

A yeast functional screen predicts new candidate ALS disease genes

Julien Couthouis^{a,1}, Michael P. Hart^{a,1}, James Shorter^{b,1}, Mariely DeJesus-Hernandez^c, Renske Erion^a, Rachel Oristano^a, Annie X. Liu^a, Daniel Ramos^{a,d}, Niti Jethava^{a,d}, Divya Hosangadi^{a,e}, James Epstein^{a,f}, Ashley Chiang^{a,g}, Zamia Diaz^b, Tadashi Nakaya^h, Fadia Ibrahim^h, Hyung-Jun Kimⁱ, Jennifer A. Solski^j, Kelly L. Williams^{j,k}, Jelena Mojsilovic-Petrovic^l, Caroline Ingre^m, Kevin Boylanⁿ, Neill R. Graff-Radfordⁿ, Dennis W. Dickson^c, Dana Clay-Falcone^{h,o}, Lauren Elman^p, Leo McCluskey^p, Robert Greene^{h,o}, Robert G. Kalb^l, Virginia M.-Y. Lee^{h,o}, John Q. Trojanowski^{h,o}, Albert Ludolph^q, Wim Robberecht^r, Peter M. Andersen^m, Garth A. Nicholson^{j,k}, Ian P. Blair^{j,k}, Oliver D. King^s, Nancy M. Bonini^l, Vivianne Van Deerlin^{h,o}, Rosa Rademakers^c, Zissimos Mourelatos^h, and Aaron D. Gitler^{a,2}

Departments of ^aCell and Developmental Biology, ^bBiochemistry and Biophysics, ^cPathology and Laboratory Medicine, and ^dNeurology, and ^eCenter for Neurodegenerative Disease Research, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; Departments of ^fNeuroscience and ^gNeurology, Mayo Clinic, Jacksonville, FL 32224; ^hJ. R. Masterman Laboratory and Preparation School, Philadelphia, PA 19130; ⁱCentral Bucks High School East, Doylestown, PA 18902; ^jRadnor High School, Radnor, PA 19087; ^kConestoga High School, Berwyn, PA 19312; ^lDepartment of Biology and the Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, PA 19104; ^mNorthcott Neuroscience Laboratory, ANZAC Research Institute, Sydney 2139, NSW, Australia; ⁿSydney Medical School, University of Sydney, Sydney 2006, NSW, Australia; ^oDepartment of Pediatrics, Division of Neurology, Abramson Research Center, Children's Hospital of Philadelphia, Philadelphia, PA 19104; ^pDepartment of Neurology, Umeå University, SE-901 87 Umeå, Sweden; ^qDepartment of Neurology, University of Ulm, 89081 Ulm, Germany; ^rVIB Vesalius Research Center and Laboratory of Neurobiology, Department of Neurology, Katholieke Universiteit Leuven, B - 3000 Leuven, Belgium; and ^sBoston Biomedical Research Institute, Watertown, MA 02472

This Feature Article is part of a series identified by the Editorial Board as reporting findings of exceptional significance.

Edited by Gregory A. Petsko, Brandeis University, Waltham, MA, and approved October 13, 2011 (received for review June 13, 2011)

Amyotrophic lateral sclerosis (ALS) is a devastating and universally fatal neurodegenerative disease. Mutations in two related RNA-binding proteins, TDP-43 and FUS, that harbor prion-like domains, cause some forms of ALS. There are at least 213 human proteins harboring RNA recognition motifs, including FUS and TDP-43, raising the possibility that additional RNA-binding proteins might contribute to ALS pathogenesis. We performed a systematic survey of these proteins to find additional candidates similar to TDP-43 and FUS, followed by bioinformatics to predict prion-like domains in a subset of them. We sequenced one of these genes, *TAF15*, in patients with ALS and identified missense variants, which were absent in a large number of healthy controls. These disease-associated variants of *TAF15* caused formation of cytoplasmic foci when expressed in primary cultures of spinal cord neurons. Very similar to TDP-43 and FUS, *TAF15* aggregated in vitro and conferred neurodegeneration in *Drosophila*, with the ALS-linked variants having a more severe effect than wild type. Immunohistochemistry of postmortem spinal cord tissue revealed mislocalization of *TAF15* in motor neurons of patients with ALS. We propose that aggregation-prone RNA-binding proteins might contribute very broadly to ALS pathogenesis and the genes identified in our yeast functional screen, coupled with prion-like domain prediction analysis, now provide a powerful resource to facilitate ALS disease gene discovery.

In the future, personalized genome sequencing will become routine, empowering us to define the genetic basis of many human diseases. Currently, however, complete genome sequencing for individuals to discover rare pathogenic mutations is still too costly and time consuming. Thus, more creative approaches are needed to accelerate the discovery of disease genes. Moreover, even once genes are revealed, the need for innovative approaches to elucidate causality remains critical.

ALS, also known as Lou Gehrig's disease, is a devastating adult-onset neurodegenerative disease that attacks upper and lower motor neurons (1). A progressive and ultimately fatal muscle paralysis ensues, usually causing death within 2–5 y of disease onset. ALS is mostly sporadic, but ~10% of cases are familial. Pathogenic mutations in several genes have been linked to familial and sporadic ALS, including *SOD1*, *TARDBP*, *FUS/TLS*, *VAPB*, *OPTN*, *VCP*, and others (2). Two of these genes, *TARDBP* (TDP-43) and *FUS/TLS* (FUS) are notable because they encode related RNA-binding proteins that harbor a prion-like domain (3–6). Moreover, both of these proteins have been identified as components of pathological inclusions in neurons of patients with ALS (7–9). Indeed, an

emerging concept suggested by the association of FUS and TDP-43 to ALS is that defects in RNA metabolism might contribute to disease pathogenesis. These observations suggested an intriguing possibility: Could TDP-43 and FUS be just the tip of an iceberg? In other words, could other human RNA-binding proteins with properties similar to those of TDP-43 and FUS also contribute to ALS?

Here we report a simple yeast functional screen, followed by bioinformatics to predict prion-like domains, to identify human proteins with similar properties to TDP-43 and FUS. We then identify mutations in human patients with ALS in one gene from this screen, *TAF15*, which ranks with the highest prion-like domain score after FUS. Importantly, we show that *TAF15* has similar in vitro and in vivo properties to TDP-43 and FUS. Moreover, the ALS-associated *TAF15* mutations are more aggregation prone in vitro, have a more severe effect on lifespan than WT when expressed in *Drosophila*, and increase cytoplasmic mislocalization in mammalian spinal cord neurons. The identification of mutations in an additional RNA-binding protein harboring a prion-like domain further underscores a key role for RNA metabolism defects in ALS and suggests that this class of aggregation-prone RNA-binding proteins might contribute very broadly to ALS and perhaps other related neurodegenerative disorders.

Results

Yeast Screen to Identify RNA-Binding Proteins with Properties Similar to TDP-43 and FUS. TDP-43 and FUS are both RNA recognition motif (RRM)-containing proteins (RRM proteins) (3) and both

Author contributions: J.C., M.P.H., J.S., M.D.-H., T.N., F.I., H.-J.K., G.A.N., I.P.B., N.M.B., V.V.D., R.R., Z.M., and A.D.G. designed research; J.C., M.P.H., J.S., M.D.-H., R.E., R.O., X.A.L., D.R., N.J., D.H., J.E., A.C., Z.D., T.N., F.I., H.-J.K., J.A.S., K.L.W., D.C.-F., L.E., L.M., R.G., N.M.B., V.V.D., R.R., Z.M., and A.D.G. performed research; J.M.-P., C.I., K.B., N.R.G.-R., D.W.D., R.G.K., V.M.-Y.L., J.Q.T., A.L., W.R., and P.M.A. contributed new reagents/analytic tools; J.C., M.P.H., J.S., M.D.-H., R.E., Z.D., T.N., F.I., H.-J.K., I.P.B., O.D.K., N.M.B., V.V.D., R.R., Z.M., and A.D.G. analyzed data; and A.D.G. wrote the paper.

Conflict of interest statement: A.D.G. is an inventor on patents and patent applications that have been licensed to FoldRx Pharmaceuticals.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

¹J.C., M.P.H., and J.S. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: gitler@mail.med.upenn.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1109434108/-DCSupplemental.

Prion-Like Domains in TDP-43, FUS, and Several Other RRM Proteins.

To focus this list further, we used a bioinformatics approach. In addition to the RRM domain, FUS and TDP-43 share a glycine-rich domain and a bioinformatics-predicted prion-like domain (4). Like prion domains found in fungal prion proteins (e.g., Sup35, Ure2, and Rnq1), these domains are enriched in uncharged polar amino acids (such as asparagine, glutamine, and tyrosine) and glycine (14). In TDP-43, the predicted prion-like

domain overlaps the glycine-rich domain; in FUS, a QGSY-rich region defines the prion-like domain, although there is some overlap with the glycine-rich domain. The prion-like domain is a shared feature that may be important, given the “prionoid” aggregation propensity of many proteins associated with human neurodegenerative disease (15). Moreover, the prion-like domains of TDP-43 and FUS are critical for the aggregation of these proteins (10–13, 16). Remarkably, using an algorithm to score 21,873

Table 1. Human RRM proteins with similar properties to FUS and TDP-43 when expressed in yeast

Name	Description	Toxicity score (1–4)	Prion domain score	Prion domain rank
BOLL	Boule-like (<i>Drosophila</i>)	2		
CPSF6	Cleavage and polyadenylation-specific factor	2.5		
DAZ1	Deleted in azoospermia 1	2.5	14	143
DAZ2	Deleted in azoospermia 2	3	14	143
DAZ3	Deleted in azoospermia 3	3.5	15	136
DAZAP1	DAZ-associated protein 1	2	12	198
ELAVL1	Embryonic lethal, abnormal vision-like 1	1		
ELAVL2	Embryonic lethal, abnormal vision-like 2	1		
ELAVL3	Embryonic lethal, abnormal vision-like 3	2.5		
ELAVL4	Embryonic lethal, abnormal vision-like 4	1		
ENOX1	Ecto-NOX disulfide-thiol exchanger 1	2.5		
EWSR1	Ewing sarcoma breakpoint region 1	3.5	32	25
FUS	Fusion (involved in malignant liposarcoma)	1.5	38	13
G3BP1	Ras-GTPase-activating protein	2		
HNRNPA0	Heterogeneous nuclear ribonucleoprotein	1	21	81
HNRNPA1	Heterogeneous nuclear ribonucleoprotein	1	28	38
HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein	1	30	32
HNRNPM	Heterogeneous nuclear ribonucleoprotein	3		
IGF2BP2	IGF-II mRNA-binding protein 2	2.5		
IGF2BP3	IGF-II mRNA-binding protein 3	2.5		
MSI2	Musashi homolog 2	2		
RALYL	RNA-binding protein-like	2.5		
RBM12B	RNA-binding motif protein	3.5		
RBM14	RNA-binding motif protein	2	16	117
RBM4	RNA-binding motif protein	3		
RBM41	RNA-binding motif protein	2.5		
RBM4B	RNA-binding motif protein	2.5		
RBM5	RNA-binding motif protein	3		
RBM9	RNA-binding motif protein	3.5		
RBMS1	RNA-binding motif, single-stranded interacting protein	2		
RBMS2	RNA-binding motif, single-stranded interacting protein	2		
RBPM5	RNA-binding motif, single-stranded interacting protein	3		
ROD1	Regulator of differentiation	1		
SNRPA	Small nuclear ribonucleoprotein polypeptide	2		
SNRPB2	Small nuclear ribonucleoprotein polypeptide	2		
TAF15	TATA box binding protein (TBP)-associated factor	3.5	33	22
TARDBP	TAR DNA-binding protein (TDP-43)	1.5	27	43
TIA1	Cytotoxic granule-associated RNA binding protein	2	23	55

Thirty-eight human RRM proteins that formed cytoplasmic aggregates and were toxic when expressed in yeast are shown. Toxicity was scored from 1 (most toxic) to 4 (not toxic). Prion domain score, based on ref. 14, indicates the maximum log-likelihood for prion-like amino acid composition vs. non-prion-like amino acid composition in any 60 consecutive aa window contained in a region parsed as prion-like by the hidden Markov model. No prion score indicates that no region of length ≥ 60 was parsed as prion-like. Prion domain rank is from 21,873 human proteins.

potentially damaging variants from benign variants (see below). Thus, nonsynonymous missense variations in *TAF15* were detected in 4 of 735 North American ALS cases and 1 of 1,328 North American controls. We also identified a fifth variant (M368T; Fig. 2E) in an independent cohort of 351 Swedish patients with ALS and a sixth variant (G452E; Fig. 24) in a cohort of 176 Australian patients with ALS. Because the *TAF15* variants were identified in sporadic ALS cases, familial evidence for segregation with disease was not possible. Notably, however, *TARDBP* and *FUS* mutations have also been confirmed in apparent sporadic ALS cases (20). In addition, the parents of the affected individuals were not available to determine whether the mutations occurred de novo or were inherited.

ALS-Specific *TAF15* Variants Form Cytoplasmic Foci in Spinal Cord Neurons. To provide evidence that the specific sequence variants that we found in ALS cases might be pathogenic, we performed an unbiased assessment of all nonsynonymous variants identified in cases and controls. We designed an assay that potentially discriminates functionally deleterious variants from benign variants. ALS-linked mutations in *TDP-43* and *FUS* have been shown to disrupt protein localization, leading to enhanced accumulation of ALS-linked variants in cytoplasmic structures (8, 9, 18, 21–23). Given this common feature, we assessed the effects of the *TAF15* variants (Fig. 24) on subcellular localization. We transfected myc-tagged WT or the mutant forms of *TAF15* into spinal cord neurons cultured from rat embryos (Fig. 3A). The transfection efficiency in these primary neuronal cultures was too low to quantify protein expression levels; however, immunoblotting of HEK293T cells transfected with these same constructs confirmed that the WT and variant constructs are expressed at similar levels (Fig. 3C). WT or mutant *TAF15* expression under these conditions did not result in

toxicity; however, we did observe significant effects of the variants on localization.

The transfected WT and mutant *TAF15* proteins were restricted to the nucleus in ~40% of cells and present in both nucleus and cytoplasm in ~60% of cells. The *TAF15* mutations did not affect nuclear localization per se, but rather promoted the formation of cytoplasmic foci. WT *TAF15* and the R388H variant, which was present in both patients and controls, localized to the nucleus and cytoplasm, in a diffuse pattern (Fig. 3A and B). In contrast, all three of the ALS-linked mutant forms of *TAF15* (G391E, R408C, and G473E) found in the North American patients with ALS showed a striking accumulation of *TAF15* in cytoplasmic foci in dendrites and axons (Fig. 3A and B). Thus, all of the *TAF15* variants that we found in patients with ALS were deleterious; that is, they caused mislocalization of the protein to cytoplasmic foci within neurons, whereas the one variant found in controls was benign and behaved like WT. This unbiased assessment of all variants provides strong support for the variants that we found in ALS cases as being risk factors for ALS (3 damaging *TAF15* variants of 735 ALS cases vs. 0 damaging *TAF15* variants of 1,328 controls, $P = 0.0451$, Fisher's exact test). The fourth ALS-specific variant (M368T; Fig. 2E), found in an independent cohort of 351 Swedish patients with ALS, also affected localization in this assay (Fig. 3A and B), but we do not include this variant in the above statistical assessment because we did not sequence an equivalent number of matched controls. The fifth ALS-specific variant (G452E; Fig. 24), in 1 Australian patient with ALS of a cohort of 176 patients with ALS, was not present in 72 sequenced Australian controls or in our collection of 1,328 sequenced North American controls. We have not yet tested the effects of this variant on *TAF15* localization in our functional assay. Unlike several of the ALS-linked *FUS* mutations, which are located in the conserved PY motif at the extreme C terminus of the

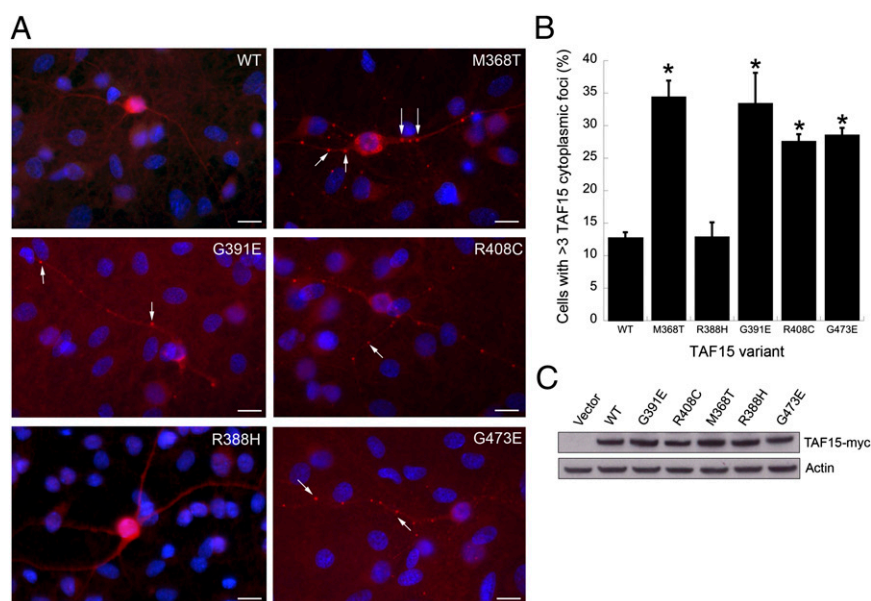


Fig. 3. A functional assay to distinguish potentially damaging *TAF15* variants from benign variants. We performed an unbiased assessment of all *TAF15* missense variants identified from sequencing of ALS cases (M368T, G391E, R408C, and G473E) and controls (R388H). (A) Primary rat embryonic neuron cultures were transfected with myc-tagged WT or mutant *TAF15* and stained with α -myc (red). Transfection of WT *TAF15* or the R388H variant found in controls results in localization within the nucleus and cytoplasm of neurons in a diffuse pattern. In contrast, the ALS-linked mutant forms of *TAF15* showed a striking accumulation of cytoplasmic foci (arrows) in dendrites and axons. (Scale bar, 20 μ m.) (B) Quantitation of transfected WT or mutant *TAF15* that accumulates in cytoplasmic puncta. Four of four *TAF15* variants found in ALS cases (M368T, G391E, R408C, and G473E) showed cytoplasmic puncta formation whereas the one variant found in both cases and controls (R388H) behaved like WT. * $P < 0.01$ (cytoplasmic puncta formation of *TAF15* variants compared with WT, Student's t test). Error bars show mean \pm SEM. (C) Because the transfection efficiency in the primary rat spinal cord neuron cultures was not high enough to detect *TAF15* overexpression by immunoblot, we transfected the same constructs of WT and *TAF15* mutants into HEK293T cells. Immunoblotting with α -myc antibodies was used to detect myc-tagged *TAF15* and α -actin antibody was used as a loading control. In contrast to the primary neuronal cultures, we did not observe a significant difference in aggregation between WT *TAF15* and the variants in HEK293T cells.

weight (Fig. 4A), we found that TAF15 rapidly aggregated at 25 °C with gentle agitation (Fig. 4B and C). Turbidity and sedimentation analysis revealed that TAF15 aggregated with kinetics similar to FUS and slightly more rapidly than TDP-43, assessed by

turbidity (Fig. 4B). If TEV protease was omitted, then little aggregation occurred (Fig. 4B). Electron microscopy studies established that TAF15 rapidly accessed oligomeric forms (Fig. 4D), which would frequently adopt a pore-like conformation [Fig. 4D]

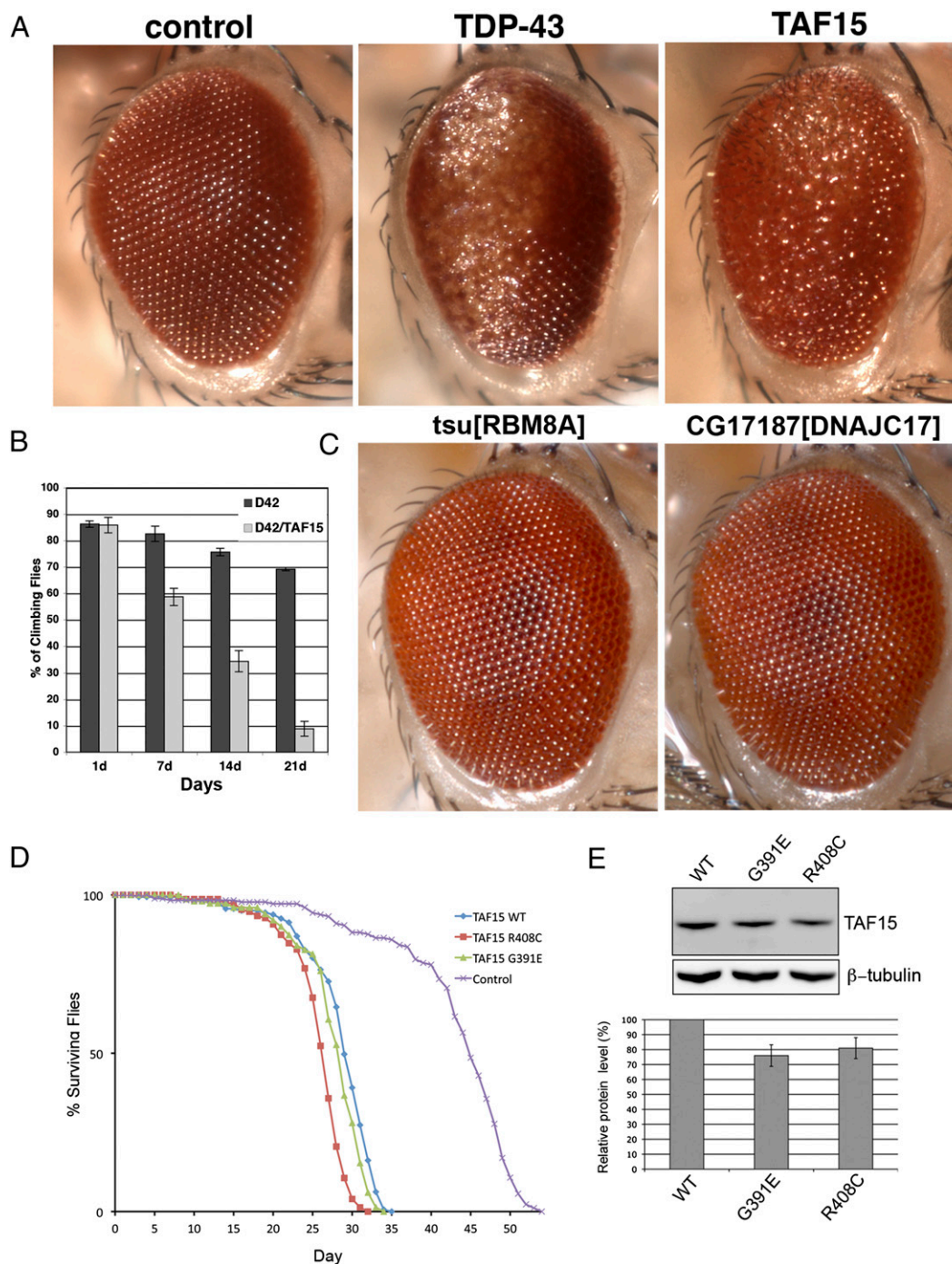


Fig. 5. TAF15 confers neurodegeneration and dysfunction in *Drosophila*. (A) Toxicity of RRM proteins in the eye. TAF15 causes degeneration and disruption of the retinal structure, akin to TDP-43 (also see ref. 24). Control is driver line alone, *gmr-GAL4/+*. TDP-43 is *gmr-GAL4/UAS-TDP-43-YFP*. TAF15 is *gmr-GAL4/UAS-TAF15* (grown at 29 °C). (B) Progressive loss of climbing behavior upon expression of TAF15 selectively in motor neurons using the motor neuron-specific D42-GAL4 driver. (C) Up-regulation of two other RRM proteins does not cause neurodegeneration in *Drosophila*. As a specificity control for the neurodegenerative phenotype conferred by up-regulation of TDP-43 and TAF15 in *Drosophila*, we tested the effects of up-regulating the fly homologs of two other human RRM proteins in the eye. Up-regulation of *tsu* and *CG17187*, which are *Drosophila* homologs of human genes *RBM8A* and *DNAJC17*, respectively, did not cause neurodegeneration. (D) Expression of TAF15 in the nervous system reduces lifespan (blue, compared with normal in purple). Up-regulation of TAF15 variants G391E and R408C causes more rapid death (red and green, compared with WT TAF15 in blue). (E) Immunoblot showing TAF15 WT and mutant expression levels in transgenic flies. β -Tubulin levels were used as loading control.

(small arrows) and *E*], similar to those formed by TDP-43 and FUS (Fig. 4 *D* and *E*) (11, 12), which are reminiscent of pathological oligomers formed by α -synuclein and amyloid- β (32). Furthermore, TAF15 also assembled into linear polymers with a cross-sectional diameter of ~ 15 – 20 nm (Fig. 4*D*, large arrows) that increased in length over time and would often become tangled into large masses by 60 min (Fig. 4*D*). In general, the morphology of TAF15 aggregates was more similar to FUS (12) than to TDP-43 (11), which over this time frame formed shorter polymers that would clump together to form large masses (Fig. 4*D*) (11). Importantly, a human RRM protein, DND1, which did not aggregate and was not toxic in yeast (Fig. 1 *C* and *D*), remained soluble and did not aggregate in this in vitro assay, providing evidence that in vitro aggregation is not a property shared by all RRM proteins. Thus, similar to TDP-43 and FUS, and concordant with the yeast data, TAF15 is an inherently aggregation-prone protein.

It is likely that sophisticated cellular proteostasis mechanisms, not recapitulated with this in vitro assay, mitigate rapid TAF15 aggregation in vivo. However, age-associated decline in these quality control measures, along with environmental triggers (for example, injury or exposure to toxins), might allow TAF15 to aggregate in disease. In vitro aggregation assays similar to these have been tremendously powerful tools in defining basic mechanisms underpinning TDP-43 and FUS aggregation (11, 12) as well as the aggregation events in Parkinson's disease and Alzheimer's disease (33–35).

ALS-Specific Variants Accelerate TAF15 Aggregation in Vitro. Next, we tested two of the ALS-linked mutant forms of TAF15 (G391E and R408C) as well as the variant that we also found in controls (R388H) in this aggregation assay (Fig. 4*F*). We noticed that if we omitted agitation during the in vitro aggregation reaction, we were able to slow down the aggregation process, allowing us to detect potential differences in aggregation between WT and mutant TAF15 proteins. R388H aggregated with similar kinetics to WT TAF15 (Fig. 4*G*). By contrast, the two ALS-linked mutants, G391E and R408C, aggregated with more rapid kinetics (Fig. 4*G*). This increased aggregation propensity might help explain why ALS-linked mutant TAF15 is more prone to accumulate in cytoplasmic inclusions in spinal cord neurons (Fig. 3) and supports the hypothesis that these are likely pathogenic variants.

TAF15 Up-Regulation Causes Neurodegeneration in *Drosophila* and ALS-Linked Mutants Have More Severe Effect on Lifespan. To extend these findings from in vitro to in vivo, and analyze the effects of TAF15 in the nervous system, we used *Drosophila*. We and others have previously shown that directing TDP-43 or FUS expression to the fly nervous system causes neurodegeneration (24–28, 30, 31). A series of transgenic lines were generated that expressed WT and mutant human TAF15. Directing expression of TAF15 to the eye of the fly caused degeneration of the structure (Fig. 5*A*) and led to progressive loss of motility when directed to motor neurons (Fig. 5*B*). As for the in vitro aggregation assay (Fig. 4), the effect was specific because two other unrelated *Drosophila* RRM proteins, the human counterparts of which did not aggregate and were not toxic in yeast, did not confer neurodegeneration when up-regulated in *Drosophila* (Fig. 5*C*). Thus, TAF15 possesses activity sufficient to confer neurodegeneration in the nervous system, in a manner similar to that of TDP-43 and FUS. TAF15 also induced a markedly shortened lifespan when expressed in the nervous system (Fig. 5*D*). Notably, expression of ALS-linked TAF15 variants G391E and R408C, inserted at the same genomic location to ensure similar protein expression (Fig. 5*E*), had a more severe effect, resulting in an even shorter lifespan than that of flies expressing WT TAF15 (Fig. 5*D*), supporting the notion that these missense variants in TAF15 are potentially deleterious.

TAF15 Is Mislocalized in Motor Neurons of Patients with ALS. We next performed immunohistochemistry on human postmortem spinal cord tissue to determine whether TAF15 was present in motor neurons and whether its localization was affected in ALS, as for TDP-43 and FUS. TAF15 was robustly expressed in spinal cord motor neurons and localized to the nucleus, as for TDP-43 and FUS (Fig. 6*A*). We next examined TAF15 localization in autopsy tissue from three sporadic ALS cases (without mutations in *TAF15*), all with confirmed TDP-43 pathology. Immunostaining of spinal cord sections from these patients with ALS revealed significant cytoplasmic staining in a punctate granular pattern (Fig. 6 *B–H*), which did not colocalize with TDP-43 inclusions. In contrast to TDP-43 (7), and similar to FUS (8, 9), we did not observe significant nuclear clearing of TAF15. The strong punctate granular localization for TAF15 was not seen in any of the control cases that we examined ($n = 3$). Because these sporadic ALS cases that we examined also contained prominent TDP-43 cytoplasmic inclusions, which did not colocalize with TAF15, future studies will be required to extend these initial findings and to determine whether TAF15 mislocalization in disease is an early initiator or rather a later consequence of motor neuron degeneration. Thus, TAF15 is expressed in a disease-relevant cell type and can be mislocalized to the cytoplasm in ALS, further supporting the notion that TAF15 can contribute to pathogenesis. Notably, a recent study has also observed TAF15 mislocalization in neurons and glial cells of patients with frontotemporal lobar degeneration (FTLD)-FUS (36).

Discussion

In an effort to streamline the identification of new ALS genetic risk factors, we devised a simple yeast functional screen to define additional RNA-binding proteins with properties shared by the known ALS disease genes FUS and TDP-43. This screen resulted in the enrichment of 38 proteins that behave like FUS and TDP-43 in yeast (cytoplasmic inclusions and toxicity), 13 of which contain a predicted prion-like domain (Table 1). Indeed, the combination of yeast screen and prion prediction algorithm enabled us to significantly focus our list of candidate genes ~ 10 -fold. As evidence of the usefulness of this approach to define genes with a role in ALS, we identified patient-specific missense variants in one of these genes, *TAF15*, in five unrelated patients with ALS (three variants from our initial cohort of North

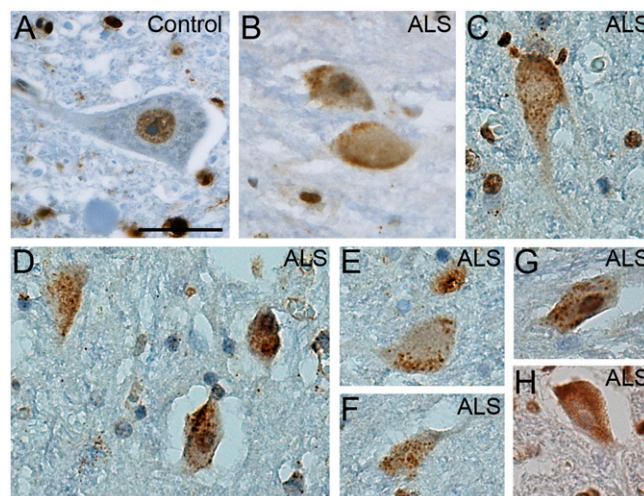


Fig. 6. TAF15 accumulates in the cytoplasm in some sporadic spinal cord neurons of patients with ALS. (*A*) Immunohistochemistry for TAF15 in a post-mortem control spinal cord neuron indicates nuclear localization, whereas, in addition to nuclear localization, TAF15 accumulated in cytoplasmic puncta of spinal cord neurons of patients with ALS (*B–H*). (Scale bar, 25 μ m.)

American Caucasian patients with ALS, a fourth variant from a cohort of Swedish patients with ALS, and a fifth variant from a cohort of Australian patients with ALS). Further, we provide in vitro and in vivo evidence that TAF15 has functional properties similar to those of TDP-43 and FUS: It is intrinsically aggregation prone and can confer neurodegeneration in *Drosophila* and the ALS-linked variants can increase aggregation in vitro, decrease lifespan in *Drosophila*, and alter protein subcellular localization in spinal cord neurons. Although familial segregation could not be assessed, the absence of the variants in a large number of healthy controls, the shared structural evidence with known ALS genes, and functional in vitro and in vivo data strongly support the notion that these variants in TAF15 represent pathogenic disease mutations for ALS.

Future studies will be required to determine the relative contribution of TAF15 variants to ALS risk compared with known genetic risk factors such as TDP-43, FUS, SOD1, and others. Our initial analyses with TAF15 in patients with ALS and control populations, as well as recent studies by Ticozzi and colleagues with TAF15 and the related gene EWSR1 (37), suggest that if indeed TAF15 mutations contribute to ALS, they will likely be rarer than FUS and TDP-43 mutations. However, as for all complicated human diseases there will very likely be common genetic contributors as well as rare genetic risk factors. For ALS, we propose that there may be a delicate balance in RNA processing within motor neurons such that slight perturbations from any one of several different aggregation-prone RNA-binding proteins could lead to neurodegeneration. An interesting additional concept that emerges from our findings is that perhaps variants in multiple RNA-binding proteins could synergize with each other to contribute to ALS. There are likely to be some variants that are extremely damaging and thus fully penetrant and aggressive on their own. Case in point: P525L and R495X mutations in FUS lead to relatively severe ALS clinical phenotypes and very early age of disease onset (18, 23), whereas other FUS mutations are less severe (e.g., R521G) (18). This result seems to be due to the effect of the mutations on FUS nuclear localization, with variants having the strongest effect on nuclear localization resulting in the earliest age of onset of ALS (18). Those aggressive FUS variants might sit at one end of a spectrum, with weaker variants at the other. Perhaps then the accumulation of multiple weaker variants in two, three, or more different aggregation-prone RNA-binding proteins (e.g., the top candidates listed in Table 1) might be required to tip the balance in RNA metabolism toward ALS. Future studies will be required to test this hypothesis and to better resolve the complexities of the ALS genetic landscape.

These findings predict that additional aggregation-prone RRM or other RNA-binding proteins, like TAF15, FUS, and TDP-43, could contribute to ALS. Notably, the prion-like domain algorithm ranked FUS and TAF15 first and second of 213 RRM proteins, respectively, and ranked TDP-43 10th. We suggest that genes ranked third through ninth should now be given top priority for genetic analysis in populations of patients with ALS, especially EWSR1, which ranked third and is a close relative of both FUS and TAF15 (38). In addition to ALS, these candidates should also be examined in related clinico-pathological disorders including FTL and inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD). For example, mutations in ALS genes TARDBP and FUS have been identified in patients with FTL and mutations in an IBMPFD gene, VCP, have been identified in patients with ALS (39–41).

Next-generation sequencing and exon capture approaches will eventually become routine in personalized medicine (42–44) and promise to identify all genetic contributors to ALS. Meanwhile, the list of ALS candidate genes that we provide here (Table 1 and Dataset S1), generated by the combination of the yeast

functional screen and prion-like domain prediction, will be a powerful resource to jumpstart efforts to identify new genetic risk factors for ALS and spur innovative new diagnostic and therapeutic approaches.

Materials and Methods

Plasmids, Yeast Strains, and Media. Strain and plasmid construction is detailed in *SI Materials and Methods*.

Human RNA-Binding Protein Plasmid Library. We constructed a library of yeast expression plasmids containing 133 unique human RRM-containing ORFs, as detailed in *SI Materials and Methods*.

Yeast Transformation and Spotting Assays. Yeast transformation and spotting assays were performed as described in ref. 10.

Microscopy. Fluorescence microscopy was performed as described in *SI Materials and Methods*.

Prion-Prediction Algorithm. Putative prion domains were predicted using a hidden Markov model as described in *SI Materials and Methods*.

Sequencing TAF15 in Patients with ALS and Controls. ALS patient and control population demographics and TAF15 mutational analysis procedures are described in *SI Materials and Methods*.

TAF15 Protein Purification and in Vitro Aggregation Assays. TDP-43 and FUS were purified as described (11, 12). WT and mutant TAF15 proteins were expressed and purified from *Escherichia coli* as GST-tagged proteins and in vitro aggregation assays were performed as described in *SI Materials and Methods*.

Drosophila Experiments. Transgenic flies expressing human TAF15 were generated by standard techniques using the pUAST vector and analyzed as described in *SI Materials and Methods*.

Rat Primary Neuron Transfection and Immunofluorescence. Primary neurons were isolated from rat embryos, cultured, and transfected as described in *SI Materials and Methods*.

Statistical Analysis. Two-tailed Fisher's exact tests were used to evaluate genetic association between TAF15 sequence variants and ALS. We tested all TAF15 nonsynonymous missense variants detected in cases and controls in an assay that potentially discriminates functionally deleterious variants from benign variants (e.g., the subcellular localization assay reported in Fig. 3), then classified the variants as deleterious or benign on the basis of their properties in this assay, and then performed a statistical comparison of the frequency of functionally deleterious variants in cases vs. controls.

Immunohistochemistry. Immunohistochemistry to detect TAF15 localization in postmortem spinal cord tissue was performed as described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank the patients and their families for their dedication and for their invaluable contributions to this research. We thank the Packard Center for ALS Research at Johns Hopkins for their generosity and collaborative spirit. We thank contributors, including the Alzheimer's Disease Centers who collected samples used in this study. We thank C. Cecere for assistance in sample collection. We thank Rose Li, Phoebe Leboy, and Marisa Bartolomei for helpful suggestions on the manuscript. This work was supported by National Institutes of Health Director's New Innovator Awards 1DP2OD004417 (to A.D.G.) and 1DP2OD002177-01 (to J.S.); National Institutes of Health Grants 1R01NS065317 (to A.D.G.), 5R21NS067354-02 (to J.S.), AG17586 (to V.V.D., J.Q.T., and R.G.), AG10124 (to V.V.D. and J.Q.T.), P01-AG-09215 (to N.M.B.), NS056070 and NS072561 (to Z.M.), T32-AG00255 [to F.I. and V.M.-Y.L. (program director)], R01 AG26251-03A1 (to R.R.), R01 NS065782 (to R.R.), and P50 AG16574 (to R.R.); the University of Pennsylvania Institute on Aging and Alzheimer's Disease Core Center Pilot Grant Program (AG10124) (to V.V.D.); the ALS Association (R.R.); a grant from the Packard Center for ALS Research at Johns Hopkins (to A.D.G. and J.S.); and an Ellison Medical Foundation New Scholar in Aging Award (to J.S.). A.D.G. is a Pew Scholar in the Biomedical Sciences, supported by The Pew Charitable Trusts, and a Rita Allen Scholar, supported by the Rita Allen Foundation. J.Q.T. is the William Maul Measey-Truman G. Schnabel, Jr., Professor of Geriatric Medicine and Gerontology. N.M.B. is an Investigator of the Howard Hughes Medical Institute. Samples from the National Cell

Repository for Alzheimer's Disease, which receives government support under a cooperative agreement grant (U24 AG21886) awarded by the National Institute on Aging, were used in this study. In Australia, the work was supported by the National Health and Medical Research Council

of Australia (1004670 and 511941) and a Peter Stearne grant from the Motor Neurone Disease Research Institute of Australia. This research was conducted while J.C. was an Ellison Medical Foundation/AFAR Post-doctoral Fellow.

- Cleveland DW, Rothstein JD (2001) From Charcot to Lou Gehrig: Deciphering selective motor neuron death in ALS. *Nat Rev Neurosci* 2:806–819.
- Van Damme P, Robberecht W (2009) Recent advances in motor neuron disease. *Curr Opin Neurol* 22:486–492.
- Lagier-Tourenne C, Cleveland DW (2009) Rethinking ALS: The FUS about TDP-43. *Cell* 136:1001–1004.
- Cushman M, Johnson BS, King OD, Gitler AD, Shorter J (2010) Prion-like disorders: Blurring the divide between transmissibility and infectivity. *J Cell Sci* 123:1191–1201.
- Udan M, Baloh RH (2011) Implications of the prion-related Q/N domains in TDP-43 and FUS. *Prion* 5:1–5.
- Gitler AD, Shorter J (2011) RNA-binding proteins with prion-like domains in ALS and FTL-D. *Prion* 5:179–187.
- Neumann M, et al. (2006) Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 314:130–133.
- Kwiatkowski TJ, Jr., et al. (2009) Mutations in the FUS/TLN gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science* 323:1205–1208.
- Vance C, et al. (2009) Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science* 323:1208–1211.
- Johnson BS, McCaffery JM, Lindquist S, Gitler AD (2008) A yeast TDP-43 proteinopathy model: Exploring the molecular determinants of TDP-43 aggregation and cellular toxicity. *Proc Natl Acad Sci USA* 105:6439–6444.
- Johnson BS, et al. (2009) TDP-43 is intrinsically aggregation-prone, and amyotrophic lateral sclerosis-linked mutations accelerate aggregation and increase toxicity. *J Biol Chem* 284:20329–20339.
- Sun Z, et al. (2011) Molecular determinants and genetic modifiers of aggregation and toxicity for the ALS disease protein FUS/TLN. *PLoS Biol* 9:e1000614.
- Ju S, et al. (2011) A yeast model of FUS/TLN-dependent cytotoxicity. *PLoS Biol* 9:e1001052.
- Alberti S, Halfmann R, King O, Kapila A, Lindquist S (2009) A systematic survey identifies prions and illuminates sequence features of prionogenic proteins. *Cell* 137:146–158.
- Aguzzi A, Rajendran L (2009) The transcellular spread of cytosolic amyloids, prions, and prionoids. *Neuron* 64:783–790.
- Furukawa Y, Kaneko K, Watanabe S, Yamanaka K, Nukina N (2011) A seeding reaction recapitulates intracellular formation of Sarkosyl-insoluble transactivation response element (TAR) DNA-binding protein-43 inclusions. *J Biol Chem* 286:18664–18672.
- Araya N, et al. (2003) Cooperative interaction of EWS with CREB-binding protein selectively activates hepatocyte nuclear factor 4-mediated transcription. *J Biol Chem* 278:5427–5432.
- Dormann D, et al. (2010) ALS-associated fused in sarcoma (FUS) mutations disrupt Transportin-mediated nuclear import. *EMBO J* 29:2841–2857.
- Lee BJ, et al. (2006) Rules for nuclear localization sequence recognition by karyopherin beta 2. *Cell* 126:543–558.
- Lagier-Tourenne C, Polymenidou M, Cleveland DW (2010) TDP-43 and FUS/TLN: Emerging roles in RNA processing and neurodegeneration. *Hum Mol Genet* 19(R1):R46–R64.
- Barmada SJ, et al. (2010) Cytoplasmic mislocalization of TDP-43 is toxic to neurons and enhanced by a mutation associated with familial amyotrophic lateral sclerosis. *J Neurosci* 30:639–649.
- Kabashi E, et al. (2010) Gain and loss of function of ALS-related mutations of TARDBP (TDP-43) cause motor deficits in vivo. *Hum Mol Genet* 19:671–683.
- Bosco DA, et al. (2010) Mutant FUS proteins that cause amyotrophic lateral sclerosis incorporate into stress granules. *Hum Mol Genet* 19:4160–4175.
- Elden AC, et al. (2010) Ataxin-2 intermediate-length polyglutamine expansions are associated with increased risk for ALS. *Nature* 466:1069–1075.
- Hanson KA, Kim SH, Wassarman DA, Tibbetts RS (2010) Ubiquitin modifies TDP-43 toxicity in a Drosophila model of amyotrophic lateral sclerosis (ALS). *J Biol Chem* 285:11068–11072.
- Li Y, et al. (2010) A Drosophila model for TDP-43 proteinopathy. *Proc Natl Acad Sci USA* 107:3169–3174.
- Lu Y, Ferris J, Gao FB (2009) Frontotemporal dementia and amyotrophic lateral sclerosis-associated disease protein TDP-43 promotes dendritic branching. *Mol Brain* 2:30.
- Ritson GP, et al. (2010) TDP-43 mediates degeneration in a novel Drosophila model of disease caused by mutations in VCP/p97. *J Neurosci* 30:7729–7739.
- Estes PS, et al. (2011) Wild-type and A315T mutant TDP-43 exert differential neurotoxicity in a Drosophila model of ALS. *Hum Mol Genet* 20:2308–2321.
- Lanson NA, Jr., et al. (2011) A Drosophila model of FUS-related neurodegeneration reveals genetic interaction between FUS and TDP-43. *Hum Mol Genet* 20:2510–2523.
- Wang JW, Brent JR, Tomlinson A, Shneider NA, McCabe BD (2011) The ALS-associated proteins FUS and TDP-43 function together to affect Drosophila locomotion and life span. *J Clin Invest* 121:4118–4126.
- Lashuel HA, Hartley D, Petre BM, Walz T, Lansbury PT, Jr. (2002) Neurodegenerative disease: Amyloid pores from pathogenic mutations. *Nature* 418:291.
- Conway KA, Harper JD, Lansbury PT (1998) Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. *Nat Med* 4:1318–1320.
- Conway KA, et al. (2000) Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: Implications for pathogenesis and therapy. *Proc Natl Acad Sci USA* 97:571–576.
- Lashuel HA, et al. (2003) Mixtures of wild-type and a pathogenic (E22G) form of Abeta40 in vitro accumulate protofibrils, including amyloid pores. *J Mol Biol* 332:795–808.
- Neumann M, et al. (2011) FET proteins TAF15 and EWS are selective markers that distinguish FTL with FUS pathology from amyotrophic lateral sclerosis with FUS mutations. *Brain* 134:2595–2609.
- Ticozzi N, et al. (2011) Mutational analysis reveals the FUS homolog TAF15 as a candidate gene for familial amyotrophic lateral sclerosis. *Am J Med Genet B Neuropsychiatr Genet* 156B:285–290.
- Tan AY, Manley JL (2009) The TET family of proteins: Functions and roles in disease. *J Mol Cell Biol* 1:82–92.
- Broustal O, et al.; French clinical and genetic research network on FTD/FTD-MND (2010) FUS mutations in frontotemporal lobar degeneration with amyotrophic lateral sclerosis. *J Alzheimers Dis* 22:765–769.
- Gitcho MA, et al. (2009) TARDBP 3'-UTR variant in autopsy-confirmed frontotemporal lobar degeneration with TDP-43 proteinopathy. *Acta Neuropathol* 118:633–645.
- Johnson JO, et al.; ITALSGEN Consortium (2010) Exome sequencing reveals VCP mutations as a cause of familial ALS. *Neuron* 68:857–864.
- Biesecker LG (2010) Exome sequencing makes medical genomics a reality. *Nat Genet* 42:13–14.
- Ng SB, et al. (2010) Exome sequencing identifies the cause of a Mendelian disorder. *Nat Genet* 42:30–35.
- Ng SB, et al. (2009) Targeted capture and massively parallel sequencing of 12 human exomes. *Nature* 461:272–276.