (2). This causes somatic damages (in red) to accumulate at a faster rate and individual lifespans to be shorter.

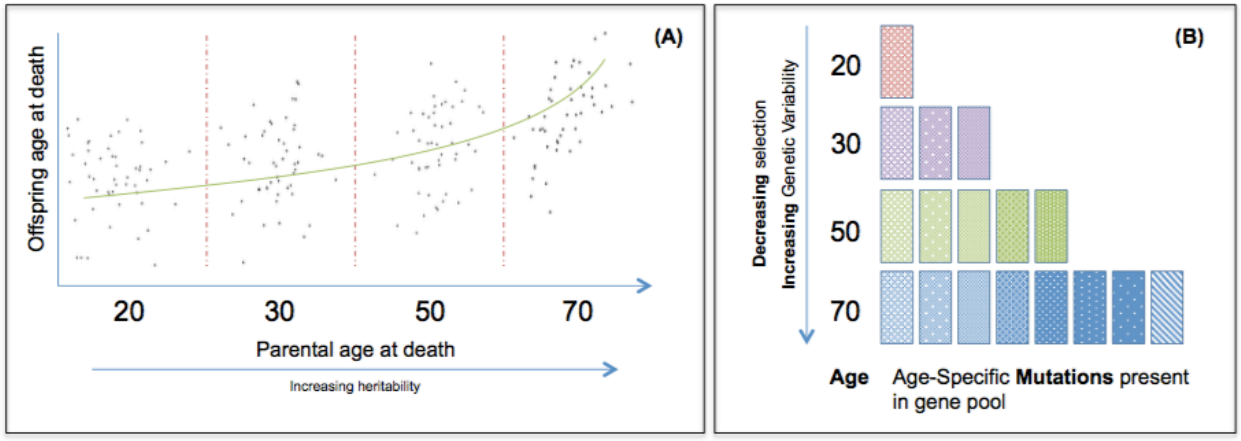

Figure 2. Prediction of Increasing Heritability of Lifespan Under Mutation Accumulation. (**A**) The heritability of lifespan is expected to increase with age under the theory of mutation accumulation (MA), as shown in the illustrative example. (**B**) According to MA, the genetic variability of age-specific mutations increases with age of mutation action due to a declining strength of selection. Mutations with effects at late ages experience only weak selection resulting in higher frequencies and higher genetic variability. Heritability is proportional to genetic variance, causing the heritability of lifespan to increase with age.

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Chapter 3: Testing two evolutionary theories of human aging with DNA methylation data

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INTRODUCTION

Evolutionary theories of aging

Aging is the progressive and general deterioration of an organism, defined by post-maturation declines in survival and fertility (Medawar 1952; Williams 1957; Hamilton 1966). Basic evolutionary theory suggests that such a trait should be selected against given its associated reductions in Darwinian fitness. However, seemingly paradoxically, aging is a universal feature of human life. The existence of aging remains an evolutionary puzzle.

Evolutionary theories provide possible explanations for the existence of human aging. Two major theories include: 1) mutation accumulation (MA) (Medawar 1952), and 2) antagonistic pleiotropy (AP) (Williams 1957), with disposable soma (DS) (Kirkwood 1977; Kirkwood and Holliday 1979; Kirkwood and Rose 1991) as a special case. Very generally, these theories suggest aging to occur because of a decline in the strength of selection with age. Even in the absence of aging, extrinsic mortality, or death due to external factors such as accident or starvation, causes fewer people to survive to higher ages (Medawar 1952). As a result, older ages matter increasingly less to lifetime reproductive success, and selection becomes increasingly ineffective (Hamilton 1966).

More specifically, MA suggests aging to be a non-adaptive consequence of the decline in the strength of selection with age (Medawar 1952). Mutations with deleterious effects confined

to late in life have only small impacts on fitness, as carriers likely reproduce before the onset of mutation action. Such mutations are hidden from the full force of selection and are essentially neutral. As a result, these mutations can grow to high frequency and accumulate within a population's germline over many generations (Hughes and Reynolds 2005). The resulting burden of late-acting deleterious mutations is suggested to cause aging under MA.

In contrast to MA, AP suggests aging to be an adaptive consequence of an evolutionary trade-off between survival and reproduction (Williams 1957). AP proposes the existence of a specific type of pleiotropic gene that has opposite effects on fitness at different ages. These genes are said to be antagonistically pleiotropic, and present a potential trade-off between early and late life, or survival and reproduction. Selection of these antagonistically pleiotropic genes depends both on the magnitude and timing of the opposing effects. Fitness advantages conferred early in life can easily selectively outweigh accompanying late-life costs due to the weakening strength of selection with age. AP suggests aging to be caused by the deleterious late-life effects of antagonistically pleiotropic genes that have accumulated in the population germline through active selection of their early-life benefits.

A special case of AP is DS (Kirkwood and Holliday 1979). Similar to AP, DS considers aging to be an adaptive consequence of the evolutionary optimization of a general trade-off between survival and reproduction (Kirkwood 1977; Kirkwood and Holliday 1979; Kirkwood and Rose 1991). In particular, DS suggests aging to result from trade-offs in the allocation of finite energy resources between biological functions such as growth, reproduction, and maintenance. Selection works to optimize energy allocation strategies to maximize fitness, and evolves energetic limits for each function. Given the limited amount of energy that can be allocated toward maintenance, mechanisms for somatic maintenance and repair mechanisms

cannot be perfect. Imperfect maintenance and repair mechanisms cause unrepaired cellular and molecular damages to accumulate over the lifetime of an individual. DS suggests aging to be caused by this accumulation of somatic damages throughout life.

DS is often considered to be a special case of AP due to their shared general trade-off framework. However, since AP and DS differ in their suggested trade-off mechanisms and underlying causes of aging (Kirkwood and Rose 1991; Kirkwood and Austad 2000), we consider them to be related but separate theories and investigate their predictions independently.

The theories of MA, AP, and DS are not mutually exclusive and large bodies of literature provide support for aspects of each individual theory (Gavrilov and Gavrilova 2002). It is possible that all three theories play some role in explaining the features and existence of human aging, but the relative importance of each theory has not yet been well established (Partridge and Barton 1993). A better understanding of each theory's contribution will help to clarify the roles of the environment and different types of genes in the aging process. Many of the methods previously used to test these theories have been able to provide support for one theory over another, but have not been able to speak to the size of the contribution each theory makes in explaining aging (Robins and Conneely 2014). Here, we have devised a unique test using DNA methylation data that will allow us to better understand the relative importance of MA and DS evolutionary models of aging.

DNA methylation and aging

DNA methylation is an epigenetic modification that is dynamic with age (Fraga and Esteller 2007; Bocklandt et al. 2011; Koch and Wagner 2011; Alisch et al. 2012; Hannum et al. 2013; Horvath 2013; Xu and Taylor 2014) and has been shown to be heritable in cross-sectional

family studies (McRae et al. 2014; Day et al. 2016). It involves the addition of a methyl group to the 5-position of a cytosine base, and typically occurs at a CpG site, where a cytosine base is directly followed by a guanine base. Functionally, DNA methylation of gene promoter regions is often associated with gene expression silencing (Razin and Riggs 1980; Jaenisch and Bird 2003; Bell et al. 2011).

Robust age-associated changes in DNA methylation occur throughout the genome (Fraga and Esteller 2007; Bocklandt et al. 2011; Koch and Wagner 2011; Alisch et al. 2012; Hannum et al. 2013; Horvath 2013; Xu and Taylor 2014). That is, numerous CpG sites consistently show variation in methylation between young and old ages. Many sites also show heritable patterns of methylation, where the measured level of methylation is more similar between closely related than unrelated individuals (Bell et al. 2012; McRae et al. 2014; Day et al. 2016). This suggests that a genetic component underlies the variation in methylation at these CpG sites.

An environmental or stochastic component to the variation in methylation is also suggested, as the nearly identical DNA methylation patterns of monozygotic twins at birth have been observed to diverge with age (Fraga et al. 2005; Martin 2005; Zampieri et al. 2015). This age-related divergence in the methylation patterns of relatives has been termed epigenetic drift (Teschendorff et al. 2013; Issa 2014; Sun and Yi 2015). The exact mechanisms driving these changes are not yet understood, but both external environmental and internal cellular events, such as imperfect methylation maintenance, have been hypothesized to contribute (Fraga and Esteller 2007; Hannum et al. 2013). The age-associated DNA methylation changes of epigenetic drift are suggested to be acquired stochastically (Jones et al. 2015), and align with DS (Kirkwood 2005).

DNA methylation has been suggested as a biomarker of human aging, or an easily repeatable measure that is descriptive of biological age (Baker and Sprott 1998). The genome-wide patterns of methylation have been observed to be dynamic throughout life, with the methylation at numerous CpG sites shown to have strong associations with age. These age-associated changes in methylation have been reported at thousands of sites across the genome in human blood samples (Alisch et al. 2012; Hannum et al. 2013; Horvath 2013; Xu and Taylor 2014). Furthermore, chronological age can be accurately predicted from the methylation measurements at just a few hundred of these CpG sites (Hannum et al. 2013; Horvath 2013). DNA methylation at these sites shows consistent changes with age across individuals, as well as cell and tissue types, and forms an "epigenetic clock" (Jones et al. 2015). Estimates of biological age derived from the methylation measurements at the clock-like CpG sites have been found to predict mortality better than chronological age (Marioni et al. 2015). Together these observations indicate that DNA methylation changes, both en masse and site-specific, reflect aspects of the aging process, and can be regarded as a biomarker of aging against which predictions of evolutionary theories can be tested.

Heritability of DNA methylation

In this study, we test the contrasting heritability predictions of MA and DS against familial DNA methylation data. We do not test heritability predictions of AP, as AP predicts a wide range of patterns of genetic variation, including patterns also expected under MA (Moorad and Promislow 2009). Unlike AP, specific predictions about genetic variation and heritability can be made for the theories of MA and DS, and these predictions are contrasting (Charlesworth and Hughes 1996; Kirkwood 2005; Moorad and Promislow 2009).

MA suggests the heritability of lifespan and other aging-related traits to increase with age. MA assumes aging to be caused by deleterious late-acting mutations that have accumulated in a population's germline over many successive generations due to a decline in strength of selection with age (Medawar 1952). Because of this weakening selection, the equilibrium population frequency of deleterious mutations is expected to increase with the age of onset of mutation action (Charlesworth 1980; Partridge and Barton 1993). Increases in the number of mutations in the population equate to increases in genetic variation. As a result of this increasing genetic variation, the heritability of lifespan and other features of aging is also predicted to increase with age (Charlesworth 1994; Gavrilova et al. 1998; Gavrilov and Gavrilova 2002). In contrast to MA, DS assumes aging to be caused by random somatic damages that accumulate over the lifetime of an individual (Kirkwood and Rose 1991). These damages result from random failure events of somatic maintenance and repair mechanisms and predict an inherently stochastic process of aging (Kirkwood 2005). This stochasticity under DS is expected to cause the phenotypic variation of aging-related traits to increase with age, which in turn causes the heritability of these traits to decrease with age.

If age-associated methylation changes are consistent with MA, we suggest that they mediate the age-specific effects of deleterious genetic mutations, and that the heritability of DNA methylation will increase with age. In contrast, if age-associated methylation changes are consistent with DS, we suggest that they result from stochastic failures in maintenance and repair mechanisms, and that the heritability of DNA methylation will decrease with age. In this study we test for increasing and decreasing heritability of DNA methylation at age-differentially methylated CpG sites. Sites where the heritability of methylation increases with age will be

considered consistent with MA, while sites where the heritability of methylation decreases with age will be considered consistent with DS.

The existence of widespread changes in DNA methylation with age is potentially consistent with both MA and DS. Our novel use of DNA methylation data in testing these theories allows us to categorize individual CpG sites across the genome as consistent with either MA or DS, or inconsistent with both theories, and to assess the ability of each theory to explain the DNA methylation changes observed in aging (Robins and Conneely 2014).

Additionally, we test for the existence of a heritable rate of aging, which is consistent with both MA and DS. Under DS, genes regulating the accuracy of somatic maintenance and repair are suggested to dictate the rate of somatic damage accumulation and imply a heritable rate of aging that is constant throughout life. Under MA, deleterious genes with age-specific effects are suggested to cause aging, and imply a heritable rate of aging that is potentially variable throughout life (e.g. slow at young ages, fast at older ages). The prediction of a heritable rate of aging can be tested using a methylation-derived measure of the aging rate. An individual's age can be estimated from DNA methylation levels at 353 CpG sites via a predictive linear model developed by (Horvath 2013). The difference between an individual's methylation-estimated age and chronological age provides a measure called age acceleration that describes that individual's rate of aging (i.e. fast or slow). Here we estimate the heritability of age acceleration using familial DNA methylation data. A significant non-zero heritability of age acceleration will indicate that changes in DNA methylation across a few hundred CpG sites are consistent with evolutionary models of aging. This will allow us to see if the patterns observed at the level of single CpG sites extend to a larger scale across the genome.

MATERIALS AND METHODS

Overview of hypotheses to be tested

We tested for increasing or decreasing heritability of methylation with age using a variance components model. At each CpG site we tested three specific hypotheses:

1) Age is a predictor of methylation level;

2) Methylation level has a heritable component;

3) The heritability of methylation has an age-dependent component.

Testing the first and second hypotheses allowed us to define a set of age-differentially-methylated CpG sites and a set of CpG sites with heritable methylation levels for further investigation. To test the third hypothesis, we restricted the analyses to sites that are age-differentially-methylated and have heritable methylation levels. Testing this hypothesis allows us to directly test for increasing or decreasing heritability of methylation with age, and to determine which CpG sites have age-associated methylation changes that are consistent with MA or DS.

Familial DNA methylation data

DNA methylation was measured in a sample of 610 individuals from 176 different families recruited for the Brisbane Systems Genetics Study (BSGS) (Powell et al. 2012). These families are all of European descent, and are comprised of adolescent MZ and DZ twin pairs, their siblings, and their parents. The age distribution for these individuals ranges from 10 years to 75 years, and has a mean age of 21 years (Figure S1).

Measuring DNA methylation

DNA methylation was measured from blood samples using the Illumina Infinium HumanMethylation450 Beadchip (Bibikova et al. 2011). This array interrogates a total of 482,421 CpG sites and 3,156 non-CpG sites across the genome using a bisulfite DNA treatment and two sets of site-specific probes binding associated methylated and unmethylated sequences (Triche et al. 2013). The proportion of DNA strands methylated at any particular site was estimated as the measured intensity of fluorescent signal from methylated probes relative to the intensity of fluorescent signal from both methylated and unmethylated probes. This ratio, with the addition of a stabilizing constant of 100 to the denominator, is referred to as a β-value. Each individual sample was measured on a randomly assigned chip and at randomly assigned position within that chip to avoid potential confounding due to family membership (McRae et al. 2014).

DNA methylation data quality control

The measured methylation state of a CpG site can be directly affected by the underlying DNA sequence. If there is a genetic variant at the cytosine or guanine of a CpG site, for instance, the site cannot be methylated. Furthermore, a genetic variant in the sequence probed by the array can impact array binding affinity and bias the measured level of methylation. To minimize the impact of these direct genetic effects on our estimates of DNA methylation heritability, we removed CpG sites with SNPs present on the 50-base CpG site probe before performing heritability analyses. Sites with underlying SNPs were identified based on data from the 1000 Genomes Project phase I release, as annotated by (Barfield et al. 2014). We further cleaned the data by removing probes annotated as binding to multiple chromosomes, probes without CpG sites, and probes with more than 11 individuals with missing data or more than 5 individuals

with detection P-values > 0.001 (McRae et al. 2014). After cleaning, a total of 373,006 probes remained for testing. A chart illustrating the data cleaning process is provided in the supplementary information (Figure S4).

Each probe was residualized using a generalized linear model with a logistic link function similar to that used by (McRae et al. 2014). The covariates in our model included chip, position on chip, and estimated proportions of the following cell types: CD8-positive T cells; CD4-positive T cells; natural killer cells; B-cells; monocytes; and granulocytes. The cell type proportions were estimated from the methylation array data using a method proposed by (Houseman et al. 2012) and reference data on the methylation signatures of purified cell types (Reinius et al. 2012). We included these estimated cell type proportions as covariates in the model to avoid potential confounding due to the heterogeneous and changing cellular composition of whole blood. After residualizing each probe we removed outlying measurements more than five interquartile ranges for the nearest quartile. (McRae et al. 2014) found these outlying data points, likely caused by rare genetic variants or measurement errors, to have a large influence on heritability estimates. After removing outliers, the residuals from the above model were used as the phenotype for all heritability analyses.

Modeling changes in the heritability of DNA methylation with age

To identify CpG sites consistent with each evolutionary theory we fit the familial BSGS data to a model of age and methylation that takes into account family structure and other relevant covariates.

For each CpG site we modeled methylation as a function of age and sex

$$X_{it} = \mu + \beta_{age}t + \beta_{sex}male + \epsilon_{it} \qquad (1)$$

where X_{it} represents the β-value for individual *i* at age *t,* residualized on chip, position on chip, and cell type proportions, and ϵ_{it} is an error term representing genetic and environmental variation. The covariance matrix for ϵ_{it} was parameterized similar to Diego *et al.* (Diego et al. 2003) with ϵ_{it} and ϵ_{js} representing the error terms for relatives *i* and *j* at ages *t* and *s*.

$$Cov(\epsilon_{it}, \epsilon_{js}) = \begin{cases} \sigma_g^2(t) + \sigma_e^2(t) & i = j \\ 2\phi_{ij}\sigma_g(t)\sigma_g(s) & i \neq j \end{cases} \qquad \begin{matrix} \sigma_g^2(t) = e^{\alpha_g + \gamma_g t} \\ \sigma_e^2(t) = e^{\alpha_e + \gamma_e t} \end{matrix} \qquad (2)$$

In this model, $\sigma_g^2(t)$ is the genetic variance at age *t*, $\sigma_e^2(t)$ is the environmental variance at age *t*, and ϕ_{ij} is the kinship coefficient between individuals *i* and *j*. The variance in this model is partitioned such that baseline heritability is reflected by α_g, while the dependence of heritability on age is reflected by γ_g. Similarly, the baseline proportion of phenotypic variance due to environmental variance is reflected by α_e, while the dependence of that proportion on age is reflected by γ_e.

For each CpG site we implemented this model in SOLAR (Almasy and Blangero 1998), a statistical genetics software package, and tested our specific hypotheses by adding restrictions to the general model described above. Each hypothesis was tested using a likelihood ratio test (LRT), comparing the fit of a full model to the fit of a restricted model. At each CpG site we tested three specific hypotheses: 1) age is a predictor of methylation level; 2) methylation has a heritable component; 3) the heritability of methylation has an age-dependent component.

To test the first two hypotheses, we did not partition the genetic and environmental variance into baseline and age-dependent terms as shown in the general model above. The full and restricted models used to test these hypotheses included σ_g^2 and σ_e^2 with no age-dependence (i.e., γ_g and γ_e set to 0), while the full and restricted models used to test the third hypothesis included $\sigma_g^2(t)$ and $\sigma_e^2(t)$ with age-dependence as shown in equation (2). To test Hypothesis 1 we tested the restriction that β_{age} = 0. Sites where β_{age} was found to be significantly non-zero after multiple test correction (FDR<0.05) were defined as age-differentially-methylated. To test Hypothesis 2 we tested the restriction that σ_g^2= 0. Sites where σ_g^2 was found to be significantly non-zero after multiple test correction (FDR<0.05) were designated as heritable.

We limited tests of Hypothesis 3 to CpG sites that are both age-differentially-methylated and moderately heritable, with h^2 > 0.2. To test Hypothesis 3 we fit two separate restricted models, with γ_g and γ_e independently restricted to zero. These two restrictions allowed us to test for age-dependent components in both genetic and environmental variance. This is necessary as heritability depends on both genetic and environmental variance, and age-dependent changes in either genetic or environmental variance will cause age-dependent changes in heritability.

We performed simulations to estimate the type I error rate and our power to detect age-dependent changes in genetic and environmental variance (for more information see Supplementary Methods). Our results indicate that our modeling approach has appropriate levels of type I error and that our power to detect age-dependent changes is approximately equivalent for the genetic and environmental variances (that is, power is similar for tests of the restrictions γ_g= 0 and γ_e = 0).

Estimating the heritability of rate of aging using DNA methylation

We used a linear model developed and tested by Horvath to predict age using DNA methylation data from 353 CpG sites (Horvath 2013). The sites included in the model were selected using elastic net regression, and have been shown to accurately predict age across many cell and tissue types. We estimated methylation-derived ages for all 610 BSGS individuals with Horvath's model and unresidualized β-values. After estimating methylation age, we calculated age acceleration, defined as the difference between methylation age and chronological age. Positive values of age acceleration suggest an increased rate of aging (i.e. fast aging), while negative values suggest a decreased rate of aging (i.e. slow aging).

To estimate the heritability of rate of aging, we modified the model shown in equations (1) and (2) to include age acceleration as the outcome. As described earlier, we tested for a heritable component by restricting σ_g^2 to zero and comparing the full and restricted models with a LRT. The model was implemented in SOLAR (Almasy and Blangero 1998).

Annotation for genomic features

All CpG sites were annotated with respect to the following genomic features: CpG islands, CpG shores, CpG shelves, strong promoters, weak promoters, poised promoters, strong enhancers, weak enhancers, insulators, transcription factor binding sites, and CTCF binding sites. For this annotation we used three data sets downloaded from the UCSC table browser for GRCh37/hg19 (ENCODE Project Consortium, 2012) (Karolchik et al. 2004): 1) CpG Islands (Gardiner-Garden and Frommer 1987); 2) Broad ChromHMM for GM12878 (Ernst and Kellis 2010); 3) Transcription factor ChIP V3 (transcription factor binding sites) (Consortium 2012). Each CpG site was annotated based on overlaps between the CpG location and the intervals of

the genomic features provided by the UCSC data sets. We defined CpG island shores to be 1.5kb out from CpG islands, and CpG island shelves to be 1.5kb out from shores. For all other genomic features we adopted corresponding ChromHMM category definitions presented in (Ernst et al. 2011).

Replication data

Familial DNA methylation data from the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study, previously described by (Corella et al. 2007; Irvin et al. 2010; Wagner et al. 2016), were used to replicate the results of our analyses. Families with at least two siblings were recruited from participants in the National Heart, Lung, and Blood Institute Family Heart Study in Minneapolis, MN and Salt Lake City, UT (Hidalgo et al. 2014). DNA methylation data were available for 1050 individuals from 182 families. The age distribution for these individuals ranges from 18 years to 88 years, and has a mean of 49 years (Figure S5).

DNA methylation was measured from isolated CD4+ T cells using the Illumina Infinium HumanMethylation450 Beadchip (Bibikova et al. 2011). This chip was also used to measure DNA methylation from the BSGS blood samples, and is described in more detail in the 'Measuring DNA methylation' section above. β-values with a detection P-value > 0.01, samples with more than 1.5% of probes missing, probes for which more than 10% of the samples had inadequate intensity, and probes mapped to more than one location or a location not matching the annotation file were removed prior to analysis (Hidalgo et al. 2014). After these quality control steps, 991 individuals and 461,281 CpG sites remained. As these data were originally prepared for another study, the quality control criteria and specific sites considered suitable for analysis

slightly differ between the GOLDN data and our original BSGS data. Only GOLDN data that passed the described quality control criteria were available for our replication analyses.

Expression data quality control and annotation

Gene expression levels were measured from the BSGS blood samples using Illumina HT12-v4.0 bead arrays (Powell et al. 2012; Powell et al. 2013). These arrays contain 47K probes designed to cover all well-characterized genes, gene candidates, and splice variants (Illumina 2011). Expression was measured for all 610 individuals for whom DNA methylation levels were also measured. Each individual sample was measured on a randomly assigned chip and at a randomly assigned position to avoid any potential confounding.

Before beginning our analyses, we removed probes where less than 10% of the samples had a detection P-value < 0.05, as well as probes with overlapping SNPs and probes of low quality (i.e. probes unlikely to match the target transcript due to sequence mismatches or sequence matches at multiple locations), as annotated by (Barbosa-Morais et al. 2010). After cleaning, the expression values of the remaining 13,222 probes were log transformed for analysis.

Gene information was annotated to each expression probe using Refseq transcript exon intervals downloaded from the UCSC table browser for hg19. For exons of the same gene with overlapping intervals, the union of the intervals was taken for consistency. Refseq gene information was annotated to an expression probe using the R Bioconductor package GenomicRanges (Lawrence et al. 2013), if there was more than a 25bp overlap between the probe and exon interval.

Identifying changes in expression associated with age-related changes in DNA methylation

To test for associations between changes in age-related methylation changes and changes in expression we modified the model shown in equations (1) and (2) to include log transformed expression values as the outcome and methylation as a covariate. For each expression probe

$$E(Y_{it}) = \alpha + \beta_{age}t + \beta_{sex}male + \beta_{CpG_j}X_{ij} \qquad (3)$$

where Y_{it} describes the expression level for individual *i* at age *t*, and X_{ij} describes the residualized methylation level for individual *i* at CpG site *j*. We implemented the model in SOLAR (Almasy and Blangero 1998), and tested if methylation level was a predictor of expression level by restricting β_{CpG_j} to zero for each CpG site *j* and comparing the full and restricted models with a LRT. β_{CpG_j} describes the change in gene expression associated with CpG site *j* becoming fully methylated from a fully unmethylated state.

Data Availability

Data from BSGS are archived at the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/), accession numbers GSE56105 (DNA methylation) and GSE53195 (gene expression). DNA methylation data from GOLDN are archived on the database of genotypes and phenotypes (dbGaP; https://www.ncbi.nlm.nih.gov/gap), accession number phs000741.v1.p1.

RESULTS AND DISCUSSION

Age-differentially-methylated CpG sites

In total, 91,261 CpG sites (24% of CpG sites tested) had significant association with age after multiple test correction (FDR<0.05). Of these sites, 47% (43,029 CpG sites) had significant increases in methylation with age, while 53% (48,232 CpG sites) had significant decreases in methylation with age (Figure 1). The large number of CpG sites showing age-differential-methylation is consistent with results observed in other epigenome-wide association studies for age. For example, we tested 600 CpG sites previously shown to have significant association with age across four independent data sets (Xu and Taylor 2014). Of these 600 CpG sites, 582 (97%) were age-differentially-methylated in a concordant direction in our data after multiple test correction. Additionally, we tested 290 of the 353 CpG sites that were included in the age prediction model of (Horvath 2013). Of these 290, 144 (~50%) of these sites were significantly age-differentially-methylated after multiple test correction.

Heritability of DNA methylation

We estimated the average heritability of DNA methylation across all CpG sites to be 0.177 (Figure 2). A heritability of 0 was estimated at 20% of sites (72,927 sites), and significant non-zero heritability was estimated at 38% of sites after multiple test correction (142,169 sites, FDR<0.05). After restricting to age-differentially-methylated CpG sites, the average estimated heritability of methylation increased to 0.272 (Figure 2). These heritability estimates are consistent with the results previously reported by (McRae et al. 2014).

We performed Fisher's exact tests to test if age-differentially-methylated sites are more likely than other sites to have heritable methylation levels. Specifically, we examined the overlap between sets of 1) age-differentially-methylated CpG sites and 2) CpG sites with heritable methylation, and tested whether this overlap is greater than expected by chance. These sets were defined after multiple test correction at varying significance levels, to ensure that results do not depend on a specific α-level (Figure 3). We observed significant overlap between the sets of age-differentially-methylated sites and sites with heritable methylation at all significance levels, indicating that age-differentially-methylated CpG sites are more likely to have heritable methylation than other sites. This observed enrichment indicates a potential genetic basis for age-related DNA methylation changes.

Our models adjusted for both age and family structure simultaneously, so the overlap between age-differentially-methylated and heritable sites is unlikely to be due to confounding between age and family structure. Furthermore, to ensure that this overlap did not reflect differences in statistical power among CpG sites with high vs. low variability, we compared the variance of methylation across sites. We observed similar distributions for the variance of methylation in the following sets of CpG sites: age-differentially-methylated sites with heritable methylation vs. age-differentially-methylated sites with non-heritable methylation; and non-age-differentially-methylated sites with heritable methylation vs. non-age-differentially-methylated sites with non-heritable methylation (Figure S6). Importantly, sites that were both age-differentially-methylated and heritable did not appear to have increased variability in methylation. This shows that the overlap between age-differentially-methylated sites and sites with heritable methylation is not simply driven by differences in phenotypic variation.

Changes in the heritability of DNA methylation with age

Under our model, described by equation (2), heritability can be defined as

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2+\sigma_e^2} = \frac{e^{\alpha_g+\gamma_g t}}{e^{\alpha_g+\gamma_g t}+e^{\alpha_e+\gamma_e t}} \quad (5)$$

where *t* represents age. Given this simplifying definition, we consider sites to be consistent with MA if: 1) γ_g is positive; and 2) γ_g is greater than γ_e. At these sites the increases in genetic variance with age are greater than the changes in environmental variance, which is suggestive of increasing heritability of DNA methylation with age. We consider sites to be consistent with DS if: 1) γ_e is positive; and 2) γ_e is greater than γ_g. At these sites the increases in environmental variance with age are greater than the changes in genetic variance, which is suggestive of decreasing heritability of DNA methylation with age. For simplicity and brevity, we will call sites found to be consistent with MA or DS based on the above criteria MA-consistent or DS-consistent respectively.

Our confidence in the estimated direction of heritability change with age for each site, and the resulting theory classification, increases with the significance of the γ_g and γ_e terms. We have increased confidence in the classification of a site as consistent with MA when that site has a significantly non-zero and positive γ_g (FDR<0.05). At these sites there are significant increases in the genetic variation underlying the variation in DNA methylation with age. Similarly, we have increased confidence in the classification of a site as consistent with DS when that site has a significantly non-zero and positive γ_e (FDR<0.05). At these sites there are significant increases in the environmental variation underlying the variation in DNA methylation with age. We are most confident in the classification of sites where both γ_g and γ_e are significant (FDR<0.05), as

in these cases we are confident in the direction of change for both the genetic and environmental variation underlying the variation in DNA methylation at those sites.

Because of this, we have divided the sites we found to be consistent with MA and DS into three different groups on the basis of the significance of the γ_g and γ_e terms (Table 1). Group 1 contains all sites consistent with MA or DS, irrespective of significance. In total, 13,467 sites were found to be consistent with MA, while 30,749 sites were found to be consistent with DS. Group 2 contains sites that are consistent with MA and have a significant γ_g term (FDR<0.05), and sites that are consistent with DS and have a significant γ_e term (FDR<0.05). A total of 102 sites found to be consistent with MA have significant γ_g terms, while a total of 2,266 sites found to be consistent with DS have significant γ_e terms. Group 3 contains sites that are consistent with MA or DS and have significant γ_g and γ_e terms (FDR<0.05). A total of 70 sites found to be consistent with MA have significant γ_g and γ_e terms, while a total of 203 sites found to be consistent with DS have significant γ_g and γ_e terms.

Figure 4 compares the significance and sign of γ_g and γ_e at each site, and shows the separation of sites into different groups. In this figure, the dotted lines represent the genome-wide significance levels for γ_g and γ_e. These significance levels, along with the axes, divide the graph into sixteen sections that can be used to visualize the categorization of sites. For instance, sites with significant positive γ_e values and significant negative γ_g values fall into the upper left section. All these sites are consistent with DS and included in the counts for groups 1, 2, and 3. Counts for each section have been superimposed on the graph, and each section has been color-coded to show theory classification and group inclusion. Red denotes sections with sites that are consistent with MA, while blue denotes sections with sites that are consistent with DS. Group membership based on significance is designated by color shade and numbered brackets. The

dashed line represents the $\gamma_g = \gamma_e$ identity line, and serves to separate counts in sections where there are sites that are consistent with both MA and DS.

Comparison of sites consistent with MA and DS

To gain a better understanding of what distinguishes MA and DS sites, we analyzed the locations, genomic features, and gene ontology associated with the CpG sites we found to be consistent with each theory.

Location: The locations of CpG sites found to be consistent with MA do not notably differ from the locations of CpG sites found to be consistent with DS. This was observed for all three significance groups, as shown in Figure 5.

Enrichment for age-methylation or age-demethylation: Fisher's exact tests were performed on the sets of sites found to be consistent with MA and DS to test if these sites are more likely than others to be age-methylated or age-demethylated. To increase the power of these tests, we compared all sites found to be consistent with MA or DS, irrespective of significance (Group 1), to the set of all sites tested for age-differential methylation. We defined age-methylated sites as those with increases in DNA methylation with age ($\beta_{age} > 0$), and age-demethylated sites as those with decreases in DNA methylation with age ($\beta_{age} < 0$).

We found MA sites to have significant depletion for age-methylation (OR=0.86, $P=1.03 \times 10^{-16}$), which is equivalent to significant enrichment for age-demethylation. In contrast, we found DS sites to have significant enrichment for age-methylation (OR=1.16, $P=7.64 \times 10^{-34}$) (Figure 6a). This means that DS-consistent sites are more likely than other

sites to be age-methylated, while MA-consistent sites are more likely to be age-demethylated.

Enrichment for genomic features: To better understand the genomic context of MA and DS sites, Fisher's exact tests were performed to test for enrichment of the following genomic features: CpG islands, CpG island shelves, CpG island shores, strong promoters, weak promoters, poised promoters, strong enhancers, weak enhancers, and insulators. We compared all sites found to be consistent with MA or DS irrespective of significance (Group 1), to the set of all sites tested for changes in heritability of DNA methylaiton with age. Previous work has shown age-methylated sites to be more likely to be located within CpG islands, and age-demethylated sites to be more likely to be located outside CpG islands (Christensen et al. 2009). We found MA sites to be significantly depleted in CpG islands (OR=0.70, $P=1.43x10^{-51}$), which is consistent with previous work given our finding that MA sites are enriched for age-demethylation.

The enrichment pattern of MA and DS sites that was observed for CpG islands extended to CpG shores, defined to be 1.5kb out from CpG islands. We found DS sites to be significantly enriched in CpG shores (OR=1.33, $P=8.81x10^{-45}$), and MA sites to be significantly depleted (OR=0.83, $P=6.98x10^{-17}$; Figure 6b). This pattern, however, did not extend to CpG shelves, defined to be 1.5kb out from CpG shores. We found no notable difference between MA and DS sites in enrichment for shelves; both sets of sites were enriched in shelves.

Weak promoters and weak enhancers were found to have similar enrichment patterns for MA and DS sites. We found MA sites to be significantly depleted in weak

promoters and weak enhancers (OR=0.81, P=2.47x10^{-8} ; OR=0.66, P=3.75x10^{-18}), and DS sites to be slightly enriched (OR=1.13, P=5.74 x10^{-14}; OR=1.09, P=0.04).

The one genomic feature we found to be significantly enriched in MA sites (OR=1.53, P=1.21x10^{-12}) and significantly depleted in DS sites (OR=0.79, P=3.54x10^{-4}) was insulators (Figure 6b). Previous work has suggested that the DNA methylation status of an insulator affects the binding of the transcriptional repressor CTCF, which may preferentially bind to unmethylated sequences (Kang et al. 2015). We found MA sites to be more likely to be located in insulators and to lose methylation with age. These results, combined with the findings of previously published work, suggest that MA sites may directly influence changes in transcription with age.

Gene Ontology: Gene ontology analyses were performed to gain a better understanding of the specific genes or type of genes that are associated with DNA methylation changes at the CpG sites consistent with MA and DS. We used the GOstats package in R (Falcon and Gentleman 2007) to assess whether any terms describing biological processes, molecular functions, or cellular components associate with the sets of genes closest to the CpG sites consistent with MA or DS. The gene closest to each CpG site was defined based on distance to transcription start site, as in (Barwick et al. 2016). We analyzed all sites found to be consistent with MA or DS, irrespective of significance (Group 1). Table S2 shows the top five significant terms after multiple test correction for the MA and DS sets for each ontology category (FDR<0.05), as well as the total number of significant terms associated with each set. Overall, the genes closest to DS sites are enriched for fewer molecular functions (3 vs. 2), cellular components (2 vs. 0), and biological processes (19 vs. 5) than the genes closest to MA sites. This is evidenced by fewer significant terms and higher P-values associated with the genes closest to

DS sites (Table S2). A lack of enrichment for functionality is consistent with stochastic age-associated DNA methylation changes and the stochastic process of aging that is suggested by DS.

Replication of results

To assess the generality of our results we tested for changes in the heritability of DNA methylation with age in an independent data set from the GOLDN study. Our analysis was restricted to sites found to be consistent with MA or DS in the BSGS data set at the Group 2 level of significance (significant γ_g for MA sites, significant γ_e for DS sites). After quality control, data from the GOLDN study were available for 101 of the 102 MA sites, and 2,164 of the 2,266 DS sites.

In the GOLDN data, 958 CpG sites were found to be consistent with MA and 1,266 CpG sites were found to be consistent with DS, irrespective of significance (Group 1). Of these sites, 56 sites were found to be consistent with MA and 1,221 sites were found to be consistent with DS in both the GOLDN and BSGS data sets. This relates to a 55% replication for MA sites and 56% replication for DS sites.

When we restricted to sites with significant γ_g or γ_e values (Group 2), 15 CpG sites were found to be consistent with MA and 247 sites were found to be consistent with DS. Of these sites, four sites were found to be consistent with MA and 229 sites were found to be consistent with DS in both the GOLDN and BSGS data sets.

Across significance groups (i.e. Groups 1 and 2), more sites were found to be consistent with DS than with MA in both the GOLDN and BSGS data sets. Although this general trend replicated well, there was little replication at the CpG site level. The low replication rates we

observed are likely due to differences in data composition. BSGS measured methylation from whole blood, while the GOLDN study measured methylation from isolated CD4+ T cells. To avoid potential confounding due to the heterogeneous cellular composition of whole blood, the BSGS methylation β-values were residualized on cell type proportions before running analyses. Since the methylation of a single cell type was measured in the GOLDN study, raw β-values were used in analyses. Additionally, the BSGS families are comprised of adolescent twins, their siblings, and their parents, while the GOLDN study families are comprised of adult siblings. This results in a bimodal age distribution with a mean age of 21 for the BSGS subjects (Figure S1), and a unimodal age distribution with a mean age of 49 for the GOLDN subjects (Figure S5).

Replicated MA sites with significant γ_g and expression analysis: To help deepen our understanding of what characterizes CpG sites with MA-consistent age-associated DNA methylation changes, we investigated the attributes of sites found to be consistent with MA in both the BSGS and GOLDN data sets. Only MA-consistent sites were tested, as we expect the DNA methylation changes at DS-consistent sites to be stochastic and to associate with a random set of genes. We limited our investigation to the four MA-consistent sites with significant γ_g values in both data sets, as we are the most confident in the theory categorization at these sites. Table 2 lists the observed direction of change in methylation with age, and the annotated location, nearest gene, and genomic features and states of these four sites. Notably, half of the replicated MA sites are located within a transcription factor binding site (TFBS). This indicates that MA sites may directly influence changes in transcription with age.

MA proposes aging to be caused by deleterious genes with age-specific effects confined to only late in life. We suggest that the effects of such genes may be influenced by age-associated changes in DNA methylation, and that these changes may be accompanied by changes

in gene expression. We tested for associations between gene expression and the methylation levels at MA-consistent sites. All 13,222 expression probes passing quality control were tested against the four replicated MA sites with significant γ_g values. After multiple test correction, 11 genes showed a significant change in expression that was associated with a change in methylation level at one of three CpG sites (FDR<0.05; Table S3). For ten of these associations, expression was found to decrease with increasing methylation, indicating that methylation at these CpG sites may be interfering with transcription. Interestingly, all 11 of the genes with significant methylation-associated expression changes are located on a different chromosome from the CpG site. This suggests that the methylation of some MA-consistent CpG sites may interfere with the transcription of distal genes. Separate chromosomes can physically interact in the 3D space of the nucleus, and the transcription of some genes have been shown to be regulated by elements (e.g. enhancers) located on separate chromosomes though such interactions (Miele and Dekker 2008). The exact role of methylation in long-range expression control has not yet been well characterized, but it is possible that the associations we observe result from a physical interruption of interchromosomal interactions by methylation. Alternatively, the associations we observe could be indirect, such that methylation interferes with the transcription of a local gene and only influences the expression of distal genes indirectly, through a common pathway.

Heritability of rate of aging

We observed age acceleration, which describes an individual's rate of aging, to have a significant non-zero estimated heritability of 0.63. This result is consistent with both MA and DS evolutionary models of aging. Horvath found the heritability of age acceleration in twins to be

100% for newborns and 39% for adults, suggesting that the importance of environmental factors increases with age (Horvath 2013). Given that the median age of the subjects in the BSGS data set is 14, our result is in line with these findings. (Marioni et al. 2015) estimated the heritability of age acceleration to be 0.43 in the BSGS data after standardizing the methylation-age estimates to correct for differences in the age-prediction ability between adolescents and adults. The disparity between these results highlights the changing predictive ability of the Horvath model based on development phase, i.e. childhood vs. adulthood.

A study by (Gentilini et al. 2013) similarly investigated the relationship between methylation and an individual's rate of aging by comparing the methylation patterns of the offspring of centenarians and the offspring of non-long-lived individuals. They found global methylation levels to decrease with age across all individuals, but found centenarians and their offspring to have significantly less global methylation loss than the offspring of non-long-lived individuals. This result suggests that a genetic component underlies the preservation of methylation patterns and that the rate of biological aging is heritable. This study also identified 217 CpGs sites that are differentially methylated in the offspring of centenarians compared to the offspring of non-long-lived individuals. We compared these 217 CpG sites to the sites we found to be consistent with MA or DS irrespective of significance (Group 1); we found 11 of these sites to be MA-consistent and 38 of these sites to be DS-consistent. This suggests that heritability in the rate of aging could be the result of both deleterious genes with age-specific effects, as suggested by MA, and genes regulating the accuracy of somatic maintenance and repair, as suggested by DS.

Using the familial BSGS data, we were able to test for the existence of a heritable rate of aging but unable to test for age-related changes in this rate. Methylation changes resulting from

drift should occur at a constant rate throughout an individual's life, while targeted methylation changes should occur at specific times and show age-dependent rates of change. This can be examined in the future using longitudinal DNA methylation data and can potentially be used to investigate if the epigenetic clock sites that have underlying changes are targeted and MA-consistent or stochastic and DS-consistent.

CONCLUSIONS

We observed age-dependent changes in the heritability of methylation at age-differentially methylated CpG sites consistent with both MA and DS. Both theories play a role in explaining human aging and the aging-related changes we observe. The number of sites found to have decreasing heritability of methylation that is consistent with DS was roughly three times the number of sites found to have increasing heritability of methylation that is consistent with MA. Decreases in the heritability of methylation with age, where the DNA methylation levels of relatives diverge with age, have previously been reported and described as epigenetic drift. DS and epigenetic drift are consistent with each other, and suggest that age-associated DNA methylation changes are stochastic and may be caused by both internal and external factors. Increases in the heritability of methylation with age, where the DNA methylation levels of relatives converge with age, have not previously been reported. The existence of such sites indicates that not all age-associated DNA methylation changes are stochastic and caused by epigenetic drift. Age-related methylation changes at these sites may instead be targeted changes that mediate the effects of deleterious age-specific mutations, as suggested by MA. Enrichment and expression analyses suggest that methylation changes at MA sites may do this by influencing transcription. Further work is needed to connect methylation changes at the CpG sites found to

be consistent with MA to specific genes or gene networks and to elucidate the role that epigenetic drift plays in the aging process.

Additionally, we found an individual's rate of aging to be heritable using a methylation-derived measure of biological age, which considers the DNA methylation at hundreds of CpG sites across the genome. A heritable rate of aging is consistent with both MA and DS. This result indicates a general agreement between the patterns observed at the small single-site scale and the larger many-site scale.

To validate our results, we repeated our analyses in an independent data set. The number of sites found to have decreasing heritability of methylation that is consistent with DS was greater than the number of sites found to have increasing heritability of methylation that is consistent with MA. Although this general trend of DS-consistent sites outnumbering MA-consistent sites replicated between studies, little replication was seen between the results at the CpG site level. This was likely due to differences in data composition between our original and replication data sets. The original BSGS data we analyzed was from families with adolescent twins (age range:10-75; mean age: 21), while the replication GOLDN study data was from families with adult siblings (age range: 18-88 years; mean age: 49).

The age distributions of our original and replication data sets are the main limitations of our study. However, loci with significant age-associated DNA methylation changes have been shown to have significant overlap in pediatric and adult populations (Alisch et al. 2012), indicating that our age-differential methylation results should not be specific to the predominant age class of the data set analyzed. Nevertheless, having an age distribution with an abundance of individuals of young ages may have biased our theory classification results against the MA model. Changes associated with the deleterious age-specific genes suggested by MA occur only

late in life, and may be missed in a data set that has predominantly adolescent individuals. Additionally, since the DS model works throughout an individual's entire life, associated changes should be detectable even at young ages. This may inflate our estimate of the relative contribution of DS in explaining the aging-related methylation changes we observe. In future work, we hope to identify additional sources of family-based data to test our hypotheses against more uniform distributions and wider age ranges. Further work using data sets with more ideal compositions will help us to better understand and differentiate between biological aging- and development-related DNA methylation changes.

In this paper, we have developed and implemented a novel approach to testing the MA and DS evolutionary models of aging using DNA methylation data. The availability of genome-wide DNA methylation data has allowed us to investigate age-related changes at sites across the genome, and to better understand their connection to the MA and DS evolutionary models of aging. Our approach, however, restricts our focus and results to only CpG sites with heritable DNA methylation levels. It is possible that age-related methylation changes at non-heritable CpG sites are also consistent with the MA and DS evolutionary models, and that the distributions of MA- and DS-consistent changes at these sites differs from those at sites with heritable methylation levels. It should also be noted that DNA methylation data differs in its suitability for testing MA and DS. DS specifically predicts epigenetic changes, such as DNA methylation changes, to be one of many types of somatic damages that occur throughout an individual's life. MA, however, predicts only the existence of germline mutations, and does not predict any specific epigenetic effects. DNA methylation data, therefore, can only test the hypothesized epigenetic impact of genes that are directly predicted by MA.

Taken together, the results of this study suggest a role for both DS and MA in explaining patterns of epigenetic change with age. We suggest that many of the methylation changes that we observe with age are acquired stochastically and equivalent to epigenetic drift, but not all. Some aging-related methylation changes may be targeted. That is, aging-related methylation changes are likely to be caused by more than one process, and not equivalent throughout the genome. Furthermore, we believe our work demonstrates the utility of DNA methylation data in evolutionary investigations of human aging. We believe that the results of this study suggest DNA methylation data will be useful in future investigations of evolutionary theories of aging.

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CONFLICTS OF INTEREST

We have no conflicts of interest to declare.

TABLES

Table 1. Categorization of heritable CpG sites. Counts of significant sites were determined after multiple test correction (FDR<0.05).

GROUP	**CONSISTENT WITH MA** Increasing h^2 with age $+\gamma_g > \gamma_e$	**CONSISTENT WITH DS** Decreasing h^2 with age $\gamma_g < +\gamma_e$
1 All consistent sites	13,467	30,749
2 Significant γ_g (if MA) or γ_e (if DS)	102	2,266
3 Significant γ_g & γ_e	70	203

Table 2. Attributes and features of the replicated MA sites with significant γ_g.

CPG SITE	LOCATION	METHYLATION CHANGE WITH AGE	NEAREST GENE	GENOMIC FEATURES & STATES
cg02914422	Chr 7: 32110145	Age-methylated	PDE1C	CpG Island TFBS Polycomb repressed CTCF binding site
cg05691152	Chr 22: 38092978	Age-methylated	TRIOBP	TFBS Weak/poised enhancer CTCF binding site
cg13672736	Chr 9: 135114066	Age-demethylated	NTNG2	CpG shelf Weakly transcribed
cg25038330	Chr 10: 463561	Age-demethylated	DIP2C	CpG shelf Hetrochromatin; low signal

FIGURES

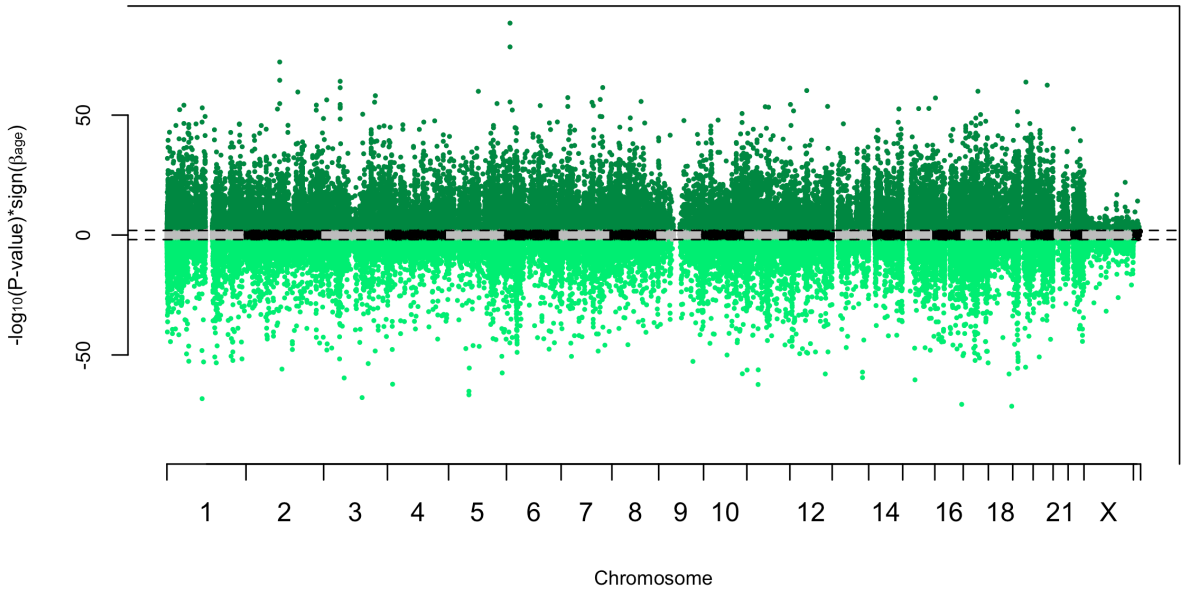

Figure 1. Manhattan plot of β_{age} P-values with sign of β_{age} estimate. Dashed lines represent genome-wide significance (FDR<0.05). Each point represents one CpG site. CpG sites with significant increases in methylation with age are colored dark green, while CpG sites with significant decreases in methylation with age are colored light green.

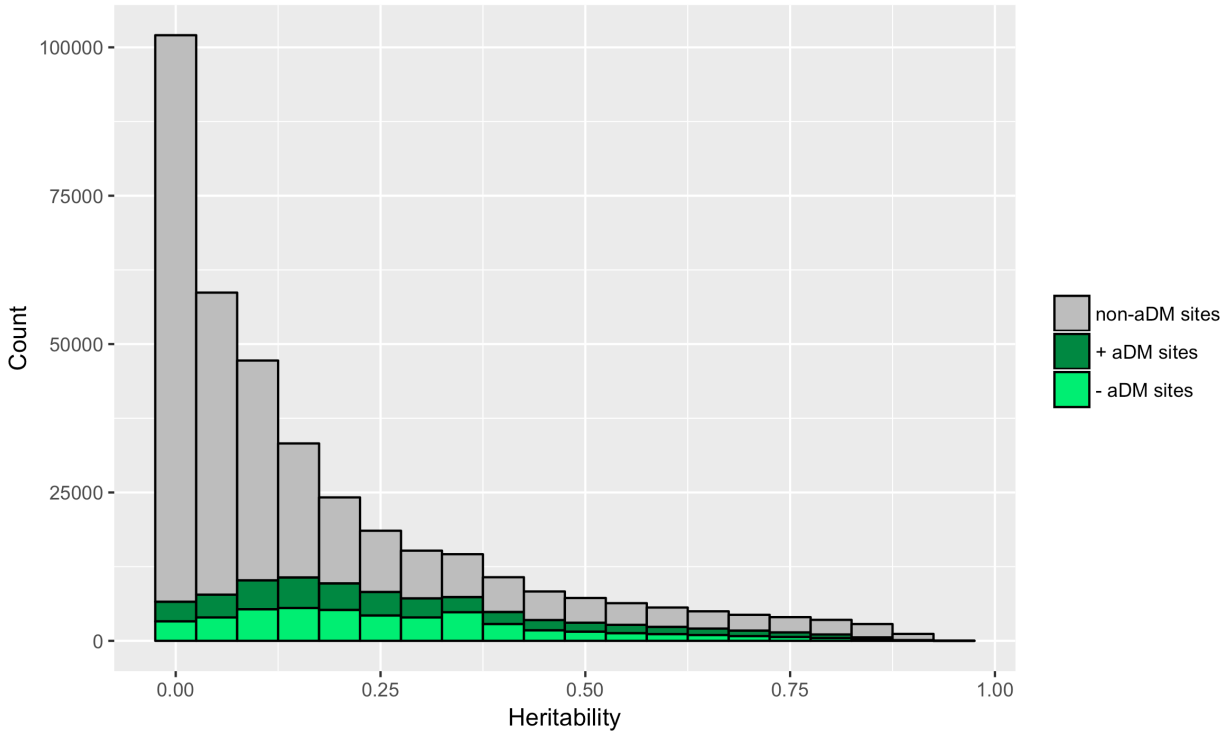

Figure 2. Distribution of heritability estimates for DNA methylation levels at age-differentially-methylated and non-age-differentially-methylated sites. The average estimated heritability of methylation across all sites is 0.177. The average estimated heritability of methylation at age-differentially-methylated CpG sites is 0.272.

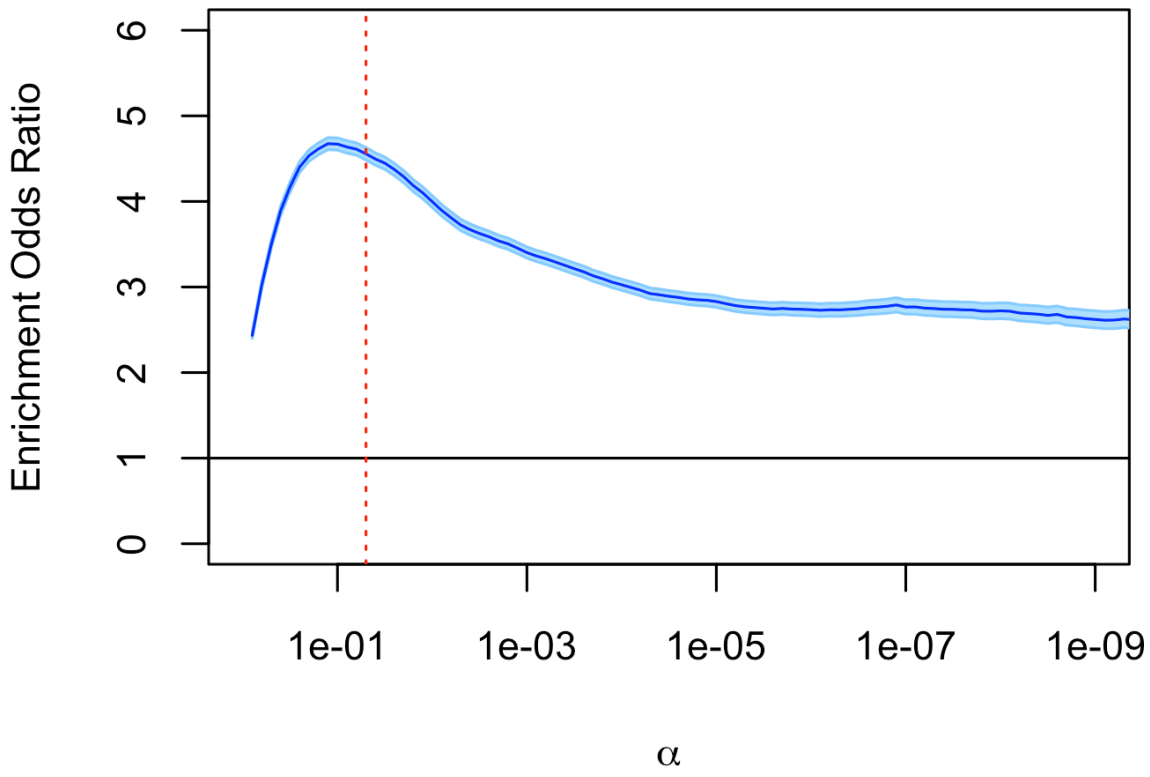

Figure 3. Results of Fisher's exact tests at varying significance levels. Sets of age-differentially-methylated CpG sites and heritable CpG sites were defined after multiple test correction. The estimated enrichment odds ratio is shown in dark blue, with the 95% confidence interval shown in light blue. The dashed red line represents $\alpha = 0.05$.

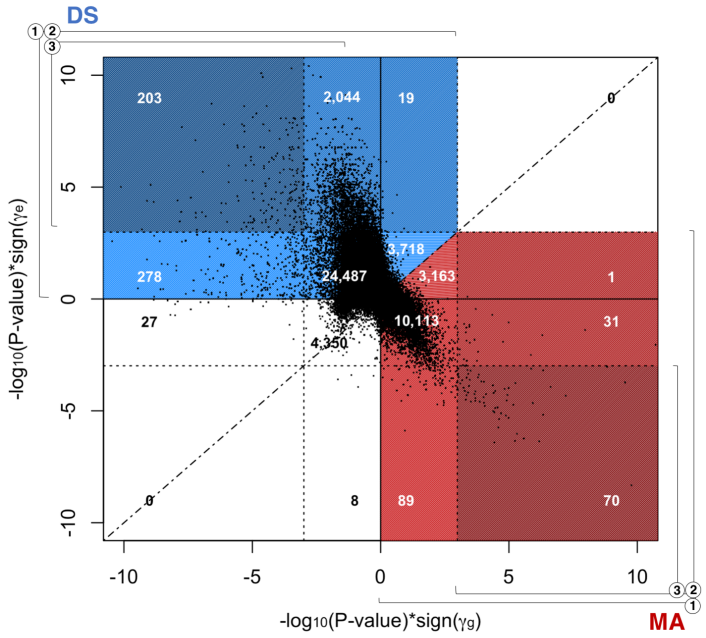

Figure 4. Scatterplot visualization of categorization of MA- and DS-consistent sites. The significance and sign of the estimated values of γ_g (x-axis) are plotted against the significance and sign of the estimated values of γ_e (y-axis). Each point represents one CpG site. The dotted lines represent the genome wide significance levels for γ_g and γ_e, and divide the graph into sixteen sections used to visualize the categorization of sites. Counts are superimposed onto color-coded sections to show theory classification and group inclusion. Red indicates a section with MA-consistent sites. Blue indicates a section with DS-consistent sites. Group membership based on significance is indicated by color shade and numbered brackets. The dashed line represents the $\gamma_g = \gamma_e$ identity line, and serves to separate counts in sections where there are sites that are consistent with both MA and DS.

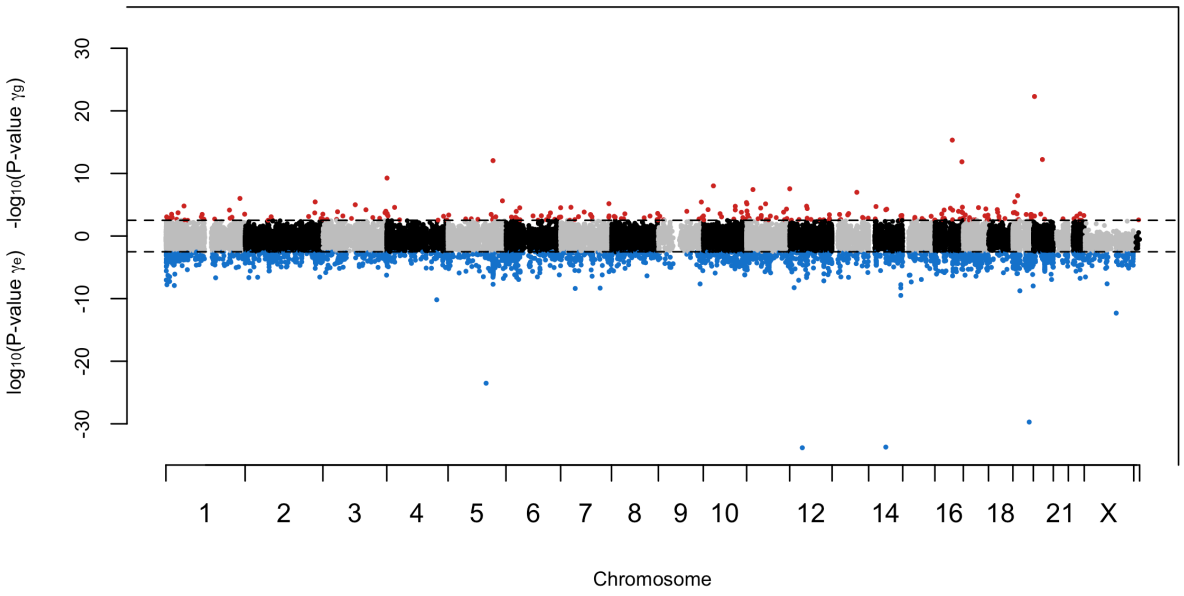

Figure 5. Manhattan plot of γ_g and γ_e P-values. Each point represents one CpG site consistent with either MA or DS. The dashed lines represent genome-wide significance (FDR<0.05). All CpG sites consistent with MA and belonging to significance groups 2 and 3 are in red. All CpG sites consistent with DS and belonging to significance groups 2 and 3 are in blue.

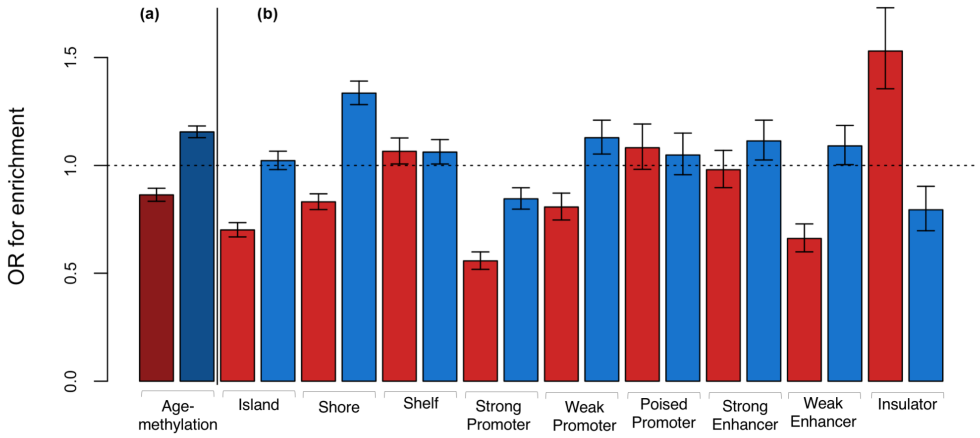

Figure 6. Histogram of enrichment in MA and DS sites. (a) Enrichment for age-methylation. MA sites are shown in red and DS sites are shown in blue. (b) Enrichment for genomic features. For each feature, MA sites are shown in red and DS sites are shown in blue.

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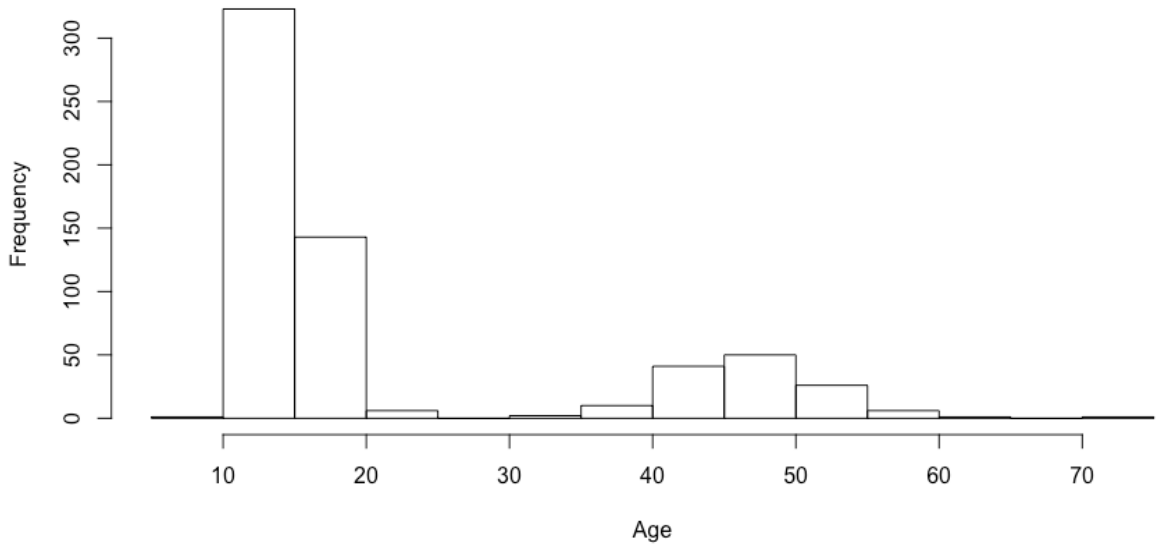

Figure S1. Age distribution of BSGS subjects with DNA methylation data.

DNA methylation data was measured in 610 individuals from the Brisbane System Genetics Study. The ages of the individuals range from 10 to 75 years, with a mean age of 21 years.

Type I Error Rate and Power

To estimate the type I error rate and our power to detect age-dependent changes in genetic and environmental variance, we performed simulations under the null and alternative hypotheses. We first estimated type I error by simulating residualized β-values at 10,000 CpG sites under the null hypothesis, where the genetic and environmental variance are independent of age, or γ_g and γ_e are equal to zero. For each CpG site a residualized β-value, $X_{it_{sim}}$, was simulated for each individual *i* at age *t*

$$X_{it_{sim}} = \alpha + \beta_{age}t + \beta_{sex}male + G_i + E_i \qquad (S1)$$

where G_i and E_i are generated random variables representing genetic and environmental contributions respectively. The environmental contributions E_i were generated from a normal distribution with mean zero and variance equal to the environmental variance estimated from the BSGS data. The genetic contributions G_i were generated from a multivariate normal distribution with mean zero and variance equal to $2\phi_{ij}\sigma_g^2$, where ϕ_{ij} is the kinship coefficient matrix from the BSGS subjects and σ_g^2 is the genetic variance estimated from the BSGS data. This formulation allows our generated genetic residuals to be correlated between relatives as in the original data. For each CpG site, the remaining coefficients in this model, α, β_{age}, and β_{sex}, take on values estimated from the BSGS data using equation (1).

At each CpG site, the generated vector $X_{it_{sim}}$ was used as the phenotype to estimate γ_g and γ_e via equation (2). At a significance level of α = 0.05, 5.94% of the CpG sites had a γ_g significantly different from 0, and 5.82% of CpG sites had a γ_e significantly different from 0 (Table S1). Figure S2 compares the distributions of γ_g and γ_e P-values, and shows nearly identical distributions at all reasonable levels of significance. These results suggest that our modeling approach has appropriate levels of type I error.

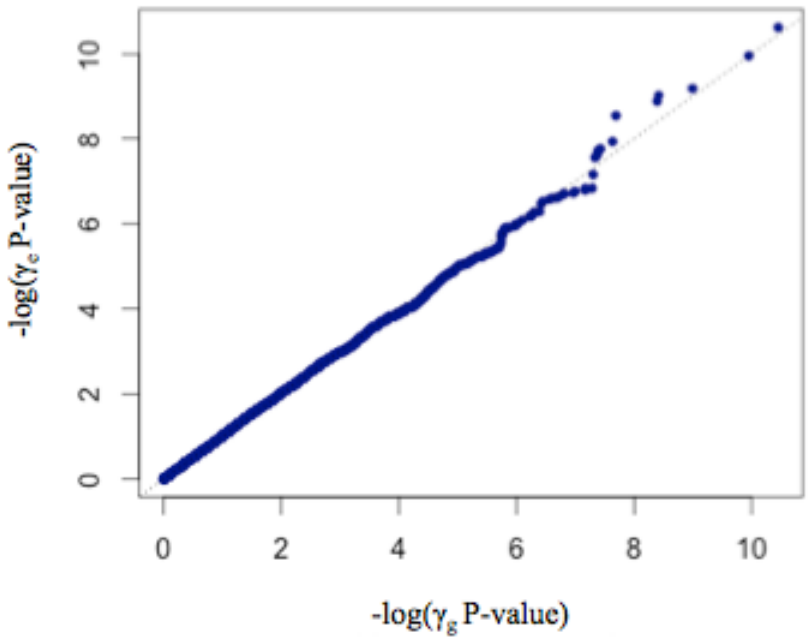

Figure S2. A quantile-quantile (Q-Q) plot for type I error simulations of γ_g and γ_e. This plot compares the distribution of γ_g p-values to the distribution of γ_e p-values. Identical distributions will lie along the identity line, shown here as a dashed line.

To estimate power we simulated normalized β-values at 10,000 CpG sites under the alternative hypothesis that the genetic and environmental variances are age-dependent, and γ_g and γ_e are non-zero. To generate these effects, we modified G_i and E_i in equation (S1) to include age-dependent terms according to the model in equation (2). The scale of the age dependence for our simulations was set to the mean of the absolute values of γ_g and γ_e estimated from the BSGS data, such that it took the same value for simulation of both G_i and E_i.

The modified G_i and E_i with age-dependent effects were used to generate new vectors of $X_{it_{sim}}$ as defined in equation (S1). As before, the generated vector $X_{it_{sim}}$ was used as the

phenotype to calculate γ_g and γ_e via equation (2) for each CpG site. At a significance level of α = 0.05, 44.9% of the CpG sites had a γ_g that was significantly different from 0, and 51.2% of the CpG sites had a γ_e that was significantly different from 0 (Table S1). Figure S3 compares the distributions of γ_g and γ_e P-values, and shows nearly identical distributions at reasonable levels of significance. This confirms that we have approximately equivalent power to detect age-dependent changes in the genetic and environmental variances with γ_g and γ_e.

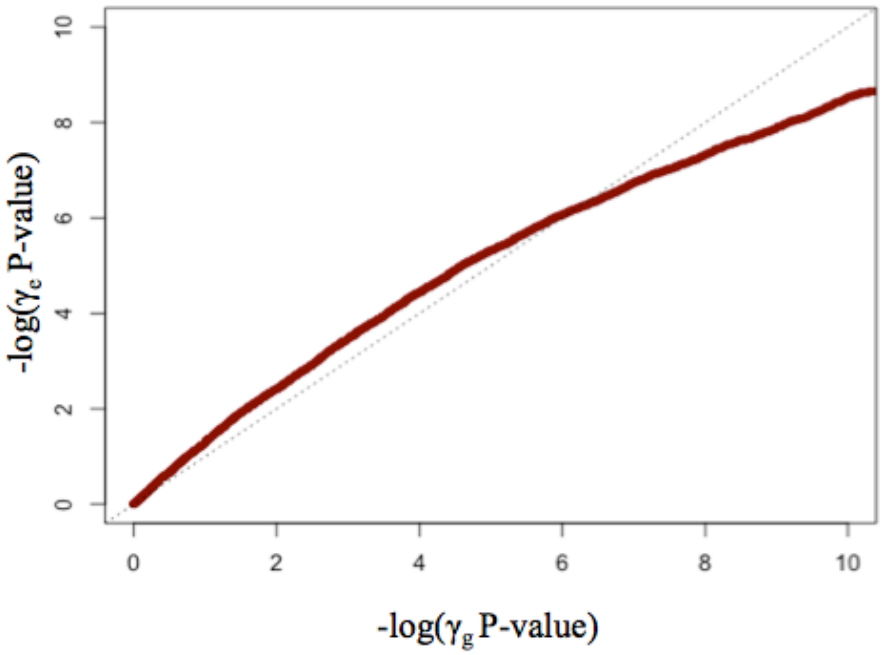

Figure S3. A quantile-quantile (Q-Q) plot for power simulations of γ_g and γ_e. This plot compares the distribution of γ_g p-values to the distribution of γ_e p-values. Identical distributions will lie along the identity line, shown here as a dashed line.

Table S1. Type I error rate and power for age-dependent changes in genetic and environmental variance.

	TYPE I ERROR $\gamma_g = 0, \gamma_e = 0$	POWER $\gamma_g \neq 0, \gamma_e \neq 0$
Proportion of significant γ_g	0.0594	0.4492
Proportion of significant γ_e	0.0582	0.5119

The age-dependence of genetic variance is reflected by γ_g.
The age-dependence of environmental variance is reflected by γ_e.

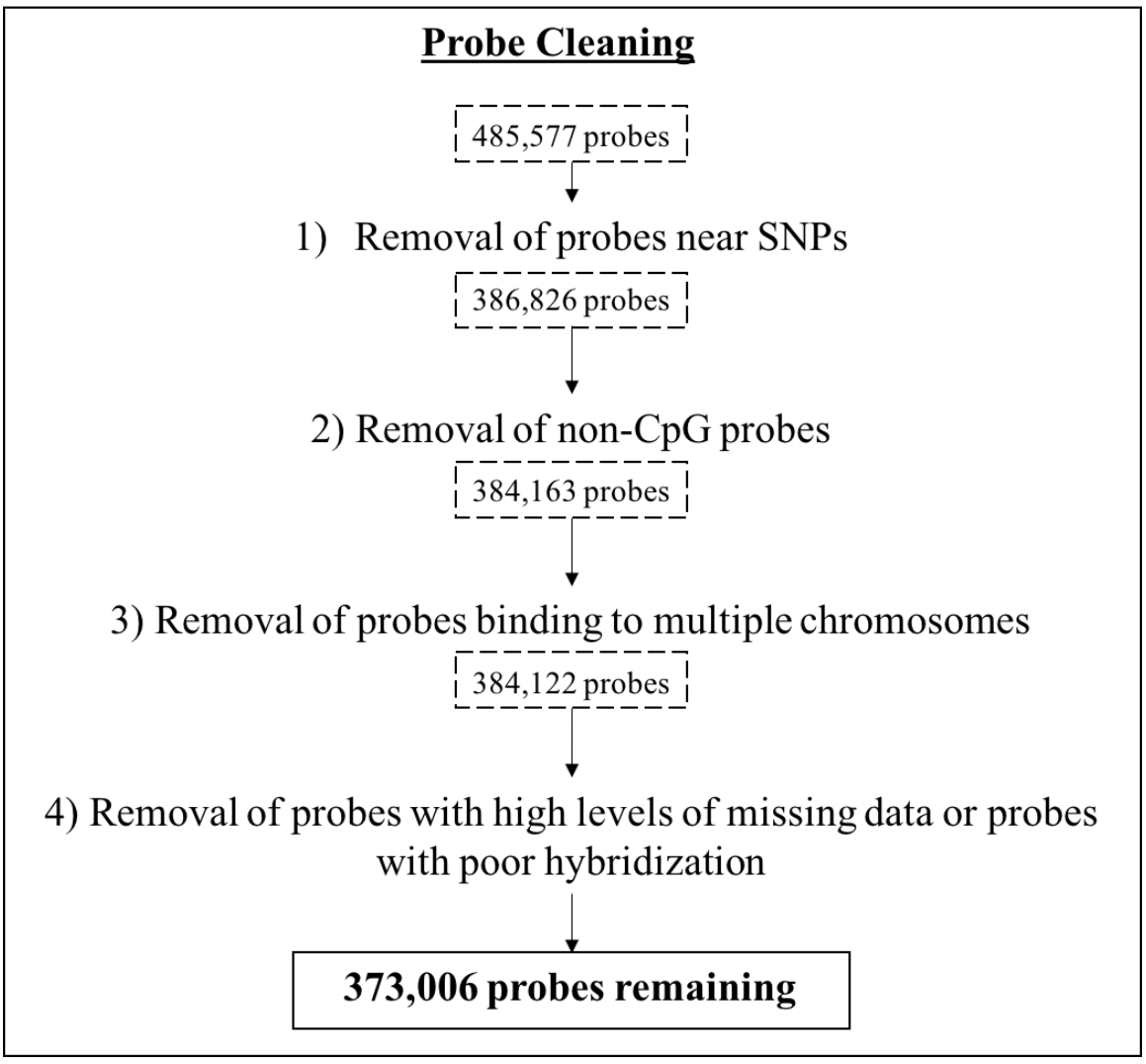

Figure S4. Data cleaning process for BSGS DNA methylation data.

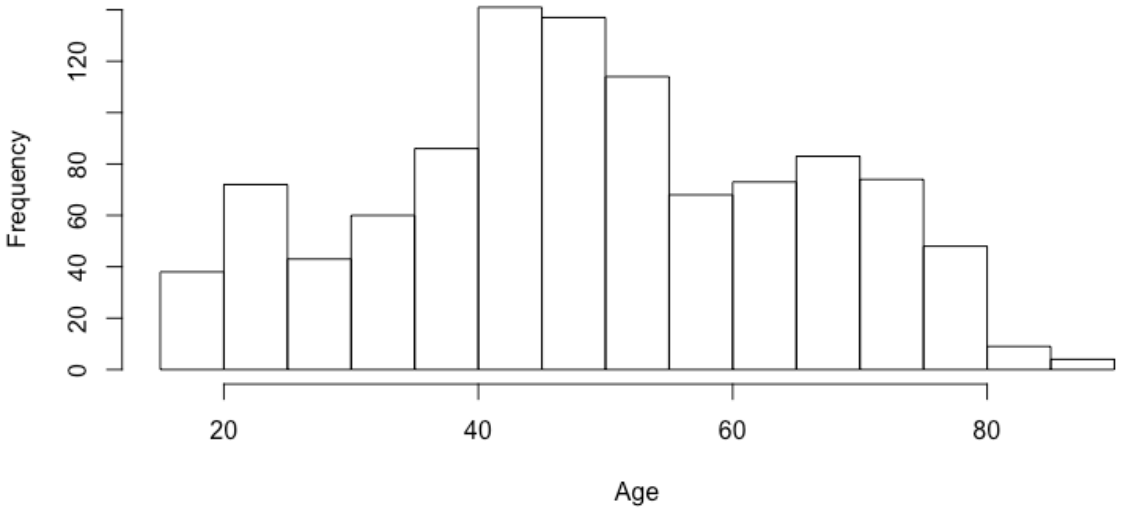

Figure S5. Age distribution of GOLDN subjects with DNA methylation data.

DNA methylation data was measured in 1050 individuals from the GOLDN Study. The ages of the individuals range from 18 to 88 years, with a mean age of 49 years.

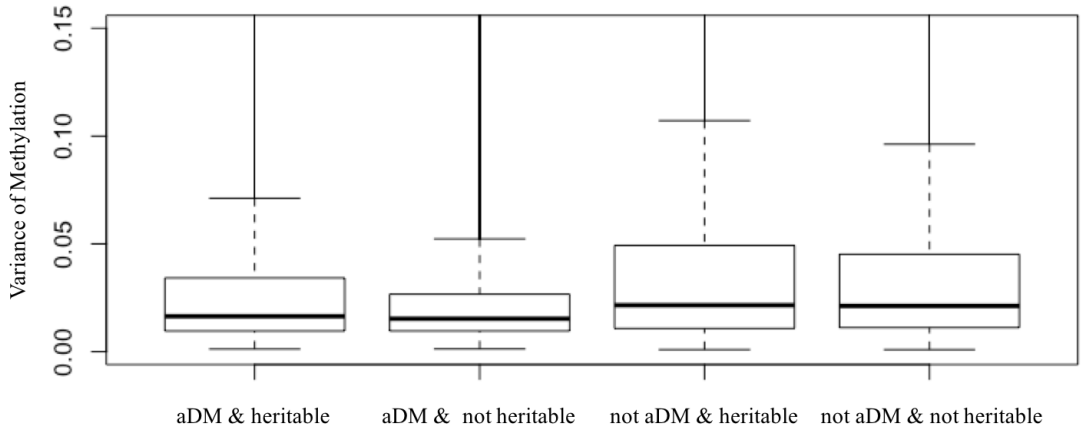

Figure S6. Boxplots comparing the distribution of methylation variance at CpG sites categorized by aDM and heritability status.

Table S2. Gene Ontology Terms. Total counts and the significant terms for MA and DS after multiple test correction (FDR<0.05).

	MA		DS	
ONTOLOGY	COUNT TERM | ADJUSTED P-VALUE		COUNT TERM | ADJUSTED P-VALUE	
MOLECULAR FUNCTION	**3**		**2**	
	Sequence-specific DNA binding	5.88E-7	Sequence-specific DNA binding	2.23E-8
	Double-stranded DNA binding	9.90E-4	Double-stranded DNA binding	2.39E-2
	Transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	9.98E-3		
CELLULAR COMPONENT	**2**		**0**	
	Integral component of plasma membrane	2.07E-4		
	Cell junction	1.80E-2		
BIOLOGICAL PROCESSES	**19**		**5**	
	Positive regulation of nucleobase-containing compound metabolic process	4.52E-5	Positive regulation of nucleobase-containing compound metabolic process	9.07E-4
	Cardiovascular system development	4.52E-5	Positive regulation of transcription from RNA polymerase II promoter	9.07E-4
	Positive regulation of RNA biosynthetic process	8.44E-5	Negative regulation of transcription from RNA polymerase II promoter	9.07E-4
	Sensory organ morphogenesis	2.04E-3	Positive regulation of RNA biosynthetic process	6.01E-3
	Multicellular organism development	2.04E-3	Pattern specification process	6.01E-3
	Tissue development	3.24E-3		
	Positive regulation of transcription from RNA polymerase II promoter	3.57E-3		
	Inner ear morphogenesis	1.13E-2		
	Embryo development ending in birth or egg hatching	1.13E-2		
	Localization	1.22E-2		
	Negative regulation of transcription from RNA polymerase II promoter	1.56E-2		
	Negative regulation of multicellular organismal process	1.56E-2		

	Embryonic forelimb morphogenesis	1.64E-2		
	Morphogenesis of a branching structure	1.84E-2		
	Positive regulation of neuron differentiation	3.34E-2		
	Regulation of cellular component movement	4.37E-2		
ALL	**24**		**7**	

Table S3. Expression analysis of replicated MA sites with significant γ_g. Significant expression and methylation associations after multiple test correction (FDR < 0.05).

CPG SITE LOCATION	ASSOCIATED EXPRESSION PROBE	DIRECTION OF ASSOCIATION	P-VALUE	GENE	LOCATION OF TSS
cg02914422 Chr 7: 32110145	ILMN_1778788	Negative	1.03×10^{-5}	AMOTL2	Chr 3: 134094259
	ILMN_3272768	Negative	4.63×10^{-6}	LINC00339	Chr 1: 22351683
	ILMN_2397199	Negative	9.67×10^{-6}	NDEL1	Chr 17: 8339169
	ILMN_1786734	Negative	1.06×10^{-5}	EIF5	Chr 14: 103800338
cg05691152 Chr 22: 38092978	ILMN_1684585	Negative	6.07×10^{-6}	ACSL1	Chr 4: 185747268
	ILMN_1694548	Negative	$8{,}40 \times 10^{-6}$	ANXA3	Chr 4: 79472741
	ILMN_1715068	Negative	1.13×10^{-5}	AQP9	Chr 15: 58430407
	ILMN_1694243	Negative	1.53×10^{-6}	LILRA6	Chr 19: 54746617
	ILMN_1714643	Negative	$4{,}87 \times 10^{-6}$	MGAM	Ch 7: 141695678
	ILMN_2114720	Negative	2.90×10^{-6}	SLPI	Chr 20: 43880879
cg13672736 Chr 9: 136114066	ILMN_1678535	Positive	7.37×10^{-6}	ESR1	Chr 6: 152011630

Chapter 4: Testing a stochastic model of epigenetic drift against longitudinal DNA methylation data

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INTRODUCTION

Epigenetic modifications are chemical additions to DNA that can regulate transcription and gene expression without changing DNA sequence. DNA methylation is an epigenetic modification that involves the addition of a methyl group to a single nucleotide base. In the human genome, this typically occurs at cytosine bases in a CpG dinucleotide sequence, referred to as CpG sites. Methylation at CpG sites in or near the promoter of a gene has been associated with gene expression silencing.

DNA methylation is known to be dynamic throughout life. Changes in methylation level with age have been observed both globally (i.e. genome-wide) and at thousands of CpG sites across the genome [1-5]. These age-related changes include patterns of both increasing and decreasing methylation [6]. DNA methylation has also been observed to increase in variability with age in populations of both related and unrelated individuals. The global methylation levels of monozygotic twins, which are nearly indistinguishable at birth, have been shown to grow increasingly different as a function of age in a cohort study of older vs. younger twins [7, 8].

Many of the observed age-related changes in methylation have been suggested to result from epigenetic drift, an accumulation of stochastic changes in DNA methylation with age [7, 9]. These stochastic methylation changes are thought to occur for many reasons, including both external environmental exposures and internal cellular events. At the cellular level, stochastic gains and losses of methyl groups may result from infidelity in methylation maintenance and

repair mechanisms. Methyl groups are added to DNA by a class of enzymes called DNA methyltransferases (DNMTs). The enzyme DNMT1 maintains methylation during replication by copying the methylation pattern of a template strand onto a newly synthesized strand, while the enzymes DNMT3A and DNMT3B add new *de novo* methylation irrespective of a previously established pattern [10-12]. Methyl groups can be lost from DNA through inexact copying and maintenance of methylation patterns by DNMT1, or removal by ten-eleven translocation (TET) enzymes and base excision repair processes.

Random cellular and molecular damages, such as the methylation changes of epigenetic drift, are suggested to underlie aging phenotypes by the disposable soma evolutionary theory of aging. This theory suggests aging to be caused the accumulation of stochastic cellular and molecular damages throughout the lifetime of an individual due to imperfect maintenance and repair mechanisms. Under this theory, the age-associated methylation changes we observe are assumed to result from random failures of methylation maintenance and repair, and to be stochastic and equivalent to epigenetic drift. Both disposable soma and epigenetic drift predict increasing variability in DNA methylation with age at the individual and population levels.

While epigenetic drift has been suggested to underlie many of the observed patterns of age-related methylation change, the stochasticity of these changes has never been directly tested. Are the DNA methylation changes we observe in aging truly stochastic and the result of epigenetic drift? Here, we test longitudinal DNA methylation data against a stochastic model of DNA methylation change with age. We define stochastic methylation changes as gains and losses of methyl groups that occur at random throughout the genome, and targeted methylation changes as gains and losses of methyl groups that occur at specific CpG sites, or within specific regions or genes. If all aging-related methylation changes are random and caused by stochastic

factors, we expect the rates of methyl group gain and loss to be similar at all sites across the genome, regardless of location or biological function. To test this hypothesis we developed a stochastic model of DNA methylation that can be used to estimate per-site rates of methylation change from longitudinal data. We investigate the stochasticity of aging-related methylation changes by testing the equivalence of estimated rates of methylation change at thousands of CpG sites across the genome.

METHODS

Overview of hypotheses to be tested

Our null hypothesis is that the DNA methylation changes we observe with age are entirely stochastic. If this is true we expect: 1) rates of methyl group gain and loss to be consistent across the genome, and 2) the direction and magnitude of methylation change at any particular CpG site to depend only on the initial level of methylation (i.e. the methylation at time zero). Sites where most DNA strands are initially unmethylated can be expected to have more opportunities to randomly gain methylation over time, while sites where most DNA strands are initially methylated can be expected to have more opportunities to randomly lose methylation over time.

We test our null hypothesis by testing the equivalence of the rate of methylation change at CpG sites across the genome. We estimate the rates of methyl group gain and loss from longitudinal methylation data using maximum likelihood estimation and a stochastic model of DNA methylation change and age. Rejection of the null hypothesis would suggest that age-related DNA methylation changes are not purely stochastic and cannot be entirely explained by drift.

Modeling stochastic changes in DNA methylation with age

To develop a stochastic model of DNA methylation change with age, we begin by considering the methylation state of a single CpG site on a single strand of DNA in discrete time. At any particular moment *t*, this CpG site can be either methylated or unmethylated. If the site is methylated at time *t*, we assume there is a probability λ_U that it will become unmethylated by time *t+1*. Similarly, if the site is unmethylated at time *t*, we assume there is a probability λ_M that it will become methylated by time *t+1*. These probabilities represent the probability of the site gaining or losing a methyl group between successive time steps through any mechanism of methylation or demethylation. **Figure 1** illustrates this two-state system and the specific transition probabilities between states at each time step.

If we consider this system in continuous time, the transition probabilities λ_U and λ_M become instantaneous rates of change. In continuous time, a CpG site in the methylated state will remain methylated for a random length of time that is exponentially distributed with parameter λ_U. This means the expected length of time the CpG site will stay methylated before losing its methyl group is $\frac{1}{\lambda_U}$. Similarly, a CpG site in the unmethylated state site will remain unmethylated for a random length of time that is exponentially distributed with parameter λ_M. This means the expected length of time the CpG site will stay unmethylated before gaining a methyl group is $\frac{1}{\lambda_M}$.

The described methylation dynamics of a single CpG site on a single strand of DNA in continuous time can be modeled as a first-order Markov chain given that: 1) the transition probabilities or rates of change λ_U and λ_M are constant over time, and; 2) the future methylation state of the CpG site satisfies the Markov property and depends only on the current state and not the sequence of past states.

If we expand our focus from a single strand of DNA to a large number of DNA strands, the methylation state of a CpG site becomes the proportion of DNA strands that are methylated at that site. Therefore, the methylation state of a CpG site when considering a large number of DNA strands is continuous and ranges from 0 to 1. The continuous-state approximation of a two-state Markov chain is an Ornstein-Uhlenbeck (OU) process [13]. An OU process is: 1) continuous in time and state space; 2) Markovian, and; 3) has Gaussian probability distributions.

The OU process suggests that the proportion of methylated DNA strands at CpG site *k* for individual *i* at age *t* (X_{kit}) has expectation

$$E[X_{kit}|\, X_{k0}] = X_{k0}e^{-\theta_k t} + \mu_k\left(1 - e^{-\theta_k t}\right) \tag{1a}$$

and variance

$$Var[X_{kit}] = \frac{\sigma_k^2}{2\theta_k}\left(1 - e^{-2\theta_k t}\right) \tag{1b}$$

where X_{k0} describes the proportion of methylated DNA strands at birth, σ_k^2 is the diffusion parameter describing the infinitesimal variance, and the terms θ_k and μ_k are functions of the instantaneous rates of methylation change λ_{U_k} and λ_{M_k}. The term θ_k equals $\lambda_{U_k} + \lambda_{M_k}$, and is the drift of the process. In the context of methylation, this represents the overall rate of methylation change in either direction. The term μ_k equals $\frac{\lambda_{M_k}}{\lambda_{U_k}+\lambda_{M_k}}$ and represents the long-term mean of the process.

The limit of the expectation and variance as $t \to \infty$ are μ_k and $\frac{\sigma_k^2}{2\theta_k}$ respectively. This indicates that after sufficient time, the probability distribution will become time-independent and have a constant mean and variance. Processes with constant probability distributions are referred to as stationary, while processes with time-dependent probability distributions are referred to as transient.

Estimating rates of methylation change

For each CpG site we used a maximum likelihood approach to estimate the OU process parameters μ, σ^2, θ, and X_0 from longitudinal data. Our approach uses a multivariate Gaussian distribution, as both the stationary and transient probability distributions of an OU process are Gaussian. At every CpG site *k=1, 2,..., K*, methylation measurements $X_{kit_{ij}}$ were collected for each individual *i*=1, 2,…,*N* at successive time points *j*=1, 2, …, *J* and ages t_{ij}.

For *J* = 3, the log-likelihood function for CpG site *k* is

$$L(\sigma_k, \mu_k, \theta_k) = \sum_{i=1}^{N} -\frac{1}{2}\left((ln|\Sigma_{\mathrm{ki}}|) + (\boldsymbol{X_{ki}} - \boldsymbol{\pi_{ki}})\Sigma_{\mathrm{ki}}^{-1}(\boldsymbol{X_{ki}} - \boldsymbol{\pi_{ki}})^T\right) \quad (2a)$$

where $\boldsymbol{X_{ki}}$ and $\boldsymbol{\pi_{ki}}$ are vectors of length *J =3* that respectively contain the observed and expected methylation at each time point *j* for each individual *i* (expected methylation is based on the OU process equation **1a**), and

$$\Sigma_{\mathrm{ki}} =$$

$$\begin{vmatrix} \frac{\sigma_k^2}{2\theta_k}(1-e^{-2\theta_k t_{i1}}) & \frac{\sigma_k^2}{2\theta_k}(e^{-\theta_k(t_{i2}-t_{i1})}-e^{-\theta_k(t_{i2}+t_{i1})}) & \frac{\sigma_k^2}{2\theta_k}(e^{-\theta_k(t_{i3}-t_{i1})}-e^{-\theta_k(t_{i3}+t_{i1})}) \\ \frac{\sigma_k^2}{2\theta_k}(e^{-\theta_k(t_{i2}-t_{i1})}-e^{-\theta_k(t_{i2}+t_{i1})}) & \frac{\sigma_k^2}{2\theta_k}(1-e^{-2\theta_k t_{i2}}) & \frac{\sigma_k^2}{2\theta_k}(e^{-\theta_k(t_{i3}-t_{i2})}-e^{-\theta_k(t_{i3}+t_{i2})}) \\ \frac{\sigma_k^2}{2\theta_k}(e^{-\theta_k(t_{i3}-t_{i1})}-e^{-\theta_k(t_{i3}+t_{i1})}) & \frac{\sigma_k^2}{2\theta_k}(e^{-\theta_k(t_{i3}-t_{i2})}-e^{-\theta_k(t_{i3}+t_{i2})}) & \frac{\sigma_k^2}{2\theta_k}(1-e^{-2\theta_k t_{i3}}) \end{vmatrix}$$

(2b)

is a covariance matrix describing the variance in methylation at each time point and covariance between each time point for individual *i.* A derivation of the covariance between the methylation at two time points at CpG site *k* for individual *i* is provided in the supplementary material.

To make optimization of the likelihood function computationally tractable, we chose site-specific starting values based on the data, and bounded σ^2 and θ. We placed a lower bound of zero on both σ^2 and θ, and an upper bound on θ of 0.5. For each CpG site *k*, we chose site-specific starting values by first minimizing the squared difference between the observed and expected correlation between the methylation data at time points two and three with respect to θ_k. Then, based on the optimized starting value of θ_k, we calculated a starting value for σ_k^2 using equation **1b** and the variance of the methylation data across individuals at CpG site *k*. Finally, the starting values for X_{k0} and μ_k were set to the mean methylation level across all individuals at the first time point and the last time point, respectively.

After estimation of the OU process parameters, values of λ_{M_k} and λ_{U_k} at each CpG site *k* were calculated from the estimated θ_k and μ_k as

$$\lambda_{M_k} = \theta_k \mu_k \qquad (3)$$

$$\lambda_{U_k} = \theta_k(1 - \mu_k)$$

Simulations were used to validate that this method can accurately estimate OU process parameters (for more information see the supplementary material). The results of these simulations showed that θ can be accurately estimated using this maximum likelihood approach across the range of reasonable θ, even with a small sample of 40 individuals.

Testing the equivalence of rates of methylation change

We test our null hypothesis of purely stochastic age-related methylation change by testing the equivalence of θ across the genome. To do this, we use likelihood ratio tests at each CpG site to compare the fit of the full model described above to the fit of a restricted model where θ is set to a single genome-wide value. We fit the full model for every CpG site before fitting the restricted model to calculate an appropriate and testable genome-wide value of θ. This value is calculated as the median full model estimate of θ. Sites where the full model fits the data significantly better than the restricted model (false discovery rate (FDR) < 0.05) will be considered to be inconsistent with epigenetic drift. The methylation changes at these sites occur at a rate that is significantly different from a genome-wide rate of random methylation change, which suggests methylation changes that may be targeted and depend on site location or biological function. Sites where the full model does not fit significantly better than the restricted model (FDR> 0.05) will be considered to be consistent with epigenetic drift. The methylation changes at these sites are consistent a model where methyl groups are gained and lost entirely randomly across the genome. For brevity and simplicity we refer to sites found to be consistent and inconsistent with genome-wide epigenetic drift based on the above criteria as drift-consistent and drift-inconsistent, respectively.

Longitudinal data

DNA methylation was measured at three time points in a sub-sample of 43 women from the Women's Health Initiative (WHI) Clinical Trials (CT) cohort. WHI is a long-term national health study focused on investigating strategies for the prevention and control of common causes of morbidity and mortality in post-menopausal women. The CT was designed as a randomized controlled study with three different interventions: 1) dietary modifications; 2) hormone therapy; and 3) calcium and vitamin D supplementation [14]. A total of 68,132 post-menopausal women between the ages 50 and 79 were enrolled into the CT at 40 clinical centers across the U.S. between September 1993 and October 1998. After enrollment, participants had a baseline clinical exam at which blood was collected and stored as buffy coat. The women were then followed annually for up to nine years. Extensive clinical data, including blood specimens, were collected during exams three years, six years, and nine years after the baseline exam. DNA methylation was measured at baseline from stored buffy coat for a random sub-sample of 2,200 CT participants. Of the 2,200 individuals with DNA methylation measurements at baseline, 43 individuals participating in the ancillary WHI Long Life Study (LLS) also had DNA methylation measured at two more time points, on average 3.5 years and 15.9 years from baseline. The data from these 43 individuals with three longitudinal DNA methylation measurements was used in this study.

The age of the study participants was self-reported in a questionnaire during screening. We approximated the chronological age of each individual at each time point as the self-reported age in years at screening plus the number of days between screening and blood sampling divided by 365.25 days and 0.5 years to increase the accuracy of the self-reported age (on average). The augmented ages range from 50.5 - 76.5 years at baseline, 54.58 - 79.88 years at the second time

point, and 67.89 - 91.74 years at the third time point. The age distributions at all three time points are shown in **Figure S2**.

Measuring DNA methylation

DNA methylation was measured from buffy coat for each individual at each time point using the Illumina Infinium HumanMethylation450 Beadchip [15]. This array interrogates a total of 485,577 sites across the genome, including 482,421 CpG sites and 3,156 non-CpG sites, using a bisulfite DNA treatment and two sets of site-specific probes binding associated methylated and unmethylated sequences [16]. For each sample, the proportion of DNA strands methylated at each site was estimated as the measured intensity of fluorescent signal from the methylated probe relative to the intensity of fluorescent signal from both methylated and unmethylated probes. This ratio measures the proportion of methylated DNA strands and is referred to as a β-value.

DNA methylation measurements were performed at the Northwestern University Genomics Core Facility in two stages. In the first stage, DNA methylation was measured from the blood collected at both the baseline and the second time point. In the second stage, DNA methylation was measured from the blood collected at the third time point. The R package ComBat was used to adjust the measured β-values to remove batch effects caused by experimental differences in methylation measurement between the two different stages [17]. These ComBat adjusted β-values were used for all analyses.

DNA methylation data filtering and quality control

DNA sequence can have direct effects on the measured methylation level at a CpG site. A genetic variant in place of either the cytosine or guanine at a CpG site, for instance, directly

prevents methylation. Furthermore, a genetic variant in the DNA sequence that is probed by the methylation array can impact binding and can alter the measured methylation level. To minimize the impact of these direct genetic effects on parameter estimates, we excluded CpG sites with a SNP present on the 50-base CpG site probe and CpG sites with a multi-modal distribution of methylation levels from analysis. CpG site probes with SNPs were identified based on data from the 1000 Genomes Project phase I release, as annotated by [18]. CpG sites with multi-modal distributions of methylation level were identified using the gaphunter function in R [19]. As additional quality control measures, we also removed probes with detection p-values >0.01 in over 10% of samples, probes on the sex chromosomes, probes annotated as binding to multiple chromosomes, probes without CpG sites, and probes with low correlations (<0.2) between successive time points. After data filtering and quality control, a total of 185,860 sites remained.

To minimize the impact of outlying methylation measurements on our parameter estimates, we removed individuals with any measurements more than five interquartile ranges from the nearest quartile at each CpG site. Outliers beyond this threshold, likely to be caused by measurement errors, were shown to have large influence on estimates by [20].

Age-differentially-methylated CpG sites

A total of 2,000 of the 185,860 filtered CpG sites were previously found to have significant changes in methylation with age (FDR<0.05) in a study on the same longitudinal WHI data used here [21]. This study by Grant *et al*. tested for associations between methylation and age using a linear mixed model that included a random effect for each individual. We restricted parameter estimation and hypothesis testing to the 2,000 sites with significant age-

associated methylation changes in our dataset, as our null hypothesis is specific to the methylation changes we observe with age.

Annotation for genomic context

Each CpG site was annotated with respect to: 1) promoters; 2) enhancers ; 3) insulators; 4) repressed genes; and 5) transcribed genes. For this annotation we downloaded the Broad ChromHMM data set for GM12878 lymphoblastoid cells and GRCh37/hg19 from the University of California, Santa Cruz (UCSC) table browser [22, 23]. Each CpG site was annotated based on overlaps between the CpG site location and the locations of the chromatin states provided by the UCSC data. After annotation, we grouped ChromHMM chromatin states describing similar genomic regions. For example, all ChromHMM promoter chromatin states were grouped into a single promoter category in our annotation. Throughout this paper we refer to these groupings of similar chromatin states as genomic contexts.

Cellular composition

The proportions of CD8-positive T cells, CD4-positive T cells, natural killer cells, B-cells, monocytes, and granulocytes in the whole blood samples were estimated from the methylation array data using a method developed by Houseman *et al.* and reference data on the methylation signatures of purified cell types [24, 25]. To conduct sensitivity analyses, the methylation data at each CpG site was residualized using a Gaussian generalized linear model that included the estimated cell type proportions as covariates to remove potential confounding due to the heterogeneous and changing cellular composition of whole blood.

RESULTS

Estimated stochastic model parameters

To investigate the stochasticity of age-related methylation changes, we fit a stochastic model to longitudinal methylation data at 2,000 age-differentially-methylated CpG sites via maximum likelihood estimation. At each site, we estimated four stochastic model parameters: X_0, μ, σ^2, and θ. These parameters respectively describe a CpG site's starting methylation, long-term mean methylation (i.e. mean methylation once the process has reached stationarity), instantaneous methylation volatility, and overall rate of methylation change. The distributions of the estimates for each of these parameters is shown in **Figure S3.** Across all sites, the average estimates of X_0, μ, σ^2, and θ are 0.56, 0.47, 0.0006, and 0.12, respectively. The estimates of σ^2 and θ are positively correlated (0.63), indicating that sites with low volatility of methylation tend to have low rates of methylation change, and sites with high volatility of methylation tend to have high rates of methylation change.

For each CpG site, we also calculated the rate of methylation (λ_M) and demethylation (λ_U) from θ and μ as shown in **Equation 3**. The distribution of λ_M ranges from 8.2×10^{-9} to 0.38 and has a mean of 0.058, and the distribution of λ_U ranges from 1.2×10^{-8} to 0.34 and has a mean of 0.067 (**Figure S4**). The mean rates of methylation and demethylation indicate that on average an age-differentially-methylated CpG site would take 17.2 years to go from fully unmethylated to fully methylated in the absence of processes to remove methyl groups, and 14.9 years to go from fully methylated to fully unmethylated in the absence of processes to add methyl groups.

Estimated stochastic model parameters and patterns of age-related methylation change

Estimated values of θ and σ^2 provide information on the age-associated methylation changes at a CpG site at the both the population and individual levels. The magnitude of σ^2 is directly related to the size of the observed methylation changes between successive time points for every individual. At sites with small σ^2, individuals are expected to have small changes in methylation between successive time points. At sites with large σ^2, individuals are expected to have large changes in methylation between successive time points. The magnitude of θ is inversely related to the expected length of time it takes methylation to reach an age-independent distribution (i.e. stationarity). At sites with large θ, the methylation of each individual is expected to reach stationarity in early life. At sites with small θ, the methylation of each individual is expected to reach stationarity late in life. When methylation is observed at the population level, different patterns of age-associated methylation change are expected before and after stationarity. Methylation observed at a site when it is far from reaching stationarity is expected to have: 1) linear changes in the population-level mean with age, and 2) increasing population-level variance with age (See equations **1a** and **1b**). Methylation observed at a site as it is transitioning to stationarity is expected to have: 1) non-linear changes in the population-level mean with age, and 2) increasing then plateauing population-level variance with age. Methylation observed at a site that has already reached stationarity is expected to have: 1) small or no linear changes in the population-level mean with age, and 2) small or no changes in the population-level variance with age.

To illustrate these patterns we split the analyzed CpG sites into six groups based on the estimated values of θ and σ^2 (**Table 1**). Within each group, CpG sites are expected to transition to stationarity at a similar time in life, and to show similar patterns of age-associated methylation

change at the both the population- and individual-level between the ages of 50 and 92 (the age range of the study population). For each group, the patterns of methylation change observed in the data closely follow our expectation based on θ and σ^2. This indicates that our stochastic model can accurately capture methylation dynamics from longitudinal data, and that the estimated values of θ and σ^2 can be used to identify and differentiate patterns of age-associated methylation change.

Equivalence of rates of methylation change across the genome

In total, 1,467 CpG sites (73% of the sites tested) did not fit a model with a site-specific rate of methylation change significantly better than a model with a genome-wide rate of methylation change (FDR>0.05). We consider the age-related methylation changes at these sites to fit a model of random genome-wide methyl group gain and loss, and to be drift-consistent. Conversely, 533 CpG sites (27% of the sites tested) fit a model with a site-specific rate of methylation change significantly better than a model with a genome-wide rate of methylation change (FDR<0.05). We consider the age-related methylation changes at these sites to be inconsistent with a model of random genome-wide methyl group gain and loss, and to be drift-inconsistent. The rates of methyl group gain and loss at these sites may depend on site location or biological function. Of the drift-inconsistent sites, 71% (380 sites) have a site-specific rate of methylation change that is less than the genome-wide rate of methylation change (0.12), while 29% (153 sites) have a site-specific rate of methylation change that is greater than the genome-wide rate of methylation change.

Methylation patterns in promoters may be better maintained than in other regions of the genome to reduce the accumulation of stochastic methylation changes with potentially

deleterious effects on gene expression. Additionally, non-random targeted methylation changes in promoters could function as a mediator of age-specific gene expression change. This means that the drift-inconsistent changes we observe may be a combination of: 1) non-random targeted methylation changes and 2) stochastic methylation changes happening at a rate based on location or biological function. However, these two possible types of drift-inconsistent changes cannot be differentiated based solely on the analysis of estimated rates of overall methylation change.

Equivalence of rates of methylation change within genomic contexts

To investigate the relationship between rate of methylation change and site location and biological function, we analyzed CpG sites in different five genomic contexts:1) promoters, 2) enhancers, 3) insulators, 4) transcribed regions, and 5) repressed regions. The set of CpG sites in each genomic context share both genomic position and biological function. All promoter sites, for example, are located in the promoter region of a gene, where methylation is expected to be negatively associated with transcription and gene expression.

We found the distribution of θ to be significantly different at CpG sites in different genomic contexts (Kruskal-Wallis test, $\chi^2 = 27.02$,p-value = 1.97×10^{-5}). Specifically, we found the median θ in enhancers to be significantly higher than in repressed regions (Nemenyi tests, $\chi^2 = 22.95$, FDR = 0.0013, **Figure 2a**). This indicates that not all age-associated DNA methylation changes are entirely stochastic, and suggests that the rate of methylation change at some CpG sites may depend on site location or biological function.

Of the CpG sites in promoters, 29% (144 sites) are drift-inconsistent and fit a model with a site-specific θ significantly better than a model with a genome-wide θ (FDR<0.05). Similarly, of the CpG sites in enhancers, insulators, repressed regions, and transcribed regions, respectively

18% (79 sites), 17% (14 sites), 24% (49 sites), and 24% (68 sites) are drift-inconsistent and fit a model with a site-specific θ significantly better than a model with a genome-wide θ (FDR<0.05, **Figure 2a**). Additionally, Fisher's exact tests showed drift-inconsistent sites to be significantly enriched in promoters (OR = 1.54, p-value = 0.012, **Figure 2b**), and depleted in enhancers (OR = 0.73, p-value = 0.012). This suggests that CpG sites with drift-inconsistent methylation changes are more likely to be in promoters, while CpG sites with drift-consistent methylation changes are more likely to be in enhancers.

Sites with increasing and decreasing heritability of methylation with age

The disposable soma (DS) theory predicts increasing variability of methylation with age at both the individual and population levels due to the accumulation of random methylation changes equivalent to epigenetic drift. As a result of this increasing variability and stochasticity, DS predicts decreasing heritability of methylation with age. In contrast to this, another evolutionary theory of aging, mutation accumulation (MA), predicts aging phenotypes to result from age-specific genes with deleterious effects confined to late in life. MA suggests that some age-associated methylation changes may mediate the effects of deleterious age-specific genes, and predicts increasing heritability of methylation with age. A previous study categorized age-differentially-methylated CpG sites as consistent with MA or DS based on the observance of age-related increases or decreases in heritability of methylation level. This study identified 13,467 MA-consistent sites where the heritability of methylation increased with age, and 30,749 DS-consistent CpG sites where the heritability of methylation decreased with age [26]. Given the predictions of each theory, we expect the methylation changes at sites found to have decreasing

heritability of methylation with age to be entirely random, and the methylation changes at sites found to have increasing heritability of methylation with age to be non-random, or targeted.

We tested the stochasticity of the sites with increasing and decreasing heritability of methylation with age by comparing the rates of methylation change between the two sets of sites and within each set of sites. Different distributions of rates of methylation change between sets of sites would indicate a difference in age-related methylation changes. Furthermore, an equivalence of the rates of methylation change across genomic contexts within each set of sites would suggest the site-specific age-related methylation changes to be random, and independent of location and biological function.

Of the 2,000 age-differentially-methylated sites we investigated, the previous study found 233 to have increasing heritability of methylation with age and 848 to have decreasing heritability of methylation with age. We found that sites with increasing heritability of methylation with age had a significantly higher median rate of methylation change than sites with decreasing heritability of methylation with age (Kruskal-Wallis test, χ^2=28.17, df=1, p-value= 1.2×10^{-7}, Figure 3). This suggests that the sets of sites with increasing and decreasing heritability of methylation experience different types of age-related methylation change. Within the set of sites with decreasing heritability of methylation with age, we found no significant difference between the median θ in any genomic context (Kruskal-Wallis test, χ^2=9.17, df=4, p-value =0.056, Figure 4a). Within the set of sites with increasing heritability of methylation with age, however, we found a significant difference in the distribution of θ in at least one genomic context (χ^2= 11.25, df=4 , p-value=0.023, Figure 4b). These results suggest that the age-associated changes in the set of sites with decreasing heritability of methylation with age are

truly random, while the age-associated changes in the set of sites with increasing heritability of methylation with age are not entirely random, and may be targeted.

We additionally tested if drift-inconsistent sites are more likely to have increasing or decreasing heritability of methylation with age. We found drift-inconsistent sites to be significantly less likely to have increasing heritability of methylation with age (Fisher's exact test, OR= 0.58, p-value=0.015). However, within the set of drift-inconsistent sites, sites with high rates of methylation change (>0.12) were found to be significantly more likely to have increasing heritability of methylation with age (Fisher's exact test, OR = 3.93, p-value = 0.0085). This indicates that drift-inconsistent sites with high rates of methylation change are more likely to have changes in the heritability of methylation that are consistent with targeted methylation changes, while drift-inconsistent sites with low rates of methylation change are more likely to have changes in the heritability of methylation that are consistent with stochastic methylation change.

DISCUSSION

We found the epigenetic drift model of methylation change to be consistent with most age-related methylation changes, but not all. Roughly one fourth of the age-related methylation changes observed here were found to be inconsistent with entirely random gains and losses of methyl groups across the genome. These drift-inconsistent methylation changes tend to occur at lower rates than drift-consistent methylation changes, and are more likely to occur within the promoter of a gene. Drift-inconsistent methylation changes are possible under two models of non-random change. First, methylation changes at drift-inconsistent sites may be non-random because the methylation changes themselves are targeted and happen at a specific sites and ages.

Second, methylation changes at drift-inconsistent sites may also be non-random because the mechanisms of stochastic methylation change depend on site location or biological function. For instance, the methylation patterns of promoters may be better maintained than in other regions of the genome, as the accumulation of stochastic methylation changes in promoters is more likely to have deleterious effects on gene expression. This means that the drift-inconsistent changes we observe are likely a combination of: 1) non-random targeted methylation changes and 2) stochastic methylation changes happening at a rate based on site location or biological function.

The evolutionary theories of DS and MA predict age-related methylation changes to be stochastic and targeted, respectively. DS suggests age-related DNA methylation changes to result from random maintenance and repair errors in epigenetic drift, while MA suggests age-related methylation changes to be targeted and to reflect or possibly mediate the deleterious effects of age-specific genes. We tested the rates of methylation change at sets of sites previously found to be consistent with DS and MA on the basis of age-related changes in the heritability of methylation. The DS-consistent sites were found to have rates of methylation change consistent with stochastic gains and losses of methyl groups. In contrast, MA-sites were found have rates of methylation change consistent with non-random gains and losses of methyl groups, suggesting targeted changes. Furthermore, drift-inconsistent sites with high rates of methylation change were found to be more likely to occur at MA-consistent sites, while drift-inconsistent sites with low rates of methylation change were found to be more likely to occur at DS-consistent sites. This suggests that drift-inconsistent changes with high rates of methylation change tend to be non-random targeted methylation changes, while drift-inconsistent changes with low rates of methylation change tend to be stochastic methylation changes happening at a rate that is dependent on site location or biological function. Further work connecting age-specific

methylation and gene expression changes is necessary to be able to fully differentiate between these two types of drift-inconsistent changes.

Four factors should be considered when interpreting the results from our analyses. First, the methylation data was extracted from whole blood, which is a heterogeneous and changing collection of cell types with different methylation patterns. Changes in cell type composition with age could potentially confound our results. However, in sensitivity analyses we found a high correlation between site-specific rates of methylation change estimated from adjusted and unadjusted data (a correlation of 0.69, **Figure S3**), which indicates that our results are not driven by a changing cellular composition. Second, since the age range of the study population spans only middle to late-life, the specific types of age-related methylation changes we observe and their relative contributions may be biased. Additional studies including data from children and young adults will help to increase our understanding of the full breadth of age-related changes that happen throughout life. Furthermore, since the study population is composed of post-menopausal women, the effects of kin selection may be observed and confound our results. The grandmother hypothesis suggests the post-reproductive period to be adaptive for women as devoting energy and resources to raising grandchildren increases fitness over continuing to reproduce [27]. Adaptive mutations that have been selected for via kin selection may not be differentiable from non-adaptive deleterious mutations that cannot be effectively selected against as suggested by MA. Finally, our model assumes that the rate of methylation change at every CpG site to be constant with age. However, it is likely that the rate of methylation change at any particular CpG site depends on a multitude of factors, both environmental and genetic, that may change over an individual's lifetime. For instance, environmental exposures such as diet, air pollution, and smoking status are known to impact methylation patterns [28], and often change

throughout an individual's life. The validity of this model assumption can be tested in future studies with longitudinal designs and available methylation, gene expression, and environmental exposure data.

In summary, we have developed a novel stochastic model of methylation and age, and used our model to test if the age-related DNA methylation changes we observe are entirely random and caused by epigenetic drift. We found evidence of both random and non-random methylation changes. Targeted methylation changes exist, but the majority of age-related DNA methylation changes are acquired stochastically as a result of epigenetic drift. We believe this work demonstrates the utility of stochastic modeling, and will enable future exploration of both the random and non-random methylation changes that occur throughout the course of human life.

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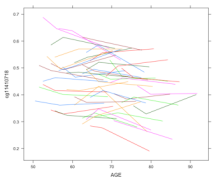
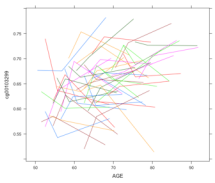
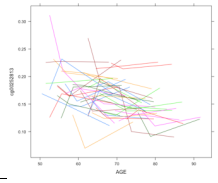
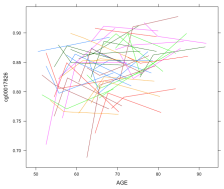
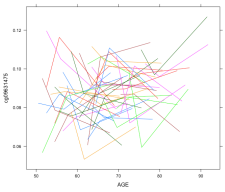
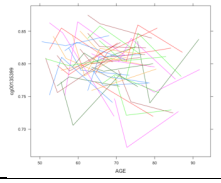
https://www.whi.org/researchers/Documents%20%20Write%20a%20Paper/WHI%20Investigator%20Long%20List.pdf.

CONFLICTS OF INTEREST

We have no conflicts of interest to declare.

TABLES

Table 1. Categorization of age-associated methylation changes based

Group θ value σ^2value	**Age at stationarity**	**Methylation dynamics** Population level age-related changes in: Mean | Variance Individual-level age-related changes	**# of sites**	**Observed methylation at a selected site**
1 θ <0.1 $\sigma^2 < 0.0005$	> 104	Linear | Increasing Smooth	646	
2 θ <0.1 $\sigma^2 > 0.0005$	> 104	Linear | Increasing Fluctuating	129	
3 0.1< θ <0.2 $\sigma^2 < 0.0005$	> 55, < 104	Non-linear | Increasing, then plateauing Smooth	415	
4 0.1<θ <0.2 $\sigma^2 > 0.0005$	> 55, < 104	Non-linear | Increasing, then plateauing Fluctuating	566	
5 θ >0.2 $\sigma^2 < 0.0005$	< 55	Negligible | Negligible Smooth	46	
6 θ >0.2 $\sigma^2 > 0.0005$	< 55	Negligible | Negligible Fluctuating	198	

This table details expected population- and individual-level patterns of methylation change with age for each group and CpG sites, and illustrates the observed methylation dynamics with an example from the data. In each example, the three repeated methylation measurements for each individual at a single CpG site are plotted against chronological age, and connected with a line. Therefore, each line within a plot represents the CpG site-specific methylation trajectory of a single individual. The calculations for the age at stationarity are provided in the supplementary material.

FIGURES

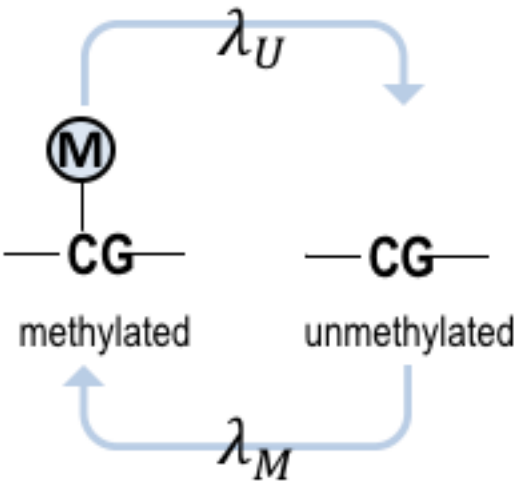


Figure 1. Two-state DNA methylation system with illustrated transition probabilities between

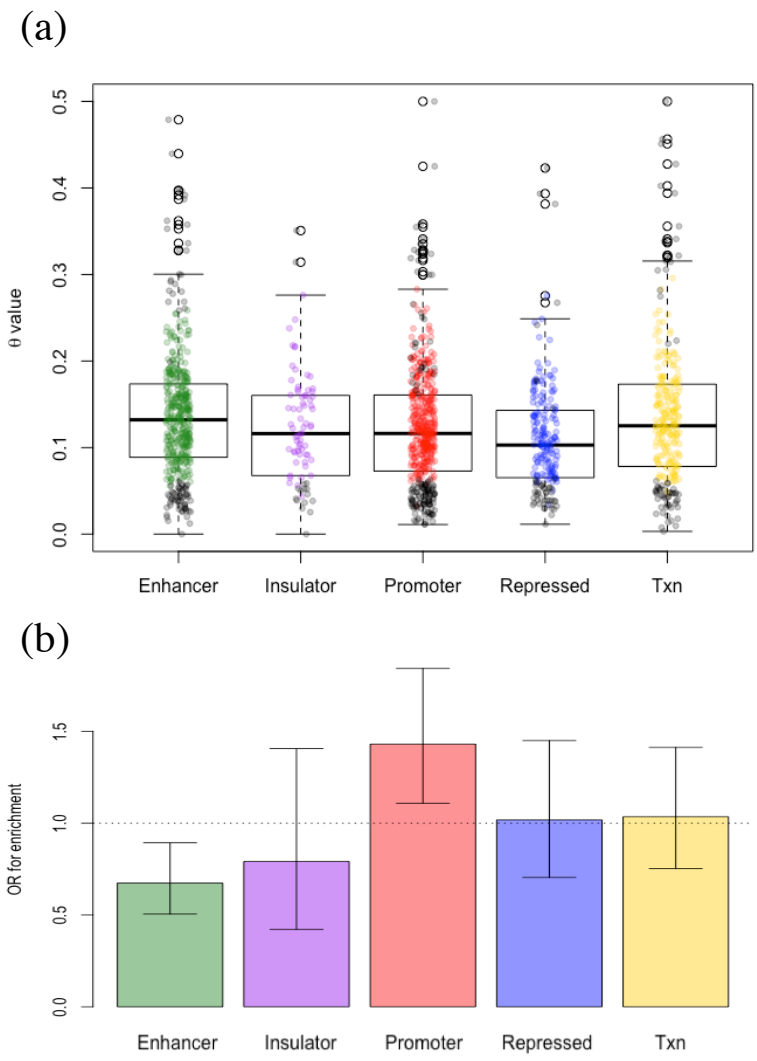


Figure 2. (a) Site-specific estimates of θ by genomic context. The distribution of θ for each genomic context is shown as a boxplot. The estimate of θ for each site in a particular genomic context is shown as a scatterplot. Each drift-consistent site is shown as a colored point, while every drift-inconsistent site is shown as a black point. (b) Enrichment for drift-inconsistent sites in five different genomic contexts.

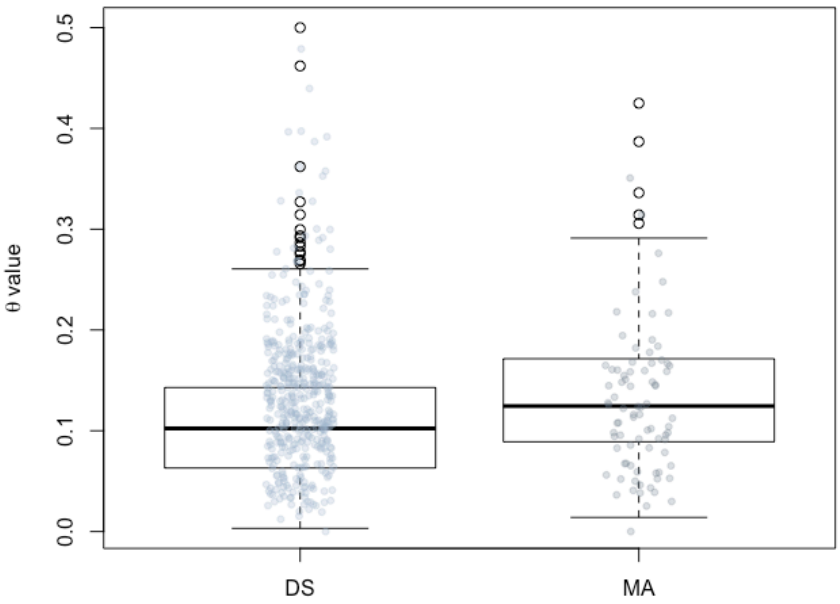


Figure 3. Distribution of estimates of rate of methylation change for sites with decreasing heritability of methylation with age (DS-consistent sites) and increasing heritability of methylation with age (MA-consistent sites).

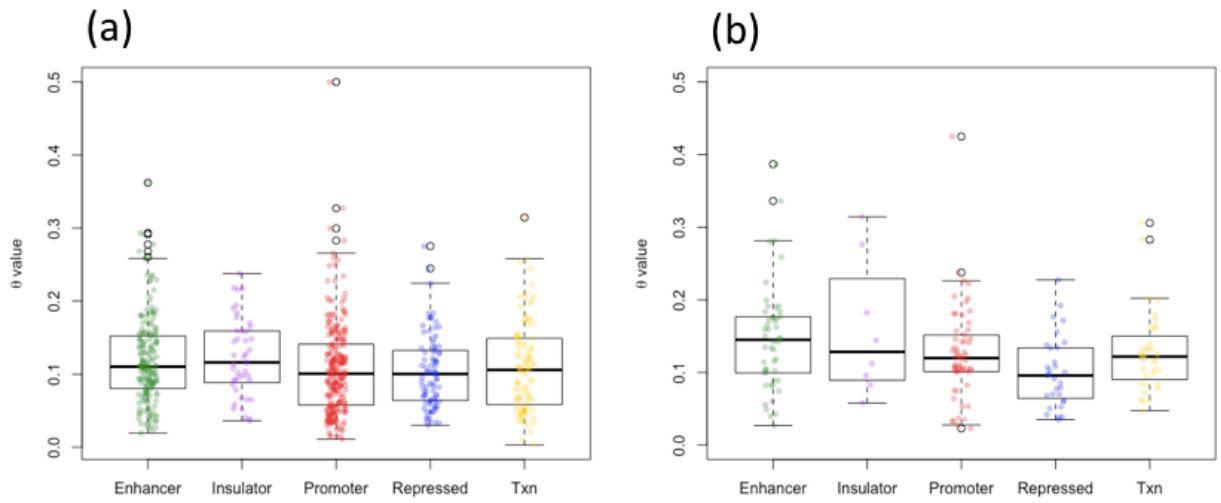


Figure 4. (a) Distribution of estimates of rate of methylation change. Sites with decreasing heritability of methylation with age (DS-consistent sites) (b) Sites with increasing heritability of methylation with age (MA-consistent sites)

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SUPPLEMENTARY MATERIAL

Derivation of covariance between methylation at time *s* and *t* for individual *i* and *s<t*

$$Cov(X_{is}, X_{it}) = E[Cov(X_{is}, X_{it}|X_{i0})]$$

$$= E[\, E[X_{is}X_{it}] - E[X_{is}]E[X_{it}]\,]$$

$$= E[\, E[\, E[X_{is}X_{it}|X_{is}]\,] - E[X_{is}\, E[\, E[X_{it}|X_{is}]\,]\,]\,]$$

$$= E[\, E[X_{is}\, E[X_{it}|X_{is}] - E[X_{is}\, E[\, E[X_{it}|X_{is}]\,]\,]\,]$$

$$= E\left[\, E\left[X_{is}\left(X_{is}e^{-\theta(t-s)} + \mu\left(1 - e^{-\theta(t-s)}\right)\right)\right] - E[X_{is}]E\left[\left(X_{is}e^{-\theta(t-s)} + \mu\left(1 - e^{-\theta(t-s)}\right)\right)\right]\right]$$

$$= E\left[\, E\left[X_{is}^2 e^{-\theta(t-s)} + X_{is}\mu\left(1 - e^{-\theta(t-s)}\right)\right] - E[X_{is}]\left(E\left[X_{is}e^{-\theta(t-s)}\right] + E\left[\mu\left(1 - e^{-\theta(t-s)}\right)\right]\right)\right]$$

$$= E\left[\, E[X_{is}^2]e^{-\theta(t-s)} + \mu\left(1 - e^{-\theta(t-s)}\right)E[X_{is}] - \mu\left(1 - e^{-\theta(t-s)}\right)E[X_{is}]^2 e^{-\theta(t-s)}\right]$$

$$= E\left[E[X_{is}^2]e^{-\theta(t-s)} - E[X_{is}]^2 e^{-\theta(t-s)}\right]$$

$$= E\left[e^{-\theta(t-s)}(E[X_{is}^2] - E[X_{is}]^2)\right]$$

$$= E[e^{-\theta(t-s)}\frac{\sigma^2(1 - e^{-\theta 2s})}{2\theta}$$

$$= \frac{\sigma^2}{2\theta}\left(e^{-\theta(t-s)} - e^{-\theta(t+s)}\right)$$

Simulation of methylation data and recovery of OU process parameters

We performed a series of simulation studies to assess the accuracy of our maximum likelihood approach for OU process parameter estimation. In each simulation study, we simulated the methylation at a single CpG site at age 50, 55, and 65 for N individuals as independent OU processes with shared parameters. To better understand how our ability to estimate OU process parameters relates to sample size, we performed simulations with sample

sizes of 40 and 1000 individuals. For each individual *i,* the methylation level at age *t,* X_{it}, was simulated from a normal distribution with mean

$$E[X_{it}|X_{is}] = X_{is} * \mathrm{e}^{-\theta(t-s)} + \mu\left(1 - \mathrm{e}^{-\theta(t-s)}\right), \quad s < t \tag{S1a}$$

And variance

$$Var[X_{it}] = \frac{\sigma^2}{2\theta}\left(1 - e^{-2\theta t}\right) \tag{S1b}$$

For each simulation study the value of θ took on a value in the set [0.0001, 0.0001, 0.01, 0.02, 0.03, 0.04, 0.05, 0.07, 0.1, 0.2], which spans the range of meaningful values for θ. A X_0 of 0.3, μ of 0.5, and σ^2 of 0.0001 was used for every simulation. Simulations at every value of θ were run ten times for N=40 and five times for N=1,000.

After simulating methylation data with equation S1, we used the maximum likelihood approach described in "Estimating rates of methylation change" to estimate values of θ, σ^2, μ, and X_0. We analyzed only the accuracy of the estimation of θ, as this is the parameter we use to test the stochasticity of age-related DNA methylation changes. Figure S1 compares the estimated and simulated θ values for each simulation study at each sample size. For both N=40 and N=1000 the percent error of the estimated θ values was less than 20% for every simulated value of θ greater than 0.01 (Table 1). This indicates that our maximum likelihood method for estimating θ is accurate at most values of θ.

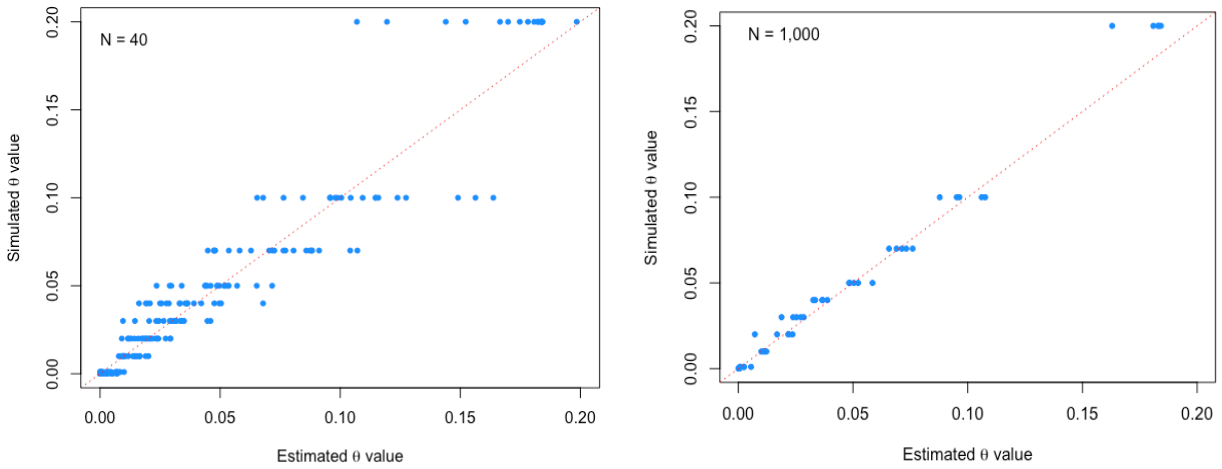

Figure S1. (a) Comparison of estimated and simulated values of θ for each simulation study at N=40 (b) Comparison of estimated and simulated values of θ for each simulation study at N=1000.

Table S1. OU process simulation parameter recovery results.

Simulated θ	Mean estimated θ	Mean percent error
N=40		
0.0001	0.0022	2125.3%
0.001	0.0033	235.8%
0.01	0.013	29.4%
0.02	0.019	2.7%
0.03	0.029	1.6%
0.04	0.034	13.8%
0.05	0.047	5.7%
0.07	0.073	4.5%
0.1	0.108	7.9%
0.2	0.176	12.2%
N=1000		
0.0001	0.00025	154.8%
0.001	0.0024	141.6%
0.01	0.011	11.5%
0.02	0.018	8.3%
0.03	0.025	17.4%
0.04	0.036	10.8%
0.05	0.052	3.2%
0.07	0.071	1.5%
0.1	0.098	1.4%
0.2	0.178	10.5%

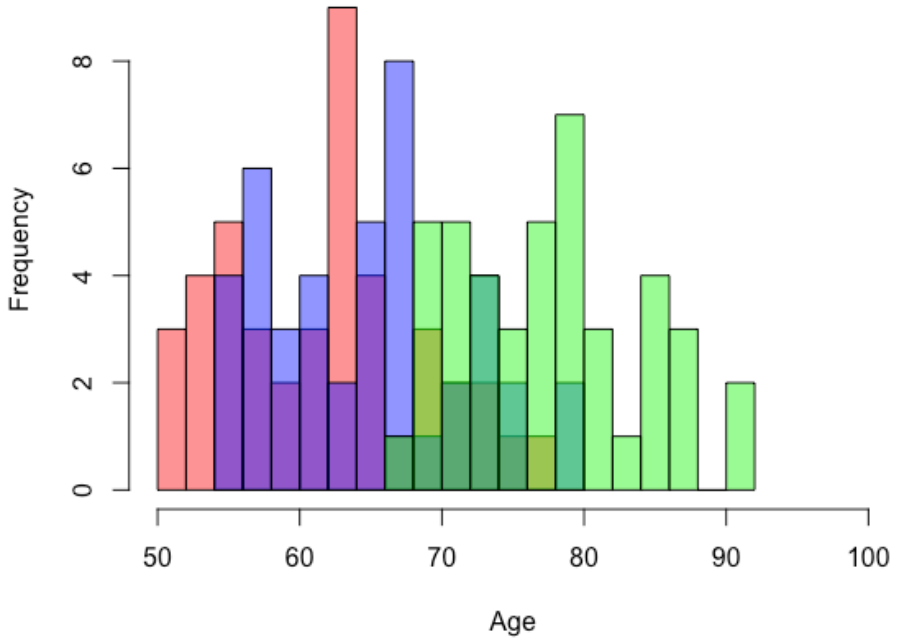

Figure S2. Age distribution at three time points for a sub-sample of 43 women from the Women's Health Initiative Clinical Trials cohort.

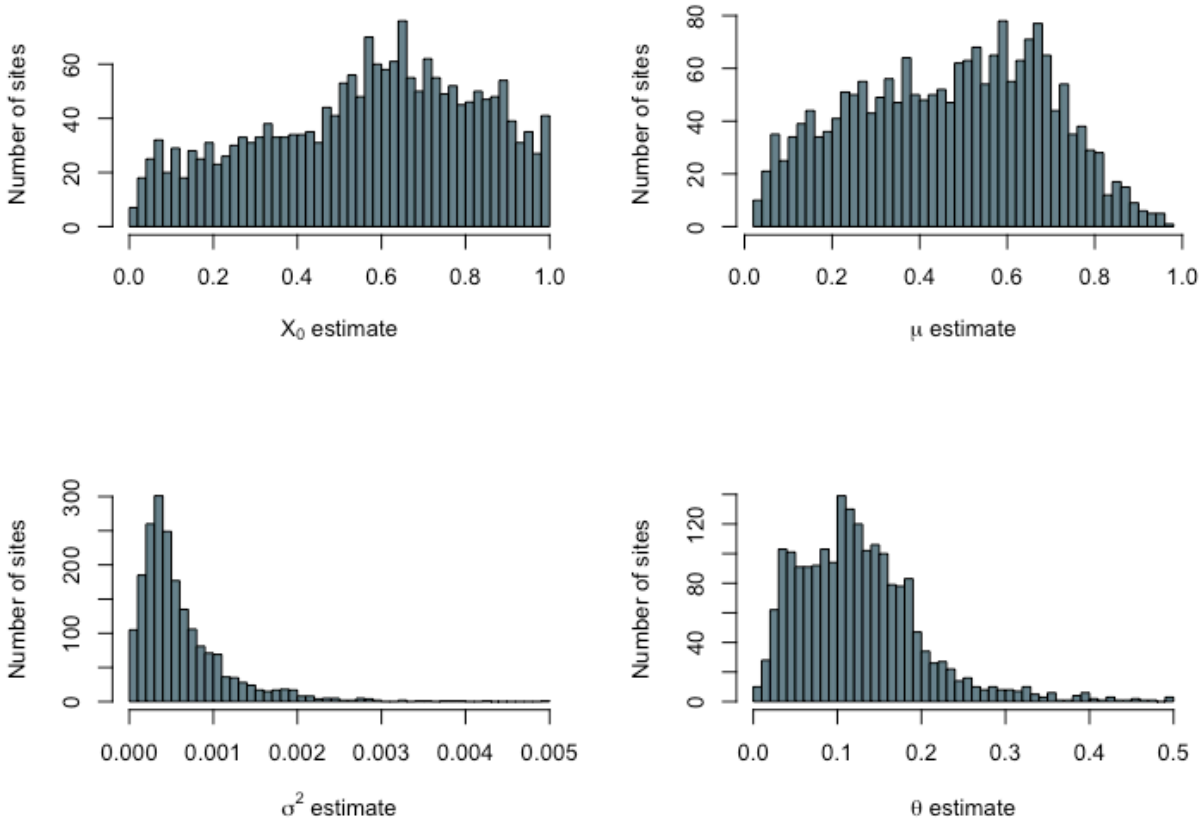

Figure S3. Distributions of OU process parameters.

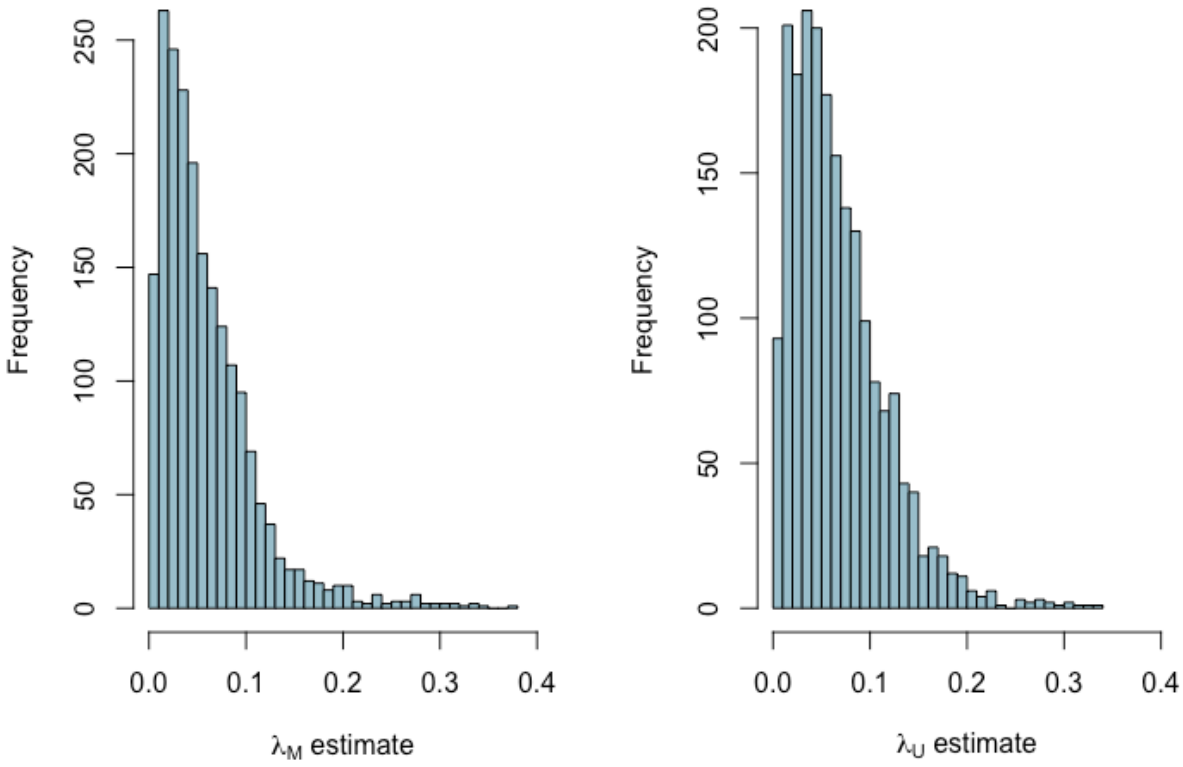

Figure S4. Distribution of rate of (a) methylation and (b) demethylaion.

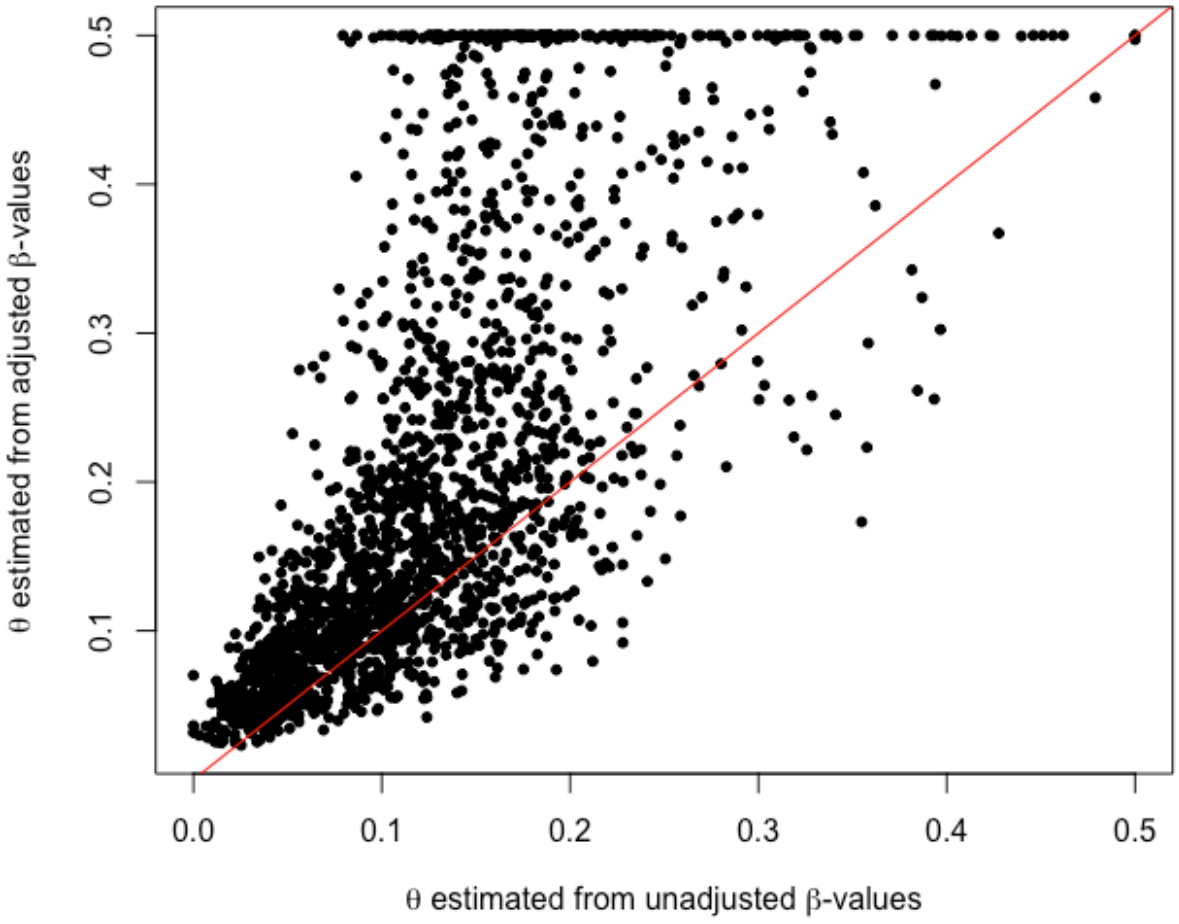

Figure S5. Comparison of estimates θ from unadjusted and adjusted methylation data. The correlation between the estimates is of 0.69.

Calculations relating θ to age of transition to stationarity

To calculate the age of a transition to stationarity based on the estimated θ we assume: 1) the smallest accurate measureable difference between β-values is 0.00001, 2) θ is constant over time, and 3) a process is stationary when equation **1a** is equal to μ and the term $X_{i0}e^{-\theta t} \rightarrow 0$ (i.e. no change in the mean over time).

When $e^{-\theta t}$=0.00001 and μ and X_{i0} are less than 1, the difference between and $X_{i0}e^{-\theta t}$ and 0 is smaller or equal to 0.000001 and cannot be accurately measured. We assume this point to be the point of transition into stationarity. The age at transition to stationarity for a particular θ is then

$$t = \frac{\ln(0.00001)}{-\theta}$$

The age at transition to stationary for a range on estimated θ values is shown in table S1. Horizontal lines separate logical grouping of θ values based on the calculated ages at transition to stationarity and the age range of our study population.

Table S2. Calculated age at stationarity given θ.

Estimated θ	**Calculated age at transition to stationarity**
0.009	1279
0.01	1151
0.011	1046
0.03	383
0.05	230
0.07	164
0.099	116
0.1	115
0.11	104
0.13	88
0.15	76
0.17	68
0.199	58
0.2	57
0.21	55
0.228	50.5

Chapter 5: Conclusions

The evolutionary theories of mutation accumulation (MA), antagonistic pleiotropy (AP), and disposable soma (DS) provide possible explanations for the existence of human aging. In Chapter 2, I reviewed the assumptions and predictions of these theories, and presented the results of previous empirical tests. Tests of theory predictions relating to the longitudinal process of aging were found to be absent from the literature. This is likely due to limitations of the frequently used measures of senescence. Lifespan, for instance, acts only as a proxy measure of an individual's overall rate of aging and provides no information about an individual's senescent state throughout life. I suggested the novel use of longitudinal and familial DNA methylation data to test the previously neglected predictions of evolutionary models of aging. DNA methylation is a newly proposed biomarker of aging that can be easily measured from blood samples collected throughout life. Furthermore, the use of microarrays to measure DNA methylation allows theory predictions to be independently tested at thousands of sites across the genome.

In Chapters 3 and 4, I used DNA methylation data to test predictions of MA and DS relating to aging as a longitudinal process. In both chapters the relative importance of each theory was investigated through tests formulated to identify MA- and DS-consistent sites across the genome. In Chapter 3, familial DNA methylation data was used to test heritability predictions of MA and DS. Sites with age-differential methylation were predicted to be MA-consistent if they had increasing heritability of methylation with age, and DS-consistent if they had decreasing heritability of methylation with age. I observed age-related changes in the heritability of methylation that were consistent with both MA and DS, suggesting that both theories play a role in explaining aging. The number of sites observed to have DS-consistent decreases in

heritability of methylation was roughly three times the number of sites observed to have MA-consistent increases in the heritability of methylation. This suggests that while the majority of age-related methylation changes are consistent with DS and randomly acquired molecular and cellular damages, age-related changes consistent with MA exist and may mediate the deleterious age-specific effects of aging genes.

In Chapter 4, longitudinal data was used to test if age-related DNA methylation changes support the stochastic aging process implied by DS. The expected methylation changes under DS are equivalent to epigenetic drift, or accumulated stochastic methylation changes with age. Both DS and epigenetic drift suggest the gains and losses of methyl groups to occur at random throughout the genome rather than at specific genes or CpG sites. I categorized age-differentially-methylated sites as drift-consistent if the methylation data fit a stochastic model with a genome-wide rate of methylation change, and drift-inconsistent if the methylation data fit a stochastic model with a site-specific rate of methylation change. The number of sites observed to have drift-consistent methylation changes was roughly three times the number of sites observed to have drift-inconsistent methylation changes. This suggests that the majority of age-related DNA methylation changes are random and consistent with DS and epigenetic drift, but that a minority of age-related DNA methylation changes are non-random and potentially targeted. I also tested the stochasticity of the sets of sites found to be consistent with MA and DS in Chapter 3. The DS-consistent sites were all found to have similar rates of methylation change (i.e. homogenous rates of methylation change) which is consistent with entirely stochastic gains and losses of methyl groups, while the MA-consistent sites were found to have significantly different rates of methylation change (i.e. heterogeneous rates of methylation change) which is consistent with non-random or targeted gains and losses of methyl groups.

Similar conclusions can be drawn from the results of Chapters 3 and 4. The results of both chapters suggest a majority of aging-related methylation changes to be consistent with DS and stochastic, and a minority of aging-related changes to be consistent with MA and potentially targeted. This means that most of the DNA methylation changes we observe with age are random and the result of environmental insults and/or methylation maintenance and repair errors. However, a small number of aging-related methylation changes are targeted and may mediate the deleterious age-specific effects of aging genes. Both MA and DS play a role in explaining aging and aging-related changes, but DS appears to be more important in understanding the age-related changes of DNA methylation. Future work connecting changes in methylation to changes in transcription and gene expression are needed to understand the effects of these stochastic and targeted changes. The results and methods presented here lay the foundation for future investigations of evolutionary theories human aging using DNA methylation data and other biomarkers of aging.