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Abstract

Background. Age-associated epigenetic changes are implicated in aging. Notably, age-associated DNA methylation changes comprise a so-called aging "clock", a robust biomarker of aging. However, while genetic, dietary and drug interventions can extend lifespan, their impact on the epigenome is uncharacterized. To fill this knowledge gap, we defined age-associated DNA methylation changes at the whole-genome single-nucleotide level in mouse liver, and tested the impact of longevity-promoting interventions, specifically the Ames dwarf *Prop1*$^{df/df}$ mutation, calorie restriction and rapamycin.

Results. In wild type mice fed an unsupplemented ad libitum diet, age-associated hypomethylation was enriched at super-enhancers in highly expressed genes critical for liver function. Genes harbouring hypomethylated enhancers were enriched for genes that change expression with age. Hypermethylation was enriched at CpG islands marked with bivalent activating and repressing histone modifications and resembled hypermethylation in liver cancer. Age-associated methylation changes are suppressed in Ames dwarf and calorie restricted mice, and more selectively and less specifically in rapamycin treated mice.

Conclusions. Age-associated hypo- and hypermethylation events occur at distinct regulatory features of the genome. Distinct longevity-promoting interventions, specifically genetic, dietary and drug interventions, suppress some age-associated methylation changes, consistent with the idea that these interventions exert their

beneficial effects, in part, by modulation of the epigenome. This study is a foundation to understand the epigenetic contribution to healthy aging and longevity and the molecular basis of the DNA methylation clock.

Introduction

Genetic, dietary and drug interventions can enhance longevity and suppress age-associated disease, such as cancer. Prominent genetic interventions that robustly extend longevity and healthspan in mammals include those that decrease growth hormone (GH) and insulin-like growth factor (IGF) signalling, for example Ames dwarf mice live more than 50% longer than their wild type siblings [1]. These diminutive mice result from a point mutation in a gene (*Prop1*$^{df/df}$) that drives development of the pituitary gland, so that mutant mice are deficient in specific hormones. The GH deficiency, in particular, has been shown to underlie their enhanced health span and extended lifespan. Ames mice are highly insulin-sensitive, resistant to some stresses and the incidence of cancer is reduced [2-4]. Dietary and drug interventions that extend lifespan include calorie restriction (CR) and the mTOR inhibitor, rapamycin [5]. Like the Ames dwarf mutation, CR and rapamycin also suppress and/or delay the incidence of cancer [5-7]. A detailed understanding of how these interventions exert their beneficial effects is essential to develop strategies to promote healthy aging in humans [8]. Currently, these interventions are thought to exert their effects by related and interconnected effects on some or all of the following: genome stability, the epigenome, telomere attrition and/or function, protein quality control, mitochondrial function, nutrient sensing, cellular senescence, stem cell exhaustion, cellular stress responses and altered intercellular communication [9]. Of note, the effects of

longevity promoting interventions on the epigenome, a key determinant of cell phenotype, are poorly understood.

Aging is associated with changes to the epigenome [10]. These changes include age-associated accumulation of histone variants, for example histone H3.3 in neurons and macroH2A in lung, liver and muscle, as well as other chromatin-associated proteins and changes to histone and DNA modifications [11-13]. Aging also affects specific gene regulatory elements, such as enhancers, promoters and CpG islands [14-22]. Underscoring the importance of such age-associated epigenetic changes, recent human studies have identified collections of specific CpGs whose age-associated change in methylation status in multiple tissues correlates strongly with chronological age. An advanced methylation age compared to actual chronological age is thought to reflect accelerated biological age, and is linked to increased mortality [23-27].

Age-associated epigenetic changes are not just biomarkers or passengers in the aging process, but can be causative in control of lifespan [28-30]. For example, in yeast, accumulation of H4K16ac at subtelomeric regions promotes replicative aging, while inactivation of the chromatin remodeler Iswi2p or the H3K36me2/3 demethylase Rph1p extends lifespan [28, 31, 32]. Decreased H3K4 methyltransferase activity can extend worm lifespan in a germline dependent manner [29]. In mice, muscle stem cells from old mice exhibit elevated repressive H3K27me3 at repressed histone genes [33], perhaps responsible for decreased proliferative potential of aged stem cells compared to young stem cells [34]. Mouse hematopoietic stem cells (HSCs) also exhibit changes in DNA methylation with age, including a small net hypermethylation both globally and at CpG islands [35, 36]. Some of these changes in aged cells are thought to promote expression of self-renewal genes and impair expression of differentiation genes, including lymphoid genes. This

can contribute to the characteristic phenotypes of aged HSCs, such as increased number, decreased function and a predisposition to myeloid differentiation [35].

Epigenetic changes linked to aging also impact specific diseases of aging, including cancer. While some age-associated epigenetic changes, such as increased abundance of histone modification H4K20me3 [10] and decreased H3K27me3 [37, 38], may activate tumor suppressor mechanisms and prevent cancer, others may be tumor promoting. Like cancer, aged tissue has been reported to exhibit global DNA hypomethylation and more focal hypermethylation at CpG islands [10]. Most notably, so-called bivalent gene promoters, marked with both activating H3K4me3 and repressing H3K27me3 (hence "bivalent") in embryonal stem (ES) cells, acquire DNA methylation in aged tissues and are also methylated and stably silenced in cancer [14-18]. In ES cells, these bivalent-marked genes are thought to be poised for activation due to loss of the repressive H3K27me3 mark during stem and progenitor cell differentiation and development. By virtue of their pro-differentiation functions these genes tend to have tumor suppressor-like properties, meaning that their methylation and stable silencing may promote proliferation, self-renewal and malignancy. In the hematopoietic system, some CpG islands progressively increase methylation from young to old to neoplasia, namely myelodysplastic syndrome (MDS) and ultimately acute myeloid leukemia [39]. *Sf3b1*, the mouse ortholog of a gene frequently mutated in human MDS, is methylated and underexpressed in aged mouse HSCs [35]. Hence, age-associated methylation changes might predispose to transformation of aged cells, by promoting silencing of tumor suppressor genes.

Given this strong and accumulating evidence that epigenetic events are important determinants of lifespan and predisposition to disease, we set out to ask whether genetic, dietary

and drug interventions that promote healthy aging and longevity suppress age-associated DNA methylation changes.

Methods

Ames dwarf mice were derived from a closed colony with a heterogeneous background (over 25 years) at the University of North Dakota [40]. Dwarf mice (and corresponding wild type (WT)) were generated by mating either homozygous (df/df) or heterozygous (df/+) dwarf males with heterozygous females (df/+). Non-genotypic intervention studies (rapamycin and CR) utilized genetically heterogeneous WT UM-HET3 mice bred at the University of Michigan. One cohort was given encapsulated rapamycin (42 pmm) from 4 months of age, and another group a CR diet initiated at 4 months of age (these mice received 60% of the intake of their age-matched controls after a two week run-in period at 80%). All cohorts contained 4 replicates (4 mice). Liver tissue was collected at 2 and 22 months of age and DNA isolated using DNeasy blood and tissue kit (Quagen). Hepatocellular carcinoma (HCC) is a relatively common disease of aging in mice. Hence, to avoid distortion of our data by neoplastic tissue, aged mice were sacrificed at 22 months (before HCC is typically apparent) and livers with overt signs of neoplasia were excluded from analysis. Where WGBS-seq was performed (by BGI, Shenzen), samples underwent a standard protocol of sonication, DNA-end repair and ligation of methylated adapter sequences prior to bisulfite conversion using ZYMO EZ DNA Methylation-Gold Kit (Zymo Research) and then 90 bp, paired-end sequencing on the Illumina Hi-Seq 4000 platform. Sequenced reads were aligned to reference genome (mm9) and methylation status of CpGs determined using Bismark and Bowtie2 [41, 42]. The bioinformatics process involved read quality assessment via FastQC, read trimming using the package Trim-Galore with alignment, read deduplication and

methylation context extraction via the Bismark suite [41]. CpG dyads were collapsed by combining the methylated and unmethylated scores at each dyad locus. There are approximately 42 million CpG loci in the mouse genome (mm9) – or 21.3 million CpG dyads. We achieved sufficient coverage to represent between 94-96% of the dyads in the young and old, WT and Ames dwarf dataset, with a mean coverage of 6.96 methylation calls per site (four biological replicates per cohort (approximately 15-fold coverage per genome), to yield approximately 1500Gbp of data (Additional File Supplementary Dataset 1: Table S1)). Despite sequencing CR and rapamycin interventions data with reduced coverage, we still observe 86-89% of all mappable CpG dyads with a mean coverage of 4.37 reads per loci. To identify differentially methylated CpG sites, a two-tailed Fisher exact test (FET) was used with p-value correction using Benjimini-Hochberg (BH-) FDR function at a rate of 5% where coverage surpassed a threshold of 10 overlapping reads. To identify DMRs, we used a sliding window-based approach operating at a range of 500bp. At each window, a two-tailed FET was performed to determine DMR significance alongside a chi-squared test of heterogeneity across the 4 mouse replicates within each cohort. Both chi-squared and FET tests were multi-sample corrected using BH-FDR with DMRs selected on the basis of significant ($p < 0.05$) BH-FDR corrected FET score and non-significant intra-cohort heterogeneity via the FDR corrected chi-squared test. We then divided DMRs into hyper- and hypomethylated, based on positive or negative changes in their respective methylation relative to their control. To determine the significance of the overlaps between regions or features, we used a permutation-based approach to assess significant enrichment over equally sized, randomly generated regions and calculated fold enrichments based on how these regions overlap, compared to an expected (random) model level of intersection. We validated findings using MeDIP-seq, an enrichment based assay that enriches methylated DNA fragments

via immunoprecipitation with anti-methyl-cytosine antibodies [60]. Additional details, including RNA- and ChIP-seq analysis information, available in Additional File Supplementary Methods).

Results

The epigenomes of wild type and Ames dwarf mice diverge with age. To investigate the relationship between age-associated epigenetic changes and healthy aging and longevity, we first set out to compare the DNA methylome of liver from young and old male WT and long-lived Ames dwarf mice. We selected liver for this study because our previous studies showed differences in liver in expression of DNMTs between WT and Ames mice and young and old mice [43]. Also, a single cell type, the hepatocyte, comprises ~80% of liver mass, and the epigenome of mouse liver has been extensively characterized, thereby aiding downstream analysis of the methylome in the context of the wider epigenetic landscape. We performed whole genome bisulfite sequencing (WGBS-seq) on the livers of young adult (2 months of age) and old (22 months of age) Ames dwarf and WT mice. We found that global levels of methylation across all CpGs in the genome were highly similar between all age groups, mouse genotypes and replicates (Figure 1a). This was also apparent from viewing whole-chromosome methylation profiles on the UCSC genome browser (Figure 1b). However principal component analysis (PCA) of the data (% methylation per CpG) suggested that local differences in methylation were present, as cohorts separated well on the 1st and/or 2nd principal components (12.38% and 7.64% of the respective cumulative variance) (Figure 1c).

Previous studies showed that epigenomes become more divergent with age [44]. Therefore, we set out to assess differences between WT and Ames dwarf mice at 2 months and 22 months of age. We determined those CpGs differentially methylated between genotypes (i.e. between WT

and Ames dwarf mice (gCpGs)). This was performed separately in both the young and old mice, using a Fisher's Exact Test method at 5%FDR (Methods). In dwarf mice, compared to WT mice, approximately 10x more CpGs were hypermethylated than were hypomethylated (Figure 1d). Strikingly, we observed a larger number of significantly hypermethylated gCpGs (Figure 1d) in the older mice as compared to the young, suggesting that the WT and Ames mice exhibit more epitype differences with age. These differences were also visualised by identifying all differentially methylated regions (gDMRs) for both the young and the old mice, using a dynamic 500bp sliding window and Fisher's Exact Test method at 5% FDR, and also removing any DMRs that are not consistent across all 4 replicates (Methods) (Figure 1e and Additional File Supplementary Statistical Data: Table 1e). We observed a significant increase in hypermethylated gDMRs, while the increase in hypomethylated gDMRs was not significant (Figure 1e and Additional File Supplementary Statistical Data: Table 1e). The consistency of the gDMRs across all four replicates within each cohort was confirmed in both gDMR heatmaps (Figure 1f) and kernel smoothed methylation plots of representative gDMRs (Figure 1g). Together, these data establish that between the Ames and WT mice there are more than 20,000 DMRs, and the number of these epigenotype differences increases markedly with age.

The Ames dwarf epigenome appears more stable and buffered against age-associated hypomethylation. To further investigate these age-associated epigenotype differences between the WT and dwarf mice, we characterised age-associated differentially methylated CpGs (i.e. CpGs whose methylation status changes with age in either WT or Ames dwarf mice (aCpGs)) in both the WT and dwarf mice, using a Fisher's Exact Test method at 5%FDR (Methods). Strikingly we observed roughly three times more significant aCpGs in the WT than the dwarf mice (Figure 2a),

suggesting that the methylome of dwarf mice is more stable through chronological aging. Similarly, we detected less than half the number of aDMRs in dwarf mice compared to WT mice (Figure 2b and Additional File Supplementary Statistical Data: Table 2b). Changes in the aDMRs were consistent across all four replicates within each cohort, as confirmed by aDMR heatmaps (Figure 2c, d) and representative kernel smoothed methylation plots (Figure 2e). Although we observed significantly more aDMRs in the WT than the dwarf mice, the magnitude of the methylation change per DMR was comparable in the WT and dwarf (Figure 2f). To confirm these aDMR loci in other mouse cohorts and strains, we performed WGBS-seq of whole liver from 2 month and 22 month of age female UM-HET3 mice from the NIA Intervention Testing Program (ITP) cohorts (4 mouse replicates per age group, approximately 5x coverage per replicate) (Additional File Supplementary Dataset 2: Table S2). Global methylation was comparable to Ames dwarf and corresponding WT and, again, between young and old (compare Figures 1a and S1a). However, PCA again separated the young and old UM-HET3, indicative of their differential methylation (Figure S1b). Hence, aDMRs were identified between young and old UM-HET3 mice (Methods). The extent of overlap of the two sets of WT hypomethylated aDMRs (i.e. the WT used for comparison to Ames dwarf throughout and WT UM-HET3 (see Methods)) was much greater than expected from random overlap and highly significant. The same was the case for the two sets of hypermethylated aDMRs. However, there was minimal overlap between hypermethylated and hypomethylated aDMRs (Figure 2g). In sum, aging of the liver generates thousands of discrete aDMRs. Significantly, the epigenome of WT mice exhibits many more such regions than that of Ames dwarf mice, suggesting that the Ames dwarf epigenome is more stable with chronological age than the wild type epigenome.

While many aDMRs were restricted to WT mice, other aDMRs were restricted to Ames mice or were shared by both genotypes (Figure S1c). Plots of mean percent methylation per mouse liver sample at each subset of DMR (i.e., WT hypermethylated aDMRs (Figure S1d), dwarf hypermethylated aDMRs (Figure S1e) and hypermethylated aDMRs shared between WT and dwarf (Figure 2h) confirmed the DMR subsets and consistency between mouse replicates (Figure 2h,i and Figure S1d-g). For example, at shared hypermethylated aDMRs, methylation increased comparably with age in both WT and dwarf (Figure 2h). The methylation changes at hypomethylated aDMRs were particularly interesting. At the hypomethylated aDMRs restricted to WT mice, the young and old Ames mice both showed methylation comparable to the young WT (Figure S1f), while at the hypomethylated aDMRs restricted to the Ames mice, the young and old WT mice both showed methylation comparable to the older Ames mice (Figure S1g). At shared hypomethylated aDMRs, while the magnitude of the change was comparable between WT and Ames, the dwarf mice showed consistently higher methylation than the WT in both age groups (Figure 2i). This phenomenon was also apparent in the population of individual aDMRs, which showed higher methylation in dwarf in both young and old mice (i.e. below right of the 45° diagonal in both plots), and an age-associated loss of methylation in both WT and dwarf (i.e. closer to zero on both x and y axes in old) (Figure S1h). At representative shared hypomethylated aDMRs, methylation declined with age in both WT and dwarf, but began at higher levels in the young dwarf (Figure 2j). In sum, in dwarf mice, hypomethylated aDMRs were biased towards a higher methylation level. This is most notable at hypomethylated aDMRs shared between WT and Ames dwarf, where the latter exhibited a higher initial level of methylation in young animals, thus potentially buffering them against the effects of age-associated hypomethylation.

Hypomethylated aDMRs are enriched at intragenic enhancers in highly expressed liver-specific genes. Next, we set out to define the location of the hypomethylated aDMRs across the genome. First, we asked how the WT and dwarf hypomethylated aDMRs are distributed across a collection of genomic features. Although there were approximately twice as many hypomethylated aDMRs in WT mice than Ames mice (Figure 2b and 3a), the proportionate distribution of these aDMRs across features of the genome was very similar (Figure 3a). Most commonly, the hypomethylated aDMRs overlap genes (~60%) and introns (~50%), although they are only modestly enriched at these features, relative to the abundance of these features in the genome (Figure 3a). Least commonly, they overlapped CpG islands (<1%) and LINE elements (~5%), and were moderately depleted at these features (Figure 3a). To further investigate, we took advantage of the many publicly available datasets for mouse liver and expanded this distribution analysis to include several ENCODE adult mouse liver histone modification ChIP-seq datasets (Figure 3a and Figure S2a and Additional Files Supplementary Datasets 3 and 4: Tables S3 and S4 respectively). Strikingly, we observed that ~55% and ~40% (Figure 3a) of all hypomethylated aDMRs overlapped the enhancer modifications H3K4me1 and H3K27ac, with an enrichment of 7-9 fold ($p<0.001$)(Figure 3a). There was a more modest overlap and enrichment, or even depletion, at other histone modifications, notably the gene body modification H3K36me3 and the repressive mark H3K27me3 (Figure 3a). In line with this enrichment at H3K4me1 and H3K27ac, there was marked overlap and enrichment at designated mouse liver enhancers, regions marked by both H3K4me1 and H3K27me3 ("Enhancers", Figure 3a). Of the ~47,000 identified mouse liver enhancers, 8,230 and 4,702 contained a hypomethylated aDMR in WT and dwarf respectively (Figure 3b), corresponding to a substantial fraction of all enhancers. Of these a

significant number (2,037) were hypomethylated in both genotypes (fold enrichment of 153, $p<0.001$ in Figure 3b). As for shared hypomethylated aDMRs generally (Figure 2i), these shared hypomethylated enhancers are apparently buffered from methylation loss in the Ames mice (Figure 3c, d). 6193 enhancers contained hypomethylated aDMRs in WT mice only (Figure 3b). These showed more marked loss of methylation in aged WT mice compared to aged dwarf mice (Figure 3e).

Since the majority of the hypomethylated enhancers are contained within genes (Figure 3a and Figure S2b), we also assessed the relationship between enhancer hypomethylation and gene expression, determined by RNA-seq (Additional File Supplementary Dataset 5: Table S5). The methylation loss per enhancer CpG was independent of expression of the gene harbouring the enhancer (Figure S2c). However, hypomethylated enhancers appeared more abundant, longer and to cover a greater fraction of the gene in highly expressed genes compared to lowly expressed genes (Figure 3f and S2d, e). Consistent with their high level of expression in liver, in both WT and dwarf mice the genes harbouring hypomethylated enhancers were highly enriched for liver specific genes (Figure S2f). Moreover, of 30 publicly available adult mouse liver transcription factor ChIP-seq datasets (Figure S2a), the factors most enriched for binding to hypomethylated aDMRs in both genotypes included key regulators of liver function (e.g., CEBPB, GR (NR3C1), RXRA, PPARA, CEBPA, HNF3A and HNF4A) ($p<0.001$)(Figure 3a). Recently, super-enhancers have been defined as clusters of enhancers that are densely bound by master transcription regulators and control expression of critical tissue specific genes [45]. Remarkably, enhancers hypomethylated during aging were greatly enriched at such super-enhancers (Figure 3g and Additional File Supplementary Statistical Data: Table 3g). Many of these trends were exacerbated in WT mice compared to Ames dwarf mice (Figure 3f, g and S2d, e). There was a

significant overlap of hypomethylated genic enhancers and changes in expression of linked genes, although the vast majority of genes containing hypomethylated enhancers did not significantly alter expression (Additional File Supplementary Dataset 6: Table S6). We conclude that hypomethylated aDMRs are most abundant at genes, introns and enhancers, and disproportionately enriched at genic super-enhancers in highly expressed genes known to play a key role in liver function. Although the distribution of hypomethylated aDMRs is similar across the WT and dwarf epigenomes, WT mice harbor a greater number of hypomethylated genes and enhancers and the potentially disruptive effects of hypomethylation [46] are seemingly buffered by a higher level of methylation in young dwarf mice.

Hypermethylated aDMRs are enriched at bivalent CpG islands. We then wanted to characterize and determine the location of hypermethylated aDMRs in the two genotypes of mice. Taking the same approach as for the hypomethylated aDMRs (Figure 3a), we asked how the hypermethylated aDMRs are spatially distributed and enriched across a range of genomic features, histone modifications and transcription factors (Figure 4a and Figure S3a). Like hypomethylated aDMRs, there were more hypermethylated aDMRs in WT mice than Ames mice (Figure 2b and Additional File Supplementary Statistical Data: Table 2b). However, in contrast to hypomethylated aDMRs, the distribution of hypermethylated aDMRs appeared different between the two genotypes. This was initially apparent in a notable disparity between fold enrichment of hypermethylated aDMRs in WT and dwarf mice; at many features these aDMRs tended to show lower fold enrichment or even depletion in WT mice, particularly at transcription factor binding sites (Figure 4a and S3a). Closer analysis showed that, while hypomethylated aDMRs were similarly distributed in WT and dwarf between regions marked or unmarked by histone

modifications and transcription factors, hypermethylated aDMRs in WT mice were disproportionately at regions lacking histone modifications and transcription factors (Figure 4b and S3b). Most of these WT-specific hypermethylated aDMRs were at regions of the genome that are relatively highly methylated even in young mice (Figure S3c).

Across more richly annotated regions of the genome, hypermethylated aDMRs were distributed similarly in WT and Ames mice. In both genotypes, hypermethylated aDMRs showed the greatest overlap with genes and introns, although like hypomethylated aDMRs this was not enriched considering the abundance of these features in the genome (Figure 4a). There was some enrichment of hypermethylated aDMRs at H3K27ac and H3K4me1-marked enhancers, although less so than for hypomethylated aDMRs (Figure 3a and 4a). However, in marked contrast to hypomethylated aDMRs, hypermethylated aDMRs showed large enrichment at CpG islands and H3K4me3 and H3K27me3 marked bivalent chromatin ($p<0.001$)(Figure 4a). There was substantial overlap between hypermethylated aDMRs at CpG islands and bivalent chromatin (fold enrichment 1,302 and $p<0.001$) (Figure 4c and Additional File Supplementary Statistical Data: Table 4c), meaning that hypermethylated aDMRs were enriched at bivalent marked CpG islands (Figure 4d). Hypermethylated bivalent regions overlapped significantly between genotypes, although there were approximately 50% more in the WT mice than Ames mice (Figure 4e and Additional File Supplementary Statistical Data: Table 4e). Interestingly, genes linked to these bivalent CpG islands tended to be expressed at relatively low levels (Figure 4f), and there was no enrichment for change in expression at these genes (Additional File Supplementary Dataset 6: Table S6). Gene ontology analysis showed that many of these bivalent CpG islands are linked to developmentally important genes that establish cell identity, similar to bivalent CpG islands in ES cells [47] (Figure S3d). In sum, hypermethylated aDMRs are enriched at bivalent CpG islands,

often of lowly expressed genes implicated in control of development and cell identity, and age-associated methylation of these islands is substantially more frequent in WT mice compared to Ames dwarf mice.

To confirm these key findings by an alternative methodology, we performed MeDIP-seq (a whole genome sequencing-based method that isolates methylated DNA sequences using an antibody to 5-methyl-cytosine (5-mC) [48]) on a single replicate of young and old WT and Ames dwarf mice. This allowed us to plot the relative enrichment of methylated DNA reads at a set of regions, in this case the hypomethylated enhancer aDMRs and hypermethylated bivalent aDMRs that are shared between WT and Ames dwarf mice (Figure 3b and 4e). This analysis confirmed a gain of methylation at hypermethylated bivalent regions and a decrease in methylation at hypomethylated enhancers (Figure S4a, b and Additional File Supplementary Statistical Data: Table S4a and S4b). Although this MeDIP-seq method cannot resolve methylation status at the single nucleotide level and our analysis of this data is limited to a single replicate, this alternative approach clearly validates key methylation changes in WT and Ames dwarf mice.

Ames dwarf mice are resistant to cancer-like methylation changes during aging. Bivalent CpG islands marked with H3K4me3 and H3K27me3 in ES cells tend to be DNA methylated in aged tissues and methylated and silenced in cancer [14-18], suggesting that age-associated DNA methylation can be a precursor to methylation and stable silencing in cancer. Therefore, we wanted to assess whether CpG islands methylated with age in mouse liver are also methylated on progression towards liver cancer and, if so, whether this trend was suppressed in cancer-resistant Ames dwarf mice. To do this, we analyzed DNA methylation data, obtained by methylated DNA immunoprecipitation (MeDIP) followed by array hybridization, from the late

precancerous stages of HCC development in 12-month old *Mdr2*/*Abcb4*-knockout (*Mdr2*-KO) male FVB strain mice, a well characterized model of chronic inflammation-mediated HCC [49, 50]. These mice typically exhibit chronic hepatitis from 2 months and HCC at 12-18 months. Enhancers that were hypomethylated in aged WT mice were comparably methylated in WT and *Mdr2*-KO mice (Figure 5a and Additional File Supplementary Statistical Data: Table 5a). In contrast, enhancers and bivalent CpG islands that were hypermethylated in aged WT mice also tended to be hypermethylated in *Mdr2*-KO mice (Figure 5b,c and Additional File Supplementary Statistical Data: Tables 5b and 5c). This phenomenon was particularly marked at bivalent CpG islands. The same marked trend was also apparent at enhancers and bivalent CpG islands methylated with age in dwarf mice (Figure 5d-f and Additional File Supplementary Statistical Data: Tables 5d-f). As noted previously, however, there were fewer such aDMRs in dwarf mice compared to WT mice (Figure 2b). These data confirm that adult liver bivalent CpG islands that are methylated during aging also tend to be methylated in pre-cancerous liver.

Age-associated DNA methylation changes are also suppressed by calorie restriction and rapamycin. As well as genetic interventions, dietary and drug interventions also promote longevity, healthy aging and suppression of cancer. To test whether dietary and drug interventions also suppress age-associated DNA methylation changes, we examined livers from female UM-HET3 mice treated with encapsulated rapamycin (42mg/kg of food) from 4 months to 22 months of age and female mice on a CR diet from 4 months to 22 months of age [51-54]. We performed WGBS-seq on DNA from whole liver, 4 replicates per cohort, approximately 5x coverage per sample (Additional File Supplementary Dataset 2: Table S2). As controls for these

UM-HET3 mice, we analyzed female UM-HET3 mice of 2 months and 22 months of age fed *ad libitum* (*ad lib*) (Figure 2g and S1a, b).

The mean global methylation level of each cohort was very similar (72.72% - 73.35%) and also very similar to the mean global methylation of the Ames dwarf mice and corresponding WT (compare Figure 1a to Figure 6a). To begin to assess the impact of CR and rapamycin on age-associated methylation changes, we compared hypomethylated and hypermethylated aDMRs between young and old UM-HET3 mice (both *ad lib*) with the same regions in rapamycin-treated and CR old mice. At hypomethylated aDMRs, methylation loss was suppressed by CR and to a lesser extent by rapamycin (Figure 6b and Supplementary Figure S5a-d and Additional File Supplementary Statistical Data: Tables 6b and S5a-d). At hypermethylated aDMRs, methylation gain was also suppressed by CR, but not significantly by rapamycin (Figure 6c and Supplementary Figure S5a, e-g and Additional File Supplementary Statistical Data: Tables S5a, e-g). At these regions, the effect of rapamycin was not significant in the whole population of hypermethylated aDMRs (Figure 6c and Supplementary Figure S5a and Additional File Supplementary Statistical Data: Table S5a), but was detectable in some individual aDMRs (Supplementary Figure S5f, g).

To assess where in the genome rapamycin and CR suppress age-associated methylation changes, we generated clustered feature interaction maps depicting the genomic distribution of regions where methylation changes were suppressed by rapamycin and CR. CR suppressed age-associated changes widely, including at genes, enhancers and CpG islands (Figure 6d, e and Additional File Supplementary Statistical Data: Tables 6d, e). Rapamycin suppressed age-associated changes at a smaller number of genes, enhancers and CpG islands (Figure 6f, g and Additional File Supplementary Statistical Data: Tables 6f, g). When averaged across a “composite”

of all enhancers hypomethylated with age, the suppression was similarly more marked by CR than rapamycin (Figure 6h). When averaged across all hypermethylated bivalent regions, the suppression by CR was readily apparent but modest, whereas the suppression by rapamycin was undetectable (Figure 6i). In sum, while both CR and rapamycin suppressed age-associated hypomethylation at enhancers, at least under these protocols CR was more efficient than rapamycin. CR also suppressed hypermethylation of some bivalent regions and CpG islands, while at these regions the effect of rapamycin was detectable at a minority of regions but not in all regions combined.

In addition to suppressing age-associated methylation events, closer analysis revealed that both CR and rapamycin caused a number of hypo and hypermethylation events that did not reflect a suppression of age-associated changes (Figure S5h). However, rapamycin caused substantially more of these than did CR. These non-age related methylation changes were widely distributed, including at genes, bivalent CpG islands and enhancers (Figure S5h).

Discussion

Here we have comprehensively mapped age-associated changes in DNA methylation across all 42 million CpGs of the genome by WGBS-seq of young and old mouse liver. In analysis and interpretation of our data, we have made extensive use of gene expression data and the many epigenomic data sets publicly available for mouse liver. Although we failed to observe global change in DNA methylation, e.g. a global hypomethylation, we did observe thousands of age-associated changes across discrete regions of the genome. The greatest number of such gains and losses of methylation occur at genes and introns, although the number of these changes is in proportion to the fraction of the genome occupied by those features. Instead, losses of

methylation are most enriched at genic enhancers, including super-enhancers, within genes highly expressed in liver, and gains of methylation are most enriched at bivalent CpG islands. In sum, age-associated changes in DNA methylation are most abundant and/or enriched at various important functional and regulatory regions of the genome.

What is the cause of these age-associated changes? It is tempting to speculate that age-associated changes are linked to the dynamic nature of these regulatory and functional regions. These dynamic regions are maintained at a steady state equilibrium that may change with age. Expressed genes and enhancers are thought to be particularly dynamic regions of the epigenome [55]. More specifically, age-associated changes in DNA methylation might be linked to age-associated changes in expression of the cellular machinery that directly controls DNA methylation, such as DNMTs and TETs. Indeed, we have previously shown an age-associated increase and decrease in expression of DNMT3a and DNMT1 respectively, in mouse liver [43]. Alternatively, age-associated changes in methylation might result from changes in metabolic substrates and cofactors important for activity of DNMTs and TETs, such as S-adenosylmethionine (SAM) and α-ketoglutarate respectively [56]. However, there must also be additional sequence and/or epigenetic determinants of methylation gains and losses to explain why some regions, such as bivalent CpG islands, gain methylation whereas others, such as enhancers, lose methylation with age.

What is the consequence of these age-associated changes in methylation? Losses of methylation at enhancers are only weakly linked to changes in expression of linked genes, and some genes increase and others decrease in expression. Recent studies suggested that DNA methylation of enhancers is required for their functional integrity [46, 57]. So, while age-associated loss of methylation at enhancers in these moderately old mice (22 months of age) is

only modestly linked to changes in gene expression, it is conceivable that this methylation loss is a precursor to more dramatic changes in methylation and expression in very old mice or perhaps after tissue stress. In contrast to enhancers, gains of DNA methylation at bivalent CpG islands are not enriched for changes in gene expression. Most of these genes are expressed at comparatively low levels even in normal young tissue, and a gain of methylation at the promoter CpG island is not expected to increase their expression. Importantly, however, age-associated changes at bivalent CpG islands are linked to hypermethylation in cancer, suggesting that age-associated gain in methylation can be a precursor to cancer, for example by blocking activation of pro-differentiation and development genes, as proposed previously [14, 17].

Age-associated changes in DNA methylation are suppressed by genetic, dietary and drug interventions that extend lifespan and delay/suppress the incidence of cancer, specifically the *Prop1* mutation in the Ames dwarf mouse, CR and rapamycin [1, 5]. Each of these interventions suppresses age-associated changes in methylation at genes, enhancers and bivalent CpG islands. Consistent with the aforementioned proposal that age-associated methylation changes are linked to control of the DNA methylation machinery and/or its metabolic regulators, Ames dwarf mice do display atypical methionine metabolism, methionine being a source of the SAM that is required for DNA methylation. Components of this amino acid pathway are upregulated in Ames mice leading to higher enzyme activities, including of glycine N-methyltransferase (GNMT), an enzyme that converts SAM to S-adenosyl-homocysteine and sarcosine. Moreover, the methyltransferase enzymes important in DNA methylation and methionine metabolism are affected by the presence or absence of GH. Methionine flux assays confirm the enhanced enzyme activities demonstrating that transmethylation and transsulfuration are markedly elevated in dwarf mice [58, 59]. Thus, elevated GNMT in Ames dwarf mice might depress age-associated

methylation of CpG islands, perhaps contributing to delayed cancer incidence [2, 3]. Similarly CR and rapamycin might suppress the incidence of cancer [5-7], at least in part, by suppressing methylation of bivalent CpG island promoters. Aside from cancer suppression, the other shared benefits of these genetic, dietary and drug interventions for maintenance of tissue and systemic function into old age might depend on suppression of super-enhancer hypomethylation and so preservation of tissue specific enhancer integrity, gene expression programs and tissue function [46]. Typically, the effect of CR on the epigenome was greater than rapamycin, in line with the greater extension of lifespan by CR than rapamycin, at least under the protocols tested here [53, 54].

Notwithstanding the more efficient suppression of age-associated epigenetic changes by CR, the epigenetic effects of CR and rapamycin were not identical and this might further underlie some of the differences between them that have been noted in previous studies, for example in endocrine and metabolic phenotypes and gene expression profiles [53, 60]. Of note, rapamycin in particular appears to induce additional changes unrelated to age-associated changes. While both CR and rapamycin induced these non age-related effects, this effect was much more marked for rapamycin. These non age-related epigenetic changes include gains of methylation at genes, enhancers and CpG islands and losses of methylation at genes and enhancers. Conceivably, such non age-related effects of rapamycin in liver and other tissues may contribute to at least some of the well-documented harmful side-effects of rapamycin, such as glucose intolerance, increased incidence of testicular degeneration and cataracts [52, 53]. Detrimental effects of rapamycin-like drugs, including dyslipidemia, hyperlipidemia and risk of diabetes, have also been noted in humans [61, 62]. Of course, such adverse consequences of rapamycin might also have a non-epigenetic basis. Regardless, this study is a first comparison of the effect of diverse genetic,

dietary and drug interventions on the epigenetic landscape, and a foundation for understanding their influence on epigenetic determinants of chronological and biological aging.

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Authors Contributions

JC and NR performed the majority of the experiments. MIR, JPT, TM, DS, TW, WC and CB performed and evaluated some individual experiments. RRM, RAM, HMBB and PDA designed and supervised individual experiments with RAM and HMBB providing the materials. HMBB and PDA conceived, designed and supervised the study; while JC, NR, HMBB and PDA wrote the manuscript with contributions from all authors.

Competing Interests.

The authors declare that they have no competing interests.

Ethics Approval

Mice were bred, housed, fed and culled according to ethical procedures overseen by IACUC at the University of North Dakota and University of Michigan.

Data Access

Data is freely available at GEO (NCBI) under the accession number GSE89275.

Figure Legends.

Figure 1. The epigenomes of wild type and Ames dwarf mice diverge with age. a) Global percent methylation per liver sample, for 2 month of age wild type (WTY), 22 month wild type (WTO), 2 month Ames dwarf (DY) and 22 month Ames dwarf (DO) mice. All P>0.05 (two tailed t-test on arcsine transformed proportions). **b)** UCSC genome browser trace of percent methylation over chromosome 1, showing pooled WTY (light blue), WTO (dark blue), DY (light red), DO (dark red) replicate tracks. **c)** Principal component analysis of CpG percent methylation, for WTY (light blue), WTO (dark blue), DY (light red), DO (dark red) liver samples. PC1 (Proportion of variance: 12.38%)" and to y-axis "PC2 (Proportion of variance: 7.64%). **d)** Bar chart of the number of significantly differentially methylated CpGs (5% FDR, Fisher's Exact test) between 4 pooled WT and 4 pooled Ames dwarf replicates (gCpGs), in liver of 2 month (young) and 22 month (old) mice. Hyper and hypo-methylated gCpGs are higher and lower in Ames dwarf mice, respectively. **e)** As d), however showing significantly differentially methylated regions (gDMRs)(5% FDR, Fisher's Exact test, 500bp windows). Regions of heterogeneity (chi-squared test <0.05) across the 4 replicates in each cohort removed. DY-WTY vs DO-WTO hyper, p<0.05 (marked with *). See also Additional File Supplementary Statistical Dataset. **f)** Heatmaps of the percent methylation across all 2 month (left) and 22 month (right) gDMRs. Replicate samples (4 mouse livers) are in rows

and the gDMRs columns. The intensity of the heatmap represents column scaled % methylation (Z-score), with value ranging from lower to higher methylation as blue to yellow. **g)** Kernel smoothed line plots of selected gDMRs, +/- 5kb. WTY, DY, WTO and DO replicates are represented by solid blue, solid red, dashed blue and dashed red lines respectively. DMRs are shown in pink and CpGs black.

Figure 2. The Ames dwarf epigenome appears more stable and buffered against age-associated hypomethylation. a) Bar chart of the number of significantly differentially methylated CpGs (5% FDR, Fisher's Exact test) between 2 month and 22 month WT (WT) and between 2 month and 22 month Ames dwarf (dwarf) mice. Hyper and Hypo-methylated aCpGs are higher and lower in 22 month mice respectively. **b)** Bar chart of the number of significantly differentially methylated regions (aDMRs)(5% FDR, Fisher's Exact test, 500bp windows), between 2 months and 22 month mice, for WT and Ames dwarf mice. Regions of heterogeneity (chi-squared test <0.05) across the 4 replicates in each cohort removed. Significance (empirical p-value) at $p < 0.05$ is indicated with *. See also Additional File Supplementary Statistical Dataset. **c)** Heatmap of the % methylation across all 2 month (DY) versus 22 month (DO) Ames dwarf differentially methylated regions (aDMRs). Replicate samples (4 mouse livers) are in rows and the aDMRs columns. The intensity of the heatmap represents column scaled % methylation (Z-score), with value ranging from lower to higher methylation as blue to yellow. **d)** Heatmap of the % methylation across all 2 month (WTY) versus 22 month (WTO) WT differentially methylated regions (aDMRs). Replicate samples (4 mouse livers) are in rows and the aDMRs columns. Columns are scaled using Z-scores. The intensity of the heatmap represents Z-score, with value ranging from negative to positive as blue to yellow. **e)** Kernel smoothed line plots of selected

aDMRs, +/- 5kb. WTY, 2 month dwarf (DY), WTO and 22 month dwarf (DO) replicates are represented by solid blue, solid red, dashed blue and dashed red lines respectively. DMRs are shown in pink and CpGs black. **f)** Histogram of the difference in mean % methylation per DMR (across all samples), between 22 and 2 month mice (O-Y), versus number of aDMRs. Showing WT aDMRs (red) and dwarf aDMRs (blue). **g)** Bar chart showing ratio of observed/expected (random) overlap between WT (from panels a-d) and UM-HET3 aDMRs. Hyper and Hypo-methylated aDMRs are higher and lower in old mice respectively. Significance (empirical p-value) at $p < 0.001$ is indicated with **. **h)** Mean % methylation per replicate across all hypermethylated aDMRs common to both WT and Ames dwarf mice (shared). WTY, WTO, DY and DO mice are light blue, dark blue, light red and dark red respectively. WTY vs WTO and DY vs DO at $p < 0.001$ indicated with ** (two tailed t-test on arcsine transformed proportions). **i)** As h), however showing shared hypomethylated aDMRs. WTY vs WTO, DY vs DO, WTO vs DO all $P<0.001$ indicated with ** and WTY vs DY $P<0.05$ indicated with * (two tailed t-test on arcsine transformed proportions). **j)** Kernel smoothed line plots of selected aDMRs common to both WT and dwarf mice, +/- 5kb. Pooled replicates for WTY, DY, WTO and DO are represented by solid blue, solid red, dashed blue and dashed red lines respectively. DMRs are shown in pink and CpGs black.

Figure 3. Hypomethylated aDMRs are enriched at intragenic enhancers in highly expressed liver-specific genes. a) Clustered feature interaction maps of spatial overlap between hypomethylated aDMRs (columns) and a selection of genomic, histone and transcription factor features (rows), showing WT (WT)(left) and dwarf (D)(center left) aDMRs. Red indicates an overlap between aDMR and feature and blue no overlap. Interaction map x axes are scaled by

number of aDMRs. The % overlap (center right) and fold enrichment observed/expected (random) overlap (right)(units of fold) for each feature is given. **b)** Venn diagram of the overlap between enhancers that contain hypomethylated aDMRs in WT and Ames dwarf mice. Enrichment of overlap obs/exp 153-fold, P<0.001. **c)** Mean % methylation per replicate across enhancers that contain hypomethylated aDMRs in both WT and dwarf mice. For 2 month WT (WTY)(light blue), 22 month WT (WTO)(dark blue), 2 month dwarf (DY)(light red) and 22 month dwarf (DO)(dark red) mice. WTY vs WTO, DY vs DO, WTO vs DO all P<0.001 (indicated with **) and WTY vs DY P<0.05 (indicated with *) (two tailed t-test on arcsine transformed proportions). **d)** Kernel smoothed line plots of selected enhancers overlapping hypomethylated aDMRs, +/- 5kb. Replicates for WTY, DY, WTO and DO are represented by solid blue, solid red, dashed blue and dashed red lines respectively. DMRs are shown in pink and CpGs black. H3K4me1 and H3K27ac enrichment (ChIP-seq) is indicated. **e)** Heatmap of the percent methylation across all enhancers containing hypomethylated aDMRs unique to WT mice (6,193 regions from Figure 3b). Replicate samples (4 mouse livers) are in columns and the aDMRs rows. The intensity of the heatmap represents row scaled % methylation (Z-score), with value ranging from lower to higher methylation as blue to red. **f)** Mean number of enhancer overlapping hypomethylated aDMRs per gene for WT (Blue) and Ames dwarf (Red). Genes are split into quartiles by expression (Q1 = highest, Q4 = lowest). Unexpressed genes (FPKM = 0) are given (U). **g)** Observed and expected overlap (in base pairs) of hypomethylated DMRs (WT, WT only; dwarf, dwarf only; shared, shared between WT and dwarf) with super-enhancers. P<0.01 indicated by **.

Figure 4. Hypermethylated aDMRs are enriched at bivalent CpG islands. a) Clustered feature interaction maps of spatial overlap between hypermethylated aDMRs (columns) and a selection

of genomic, histone and transcription factor features (rows), showing WT (WT)(left) and dwarf (D)(center left) aDMRs. Red indicates an overlap between aDMR and feature and blue no overlap. Interaction map x axes are scaled by number of aDMRs. The % overlap (center right) and fold enrichment observed/expected (random) overlap (right)(units of fold) for each feature is given. **b)** Percentage of WT and Ames dwarf aDMRs that overlap with either histone modifications or panel of 30 transcription factors (Histone or TF) (blue) or neither (Neither)(red). **c)** Venn diagram of the base pair (in mega bp) overlap between hypermethylated aDMR containing CpG islands and hypermethylated aDMR containing bivalent regions in Ames WT. Enrichment of overlap obs/exp 1302-fold, $P<0.001$. **d)** Kernel smoothed line plots of selected bivalent CpG island (CpGI) overlapping hypermethylated aDMRs, +/- 5kb. Replicates for 2 month WT (WTY) and 22 month WT (WTO) mice are represented by solid blue and dashed blue lines respectively. DMRs are shown in pink and CpGs black. H3K4me3 and H3K27me3 enrichment (ChIP-seq) is indicated. **e)** Venn diagram of liver bivalent regions that contain hypermethylated aDMRs in WT and dwarf mice. Enrichment of overlap obs/exp 173-fold, $P<0.001$. **f)** Mean number of hypermethylated bivalent aDMRs per gene for WT (Blue) and Ames dwarf (Red). Genes are split into quartiles by expression (Q1 = highest, Q4 = lowest). Unexpressed genes (FPKM = 0) are given (U).

Figure 5. Ames dwarf mice are resistant to cancer-like methylation changes during aging. **a)** Mean methylation enrichment per probe (top panel) at all probes within enhancers that contain WT hypomethylated aDMRs, for control (x-axis) and *Mdr-2* knock out (y-axis) mice. Numbers top left and bottom right show the number of probes above and below the dashed diagonal. Bottom panel shows same data per mouse replicate. $P<0.05$ indicated by * (two tailed t-

test). **b)** as a), however using enhancers containing WT hypermethylated aDMRs. P<0.01 indicated by ** (two tailed t-test). **c)** as a), however using bivalent regions that contain Ames WT hypermethylated aDMRs. P<0.01 indicated by ** (two tailed t-test). **d)** as a), however using enhancers that contain Ames dwarf hypomethylated aDMRs. P>0.05 (two tailed t-test). **e)** as a), however using enhancers that contain Ames dwarf hypermethylated aDMRs. P<0.01 indicated by ** (two tailed t-test). **f)** as a), however using bivalent regions that contain Ames dwarf hypermethylated aDMRs. P<0.01 indicated by ** (two tailed t-test).

Figure 6. Age-associated DNA methylation changes are also suppressed by calorie restriction and rapamycin. a) Global % methylation per sample, for 2 month (Y) and 22 month (O) control, 22 month caloric restricted (CR) and 22 month Rapamycin treated (Rapa) UM-HET3 mice. Part of this panel is reproduced from Figure S1c. All P>0.05 (two tailed t-test on arcsine transformed proportions). **b)** Mean % methylation per sample across all O-Y hypomethylated aDMRs for the samples in a)**.** Y vs O, Y vs Rapa, O vs CR all P<0.001 (selected indicated by **) and Y vs CR, O vs Rapa , CR vs Rapa all P<0.05 (selected indicated by *) (two tailed t-test on arcsine transformed proportions). **c)** Mean % methylation per sample across all O-Y hypermethylated aDMRs for the samples in a). Y vs O, Y vs Rapa, Y vs CR, all P<0.001 (selected indicated by **), O vs CR, CR vs Rapa P<0.05, O vs Rapa P>0.05 (selected indicated by *) (two tailed t-test on arcsine transformed proportions). **d)** Clustered feature interaction maps of spatial overlap between O-Y aDMRs (columns) and a selection of genomic, histone and transcription factor features (rows), showing hypomethylated aDMRs that are also CR-O hypermethylated DMRs (i.e. suppressed by CR). 1116 regions. **e)** As d), for O-Y hypermethylated aDMRs that are also CR-O hypomethylated DMRs (i.e. suppressed by CR). 359 regions. **f)** As d), for O-Y hypomethylated aDMRs that are also

Rapa-O hypermethylated DMRs (i.e. suppressed by rapamycin). 330 regions. **g)** As d), for O-Y hypermethylated aDMRs that are also Rapa-O hypomethylated DMRs (i.e. suppressed by rapamycin). 225 regions. In d)-g), interaction map x axes are scaled by number of aDMRs. **h)** Composite profiles of mean percent methylation at all hypomethylated aDMR (Old-Young, UM-HET3 mice) enhancer regions (n=1867), showing Young (blue), Old (black), CR (orange) and rapamycin (red). **i)** Composite profiles of mean percent methylation at hypermethylated aDMR (Old-Young) bivalent regions (n=536), showing Young (blue), Old (black), CR (orange) and rapamycin (red).

Figure S1. Supplementary data to Figure 2. a) Global % methylation per sample, for 2 month (Y), 22 month (O) UM-HET3 mice. P>0.05 (two tailed t-test on arcsine transformed proportions). **b)** Principal component analysis of Bisulphite sequencing % methylation, for Y and O UM-HET3 mice. **c)** Venn diagrams of overlap in mega bp between WT and dwarf hypermethylated aDMRs (left) and hypomethylated aDMRs (right). Both WT and dwarf, P < 0.001. **d)** Mean % methylation per sample across all hypermethylated aDMRs that present in WT but not dwarf mice. WTY vs WTO, WTY vs DY, WTO vs DO P<0.001 indicated by ** and DY vs DO P>0.05 (two tailed t-test on arcsine transformed proportions). **e)** Mean % methylation per sample across all hypermethylated aDMRs that present in dwarf but not WT mice. DY vs DO, WTO vs DO P<0.001 indicated by **, WTY vs DY P<0.05 indicated by *, WTY vs WTO P>0.05 (two tailed t-test on arcsine transformed proportions). **f)** Mean % methylation per sample across all hypomethylated aDMRs that present in WT but not dwarf mice. WTY vs WTO, WTO vs DO P<0.001 indicated by **, DY vs DO P<0.05 indicated by *, WTY vs DY P>0.05 (two tailed t-test on arcsine transformed proportions). **g)** Mean % methylation per sample across all hypomethylated aDMRs that present

in dwarf but not WT mice. DY vs DO, WTY vs DY P<0.001 indicated by **, WTY vs WTO P<0.05 indicated by *, WTO vs DO P>0.05 (two tailed t-test on arcsine transformed proportions). **h)** Left panel : Scatter plot of all hypomethylated aDMRs common to both WT and Ames dwarf mice, showing mean % methylation (per region) in 2 month old WT mice (y-axis) and 2 month old dwarf mice (x-axis). Regions that are significantly different in % methylation between WT and dwarf are shown in red. Right panel : As left, but in old mice.

Figure S2: Supplementary data to Figure 3. a) Clustered feature interaction maps of spatial overlap between hypomethylated aDMRs (columns) and a panel of 30 transcription factors (rows), showing WT (WT)(left) and dwarf (D)(center left) aDMRs. Red indicates an overlap between aDMR and feature and blue no overlap. Interaction map x axes are scaled by number of aDMRs. The % overlap (center right) and fold enrichment observed/expected (random) overlap (right)(units of fold) for each feature is given. **b)** Proportion of enhancers overlapping WT (outer ring) and dwarf (inner ring) hypomethylated aDMRs that are: promoter (blue), genic (and not promoter)(red) and intergenic (and not promoter)(green). **c)** Mean difference in % methylation (22 month (O) – 2 month (Y)) per CpG within hypomethylated aDMRs that overlap genic enhancers. CpGs are split into quartiles by expression of the nearest gene (Q1 = highest, Q4 lowest). Unexpressed genes (FPKM = 0) are given (U). WT (blue) and dwarf (red) values are shown. **d)** Mean percent of gene that is a hypomethylated aDMR that overlaps genic enhancers for WT (Blue) and Ames dwarf (Red). Genes are split into quartiles by expression (Q1 = highest, Q4 = lowest). Unexpressed genes (FPKM = 0) are given (U). WT (blue) and dwarf (red) values are shown. **e)** Mean size in bp that is a hypomethylated aDMR that overlaps genic enhancers for WT (Blue) and Ames dwarf (Red). Genes are split into quartiles by expression (Q1 = highest, Q4

lowest). Unexpressed genes (FPKM = 0) are given (U). WT (blue) and dwarf (red) values are shown. **f)** Most highly enriched tissue expression profiles (David, UP tissue) amongst all WT (top table) and dwarf (bottom table, italicized) genes with aDMR overlapping enhancers.

Figure S3. Supplementary data to Figure 4. a) Clustered feature interaction maps of spatial overlap between hypermethylated aDMRs (columns) and a panel of 30 transcription factors (rows), showing WT (WT)(left) and dwarf (D)(center left) aDMRs. Red indicates an overlap between aDMR and feature and blue no overlap. Interaction map x axes are scaled by number of aDMRs. The % overlap (center right) and fold enrichment observed/expected (random) overlap (right)(units of fold) for each feature is given. **b)** Barchart of the number of WT (blue) and Ames dwarf (red) aDMRs that overlap with either histone modifications or panel of 30 transcription factors (TF and Histone) or neither (Neither). **c)** Histograms of number of hypermethylated aDMRs (y-axis) by % methylation (x axis) in 2 month WT (left) and Ames dwarf (right) mice. Showing aDMRs that overlap with either histone modifications or panel of 30 transcription factors (blue) or neither (red). **d)** Top enriched IPA molecular functions for all genes containing WT (left) and Ames dwarf (right) hypermethylated aDMRs overlapping bivalent regions.

Figure S4: Supplementary data to Figures 2-4. a) Box-plot of mean MeDIP enrichment at shared hypermethylated aDMR bivalent regions. We observe significant increase of mean enrichment across all regions with strain and age in both instances. WTY vs. WTO ($P=0.011$) and DY vs. DO ($P=0.011$) all $P<0.05$ indicated by *(significance was determined using a Mann-Whitney test of median shift). **b)** Box-plot of mean MeDIP enrichment at a shared set of hypomethylated aDMR enhancer regions. We observe significant decrease in enrichment in both

dwarf and WT strains with age. WTY vs. WTO and DY vs. DO all P<0.001 indicated by **(significance was determined using a Mann-Whitney test of median shift).

Figure S5: Supplementary data to Figure 6. a) Bar chart of the number of significantly differentially methylated regions (5% FDR, Fisher's Exact test, 500bp windows), between UM-HET3 young (2 months) and old (22 month) control mice (Old-Young), young and 22 month calorie restricted mice (CR-Young) and young and 22 month Rapamycin treated mice (Rapa-Young). Regions of heterogeneity (chi-squared test (<0.05) across the 4 replicates in each cohort removed. See also Supp Dataset 2. Hyper and Hypo-methylated DMRs are higher and lower in 22 month mice respectively. **b)** Scatter plot of all hypomethylated aDMRs (significant as defined in a)) between 22 month (O) and 2 month (Y) control UM-HET3 mice, showing mean percent methylation (per region) in 22 month old control mice (x-axis) and 22 month old calorie restricted mice (CR)(y-axis). Regions that are significantly different in % methylation between control and CR mice are given in red. Only regions with >=10 reads in CR were included. **c)** As b), however using percent methylation in Rapamycin treated (Rapa) instead of CR. **d)** Histogram of hypomethylated aDMR (significant as defined in a)) counts (O-Y) versus relative difference in mean % methylation per aDMR, for: (O-Y)/Y (blue), (Rapa-Y)/Y (red) and (CR-Y)/Y (green). **e)** As b), however using all hypermethylated aDMRs between 22 month (O) and 2 month (Y) control mice. **f)** as b) however using all hypermethylated aDMRs between 22 month (O) and 2 month (Y) control mice, and % methylation in Rapamycin treated (Rapa) instead of CR. **g)** Histogram of hypermethylated aDMR (significant as defined in a)) counts (O-Y) versus relative difference in mean % methylation per aDMR, for: (O-Y)/Y (blue), (Rapa-Y)/Y (red) and (CR-Y)/Y (green). **h)** Left : Clustered feature interactions maps of spatial overlap between DMRs (significant as defined

in a)) that are hypomethylated in CR-Young but not Old-Young. Red indicates an overlap between DMR and feature and blue no overlap. 1301 regions. Other 3 interaction maps as indicated. Interaction map x-axes are scaled by number of DMRs indicated.

References

1. Brown-Borg, H.M., *The somatotropic axis and longevity in mice.* Am J Physiol Endocrinol Metab, 2015. **309**(6): p. E503-10.
2. Ikeno, Y., et al., *Delayed occurrence of fatal neoplastic diseases in ames dwarf mice: correlation to extended longevity.* J Gerontol A Biol Sci Med Sci, 2003. **58**(4): p. 291-6.
3. Ikeno, Y., et al., *Reduced incidence and delayed occurrence of fatal neoplastic diseases in growth hormone receptor/binding protein knockout mice.* J Gerontol A Biol Sci Med Sci, 2009. **64**(5): p. 522-9.
4. Salmon, A.B., et al., *Fibroblast cell lines from young adult mice of long-lived mutant strains are resistant to multiple forms of stress.* Am J Physiol Endocrinol Metab, 2005. **289**(1): p. E23-9.
5. Mercken, E.M., et al., *Of mice and men: the benefits of caloric restriction, exercise, and mimetics.* Ageing Res Rev, 2012. **11**(3): p. 390-8.
6. Harrison, D.E., et al., *Rapamycin fed late in life extends lifespan in genetically heterogeneous mice.* Nature, 2009. **460**(7253): p. 392-5.
7. Anisimov, V.N., et al., *Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice.* Cell Cycle, 2011. **10**(24): p. 4230-6.
8. Longo, V.D., et al., *Interventions to Slow Aging in Humans: Are We Ready?* Aging Cell, 2015.
9. Lopez-Otin, C., et al., *The hallmarks of aging.* Cell, 2013. **153**(6): p. 1194-217.
10. Berdasco, M. and M. Esteller, *Hot topics in epigenetic mechanisms of aging: 2011.* Aging Cell, 2012. **11**(2): p. 181-6.
11. Kreiling, J.A., et al., *Age-associated increase in heterochromatic marks in murine and primate tissues.* Aging Cell, 2011. **10**(2): p. 292-304.
12. Maze, I., et al., *Critical Role of Histone Turnover in Neuronal Transcription and Plasticity.* Neuron, 2015. **87**(1): p. 77-94.
13. Booth, L.N. and A. Brunet, *The Aging Epigenome.* Mol Cell, 2016. **62**(5): p. 728-44.
14. Rakyan, V.K., et al., *Human aging-associated DNA hypermethylation occurs preferentially at bivalent chromatin domains.* Genome Res, 2010. **20**(4): p. 434-9.
15. Ohm, J.E., et al., *A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing.* Nat Genet, 2007. **39**(2): p. 237-42.
16. Schlesinger, Y., et al., *Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer.* Nat Genet, 2007. **39**(2): p. 232-6.

17. Teschendorff, A.E., et al., *Age-dependent DNA methylation of genes that are suppressed in stem cells is a hallmark of cancer.* Genome Res, 2010. **20**(4): p. 440-6.
18. Widschwendter, M., et al., *Epigenetic stem cell signature in cancer.* Nat Genet, 2007. **39**(2): p. 157-8.
19. Avrahami, D., et al., *Aging-Dependent Demethylation of Regulatory Elements Correlates with Chromatin State and Improved beta Cell Function.* Cell Metab, 2015. **22**(4): p. 619-32.
20. Bochkis, I.M., et al., *Changes in nucleosome occupancy associated with metabolic alterations in aged mammalian liver.* Cell Rep, 2014. **9**(3): p. 996-1006.
21. Day, K., et al., *Differential DNA methylation with age displays both common and dynamic features across human tissues that are influenced by CpG landscape.* Genome Biol, 2013. **14**(9): p. R102.
22. Raddatz, G., et al., *Aging is associated with highly defined epigenetic changes in the human epidermis.* Epigenetics Chromatin, 2013. **6**(1): p. 36.
23. Horvath, S., *DNA methylation age of human tissues and cell types.* Genome Biol, 2013. **14**(10): p. R115.
24. Hannum, G., et al., *Genome-wide methylation profiles reveal quantitative views of human aging rates.* Mol Cell, 2013. **49**(2): p. 359-67.
25. Weidner, C.I., et al., *Aging of blood can be tracked by DNA methylation changes at just three CpG sites.* Genome Biol, 2014. **15**(2): p. R24.
26. Marioni, R.E., et al., *DNA methylation age of blood predicts all-cause mortality in later life.* Genome Biol, 2015. **16**(1): p. 25.
27. Gross, A.M., et al., *Methylome-wide Analysis of Chronic HIV Infection Reveals Five-Year Increase in Biological Age and Epigenetic Targeting of HLA.* Mol Cell, 2016. **62**(2): p. 157-68.
28. Dang, W., et al., *Histone H4 lysine 16 acetylation regulates cellular lifespan.* Nature, 2009. **459**(7248): p. 802-7.
29. Greer, E.L., et al., *Members of the H3K4 trimethylation complex regulate lifespan in a germline-dependent manner in C. elegans.* Nature, 2010. **466**(7304): p. 383-7.
30. Maures, T.J., et al., *The H3K27 demethylase UTX-1 regulates C. elegans lifespan in a germline-independent, insulin-dependent manner.* Aging Cell, 2011. **10**(6): p. 980-90.
31. Dang, W., et al., *Inactivation of yeast Isw2 chromatin remodeling enzyme mimics longevity effect of calorie restriction via induction of genotoxic stress response.* Cell Metab, 2014. **19**(6): p. 952-66.
32. Sen, P., et al., *H3K36 methylation promotes longevity by enhancing transcriptional fidelity.* Genes Dev, 2015. **29**(13): p. 1362-76.
33. Liu, L., et al., *Chromatin modifications as determinants of muscle stem cell quiescence and chronological aging.* Cell Rep, 2013. **4**(1): p. 189-204.
34. Marzluff, W.F., E.J. Wagner, and R.J. Duronio, *Metabolism and regulation of canonical histone mRNAs: life without a poly(A) tail.* Nat Rev Genet, 2008. **9**(11): p. 843-54.

35. Sun, D., et al., *Epigenomic profiling of young and aged HSCs reveals concerted changes during aging that reinforce self-renewal.* Cell Stem Cell, 2014. **14**(5): p. 673-88.
36. Beerman, I., et al., *Proliferation-dependent alterations of the DNA methylation landscape underlie hematopoietic stem cell aging.* Cell Stem Cell, 2013. **12**(4): p. 413-25.
37. Dhawan, S., S.I. Tschen, and A. Bhushan, *Bmi-1 regulates the Ink4a/Arf locus to control pancreatic beta-cell proliferation.* Genes Dev, 2009. **23**(8): p. 906-11.
38. Morgan, M.A. and A. Shilatifard, *Chromatin signatures of cancer.* Genes Dev, 2015. **29**(3): p. 238-49.
39. Maegawa, S., et al., *Age-related epigenetic drift in the pathogenesis of MDS and AML.* Genome Res, 2014. **24**(4): p. 580-91.
40. Brown-Borg, H.M., et al., *Growth hormone signaling is necessary for lifespan extension by dietary methionine.* Aging Cell, 2014. **13**(6): p. 1019-27.
41. Krueger, F. and S.R. Andrews, *Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications.* Bioinformatics, 2011. **27**(11): p. 1571-2.
42. Langmead, B., et al., *Ultrafast and memory-efficient alignment of short DNA sequences to the human genome.* Genome Biol, 2009. **10**(3): p. R25.
43. Armstrong, V.L., et al., *Expression of DNA methyltransferases is influenced by growth hormone in the long-living Ames dwarf mouse in vivo and in vitro.* J Gerontol A Biol Sci Med Sci, 2014. **69**(8): p. 923-33.
44. Fraga, M.F., et al., *Epigenetic differences arise during the lifetime of monozygotic twins.* Proc Natl Acad Sci U S A, 2005. **102**(30): p. 10604-9.
45. Whyte, W.A., et al., *Master transcription factors and mediator establish super-enhancers at key cell identity genes.* Cell, 2013. **153**(2): p. 307-19.
46. Charlet, J., et al., *Bivalent Regions of Cytosine Methylation and H3K27 Acetylation Suggest an Active Role for DNA Methylation at Enhancers.* Mol Cell, 2016. **62**(3): p. 422-31.
47. Bernstein, B.E., et al., *A bivalent chromatin structure marks key developmental genes in embryonic stem cells.* Cell, 2006. **125**(2): p. 315-26.
48. Kurdyukov, S. and M. Bullock, *DNA Methylation Analysis: Choosing the Right Method.* Biology (Basel), 2016. **5**(1).
49. Stoyanov, E., et al., *Chronic liver inflammation modifies DNA methylation at the precancerous stage of murine hepatocarcinogenesis.* Oncotarget, 2015. **6**(13): p. 11047-60.
50. Mauad, T.H., et al., *Mice with homozygous disruption of the mdr2 P-glycoprotein gene. A novel animal model for studies of nonsuppurative inflammatory cholangitis and hepatocarcinogenesis.* Am J Pathol, 1994. **145**(5): p. 1237-45.
51. Harrison, D.E., et al., *Acarbose, 17-alpha-estradiol, and nordihydroguaiaretic acid extend mouse lifespan preferentially in males.* Aging Cell, 2014. **13**(2): p. 273-82.
52. Wilkinson, J.E., et al., *Rapamycin slows aging in mice.* Aging Cell, 2012. **11**(4): p. 675-82.

53. Miller, R.A., et al., *Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction.* Aging Cell, 2014. **13**(3): p. 468-77.
54. Flurkey, K., C.M. Astle, and D.E. Harrison, *Life extension by diet restriction and N-acetyl-L-cysteine in genetically heterogeneous mice.* J Gerontol A Biol Sci Med Sci, 2010. **65**(12): p. 1275-84.
55. Schubeler, D., *Function and information content of DNA methylation.* Nature, 2015. **517**(7534): p. 321-6.
56. Berger, S.L. and P. Sassone-Corsi, *Metabolic Signaling to Chromatin.* Cold Spring Harb Perspect Biol, 2015.
57. Rinaldi, L., et al., *Dnmt3a and Dnmt3b Associate with Enhancers to Regulate Human Epidermal Stem Cell Homeostasis.* Cell Stem Cell, 2016. **19**(4): p. 491-501.
58. Uthus, E.O. and H.M. Brown-Borg, *Altered methionine metabolism in long living Ames dwarf mice.* Exp Gerontol, 2003. **38**(5): p. 491-8.
59. Uthus, E.O. and H.M. Brown-Borg, *Methionine flux to transsulfuration is enhanced in the long living Ames dwarf mouse.* Mech Ageing Dev, 2006. **127**(5): p. 444-50.
60. Karunadharma, P.P., et al., *Subacute calorie restriction and rapamycin discordantly alter mouse liver proteome homeostasis and reverse aging effects.* Aging Cell, 2015. **14**(4): p. 547-57.
61. Barlow, A.D., M.L. Nicholson, and T.P. Herbert, *Evidence for rapamycin toxicity in pancreatic beta-cells and a review of the underlying molecular mechanisms.* Diabetes, 2013. **62**(8): p. 2674-82.
62. Peddi, V.R., et al., *Review of combination therapy with mTOR inhibitors and tacrolimus minimization after transplantation.* Transplant Rev (Orlando), 2013. **27**(4): p. 97-107.