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Systems genetics identifies *Hp1bp3* as a novel modulator of cognitive aging

Sarah M. Neuner [a], Benjamin P. Garfinkel [b,c,1], Lynda A. Wilmott [a,1], Bogna M. Ignatowska-Jankowska [c], Ami Citri [c], Joseph Orly [b], Lu Lu [d], Rupert W. Overall [e], Megan K. Mulligan [d], Gerd Kempermann [e,f], Robert W. Williams [d], Kristen M.S. O'Connell [g], Catherine C. Kaczorowski [a,*]

[a] Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, Memphis, TN 38163, USA
[b] Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
[c] The Edmond and Lily Safra Center for Brain Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
[d] Department of Genetics, Genomics, and Informatics, University of Tennessee Health Science Center, Memphis, TN 38163, USA
[e] CRTD—Center for Regenerative Therapies Dresden, Technische Universität Dresden, Dresden 01307, Germany
[f] German Center for Neurodegenerative Diseases (DZNE) Dresden, Dresden 01307, Germany
[g] Department of Physiology, University of Tennessee Health Science Center, Memphis, TN 38163, USA

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ABSTRACT

An individual's genetic makeup plays an important role in determining susceptibility to cognitive aging. Identifying the specific genes that contribute to cognitive aging may aid in early diagnosis of at-risk patients, as well as identify novel therapeutics targets to treat or prevent development of symptoms. Challenges to identifying these specific genes in human studies include complex genetics, difficulty in controlling environmental factors, and limited access to human brain tissue. Here, we identify *Hp1bp3* as a novel modulator of cognitive aging using a genetically diverse population of mice and confirm that HP1BP3 protein levels are significantly reduced in the hippocampi of cognitively impaired elderly humans relative to cognitively intact controls. Deletion of functional *Hp1bp3* in mice recapitulates memory deficits characteristic of aged impaired mice and humans, further supporting the idea that *Hp1bp3* and associated molecular networks are modulators of cognitive aging. Overall, our results suggest *Hp1bp3* may serve as a potential target against cognitive aging and demonstrate the utility of genetically diverse animal models for the study of complex human disease.

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1. Introduction

Aging is associated with a decline in cognitive performance that begins around midlife (i.e., 45–50 years), but the onset and severity varies greatly among individuals (Davies et al., 2015; Singh-Manoux et al., 2012). Although variation in cognitive performance at midlife is highly heritable [i.e., 62%–78% of variance is attributable to genetic factors (McClearn et al., 1997; Plomin and Deary, 2015)], a large portion of this heritability remains unexplained by currently identified gene variants (Johnson et al., 2016). Several studies have identified associations between apolipoprotein E, brain-derived neurotrophic factor, and catechol-O-methyltransferase with either cognitive ability or rate of cognitive decline in older people (Harris and Deary, 2011; Laukka et al., 2013; Payton, 2009; Tapia-Arancibia et al., 2008; Wisdom et al., 2011). However, even when polymorphisms in these genes are significantly associated with cognitive phenotypes, effect sizes are typically small (Harris and Deary, 2011), indicating that additional genes contribute significantly to the regulation of cognitive decline in human populations. The identification of specific genes that modify the development and progression of cognitive decline may aid in early diagnosis of at-risk patients, as well as identify novel targets for the development of therapeutics to prevent or delay the onset of disease.

Challenges to identifying DNA variants that modify cognitive aging in humans include substantial genetic heterogeneity, difficulty in controlling environmental factors, and limited molecular data from disease-relevant human brain tissue. To circumvent some of these challenges, murine genetic reference panels (GRPs) have

* Corresponding author at: Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, 855 Monroe Ave Suite 515, Memphis, TN 38163, USA. Tel.: +1 901 448 2178; fax: +1 901 448 7193.
E-mail address: ckaczoro@uthsc.edu (C.C. Kaczorowski).
[1] Authors contributed equally.

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been designed to model some of the genetic and phenotypic complexity of human populations (International HapMap et al., 2010; Peirce et al., 2004; Williams et al., 2001), allowing a researcher to exploit phenotypic heterogeneity across a population while controlling for environmental factors (Williams and Auwerx, 2015). One such model, a well-characterized GRP known as the BXDs (Peirce et al., 2004; Taylor, 1978; Taylor et al., 1999), was derived by crossing 2 common inbred strains, C57BL/6J (B6) and DBA/2J (D2). The BXDs have been successfully used to identify genomic regions important for determining learning and memory capabilities early in life (Wehner et al., 1997) but have not yet been used to study cognitive aging. To identify genes and molecular pathways regulating memory capabilities during aging, here we perform a forward systems genetic analysis on an aged cohort of strains from the BXD GRP.

2. Methods

2.1. Animals

Male and female mice were group housed (2–5 per cage) and maintained in colony housing (12-hour light/dark cycle) with ad libitum access to food and water. All mouse experiments were conducted in accordance with the University of Tennessee Health Science Center Animal Care and Use Committee, the Institutional Committee on Animal Care and Use at the Hebrew University of Jerusalem, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Middle-aged mice (15 ± 0.3 months, 2–8 mice per strain) from 21 BXD recombinant inbred strains were phenotyped. Although experiments in rodents using a single inbred strain are often carried out using 7–12 replicates (Kaczorowski and Disterhoft, 2009; Kaczorowski et al., 2011, 2012), mapping studies using the BXD panel gain much more power by increasing numbers of unique genotypes rather than replicates per strain (Belknap, 1998). This is because at each locus, roughly half of the lines inherit B/B genotypes and the other half D/D genotypes (see Fig. 1B in Andreux et al., 2012). The BXDs were derived by inbreeding the F2 progeny of a C57BL/6J (B6) and DBA/2J (D2) intercross to create a panel that models some of the genetic complexity of human populations (Chesler et al., 2005; International HapMap et al., 2010). The parental strains, B6 and D2, differ in a variety of traits, including memory function (Balogh et al., 2002), amyloid precursor protein processing (Lehman et al., 2003), adult hippocampal neurogenesis (Kempermann et al., 2006), and hippocampal excitability (Oksman et al., 2005), which confers wide phenotypic variability to the resulting BXD strains. Each BXD line has been inbred for >20 generations, and their genomes are stable, which allows for replication studies across time and laboratories (Peirce et al., 2004). In addition to collected "health records" at GeneNetwork.org, both genotype and transcriptome data for ~40 tissues and cells, including hippocampus, have been generated for many of the strains, which we combined with full-sequence genome data for both B6 and D2 to facilitate genetic mapping to identify individual genetic variants that correlate with traits of interest (Chesler et al., 2005;

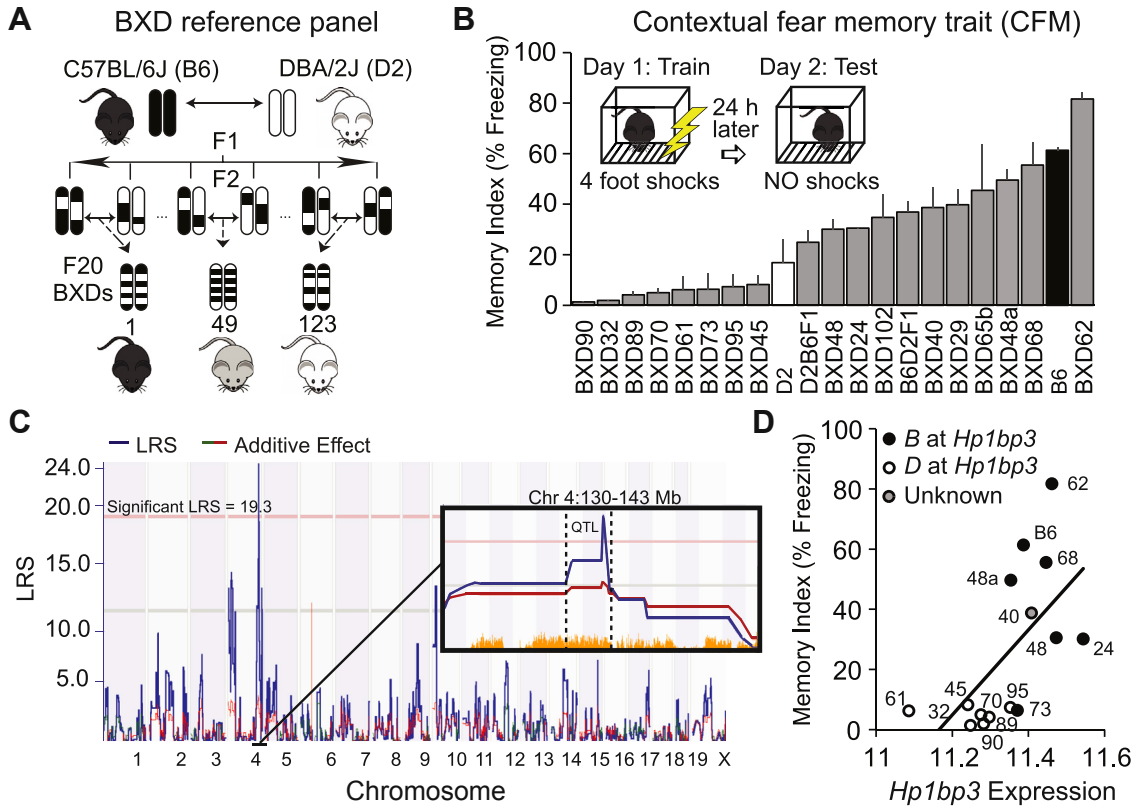

Fig. 1. Identification of *Hp1bp3* as top candidate modulator of cognitive aging. (A) The BXD panel was derived by inbreeding the F2 progeny of a C57BL/6J (B6) and DBA/2J (D2) intercross. (B) Contextual fear memory (CFM) varies widely across 21 BXD strains (n = 2–8 per strain, mean age = 15 ± 0.3 months). Memory index was quantified by percent time spent freezing during 10 minute test. (C) Genetic interval mapping revealed a significant CFM QTL on mouse chromosome 4 (Chr4: 137.5–140.5 Mb). Pink horizontal line: genome-wide statistical significance ($p = 0.05$), green additive effect line: *D* parental allele increases trait values, red: *B* allele increases trait values. Yellow tick marks: presence of genome sequence variant. (D) *Hp1bp3* expression is highly correlated with CFM across BXD strains (Pearson $r = 0.6$, $p < 0.05$), with strains inheriting the *D* allele having lower levels of hippocampal *Hp1bp3* and poorer CFM. Abbreviation: QTL, quantitative trait locus.

Houtkooper et al., 2013; Wang et al., 2016). Hp1bp3$^{tm1a(EUCOMM)Wtsi}$ mice (n = 6/group) were acquired from the European Conditional Mouse Mutagenesis program and are described elsewhere (Garfinkel et al., 2015a).

2.2. Contextual fear conditioning

All mice were habituated to transport for at least 3 days before behavioral tests. On the fourth day, mice were trained on a standard contextual fear conditioning paradigm (Neuner et al., 2015). Briefly, contextual fear conditioning consisted of a 180-second baseline period followed by 4 mild foot shocks (1 second, 0.9 mA, intershock interval 115 ± 20 seconds). The average activity burst following each shock was determined by manual timing and used to quantify the postshock reactivity for each mouse to ensure no difference in pain sensitivity. Twenty-four hours later, hippocampus-dependent contextual fear memory (Chen et al., 1996) was tested in a 10-minute session. Behavioral freezing, an index of conditioned fear, was measured using Freeze Frame software (Coulbourn Instruments, PA, USA) at the University of Tennessee or EthoVision software (Noldus Information Technology, Netherlands) at the Hebrew University of Jerusalem. Our laboratory has previously demonstrated that measures of freezing obtained via video monitoring software correlates well with hand-scored measures of freezing (Kaczorowski et al., 2012). Experimenters were blind to the genotype during behavioral tests and data analysis.

2.3. Calculation of memory index for adult BXD strains

BXD strains aged 8–9 weeks had previously been subjected to contextual fear conditioning by Philip et al. (2010). The data set used in our study (including males and females) is publically available on GeneNetwork.org as BXD Published Phenotypes Record ID 11908. The memory index used in Trait 11908 is a measure of activity (beam breaks) throughout contextual fear memory (CFM) testing. This measure of activity has been previously shown to correlate well with measures of hand-scored freezing behavior (Valentinuzzi et al., 1998). As the dynamic range of human-observed freezing behavior is 0%–100% (Anagnostaras et al., 2000), and an activity score of zero is equivalent to 100% freezing, we subtracted these activity measures from 100 to obtain a measure of inactivity that parallels the CFM index (% freezing) calculated for middle-aged strains. Specifically, the mean time each adult BXD strain spent active during testing was subtracted from 100 to obtain a measure of inactivity. The genotype of each strain at the *Hp1bp3* locus was then obtained from GeneNetwork.org and used for genotype-by-memory analyses.

2.4. T-maze

The T-maze test was performed as described previously (Deacon and Rawlins, 2006). Mice were given 10 minutes to habituate to the testing room. The T-maze floor was coated with fresh woodchips before each round of trials. Mice were placed into the T-maze and allowed to choose 1 of 2 "goal" arms. The mouse was then confined to that arm for 30 seconds using a central partition. The mice were removed from the maze and immediately placed back at the starting point, the partition removed, and the mouse allowed to choose between the 2 goal arms again (Deacon and Rawlins, 2006). An "alternation" occurred when the mouse entered the opposite arm than that to which it was just confined. The criterion point used was whole animal in goal arm, including tail tip. A total of 6 trials were performed, with animals tested no more than twice a day. The alternation scores across all 6 trials were averaged to obtain an average alternation score for each mouse.

2.5. Quantitative trait locus (QTL) identification and candidate gene selection

After CFM tests for 21 BXD strains were completed, the mean time spent freezing was averaged for each strain. Strain means were entered in GeneNetwork.org and are publically available as BXD Published Phenotypes Record ID: 18395. Hippocampal transcript data from aged BXD strains available on GeneNetwork.org as *UTHSC BXD Aged Hippocampus Affy MoGene1.0ST (May15) RMA Gene Level*, were used to identify cis (locally)-regulated genes (Andreux et al., 2012; Chesler et al., 2005) from within intervals of interest. The same transcript data were used to identify genes whose expression correlated with memory function in aged animals. Only probes that did not overlap single nucleotide polymorphisms (SNPs) were used to avoid hybridization artifacts that may cause apparent differences in expression due to technical rather than biological variance. When multiple SNP-free gene level probes were available (e.g., *Hp1bp3*), the mean expression of exon-targeting probes was used. In cases where SNP-free gene level probes were not available, exon-level hippocampal transcript data from aged BXD strains were used (*UTHSC BXD Aged Hippocampus Affy Mouse Gene1.0ST (Sep12) RMA Exon Level,* data set accession ID GN392). If exon level probes were used, the probe with highest average expression across all strains was selected. All probes were verified by BLAT (UCSC Genome Browser). Correlation analyses for initial candidate gene prioritization used Spearman's rho and were adjusted for multiple comparisons using Bonferroni correction (95% CI).

2.6. Western blots

Western blots were performed as previously described (Hatfield et al., 2015; Neuner et al., 2015). Frozen human hippocampal samples (n=3/group) collected postmortem were obtained from the University of Kentucky Sanders-Brown Center on Aging and stored at −80 °C until use. Briefly, hippocampal lysates were prepared from frozen tissue, protein concentration was determined using a Nanodrop2000 Spectrophotometer (ThermoScientific), and 20 μg of total protein was loaded and separated on a 10% SDS-PAGE gel. Proteins were transferred using the Bio-Rad TurboTransfer system and blocked for 30 minutes at room temperature. Primary antibodies for HP1BP3 and GAPDH (ProteinTech #24556-1-AP and Fitzgerald #10R-G109a, respectively) were incubated overnight and detected by anti-mouse and anti-rabbit fluorescent-conjugated antibodies. Visualization was performed using an Odyssey image scanner and blots were quantified using the Odyssey software version 5.0 (LiCOR). Results were replicated in 2 independent Western blots. Observed double-band staining is the typical expression pattern for HP1BP3 (Garfinkel et al., 2015b) and overlaps with positive control HP1BP3 overexpression lysate from human 293T cells (Abnova #H00050809-T02), which was used as a positive control. Loading dye–only lanes served as negative control.

2.7. Gene set enrichment analysis

Gene set enrichment analysis was performed as described previously using version 2.2.0 (Mootha et al., 2003; Subramanian et al., 2005). Briefly, all nominally significant correlates of hippocampal *Hp1bp3* were extracted from whole-genome hippocampal transcript data from BXD strains age-matched to those used for contextual fear conditioning. Correlates were ranked based on correlation coefficient, and this preranked list was used to calculate an enrichment score for gene sets obtained from the Molecular Signatures Database version 5.0. Enrichment scores are obtained by calculating a cumulative ("running-sum") statistic, which is increased when a gene is present in the set being tested and

decreased when a gene is not. The maximum deviation of this cumulative score from 0 is the enrichment score. Then, normalized enrichment scores were obtained to account for the size of the gene set being tested. The proportion of false positives was controlled using established methods (Mootha et al., 2003; Subramanian et al., 2005). Based on these criteria, any gene set with a false discovery rate of $\leq$0.25 was accepted as significantly enriched in our data set.

2.8. Statistical analysis

Statistical analysis was performed using SPSS software (IBM) and Microsoft Excel. Analyses included independent *t*-tests, repeated measures, 1-way and 2-way analysis of variance, and Pearson correlation with confidence level set at $p < 0.05$. All analyses were corrected for multiple comparisons and described in the Section 3. Unless otherwise stated, data values reported here are given as mean ± standard error of the mean. The order of behavioral tests and mice allocated to each condition were randomized. The experimenter was blind to group allocation for all behavioral studies, and analysis of raw data was conducted blind to experimental groups.

3. Results

3.1. Genetic mapping identifies an interval on chromosome 4 associated with memory status at midlife

To identify genes involved in the regulation of cognitive aging, we analyzed hippocampus-dependent memory function across a cohort of middle-aged mice (15 ± 0.3 m) that model the genetic and phenotypic variation of human populations (the BXD genetic reference panel, Fig. 1A). Specifically, mice were trained on standard contextual fear conditioning (Kaczorowski and Disterhoft, 2009). During training on day 1, mice received 4 scrambled foot shocks in the conditioning chamber over a 10-minute training session. Twenty-four hours later, mice were returned to the original conditioning chamber, and the percentage of time each mouse spent freezing over the 10-minute test was measured as an index of CFM. CFM is highly variable across this aged BXD family (21 strains tested, n = 2–8 mice/strain, Fig. 1B), and heritability estimates that compare the observed genetic (between-strain) variance to technical (within-strain) variance (heritability, $h^2 \approx 0.7$) demonstrate that much of this variability is attributable to genetic factors. Although strain-specific differences in acquisition were also observed, the variance in acquisition was not sufficient to explain the differences in CFM across the panel.

Subsequent interval mapping using the average memory index for each strain highlighted a region of chromosome 4 (Chr 4, 137.7–140.5 Mb) that was significantly associated with variation in CFM in middle-aged mice (Fig. 1C). This QTL had not associated with memory function in studies using younger adult sex-matched BXDs (Philip et al., 2010), suggesting the QTL contains variants that contribute to variation in cognitive aging as opposed to general memory function.

3.2. Prioritization of positional candidates identifies Hp1bp3 as putative regulator of cognitive aging across BXD panel

To identify genes located in the QTL that may be causally involved in regulating CFM abilities at midlife, positional gene candidates were prioritized based on the following: (1) annotated sequence differences segregating among the BXD strains, (2) local control of gene expression as determined by expression QTL analysis using hippocampal transcriptome data from aged BXD strains, and (3) significant correlation between hippocampal gene expression and CFM. The gene *heterochromatin protein 1 binding protein 3* (*Hp1bp3*) emerged as the single best positional candidate (Table 1).

Hp1bp3 contains multiple missense variants in coding regions, numerous noncoding variants, and insertions and/or deletions predicted to impact protein function, transcriptional regulation and/or splicing (McLaren et al., 2010; Supplementary Table 1). Hippocampal *Hp1bp3* expression was significantly correlated with CFM status, with those BXD strains inheriting the *D* parental allele exhibiting lower levels of *Hp1bp3* transcript and worse CFM performance relative to the *B* allele (Fig. 1D). Although *Hp1bp3* genotype had no effect on memory status in younger adult mice (age of 8–9 weeks, Fig. 2A, secondary analysis of data from Philip et al., 2010), strains inheriting the *D* allele exhibited significantly impaired CFM at midlife (Fig. 2B). Because variation in the CFM index at midlife was not due to genetic differences in baseline anxiety or postshock pain sensitivity confounds (Fig. 3), these data suggest a genotype-by-age interaction in which reductions in *Hp1bp3* expression correspond specifically to aging-related cognitive impairment (Figs 1–3). In support, the genotype at *Hp1bp3* can account for as much as 52% of the strain variance in CFM performance at midlife. However, calculations of genetic variance

Table 1
Locally (*cis*) regulated positional candidates

Gene symbol	Description	SNP count	Trait ID in gene network	Location	Max LRS	Correlation with CFM
Hp1bp3	Heterochromatin protein 1 binding protein 3	106	10338180,[a] 10344447,[a] 10342675[a]	Chr 4: 137.7	Chr4: 139.3	r = 0.7, $p < 0.05$
Eif4g3	Eukaryotic translation initiation factor 4 gamma 3	763	10509463[a]	Chr4: 137.5	Chr4: 140.2	r = 0.5, N.S.
Capzb	Capping protein (actin filament) muscle Z-line, beta	448	10509620, 10509628[a]	Chr4: 138.7	Chr4: 138.2	r = 0.4, N.S.
Iffo2	Intermediate filament family orphan 2	225	10509777, 10509781[a]	Chr4: 139.1	Chr4: 139.3	r = −0.4, N.S.
Padi2	Peptidyl arginine deiminase, type II	132	10509838, 10509846[a]	Chr4: 140.5	Chr4: 140.5	r = 0.4, N.S.
Kif17	Kinesin family member 17	119	10509526, 10509532[a]	Chr4: 137.8	Chr4: 138.2	r = −0.1, N.S.
Pla2g2f	Phospholipase A2, group IIF	40	10517646, 10517654[a]	Chr4: 138.3	Chr4: 140.2	r = 0.2, N.S.
Mrto4	MRT4 (messenger RNA turnover 4) homolog (*Saccharomyces cerevisiae*)	32	10517706, 10517711[a]	Chr4: 138.9	Chr4: 139.9	r = −0.5, N.S.
Nbl1	Neuroblastoma, suppression of tumorigenicity 1	10	10517677, 10517681[a]	Chr4: 138.6	Chr4: 138.2	r = −0.5, N.S.
Camk2n1	Calcium/calmodulin-dependent protein kinase II inhibitor 1	4	10509568, no SNP-free probe	Chr4: 138.0	Chr4: 140.5	—

Genes located under the significant CFM QTL were first prioritized for presence of annotated sequence variants between parental strains B6 and D2. Whole-genome transcript data from aged BXD strains were then used to perform expression QTL mapping, and those genes whose expression had a peak LRS at the location of the gene (*cis*, or locally, regulated genes) were given higher priority. These genes were tested for correlation with the CFM trait using SNP-free probes from gene-level analyses (or exon-level analyses where necessary, see Section 2.5). Spearman's rho correlations are reported.
Key: CFM, contextual fear memory; LRS, likelihood ratio statistic; N.S., not significant; QTL, quantitative trait locus.
[a] Denotes SNP-free probe.

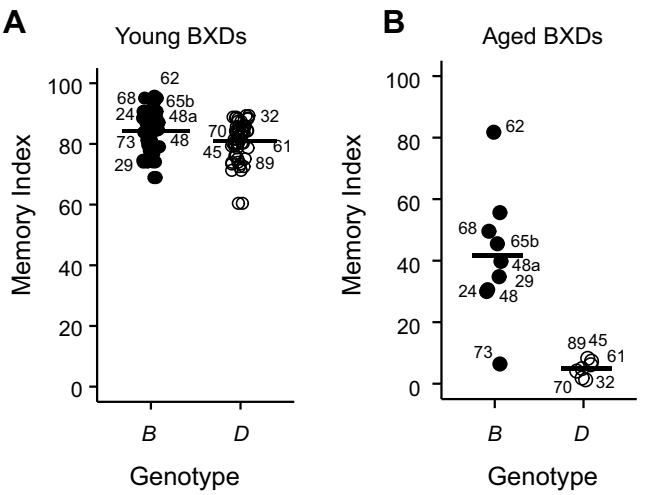

Fig. 2. Effect of *Hp1bp3* genotype on CFM status is age-dependent. (A) Memory index for adult BXDs (age = 8–9 weeks) was calculated using CFM data generated by Philip et al. (2010) and is publically available on GeneNetwork.org (Trait ID 110908, see Section 2.3). Parental origin of *Hp1bp3* allele did not significantly affect memory status in adulthood (*D* allele memory index = 81.0 ± 1.3, *B* =84.3 ± 1.3, t[54] = −1.86, p = 0.068). (B) Middle-aged (age = 15.0 ± 0.3 months) BXD strains inheriting the *D* parental allele performed significantly worse on CFM tests than strains inheriting the *B* allele (*D* = 4.8 ± 1.0, *B* = 41.5 ± 20.7, t[14] = −4.6, $p < 0.001$). Two-way analysis of variance revealed a significant interaction between genotype and age (F[1,66] = 33.5, $p < 0.001$), suggesting that *Hp1bp3* genotype influences memory status in an age-dependent manner. Abbreviation: CFM, contextual fear memory.

explained by a QTL are biased upward by an average of 30% due to a combination of epistatic, genotype-by-environment interactions, and small sample size (n < 1000) (Beavis, 1998; Wellenreuther and Hansson, 2016; Wurschum and Kraft, 2014). Therefore, we estimate that the actual biological heritable variation in CFM at midlife explained by *Hp1bp3* genotype is more likely in the range of 20%–25%.

3.3. Functional validation in a knockout (KO) mouse model confirms novel role for Hp1bp3 in cognitive function

To assess the functional consequence of loss of *Hp1bp3* on hippocampus-dependent learning and memory, we employed a reverse genetics approach. During CFM training, *Hp1bp3* KO and wild-type (WT) mice (Garfinkel et al., 2015a) showed no differences in baseline activity (Fig. 4A) or shock reactivity (Fig. 4B), and KO mice exhibited comparable acquisition of conditioned fear, indicating they successfully learned the context-shock association (Fig. 4A). Nevertheless, KO mice exhibited long-term CFM deficits when tested 24 hours later (Fig. 4C). Notably, the effect of *Hp1bp3* KO on CFM was similar to the effect of genotype at the *Hp1bp3* locus. As the KO mice were generated on a B6 background, the WT mice carry the *B* version of the Hp1bp3 allele, and as expected, perform comparable to B6 mice from the BXD GRP. However, KO mice performed more similarly to BXDs harboring the *D* allele, which exhibited a 20%–40% reduction in conditioned freezing during CFM tests compared to strains with the *B* allele (Fig. 2B). This suggests the *D* allele functions similarly to a loss-of-function mutation.

To determine if loss of *Hp1bp3* mimics aging-related effects on additional hippocampus-dependent cognitive domains (Zornetzer et al., 1982), working memory was assessed using the T-maze test of spontaneous alternation (Deacon and Rawlins, 2006). KO mice were significantly impaired relative to WT mice, and like aged mice (Lalonde, 2002), performed at chance levels (Fig. 4D). Taken together, these results demonstrate, for the first time, that *Hp1bp3* is necessary for successful hippocampus-dependent memory function spanning multiple cognitive domains and suggest that therapeutic interventions to restore levels of *Hp1bp3* may improve cognitive function.

3.4. Hp1bp3 is associated with memory function in human cognitive aging

To test the hypothesis that *Hp1bp3* is also involved in human cognitive aging and to evaluate the translational relevance of our murine results, we next tested whether hippocampal *Hp1bp3* expression correlated with cognitive performance in elderly humans (Table 2). Caveats to working with human brain samples include variable postmortem intervals and undefined brain pathology (Bennett et al., 2014; Hargis, 2016). Therefore, to limit potential confounds we analyzed hippocampal tissue from humans with “normal” cognitive aging (i.e., no distinct Alzheimer’s disease [AD] pathology), which is not typically associated with gross neurodegeneration (Burke and Barnes, 2006; Korbo et al., 2004). Quantitative Western blots for HP1BP3 were performed on postmortem hippocampal tissue lysates prepared from cognitively intact (mini-mental state examination [MMSE] score = 29.3 ± 0.3, age = 78 ± 9.7 years) and cognitively impaired humans (MMSE = 22.6 ± 3.0, age = 84 ± 4.4 years). HP1BP3 levels were significantly

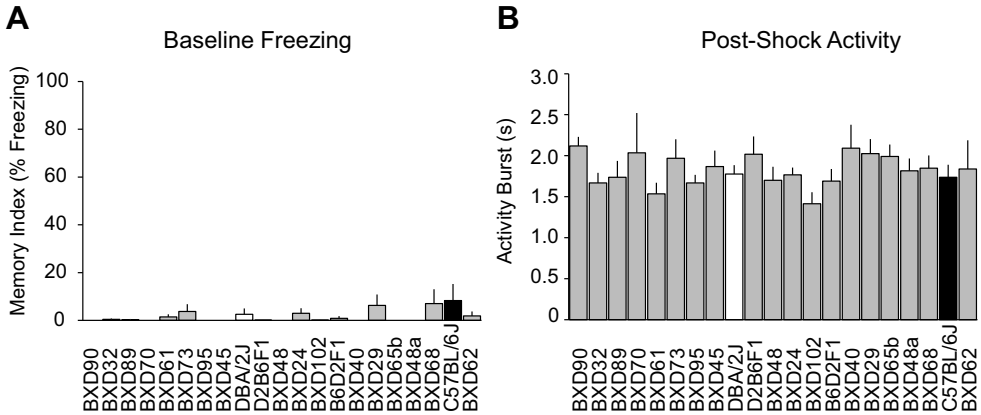

Fig. 3. Differences in CFM were not attributable to baseline freezing or postshock reactivity. There were no significant effects of strain on either (A) baseline freezing (F[20,50] = 1.009, p = 0.47) or (B) length of postshock activity burst (F[20,49] = 1.668, p = 0.07), indicating no strain-specific differences in baseline fear and/or anxiety or pain sensitivity, respectively, across 21 BXD strains tested. Abbreviation: CFM, contextual fear memory.

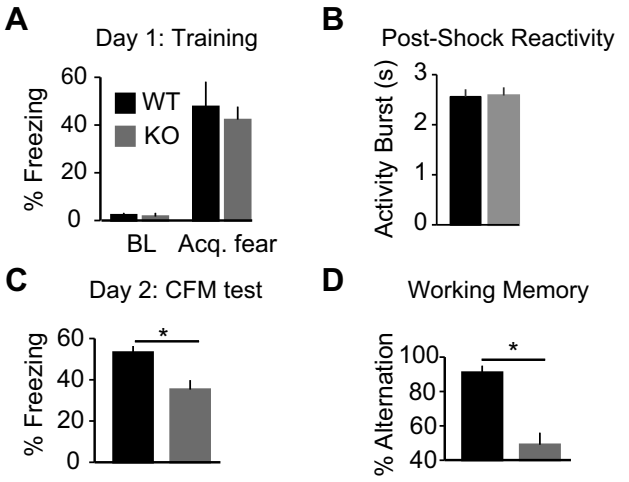

Fig. 4. Hp1bp3 knockout (KO) mice exhibit impairment on hippocampus-dependent long-term CFM and working memory tasks. (A) Comparable baseline (BL) freezing and acquisition of conditioned fear (Acq. fear) by the final trial in wild-type (WT) and *Hp1bp3* KO mice (n = 6 per group) during contextual fear conditioning. (B) There were no differences in postshock reactivity during fear conditioning training between WT and *Hp1bp3* KO mice, indicating no differences in pain sensitivity. Videos were manually analyzed to determine the average length of activity burst for each mouse. WT = 2.6 ± 0.2 seconds, KO = 2.6 ± 0.2 seconds; t(1,10)= −0.11, $p = 0.91$. (C) KO mice were significantly impaired on CFM (t[10] = 3.10, $p < 0.001$). (D) KO mice also exhibited spatial working memory deficits (t[12] = 5.095, $p < 0.001$). *$p < 0.05$. Abbreviation: CFM, contextual fear memory.

lower in humans diagnosed with cognitive impairment compared with age-matched controls (Fig. 5). Double-band staining is typical of HP1BP3 expression, indicative of multiple splice variants present in brain tissue (Garfinkel et al., 2015b). These results suggest either lower or reduced expression of *Hp1bp3* may underlie cognitive deficits observed in human populations and highlight the value and potential relevance of aged cohorts of diverse strains of mice for identifying molecular correlates of cognitive decline in humans.

3.5. *Genetic correlation analyses elucidate functional roles for* Hp1bp3 *in cognitive aging*

There is evidence that groups of highly correlated genes are likely to play a similar biological function and/or act as part of the same biological pathway (Eisen et al., 1998; Zhang and Horvath, 2005). To identify mechanisms through which *Hp1bp3* may act to regulate cognitive abilities at midlife, genes whose hippocampal expression significantly correlated with that of *Hp1bp3* were identified using whole-genome hippocampal transcript data from middle-aged BXD strains. All nominally significant correlates (uncorrected $p \leq 0.05$, 2074 genes) were sorted into a ranked list, with the most highly positively correlated genes at the top (Supplementary Table 2). This list was then used for gene set enrichment analysis (Broad Institute (Subramanian et al., 2005); and tested against gene sets publicly available from Broad Institute's Molecular Signatures Database, including Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto, 2000), Reactome pathways (Matthews et al., 2009), and Gene Ontology (GO) classification (Harris et al., 2004), using a false discovery rate of ≤0.25 to identify significantly enriched gene sets per standard methods (Subramanian et al., 2005).

Table 2
Demographics for elderly humans used for postmortem hippocampal tissue analysis

Patient	Age	Sex	MMSE	Group	Postmortem interval (h)
H1	79	Female	29	Intact	1.75
H2	93	Female	30	Intact	2.25
H3	81	Male	29	Intact	2.83
H4	86	Female	27	Impaired	2.48
H5	90	Female	24	Impaired	2.5
H6	59	Male	17	Impaired	5.85

Key: MMSE, mini-mental state examination score (maximum score indicating normal cognition = 30).

Functions of *Hp1bp3* that have been identified include regulation of chromatin structure (Dutta et al., 2014), gene expression (Garfinkel et al., 2015b), cell cycle progression (Dutta et al., 2014), and insulin signaling (Garfinkel et al., 2015a), several of which were supported by our analyses. For example, positive correlates of *Hp1bp3* were significantly enriched both in nuclear localization (Fig. 6A) and insulin signaling (Fig. 6B). In addition, we found *Hp1bp3* gene correlates enriched for terms related to plasma membrane localization and for functions related to neuronal function and excitability, including voltage-gated channel activity, ion channel activity, and G-protein coupled receptor activity (Fig. 6C). Together, these results support the hypothesis that *Hp1bp3* may critically influence expression of ion channels and receptors located in the plasma membrane that mediate neurogenesis, neuronal excitability, and plasticity; all of which are mechanisms that are disrupted in hippocampal neurons of aged rodent models with cognitive deficits (Burke and Barnes, 2006; Kaczorowski and Disterhoft, 2009; Mattson and Magnus, 2006). Finally, *Hp1bp3* gene correlates also show significant overlap with genes differentially expressed between AD patients and controls (Blalock et al., 2004; Fig. 6D), suggesting the role of Hp1bp3 in cognitive aging may extend into the regulation of cognitive deficits observed in AD.

4. Discussion

The goal of this study was to use a genetically diverse panel of mice to identify genetic factors involved in the regulation of cognitive aging that may have gone undetected in either complex human studies or murine studies utilizing only a single genetic background. Aging is a leading risk factor for age-associated dementias such as AD, and our work and others suggest that genetic factors and mechanisms underlying biological processes during midlife play a key role in determining an individual's susceptibility or resilience to transitioning between healthy brain aging and pathological brain aging (Douaud et al., 2013; Jack et al., 2013; Kaczorowski et al., 2011; Keller, 2006; Miller et al., 2008). Thus, there is a critical need to understand gene variants that play a role in determining memory capabilities at this midlife transition point. To directly address this need, and to overcome some of the barriers inherent to human studies, we turned to a well-characterized murine GRP, which allows for the exploitation of phenotypic heterogeneity across a population while exerting precise control of environmental conditions.

4.1. *Identification and validation of* Hp1bp3 *as top positional candidate modulating cognitive aging*

By combining forward and reverse murine genetic approaches and by joint analysis of mouse and human cohorts, we progressed from the identification of a significant QTL containing variants regulating CFM at midlife to the demonstration that our top positional candidate, *Hp1bp3*, is important for hippocampus-dependent long-term CFM and spatial working memory. Knockout of *Hp1bp3* has been associated with viability and growth abnormalities (Garfinkel et al., 2015b) that may affect behavior. Therefore, we examined the behavior of *Hp1bp3* KO mice on 3 tasks that may confound CFM testing (e.g., baseline exploratory activity, postshock reactivity, and acquisition of contextual fear). We found no

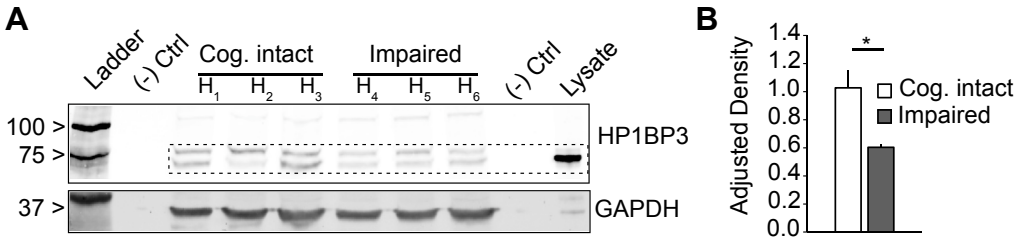

Fig. 5. HP1BP3 protein is correlated with memory status in aging humans. (A) Western blot shows hippocampal HP1BP3 protein is reduced in cognitively impaired elderly humans (n = 3, mini-mental state examination [MMSE] = 22.6 ± 3.0, age = 84 ± 4 years) relative to cognitively intact controls (n = 3, MMSE = 29.3 ± 0.3, age = 78 ± 10 years). (B) Quantification of hippocampal HP1BP3 protein (cog. intact = 1.02 ± 0.21 adjusted density, impaired = 0.60 ± 0.04, t(1,4) = −3.344, $p = 0.03$). *$p < 0.05$.

differences between the *Hp1bp3* KO and WT mice on these measures, suggesting that the effects of *Hp1bp3* KO reported herein are not due to gross behavioral abnormalities. Other positional candidates identified in the CFM QTL, such as *Pink1* and *Kif17* (Table 1), have been linked to cognitive deficits (Roberson et al., 2008) and neurodegeneration (Moisoi et al., 2014) and contain variants that could impact gene function or expression. Here, we focused on genetic correlates of CFM expressed in the hippocampus due to the

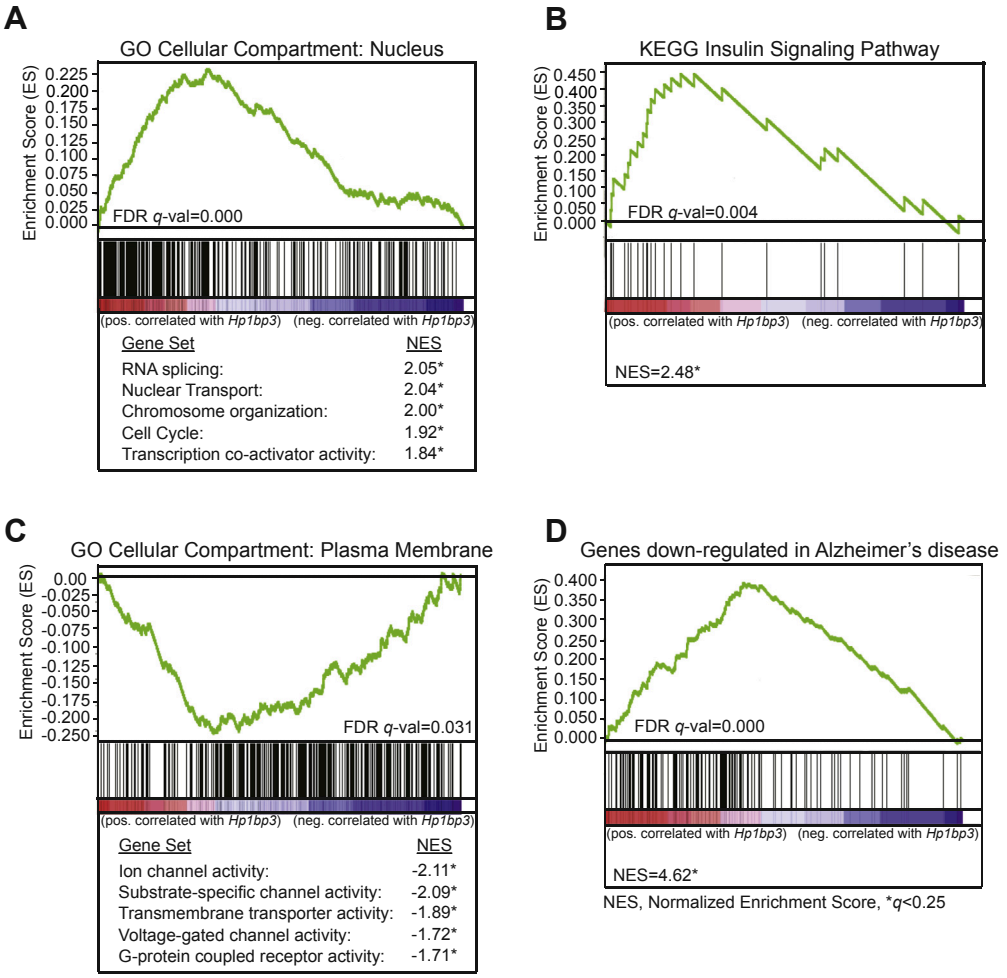

Fig. 6. Gene set enrichment analysis. All nominally significant hippocampal correlates (uncorrected $p \leq 0.05$, n = 2074, see Supplementary Table 2) of *Hp1bp3* were extracted from whole-genome transcriptome data from BXD strains age-matched to those used for contextual fear conditioning. The genes were sorted based on correlation coefficient, with the most highly positively correlated genes at the top of the list. Graphs illustrate calculation of enrichment score or deviation from random distribution throughout the preranked list. Each vertical black line below the graph represents a gene set member and its position in the preranked list (red = genes positively correlated with *Hp1bp3*, blue = genes negatively correlated with *Hp1bp3*). A false discovery rate (FDR) cutoff of $q \leq 0.25$ was used to identify significantly enriched sets according to established methods. (A, top) Positive correlates were significantly enriched for localization to the nucleus. (A, bottom) Genes localized to the nucleus were also present in significantly enriched functional gene sets including RNA splicing, nuclear transport, chromosome organization, cell cycle, and transcription cofactor activity. Normalized enrichment score represents enrichment score after adjustment to account for size of the given gene set. (B) Positive correlates were significantly enriched for the KEGG pathway corresponding to insulin signaling. (C, top) Negative correlates of *Hp1bp3* were significantly enriched for localization to the plasma membrane. (C, bottom) Genes localized to the plasma membrane were also present in significantly enriched functional gene sets including ion channel activity, substrate-specific channel activity, transmembrane transporter activity, voltage-gated channel activity, and G-protein–coupled receptor activity. (D) Correlates of *Hp1bp3* also show significant overlap with a set of genes determined by Blalock et al. (2004) to be significantly differentially expressed between AD patients and corresponding controls. Abbreviation: GO, gene ontology.

hippocampal-dependent nature of contextual fear conditioning (Chen et al., 1996) and the fact that the hippocampus is one of the first structures affected in aging (Burke and Barnes, 2006; Gant et al., 2006). However, given that the formation and recall of CFM involves a distributed network of brain regions (Tovote et al., 2015), it is possible that altered gene expression in other regions (e.g., prefrontal cortex) may also contribute to variation in cognitive aging. As variation in *Hp1bp3* genotype was estimated to account for ~20%–25% of the heritable variation in CFM at midlife, it is possible that additional genes, acting alone or in combination with *Hp1bp3*, also influence some of the observed variation in cognitive decline, and further investigation into these candidates is warranted.

4.2. Hp1bp3 *likely plays conserved role in humans*

Reduced expression of *Hp1bp3* is observed in both cognitively impaired aged mice and humans, suggesting that decreased expression of *Hp1bp3* contributes to cognitive aging in both species. In support of this idea, *Hp1bp3* is among the top 100 genes upregulated in response to metformin hydrochloride (Lamb et al., 2006), a drug known to enhance memory in mice (Wang et al., 2012), which was recently approved for clinical trial to prevent or reduce effects of aging, including cognitive decline (Targeting Aging with Metformin, TAME study). In addition, *Hp1bp3* is highly conserved in mammals, with 93% similarity between the human and murine primary sequences (Garfinkel et al., 2015b) indicating *Hp1bp3* likely plays an important functional role in both species. Deletion of the region of the human genome syntenic to our QTL results in a condition known as deletion 1p36 syndrome (Battaglia et al., 2008). Although specific phenotypes vary according to size and location of deletion breakpoint (Gajecka et al., 2007), many with this syndrome exhibit cognitive deficits and mental retardation (Battaglia et al., 2008), supporting the idea that genes in this area are necessary for cognitive function in both humans and animals.

4.3. *Gene set enrichment analyses highlight putative functional roles for* Hp1bp3

Hp1bp3 has been shown to be evolutionarily and structurally related to the linker histone H1 family, members of which confer higher-order organization to chromatin by binding to the surface of nucleosomes (Garfinkel et al., 2015b). A number of studies suggest this family of proteins plays a highly specific role in gene expression, possibly because of their role in chromatin organization (Garfinkel et al., 2015b). It is thought that *Hp1bp3* contributes to the interconversion of heterochromatin and euchromatin (Dutta et al., 2014), thereby activating or silencing specific genes as needed. As long-term memory and cognition depend on de novo gene expression in response to learning and/or training event (Cavallaro et al., 2002), it is possible that *Hp1bp3* is regulating the transcription of specific genes necessary for successful cognitive function. Genes significantly positively correlated with *Hp1bp3* were enriched for localization in the nucleus, with functions including transcription and RNA processing. Genes negatively correlated with *Hp1bp3* include genes with known links to cognition and neuron function, such as channel and transporter activity (Fig. 6C). In addition, genes effected by *Hp1bp3* knockdown in cell lines are known to have important neuronal and/or memory functions in vivo, including the regulation of neuronal excitability (Averaimo et al., 2014; Gulledge et al., 2013; Richards et al., 2007), Ca^{2+} homeostasis (Jia et al., 2015), synaptic plasticity (Lee et al., 2012), and inhibitory neurotransmission (Marsden et al., 2007) in the hippocampus (e.g., Ca^{2+} ATPase, Na^{+}/K^{+} ATPase, CLCC1 and CLIC channels, and GABARAP). However, our gene set enrichment analysis results do not differentiate whether changes in nuclear or plasma membrane proteins occur first, so it is possible that compensatory changes in transcription and RNA processing are occurring due to aging-induced alterations in plasma membrane ion channels and receptors. A targeted in vivo knockdown of *Hp1bp3* and subsequent gene expression analysis will help to clarify the role of *Hp1bp3* in gene expression under physiological conditions.

5. Conclusion

Here, we demonstrate for the first time that *Hp1bp3* is a key modulator of cognitive aging. In addition, although the BXD family has previously been used to study cognition in young mice (Philip et al., 2010; Wehner et al., 1997), but *Hp1bp3* had not emerged as a key regulator of CFM, our results suggest that variation in *Hp1bp3* genotype may influence memory in an age-dependent manner. As biological processes regulating memory function at midlife may play a critical role in the development of AD dementia (Douaud et al., 2013; Jack et al., 2013; Kaczorowski et al., 2011; Keller, 2006; Miller et al., 2008) and *Hp1bp3* gene correlates also show significant overlap with genes differentially expressed between AD patients and controls (Blalock et al., 2004; Fig. 6D), we speculate that treatments that restore *Hp1bp3* expression and/or function may improve cognition in patients with normal cognitive decline as well as AD dementia. This prediction is further supported by evidence that metformin hydrochloride, a drug known to increase *Hp1bp3* expression (Lamb et al., 2006), has been shown to enhance memory in mice (Wang et al., 2012) and is approved for an anti-aging clinical trial in humans (Targeting Aging with Metformin, TAME study). Overall, our results suggest *Hp1bp3* and related networks may serve as potential targets against cognitive aging and demonstrate the utility of genetically diverse mouse models for the study of complex human disease.

Disclosure

The authors have no conflicts of interest to disclose.

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the article. All authors reviewed and contributed intellectually to the article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2016.06.008.

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