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Title: Caloric-restriction induced alterations in CG and non-CG methylation attenuate age-associated changes in the old brain.

Abbreviated title: CR attenuates age-related DNAm in the old brain

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Abstract

Brain aging is marked by cognitive decline and increased susceptibility to neurodegeneration. Epigenetic mechanisms, including DNA methylation, are vital to CNS cellular function and memory formation, and are dysregulated with aging and age-related neurodegenerative disease. Caloric-restriction (CR), an established pro-longevity intervention, increases neurogenesis, improves memory function, and protects from age-associated pathologies. However, the molecular mechanisms promoting the neuroprotective effect of CR remain largely unknown. We tested the role of DNA methylation as a mechanism for CR-induced neuroprotection in the old hippocampus. Hippocampal DNA from young (3M) and old (24M) male mice fed *ad libitum* and 24M old mice fed 40% calorie-restricted diet from 3M of age were examined by genome-wide bisulfite sequencing to measure methylation levels at base-specific resolution. Over 22 million CG and CH (non-CG) sites were examined. Of the ~40,000 differentially methylated CGs (dmCGs) and ~80,000 CHs (dmCHs) observed with aging, 35% and 38%, respectively, were prevented by CR. Unique to dmCHs, CR preferentially prevented age-related hypermethylation. A diet-specific methylation response was observed in both CG and CH contexts. Diet-induced dmCHs were enriched in unexpected genomic locations including promoters and CG islands including hypermethylation of DNMT1 and Tet3 promoters corresponding to reduced gene expression. These findings demonstrate for the first time that caloric-restriction prevents age-induced cytosine methylation changes in the old brain in combination with diet-specific methylation changes that may function to maintain epigenetic homeostasis through epigenetic auto-regulation. The prevention of age-dmCGs/CHs by CR emphasizes the prominent role of DNA methylation as a driver of the aging process.

Significance Statement

DNA methylation is a central epigenetic regulator of genomic structure and function. Aberrant control of DNA methylation is associated with brain aging and contributes to cognitive decline and age-related

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neurodegenerative disease. Unexplored though is whether anti-aging treatments can maintain 'youthful' methylation patterns in the brain. Our findings demonstrate that caloric-restriction attenuates age-related changes in DNA methylation and induces changes in DNA methylation at specific sites independent of aging in both neuroprotective pathways and pathways shared by different tissues responsive to calorie-restriction, potentially through a combination of epigenetic auto-regulation and uniquely targeted changes in methylation. These findings contribute to our understanding of the aging process and the molecular mechanisms underlying anti-aging treatments.

Introduction

With increased life-expectancy, incidence of age-related neurodegenerative diseases such as Alzheimer's, Parkinson's, and other dementias, has increased and is expected to double by 2040 (Reitz et al., 2011; Reeve et al., 2014; Hickman et al., 2016). Anti-aging interventions offer promise in delaying age-related impairments and neurological disease development. The most established anti-aging intervention is calorie-restriction (CR), where caloric intake is reduced by 10-40%. CR consistently increases lifespan and improves health span across model organisms including invertebrates (Lee et al., 2006), rodents (Turturro et al., 1999), and primates (Colman et al., 2014). Several conserved mechanisms have been proposed to underline the beneficial effects observed following CR including inhibition of mTOR signaling (Kaeberlein et al., 2005), decreased insulin/IGF-1 signaling (Sonntag et al., 1999) activation of Sirt1/Sirt2 (Bordone and Guarente, 2005) and activation of redox signaling (Hyun et al., 2006), however CR is also proposed to act through cell-autonomous and tissue specific mechanisms (Dacks et al., 2013; Schafer et al., 2015; Barger et al., 2017).

Short- and long-term CR have a protective effect against brain aging. CR increases expression of DNA repair and anti-stress proteins (Kisby et al., 2010), improves glucose metabolism efficiency (Willette et al., 2012b) and delays onset of inflammatory cytokines (Swindell, 2009). Specifically to the brain, CR

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delays neurodegeneration and synaptic dysfunction (Dietrich et al., 2010; Schafer et al., 2015), improves neuroendocrine function (Redman and Ravussin, 2009; Willette et al., 2012a) and promotes induction of genes active in neuroprotection, neural growth (Park et al., 2013) and synaptic and neuronal function (Adams et al., 2008; Fontan-Lozano et al., 2008; Schafer et al., 2015; Wood et al., 2015). At the functional level, CR improves learning and memory and is beneficial in reducing anxiety and depression in both rodents and humans (Inoue et al., 2004; Lutter et al., 2008; Willette et al., 2012a). CR is also beneficial in alleviating pathology of age-related neurodegenerative disease, improves cognitive function (Halagappa et al., 2007) and decreases hippocampal beta-amyloid accumulation in Alzheimer's models (Wang et al., 2005). Despite these positive effects on brain aging and age-related disease, the molecular mechanisms underlying brain aging processes and those responsive to CR remain elusive.

Epigenetic modifications in the form of DNA methylation and histone modifications are proposed as central regulators of the aging process (Lopez-Otin et al., 2013). DNA methylation is a malleable epigenetic modification in which a methyl group is added to the 5-position of a cytosine ring and plays a complex role in genomic regulation. Proper control of DNA methylation is instrumental to neural differentiation, neural growth, synaptic function and intact cognitive function (Feng et al., 2010; Lister et al., 2013; Grigorenko et al., 2016), key processes hampered by the aging process. Altered methylation at specific genomic locations is associated with the normal aging process (Xekardaki et al., 2015; Wyss-Coray, 2016). Recent studies have shown a protective epigenetic effect of anti-aging interventions in the liver and kidney (Kim et al., 2016; Cole et al., 2017; Hahn et al., 2017). However, whether the response by DNA methylation to aging is common tissues or demonstrates tissue specific patterns is unclear.

We previously described extensive age-related DNA methylation changes in the old brain (Masser et al., 2017). In the current study we investigated whether these changes are prevented by CR by focusing on the hippocampus, a tissue demonstrating molecular, cellular and functional impairment with old age

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(Vanguilder and Freeman, 2011; Gray and Barnes, 2015; Seib and Martin-Villalba, 2015). Using a genome-wide sequencing approach we examined the response of age-related CG and for the first time CH (non-CG) methylation to CR. Additionally, we characterize unique CR-specific changes in both CG and CH methylation, highlighting potential functional similarities and dissimilarities between CG and CH methylation. Lastly, we show that enzymes regulating DNA methylation and demethylation are suppressed by CR but not aging, in part through alteration in promoter methylation induced by CR, indicating a potential mechanism for CR modulation of the neuroepigenome.

Materials and methods

Animals

Male C57BL/6 mice were obtained from the NIA aging and caloric restriction colony at 3 months and 24 months of age. Mice were housed at the University of Oklahoma Health Sciences Center animal facility and maintained under SPF conditions in a HEPA barrier environment. Mice were fed irradiated NIH-31 mouse/rat diet (Teklad, Envigo). Both CR and AL mice were individually housed. CR was initiated at 14 weeks of age at 10% restriction, increased to 25% restriction at 15 weeks, and to 40% restriction at 16 weeks of age. Young (3 months) and old (24 months) mice were euthanized and both right and left hippocampus were harvested, snap frozen in liquid nitrogen, and stored at -80°C. All animal experiments were performed according to protocols approved by the OUHSC Institutional Animal Care and Use Committee. Tissues from young and old *ad libitum* animals were used in our previous study (Masser et al., 2017).

Library preparation and sequencing

Hippocampal gDNA was isolated by spin columns (Zymo duet) and genome-wide bisulfite capture sequencing in accordance to previously described and validated methods (Hing et al., 2015; Masser et

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al., 2016). gDNA was quantified by qubit (Invitrogen) and sheared by sonication (Covaris e220) to an average 200bp fragment size. Fragment size was confirmed by capillary electrophoresis (DNA1000, Agilent). Bisulfite sequencing library preparation was performed with 3µg DNA using SureSelect Methyl-seq XT per manufacturer instructions (Agilent). In brief, sheared DNA was end-repaired, adenylated and adapter ligated prior to bisulfite conversion. Libraries were sized and quantified by capillary electrophoresis to ensure recovery of at least 350 ng of library. Libraries were hybridized to mouse SureSelect Methyl-Seq capture library. Hybridized libraries were captured with Dynabeads MyOne Streptavidin T1 magnetic beads and then bisulfite converted (EZ DNA Methylation-Gold, Zymo Research), amplified according to manufacturer recommendations and purified with AMPure XP beads. Following bisulfite conversion libraries were indexed and confirmed for fragment size by capillary chip (DNA high sensitivity, Agilent). Prior to sequencing, libraries were quantified by PCR (KAPA library quantification kit, Kapa Biosystems). Libraries were diluted to 4nM and pooled in equimolar concentrations. Pooled libraries were diluted to 12 pM and then sequenced at 100 bp paired end reads on the Illumina HiSeq2500 platform. Raw fastq files are available through the Sequencing Read Archive (bioproject ID PRJNA397575).

Informatics

Paired-end reads were trimmed using CLC Genomics Workbench v10.1.1 (RRID:SCR_011853). Reads with a Q-score <30 or with >1 ambiguous nucleotide were filtered. Reads passing filter were adapter trimmed and an additional 3 bp were removed from the 3’ and 5’ end of each read. All computational analysis was performed in UNIX and R using custom scripts or published tools (where specified). Trimmed PE reads were aligned as PE to the mouse GRCm38/mm10 assembly using Bismark Bisulfite Mapper version 0.14.4 (RRID:SCR_005604) (Krueger and Andrews, 2011). Following read mapping, methylation calling was performed with Bismark methylation extractor and % methylation was determined by calculating

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(total C methylated/total C covered) per cytosine in the genome. Only cytosines covered in all groups with a cumulative coverage of 35 were used for analysis, exceeding the coverage recommendations in the field for BS-seq studies (Roadmap Epigenomics et al., 2015; Ziller et al., 2015). Greater than 22M CG and non-CG sites were carried forward for downstream analysis. Differential methylation between groups was determined using Kruskal-Wallis H test in R, significant sites were tested for pairwise multiple comparisons using Conover post hoc test using 'lsmeans' library, p-values were adjusted for false discovery using the Benjamini-Hochberg procedure. Age-changes were defined as all differences found between young-AL and old-AL animals. Diet-changes were defined as all differentially methylated cytosines between Old-AL and Old-CR animals.

Annotation and enrichment

Coordinates for genic features including exons, introns and promoters (defined as ±1kb from TSS) were downloaded from UCSC genome browser for GRCm38/mm10 reference genome. CG island shores were defined as 2kb upstream and downstream from the CG island borders and shelves were defined as 2kb upstream and downstream from shores. Coordinates for annotated gene regulatory regions for mouse were downloaded from ENSEMBL open database (Zerbino et al., 2015). Over- and under- representation of differentially methylated cytosines was determined by overlapping all analyzed sites with the genic feature list and gene regulatory regions using 'bedtools' (Quinlan and Hall, 2010). Statistical significance of over- and under- representation was determined using hypergeometric test. For pathway analysis genes were annotated for differentially methylated cytosines, only genes with >2 differentially methylated sites were included in the analysis. Analysis of pathways enriched for genes affected for differential methylation was performed with the 'ReactomePA' R package. Pathways with broad (>100 genes) or narrow (<7 genes) definitions were excluded. Additionally, pathways with similar gene lists were consolidated. A full unedited list will be available upon request.

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Real-Time PCR (qPCR)

Expression of Dnmts (pan-Dnmt1, Mm01151063_m1; E1-Dnmt1, Mm00599763_m1; Alt. E1-Dnmt1, Mm01174085_m1; Dnmt3a1, Mm00432870_m1) and Tets (Tet1, Mm01169087_m1; Tet2, Mm00524395_m1; Tet3, Mm00805756_m1) was determined by real-time qPCR as described previously (Mangold et al. 2017b). β-actin (Mm02619580_g1) was used as the endogenous control and each sample was analyzed in technical triplicates.

Experimental Design and Statistical Analysis

Genome-wide methylation analysis was performed with n=4/group. Gene expression experiment were performed with n=7-8/group. All statistical analysis was performed in R. Box plots represent the 25th and 75th percentiles. Differential methylation and enrichments were determined as detailed above. Global methylation differences were compared using linear regression and are written as mean ± SD. Analysis of gene expression using RT-qPCR was compared using One-Way ANOVA with pairwise comparisons and Benjamini-Hochberg multiple testing corrections.

Results

Age related CG methylation is attenuated by life-long caloric restriction

To examine the effect of life-long caloric-restriction on CG methylation in the old brain,. Hippocampal DNA was isolated from Young (3 months old, Y-AL, n=4) and old (24 months old, O-AL, n=4) male mice that were fed *ad libitum* (AL) throughout life and old animals fed a 40% caloric restriction (CR) diet (24 months old, O-CR, n=4) starting at 3 months of age. Using genome-wide bisulfite sequencing, base-specific methylation levels across 3 million CpGs were compared between the 3 groups. Global DNA methylation, i.e. the methylation averages across all analyzed CpG sites did not change with age or diet (Y-AL: 42.3% ± 0.7; O-AL: 42.6% ±0.5; O-CR: 42.6%±0.6; p=0.45 by linear regression). We defined age-

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associated dmCGs (age-dmCGs), as any methylation difference between young-AL and old-AL animals that met statistical criteria (methylation difference >|5%| and adjusted p-value < 0.05, for more details see differential methylation in Methods). Comparing young-AL animals to old-AL animals, 41,586 age-related age-dmCGs were identified. Examination of the methylation profile of all age-dmCGs for differences between young-AL and old-CR mice reveals a pattern of regression towards that of young-AL, indicating a "younger" methylation profile in age matched animals fed CR diet (Fig. 1A, B). At single base resolution, life-long calorie-restricted diet prevented ~32% hypermethylated and 36% hypomethylated age-dmCGs (Fig. 1C). The distribution of age-dmCGs shows higher number of hypermethylated dmCGs than hypomethylated dmCGs. Prevented age-dmCGs followed a similar distribution that was proportional to that observed for age-dmCGs. (Fig. 1D). Principal component analysis of age-dmCGs unaffected by diet demonstrated clustering of all old animals as compared to young-AL (Fig. 1E). Parallel analysis of prevented age-dmCGs separates young-AL and old-CR from old-AL on the first component, while young-AL and old-CR separate on the second component (Fig. 1F). The partition of young-AL and old-CR may be attributed to a subset of 416 loci that present differential methylation between Y-AL and O-CR with both diet and aging, e.g. gain in methylation with age and loss in methylation with age and diet as compared to Y-AL.

The localization of differentially methylated sites by genomic context and their interaction with other epigenomic marks can aid in understanding the role of DNA methylation changes in genomic regulation. Methylation changes were mapped with known coordinates of gene-centric features to annotate the enrichment of differentially methylated sites. In genic features, including CG islands, shores and shelves, introns, exons and promoters there was no observable difference in enrichment pattern between hyper- and hypo- methylated age-dmCGs or prevented age-dmCGs (Fig. 1G). Age-dmCGs and prevented age-dmCGs were enriched in introns and CGI shores while under-represented in islands, CGI shelves, exons, and promoters. Depletion of age-dmCGs in promoters was more pronounced in promoters containing

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CGI. Previous reports demonstrated that DNA methylation may alter gene expression through interaction with gene regulatory regions (Roadmap Epigenomics et al., 2015). To identify enrichment at specific gene regulatory regions we used the ENSEMBL brain specific regulatory build (Zerbino et al., 2015) which was constructed using sequencing methods to identify CTCF binding sites enhancers, open chromatin regions, promoters, promoter flanks, and transcription factor binding sites and their specific activation states (active, inactive, poised, repressed). Enrichment of age-dmCGs was predominantly observed in inactive state gene regulatory regions. Inactive regions are defined as regions not associated with other epigenetic marks, indicating that the effect of differential methylation at these regions is independent of interaction with other known epigenetic marks. Age-dmCGs were enriched in active state regulatory regions and occurred in CTCF binding sites (hypo only), enhancers (hypo and hyper), open chromatin regions (hyper only), and promoter flank (hyper only). Prevented age-dmCG generally demonstrated a similar enrichment pattern (Fig. 1H).

The interaction between gene expression and DNA methylation is still an active area of exploration. The canonical view of DNA methylation regulation of gene expression through promoter or gene body methylation has been challenged recently both in neurons (Sharma et al., 2016) and following CR (Hahn et al., 2017). Therefore, we asked which pathways are affected by DNA methylation without assumptions as to whether methylation is positively or negatively regulating a specific gene. Only genes containing more than 2 dmCGs in their gene body (inclusive of promoter, introns and exons) were included in this analysis. Genes were separated by those affected by hyper- and hypo- methylation. Hypermethylated age-dmCGs were found in 3,266 genes and hypomethylated age-dmCGs were found in 1,925. Of those genes 948 were found to contain prevented hypomethylated age-dmCGs and 548 contained prevented hypermethylated age-dmCGs. Pathways involved in neural cell communication, neural cell growth, cellular integrity, cell metabolism and inflammatory pathways were affected by both hyper- and hypomethylation with aging (Fig. 1I). The Rho GTPase pathway, which is involved in neurite

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growth and axon guidance (Hall and Lalli, 2010) and downstream of cognitive decline-related myelin associated inhibitors of synaptic plasticity (Vanguilder et al., 2012) was enriched in prevented hypomethylated age-dmCGs while no pathways were enriched amongst the prevented hypermethylated age-dmCGs. These findings are consistent with the negative effect of aging on neuronal integrity and positive cellular effect of CR on neuronal survival and growth (Seib and Martin-Villalba, 2015; Hornsby et al., 2016).

Age related non-CG methylation is attenuated by life-long caloric restriction

The effect of calorie restriction on CH methylation has not been previously examined. CH methylation is higher in the brain relative to other tissues (Lister et al., 2013), is differentially regulated as compared to CG methylation in the brain (He and Ecker, 2015), and has unique functional impacts on gene regulation (Lister and Mukamel, 2015). We analyzed >22M CHs sites and asked whether age-dmCHs respond similarly to diet as age-dmCGs. Differential methylation of CHs was determined in a base-by-base fashion, similar to CGs. The absolute methylation level of most dmCHs was generally lower than that of CGs. Average global CH methylation levels did not change with age or with diet (Y-AL: 1.59% ± 0.07; O-AL: 1.64% ±0.05; O-CR: 1.58%±0.8; F = 0.61, p=0.83 by linear regression). More dmCHs (79,058 age-dmCHs) were identified to change with age compared to CGs. The methylation profile of age-dmCHs in old-CR animals was closer to that of young-AL animals compared to that of old-AL animals (Fig. 2A). The number of hypermethylated age-dmCHs was higher than hypomethylated dmCHs in a manner similar to CGs. Life-long calorie restriction prevented 59% of all hypermethylated age-dmCHs and 11% of hypomethylated age-dmCHs. Prevention of hypermethylated age-dmCHs by CR was 10 times more likely than diet induced preventions of hypomethylated age-dmCHs (Fig. 2B-C). Principal component analysis of age-dmCHs unaffected by diet shows a distinct clustering of both AL and CR old animals compared to

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young-AL animals (Fig. 2D). Clustering of samples by the methylation profile of prevented age-dmCHs shows that old-AL animals separate from young-AL and old-CR animals (Fig. 2E).

Differentially methylated CHs were generally under-represented in CG islands and shelves, and in gene-centric elements. The exception being an enrichment of prevented hypomethylated age-dmCH in CGI shores (Fig. 2F). Taken together, under-representation of dmCHs in in CG islands and genic regions indicate that changes in methylation with age, and those prevented by CR, occur primarily in intragenic regions and are partially excluded from CG rich regions. Enrichment analysis of age-dmCHs in gene-regulatory regions reveals that age-dmCHs and prevented age-dmCHs, regardless of gain or loss in methylation, are enriched mostly in active and inactive enhancers. Hypomethylated age-dmCHs and prevented age-dmCHs were also enriched in poised TF binding sites and repressed promoters (Fig. 2G).

Pathways enriched for genes affected by age-dmCH were similar to those enriched by age-dmCG, including pathways involved in metabolic regulation, cellular signaling, and regulation of neural cell structure and growth. Pathways enriched for prevented age-dmCHs were primarily involved in energetics, metabolism and neurite growth (Fig. 2H, a full pathway list can be found in additional table 2). Of all the pathways found to be affected by differential methylation 18 were common to age-dmCHs and age-dmCGs (Fig. 2I).

Gene regulatory regions are affected differentially by CR induced CG methylation

Caloric-restriction was sufficient to prevent ~1/3 of all age-dmCs (CGs and CHs). CR also induced DNA methylation changes independent of aging as evident when comparing old-AL and old-CR animals. Methylation of these diet-induced dmCGs (diet-dmCGs) is not different between young-AL and old-AL animals (colored green in Fig. 3A-left scatterplot) but are differentially methylated between old-AL and old-CR animals (Fig. 3A-right scatterplot). Of all differentially methylated dmCGs between old-AL and old-CR, 69.4% were induced by CR and were independent of the aging process (Fig. 3B). A single base

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example of a diet-induced change in methylation is depicted in Fig. 3C. The distribution of all diet-dmCGs shows significant preference towards hypermethylation (Fig. 3D), though the magnitude of methylation change was similar between hyper- and hypo- methylation (12.3% vs -12.5%, Fig 3D).

The genomic localization of diet-dmCGs is different from age-dmCGs. Hypermethylated diet-dmCGs were over-represented in CG islands and shores and under-represented in CG shelves. Hypomethylated diet-dmCGs were only enriched in shores. With respect to genic regions diet-dmCGs were primarily depleted with the exception of a small but significant over representation of hypermethylated sites in exons and hypomethylated sites in introns (Fig.3E). The same distinction between hyper- and hypo- diet-dmCGs was observed in gene regulatory regions. Enrichment of diet-dmCGs was primarily in poised regulatory regions while enrichment of hypomethylated diet-dmCGs was observed in active regulatory features, including CTCF binding sites, enhancers, open chromatin and promoter flanks. Active promoters were not enriched for hyper- and hypo- methylated diet-dmCGs; however, hypermethylated diet-dmCGs were enriched in inactive and repressed promoters (Fig. 3F). Genes affected by diet-dmCGs were enriched for pathways involving energy regulation, inflammatory pathways and phagocytosis (Fig. 3G). Interestingly, we found that genes containing diet-dmCGs were enriched in DNA methylation and senescence-associated secretory phenotype pathways; both pathways have been proposed as potential regulators of the aging process (Lopez-Otin, 2013).

Non-CG diet induced changes are clustered

The diet-specific effect on methylation was also observed in non-CG sites (Fig. 4A). Changes in CH methylation with diet occurred generally at lower methylation levels with the majority of changes occurring under 50% (Fig. 4A – right panel), but showed no difference between young-AL and old-CR (Fig. 4A – left panel). Most diet-dmCHs (75% of all differential methylation observed in CHs) were independent of the aging process (Fig. 4B). Twice as many hypermethylated (64,191 sites) than

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hypomethylated diet-dmCHs (26,237 sites) were observed, similar to diet-dmCGs. Hypermethylation of diet-dmCHs was higher than hypomethylated diet-dmCHs (10% vs -8%, Fig. 4C).

CG dinucleotides are generally depleted from large regions of the genome but occur at specific, high density regions such as in CG islands and promoters. On the other hand, the distribution of CHs is relatively even across the genome (He and Ecker, 2015). We investigated whether age and diet induced dmCHs are sporadically distributed across the genome or clustered as a result of targeted changes at specific genomic loci. We computed the distance between dmCHs, 62% of all dmCHs (age and diet) occurred within 10kb of one another; therefore we focused our analysis to 10kb segments. The proximity of diet-dmCHs to the nearest dmCH was higher than that observed for age-dmCHs (67% diet-dmCHs compared to 56% age-dmCHs, Fig. 4D). This difference was even more pronounced when calculating the distance between dmCHs within 1kb (49% vs. 29%). Further examination of the clustering pattern of dmCHs in 10kb and 1 kb segments revealed that the distance to the next proximal dmCH (2nd dmCH, 3rd dmCH, etc.) decreased gradually for both age and diet, but more diet-dmCHs clustered together between the 1st and the 6th diet-dmCHs than the 1st and 3nd age-dmCHs in 10kb segments (Figure 4E). This clustering of diet-dmCHs suggests differential regulation/targeting mechanisms of CH methylation in response to diet and between diet and age.

Diet dmCHs are over-represented in CGI-promoters and depleted from non-CGIs promoters.

Hypermethylation of diet-dmCHs was over-represented in CG islands, shores and exons, while hypomethylation was over-represented in introns, a localization pattern similar to diet-dmCGs. Interestingly, hypermethylated diet-dmCHs were also over-represented in promoters containing CGIs but were absent from non-CGI promoters (Fig. 5A). This finding is unexpected since CH methylation is often precluded from high-CG-rich regions and promoters (Lister et al., 2009; Laurent et al., 2010; Xie et al., 2012; Guo et al., 2014), further supporting a regulated control of methylation by diet. Enrichment of

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diet-dmCHs in gene regulatory regions confirms the observation of over-representation of hypermethylated diet-dmCHs in promoters. Using the ENSEMBL regulatory build we further divided promoters by their activation state and found that hypermethylated diet-dmCHs are enriched in active and poised promoters and promoter flanks, and are under-represented in repressed and inactive promoters. Enrichment of hypomethylated diet-dmCHs was mainly observed in inactive gene regulatory elements (Fig. 5B).

Higher numbers of genes contained diet-induced CH hypermethylation (5,340 genes) than hypomethylation (2,981 genes), consistent with the distribution of diet-dmCHs (Fig. 4C); however genes with hypomethylated diet-dmCHs were enriched in a greater number of pathways (40 pathways by hypomethylation vs 21 pathways by hypermethylation). Top enrichment pathways included those regulating cell structure, neural cell communication and growth and energy metabolism. Inflammatory pathways were mostly enriched for genes containing hypomethylated dmCHs (Fig. 5C). Of the 69 pathways affected by both dmCHs and dmCGs we found 12 common pathways regulating inflammation and neuronal integrity (Fig. 5D).

Caloric restriction changes in methylation auto-regulates the DNA methylation machinery.

DNA methylation regulatory enzymes (DNA methyltransferases; Dnmts and Ten-eleven Translocation; Tets) mRNA and protein expression do not change in the hippocampus with aging (Hadad et al., 2016). Given that diet-dmCGs were enriched in the DNA methylation pathway; we examined whether any of the CR-induced changes in methylation observed in the current study mapped to Dnmts and Tets genic elements. We identified 6 hypermethylated diet-dmCs (2 dmCGs and 4 dmCHs) in the promoter region of Tet3 (Fig. 6A), corresponding to a decrease in Tet3 mRNA expression following CR (Fig. 6B). We also found 9 hypermethylated diet- dmCs (6 dmCHs and 3 dmCGs) in the promoter region of Dnmt1 (Fig. 6C), but an examination of Dnmt1 with a pan-isoform assay inclusive of all known isoforms revealed no

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changes in expression (Fig. 6D). Promoter methylation of Dnmt1 is located near two known start sites; therefore we examined the expression of each isoform separately by qPCR. Expression of the exon-1 containing mRNAs declined significantly with diet, and minimally with aging, while the alternate exon-1 isoform was unchanged (Fig. 6E).

No other dmCs induced by CR found in our analysis were observed in the remaining DNA methylation regulatory enzymes, however examination of the expression levels of these enzymes show a small but significant decrease with age and diet of Dnmt3a1 and a decrease in Tet2 expression with diet (Fig. 3F). Tet1 expression did not differ with age or diet as well. These findings suggest that while Dnmts and Tets are generally not regulated with age, the epigenetic effects of CR may occur through regulation of Dnmts and Tets.

Discussion

This study examined whether DNA methylation is a neuroprotective mechanism of action of CR in retarding brain aging. Given that calorie-restriction improves cognition and cellular function and that altered methylation may underline age-related dysfunction in these processes, hippocampal DNA methylation was quantified at base-specific resolution to identify age-associated changes in DNA methylation and the effect of CR in preventing these changes, as previously reported for CG methylation in the kidney and liver (Kim et al., 2016; Cole et al., 2017; Hahn et al., 2017). Our findings demonstrate for the first time that base-specific prevention of age-related differential methylation by CR is observed in the brain, not only in CG context but also CH. This is the first study to evaluate the effect of CR on CH methylation, an observation that may be unique to neural tissues.

DNA methylation changes in the old hippocampus are observed in both CG and CH contexts (Masser et al., 2017). These changes were enriched in in inflammation related pathways, neuronal integrity, neuronal function and synaptic transmission. Previous studies examining gene expression change with

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age in the hippocampus showed enrichment in similar pathways (Keleshian et al., 2013; Stilling et al., 2014; Meng et al., 2016; Mangold et al., 2017a; 2017b). Studies examining the relationship between gene expression and methylation with age and CR (Hahn et al., 2017), and in the brain (Sharma et al., 2016) failed to identify a simple correlation. While demonstrating causality between differential methylation of thousands of sites and gene expression remains unfeasible, we hypothesize that methylation changes with age contribute to the dysregulation of genes in these pathways. Based on our data, enrichment of methylation changes with age are commonly occurred in introns and genomic regulatory regions such as open chromatin, CGI shores and CTCF binding sites and were under-represented in promoters and CG islands. These findings may support a more complex role to methylation in regulating gene expression during the aging process than simplistic alterations in promoters. This may also explain the discrepancies between current studies and the canonical mechanisms by which methylation regulates expression. Further investigation is required to discover the many intricate mechanisms by which methylation acts as a genomic regulator with aging.

Over a third of all age-related differentially methylated cytosines were prevented by CR in age-matched animals. The distribution of prevented sites in CG context was comparable to that of age-dmCGs, however for CH sites CR preferentially prevented age-related hypermethylation. This may be caused by the diet-specific reduction in expression of the DNA methylation regulatory enzymes, or via targeted mechanisms such as recruitment of Dnmts and Tets to specific genomic locations by targeting co-factors and changes to histone modifications and genome occupancy. In light of our results in which both the methylation and demethylation machinery changed with diet and previous findings of altered genomic regulation following CR (Chouliaras et al., 2013; Gong et al., 2015) we consider the later more likely. It is worth noting that CR effects on age-related dmCs are classified as ‘prevented’, however the possibility remains that these changes are delayed or reversed at different points throughout life. To further

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elucidate the dynamics by which CR prevents, delays or reverses changes in DNA methylation with aging, future studies examining time points across the lifespan are necessary.

The majority of CR-induced differentially methylated sites were independent of aging. These diet-specific changes demonstrated different distributions in promoters, genes, and enhancers compared to CR prevented age-related sites. Previous studies in the liver (Cole et al., 2017; Hahn et al., 2017) reported enrichment of diet-induced CG hypermethylation in CG islands and gene bodies while CG hypomethylation occurred in gene bodies and enhancers. Interestingly, we observed a similar enrichment pattern in the brain. The commonalities in enrichment of diet-dmCGs in both liver and hippocampus suggest common regulation of DNA methylation by diet across tissues that may be caused by changes in regulation of common core proteins required for the targeting and recruitment of the DNA methylation machinery to specific locations within the genome. Targeting of Dnmts and Tets to specific genomic loci is a complex process that is not fully understood (Wu and Zhang, 2017). Post translational histone modifications, have been positively and negatively correlated with DNA modification (Lehnertz et al., 2003; Cedar and Bergman, 2009; Balasubramanian et al., 2012) and may be altered through diet, thus changing recruitment of Dnmts and Tets. In the brain, CR attenuates age-changes of Dnmt3a and other epigenetic marks (Bordone and Guarente, 2005; Chouliaras et al., 2013; Gong et al., 2015). Additionally, insulin signaling, specifically the PI3K/AKT pathway, a conserved pathway altered by CR, was shown to modify DNA methylation through destabilization of Dnmts (Popkie et al., 2010)

Genomic CH methylation is higher in the brain compared to other tissues (Lister et al., 2009), and has been show to regulate transcription of genes involved in synaptic and neural growth (Chen et al., 2015; Jang et al., 2017). Differential CH methylation has not been examined previously in the context of CR and aging. Here we demonstrated that changes induced by diet in CH methylation are not sporadic and

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are clustered at higher density than age-associated changes. Surprisingly diet-induced gain of methylation at CH loci is observed in regions where CH methylation is often excluded such as promoters, CG islands, TF binding sites and active enhancers (Lister et al., 2009; Laurent et al., 2010; Xie et al., 2012; Guo et al., 2014). These findings provide evidence for a more controlled regulation of DNA methylation following CR, potentially lost with aging. Genomic regulation of CH methylation is different than CGs but remains largely unknown due to the lack of CHs data from DNA methylation analyses. In the context of CR, methylation of CHs appears to respond differently than CGs and serve a non-redundant role in genomic regulation because 1) enrichment of dmCGs and dmCHs was different at promoters, TF binding sites, enhancers and open chromatin and 2) the affinity of the Dnmts and Tets to CHs is lower than that of CGs (Ramsahoye et al., 2000; Suetake et al., 2003; He and Ecker, 2015). The differential localization of dmCHs as compared to dmCGs provides evidence that different, and as yet unknown, targeting mechanisms are being used to regulate CH and CG methylation with diet.

Several pathways are known to be upregulated by food-restriction and its mimetics (Fusco and Pani, 2013). Prior gene expression studies in the brain have shown that mTOR signaling and insulin signaling are downregulated by CR, as they are in other tissues, while brain-specific induction of pathways including calcium signaling, synaptic vesicle regulation, neurotrophic signaling, neural growth, differentiation and function and neuronal structure are also observed (Schafer et al., 2015; Wood et al., 2015). We observed diet-induced methylation changes in pathways previously reported to change following CR in the brain, specifically calcium signaling, energy metabolism, NGF signaling, FGFR signaling and pathways involved in axon guidance and growth. While a direct effect was not established, the common findings between our study and previous gene expression studies suggest a role for DNA methylation as a possible mechanism for the protective effect of CR against brain aging.

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Altered methylation patterns were also observed in DNA methylation regulatory pathways. Long-term CR induced hypermethylation of both CGs and CHs in the promoters of Tet3 and Dnmt1, accompanied by reduced expression with diet indicating a potential auto-regulation of the DNA methylation machinery with diet. CR was reported to decrease Tet2 and Dnmt3b and increase Tet3 expression in the liver (Hahn et al., 2017) and decrease Dnmt3a in the hippocampus (Chouliaras et al., 2011). Both Tet3 and Dnmt1 were not previously reported to change with diet in the brain. Consistent with previous results, Dnmt3a1 mRNA expression decreased following CR. Dnmt3a1 is considered the primary enzyme for *de novo* CH methylation along with Dnmt3b, however Dnmt3b is expressed at low levels in the brain (He and Ecker, 2015). Based on our findings of brain-specific differential expression of Dnmts and Tets with CR and enrichment of differential methylation in pathways involved in neuronal function, we propose that DNA methylation underlies brain-specific protective effects of CR, however the targeting and recruitment of Dnmts and Tets to their genomic locations shares commonalities across tissues as is evident by enrichment of methylation at gene regulatory regions. We are the first to report auto-regulation of DNA methylation by diet which appears to be specific to the brain.

Taken together our data supports a role of calorie-restriction in maintaining homeostasis through DNA methylation. Changes in methylation were enriched in pathways shown to improve following CR and are beneficial to brain aging. By including CH methylation analysis, we demonstrate similarities and dissimilarities in the functions of CG and CH methylation in the protective effect induced by CR. These findings highlight the importance in investigating both CG and CH methylation and propose a prominent systemic role for DNA methylation in response to CR. Future studies are required in order to further parse the specific mechanisms of diet-induced DNA methylation regulation and how these promote brain health in old age.

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Figure Legends

Figure 1: Caloric-restriction prevents age-associated differential CG methylation. **A.** Scatterplot depicting significant age-dmCG (n= 41,586, adjusted p-value<0.05 & methylation difference between Old-AL and Young-AL>|5%|, Kruskal-Wallis H test with Conover pairwise comparisons followed by false-discovery rate multiple testing corrections) methylation levels between young *ad lib* (Y-AL) and old *ad lib* (O-AL) groups (red dots) and young *ad lib* (Y-AL) and old calorie-restricted (O-CR) groups (blue dots). Linear regression lines pass through Y-AL~Y-AL (black), O-AL~Y-AL (blue) and O-CR~Y-AL (dotted green) age-dmCG methylation values separated by hyper- and hypo- age-dmCHs. Calorie-restriction diet causes a 'younger' methylation profile in age-associated differentially methylated sites. **B.** Single base representatives of an age-dmCG (left) and a prevented age-dmCG (right). **C.** Quantification of the number of differential CG methylation in old animals fed *ad lib* (AL) and calorie-restricted (CR) diets. Grey regions indicate age-dmCGs prevented by caloric-restriction (n=13,950). **D.** Distribution plot of age-dmCGs and prevented age-dmCGs. Age-dmCGs and those prevented by diet follow a similar distribution. **E, F.** PCA plots of unique age-dmCGs **(E)** and prevented age-dmCGs (**F**) showing clustering of old animals in age-dmCGs but separation in prevented age-dmCGs. **G.** Enrichment of age-dmCGs and prevented age-dmCGs in gene-centric regions, only significant enrichments is shown (p<0.05, hypergeometric test). **H.**

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Enrichment of age-dmCGs and prevented age-dmCGs in gene-regulatory regions. Red indicates under-representation (log(odds ratio) < 0 & p-value<0.05, hypergeometric test), blue indicates over-representation (log(odds ratio)>0 & p-value<0.05, hypergeometric test) and white shows no significance (p-value>0.05). **I.** Pathway analysis of genes containing >2 significant age-dmCGs or prevented age-dmCGs. Top enrichment pathways are shown (FDR adjusted p-values < 0.01). Circle size indicates the ratio of genes in pathway containing diet-dmCGs, colored by p-values. No pathways were enriched for genes containing prevented hypermethylated age-dmCGs.

Figure 2: Caloric-restriction prevents age-associated differential CH methylation. **A.** Scatterplot depicting significant age-dmCH (n= 79,056, adjusted p < 0.05 & methylation difference between Old-AL and Young-AL > |5%|, Kruskal-Wallis H test with Conover pairwise comparisons followed by false-discovery rate multiple testing corrections) methylation levels between young *ad lib* (Y-AL) and old *ad lib* (O-AL) groups (red dots) and young *ad lib* (Y-AL) and old calorie-restricted (O-CR) groups (blue dots). Linear regression lines pass through Y-AL~Y-AL (black), O-AL~Y-AL (blue) and O-CR~Y-AL (dotted green) age-dmCH methylation values separated by hyper- and hypo- age-dmCHs. **B.** Quantification of the number of differential CH methylation in old animals fed *ad lib* (AL) and calorie-restricted (CR) diets. Grey regions indicate age-dmCHs prevented by caloric-restriction (n=29,880). **C.** Distribution plot of age-dmCHs and prevented age-dmCHs. The probability of hypermethylation age-dmCHs to be prevented by diet was higher than that of hypomethylated age-dmCH (0.59 prevented hyper compared to 0.11 prevented hypo). **D, E.** PCA plots of age-dmCHs (**D**) and prevented age-dmCHs (**E**). **F.** Enrichment of age-dmCHs and prevented age-dmCHs in gene-centric regions, only significant enrichments are shown (p<0.05, hypergeometric test). **G.** Enrichment of age-dmCHs and prevented age-dmCHs in gene-regulatory regions. Red indicates under-representation (log(odds ratio)<0 & p-value<0.05, hypergeometric test), blue indicates over-representation (log(odds ratio)>0 & p-value<0.05, hypergeometric test) and white blocks did not reach statistical significance **H.** Pathway analysis of genes containing >2 significant age-

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dmCHs or prevented age-dmCHs. Top enrichment pathways are shown (FDR adjusted p-values<0.01). Circle size indicates the ratio of genes in pathway containing diet-dmCGs, colored by p-values. I. Overlap between pathways enriched for age-dmCHs and age-dmCGs.

Figure 3: Diet induces changes in CG methylation independent of aging. **A.** *Left*: 3D scatterplot showing the methylation levels of significant diet-dmCGs including unique diet-dmCGs (green) and age-dmCGs prevented by diet (blue) (adjusted p < 0.05 & methylation difference between Old-AL and Old-CR > |5%|, Kruskal-Wallis H test with Conover pairwise comparisons followed by false-discovery rate multiple testing corrections) focusing on young-AL and old-AL. *Right*: 90° rotation of the 3D plot showing unique diet-dmCG (green) regress away from the midline when comparing old-AL and Old-CR methylation levels. **B.** Proportion of diet-dmCGs unique to diet (green) and those with an age interaction (prevented age-dmCGs, blue) (N = 46,052). **C.** Single base example of a unique diet-dmCG. **D.** Distribution of diet-dmCGs. Dotted lines mark the average methylation differences of hypomethylated diet-dmCGs (red, -12.5%) and hypermethylation diet-dmCGs (green, 12.3%). **E.** Enrichment of diet-dmCGs in gene-centric regions (p<0.05, hypergeometric test). **F.** Enrichment of diet-dmCGs in gene-regulatory regions. Red indicates under-representation (log odds ratio < 0 & p-value < 0.05, hypergeometric test), blue indicates over-representation (log odds ratio > 0 & p-value < 0.05, hypergeometric test) and white show no significance **G.** Pathway analysis of genes containing >2 significant diet-dmCGs. Top enrichment pathways are shown (FDR adjusted p-values < 0.01). Circle size indicates the ratio of genes in pathway containing diet-dmCGs, colored by p-values.

Figure 4: Diet induced differential CH methylation is clustered at specific genomic loci**.** A. *Left*: 3D scatterplot showing the methylation levels of significant diet-dmCHs including unique diet-dmCHs (green) and age-dmCHs prevented by diet (blue) (adjusted p < 0.05 & methylation difference between Old-AL and Old-CR > |5%|, Kruskal-Wallis H test with Conover pairwise comparisons followed by false-

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discovery rate multiple testing corrections) focusing on young-AL and old-AL. *Right*: 90° rotation of the 3D plot showing unique diet-dmCH (green) regress away from the midline when comparing old-AL and Old-CR methylation levels. **B.** Proportion of diet-dmCHs unique to diet (green) and those with age interaction (prevented age-dmCHs, blue) (N = 90,428). **C.** Distribution of diet-dmCHs. Dotted lines mark the average methylation differences of hypomethylated diet-dmCHs (red, 8%) and hypermethylation diet-dmCHs (green, 10%). **D.** Distribution plot comparing the proximity between dmCHs by age and diet focusing on 10 kb segments. **E.** Line graph representing % of dmCHs by degree of proximity within 10kb (solid lines) or 1kb (dotted line) by age or by diet.

Figure 5: Diet-induced differential CH methylation is enriched in CG-rich regions of the genome. **A.** Enrichment of diet-dmCHs in gene-centric regions, only significant enrichments are shown ($p<0.05$, hypergeometric test). Diet-dmCHs were found to be enriched in CG-rich regions, promoters and gene bodies. **B.** Enrichment of diet-dmCHs in gene-regulatory regions. Red indicates under-representation (log(odds ratio) < 0 & p-value < 0.05, hypergeometric test), blue indicates over-representation (log(odds ratio) > 0 & p-value < 0.05, hypergeometric test) and white show no significance **C.** Pathway analysis of genes containing >2 significant diet-dmCHs. Top enrichment pathways are shown (FDR adjusted p-values < 0.01). Circle size indicates the ratio of genes in pathway containing diet-dmCGs, scale bar represents p-values. **D.** Overlap between pathways enriched for diet-dmCHs and diet-dmCGs.

Figure 6: Diet induced differential methylation involved in auto-regulation of DNA regulatory enzymes. **A.** Calorie-restriction induces hypermethylation of CGs (red) and CHs (green) in the promoter regions of Tet3 **B.** mRNA expression of Tet3 decreases with diet but not age (n=7-8/group, ** $p < 0.005$, One-way ANOVA with Benjamini-Hochberg correction). **C.** Calorie-restriction induces hypermethylation of CGs (red) and CHs (green) in the promoter regions of DNMT1. **D.** mRNA expression of DNMT1 using an assay inclusive of all known DNMT1 isoforms show no significant differences with age or diet (n=7-8/group, p

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> 0.05, One-way ANOVA with Benjamini-Hochberg correction). **E.** Isoform specific assays were used to assess expression of DNMT1 shown significant decrease in DNMT1 expression with age and diet in isoforms utilizing exon 1 but no difference in expression when examining isoforms using the alternative exon 1 (n=7-8/group, * $p < 0.05$ ** $p < 0.001$, One-way ANOVA). **F.** mRNA expression of Dnmt3a1 decreases with diet and age. Tet1 expression did not change in any condition and Tet2 expression decreased with diet but not age (n=7-8/group, ** $p < 0.001$, One-way ANOVA with Benjamini-Hochberg correction).

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743 **Figure 1**

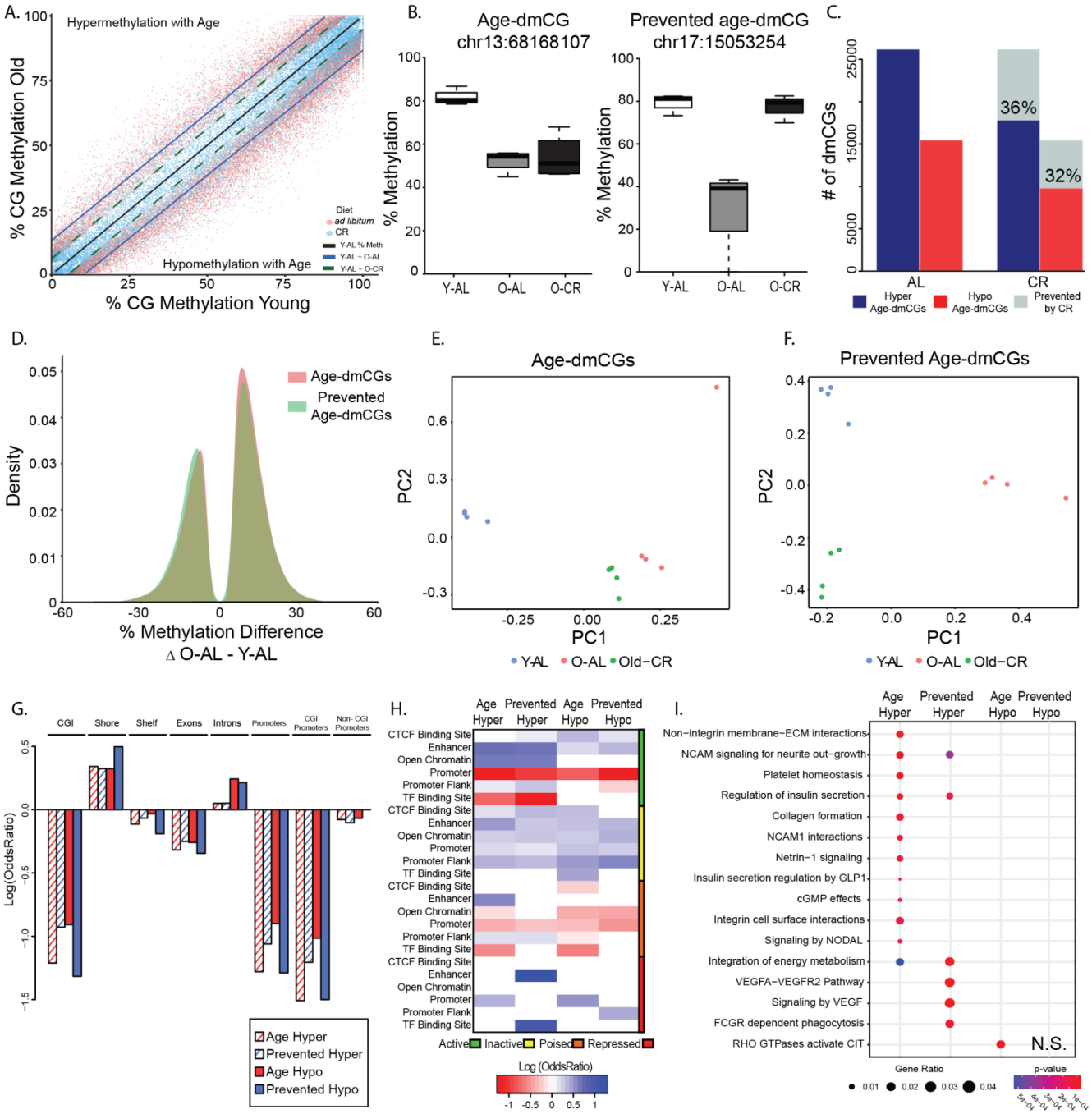

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Figure 2

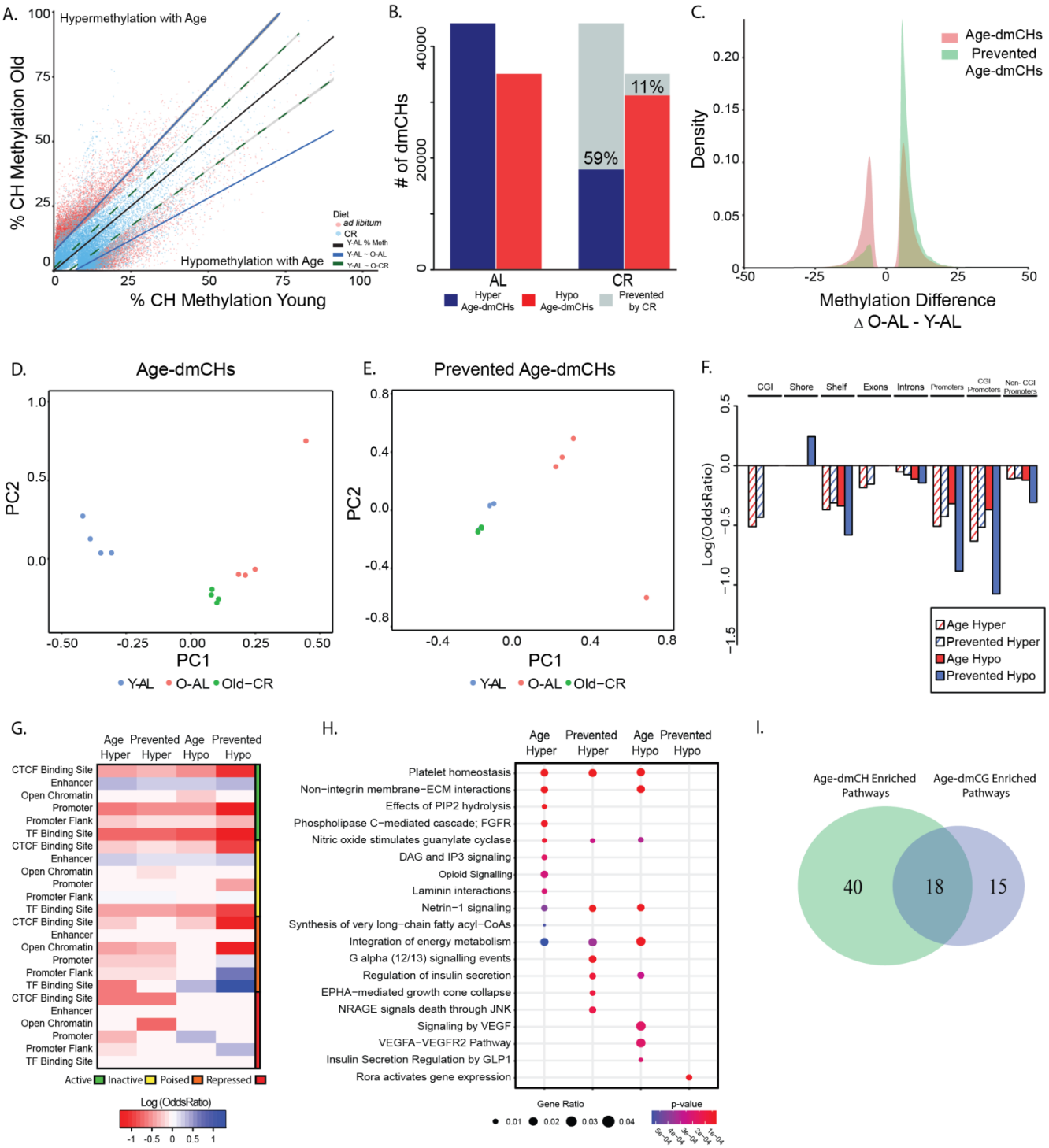

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Figure 3

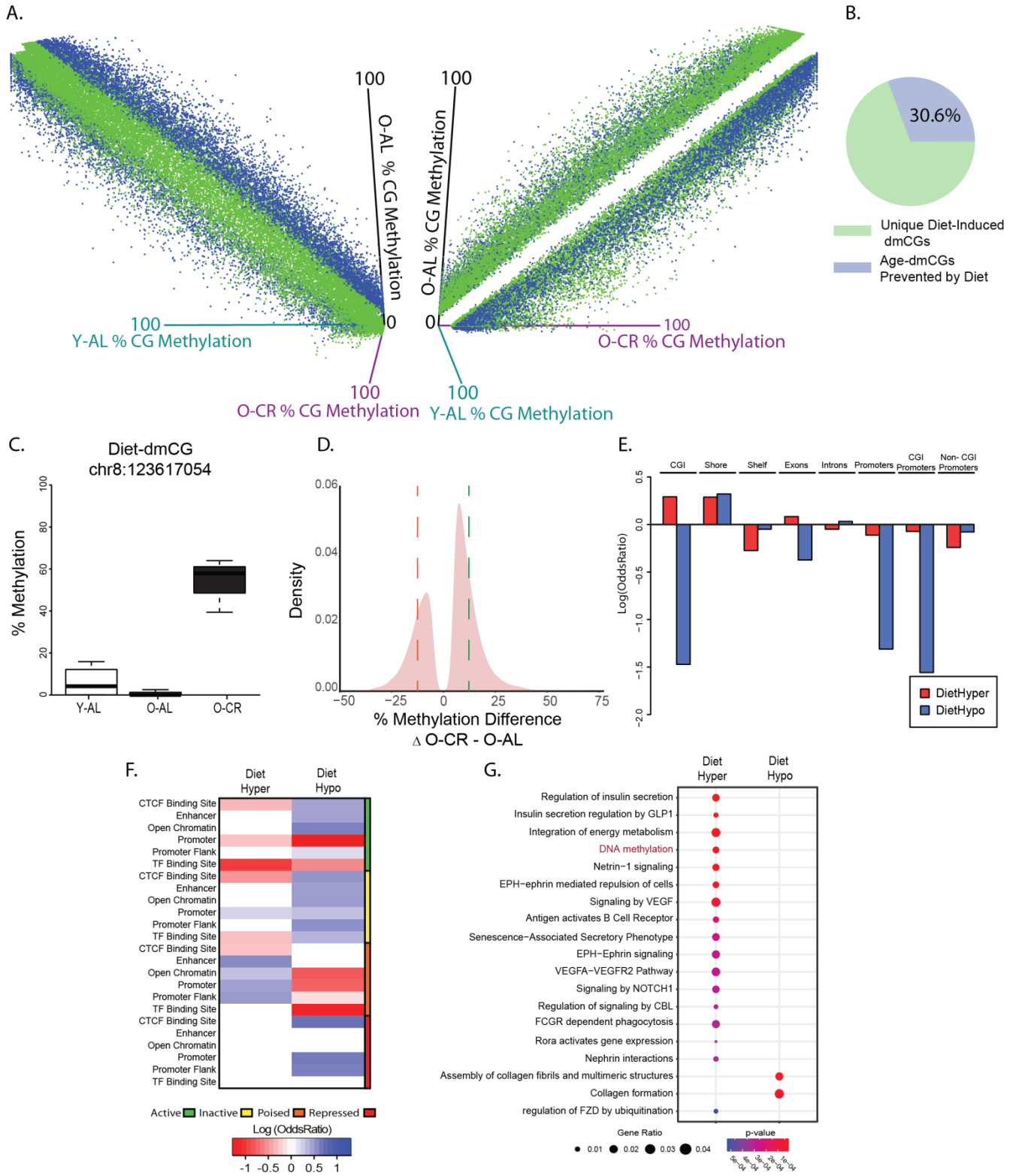

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Figure 4

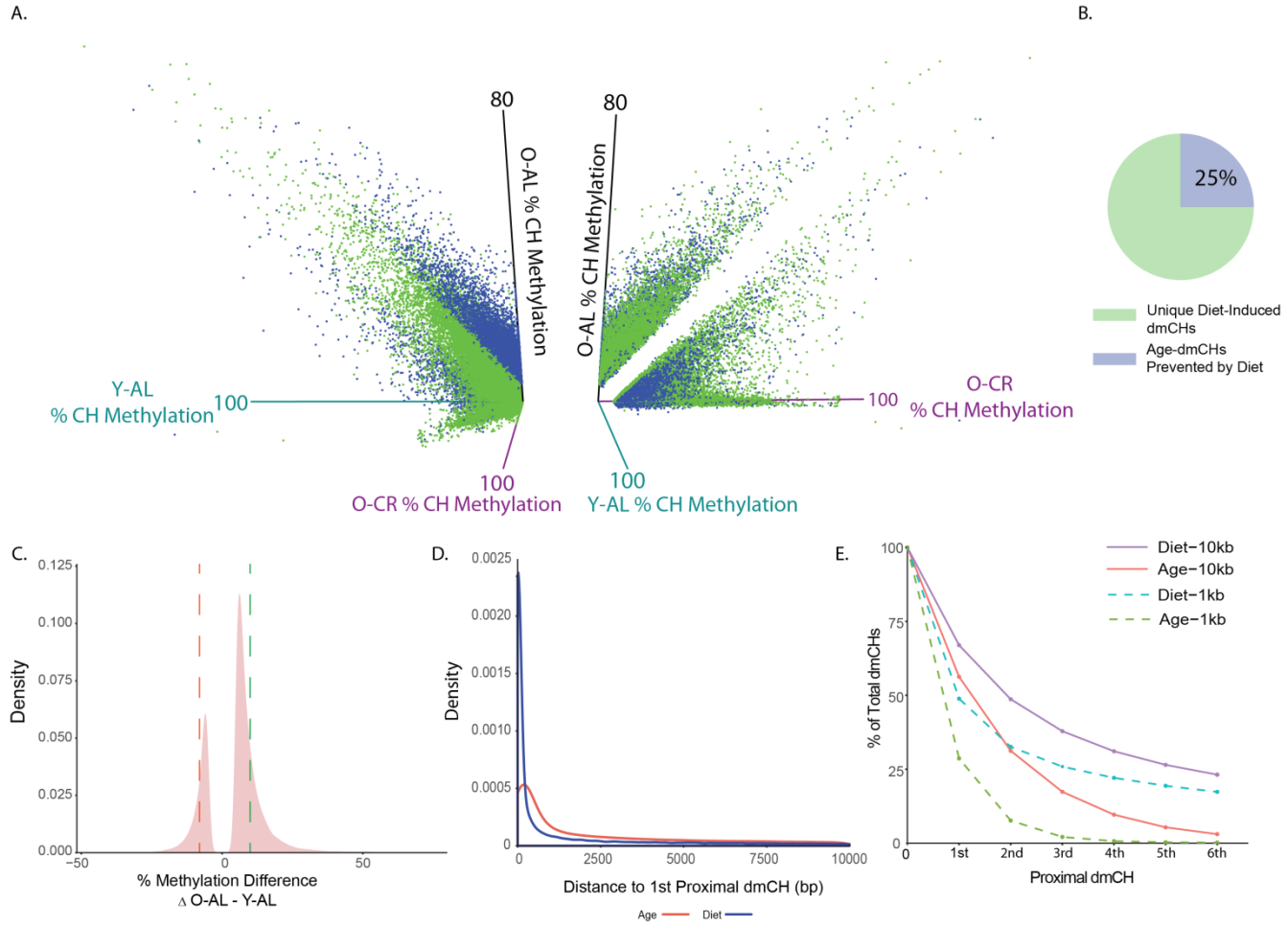

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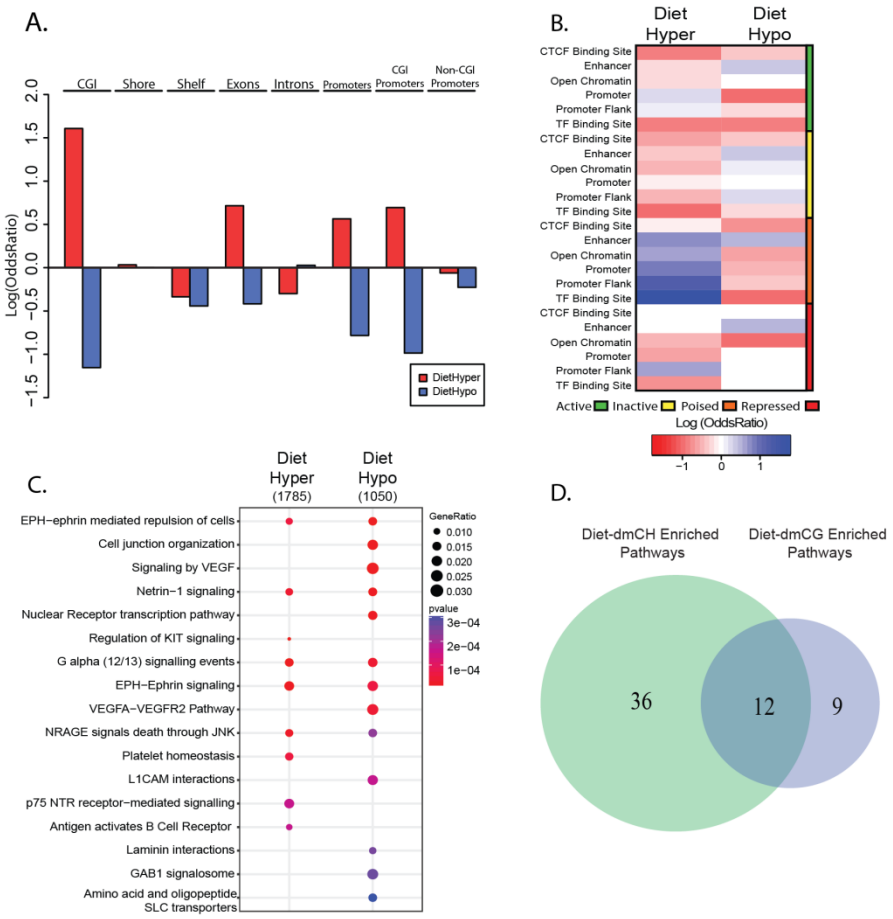
Figure 5



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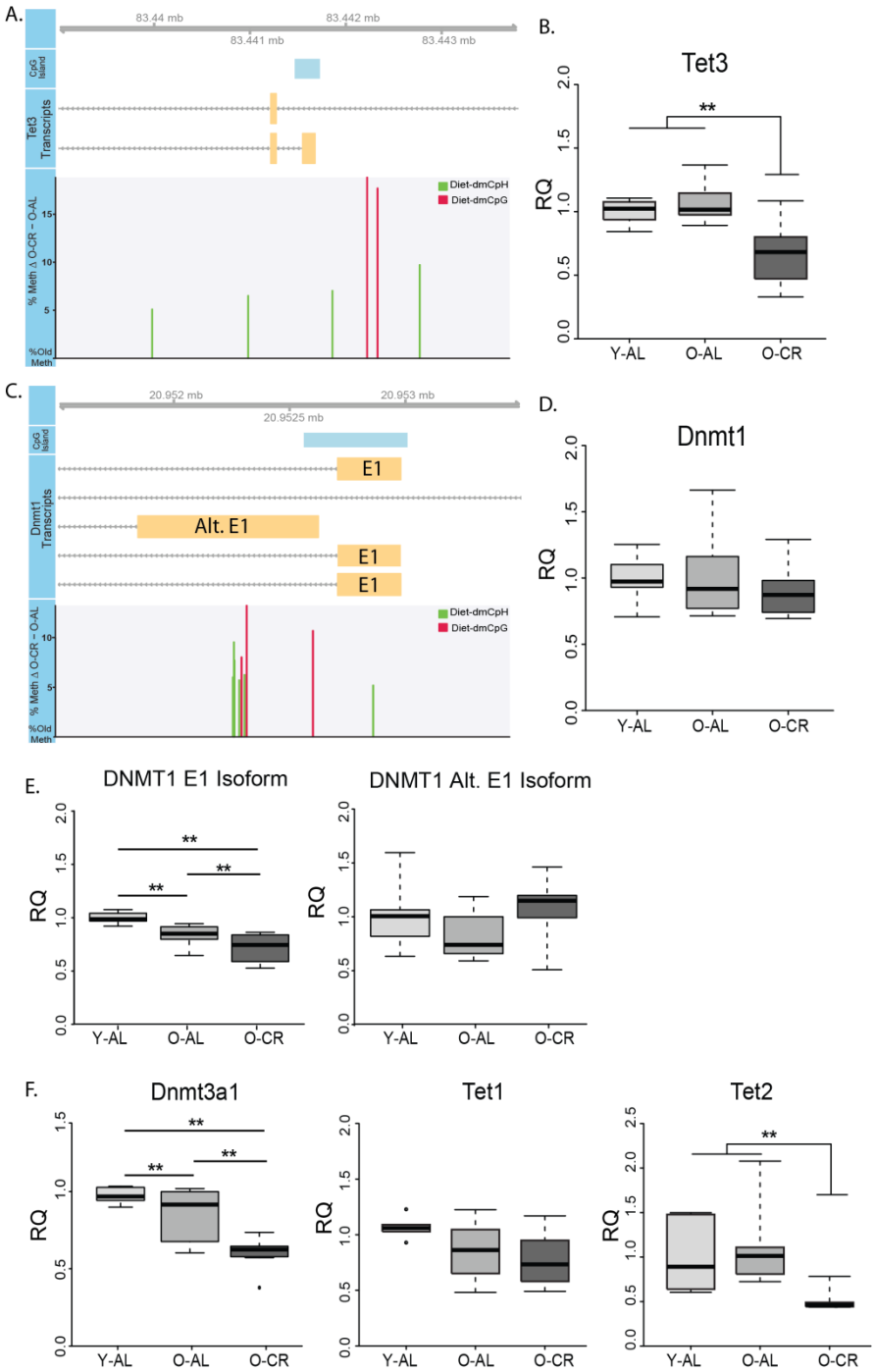
Figure 6