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Tau promotes neurodegeneration through global chromatin relaxation

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Abstract

The microtubule-associated protein tau is involved in a number of neurodegenerative disorders, including Alzheimer's disease (AD). Previous studies link oxidative stress and subsequent DNA damage to neuronal death in AD and related tauopathies. Since DNA damage can significantly alter chromatin structure, we examined epigenetic changes in tau-induced neurodegeneration. We have found widespread loss of heterochromatin in tau transgenic *Drosophila* and mice, and in human AD. Importantly, genetic rescue of tau-induced heterochromatin loss substantially reduced neurodegeneration in *Drosophila*. We identified oxidative stress and subsequent DNA damage as a mechanistic link between transgenic tau expression and heterochromatin relaxation, and found that heterochromatin loss permits aberrant gene expression in tauopathies. Furthermore, large-scale analyses from human AD brains revealed a widespread transcriptional increase in genes that are heterochromatically silenced in controls. Our results establish heterochromatin loss as a toxic effector of tau-induced neurodegeneration, and identify chromatin structure as a potential therapeutic target in AD.

Tauopathies, including AD, are characterized pathologically by the deposition of neurofibrillary tangles composed of the microtubule-associated protein tau in the brains of affected individuals¹. The discovery of dominant mutations in the *tau* gene linked to a familial neurodegenerative disorder, frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), confirms tau as a causal factor in neurodegenerative disease pathogenesis²⁻⁴.

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AUTHOR CONTRIBUTIONS

B.F. and M.B.F. conceptualized the study. B.F. performed experiments and ChIP-seq analysis. M.H. performed human expression analysis, principal component analysis, and provided guidance for ChIP-seq analysis. J.L. provided critical reagents and contributed to research design. B.F., M.B.F., and M.H. participated in interpreting data and wrote the manuscript. M.B.F. supervised the research.

COMPETING FINANCIAL INTERESTS

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The precise mechanism of tau-induced neurotoxicity remains a critical question in tauopathy research, and understanding the molecular and cellular pathways perturbed by tau in disease states is essential for the development of effective therapies⁵. Although multiple lines of evidence implicate oxidative DNA damage and aberrant neuronal cell cycle activation in the toxic cascade triggered in tauopathies⁶⁻⁸, the mechanisms controlling the interplay between these processes in terminally differentiated neurons are unknown. Based on recovery of chromatin regulators in unbiased forward genetic screens for modifiers of tau neurotoxicity^{9,10} (M.B.F., unpublished data), and because chromatin remodeling is associated with oxidative DNA damage and cell cycle regulation^{11,12}, we hypothesized that oxidative stress-induced DNA damage causes chromatin alterations that promote cell cycle reentry in tauopathy.

An appropriate balance and distribution of active and repressed chromatin is required for proper transcriptional control, maintaining nuclear architecture and genomic stability¹³, and regulating the cell cycle¹⁴. Within the nucleus, DNA is packaged into specific chromatin domains termed euchromatin and heterochromatin. Euchromatin is generally gene rich and permissive for gene activation, while heterochromatin is relatively gene sparse and transcriptionally quiescent¹². A set of highly conserved proteins and post-translational histone modifications maintain these distinct chromatin domains along chromosomes in both insects and vertebrates. In particular, dimethylated lysine 9 of histone 3 (H3K9me2), and the heterochromatin protein 1 α (HP1 α) are enriched in constitutive telomeric and pericentromeric heterochromatin¹². The experiments described in our study establish a role for H3K9me2, HP1 α , and heterochromatin loss in tau-mediated neurodegeneration in insect and vertebrate systems.

RESULTS

Tau transgenic *Drosophila* have widespread alterations in chromatin structure

To explore an association between tau neurotoxicity and chromatin alterations, we first determined if histone methylation patterns or chromatin-associated proteins are altered in the brains of tauopathy model *Drosophila*. Pan-neuronal transgenic expression of an FTDP-17 mutant form of human tau, tau^{R406W}, in *Drosophila* causes progressive neurodegeneration and provides a well-characterized *in vivo* genetic model of tau neurotoxicity^{7,15,16}. We found that total levels of H3K9me2 and HP1 α were significantly decreased in tau transgenic *Drosophila* heads compared to controls (Fig. 1a, b). Reverse transcription followed by PCR (RT-PCR) indicated that mRNA levels of *Su(var)3-9*, which encodes the main histone methyltransferase responsible for H3K9 dimethylation, and *Su(var)205*, which encodes HP1 α , were equal between control and tau transgenic *Drosophila* heads, indicating that loss of H3K9me2 and HP1 α in tauopathy was not due to transcriptional changes in these genes (Supplementary Fig. 1a). To determine if heterochromatin loss is specific to R406W mutant tau versus a general feature of tau pathology, we utilized flies transgenic for human wild type tau (tau^{WT})¹⁵ or a pseudohyperphosphorylated form of human tau (tau^{E14})⁷. Tau phosphorylation is an important pathogenic event in AD and related tauopathies, and the tau^{E14} transgene causes significantly more toxicity in *Drosophila* than tau^{WT} or tau^{R406W}^{7,16-18}. Neuronal expression of tau^{WT} or tau^{E14} reduced H3K9me2 and HP1 α

levels (Supplementary Fig. 1b, c), indicating that heterochromatin defects are a general feature of tau-induced toxicity, and are downstream of aberrant tau phosphorylation. The extent of heterochromatin loss caused by expression of wild type or pseudohyperphosphorylated tau correlated with their respective toxicities. Since expression of tau^{R406W} provides a level of toxicity well suited for genetic manipulation and biochemical analysis, the R406W mutant tau is used primarily in our subsequent experiments, and is termed “tau” hereafter for simplicity. To visualize heterochromatin structure directly, we stained control and tau transgenic brain sections with antibodies recognizing H3K9me2 or HP1 α . Nuclei of control brains contained prominent H3K9me2- and HP1 α -rich heterochromatic foci, termed chromocenters. Tau transgenic *Drosophila*, however, had significantly reduced chromocenter labeling, and instead had diffusely distributed H3K9me2 and HP1 α (Fig. 1c, d, Supplementary Fig. 1d).

The highly condensed packaging of heterochromatic DNA renders it less accessible to polymerases and transcription factors, and genes embedded within heterochromatin are generally less highly transcribed than genes located in euchromatin. To determine if chromatin relaxation permits expression of genes normally silenced by heterochromatin, we used *Drosophila* lines transgenic for either *LacZ* (BL2 reporter¹⁹) or *GFP* (Y10C reporter²⁰) embedded in and silenced by heterochromatin. These reporters were silenced in controls, but were expressed in tau transgenic *Drosophila* brains (Fig. 1e). Transgenic expression of tau^{WT} or tau^{E14} also activated reporter expression in *Drosophila* (Supplementary Fig. 1e). 80% of β -galactosidase positive foci in tau transgenic *Drosophila* were also positive for a disease-associated tau phosphoepitope (AT8) (Fig. 1f) and 70% of β -galactosidase positive foci were positive for cleaved PARP, a marker of caspase activation²¹ (Fig. 1g). A number of studies in this model of tauopathy have demonstrated that reentry of postmitotic neurons into the cell cycle is a mechanism whereby tau promotes neurotoxicity^{7,8,16}. Accordingly, we found that 60% of β -galactosidase positive foci in tau transgenic *Drosophila* were also positive for proliferating cell nuclear antigen (PCNA) (Fig. 1h), an S phase marker of cell cycle activation. Together, these findings suggest that tau-induced heterochromatin loss permits aberrant gene expression that is coincident with tau neurotoxicity, cell cycle reentry, and subsequent apoptosis of postmitotic neurons.

Genetic manipulation of chromatin structure modifies tau neurotoxicity

To determine if heterochromatin loss directly mediates cell death in our tauopathy model, we first asked if restoring heterochromatin suppresses tau-induced toxicity. We stimulated heterochromatin formation in tau transgenic *Drosophila* by genetically manipulating genes that promote euchromatin. Ash1 is a histone methyltransferase that promotes euchromatin formation and transcriptional activation by methylating histones such as H3K4 and H3K36²². The nucleosome remodeling factor (NURF) complex catalyzes ATP-dependent nucleosome sliding and is required for transcriptional activation²³. In the context of transgenic tau expression, loss of ash1 or NURF complex function significantly increased heterochromatin based on several assays. Total H3K9me2 and HP1 α levels (Fig. 2a, b, Supplementary Fig. 1f, g) and H3K9me2 and HP1 α chromocenter labeling (Fig. 2c) were significantly increased in tau transgenic flies harboring a loss of function mutation in *ash1* or RNAi targeted to *NURF301*. Furthermore, loss of ash1 function or RNAi-mediated

reduction of *NURF301* significantly silenced expression of the heterochromatin-embedded reporter in tau transgenic flies (Fig. 2d). We found that genetically restoring heterochromatin in tau transgenic flies suppressed neuronal apoptosis (Fig. 2e) and reduced the number of PCNA positive foci (Fig. 2f) by approximately 50%, directly implicating heterochromatin loss as a cause of cell cycle activation and neuronal death in tauopathy. RNAi transgenes targeted to *ash1*, *NURF301*, and *NURF38* were not toxic in the absence of tau, nor was loss of *ash1* function. To assess the overall health of tau transgenic *Drosophila* in which heterochromatin is genetically restored, we measured the locomotor activity of control and transgenic flies. Tau transgenic *Drosophila* had a significant decrease in locomotor activity at ten days of age compared to control, and this locomotor deficit was significantly rescued by RNAi-mediated reduction of *ash1* or *NURF301* (Fig. 2g). Tau transgenic *Drosophila* had similar mRNA levels of *ash1*, *NURF301*, and *NURF38* compared to control, suggesting that tau did not cause heterochromatin changes by altering expression of these genetic modifiers (Supplementary Fig. 1h). We used RT-PCR to confirm knockdown of *ash1*, *NURF301*, and *NURF38* mRNA levels in heads of transgenic flies (Supplementary Fig. 1i).

We next determined if further relaxation of heterochromatin enhances tau neurotoxicity. We reduced H3K9me2 and HP1 α levels by expressing tau with heterozygous loss-of-function alleles of *Su(var)3-9* or *Su(var)205*. In the context of transgenic human tau, mutation of *Su(var)3-9* or *Su(var)205* further reduced H3K9me2 and HP1 α levels (Fig. 2h, i, Supplementary Fig. 1j, k), caused additional loss of H3K9me2 and HP1 α chromocenter labeling (Fig. 2j), and increased expression of the heterochromatin-embedded reporter (Fig. 2k) compared to tau expressed alone, indicating that these genetic manipulations exacerbate tau-induced heterochromatin loss. Tauopathy model flies harboring mutations in *Su(var)3-9* and *Su(var)205* had double the levels of neuronal apoptosis (Fig. 2l) and PCNA positive foci (Fig. 2m) compared to tau expressed alone. Importantly, mutant *Su(var)3-9* and *Su(var)205* transgenes were not toxic in the absence of tau. Loss of function mutations in *Su(var)205* or *Su(var)3-9* also significantly enhanced the locomotor deficits of tau transgenic *Drosophila* (Fig. 2n). In total, these genetic data imply that heterochromatin loss plays a causal role in tau-induced neurotoxicity *in vivo*.

A major pathological feature of AD and other tauopathies is the accumulation of abnormally phosphorylated forms of tau in the brains of affected individuals¹. Genetically manipulating chromatin in the context of transgenic tau did not alter tau levels or the levels of disease-associated tau phosphoepitopes PHF1, AT270, or AT8 (Supplementary Fig. 1l), despite significant modification of tau-induced toxicity. These results suggest that heterochromatin loss in tau transgenic *Drosophila* is downstream of tau phosphorylation, and that suppression or enhancement of tau toxicity is not simply a result of altered tau expression.

Oxidative stress promotes DNA damage and heterochromatin loss

We next considered mechanisms whereby tau induces heterochromatin loss. Oxidative DNA damage and activation of the cell cycle are known to promote chromatin remodeling^{11,12}, are present in human AD brains^{24,25}, and are critical components of tau-induced neurodegeneration in our *Drosophila* model of tauopathy^{7,8,16}. We first determined if

oxidative stress causes DNA damage and heterochromatin loss in *Drosophila*. We genetically induced oxidative stress in *Drosophila* using either a strong loss-of-function mutation in *Thioredoxin reductase-1* (*Trxr-1⁴⁸¹*)²⁶ or *Superoxide dismutase 2* (*Sod2⁰²*)²⁷. We have previously shown that loss of *Trxr-1* or *Sod2* function enhances tau neurotoxicity¹⁶. We assessed DNA damage in *Trxr-1⁴⁸¹* and *Sod2⁰²* mutant flies based on phosphorylation of Ser-139 of the histone variant H2Av (pH2Av), a marker of DNA double strand breaks. Immunofluorescence indicated that pH2Av was present in the brains of *Trxr-1⁴⁸¹* and *Sod2⁰²* mutant flies (Fig. 3a, b), but not in controls. As a second measure of DNA damage, we performed the comet assay, which utilizes single-cell gel electrophoresis to quantify DNA single and double strand breaks. We observed an increase in the percent of DNA in comet tails in *Trxr-1* and *Sod2* mutant brains, confirming that these genetic manipulations damage DNA (Fig. 3c, d). Since DNA damage can cause widespread heterochromatin loss^{28,29}, and reduced *Trxr-1* or *Sod2* function causes DNA damage, we asked if loss of *Trxr-1* or *Sod2* function causes heterochromatin relaxation. Indeed, whole head homogenates from *Trxr-1⁴⁸¹* and *Sod2⁰²* flies had reduced levels of H3K9me2 and HP1 α (Fig. 3e, f), and increased activation of the heterochromatin-embedded reporter in the brain (Fig. 3g, h), supporting the idea that oxidative stress can cause heterochromatin loss in tauopathy.

Since tau is known to aberrantly activate the cell cycle in neurons⁷, we next determined if neuronal cell cycle activation is upstream of heterochromatin loss. Neuronal overexpression of *CycA* or *Rheb*, two proteins known to promote the cell cycle, did not reduce H3K9me2 or HP1 α levels (Fig. 3i, j), or activate the heterochromatin-embedded reporter (data not shown), suggesting that cell cycle activation does not cause widespread heterochromatin loss. Because cell cycle activation in postmitotic neurons causes apoptosis in *Drosophila*⁷, these data also indicate that heterochromatin loss is not a result of general cellular toxicity. We next determined if cell cycle activation is downstream of heterochromatin loss. Brains of *Drosophila* with RNAi-mediated neuronal knockdown of *Su(var)205* or *Su(var)3-9* were positive for PCNA (Fig. 3k), suggesting that heterochromatin loss is indeed upstream of aberrant cell cycle activation in neurons. We used RT-PCR to confirm RNAi-mediated knockdown of *Su(var)205* and *Su(var)3-9* (Supplementary Fig. 2a). Taken together, these data place oxidative stress-induced DNA damage upstream of heterochromatin loss in a sequence of cellular events that lead to aberrant cell cycle activation and subsequent neuronal apoptosis.

Heterochromatin loss permits aberrant gene expression in tau transgenic *Drosophila*

To identify specific genes with reduced H3K9me2 levels, we performed H3K9me2-based ChIP-sequencing (ChIP-seq) in biological duplicate (Fig. 4a, <http://genome.ucsc.edu>³⁰), and validated significantly H3K9me2 depleted genes with quantitative PCR (qPCR) (Fig. 4b). Given the known role of H3K9me2 in gene silencing, we expected that H3K9me2 loss at these loci would correlate with increased gene expression. Six of the genes (*Ago3*, *CG15115*, *CG15661*, *Ir41a*, *nvd*, and *uif*) were significantly upregulated in tau transgenic flies (Fig. 4c), as predicted. However, expression of the remaining five genes (*CG40006*, *Dscam*, *Gprk1*, *IntS3*, and *Snap25*) was unaffected by heterochromatin loss. These five genes are classified as moderately to highly expressed (modEncode Project³¹) in *Drosophila*

heads despite their heterochromatic environment, while the six genes with increased expression in tau transgenic *Drosophila* are classified as heterochromatically silenced, with no to low baseline expression in *Drosophila* heads (Fig. 4c). These data suggest that baseline levels of gene transcription control how a gene embedded within heterochromatin will respond to heterochromatin loss. Thus, tau-induced heterochromatin loss only affects the expression of genes that are normally transcriptionally silenced by heterochromatin. Of the six genes that are both H3K9me2 depleted and transcribed at higher levels in tau transgenic *Drosophila*, two have human homologs. *Ago3* is homologous to the human gene *PIWIL1*, and *CG15661* is homologous to the human gene *UGT1A10*.

We next determined if aberrant gene expression resulting from heterochromatin loss contributes significantly to tau-induced toxicity. As a proof of principle, we focused on *Ago3* due to its well-conserved human homolog and intriguing biological function. *Ago3* regulates PIWI-associated RNAs (piRNAs)³², a highly conserved class of small RNAs that are required for post-transcriptional gene silencing of transposons^{33,34}. Consistent with increased transcription, *Ago3* protein levels were increased over 4-fold in tau transgenic *Drosophila* heads compared to control (Fig. 4d, e). To determine if *Ago3* overexpression directly contributes to tau neurotoxicity, we reduced *Ago3* levels in tau transgenic *Drosophila* using RNAi. We found significantly fewer apoptotic and PCNA-positive cells in tau transgenic *Drosophila* brains expressing either of two nonoverlapping RNAi lines targeted to *Ago3* (Fig. 4f, g). RNAi mediated reduction of *Ago3* also significantly suppressed the locomotor defects of tau transgenic *Drosophila* (Fig. 4h). Transgenic RNAi targeted to *Ago3* did not affect levels of transgenic tau or tau phosphorylation (Supplementary Fig. 2b), indicating that suppression of tau-induced toxicity was not due to decreased tau expression or alterations in tau phosphorylation. RT-PCR confirmed RNAi-mediated knockdown of *Ago3* in the brains of transgenic flies (Supplementary Fig. 2c). These data strongly suggest that aberrant gene expression resulting from heterochromatin relaxation is a toxic mediator of tauopathy.

Heterochromatin loss in vertebrate tauopathies

Drosophila models of disease are valuable tools for generating and genetically testing hypotheses that can then be validated in higher organisms. We thus determined if heterochromatin loss is a conserved mechanism of tau-induced neurotoxicity. JNPL3 mice express human tau carrying the FTDP-17 mutation P301L (tau^{P301L}). These tau transgenic mice undergo progressive neurodegeneration, and develop neurofibrillary tangles in the brain and spinal cord³⁵. We investigated H3K9me2 and HP1 α changes specifically in motor neurons, since they are a specifically vulnerable cell type in the JNPL3 model. In control mice, H3K9me2 and HP1 α were concentrated in chromocenters at the nucleolar periphery. In tauopathy mice, however, ring-like perinucleolar chromocenter staining was significantly reduced for both H3K9me2 and HP1 α (Fig. 5a-c), similar to our observations in tau transgenic *Drosophila*. We next asked if the aberrant expression of *Ago3* present in tau transgenic *Drosophila* is conserved for its mammalian homolog, *PIWIL1*. While *PIWIL1* levels were very low in control mice, we observed approximately double the levels of *PIWIL1* staining in motor neurons of tau transgenic mice (Fig. 5d). These data suggest that

heterochromatin loss and aberrant gene expression are features of tauopathy that are conserved between *Drosophila* and mice.

The ultimate goal of our research is to identify mechanisms of tau-induced toxicity in human disease. Having demonstrated heterochromatin loss and aberrant gene expression in *Drosophila* and mouse tauopathy models, we next investigated heterochromatin in AD, the most common human tauopathy. We first used immunostaining to determine if there are significant differences in H3K9me2, HP1 α , and PIWIL1 between human control and AD brains. In pyramidal hippocampal neurons from postmortem control brains, H3K9me2 was concentrated in perinucleolar chromocenters. In AD, however, H3K9me2 staining was diffuse (Fig. 6a), similar to our observations in tau transgenic *Drosophila* and mice. For HP1 α , diffuse staining was restricted to pyramidal neurons in AD brains that were positive for a disease-associated tau phosphoepitope (AT8), consistent with a link between tau toxicity and HP1 α loss (Supplementary Fig. 3a). Immunofluorescence also revealed a widespread increase in PIWIL1 staining in AD hippocampal neurons (Fig. 6b). We next used quantitative methods to validate the loss of H3K9me2 and increase of PIWIL1 levels in AD in age-, gender-, and postmortem interval-matched cases and controls. Neurons outnumber glia ten to one in *Drosophila*³⁶. In humans, however, the reverse is true with glia being significantly more abundant than neurons. To eliminate the confounding effects of glia in humans, we purified nuclei from postmortem brain tissue and separated neuronal and glial nuclei via Fluorescence Activated Cell Sorting (FACS) (Fig. 6c). Western blots with neuron- (NeuN) and astrocyte-specific (S100 β) antibodies confirmed the purity of each nuclear population (Fig. 6d). We found that H3K9me2 was significantly depleted in human AD neurons (Fig. 6e, f). Similarly, RT-PCR of *PIWIL1* in FACS-purified neuronal nuclei showed significantly increased levels in human AD neurons compared to controls. The geometric mean of *PIWIL1* levels relative to the endogenous control gene *CYC1* were 0.054 (s.e.m. = 0.022) in control brains, versus 0.216 (s.e.m. = 0.088) in AD brains (n = 6 brains, p < 0.05 Wilcoxon Rank-Sum).

Having established that heterochromatin loss and *PIWIL1* overexpression are conserved from our *Drosophila* model to human AD, we took a global approach to determine if heterochromatically silenced genes in human control brains are expressed at higher levels in AD brains. Based upon our findings in *Drosophila*, we predicted that heterochromatin loss in AD permits expression of genes that are heterochromatically silenced in control brains. Such changes most likely would not be detected in traditional analyses of gene expression because these analyses are often focused on genes with higher expression levels. In order to classify genes as heterochromatic or euchromatic, we utilized ChIP-seq data sets for all six publicly available histone modifications in the human hippocampus: H3K4me1, H3K4me3, H3K36me3, H3K9me3, H3K27me3, and H3K9ac (NIH Epigenomics Roadmap Project³⁷). We used ChromHMM³⁸ (default settings), along with data from these six histone modifications in the adult substantia nigra, midfrontal lobe, liver, and fetal brain to define five different chromatin states (Supplementary Fig. 3b). Based upon the presence of state three in known heterochromatic regions and the predominance of H3K9me3 and H3K27me3 in state four, we assigned states three and four as heterochromatic. Supplementary Dataset 1 provides a list of chromatin states throughout the human genome as a file that can be

uploaded into genome browsers. To compare expression levels of heterochromatic and euchromatic genes between control and AD, we used publicly available microarray data from laser-captured hippocampal neurons³⁹. We used the present/absent detection call for each microarray probe to assign high or low gene expression for heterochromatic and euchromatic genes. We considered a gene highly expressed if it was called as present in at least 80% of the control and AD samples. Notably, over one third of the genes that are heterochromatic and expressed at low levels in control hippocampal neurons were expressed at least 50% higher in AD, including *PIWILI* and *UGT1A10* (Fig. 6g, genes listed in Supplementary Dataset 2), while only 13% of euchromatic genes with low expression were expressed at least 50% higher in AD (Fig. 6h). The chi-square test indicated that the most significant transcriptional increase in AD was for genes classified as heterochromatic and expressed at low levels in control (Fig. 6i). Significant large-scale gene expression changes were not observed in an unrelated liver disease (Supplementary Fig. 3c, d), suggesting that our results do not represent nonspecific effects of cellular toxicity.

Cellular differentiation is accompanied by a marked transition from a permissive chromatin state to a restrictive state that is rich in heterochromatin⁴⁰. We hypothesized that heterochromatin relaxation in AD causes a shift in gene expression back towards a dedifferentiated state. Indeed, the list of genes with increased expression in AD reveals pluripotency-associated genes such as *POU1F1*, *NOG*, and *NR5A2*. Pathway analysis using the PANTHER Classification System indicated that genes associated with developmental processes were significantly overrepresented in the list of genes that were heterochromatically silenced in controls and expressed at least 50% higher in AD (Supplementary Table 1). We performed principal component analysis, a common technique for identifying patterns in complex datasets, to determine if the gene expression profile in AD has similarities to fetal brain. We compared gene expression profiles of fetal brains, human control hippocampal neurons, hippocampal neurons from AD, and whole hippocampal tissue from control and three stages of AD. The first principal component that could not be trivially explained by different experimental conditions is the fourth, which assigned AD hippocampal neurons, severe AD hippocampi, and fetal brains to the same principal component (Supplementary Fig. 3e). These analyses indicate that the expression profile of fetal brains is more similar to AD hippocampi and hippocampal neurons than to control hippocampi or hippocampal neurons, consistent with a major global shift in gene expression in AD toward a more fetal state.

Given that gene expression signatures in neurodegenerative diseases can be conserved from brain to peripheral tissues⁴¹, we next compared expression levels of heterochromatic and euchromatic genes between control and AD peripheral blood mononuclear cells (Supplementary Fig. 3f, g). Similar to AD brain, 34.3% of genes that are heterochromatically silenced in control peripheral blood mononuclear cells were expressed at least 50% higher in AD (genes listed in Supplementary Dataset 3), while only 17.0% of silenced genes in euchromatin were expressed at least 50% higher in AD (Supplementary Fig. 3h), suggesting that changes in heterochromatin present in AD brain are also present in peripheral blood.

DISCUSSION

We have found that loss of normal heterochromatin promotes tau-mediated neurodegeneration *in vivo*. Our current data, together with previous work from our laboratory and others, support a working model in which aberrant tau phosphorylation and/or aggregation is caused by amyloid β ⁴², mutations in the *tau* gene²⁻⁴, pathologic tau transfer⁴³, or as yet uncharacterized mechanisms. Pathologic tau causes an increase in actin stabilization¹⁸ that inhibits association of the fission protein Drp1 with mitochondria, leading to mitochondrial elongation and the production of reactive oxygen species⁴⁴. We have found that oxidative stress causes DNA damage, heterochromatin loss and consequent aberrant gene expression in *Drosophila*. In cultured rat embryonic fibroblasts and in the adult rat liver, metabolic stress induced by caloric restriction increases Suv39h1, the mammalian homolog of Su(var)3-9⁴⁵. These data raise the possibility that the stress and DNA damage control mechanisms differ in mitotic cells and postmitotic neurons. There is an extensive and evolving literature that documents mechanisms of DNA-damage induced chromatin relaxation, and we have previously implicated a number of proteins that regulate this process in the pathogenesis of tauopathy, including pH2Ax, ATM and p53⁸. Our data suggest that oxidative stress-induced DNA damage is one mechanism whereby pathologic tau causes global heterochromatin relaxation; however, further work may reveal additional mechanisms of tau-induced heterochromatin loss. *In vitro* and in cultured cells, tau binds the minor groove of the DNA double helix and is thought to protect DNA from oxidative damage⁴⁶⁻⁴⁸. Our findings do not preclude a possible role for nuclear tau in the pathway of heterochromatin loss, as it is possible that tau dissociates from DNA and renders it more sensitive to reactive oxygen species caused by pathogenic tau in the cytoplasm. Our data suggest that DNA-damage induced heterochromatin loss causes a global shift in gene expression towards a more developmental state that results from neuronal dedifferentiation, triggering inappropriate cell cycle reentry and subsequent apoptosis (Supplementary Fig. 4). Since oxidative stress caused heterochromatin loss in our *Drosophila* tauopathy model, our findings raise the possibility that heterochromatin relaxation and subsequent aberrant gene expression are relevant to other neurodegenerative diseases linked to oxidative stress.

Despite spatial obstacles, many genes embedded within heterochromatin are actively transcribed. Notably, we found that only those genes with no to low baseline levels of transcription in controls are upregulated in tau transgenic *Drosophila* and in human AD. This observation may have implications for understanding chromatin biology, since it suggests that heterochromatically silenced genes are transcriptionally more sensitive than active genes to changes in the local chromatin environment. Although we focus our experiments here on protein coding loci, heterochromatin loss likely permits increased expression of non-protein coding RNA transcripts. Indeed, increased expression of a regulatory RNA has already been implicated in the pathogenesis of AD⁴⁹.

Heterochromatin loss and aberrant gene expression are conserved among *Drosophila*, mouse, and human tauopathy, emphasizing the potential clinical relevance of these data. Within a group of genes with significant H3K9me2 loss in tau transgenic *Drosophila*, we focused specifically on *Ago3*, the *Drosophila* homolog of human *PIWIL1*. *Ago3* regulates PIWI-interacting RNAs, or piRNAs, a large class of highly conserved small noncoding

RNAs that are responsible for silencing transposons^{33,34}. Genetic reduction of Ago3 suppressed tau neurotoxicity in *Drosophila*, and Ago3/PIWIL1 was overexpressed in tau transgenic *Drosophila* and mice, and in human AD. These data suggest that Ago3/PIWIL1-mediated piRNA biogenesis and subsequent transposable element control are deregulated in tauopathy. In addition, components of the piRNA pathway are associated with stem cell self-renewal and are aberrantly expressed in several types of somatic tumors⁵⁰, suggesting that dysregulation of the piRNA pathway leads to loss of proliferative control. PIWIL1, and possibly other aberrantly transcribed coding and noncoding loci, represent novel targets for therapeutic intervention in AD. Furthermore, widespread changes in heterochromatic gene expression are conserved in the blood as well as the brain in patients with AD, identifying a new class of candidate biomarkers.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes

The raw sequence data obtained by ChIP-seq are available at NCBI GEO under series accession number GSE53719.

ONLINE METHODS

Genetics and Animal Models

Drosophila melanogaster crosses and aging were performed at 25°C. Flies expressing tau^{R406W}, tau^{WT} and tau^{E14} have been described previously^{7,15,16}. The pan-neuronal *elav-GAL4* driver was used to drive tau and RNAi transgene expression. The following *Drosophila* lines were obtained from the Bloomington *Drosophila* Stock Center: *Sod2*⁰², *ash1*^{B1}, *ash1*^{JF01498}, *NURF38*^{JF01299}, *NURF301*^{JF01709}, *Su(var)205*⁵, *Su(var)205*^{MB11439}, *Su(var)3-9*¹, *Su(var)3-9*², *Ago3*^{GL00117}, *Ago3*^{HMS00125}. *Su(var)3-9* and *Su(var)205* RNAi lines were obtained from the Vienna *Drosophila* RNAi center (lines number 39377 and 31995). The following investigators kindly contributed fly stocks: BL2 chromatin reporter, J. Eissenberg; Y10C chromatin reporter, K. Maggert; *Trxr-1*⁴⁸¹, F. Missirlis; UAS-CycA, C. Lehner; UAS-Rheb, P. Gallant. JNPL3 mice (*Mus musculus*) were originally developed on a C57BL/DBA2/SW background. JNPL3 mice are housed 2-5 per cage in a vivarium with a 12 hour off/on light/dark cycle and are used compliance with ethical guidelines and regulations. These mice underwent non-invasive behavioral testing to assess motor function and disease progression, including: assessment of escape extension during tail elevation, the righting reflex when turned on their backs, and the ability to grasp and hang from a rope during rope hang tests. Spinal cords of 8.5-14 month old JNPL3 tau transgenic mice (five males and one female) and nontransgenic control littermates were analyzed (three males and three females).

Immunostaining

Antibodies are summarized in Supplementary Table 2. Formalin-fixed, paraffin-embedded sections from *Drosophila* heads, mouse spinal cord, or human brain were used for

immunostaining experiments. Sodium citrate-based antigen retrieval was performed prior to staining. For cPARP, pH2Av, and elav staining, *Drosophila* brains were dissected in PBS and fixed in methanol for 20 minutes prior to staining. A commercially available kit was used for TUNEL staining (Calbiochem, TdT FragEL). β -galactosidase, TUNEL, and PCNA-positive nuclei were counted throughout the entire brain. Secondary antibodies were fluorescent except in the case of H3K9me2 and HP1 α staining in Supplementary Fig. 1d, and quantification of β -galactosidase, TUNEL and PCNA, in which secondary detection was performed with DAB. Brains were imaged on a Nikon Eclipse E600 fluorescent microscope with SPOT software or a Zeiss LSM 510 META upright confocal microscope. Images were analyzed with ImageJ. To quantify chromocenters in *Drosophila*, 100 nuclei were counted in three brains per genotype and scored for presence or absence of H3K9me2 or HP1 α chromocenters. For quantification of experiments in control and JNPL3 mice, all motor neurons in spinal cord sections were counted and scored for presence or absence of H3K9me2 or HP1 α perinucleolar staining. For quantification of PIWIL1 immunostaining, ImageJ was used to measure PIWIL1 fluorescence intensity in motor neurons from at least five nonoverlapping microscopic fields at 40X.

Western blotting

Frozen *Drosophila* heads were homogenized in Laemmli sample buffer (Sigma), boiled for 10 minutes, and analyzed by 15% SDS-PAGE (Lonza). While whole heads contain a limited number of nuclei of non-nervous system origin, including epithelial- and muscle-derived nuclei, the simple external exoskeleton structure of the fly results in head preparations composed of primarily nervous system tissue. For western blots on neuronal nuclei from postmortem tissue, 150,000 sorted nuclei were loaded per lane.

Locomotor behavioral assay

Flies were collected in fresh vials on the day of eclosion and were transferred without anesthesia to fresh vials on days 3, 5, 7, and 9. The locomotor assay was performed in the afternoon of day 10. To count the number of centimeters walked in 30 seconds, a single fly was tapped gently to the same initial starting position within its vial and the vial was placed over a gridded surface. The number of centimeters traversed was counted four times per fly, and 18 flies of each genotype were assayed.

Comet

Two brains per trial were dissected from adult flies in PBS, homogenized with a plastic pestle and subjected to comet using commercially available reagents (CometAssay, Trevigen). Fifty nuclei were quantified per trail using the ImageJ plugin Comet_Assay.

Chromatin immunoprecipitation and sequencing

Chromatin was prepared⁵¹ and immunoprecipitated⁵² as described with the following modifications. The heads of 2 g of male flies were ground in liquid nitrogen with a mortar and pestle prior to crosslinking with formaldehyde. Chromatin was sheared to 500 base pairs by fifteen 30 second bursts of sonication at 100% duty and level 5 power with a Branson Sonifier 450. IgG was used as a negative control. Libraries were prepared using the

NEBNext ChIP-Seq Library Prep Reagent Set for Illumina according to the manufacturer's recommendations. DNA quality was measured at the Harvard Biopolymers Facility on an Agilent 2100 Bioanalyzer with a high sensitivity chip.

ChIP-seq Analysis

Input and H3K9me2-ChIPped DNA was sequenced using an Illumina platform with a sequencing read length of 35 base pairs (Elim Biopharm). Experiments were carried out in biological duplicates. The number of reads for each experiment is summarized in Supplementary Table 3. Reads were aligned to the *Drosophila* genome using Bowtie⁵³ with the command:

```
bowtie -tS -n 2 -l 28 -e 70 -p 1 dmel_genome.fasta file.txt file.sam
```

To identify genomic regions with reduced H3K9me2 levels in tau transgenic *Drosophila*, we developed a trough-finding algorithm. Conceptually, detecting a reduction in the amount of binding is similar to the widely studied problem of finding enriched regions, or “peak-calling”.

As a pre-processing step, we first extended each read to 350 base pairs since that is the average size of the ChIPped DNA fragment that was sequenced. The total read count was then normalized to 10,000,000 reads for each sample. Next, we subtracted the input from the ChIP for both tau and control experiments

$$\text{input_normalized_ChIP} = \text{ChIP} - \alpha \times \text{input}$$

where α is chosen such that the absolute value of the covariance between input and input_normalized_ChIP is minimized

$$\alpha = \text{argmin Cov}[\text{ChIP} - \alpha \times \text{input}, \text{input}]$$

This procedure ensures that the input is minimally informative about the normalized ChIP. We refer to these as the normalized, input subtracted data, and we label them t and c for tau and control, respectively.

Next, we search for regions where t is significantly lower than c by scanning the genome with a window of size W base pairs that is moved using a step-size of w base pairs. For each window we compare the number of reads in the two samples, and if the difference, $Z = c - t$, is larger than a critical value, z^* , the window is considered significant. To test if a region is de-enriched, we used the null hypothesis that the normalized read counts for both tau and control follow normal distributions with mean and variance calculated based on the sample average and sample variance from all windows. The critical value, z^* , is determined using a false-discovery rate approach. Since the distribution of reads is different for heterochromatic and euchromatic regions, we ran the algorithm for the two domains separately with different

parameters for the null distribution. Euchromatic and heterochromatic regions were defined as by GenBank. Below is an outline of our algorithm in pseudo-code:

```
Pseudo-code for trough finding

reduced_regions ← ∅
genes_affected ← ∅
for all windows  $w_i$ 
do
 $z_i \leftarrow e_i - t_i$ 
if  $z_i < z^*$  then
add_item(reduced_regions,  $w_i$ )
gene ← ∅ find_overlapping_gene( $w_i$ )
if gene ≠ ∅ then
add_item(genes_affected, gene)
end if
end if
end for
```

The method `add_item` takes a list and an element as arguments and adds the element to the list if it is not already present. The method `find_overlapping_gene` takes a region of the *Drosophila* genome as input and returns the list of genes where at least one base pair overlaps the region. If no genes overlap the region, the empty set is returned. Our final list used an FDR of 0.1, a window size of 1,000 base pairs and a step size of 100 base pairs. For the first experiment, we found 2,456 significant regions covering 167 different genes, and for the biological replicate we found 143 significant regions covering 22 different genes. There were eleven genes that were identified as significant in both experiments, and those were selected for further validation as described in the main text.

Quantitative PCR

H3K9me2 ChIP was performed on heads from 200 mg of whole *Drosophila* as described above. SYBR Green (Life Technologies) based qPCR was performed on an Applied Biosystems StepOnePlus Real-Time PCR System (primers listed in Supplementary Table 4). For reverse transcription, RNA was extracted from 10 *Drosophila* heads using QIAzol (Qiagen), or from sorted NeuN positive human neuronal nuclei. RNA concentrations were measured with a Nanodrop ND-1000 Spectrophotometer. Equal amounts of RNA were reverse transcribed using a cDNA Reserve Transcription Kit (Applied Biosystems), followed by qPCR (primers listed in Supplementary Table 4). Each data point is the result of at least three biological replicates each composed of three technical replicates. *RpL32* is the internal control gene for *Drosophila* RT-PCR. *EIFA4* and *CYCI* were selected as internal controls for human RT-PCR based upon their stability in postmortem brain of AD patients⁵⁴. Similar results were obtained with both internal controls.

Human brain analyses

For FACS analysis, human control and AD brains with a postmortem interval of less than 24 hours were collected through the autopsy service at Brigham and Women's Hospital under the appropriate IRB. Control brains included one male and five females, Braak stages I/II, with a median age of 70.5 and an age range of 65-80 years. AD brains were from one male and five females, Braak stages V/VI, with a median age of 74.5 and an age range of 69-81 years. Nuclei were extracted and labeled with NeuN as described previously⁵⁵. Nuclei were sorted at the Children's Hospital of Boston Flow Cytometry Research Facility on a BD Biosciences FACSAria Cell Sorter with BDFACS Diva software.

For large-scale expression analysis of human brain, we identified chromatin states as described in the main text. For blood, histone modifications in CD4+ cells were used to identify chromatin states. We used publicly available microarray data (GSE28146³⁹, GSE16593⁵⁶, GSE4226⁵⁷) and g:profiler⁵⁸ to map the microarray probes to genes. For genes with multiple probes, we calculated the geometric mean to associate one expression value with each gene. If the 5' transcription start site of a gene overlapped one of the two heterochromatic states the gene was considered heterochromatic, otherwise it was considered euchromatic. For hippocampal neurons, 4,412 genes represented on the microarray were assigned to heterochromatin and 12,976 were assigned to euchromatin. For liver, 4,164 genes were assigned to heterochromatin and 13,214 were assigned to euchromatin. For blood, 1,474 genes were assigned to heterochromatin and 5,893 were assigned to euchromatin. Present/absent microarray calls were not available for the liver, so we set an arbitrary threshold and required that the mean of the log₂ expression exceeded three in either sample.

Principal component analysis of gene expression from multiple brain samples

Using microarray data sets from hippocampal neurons from control and AD brain, whole hippocampi of control brains and brains with different stages of AD (GSE1297³⁹), and whole fetal brain (GSE30803⁵⁹), we defined heterochromatic genes as described above. Since different array platforms were used for the samples, we only considered the 2,844 heterochromatic genes that were present on both platforms. To compare the expression levels between different samples, we first standardized the expression level for each sample by subtracting the mean and dividing by the standard deviation. We then calculated the principal components for the $g \times s$ matrix Y , where g represents the 2,844 genes and s represents the 7 different tissues. We next determined how the coefficients of the 'eigentissues' separated the different samples. We based similarity on whether each tissue was assigned a positive or negative coefficient for each principal component. The first component has positive coefficients for all samples, suggesting that it does not provide much relevant information. The second and third components group tissues from the same experiments, suggesting that a large degree of the variance is due to different experimental conditions. The first component that cannot be trivially explained by different experimental conditions is the fourth. It assigns AD hippocampal neurons, severe AD hippocampi, and fetal brains to the same category, suggesting that gene expression in these tissues is more similar than the other tissues.

Statistical Analysis

All n's reported are biological replicates. No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported in previous publications^{7,16,44}. For experiments in mice and human tissue, sample sizes were based on power analysis calculations, which demonstrated that a 30% difference between 6 cases and controls will achieve a power of 80%. Variance is similar between groups that are being compared. Data collection and analysis were not routinely performed blind to the conditions of the experiments. Data were collected and processed side by side in randomized order for all experiments. Data distribution was assumed to be normal but this was not formally tested except for ChIP-seq analysis. Statistical analysis was performed using a one-way ANOVA when making multiple comparisons, and an unpaired student's t-test when making comparisons between two samples. The Wilcoxon rank-sum test was used for statistical analysis of RT-PCR from human NeuN-purified nuclei. This non-parametric test is the most appropriate to use when the underlying distribution is unknown and the sample size is small. The chi-square test was used for statistical analysis of microarray data. We tested for independence between both expression and chromatin states with a large sample size, and thus fulfilled the criteria for this non-parametric test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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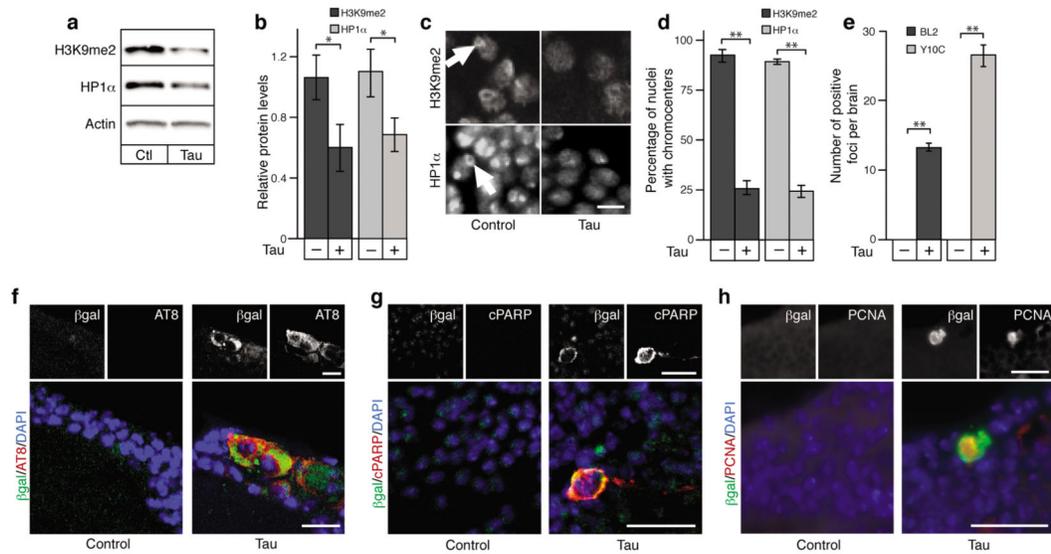


Figure 1.

Tau transgenic *Drosophila* have widespread alterations in chromatin structure. **(a)** H3K9me2 and HP1α levels in control and tau transgenic fly head homogenates. The full-length blot is shown in Supplementary Figure 5a **(b)** Quantification of **a**, $n = 5$ flies, (for H3K9me2, $*p = 0.04$, $t = 3.14$ for HP1α, $*p = 0.04$, $t = 3.08$ for four degrees of freedom, unpaired t-test). **(c)** H3K9me2 and HP1α immunostaining of tau transgenic fly brains. Arrows indicate chromocenters. The region presented is cortex. Scale bar is $3 \mu\text{m}$. **(d)** Quantification of experiments in **c**, $n = 3$ brains (for H3K9me2, $**p = 9e-05$, $t = 105.36$ for two degrees of freedom, for HP1α, $**p = 4.7e-05$, $t = 145.24$ for two degrees of freedom, unpaired t-test). Control is *elav-GAL4/+* in **a-d**. **(e)** Quantification BL2 (β -galactosidase) and Y10C (GFP) chromatin reporter activation in control and tau transgenic flies. Controls are *elav-GAL4/BL2* and *elav-GAL4/Y10C*, $n = 6$ brains (for BL2, $**p = 5.12e-09$, $t = 81.94$ for five degrees of freedom, for HP1α, $**p = 1.13e-05$, $t = 17.47$ for five degrees of freedom, unpaired t-test). β -galactosidase and AT8 **(f)** cleaved PARP (cPARP) **(g)** or PCNA **(h)** immunostaining in flies transgenic for the BL2 chromatin reporter, $n = 3$ brains. Control is *elav-GAL4/BL2* in **f** and **h**, and *elav-GAL4/BL2;UAS-PARP/+* in **g**. Scale bars are $10 \mu\text{m}$ in **f-h**. Data are mean \pm s.e.m., all flies are 10 days old.

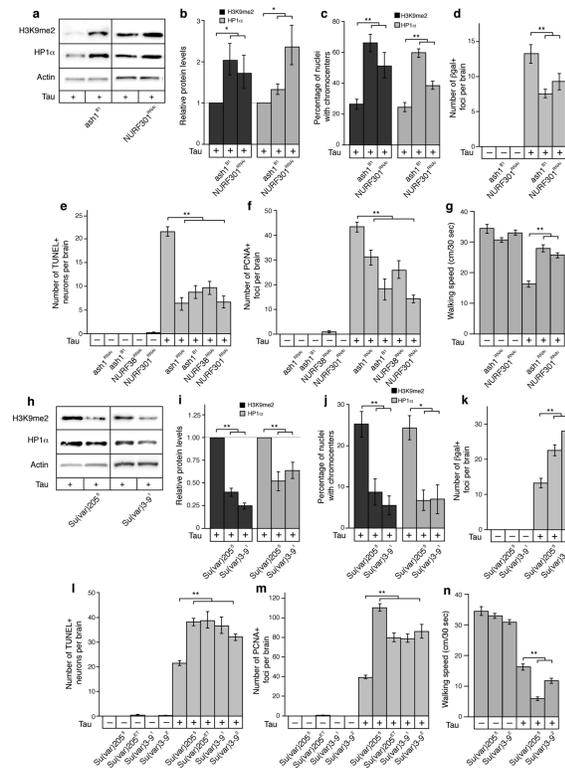


Figure 2.

Genetic manipulation of chromatin structure modifies tau-induced toxicity in *Drosophila*. **(a)** H3K9me2 and HP1 α levels in tau transgenic flies heterozygous for a loss-of-function mutation in *ash1* or expressing an RNAi transgene targeted to *NURF301*. Full-length blots are shown in Supplementary Figure 5b. **(b)** Quantification of **a**, $n = 3$ heads, (for H3K9me2, $*p = 0.04$, $F = 4.38$ for two degrees of freedom, for HP1 α , $*p = 0.02$, $F = 5.6$ for two degrees of freedom, one-way ANOVA). **(c)** Quantification of H3K9me2- and HP1 α -positive chromocenters in tau transgenic flies heterozygous for a loss-of-function mutation in *ash1* or expressing an RNAi transgene targeted to *NURF301*, $n = 3$ brains (for H3K9me2, $**p = 0.01$, $F = 11.64$ for two degrees of freedom, for HP1 α , $**p < 0.001$, $F = 46.2$ for two degrees of freedom, one-way ANOVA). **(d)** Quantification of BL2 reporter activation in control and tau transgenic flies heterozygous for a loss-of-function mutation in *ash1* or expressing an RNAi transgene targeted to *NURF301* ($**p = .001$, $F = 8.85$ for two degrees of freedom, one-way ANOVA). Neuronal degeneration assayed by TUNEL staining **(e)** and cell cycle activation assayed by PCNA staining **(f)** in brains of control and tau transgenic flies heterozygous for a loss-of-function mutation in *ash1* or expressing an RNAi transgene targeted to *ash1*, *NURF301*, or *NURF38* (for TUNEL, $**p < 0.001$, $F = 22.1$ for two degrees of freedom, for PCNA, $**p < 0.001$, $F = 14.569$ for two degrees of freedom, one-way ANOVA). **(g)** Locomotor activity in control and tau transgenic flies expressing an RNAi transgene targeted to *ash1* or *NURF301*, $n = 18$ flies, $**p < 0.001$, $F = 11.66$ with two degrees of freedom, one-way ANOVA. **(h)** H3K9me2 and HP1 α levels in homogenates from heads of tau transgenic flies heterozygous for loss-of-function mutations in *Su(var)205* or *Su(var)3-9*. Full-length blots are shown in Supplementary Figure 5c. **(i)** Quantification of **h**, $n = 3$ heads (for H3K9me2, $**p = 0.0002$, $F = 1,080.2$ for two degrees of freedom, for HP1 α , $**p = 0.01$, $F = 16.83$ for two degrees of freedom, one-way ANOVA). **(j)** Quantification of H3K9me2- and HP1 α -positive chromocenters in tau transgenic flies heterozygous for a loss-of-function mutation in *Su(var)205* or *Su(var)3-9* based on immunofluorescence, $n = 3$ brains (For H3K9me2, $**p = 0.01$, $F = 13.16$ for two degrees of freedom, for HP1 α , $*p = 0.02$, $F = 8.964$ for two degrees of freedom, one-way ANOVA). **(k)** Quantification of BL2 reporter activation in control and tau transgenic flies heterozygous for loss-of-function mutations in *Su(var)205* or *Su(var)3-9*, $**p < 0.001$, $F = 21.2$ for two degrees of freedom, one-way ANOVA. Neuronal apoptosis **(l)** and

PCNA positive foci (**m**) in control and tau transgenic flies heterozygous for loss-of-function mutations in *Su(var)205* or *Su(var)3-9* (for TUNEL, **p < 0.001, F = 10.26 for four degrees of freedom, for PCNA, **p < 0.001, F = 34.48 for four degrees of freedom, one-way ANOVA). (**n**) Locomotor activity in control and tau transgenic flies heterozygous for a loss-of-function mutation in *Su(var)205* or *Su(var)3-9*, n = 18 flies, **p < 0.001, F = 12.84 for two degrees of freedom, one-way ANOVA. Flies are 10 days old and n = 6 unless otherwise specified. Controls are *elav-GAL4/+*. Data are mean ± s.e.m.

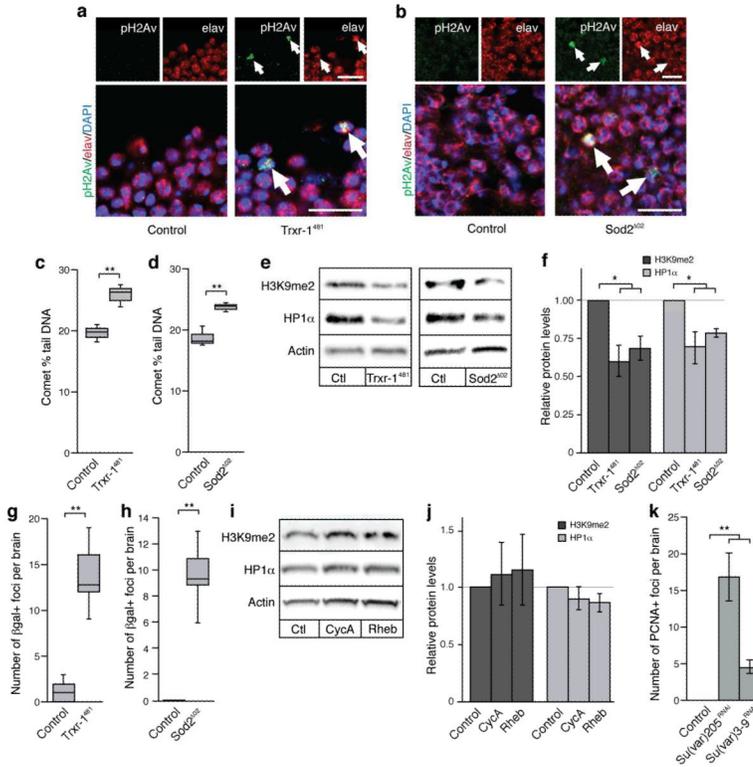


Figure 3.

Oxidative stress causes DNA damage and heterochromatin loss in *Drosophila*. pH2Av and elav staining in neurons of control and *Trxr-1⁴⁸¹* hemizygous (a) or *Sod2⁰²* heterozygous (b) mutant flies, n = 3 brains. Arrows indicate pH2Av positive nuclei. Scale bar is 10 μm. Quantification of comet tails in *Trxr-1⁴⁸¹* hemizygous (c) and *Sod2⁰²* heterozygous (d) mutant flies compared to control, n = 6 brains (for c, **p = 0.01, t = 9.2 for two degrees of freedom, for d, **p = 0.01, t = 10.84 for two degrees of freedom, unpaired t-test). (e) H3K9me2 and HP1α levels in homogenates from heads of flies harboring loss-of-function mutations in *Trxr-1* or *Sod2*. Full-length blots are shown in Supplementary Figure 5d. (f) Quantification of experiments in e, n = 3 heads (for H3K9me2, *p = 0.02, F = 7.98 for two degrees of freedom, for HP1α, *p = 0.03, F = 6.5 for two degrees of freedom, one-way ANOVA). Quantification of BL2 reporter activation in brains of *Trxr-1⁴⁸¹* (g) and *Sod2⁰²* (h) flies; control is hemizygous for the BL2 reporter. n = 6 brains (for g, **p < 0.001, t = 20.19 for five degrees of freedom, for h, **p < 0.001, t = 26.56 for five degrees of freedom, unpaired t-test). (i) H3K9me2 and HP1α levels in homogenates from heads of flies with neuronal overexpression of CycA or Rheb. Full-length blots are shown in Supplementary Figure 5e. (j) Quantification of experiments in i, n = 3 heads (for H3K9me2, p = 0.92, F = 0.08 for two degrees of freedom, for HP1α, p = 0.54, F = 0.71 for two degrees of freedom, one-way ANOVA). (k) Cell cycle activation assayed by PCNA staining in brains of flies with RNAi-mediated knockdown of *Su(var)205* or *Su(var)3-9*. n = 6 brains, **p < 0.001, F = 23.52 for two degrees of freedom, one-way ANOVA. Control is *w¹¹¹⁸* in a-h, and *elav-GAL4/+* in i-k. *Trxr-1⁴⁸¹* hemizygous flies and respective controls are 2 days old, all other flies are 10 days old. Data are mean ± s.e.m., except in c, d, g and h in which data are represented as box and whisker plots comprised of minimum, lower quartile, median, upper quartile and maximum values.

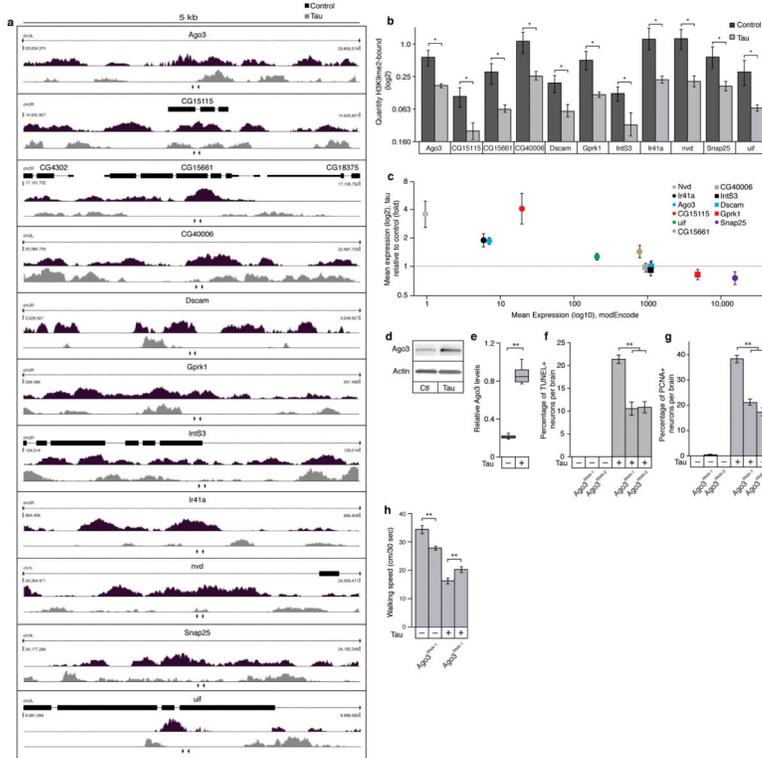


Figure 4.

H3K9me2 loss and increased gene expression in tau transgenic *Drosophila*. **(a)** H3K9me2 distribution in genes with significant H3K9me2 loss in tau transgenic flies based on H3K9me2 ChIP-seq, $n = 2$ rounds of ChIP-seq on biological replicates, (FDR < 0.01). Arrows indicate the locations of primers used for qPCR. Rectangles indicate exons, dashed lines indicate introns. **(b)** qPCR of each gene identified by ChIP-seq as H3K9me2 depleted in tau transgenic flies, $n = 3$ trials, 10 heads per trial (for Ago3, $*p = 0.02$, $t = 6.85$, for CG15115, $p = 0.004$, $t = 15.6$, for CG15661, $*p = 0.01$, $t = 8.25$, for CG40006, $*p = 0.01$, $t = 9.56$, for Dscam, $*p = 0.0003$, $t = 54.42$, for Gprk1, $*p = 0.004$, $t = 15.07$, for IntS3, $*p = 0.0003$, $t = 62.71$, for Ir41a, $*p = 0.005$, $t = 14.38$, for nvd, $*p = 0.007$, $t = 12.31$, for Snap25, $*p = 0.004$, $t = 15.75$, for uif, $*p = 0.003$, $t = 19.15$, for two degrees of freedom, unpaired t-test). **(c)** Expression levels of H3K9me2 depleted genes in tau transgenic flies versus baseline gene expression (modEncode). Transcript levels are relative to both control and the endogenous control gene *RpL32*. $n = 3$ trials, 10 heads per trial, circles are significantly different from control, which is set to 1, (for Ago3, $p = 0.0003$, $t = 59.92$, for CG15115, $p = 0.05$, $t = 4.33$, for CG15661, $p = 0.04$, $t = 5.09$, for CG40006, $p = 0.35$, $t = 1.2$, for Dscam, $p = 0.48$, $t = 0.86$, for Gprk1, $p = 0.053$, $t = 4.15$, for IntS3, $*p = 0.38$, $t = 1.13$, for Ir41a, $p = 0.01$, $t = 9.79$, for nvd, $p = 0.04$, $t = 4.91$, for Snap25, $p = 0.05$, $t = 4.5$, for uif, $p = 0.02$, $t = 6.29$, for two degrees of freedom, unpaired t-test). **(d)** Ago3 levels in homogenates from control and tau transgenic fly heads. Full-length blots are shown in Supplementary Figure 5f. **(e)** Quantification of **d**, $n = 5$ heads, $**p = 3.57e-07$, $t = 63.98$ for four degrees of freedom, unpaired t-test. Neuronal degeneration assayed by TUNEL staining **(f)** and cell cycle activation assayed by PCNA staining **(g)** in brains of control and tau transgenic flies with RNAi-mediated reduction of *Ago3*. $n = 6$ brains (for TUNEL, $**p < 0.001$, $F = 29.92$ for two degrees of freedom, for PCNA, $**p < 0.001$, $F = 57.67$ for two degrees of freedom, one-way ANOVA). **(h)** Locomotor activity in control and tau transgenic flies with RNAi-mediated reduction of *Ago3*, $n = 18$ flies (for Ago3^{RNAi-1} compared to control, $**p = 4.8e-16$, $t = 6.56$ for 17 degrees of freedom, for tau + Ago3^{RNAi-2} compared to tau expressed alone, $**p = 0.001$, $t = 3.865$ for 17 degrees of freedom, unpaired t-test). Control is *elav-GAL4/+*. Data are mean \pm s.e.m., unpaired t-test or ANOVA. All flies are 10 days old.

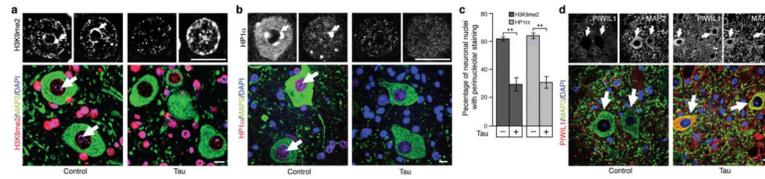


Figure 5.

Tauopathy model mice have heterochromatin loss and increased PIWIL1 expression. Perinucleolar chromocenter staining (arrows) of H3K9me2 (**a**) and HP1α (**b**) in control and JNPL3 motor neurons. Upper boxes are H3K9me2 or HP1α staining in MAP2-positive neurons at higher magnification. (**c**) Quantification of experiments in **a** and **b** (for **a**, $**p = 0.002$, $t = 6.09$ for five degrees of freedom, for **b**, $**p = 0.00002$, $t = 16.97$ for five degrees of freedom, unpaired t-test). (**d**) PIWIL1 immunostaining (arrows) in control and JNPL3 motor neurons. $n = 6$ spinal cords per genotype. Scale bars are $10 \mu\text{m}$. Data are mean \pm s.e.m.

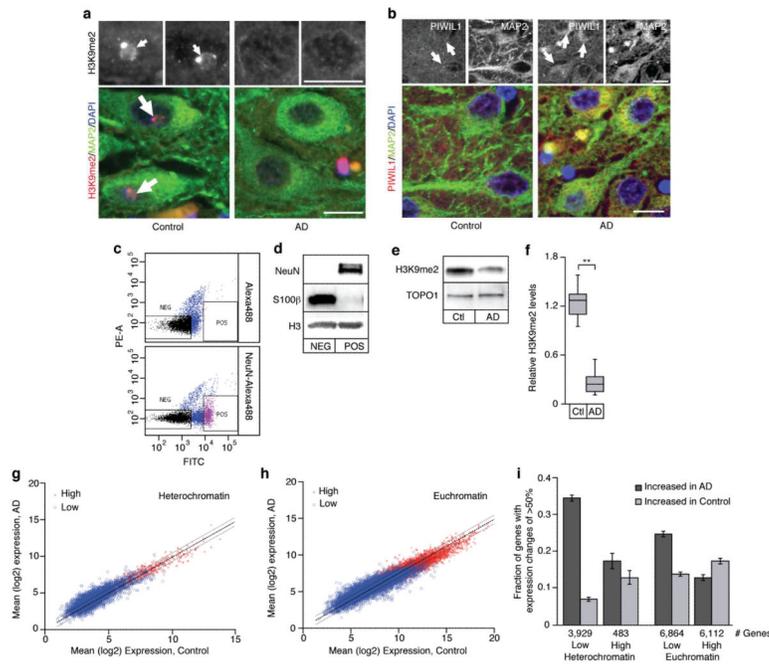


Figure 6.

H3K9me2 loss and increased gene expression in human AD brains. **(a)** Perinucleolar chromocenter staining of H3K9me2 (arrows) in human control and AD hippocampal neurons. $n = 6$ brains. **(b)** PIWIL1 immunostaining (arrows) in control and human AD hippocampal neurons. $n = 6$ brains. Upper boxes are H3K9me2 or PIWIL1 staining in MAP2-positive neurons at higher magnification in **a** and **b**. **(c)** FACS of postmortem human cortical nuclei stained with secondary antibody AlexaFluor488, or the neuron-specific NeuN antibody and secondary antibody AlexaFluor488. **(d)** S100 β and NeuN levels in NeuN negative (NEG) and NeuN positive (POS) nuclear populations purified via FACS. Full-length blots are shown in Supplementary Figure 5g. **(e)** H3K9me2 levels in FACS-purified NeuN positive neuronal nuclei from control and AD brains. Full-length blots are shown in Supplementary Figure 5h. **(f)** Quantification of **e**, $n = 6$ brains, data are represented as box and whisker plots comprised of minimum, lower quartile, median, upper quartile and maximum values for H3K9me2 levels, $p = 0.00002$, $t = 15.95$ for two degrees of freedom, unpaired t-test. Average mRNA levels of heterochromatic **(g)** and euchromatic **(h)** genes in laser-captured neurons from control ($n = 13$) and AD ($n = 10$) postmortem hippocampi. Gray lines indicate a 50% gene expression threshold. **(i)** Bar plot of genes from **g** and **h** with expression changes greater than 50%. For increased expression of genes in AD that are classified as heterochromatic and expressed at low levels in control, $p < 10^{-16}$, chi-square. Error bars in **i** reflect standard deviation from 1,000 bootstraps.