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LEARNING AND AGE-RELATED CHANGES IN GENOME-WIDE H2A.Z BINDING IN THE MOUSE HIPPOCAMPUS

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Summary

Histone variants were recently discovered to regulate neural plasticity, with H2A.Z emerging as a memory suppressor. Using whole-genome sequencing of the mouse hippocampus, we show that basal H2A.Z occupancy is positively associated with steady-state transcription, whereas learning-induced H2A.Z removal is associated with learning-induced gene expression. AAV-mediated H2A.Z depletion enhanced fear memory and resulted in gene-specific alterations of learning-induced transcription, reinforcing the role of H2A.Z as a memory suppressor. H2A.Z accumulated

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Author contributions

I.B.Z. conceived and designed the experiment, and co-wrote the manuscript with G.S., J.J.D., A.J.K., and B.J.W. A.J.K., C.P.G., and J.J.D. carried out analyses and figure preparation. G.S. and A.B.A. carried out the experiments. K.N. assisted with data interpretation and manuscript preparation. MAB and AMD produced AAV viruses. P.B-C. and H-Y.M.C assisted with imaging.

Declaration of interests

The authors declare no competing interests.

with age, although it remained sensitive to learning-induced eviction. Learning-related H2A.Z removal occurred at largely distinct genes in young *vs* old mice, suggesting that H2A.Z is subject to regulatory shifts in the aged brain despite similar memory performance. When combined with prior evidence of H3.3 accumulation in neurons, our data suggest that nucleosome composition in the brain is reorganized with age.

Keywords

H2A.Z; fear conditioning; memory; learning; ChIP-seq; RNA-seq; gene-expression; epigenetics; chromatin; hippocampus; histone variants

Introduction

Chromatin modifications are crucial for learning and memory and serve as key regulators of transcription (Walters and Zovkic 2015). Although DNA methylation and post-translational modifications of histones have well-established roles in memory (Day and Sweatt 2011), a role for histone variants was only recently identified (Dunn et al., 2017, Michod et al., 2012, Zovkic et al., 2014, Maze et al., 2015). H2A.Z, a variant of histone H2A, is actively exchanged in response to learning and H2A.Z depletion in the hippocampus or the medial prefrontal cortex enhances memory, suggesting that H2A.Z is a memory suppressor (Zovkic et al., 2014). However, many aspects of H2A.Z regulation in the hippocampus remain unknown, particularly the extent to which genome-wide H2A.Z binding is altered in response to learning.

The effect of H2A.Z on transcription is also unclear, with studies outside the brain reporting both a positive and a negative association with gene expression (e.g. Bonisch and Hake, 2012, Chen et al., 2013). We previously showed that learning-induced eviction of H2A.Z from nucleosomes immediately downstream of the TSS is positively associated with the expression of memory-related genes, whereas H2A.Z depletion resulted in both up- and down-regulation of gene expression under basal conditions (Zovkic et al., 2014). Moreover, H2A.Z depletion produced context-specific effects on transcription in rat primary cortical neurons (Dunn et al., 2017), reinforcing a complex regulatory function of H2A.Z in the brain.

Whereas canonical histones are selectively synthesized during cell division, histone variants are replication-independent, a property that has been linked with age-related accumulation of the histone variant H3.3 in neurons (Maze et al., 2015). Age-related accumulation of H2A.Z is of particular interest, given the increased prevalence of cognitive decline with age (Castellano et al., 2012) and our evidence that H2A.Z has a negative effect on memory (Zovkic et al., 2014). In contrast to H3.3, which is the only replication-independent H3 variant, H2A variants are much more diverse, such that the potential for age-related accumulation of H2A.Z is less clear (Zovkic and Sweatt, 2015).

Here, we carried out the first genome-wide characterization of H2A.Z dynamics in the hippocampus of young (4-month-old mice) and aged (15.5 months) mice to determine where H2A.Z binds in the genome, whether binding is modified by age, and how H2A.Z binding is

affected by contextual fear conditioning, a hippocampus-dependent associative learning task. To determine the link with transcription, we compared H2A.Z binding to basal and learning-induced changes in gene expression, allowing for direct comparison of H2A.Z's association with steady-state *vs.* stimulus-induced transcription. These questions are fundamental to understanding the basis of H2A.Z function in stimulus-induced plasticity and memory formation.

Results

Distribution of H2A.Z binding in the hippocampus

Although H2A.Z binding in non-neuronal cells has been widely studied (e.g. Li et al., 2005, Raisner et al., 2005, Ku et al., 2012), the distribution of H2A.Z in the hippocampus, a brain region critical for memory formation, is not well characterized (but see Soboleva et al., 2017). To determine the extent of hippocampal H2A.Z binding and identify regulatory regions bound by H2A.Z, we combined H2A.Z chromatin immunoprecipitation (ChIP) with next-generation sequencing. We identified 20,213 H2A.Z peaks (for example, see Figure 1a) distributed across 12,696 unique genes (for a list of genes, see Supplemental Table 1). In line with studies in non-neuronal cells (Weber et al., 2010, Coleman-Derr and Zilberman, 2012), the majority (~50%) of H2A.Z was found in exons, with fewer peaks in intergenic and 5'UTR regions (Figure 1b). In contrast, introns and non-coding RNA represented a minor proportion of H2A.Z-bound regions. In non-neuronal cells, H2A.Z occupies enhancers, promoters, and gene bodies, with distinct bindings sites implicated in distinct functional outcomes (Ku et al., 2012, Hu et al., 2013, Brunelle et al., 2015, Subramanian et al., 2015). Thus, we compared H2A.Z binding in our data set against published data of hippocampal histone PTMs that serve as an index of regulatory regions [H3K27Ac was used as an index of enhancers; H3K4me3 was used as an index of promoters (Gjoneska et al., 2015)]. H2A.Z was enriched at gene bodies and promoters, with promoter enrichment dramatically exceeding gene body enrichment (Figure 1c and Supplemental Figure 1).

Consistent with data across species and cell types (Coleman-Derr and Zilberman, 2012, Ku et al., 2012), H2A.Z is strongly positioned at sites flanking the TSS (Figure 1d), suggesting that H2A.Z localization is conserved in the hippocampus. H2A.Z selectively occupied TSS-flanking CpG islands, such that non-CpG regions were virtually devoid of H2A.Z (Figure 1d). CpG islands are generally unmethylated (Deaton and Bird, 2011) and DNA methylation in non-neuronal cells interferes with H2A.Z deposition (Zilberman et al., 2008, Coleman-Derr and Zilberman, 2012, To and Kim, 2014). Thus, preferential H2A.Z occupancy at CpG islands suggests that H2A.Z deposition at TSS-flanking nucleosomes may at least in part be attributed to low levels of DNA methylation.

H2A.Z accumulates with age

Whereas canonical histones are replication-coupled and thus synthesized only during cell division, histone variants are replication-independent, allowing for their accumulation in non-dividing cells, such as neurons (Maze et al., 2015). To determine if H2A.Z accumulates with age, we compared H2A.Z binding in the hippocampus of young (4 months old) and aged (15.5 months old) mice. We found that 699 H2A.Z peaks that were common to young

and aged mice exhibited increased binding with age, whereas only 10 peaks had reduced binding in aged mice (Supplemental Table 1 and Figure 2 a,c,d). H2A.Z accumulated primarily in exons and 5'UTR regions, with minimal changes occurring in introns, intergenic regions, and non-coding RNA (Figure 2b). A GO enrichment analysis revealed H2A.Z accumulation in categories that include positive and negative regulators of transcription, as well as ubiquitin protein ligase activity (Figure 2e), consistent with a recent study demonstrating H2A.Z-mediated regulation of the ubiquitin proteasome system (Dunn et al., 2017). Combined with evidence for age-related accumulation of H3.3 (Maze et al., 2015), our data suggest that the composition of histone subtypes that make up nucleosomes is subject to age-related changes, which may influence the overall chromatin landscape in aged mice.

Fear conditioning causes widespread H2A.Z removal

We previously showed that H2A.Z is subject to regulation by learning, but the extent to which H2A.Z is modified across the genome and outside of TSS-flanking nucleosomes is unknown. To investigate learning-induced H2A.Z dynamics, we compared H2A.Z binding 30 min after contextual fear conditioning to naïve (untrained) control mice at 4 and 15.5 months of age (see Supplemental Figure 2a for PCA plot). In young adults, fear training produced an overwhelmingly unidirectional effect on H2A.Z binding, with H2A.Z peaks declining at 3048 loci and increasing at only 25 loci (Figure 3 a–c). Based on GO enrichment analyses, H2A.Z eviction was associated primarily with loci involved in microtubule binding, protein dimerization, phosphorylation, as well as ATP-, RNA- and DNA-binding (Supplemental Figure 3).

Given the age-related accumulation of H2A.Z, we conducted additional comparisons to determine if learning-induced H2A.Z dynamics differ in aged mice. As in young mice, aged mice exhibited wide-spread H2A.Z eviction, with 2901 decreased and only 9 increased H2A.Z peaks in response to training (Figure 3d). Moreover, the magnitude of learning-induced H2A.Z eviction was lower (Mann-Whitney $U = 1536275$, $p < 0.0001$) in aged (1.3 mean fold change; Figure 3d) compared to young (2.2 mean fold change; Figure 3a) mice, suggesting that learning-induced changes in H2A.Z binding were less pronounced with age.

Despite extensive H2A.Z eviction at both ages, there was minimal overlap between genes (226) and peaks (299) on which eviction occurred in young and aged mice (Figure 3g). To determine the extent to which changes in learning-induced H2A.Z binding were age-specific, we compared significantly altered genes in aged mice with all genes (i.e. irrespective of significance) altered in young mice and vice versa. Using this comparison, we found that the majority of peaks that were modified in young mice were modified in the same direction in aged mice, even though many peaks that were significantly altered in young mice remained statistically unchanged in aged mice (Supplemental Figure 4a,b). Thus, the most prominent changes in H2A.Z binding occurred for different genes in young and aged mice, but the overall trend was similar across genes. Together, these data suggest that aging is associated with shifts in H2A.Z regulation that are characterized by its basal accumulation, reduced eviction magnitude, and eviction from distinct genes compared to young mice in response to learning.

H2A.Z regulation at the TSS

H2A.Z is particularly abundant at the TSS and H2A.Z dynamics at the TSS are especially important for gene regulation (Weber et al., 2014). We found that H2A.Z occupancy was higher downstream (+1 nucleosome) than upstream (−1 nucleosome) of the TSS under basal conditions, with both nucleosomes exhibiting a significant reduction in binding after fear conditioning and a significant increase in binding with age (Supplemental Figure 5 a–c). Thus, despite higher H2A.Z occupancy downstream of the TSS, both nucleosomes are equally sensitive to learning- and age-related regulation.

Young and aged mice exhibit similar fear memory

Despite age differences in the pattern of H2A.Z eviction, there were no age differences in post-shock freezing during the training session in mice whose brains were used for sequencing, with mice of both ages showing significant learning over repeated shock administrations (Supplemental Figure 4 c,d). Similarly, we did not observe differences in fear memory 24h after training in a separate group of mice (Supplemental Figure 4e), suggesting that age differences in H2A.Z eviction patterns are not attributable to differences in learning capacity or learning thresholds, and instead reflect age differences in molecular underpinnings of memory processing.

Age- and learning-related changes in gene expression

A comparison of basal transcription in young and aged mice revealed 1036 differentially-expressed genes (DEGs), of which 776 were downregulated and 226 were upregulated with age (Supplemental Table 2; see Supplemental Figure 2b for PCA analysis). As with H2A.Z eviction, a comparable number of genes were modified by training in young (314 genes; Supplemental Table 3) and aged (357 genes; Supplemental Table 4) mice, but there was minimal overlap (83 genes: 67 in the same direction in both ages, 16 in opposite direction) between genes that were altered in each age group (Figure 4a). Of the 357 genes modified by fear conditioning in aged mice, 155 were also altered by aging under basal conditions, suggesting that age differences in learning-induced gene expression are tied to altered regulation of basal gene activity.

H2A.Z is positively correlated with steady-state, and negatively correlated with learning-induced transcription

The relationship between H2A.Z and transcription is stimulus-dependent. Under steady-state conditions (i.e., in naïve mice), H2A.Z is positively associated with basal gene expression, such that genes with the highest H2A.Z occupancy also have the highest expression (Figure 4c). To determine how learning-induced H2A.Z eviction relates to the expression of memory-related genes, we assessed whether H2A.Z eviction occurred on genes that are altered 30 min (data presented) and 1hr (Kennedy et al., 2016) after fear conditioning. Compared to all genes, H2A.Z peaks were significantly more associated with genes that are upregulated, but not genes that are downregulated 30 min after fear conditioning (Figure 4e). When only the peaks that are significantly altered by training are included in the analysis, there is a significant overrepresentation of H2A.Z eviction on genes upregulated by fear conditioning at both 30 min and 60 min in young mice (Figure 4f). That is, significant

changes in learning-induced H2A.Z eviction are disproportionally associated with genes that are upregulated by training at both 30 min (11 of 78, 14%) and 60 min (134 of 680, 20%) (Figure 4d), and 96% of the time this occurs at a promoter, whereas no such relationship was found with genes that are downregulated after training. The same association was found for aged mice, whereby H2A.Z eviction was associated with upregulated, but not with downregulated genes (Figure 4g). Of note, there are considerably more DEGs at 60 than at 30 min, indicating that significant learning-induced H2A.Z eviction predicts increased gene expression 30 min later. Maze et al. (2015) also reported that depolarization-induced H3.3 dynamics in neurons were selectively correlated with transcription at late (2 and 5h), but not at early (30 min) time points, suggesting that dynamic regulation of histone variants can have prolonged effects on transcription. Overall, these data suggest that learning-induced H2A.Z eviction regulates the induction of memory-promoting genes, but not the inhibition of genes that are downregulated during memory formation.

Hippocampal H2A.Z depletion regulates gene expression at 1h

We previously showed that H2A.Z depletion affects the expression of a small number of genes 30 min after training (2 of 8 genes studied; Zovkic et al., 2014). Given the relationship between H2A.Z eviction and learning-induced gene expression after 1h (Figure 4 d,f), we selected several of these genes to determine how their expression is affected by AAV-mediated H2A.Z depletion in the hippocampus (Figure 4 h,i,j). Using the same shRNA sequence as in our published work (Zovkic et al., 2014), we replicated our previous observation that the depletion of *H2afz*, a gene encoding H2A.Z, enhances fear memory (Figure 4k), supporting the hypothesis that H2A.Z is a memory suppressor.

Using a separate group of mice, we collected infected hippocampal tissue 1h after training and showed that only *Use1* was affected by H2A.Z depletion at baseline (Virus × Training interaction: $F_{1,15} = 7.94$, $p = 0.01$), with higher expression observed in H2A.Z-depleted mice. However, H2A.Z depletion reduced the learning-induced induction of *Use1*, and of other genes that were not affected by H2A.Z depletion at baseline, including *Lars2* (Main effect of Virus: $F_{1,15} = 4.45$, $p = 0.05$; Main effect of Training: $F_{1,15} = 21.89$, $p < 0.0001$), *Rxrg* (Virus × Training interaction: $F_{1,15} = 7.43$, $p = 0.02$), and *Tmem143* (Virus × Training interaction: F1,15 = 10.87, p = 0.05) (Figure 4l), suggesting that H2A.Z is especially important for learning-induced gene expression. In contrast, *Rps11* was induced by learning irrespective of treatment, but overall expression levels were lower in H2A.Z-depleted mice compared to scramble controls (Main effect of Training: $F_{1,16} = 65.85$, $p < 0.0001$; Main effect of Virus: $F_{1,16} = 6.47$, $p = 0.02$) (Figure 4l).

We previously found minimal effects of H2A.Z depletion on immediate early gene expression at 30 min, with the exception of increased learning-induced *Arc* induction (Zovkic et al., 2014). Consistent with those findings, we now show that *Arc* (Virus × Training interaction: $F_{1,15} = 7.39$, $p = 0.02$) expression is also elevated 1h after fear conditioning in H2A.Z-depleted mice. Such bidirectional effects of H2A.Z depletion were previously reported (Dunn et al., 2017, Weber et al., 2014), and suggest that H2A.Z can promote or repress learning-induced gene expression, while producing minimal effects on basal transcription.

Discussion

Our data show that H2A.Z accumulates in aged mice, but is widely removed from chromatin during learning irrespective of age, whereas H2A.Z incorporation is a rare event at either age. Although H2A.Z-mediated eviction occurred on largely distinct genes in the two age groups, both ages demonstrated a similar relationship between H2A.Z and transcription, whereby H2A.Z removal only predicted the expression of upregulated genes, suggesting that learning-induced H2A.Z removal promotes gene induction, but not gene repression.

Interestingly, H2A.Z depletion affected learning-induced induction of several genes despite minimal effects on their basal expression, suggesting that H2A.Z primarily regulates stimulus-induced gene activity. This may be especially true for genes expressed 1h after training, as H2A.Z eviction at 30 min predicts gene expression at 60 min, and gene induction 60 min after learning is affected by virally-mediated H2A.Z depletion. However, instead of potentiating learning-induced gene activity, we found that virally-mediated H2A.Z depletion impaired learning-induced gene-expression, suggesting that global H2A.Z depletion does not recapitulate the effects of learning-induced H2A.Z eviction on transcription. Indeed, these data indicate that dynamic H2A.Z removal is required for the induction of certain memory-related genes, consistent with evidence that dynamic H3.3 turnover is essential for gene induction (Maze et al., 2015).

Despite impaired learning-induced gene induction, H2A.Z depletion nevertheless resulted in improved memory, supporting our previous evidence that H2A.Z is a memory suppressor. These data suggest that H2A.Z does not promote memory through overall potentiation of learning-induced gene expression, but instead produces its effects through a small number of memory-related genes, such as *Arc*.

In contrast to H3.3, which becomes the predominant H3 in the adult brain, H2A.Z accumulation in the hippocampus is more modest and is not reflected in altered bulk levels of H2A.Z (Maze et al., 2015). This is likely because H2A variants are more numerous compared to H3, such that age-related changes may be more evenly distributed among different H2A subtypes (Zovkic and Sweatt, 2015). Combined with evidence for H3.3 accumulation, our data suggest that histone subunit composition of nucleosomes is altered with age, which may have broad effects on surrounding chromatin. For example, H2A.Z tends to occupy nucleosomes containing distinct H3 modifications compared to canonical H2A (Sevilla and Binda, 2014), such that age-related shifts in H2A.Z may be accompanied by shifts in other histone marks, many of which are also altered in aged mice (Peleg et al., 2010, Walker et al., 2013, Morse et al., 2015).

We observed extensive changes in gene expression and H2A.Z induction despite normal memory formation in aged mice. We focused on late-middle age because this is a time when memory deficits begin to emerge on some tasks (Verbitsky et al., 2004), but not others (Cuppini et al., 2006). Similar performance in fear memory using a relatively robust training protocol suggests that H2A.Z accumulation may be a normal part of aging, which is supported by evidence that H3.3 accumulation reaches saturation by adolescence in the human brain and adulthood in mouse brain (Maze et al., 2015) and as such, changes in

chromatin composition can occur without dramatic shifts in memory. In support of this hypothesis, human studies find age-related shifts in gene expression in cognitively intact adults (Berchtold et al., 2008, Lu et al., 2004), suggesting that some shifts in gene expression and chromatin regulation are a normal part of aging.

This discovery is in line with evidence that aged mice are capable of comparable memory formation as young mice, but that this process becomes less efficient with age (Schimanski and Barnes, 2010). Indeed, age-related changes in chromatin-regulating factors may be a protective part of normal aging, as described for increased levels of the transcriptional repressor REST (Hwang et al., 2017), or they may be detrimental, as reported for various histone PTMs(Peleg et al., 2010, Morse et al., 2015, Snigdha et al., 2016). Together, these data suggest that neural chromatin is subject to age-related reorganization in which histone variants become overrepresented, but remain dynamic to regulate gene-transcription during learning.

Experimental procedures

Further details and an outline of resources used in this work can be found in Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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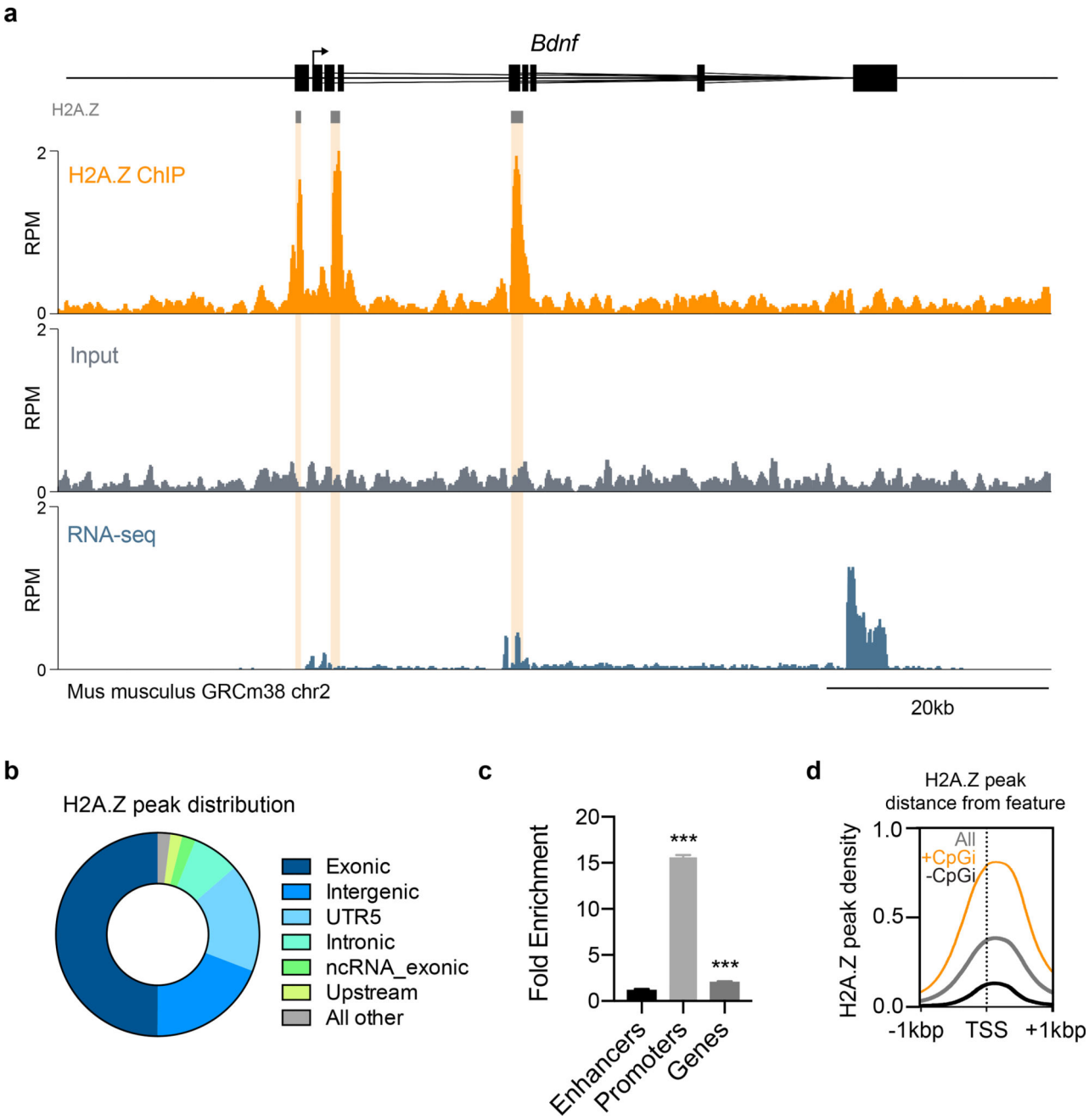
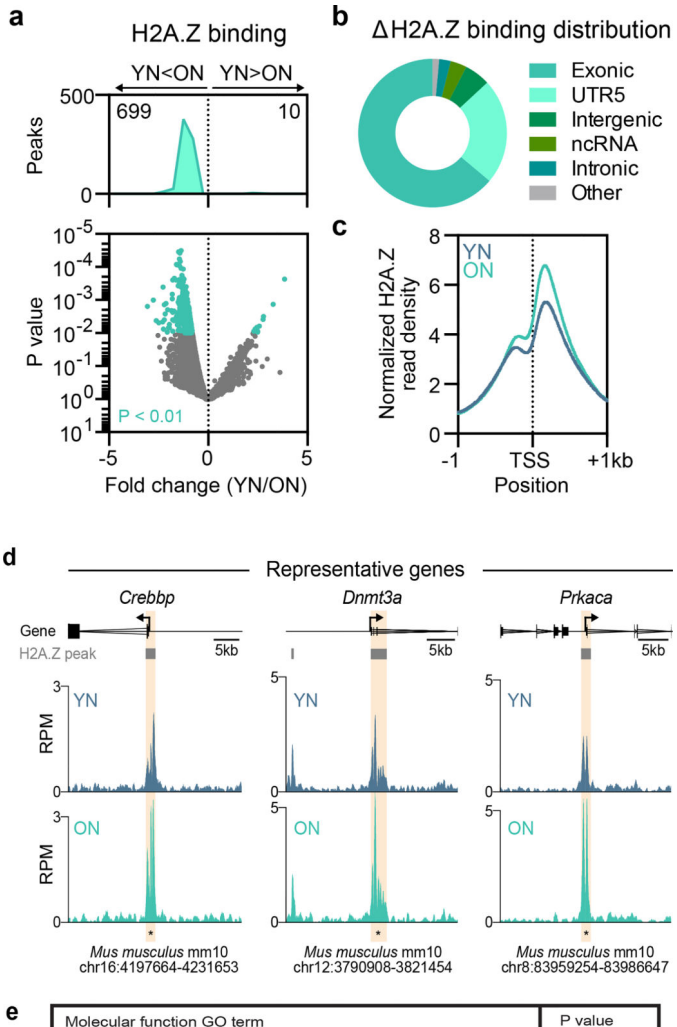


Figure 1. Genome-wide H2A.Z distribution

(a) H2A.Z binding and *Bdnf* expression relative to input in Young-Naïve (YN) mice. (b) A donut plot depicting regions of H2A.Z binding. (c) H2A.Z binding in our data set was compared against published data of hippocampal histone PTMs (H3K27Ac was used as an index of enhancers; H3K4me3 was used as an index of promoters; Gjoneska et al. 2015). H2A.Z was enriched at gene bodies and promoters, with promoter enrichment dramatically exceeding gene body enrichment (d) H2A.Z preferentially localized to TSS-flanking CpG islands.



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Molecular function GO term	P value
R-SMAD binding	4.36E-02
Ubiquitin protein ligase activity	9.53E-08
Ubiquitin protein transferase activity	1.98E-08
Ubiquitin-like protein ligase activity	1.45E-07
Transcription corepressor activity	4.74E-03
Ras GTPase binding	2.38E-04
Chromatin binding	7.39E-07
Protein serine/threonine kinase activity	1.95E-04
Transcription factor binding	1.93E-04
Transcription factor activity, RNA Pol II Core promoter proximal region sequence-specific binding	1.43E-02
RNA Polymerase II core promoter proximal region sequence-specific DNA binding	4.03E-02
Protein domain specific binding	1.53E-05

Figure 2. Age-related changes in H2A.Z binding

(a) 699 H2A.Z peaks increased and only 10 peaks decreased in Old-Naïve (ON) compared to Young-Naïve (YN) mice. (b) Regions at which age-related changes in H2A.Z occurred. (c) H2A.Z accumulation was evident both up- and down-stream of the TSS. (d) Examples of age differences in H2A.Z binding at representative genes. (e) Results of top ten categories identified with GO Enrichment analysis of the 699 H2A.Z peaks that increased with age.

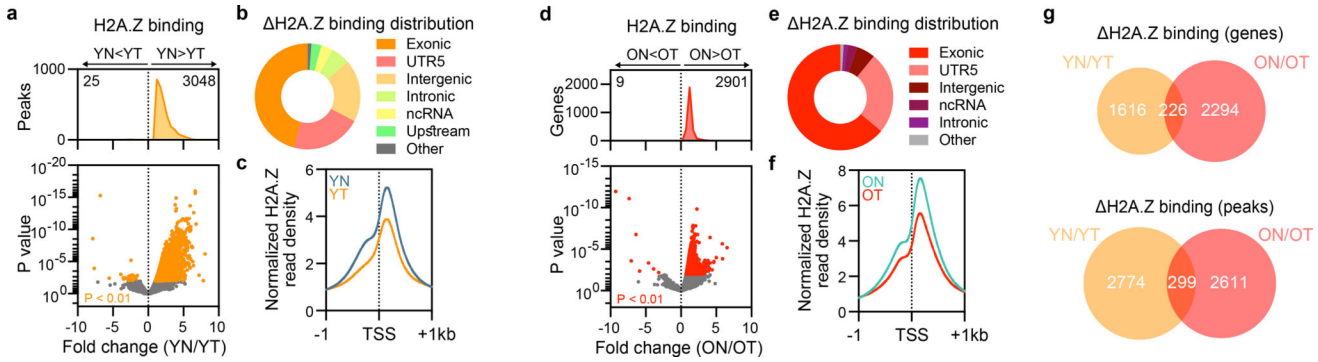

Figure 3. Fear conditioning results in wide-spread H2A.Z removal

(a) TOP: Fear conditioning resulted in extensive removal of H2A.Z, as evidenced by reduced H2A.Z binding at 3048 peaks in Young-Trained (YT) compared to Young-Naïve (YN) mice. In contrast, H2A.Z binding increased at only 25 peaks. BOTTOM: The same pattern is seen when peaks that are not statistically significant are visualized. (b) Regions of learning-induced changes in H2A.Z in young mice. (c) H2A.Z was removed both up- and down-stream of the TSS in response to training in young mice. (d) TOP: Fear conditioning resulted in extensive H2A.Z removal in Old-Trained (OT) compared to Old-Naïve (ON) mice. BOTTOM: This pattern is also seen when peaks that were not statistically significant are visualized. (e) Donut plot depicting key regions of learning-induced changes in H2A.Z in old mice. Compared to young mice, a greater proportion of H2A.Z eviction occurred in exons. (f) H2A.Z was removed both up- and down-stream of the TSS in response to training in old mice. (g) Despite similar genome-wide patterns of H2A.Z eviction, there was minimal overlap between the peaks and unique genes at which H2A.Z changes occurred in young and old mice.

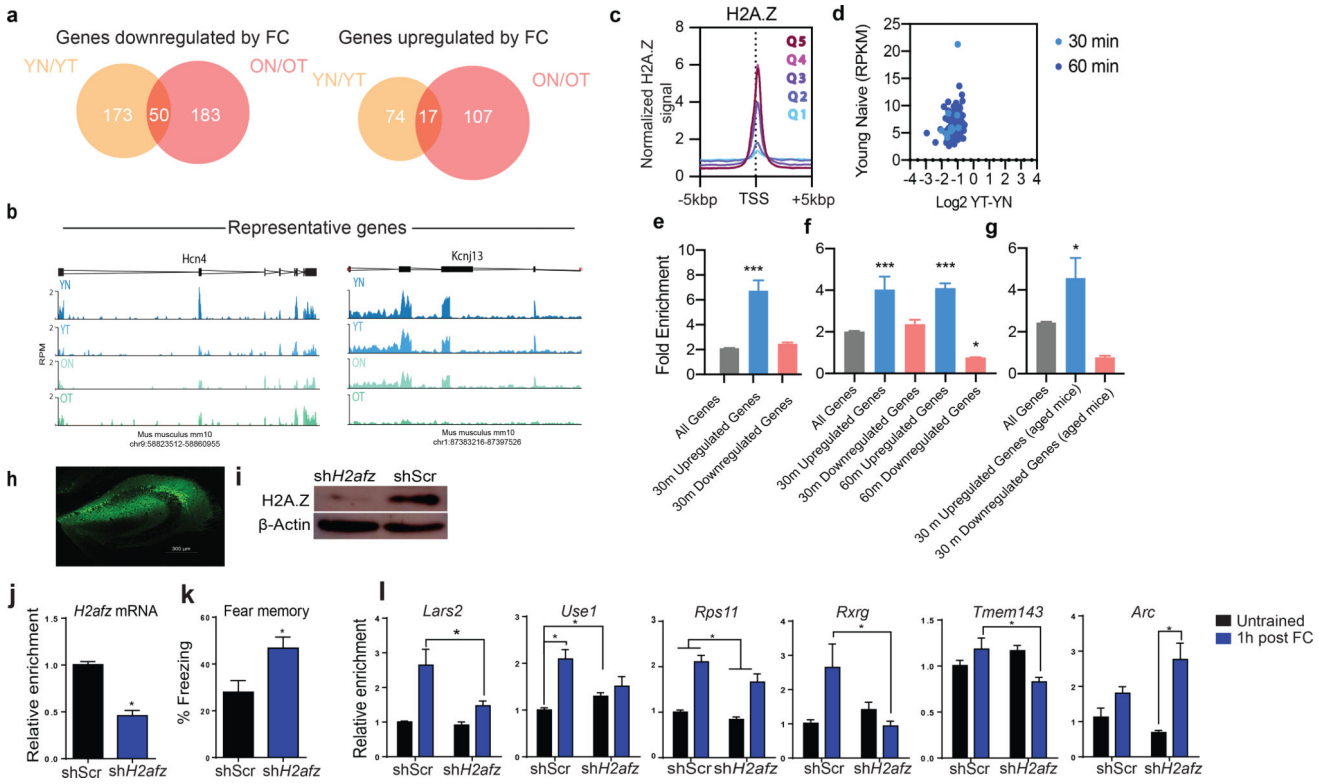

Figure 4. H2A.Z is associated with memory-related genes

(a) There was minimal overlap for learning-modified genes between young and old mice. (b) Representative genes demonstrating learning-induced changes in young, but not in old mice (Hcn4) and in old, but not in young mice (Kcnj13). (c) Genes with higher steady state expression (shown as quintiles based on expression level) had higher levels of H2A.Z binding at baseline. (d) In every case where a significant change in H2A.Z occupancy due to fear conditioning is observed at a memory gene, H2A.Z is found in lower amounts than in the naïve/untrained group. (e) As compared to all genes, H2A.Z peaks are significantly more associated with genes upregulated 30 min after fear conditioning, but not genes downregulated after fear conditioning. (f) H2A.Z peaks that are significantly changed 30 min after fear conditioning are disproportionally associated with upregulated and not with downregulated genes. The same trend is observed for memory genes upregulated 60 min after fear conditioning (data set from Kennedy et al. 2016) (g) H2A.Z peaks found to be significantly altered in old mice are also disproportionally associated with upregulated, and not with downregulated genes in old mice. (h) Representative image of AAV spread in the hippocampus. (i) Western blot confirming AAV-mediated H2A.Z depletion. (j) Expression of *H2afz* is reduced with AAV-mediated depletion, thus validating AAV-mediated knockdown. (k) AAV-mediated H2A.Z depletion enhances fear memory. (l) AAV-mediated gene-depletion results alters learning induced gene-expression 1h after fear conditioning.