Identification of PSMB5 as a genetic modifier of fragile X–associated tremor/ataxia syndrome

Stephen Warren, Emory University
Jorge Juncos, Emory University
Emily Allen, Emory University
Stephanie Sherman, Emory University
Peng Jin, Emory University
David Cutler, Emory University
Thomas Wingo, Emory University
Zhaohui Qin, Emory University
Michael Epstein, Emory University
Michael Zwick, Emory University

Only first 10 authors above; see publication for full author list.

Journal Title: Proceedings of the National Academy of Sciences of the United States of America

Volume: Volume 119, Number 22

Publisher: (publisher) | 2022-05-31, Pages e2118124119-e2118124119

Type of Work: Article

Publisher DOI: 10.1073/pnas.2118124119

Permanent URL: https://pid.emory.edu/ark:/25593/w440k

Final published version: http://dx.doi.org/10.1073/pnas.2118124119

Accessed July 8, 2023 2:09 AM EDT
Identification of PSMB5 as a genetic modifier of fragile X–associated tremor/ataxia syndrome

Ha Eun Kong,† Junghwa Lim, Alexander Linsalata, Yunhee Kang, Indranil Malik, Emily G. Allen, Yiqiu Cao, Lisa Shubecka, Rich Johnston, Yiqu Cao, Lisa Shubecka, Rich Johnston, Junting Huang, Yanghong Gu,† Xiangxue Guo, Michael E. Zwick, Zhaozhi Qin, Thomas S. Wingo, Jorge Junco, David L. Nelson, Michael P. Epstein, David J. Cutler, Peter K. Todd, Stephanie L. Sherman, Stephen T. Warren, and Peng Jin

Our current understanding of the pathogenesis of FXTAS stands by two main proposed mechanisms: RNA toxicity and repeat-associated non-AUG (RAN) translation. The RNA toxicity mechanism of FXTAS pathogenesis results from sequestration of key RNA binding proteins to the expanded CGG repeats, preventing them from performing their normal physiological function (10). The RNA-binding proteins that have been shown to be sequestered include the heterogeneous nuclear ribonucleoprotein (hnRNP A2/B1), which results in alteration of dendritic transport upon sequestration via the CGG repeats, and Pur α, which plays a significant role in DNA replication, neuronal messenger RNA (mRNA) transport, and translation as well as Sam68, TDP43, and the DiGeorge syndrome critical region 8 protein, DGC88 (11–15).

The second mechanism of FXTAS pathogenesis is via RAN translation of the CGG repeats into polypeptides, the predominant species being FMRpolyG (16, 17). First discovered in the CAG repeat expansion in human spinocerebellar ataxia type 8 and myotonic dystrophy type 1 (18), RAN translation has since been shown to play a potentially pathogenic role in amyotrophic lateral sclerosis (ALS)/frontotemporal dementia (FTD) as well as FXTAS (19). Recently, Sellier et al. have shown that FMRpolyG interacts with the nuclear lamina protein LAP2β and that the mechanism of pathogenesis may act through the resulting perturbation of the lamina architecture (16). Both FMR1 mRNA and FMRpolyG peptide have been found in the human postmortem brain inclusions.

Significance

Expansion of 55–200 CGG repeats in the 5′ untranslated region of FMR1 predisposes carriers to fragile X–associated tremor/ataxia syndrome (FXTAS), a late-onset neurodegenerative disorder. FXTAS demonstrates incomplete penetrance, which strongly suggests the presence of genetic modifiers. We performed whole-genome sequencing (WGS) on male premutation carriers (CGG<200) and prioritized candidate variants to screen for candidate genetic modifiers using a Drosophila model of FXTAS. We found 18 genes that genetically modulate CGG-associated neurotoxicity in Drosophila, such as Probeta5 (PSMB5), pAbp (PABPC1L), e(y1) (TAF9), and CG14231 (OSGEP1). Among them, knockdown of Probeta5 (PSMB5) suppressed CGG-associated neurodegeneration in the fly as well as in N2a cells. Interestingly, an expression quantitative trait locus variant in PSMB5, PSMB5e11543947:A, was found to be associated with decreased expression of PSMB5 and delayed onset of FXTAS in human FMR1 premutation carriers. Finally, we demonstrate that PSMB5 knockdown results in suppression of CGG neurotoxicity via both the RAN translation and RNA-mediated toxicity mechanisms, thereby presenting a therapeutic strategy for FXTAS.
that are characteristic of FXTAS pathology, suggesting that both mechanisms may contribute to the disease (17, 20).

Both mouse and fly models have been developed to study the molecular and genetic basis of FXTAS (16, 21–24). Our group has previously established a Drosophila model of FXTAS that expresses the premutation CGG repeat in the context of the human FMR1 5′UTR. Owing to its advantages of rapid reproduction and affordability as well as facile genetics, the FXTAS Drosophila model has been instrumental in showing that the premutation CGG repeats are sufficient to cause FXTAS pathology, as well as in the identification of hnrRNPA2B1 and Purα as RNA binding proteins sequestered by the CGG premutation expansion (11, 15, 22). Expression of r(CGG)90 under the eye-specific driver in Drosophila, gmr-GAL4, leads to a rough eye phenotype in the fly, characterized by cell death, loss of pigmentation, and ommatidial disruption (22).

Here, by combining whole-genome sequencing (WGS) with genetic screening using Drosophila model, we have identified 18 genes as potential genetic modifiers of FXTAS. We show that knockdown of one of the identified genetic modifiers, Proteasome subunit beta-type 5 (PSMB5), ameliorates CGG-associated neurotoxicity in Drosophila as well as in mammalian cells. Through the PSMB5 expression quantitative trait locus (eQTL) variant PSMB5rs11543947-A, we demonstrate a correlation between decreased PSMB5 mRNA expression and delayed onset of FXTAS in human premutation carriers. Finally, we show that both mechanisms of FXTAS pathogenesis—RNA toxicity and RAN translation—may account for the suppression of CGG-associated toxicity in FXTAS upon lowering the expression of PSMB5. Our works suggest that using FXTAS Drosophila as a genetic screening tool can be powerful in the validation of candidate genes from WGS of FXTAS premutation carriers and controls, and consistent with previous studies, we have identified a role for the proteasome in FXTAS pathogenesis (25–27).

Results

Combination of WGS and Drosophila Screen Identifies 18 Genes as Genetic Modifiers of FXTAS. With the goal of identifying genetic modifiers of FXTAS, we recruited and collected whole blood samples from 108 FMR1 premutation carriers for WGS (SI Appendix, Fig. S1 and Table S1). Following our pipeline outlined in Materials and Methods, we mapped, called, and annotated the variants using PEMapper, PECaller, and Bystro (28, 29). Given our limited statistical power, instead of taking a statistical approach for candidate gene selection, we employed a carefully curated criteria to select candidate genes (Fig. 1A), prioritizing variants based on combined annotation dependent depletion (CADD) score (31), phenotype severity of the premutation carriers harboring the variant, and any reported interaction of the gene with known ataxia genes as well as existence of Drosophila orthologs and RNA interference (RNAi) lines (Materials and Methods) (30).

As a result, we selected 97 genes to screen in the FXTAS Drosophila model by knocking down the candidate gene in the FXTAS fly eye using the UAS-GAL4 system, then screening for enhancement or suppression of the rough eye phenotype (Fig. 1A and SI Appendix, Table S2). The corresponding RNAi lines were obtained from the Transgenic RNAi Project (TRIP) (32). Out of the 97 candidate genes screened, 18 genes exhibited genetic modulation of CGG toxicity, such as Probeta5 (human PSMB5), pabp (human PABPC1L), e(1)y1 (human TAF9), and CGI4231 (human OSGEPL1) (Fig. 1B and C and SI Appendix, Table S2).

The eQTL Variant PSMB5rs11543947-A Is Associated with Decreased Expression of PSMB5 mRNA and Correlates with Delayed Onset of Ataxia and Tremor in Human Premutation Carriers. Proteasome Subunit Beta-5 (PSMB5) was selected for the Drosophila screen based on a variant (PSMB5rs11543947-A) that we found in our FMR1 premutation carrier population (Fig. 2A). PSMB5rs11543947-A has a high CADD score of 34, indicating that it is predicted to be among the top 0.1% deleterious variants in the genome (Fig. 1B) (31). Intriguingly, as data from the Genotype-Tissue Expression Project (GTEx) indicate, PSMB5rs11543947-A is also an eQTL associated with decreased expression of PSMB5 mRNA across a vast spectrum of tissues (SI Appendix, Fig. S2). In cortex tissue from the brain, we found that on average, individuals harboring the variant allele of PSMB5rs11543947-A demonstrate decreased expression of PSMB5 (Fig. 2B). In addition, although PSMB5rs11543947-A was in Hardy–Weinberg equilibrium in our population, we were interested to find that in the comparison of premutation carriers exhibiting delayed onset of both tremor and ataxia with premutation carriers with early onset, PSMB5rs11543947-A was associated with delayed onset of both core phenotypes of FXTAS: tremor and ataxia (Fig. 2C). Early onset was defined as onset of both tremor and ataxia before age 65, and delayed onset was defined as the absence of both tremor and ataxia above age 69 (SI Appendix, Table S1). We further examined the expression of PSMB5 in both FXTAS mouse model and FMR1 premutation-derived neural progenitor cells and did not observe the differential expression (SI Appendix, Fig. S3 A and B). Based on these findings, we hypothesized that premutation carriers with a lower baseline expression of PSMB5 may be protected against the CGG-associated neurodegeneration associated with the premutation expansion in FMR1.

Knockdown of Proteasome Subunit beta-5 (PSMB5) Suppresses rCGG-Associated Neurodegeneration in Drosophila. We set out to test our hypothesis by taking advantage of the UAS-GAL4 system to modulate the expression of the fly ortholog of PSMB5 (Probeta5) in the FXTAS Drosophila eye. We observed strong suppression of CGG-associated neurodegeneration upon Probeta5 knockdown in the 90-CGG fly (Fig. 2D and SI Appendix, Table S2 and Fig. S3C). Conversely, overexpression of Probeta5 resulted in inversion of the effect, such that the fly eyes exhibited enhancement of the rough eye phenotype (Fig. 2D). Knockdown of Probeta5 in the wild-type (WT) fly eye resulted in enhancement of the rough eye phenotype, suggesting that knockdown of Probeta5 is inherently toxic in the absence of CGG repeats. However, interestingly, in the background of CGG-associated neurotoxicity, knockdown of Probeta5 ameliorates the toxicity (Fig. 2D). Taken together, the genetic evidence in the fly and human premutation carriers suggests that the PSMB5 expression could modulate CGG repeat-induced neuronal toxicity and the knockdown of PSMB5 may ameliorate of CGG-induced neurodegeneration.

Amelioration of CGG Toxicity Is Specific to PSMB5. Intrigued by these findings, we wanted to verify whether the amelioration of CGG-associated neurotoxicity is specific to PSMB5 knockdown or whether it also involves other subunits of the proteasome complex. The 26S proteasome consists of the 20S core particle and the 19S regulatory particle (RP) (33). Therefore, we tested all genes related to the 20S core particle subunits and the 19S regulatory particle that have fly orthologs as well as Drosophila TRIP lines available for screening (SI Appendix, Table S3). Thirty-five out of 41 human genes that encode
components of the 26S complex were screened based on the presence of fly orthologs and availability of TRIP lines. Four of the 35 genes demonstrated enhancement of CGG-associated neurodegeneration upon knockdown: Prosalpha3 (PSMA4), Prosalpha6T (PSMA1), Prosalpha7 (PSMA3), and Rpn3 (PSMD3) (Fig. 3A and SI Appendix, Table S3). Over 88% of the genes tested showed no genetic modulation of the CGG-associated neurotoxicity upon knockdown, and significantly, none of the

Fig. 1. Identification of genetic modifiers of FXTAS by combining WGS with Drosophila genetic screening. (A) Schematic depicting the analysis workflow from WGS analysis to Drosophila screen. Candidate genes were selected based on the variant CADD score, inclusion in the ataxia interactome (30), and clinical phenotype data of the variant carriers as well as the existence of Drosophila orthologs and availability of RNAi lines. A total of 97 genes were selected to test for genetic modulation in FXTAS Drosophila. Eighteen genes demonstrated significant modulation of CGG-associated neurodegeneration in the FXTAS fly eye. (B) List of 18 human genes and corresponding Drosophila orthologs that demonstrated genetic modulation of CGG-associated neurodegeneration in the FXTAS Drosophila screen. The table indicates the genomic location of the variant in the premutation carrier population. Thirteen genes had variants with high CADD scores, two had indel/frameshift variants, and three genes were part of the ataxia interactome (30). (C) Drosophila screen identifies 18 genetic modifiers of CGG-associated neurotoxicity. Representative light microscopy (first and third rows) and SEM images (second and fourth rows) from Drosophila of the indicated genotypes crossed to gmr-GAL4, UAS-(CGG)90 EGFP (first and second rows) or gmr-GAL4 flies (third and fourth rows) as control. After performing the crosses, progeny were collected and aged to 7 d. The screen was performed by scoring eye phenotype in crosses with n > 10 progeny, which was visualized using light microscopy and confirmed with SEM. Representative images are shown. RNAi knockdown of Ap-1gamma, CG14231, CG4393, ci, pAbp, lark, and e(y)1 results in enhancement of CGG-associated neurodegeneration.

PNAS 2022 Vol. 119 No. 22 e2118124119 https://doi.org/10.1073/pnas.2118124119
screened genes showed suppression of CGG-associated toxicity upon knockdown. Our results clearly suggest that the alleviation of CGG toxicity is specific to Prosbeta5, and knockdown of other subunits of the Drosophila proteasome complex did not recapitulate the suppression of CGG toxicity seen in Prosbeta5 knockdown.

Psmb5 Knockdown and Inhibition Alleviate CGG-Associated Toxicity in Mammalian Neuronal Cells. To further test this hypothesis in a mammalian model system, we transfected murine Neuro2A cells with the 5'UTR CGG 99x FMR1-EGFP plasmid expressing 99 CGG repeats in the context of the human 5'UTR FMR1, in frame with EGFP, as well as an empty vector control. Notably, upon small interfering RNA (siRNA) knockdown of Psmb5, we observed a significant amelioration of CGG-associated toxicity in N2A cells after 96 h (n = 3 independent replicates; Fig. 3B and SI Appendix, Fig. S3D). Consistent with the finding in the flies, knocking down Psmb5 in Neuro2A cells transfected with empty vector control resulted in decreased cell viability (Fig. 3B).

We further validated whether this amelioration of CGG-associated toxicity is specific to Psmb5 using a US Food and Drug Administration (FDA)–approved selective inhibitor of Psmb5, ixazomib citrate (34). We administered 0.25 nM of ixazomib citrate to Neuro2A mammalian cells following transfection of the 5'UTR CGG 99x FMR1-EGFP plasmid expressing...
CGG repeats in the context of the human 5′UTR (SI Appendix, Fig. S4). In Neuro2A cells treated with dimethyl sulfoxide (DMSO) after expression of the rCGG repeats, only ∼50% of control cells survived after 84 h (n = 3 independent replicates) (Fig. 3C). However, upon administration of ixazomib citrate for 84 h, we observed a significant alleviation of CGG-associated toxicity with 85% of cells surviving (Fig. 3C). Intriguingly, the administration of MG-132, another 26S proteosome inhibitor that blocks the proteolytic activity of the 26S proteosome complex, did not result in the reduced CGG-associated toxicity, suggesting that PSMB5 could act in a manner that is independent of the 26S proteosome (Fig. 3C).

**Knockdown of PSMB5 Significantly Diminishes RAN Translation in a Frame-Independent Manner.**

Given that our genetic findings of CGG toxicity alleviation upon PSMB5 knockdown were recapitulated in the Drosophila as well as in Neuro2A cells through various methods, we sought a mechanistic explanation and asked whether knockdown of PSMB5 alters RAN translation. RAN translation has been shown to play a potentially pathogenic role in FXTAS, with the predominant species being FMR1PolyG (16, 17). To test this, we utilized a set of transflectable, plasmid-based nanoluciferase (NL) reporters (36) for canonical and RAN translation. The reporter for canonical translation, AUG-NL-3xF, comprises a short, unstructured 5′UTR; an AUG-initiated NL open reading frame; and a C-terminal 3xF tag (3xF) tag to enable detection by Western blotting. The reporter for RAN translation, AUG-NL-3xF, initiates within the 5′UTR of the FMR1 mRNA, then upon sequestration of DGCR8 to the expanded CGG repeat. Based on a database of high-throughput data such as cross-linking and immunoprecipitation sequencing (CLIP-seq), Ribosome sequencing (Ribo-seq), and RNA sequencing (RNA-seq) POST-trAnscriptional Regulation database 2 (POSTAR2), we found that PSMB5 mRNA has been shown to be bound by DGC8R8, an RNA binding protein already reported to be sequestered by the FMR1 premutation repeats (14, 38).

When PSMB5 was knocked down by siRNAs against PSMB5, we saw a decline in canonical translation but a significantly steeper decline in RAN translation (Fig. 4A and SI Appendix, Fig. S5). Furthermore, we observed that the decline in RAN translation is frame-independent; knockdown of PSMB5 resulted in significant decline of RAN translation in both the +1 and +2 frames (Fig. 4B).

**Knockdown of PSMB5 May Ameliorate CGG-Associated Toxicity by Alleviating the Effects of DGCR8 Sequestration to the Expanded FMR1 Premutation CGG Repeat.**

Besides RAN translation, we also determined whether the alleviating effects of PSMB5 knockdown may be associated with the sequestration of RNA binding proteins to the expanded CGG repeat. Based on a database of high-throughput data such as cross-linking and immunoprecipitation sequencing (CLIP-seq), Ribosome sequencing (Ribo-seq), and RNA sequencing (RNA-seq) POST-trAnscriptional Regulation database 2 (POSTAR2), we found that PSMB5 mRNA has been shown to be bound by DGC8R8, an RNA binding protein already reported to be sequestered by the FMR1 premutation repeats (14, 38).

We hypothesized that if DGC8R8 normally binds PSMB5 mRNA, then upon sequestration of DGC8R8 to the expanded CGG repeats, we may be able to observe a significant decline of PSMB5 mRNA bound to DGC8R8 (Fig. 4C). To test this, we transfected HEK 293T cells with a plasmid expressing FLAG-tagged human DGC8R8. Forty-eight hours later, we subsequently transfected the cells with either a plasmid expressing rCGG99 in the context of the human FMR1 5′UTR or an empty vector control (Fig. 4D). After 24 h, we performed immunoprecipitation for DGC8R8 using anti-FLAG antibody and found that upon expression of the FMR1 premutation CGG repeats, the level of PSMB5...
mRNA bound to DGCR8 dropped significantly (Fig. 4E). As a positive control, we also tested the levels of the small nuclear RNA (snoRNA) U16, a well-validated target of DGCR8 (Fig. 4E). Overall, these findings lend support to the possibility that DGCR8 plays a role in PSMB5 mRNA processing that is perturbed upon sequestration of DGCR8 to the CGG repeats (Fig. 4 D and E).

**Discussion**

FXTAS is a late-onset neurodegenerative disorder caused by the 55 to 200 expanded CGG repeats in the 5’ UTR of *FMR1*. Fragile X premutation carriers have an estimated prevalence of 1:250 women and 1:800 men (7–9). It is not at all understood why only some premutation carriers develop FXTAS; ∼40% of males and 16% of females develop FXTAS in late adulthood (1, 2). The incomplete penetrance of FXTAS suggests the presence of genetic modifiers. Here we performed WGS on male premutation carriers (CGG<sub>55–200</sub>) and demonstrate that using FXTAS as a genetic screening tool can be powerful in the validation of candidate genes discovered through WGS. We report 18 candidate genes as genetic modifiers of FXTAS using this approach, which can serve as promising candidates for biomarker discovery and therapeutic development.

The pathogenesis of neurodegenerative diseases is commonly linked to defects in the degradation of misfolded proteins. Three mechanisms promote effective removal of misfolded proteins, namely, the ubiquitin (Ub)-proteasome system (UPS), chaperone-mediated autophagy, and macroautophagy (39). In many neurodegenerative diseases such as Huntington’s disease, Parkinson’s disease (PD), Alzheimer’s disease (AD), and ALS, the pathology stems from misfolded aggregates that are resistant to degradation via these mechanisms, and their accumulation particularly affects postmitotic neurons (40). Capitalizing on this pathologic mechanism, therapeutic strategies on many neurodegenerative diseases have been designed to augment the degradation of protein aggregates by further activating the mechanisms of protein clearance (41–43). In FXTAS, activation of proteolytic mechanisms has yet...
to be implemented as a therapeutic approach. However, previous studies have indicated the potential of UPS as a therapeutic target for FXTAS (26). In Drosophila, impairment of β2 and β6 subunits of the proteasome were shown to enhance CGG-associated ommatidial degeneration (26), whereas overexpression of Hsp70, a molecular chaperone that assists in refolding, was shown to suppress the neurotoxicity even in the absence of misfolded proteins (22). In addition to the UPS, many have shown that activating autophagy via rapamycin may rescue neurodegenerative phenotypes (44, 45). However, contrary to other neurodegenerative disorders, previous reports clearly demonstrate that in FXTAS, activating autophagy via rapamycin actually enhances the CGG-associated neurodegeneration in Drosophila (26, 46). Instead, activating the mTOR pathway, rather than inhibiting mTOR via rapamycin, ameliorated CGG-associated neurotoxicity in Drosophila. These findings were the first indication that although FXTAS is a neurodegenerative disease with a protein accumulation problem, the pathogenic mechanism may differ significantly from the other neurodegenerative pathologies, which could be alleviated via rapamycin.

We report that while knockdown of most components of the 26S proteasome resulted in enhancement or no change in CGG-associated neurodegeneration, knockdown of PSMB5, one of the core catalytic subunits of the 26S proteasome complex, significantly ameliorates CGG-induced neurodegeneration in various models—as demonstrated in Drosophila, mammalian cells, as well as a correlation of delayed onset FXTAS phenotypes in human premutation carriers. Importantly, this study has investigated both potential mechanisms of FXTAS toxicity to explain this finding. We have shown in mammalian cells that RAN translation is significantly diminished by knockdown of PSMB5, although excessive knockdown of PSMB5 will even affect global translation. We have also shown that at the mRNA level, the level of PSMB5 mRNA bound to DGCGR8 is significantly diminished in the presence of the premutation CGG repeats, suggesting that upon sequestration of DGCGR8 to the CGG repeats, the role of DGCGR8 in the regulation of PSMB5 mRNA may be perturbed (14).

Our findings have significant value for the development of future therapeutics as well as potential biomarkers for disease prognosis. Although not a curative strategy, our data from Drosophila and mammalian cells suggest that modestly diminishing PSMB5 expression may be a promising therapeutic approach to delay the onset of a debilitating neurodegenerative disorder. Several FDA-approved proteasome inhibitors are readily available, such as bortezomib (Velcade), carfilzomib (Kyprolis), and ixazomib citrate (Ninlaro), the first oral proteasome inhibitor approved by the FDA (47, 48). According to the evidence presented in this study, low-dose inhibition of PSMB5 at the protein level, via ixazomib citrate, may be a potential therapeutic strategy for the treatment of FXTAS.

We hypothesize based on our model that in the setting of the expanded CGG repeats in FXTAS, CGG-mediated sequestration of DGCGR8 may diminish DGCGR8-mediated regulation of PSMB5 mRNA, resulting in pathology. Future experiments will be necessary to explore the mechanism behind the DGCGR8 processing of PSMB5 mRNA and CGG-associated toxicity. Our observations may also point toward a potential unique role of the PSMB5 subunit, especially in RAN translation. To the best of our knowledge, studies have yet to explore potential independent roles of the proteasome subunits, including PSMB5. Given our findings, it would be valuable to further investigate any potential noncanonical roles of PSMB5 and to explore its role in RAN translation.

It remains to be seen whether manipulating the RNA expression or protein level of PSMB5 may also ameliorate neurotoxicity in other neurodegenerative diseases, especially repeat-associated disorders, or whether this is a unique mechanism in FXTAS. Notably, we have recently begun to uncover additional disorders that are associated with CGG expansions. Recent studies have identified novel associations of CGG/GCC repeat expansions in LOC642361/NUTM2B-AS1 and LRP12 with oculopharyngeal myopathy with leukoencephalopathy and oculopharyngodistal myopathy, respectively (49). In addition, GCC repeat expansions have been associated with neuronal intranuclear inclusion disease (NIID) (50) as well as a larger family of NIID-related disorders (NIIDRD) that include AD and PD (51). Our identification of PSMB5 as a key player in the modification of CGG-associated neurodegeneration may have implications for these NIID, NIIDRD, and ocular disorders caused by CGG/GCC repeat expansions.

Our finding that PSMB5 is enriched in premutation carriers with delayed onset of FXTAS was limited in statistical significance due to the small sample size. Attaining adequate statistical power to identify such variants that are not independently causative but may predispose or protect against a disease remains a challenge in the study of rare diseases. In light of these limitations, future studies are needed to further validate the potential of PSMB5 as a biomarker for disease prognosis in FXTAS. Nonetheless, combining our findings among premutation carriers with those from model systems provides strong evidence to continue with this line of investigation.

In conclusion, we have demonstrated that knockdown of PSMB5 suppresses CGG-associated neurodegeneration in Drosophila as well as in a mammalian in vitro model. Inhibiting the β5 subunit at the protein level by the proteasome inhibitor ixazomib citrate also suppresses the CGG-generated toxicity in vitro. Further, we have shed light on a possible mechanism of the suppression of CGG-associated neurotoxicity, via significant suppression of RAN translation. Last, through WGS, we have identified PSMB5 as a variant that has high potential as a biomarker for delayed onset of disease. The association between PSMB5 and delayed onset of FXTAS aligns well with the molecular findings in various model systems due to its role as an eQTL that correlates with decreased expression of PSMB5. Taken together, our data suggest that PSMB5 offers a promising therapeutic target for alleviating the CGG-associated neurodegeneration in FXTAS.

Materials and Methods

Study Population. The protocols and consent forms were approved by the Institutional Review Board at Emory University, and written informed consent was obtained from all subjects (IRB00074941). Subjects were identified from previous FX research projects at Emory, through recruitment efforts at scientific conferences, and through collaborations with other research groups. Subjects were screened for eligibility based on premutation carrier status, presence or absence of symptoms of tremor and/or ataxia, age, and gender. Only one family member per pedigree was enrolled in the study. After a subject was determined to be eligible, either a blood or saliva sample was collected. In addition, a brief medical history was collected from the subject or a family member, and pertinent medical records were collected. If medical records did not provide sufficient information for determining eligibility, a video examination was conducted by study personnel and reviewed by a neurologist. Early onset subjects were defined as male or female premutation carriers with symptoms of tremor or ataxia before age 65, as reviewed by a neurologist. Control individuals were defined as male premutation carriers that reached age 69 without significant tremor or ataxia symptoms, as reviewed by a neurologist.
Sample Preparation for WGS. DNA was extracted from biological samples using Qiagen Giamp DNA Blood Mini Kit, Gentra Puregene extraction kit, or prepIt-L2P protocol from Oragen. Fragile site, folic acid type, rare, Fa(X)(Q27.3) A (FRAXA) CGG repeat numbers were determined by a fluorescent sequencing method (52). For male samples that did not amplify or females with only one allele, a second PCR protocol was used (53). The PCR for FRAXA consisted of 1× PCR Buffer (Gibco/BRL), 10% DMSO, 370 μM deazaG, 500 μM d(ACT), 0.3 μM each primer, 15 ng T4 gene 32, and 1.05 U Roche Expand Long Taq. Primers for the FRM1 gene were C, 5’CG5GCTAGCTCGTTGTGGTTTCTAGCTTCCG3’; and F, 5’AGCCCCGGACTTACCGAGTCCCTCCCA3’ (54). Samples were stored and sent in batches for WGS at Hudson Alpha.

WGS. For HiSeq X Ten sequencing, DNA samples were normalized to 1,000 ng DNA in 50 μL water, sheared to ~350 to 400 bp fragments with a Covaris LE-220 instrument, and end-repaired and A-tailed using New England Biolabs End-Enzyme and A-Tailing kits, under the manufacturer’s recommended conditions. Following each step, the library was purified via Agencourt AMPure XP beads and eluted in water. Standard Illumina paired-end adapters were ligated to the A-tailed DNA via New England Biolabs Rapid Ligation kit, purified using AMPure XP beads, and amplified with KAPA Biosystems HIFI PCR kit using six cycles of PCR. The primers were standard Illumina primers with a custom seven-base sample barcode in the i7 position. The final library was quality controlled using size verification via Perki-

Drosophila. Screen. Transgenic flies expressing r(CGG)90 were previously described (22). The GMR-GAL4 and UAS-TRIP lines were obtained from Bloomington Stock Center. All Drosophila lines were maintained, and crosses were performed in standard medium at 25 °C. Following the crosses, progeny were collected and aged to 7 d. The screen was performed by scoring eye phenotype in progeny with n > 10, which was visualized using light microscopy and confirmed with scanning electron microscopy (SEM).

SEM. Following dehydration in increasing concentrations of ethanol (25, 50, 75, and 100%), whole flies were incubated for 1 h with hexamethyldisilazane (Elec-

Cell Culture, Transfection, and Cell Viability Assays. For transfection of FLAG-DGR8, HEK293T cells were plated in Dulbecco’s Modified Eagle Medium. After 24 h, cells were transfected with pFLAG/HA-DGCR8 (Addgene no. 10921) according to manufacturer instructions. Forty-

Immunoprecipitation. The immunoprecipitation (IP) protocol for FLAG-DGR8 was based on a described protocol with modifications (55). HEK293T cells transfected with pFLAG/HA DGCR8 (Addgene no. 10921) were grown in 60-mm plates. Forty-eight hours after transfection, cells were washed with ice-cold PBS, then scraped and centrifuged at 1,000 rpm for 5 min at 4 °C. For each immunopre-

 Luciferase Assays and Western Blotting. HEK293 (CRL-1573; American type culture collection [ATCC]) cells were cultured and passed at 37 °C, 5% CO2 in DMEM supplemented with 10% FBS without antibiotics. For luciferase assays, HEK293 cells were plated on 96-well plates at 2.0 × 104 cells per well in 100 μL media and reverse transfected with ON-TARGET siRNAs against human PSMB5 (nos. 1 and 2; J-004522-05-0002, J-004522-06-0002) or nontargeting controls (D-001810-10; Dharmacon) at 15 μM using Lipofectamine RNAiMAX (ThermoFisher Scientific). In brief, siRNA and RNAiMAX were diluted in Opti-

For Western blotting experiments, HEK293 cells were plated in 12-well plates at 2 × 105 cells per well in 1 mL media and reverse transfected, as described above, with siRNAs at 15 μM. Forty-eight hours after plating, cells were transfected with 800 ng per well pcDNA3.1(+)-XL.3x Flag using Vivafect (Promega). Luciferase assays were performed 24 h after plasmid transfection, as described by Kneze et al. (36) and Green et al. (37).

8 of 10  https://doi.org/10.1073/pnas.2118124119
(final concentration 0.5 mg/mL) for 2 h at 4 °C, rotating, to elute the protein from the beads. For RNA extraction, 200 μL 1x RO1 RNase-free DNase buffer was added to each tube, and the samples were treated with RO1 RNase-free DNase (Promega; 6106) according to the manufacturer’s instructions. SDS was added to yield a final concentration of 1%, then the samples were treated with proteinase K for 30 min at 55 °C, flicking the tube occasionally using a finger. Total RNA was extracted using Phenol:Chloroform:Isomyl Alcohol 25:2:1 (P3803; Sigma). For Western blotting, samples were denatured in 2x Laemml Sample Buffer (Bio-Rad) at 95 °C, separated by SDS-PAGE on Mini-PROTEAN TGX Precast Mini Gels (Bio-Rad), transferred onto 0.2 μm PVDF membrane by Trans-Blot blotting System and Mini Transfer Pack (Bio-Rad), and probed with primary antibodies against FLAG (1:4,000, mouse pAb, F1804; Sigma) at 4 °C overnight. Primary antibodies were labeled by horseradish peroxidase (HRP)-linked secondary anti-mouse antibody (1:4,000; Cell Signaling Technology). The enhanced chemiluminescent signals were detected using HyGLO Quick Spray Chemiluminescent HRP Antibody Detection Reagent (Thomas Scientific) and were visualized by the Chemidoc Touch Imaging System (Bio-Rad). For real-time PCR, cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen) using random hexamer primers, and real-time PCR was performed with primers in SI Appendix, Table S4.

Data Availability. Genomic data have been deposited in NIMH Data Archive (https://nda.nih.gov/edit_collection?item_id=2380). All other study data are included in the article and/ or Appendix S1.
49. H. Ishiura et al., Noncoding CGG repeat expansions in neuronal intranuclear inclusion disease, oculopharyngodistal myopathy and an overlapping disease. Nat. Genet. 51, 1222–1232 (2019).

50. J. Sone et al., Long-read sequencing identifies GGC repeat expansions in NOTCH2NL associated with neuronal intranuclear inclusion disease. Nat. Genet. 51, 1215–1221 (2019).

51. Y. Tian et al., Expansion of human-specific GGC repeat in neuronal intranuclear inclusion disease-related disorders. Am. J. Hum. Genet. 105, 166–176 (2019).

52. K. L. Meadows et al., Survey of the fragile X syndrome and the fragile X E syndrome in a special education needs population. Am. J. Med. Genet. 64, 428–433 (1996).

53. W. T. Brown et al., Rapid fragile X carrier screening and prenatal diagnosis using a nonradioactive PCR test. JAMA 270, 1569–1575 (1993).

54. Y. H. Fu et al., Variation of the CGG repeat at the fragile X site results in genetic instability: Resolution of the Sherman paradox. Cell 67, 1047–1058 (1991).

55. S. Macias et al., DGCRR8-HITS-CLIP reveals novel functions for the Microprocessor. Nat. Struct. Mol. Biol. 19, 760–766 (2012).