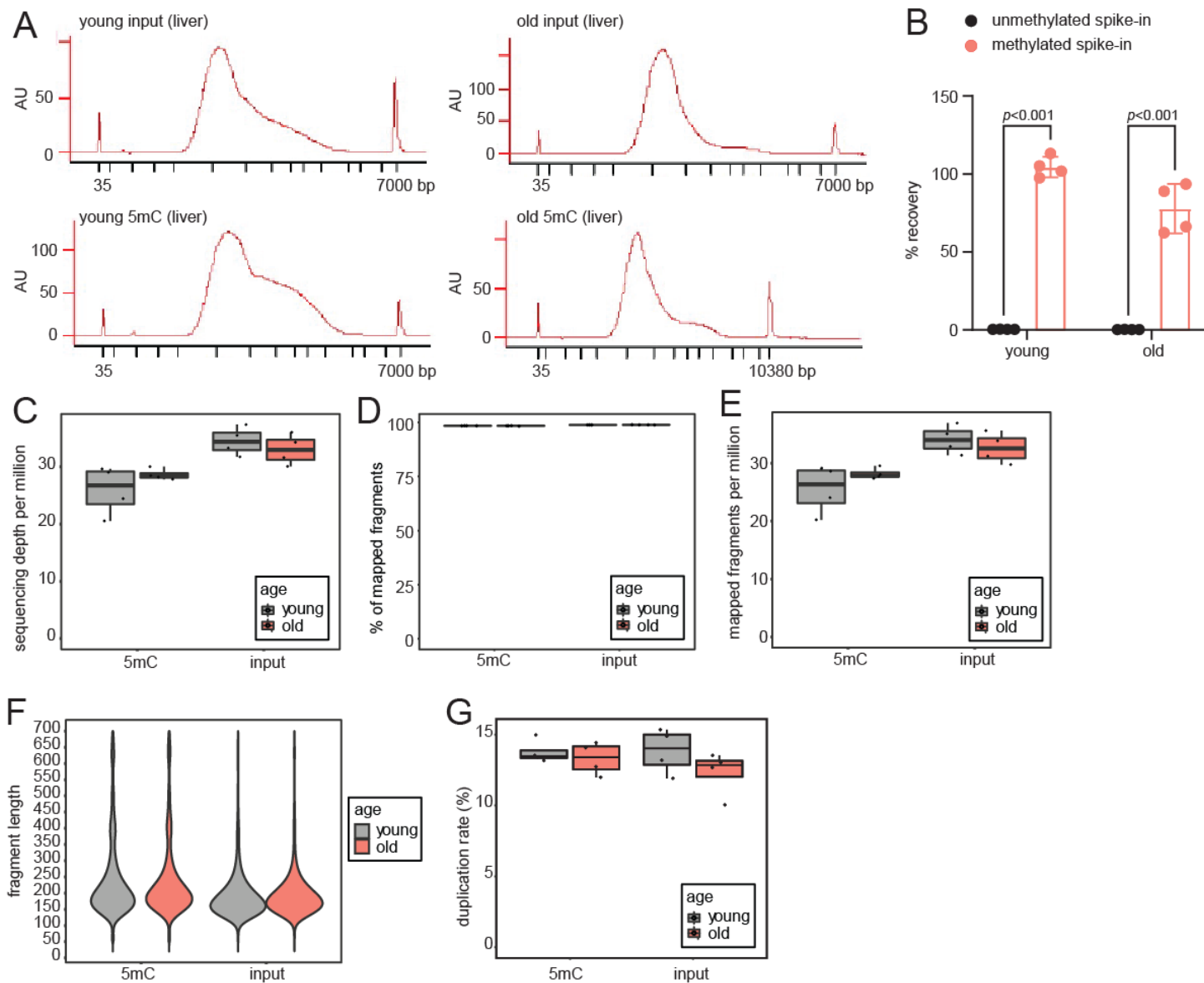


Supp. Figure 1: Quality control metrics of mouse liver hMeDIP-seq libraries

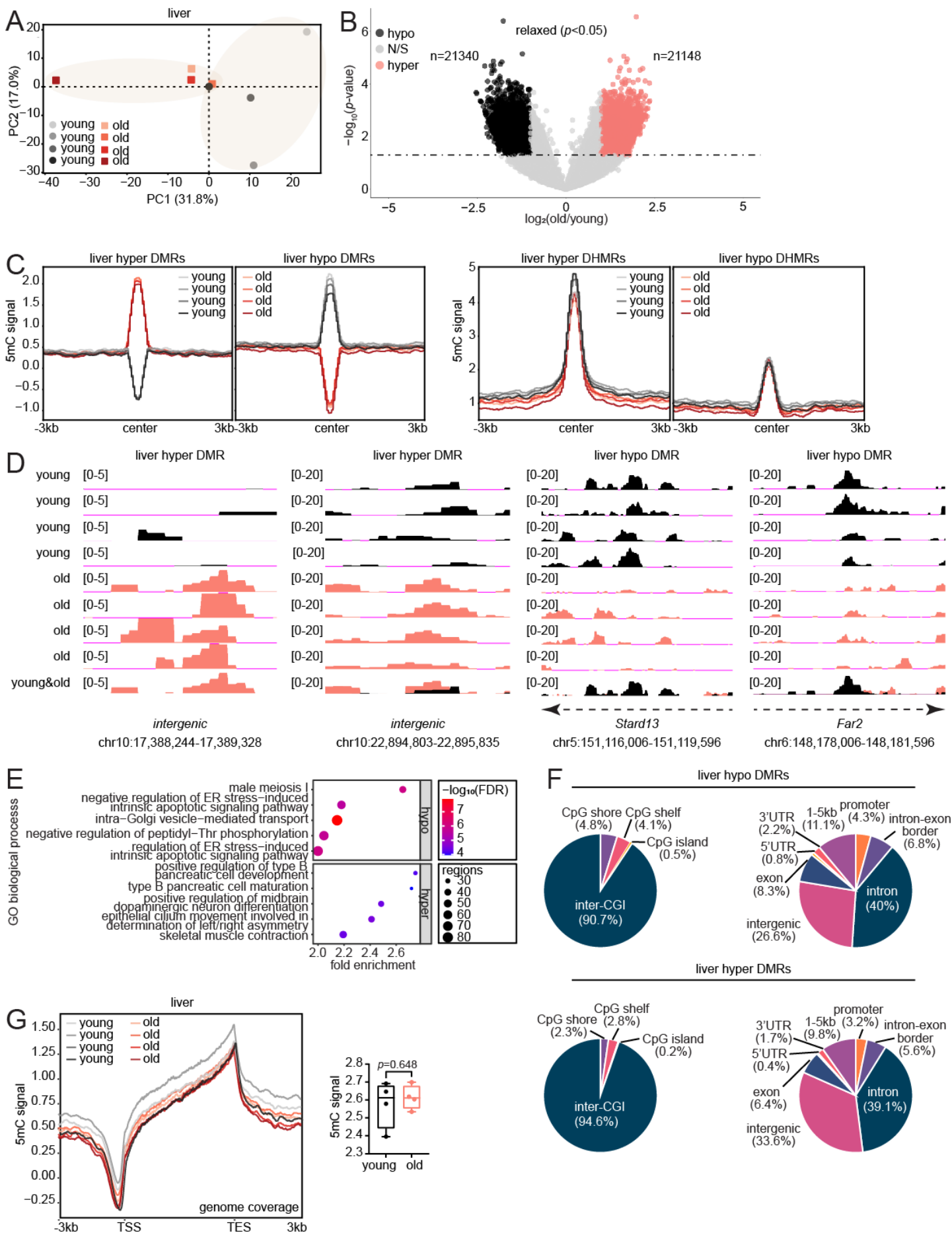
(A) (Left) Representative Bio-analyzer electropherogram of liver hMeDIP-seq library from young input and 5hmC samples (n=4). (Right), same as left but for old liver samples (n=4). AU represents arbitrary fluorescence units. (B) qPCR analysis for exogenous unmethylated and hydroxymethylated DNA control spike-ins included in hMeDIP-seq for young and old (n=4 each) samples. Data are presented as mean \pm SD; statistical significance was assessed using two-way ANOVA with Šídák's multiple comparisons post-hoc test. Figure panels (C-G) present QC data from 5hmC and input libraries of young and old (n=4 each) mouse liver samples, including (C) sequencing depth, (D) percent of alignment rates of 5hmC and input libraries to the GRCm38/mm10 genome, (E) the number of fragments mapped to the GRCm38/mm10 genome, (F) the length of mapped fragments extracted from SAM file, and (G) duplication rates. For (C-G), statistical significance was assessed using two-sided unpaired Welch's t-test. (H) Lisa MOTIF analysis using the top 500 genes in young and old (ranked by gene body 5hmC signal). The top 20 young and old TFs (sorted by p -value) are labeled. (I) Spearman-rank correlation between young (panel 1) and old (panel 2) gene body 5hmC signal and mRNA FC (old vs young). Correlation between old vs young (n=4 each) mean gene body 5hmC signal and mRNA FC between old and young (n=3 each), panel 3. Correlation between random gene body 5hmC values and mRNA FC (old vs young, panel 4). ρ = Spearman's correlation coefficient. p -values were derived from Spearman's rank correlation. For all box plots (C-G), the horizontal line within each box represents the 50th, while the bounds of the box depict the 25th and 75th percentile of the data. The whiskers extend to the minima (the smallest value within 1.5 times the IQR below the first quartile, excluding outliers) and the maxima (the largest value within 1.5 times the IQR above the third quartile, excluding outliers). Source data are provided as a Source Data file.



Supp. Figure 2: Quality control metrics of mouse liver MeDIP-seq libraries

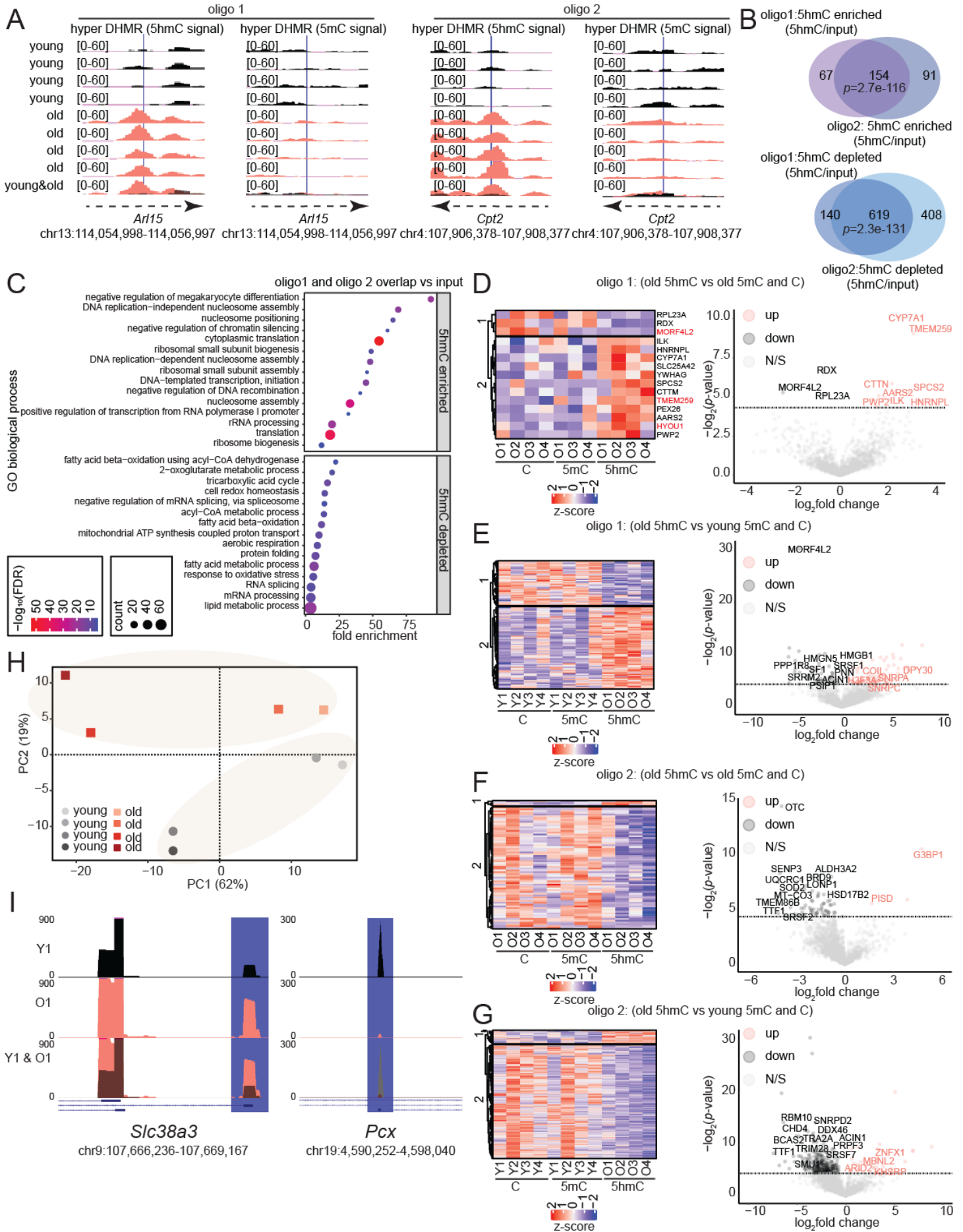
(A) (Left) Representative Bio-analyzer electropherogram of liver MeDIP-seq library from young input and 5mC samples (n=4). (Right), same as left but for old liver samples (n=4). AU represents arbitrary fluorescence units.

(B) qPCR analysis for exogenous unmethylated and hydroxymethylated DNA control spike-ins included in MeDIP-seq for young and old (n=4 each) mouse liver samples. Data are presented as mean \pm SD; statistical significance was assessed using a two-way ANOVA with Šídák's multiple comparisons post-hoc test. Figure panels (C-G) present QC data from 5mC and input libraries of young and old (n=4 each) mouse liver samples, including (C) sequencing depth, (D) percent of alignment rates of 5mC and input libraries to the GRCm38/mm10 genome, (E) the number of fragments mapped to the GRCm38/mm10 genome, (F) the length of mapped fragments extracted from SAM file, and (G) duplication rates. For (C-G), statistical significance was assessed using two-sided unpaired Welch's t-test. For all box plots (C-G), the horizontal line within each box represents the 50th, while the bounds of the box depict the 25th and 75th percentile of the data. The whiskers extend to the minima (the smallest value within 1.5 times the IQR below the first quartile, excluding outliers) and the maxima (the largest value within 1.5 times the IQR above the third quartile, excluding outliers). Source data are provided as a Source Data file.



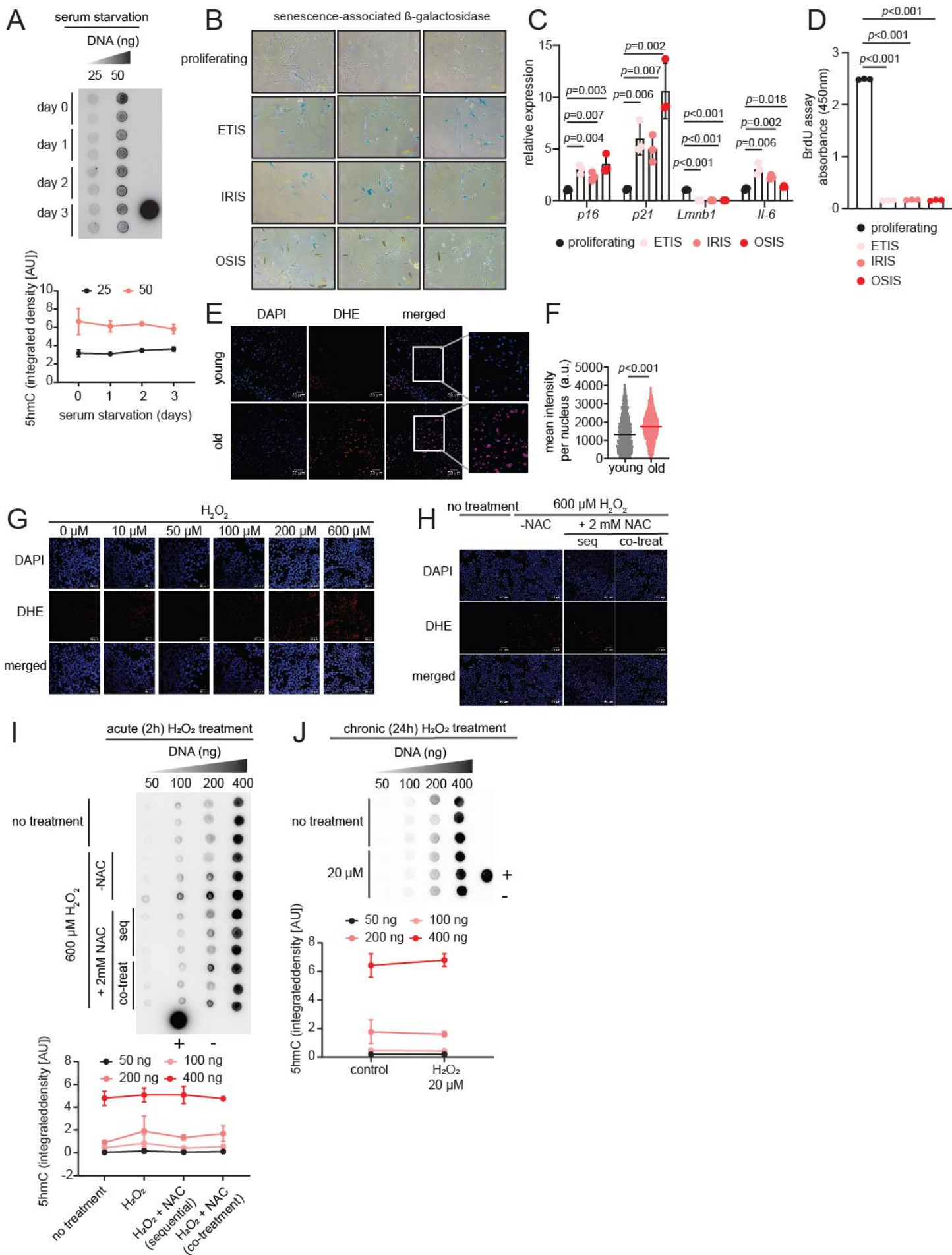
Supp. Figure 3: Age-related differences in 5hmC occur without detectable differences in 5mC

(A) PCA plot obtained using input subtracted 5mC bigWig files of young and old (n=4 each) mouse liver. (B) Volcano plot of differentially methylated regions (DMRs) between old and young (n=4 each) mouse liver; identified by QSEA with $p < 0.05$. Hypo DMRs ($FC \leq -2$) are regions with less enrichment in the old and hyper DMRs ($FC \geq 2$) are regions with higher enrichment in the old. (C) Metaplots of young and old (n=4 each) 5mC signal at the DMRs identified by QSEA. (D) Example genome browser tracks for liver hyper DMRs (two intergenic regions) and hypo DMRs (*Stard13* and *Far2*). (E) GO terms associated with the DMRs from (B) using GREAT. The top 5 biological process terms with $FDR < 0.05$ are shown. (F) Pie charts showing CpG and genic/intergenic annotations of the DMRs from (B). (G) Metaplot of young and old (n=4 each) mouse liver 5mC signal over the gene bodies of all mm10 genes; signal quantifications are shown on the side. Statistical significance was assessed using two-sided unpaired Welch's t-test. Source data are provided as a Source Data file.



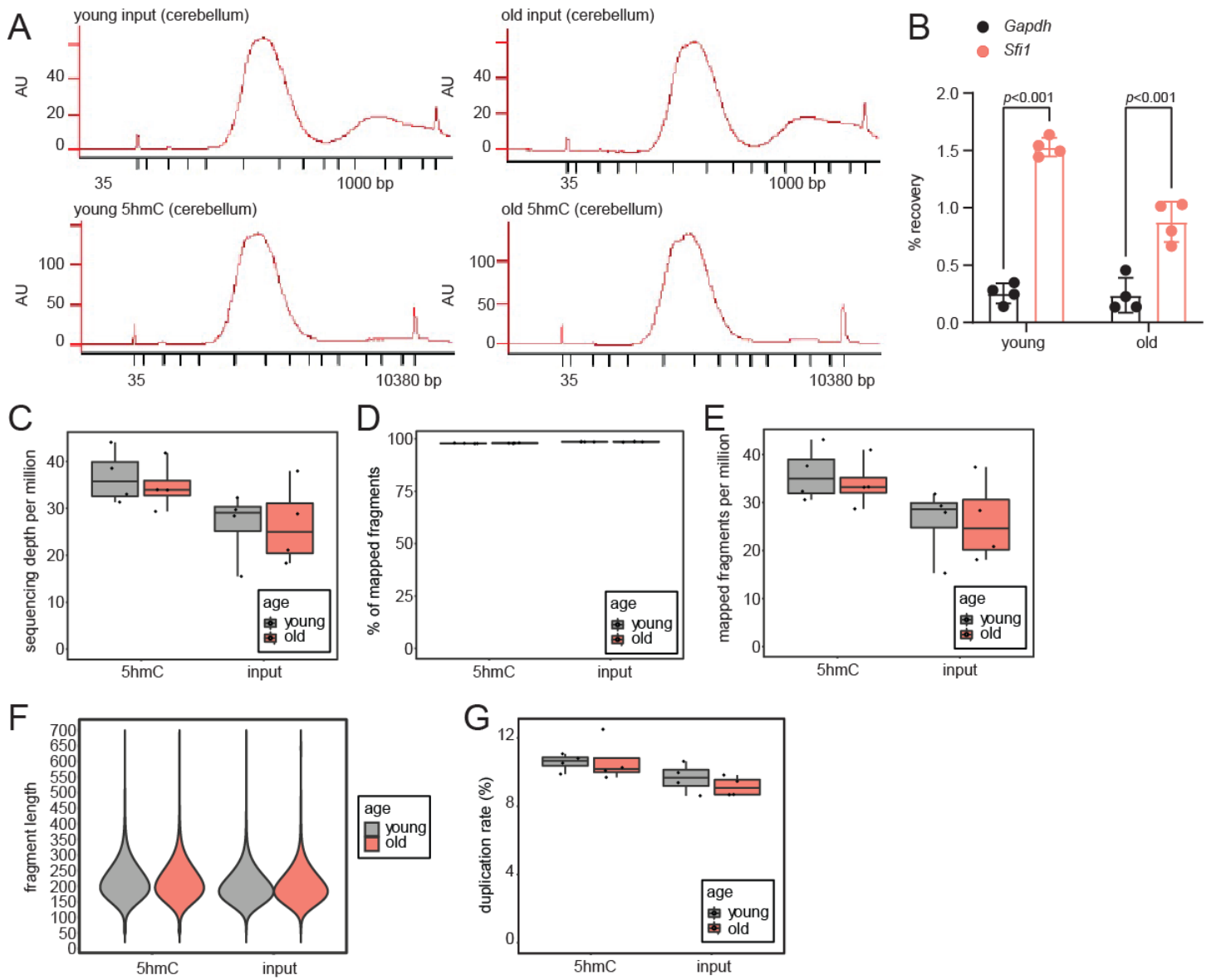
Supp. Figure 4: Supplementary for alternative splicing mediates 5hmC's transcriptionally restrictive function through decreased binding of splicing factors

(A) Genome browser views of young and old (n=4 each) 5hmC and 5mC signal at endogenous regions used to design oligo 1 (left) and oligo 2 (right). (B) Overlap between oligo 1 and oligo 2 for 5hmC-enriched proteins (top) and input-enriched proteins (bottom) when comparing 5hmC vs input. (C) GO analysis using DAVID for proteins overlapping between oligo 1 and oligo 2 in (B). (D) Heatmap (left) and volcano plot (right) of differential proteins ($p < 0.05$) in oligo 1 for the comparison of input-subtracted old 5hmC vs old 5mC and C. (E) Heatmap (left) and volcano plot (right) of differential proteins ($p < 0.05$) in oligo 1 for the comparison of input-subtracted old 5hmC vs young 5mC and C. (F) Heatmap (left) and volcano plot (right) of differential proteins ($p < 0.05$) in oligo 2 for the comparison of input-subtracted old 5hmC vs old 5mC and C. (G) Heatmap (left) and volcano plot (right) of differential proteins ($p < 0.05$) in oligo 2 for the comparison of input-subtracted old 5hmC vs young 5mC and C. (H) PCA plot of dRNA-seq data based on normalized transcript counts for young and old (n=4 each) mouse liver samples. (I) Examples of alternative splicing events in genes with high 5hmC. Shaded area shows a differentially used exon. Source data are provided as a Source Data file.



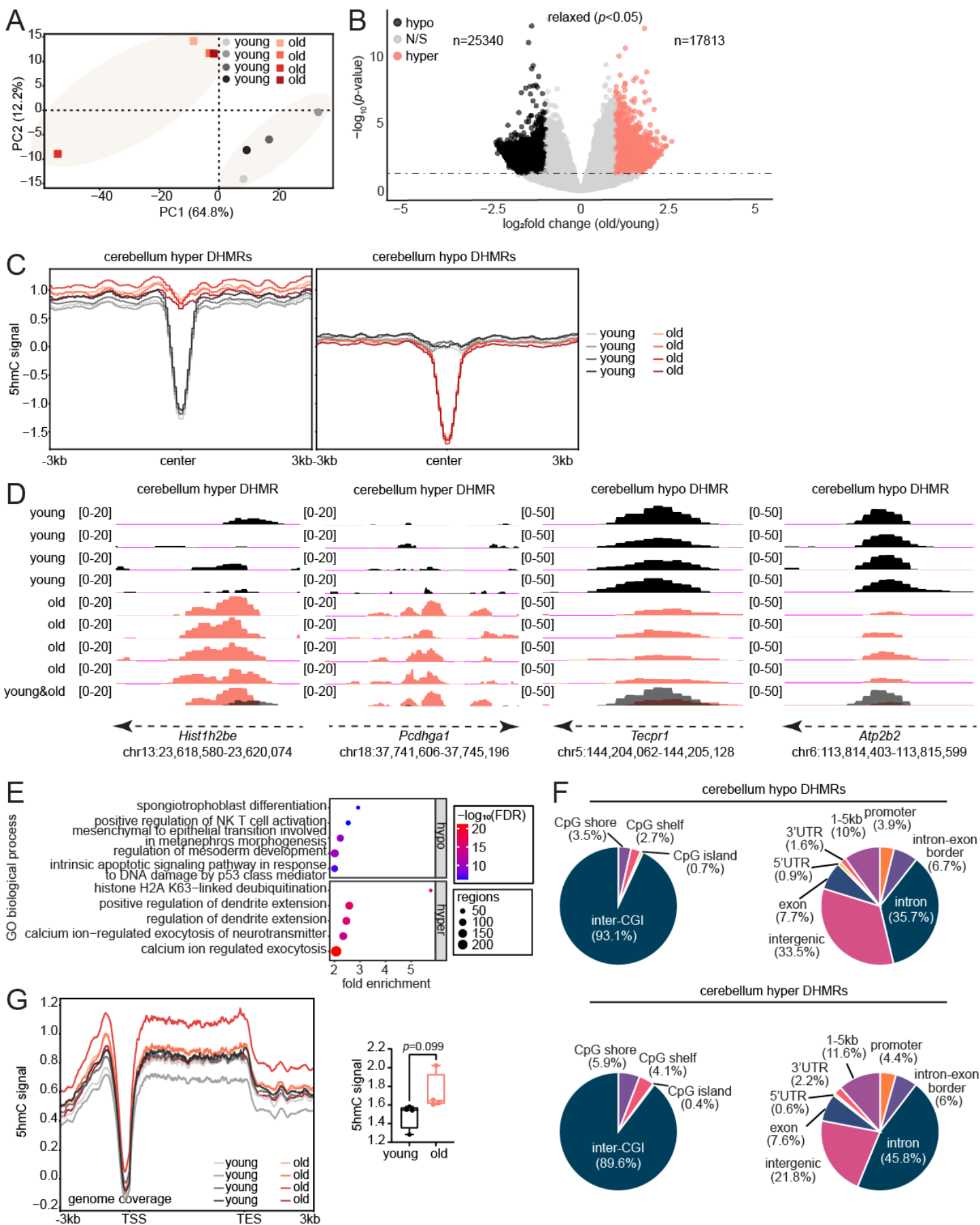
Supp. Figure 5: Supplementary for quiescence and senescence drive the increase of 5hmC with age and impact cellular function

(A) Dot blot for 5hmC signal using gDNA from serum starvation induced quiescent HepG2 cells; n=2 independent cell cultures sourced from a single vial. + control is 25 ng of young mouse hippocampus gDNA, – control is water. Quantifications are depicted below. Statistical significance was assessed using two-way ANOVA with Tukey's multiple comparisons post-hoc test. (B) Representative images of SA- β -gal staining in proliferating and ETIS, IRIS, and OSIS cells. (C) qPCR analysis of *p16*, *p21*, *Lmn1*, and *Il-6* in proliferating and ETIS, IRIS, and OSIS WI-38 cells. For all groups, n=3 independent cell cultures sourced from a single vial; statistical significance was assessed using multiple two-sided unpaired t-test with FDR correction (Benjamini, Krieger, and Yekutieli). (D) BrdU assay of proliferating and ETIS, IRIS, and OSIS WI-38 cells. Statistical significance was assessed using multiple two-sided unpaired t-test with FDR correction (Benjamini, Krieger, and Yekutieli). (E) Representative images of DHE staining of young and old (n=1 each, male) mouse liver sections. (F) DHE mean intensity per nucleus from (E), using data from 5 fields of view for both young and old liver tissue sections. Horizontal bars represent median; statistical significance was assessed using two-sided unpaired Welch's t-test. (G) Representative images of DHE staining of HepG2 cells treated with indicated concentrations of H₂O₂ for 2 h. (H) Representative images of DHE staining of HepG2 cells treated with 600 μ M H₂O₂ for 2 h without NAC, sequential 24 h treatment with NAC after 2 h H₂O₂ treatment, or 2 h co-treatment with NAC followed by 24 h treatment with NAC only. (I) 5hmC dot blot of HepG2 cells from (H). Quantifications are depicted below. (J) 5hmC dot blot of HepG2 cells treated with H₂O₂ for 24 h. Quantifications are depicted below. For both (I-J), statistical significance was assessed using a two-way ANOVA with Tukey's multiple comparisons post-hoc test. Unless noted, all data are presented as mean \pm SD. For (A, I, J), AU represents arbitrary fluorescence units. Source data are provided as a Source Data file.



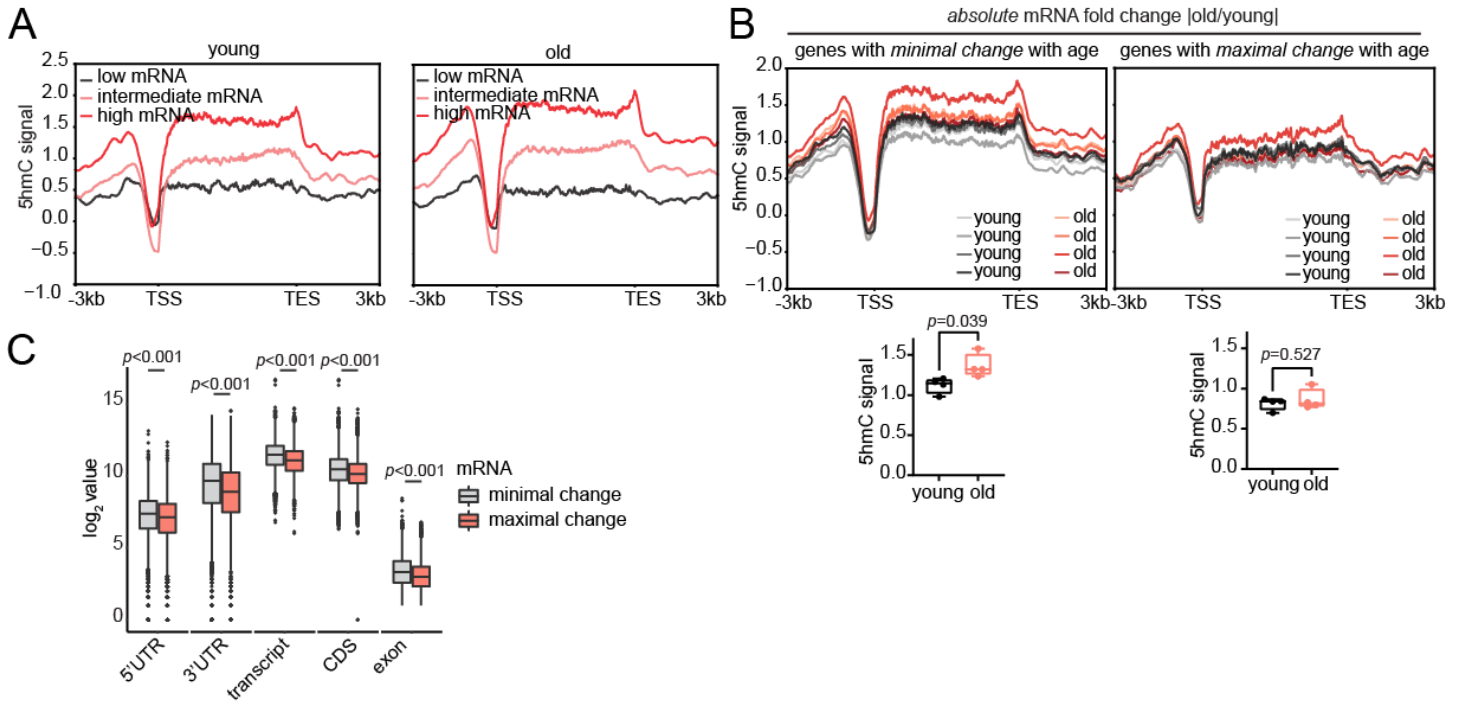
Supp. Figure 6: Quality control metrics of mouse cerebellum hMeDIP-seq libraries

(A) (Left) Representative Bio-analyzer electropherogram of cerebellum hMeDIP-seq library from young input and 5hmC samples (n=4 each). (Right), same as left but for old cerebellum samples (n=4 each). AU represents arbitrary fluorescence units. (B) qPCR analysis of endogenous controls, *Gapdh* (negative control) and *Sfi1* (positive control) for young and old (n=4 each) mouse cerebellum samples. Data are presented as mean \pm SD; statistical significance was assessed using two-way ANOVA with Šídák's multiple comparisons post-hoc test. Figure panels (C-G) present QC data of 5hmC and input libraries from young and old cerebellum samples, including (C) sequencing depth, (D) percent of alignment rates of 5hmC and input libraries to the GRCm38/mm10 genome, (E) the number of fragments mapped to the GRCm38/mm10 genome, (F) the length of mapped fragments extracted from the SAM file, and (G) duplication rates. For (C-G), statistical significance was assessed using two-sided unpaired Welch's t-test. For all box plots (C-G), the horizontal line within each box represents the 50th, while the bounds of the box depict the 25th and 75th percentile of the data. The whiskers extend to the minima (the smallest value within 1.5 times the IQR below the first quartile, excluding outliers) and the maxima (the largest value within 1.5 times the IQR above the third quartile, excluding outliers). Source data are provided as a Source Data file.



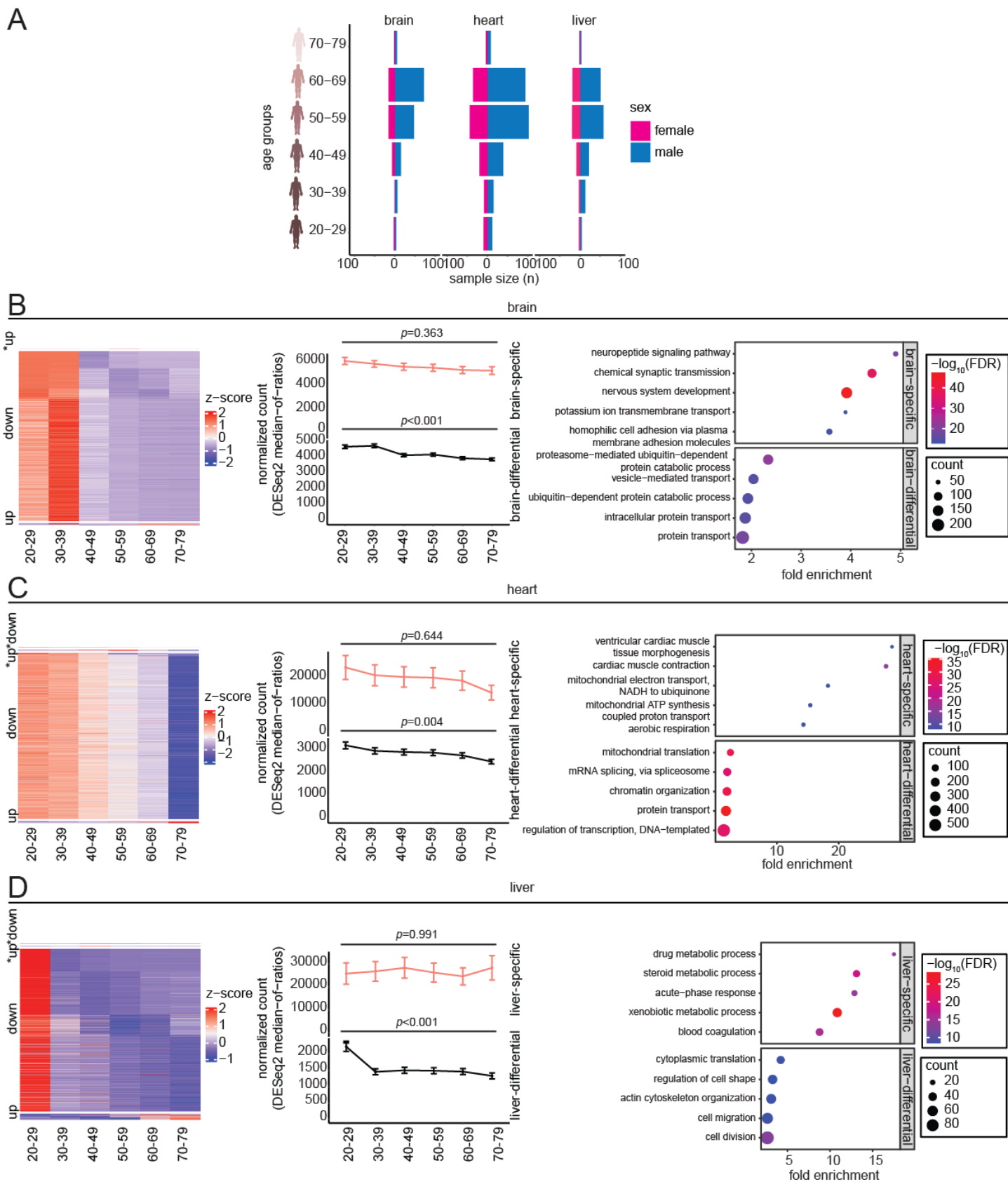
Supp. Figure 7: 5hmC's transcriptionally restrictive function extends to mouse cerebellum

(A) PCA plot obtained using input subtracted 5hmC bigWig files of young and old (n=4 each) mouse liver. (B) Volcano plot of DHMRs between old and young (n=4 each) mouse cerebellum; identified by QSEA with $p < 0.05$. Hypo DHMRs ($FC \leq -2$) are regions with less enrichment in the old and hyper DHMRs ($FC \geq 2$) are regions with higher enrichment in the old. (C) Metaplots of young and old (n=4 each) 5hmC signal at the DHMRs in (B). (D) Example genome browser tracks for cerebellum hyper DHMRs (*Hist1h2be* and *Pcdhga1*) and hypo DHMRs (*Tecpr1* and *Atp2b2*). (E) GO terms associated with the DHMRs from (B) using GREAT. The top 5 biological process terms with $FDR < 0.05$ are shown. (F) Pie charts showing CpG and genic/intergenic annotations of the DHMRs from (B). (G) Metaplot of young and old (n=4 each) 5hmC signal over the gene bodies of all mm10 genes; 5hmC signal quantifications are shown on the side. Statistical significance was assessed using two-sided unpaired Welch's t-test. Source data are provided as a Source Data file.



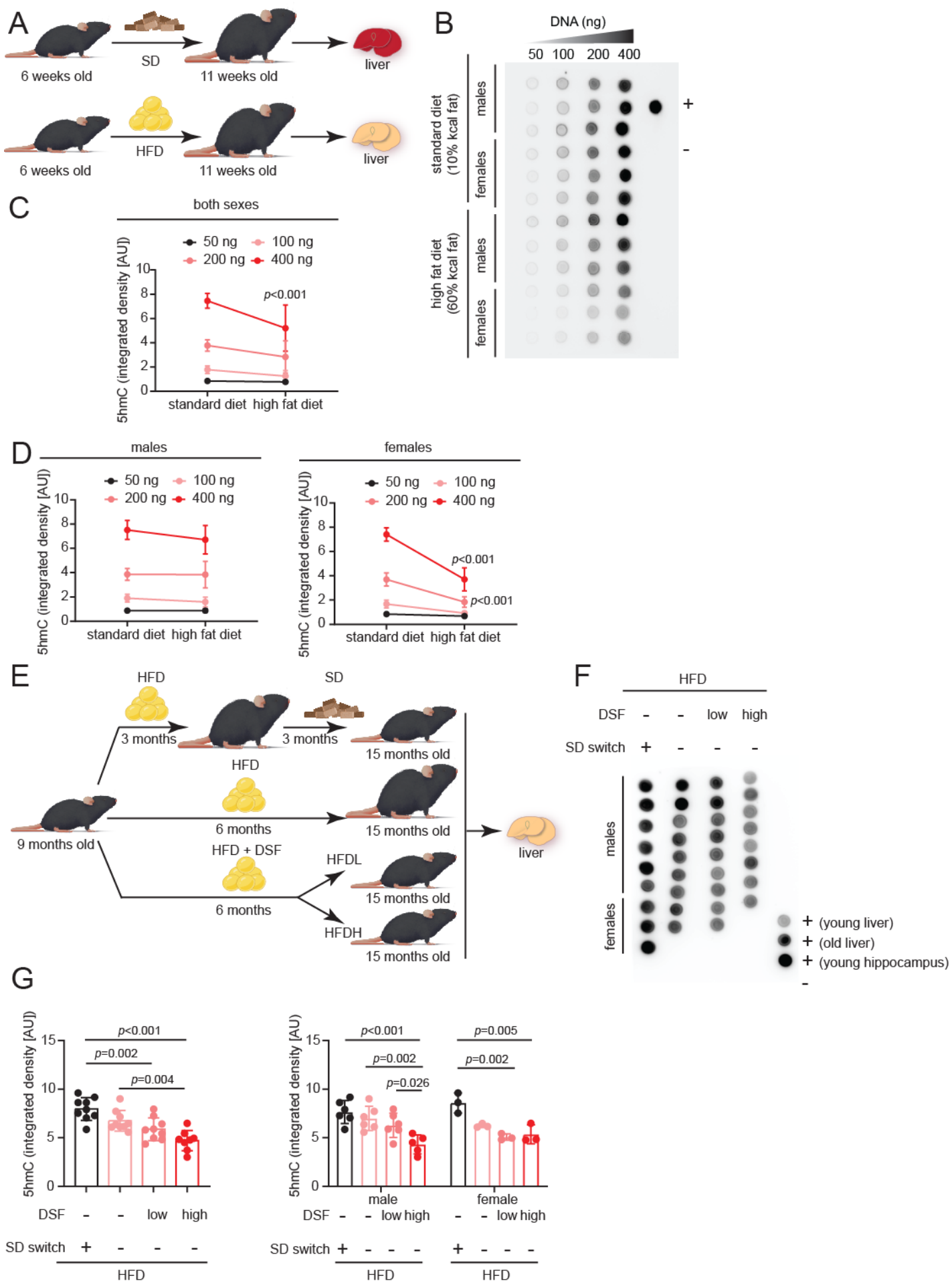
Supp. Figure 8: Gene body 5hmC restricts the magnitude of transcriptional changes during cerebellum aging

(A) Metaplots of young and old (n=4 each) merged 5hmC signal over the gene bodies with low (n=5900), intermediate (n=5900), and high (n=5900) average mRNA counts for young (left) and old (right) samples (n=4 each). (B) Metaplots of young and old (n=4 each) cerebellum 5hmC signal over gene bodies with minimal (left) and maximal (right) expression change between old and young (n=4 each). Quantifications are depicted below; statistical significance was assessed using two-sided unpaired Welch's t-test. (C) Box plots showing the distribution of various genic features for the genes with minimal and maximal expression changes between old and young (n=4 each) mice. Statistical significance was assessed using two-sided unpaired Welch's t-test. Source data are provided as a Source Data file.



Supp. Figure 9: Supplementary for human tissues also show 5hmC-mediated transcriptional restriction

(A) Distribution of sex and age groups among GTEx donors analyzed in this study. **(B)** (Left) Heatmap output from ImpulseDE2 with monotonous and transiently changing genes with age in the brain. (Middle) normalized mRNA count of brain-specific and brain-differential genes from (left). (Right) GO plots associated with the brain-specific and brain-differential genes. **(C)** (Left) Heatmap output from ImpulseDE2 with monotonous and transiently changing genes with age in the heart. (Middle) normalized mRNA count of heart-specific and heart-differential genes from (left). (Right) GO plots associated with the heart-specific and heart-differential genes. **(D)** (Left) Heatmap output from ImpulseDE2 with monotonous and transiently changing genes with age in the liver. (Middle) normalized mRNA count of liver-specific and liver-differential genes from (left). (Right) GO plots associated with the liver-specific and liver-differential genes. Data are presented as mean \pm SEM. Statistical significance was assessed using one-way ANOVA test (without post-hoc comparisons). An asterisk (*) indicates transiently downregulated (*down) or upregulated (*up) genes. Terms without asterisks denote monotonously downregulated (down) or upregulated (up) genes. Source data are provided as a Source Data file.



Supp. Figure 10: 5hmC is downregulated in response to high-fat diet and disulfiram

(A) Schematic of diet regimen for standard and HFD. (B) Dot blot for 5hmC signal in gDNA isolated from mice liver fed on either a SD or HFD (n=6 each). + control is 200 ng of young mouse hippocampus gDNA, – control is water. (C) 5hmC quantification from dot blot (B) stratified by type of diet. (D) Same as (C) except stratified by type of diet and sex. For (C-D), data are presented as mean \pm SD; statistical significance was assessed using two-way ANOVA with Šídák's multiple comparisons post-hoc test. AU represents arbitrary fluorescence units. (E) Schematic of diet regimen for HFD and DSF drug treatment. (F) Dot blot for 5hmC signal in gDNA isolated from mice liver according to the procedure outlined in (E); 3-month HFD, 3-month SD (n=9), 6-month HFD (n=9), 3-month HFD with low DSF (n=9), and 3-month HFD with high DSF (n=8). (G) (Left), quantification of 5hmC signal from (F) stratified by type of diet; statistical significance was assessed using one-way ANOVA with Tukey's multiple comparisons post-hoc test. (Right), same as (left) except stratified by type of diet and sex. Statistical significance was assessed using two-way ANOVA with Tukey's multiple comparisons post-hoc test. For (G, both panels), data are presented as mean \pm SD. AU represents arbitrary fluorescence units. Source data are provided as a Source Data file. Illustration credit: Endosymbiont GmbH.