Inhibition of RNA lariat debranching enzyme suppresses TDP-43 toxicity in ALS disease models

Maria Armakola1,2,12, Matthew J Higgins3,12, Matthew D Figley1, Sami J Baranada4,5, Emily A Scarborough6, Zamia Diaz7, Xiaodong Fang1, James Shorter6, Nevan J Krogan3,7,8, Steven Finkbeiner4,5,9, Robert V Farese Jr4,10,11,13 & Aaron D Gitler1,13

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease primarily affecting motor neurons. Mutations in the gene encoding TDP-43 cause some forms of the disease, and cytoplasmic TDP-43 aggregates accumulate in degenerating neurons of most individuals with ALS. Thus, strategies aimed at targeting the toxicity of cytoplasmic TDP-43 aggregates may be effective. Here, we report results from two genome-wide loss-of-function TDP-43 toxicity suppressor screens in yeast. The strongest suppressor of TDP-43 toxicity was deletion of DBR1, which encodes an RNA lariat debranching enzyme. We show that, in the absence of Dbp1 enzymatic activity, intronic lariats accumulate in the cytoplasm and likely act as decoys to sequester TDP-43, preventing it from interfering with essential cellular RNAs and RNA-binding proteins. Knockdown of Dbp1 in a human neuronal cell line or in primary rat neurons is also sufficient to rescue TDP-43 toxicity. Our findings provide insight into TDP-43-mediated cytotoxicity and suggest that decreasing Dbp1 activity could be a potential therapeutic approach for ALS.

ALS, also known as Lou Gehrig’s disease, is a late-onset neurodegenerative disease characterized by loss of motor neurons, progressive weakness and eventual paralysis and death within 3–5 years of diagnosis1. Most ALS cases are sporadic (SALS), but 10% are familial (FALS), of which ~20% result from mutations in the SOD1 gene (encoding Cu/Zn superoxide dismutase 1)1,2. SOD1 mutations are thought to cause disease by a toxic gain of function1, and, thus, strategies to lower SOD1 levels are being pursued1. However, SOD1 mutations account for only a small percentage of ALS cases, and additional therapeutic strategies are needed.

RNA-binding proteins and RNA-processing pathways have recently been implicated in ALS5–6. The RNA-binding protein TDP-43 has been found in cytoplasmic inclusions in the spinal cord neurons of most ALS cases without SOD1 mutation7–8, and mutations in TARDBP, which encodes TDP-43, have been identified in FALS and SALS cases9–13. Mutations in FUS, which encodes another RNA-binding protein (FUS, also called TLS), have also been found in some ALS cases14–18. Therefore, therapies targeting TDP-43 and/or FUS could be effective in cases not caused by SOD1 mutation. Notably, TDP-43 inclusions occur in many frontotemporal dementia cases, and targeting TDP-43 might be an effective therapeutic strategy for these patients. Efforts are under way to define the mechanisms by which TDP-43 and FUS and defects in RNA processing pathways contribute to ALS.

We have used yeast models to illuminate mechanisms underpinning TDP-43 and FUS aggregation and cellular toxicity19–21. TDP-43 forms aggregates in the cytoplasm of yeast cells and is toxic, recapitulating two key features of TDP-43 relevant to human disease19. We used a genome-wide plasmid overexpression screen to identify modifiers of aggregation and toxicity. One modifier of toxicity was Ppb1, whose human homolog, ataxin 2, harbors a polyglutamine tract that is expanded (>34 glutamines) in spinocerebellar ataxia type 2. We found that intermediate-length polyglutamine expansions (~27–33 glutamines) in ataxin 2 are a genetic risk factor for ALS22,23. Additional genetic modifiers of TDP-43 toxicity in yeast might provide further insight into pathogenic mechanisms and could represent novel therapeutic targets.

Here, we report results from a genome-wide loss-of-function screen to identify yeast genes that modify TDP-43 toxicity. In our previous screen (ref. 22 and A.D.G., unpublished data), we used a library of yeast overexpression plasmids. Here, we interrogated collections of nonessential yeast knockouts and knockdowns of essential genes. Loss-of-function screens may be useful to identify therapeutic targets for which inhibitors could be developed. Among the most potent

1Department of Genetics, Stanford University School of Medicine, Stanford, California, USA. 2Neuroscience Graduate Group, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania, USA. 3Gladstone Institute of Cardiovascular Disease, J David Gladstone Institutes, San Francisco, California, USA. 4Gladstone Institute of Neurological Disease, Taube-Koret Center, Hellman Family Foundation Program, J David Gladstone Institutes, San Francisco, California, USA. 5Department of Neurology, University of California, San Francisco, San Francisco, California, USA. 6Department of Biochemistry & Biophysics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania, USA. 7Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, California, USA. 8California Institute for Quantitative Biosciences, San Francisco, California, USA. 9Department of Physiology, University of California, San Francisco, San Francisco, California, USA. 10Department of Medicine, University of California, San Francisco, San Francisco, California, USA. 11Department of Biochemistry & Biophysics, University of California, San Francisco, San Francisco, California, USA. 12These authors contributed equally to this work. 13These authors jointly directed this work. Correspondence should be addressed to A.D.G. (agitler@stanford.edu) or R.V.F. (bfarese@gladstone.ucsf.edu).

Received 10 July; accepted 10 September; published online 28 October 2012; doi:10.1038/ng.2434
knockout suppressors of TDP-43 toxicity discovered in our screen was dbr1Δ. Dbr1 catalyzes the debranching of lariat introns that are formed during pre-mRNA splicing. We show that inhibiting the debranching enzymatic activity of Dbr1 is sufficient to rescue TDP-43 toxicity. We also provide evidence that intrinsic lariat species that accumulate in the cytoplasm of dbr1Δ cells act as decoys to sequester toxic cytoplasmic TDP-43, possibly preventing it from interfering with other essential cellular RNA targets and RNA-binding proteins. Knockdown of Dbr1 in a human neuronal cell line and in primary rat neurons is also sufficient to rescue TDP-43 toxicity, suggesting that the effect of Dbr1 on TDP-43 toxicity is conserved from yeast to mammals. We propose that the debranching enzymatic activity of Dbr1 could be a potential therapeutic target for ALS and related TDP-43 proteinopathies.

RESULTS

**DBR1 is a potent modifier of TDP-43 toxicity in yeast**

To identify genes that modify TDP-43 toxicity, we performed two independent, unbiased genome-wide yeast deletion screens in two different laboratories (Supplementary Fig. 1). Similar approaches have been used to discover modifiers of the neurodegenerative disease proteins α-synuclein, huntingtin and FUS.21,24,25 In the first screen, we used synthetic genetic array (SGA) analysis26 to introduce a galactose-inducible plasmid expressing TDP-43 into each nonessential yeast deletion strain by mating (Supplementary Fig. 1a). The second screen included the deletion genes and a library of essential genes generated by decreased abundance by mRNA perturbation (DAmP) technology27 (Supplementary Fig. 1b–e). We selected yeast deletion strains in which TDP-43 was more (enhancer) or less (suppressor) toxic than in wild-type cells. Screen 1 was repeated three independent times, and only gene deletions that were reproduced all three times were confirmed by immunofluorescence and immunoblotting (Supplementary Fig. 1a). In analyzing these results, we chose to focus on suppressors of TDP-43 toxicity because these could represent attractive therapeutic targets for small molecule inhibitors or RNA interference (see the Supplementary Note for discussion of additional yeast TDP-43 toxicity modifier genes).

One of the most effective deletion suppressors of TDP-43 toxicity identified independently in both screens was dbr1Δ, and we focused further efforts on the DBR1 gene. DBR1 deletion suppressed the toxicity of wild-type TDP-43, as well as that of an ALS-linked mutant form of TDP-43 (Gln331Lys; Fig. 1a and Supplementary Fig. 2). Immunoblotting confirmed that toxicity was not suppressed because of lower TDP-43 expression in the dbr1Δ strain (Fig. 1b). Deletion of DBR1 did not suppress the toxicity of two other neurodegenerative disease proteins, α-synuclein and mutant huntingtin. Indeed, α-synuclein toxicity was slightly enhanced in the dbr1Δ strain (Fig. 1a). However, DBR1 deletion suppressed toxicity of another RNA-binding protein linked to ALS, FUS (Fig. 1a), showing specificity of the DBR1 genetic interaction for ALS-linked RNA-binding proteins TDP-43 and FUS.

Inhibiting DBR1 suppresses TDP-43 toxicity in mammalian cells

Identification of Dbr1 as a potent modifier of TDP-43 toxicity suggests the possibility that inhibiting Dbr1 enzymatic activity (Fig. 1c) could be a promising therapeutic strategy for ALS, especially as TDP-43 is thought to contribute broadly to almost all ALS cases without SOD1 mutation28. Therefore, we tested whether inhibiting Dbr1 function could rescue TDP-43 toxicity in mammalian cells. We generated a stable human M17 neuroblastoma cell line that inducibly expresses epitope-tagged ALS-linked mutant TDP-43 (Gln331Lys) under the control of a doxycycline-regulated promoter (Fig. 2a). Inducible expression of TDP-43 Gln331Lys was confirmed by immunofluorescence and immunoblotting (Fig. 2b and data not shown). Upregulating mutant TDP-43 was toxic in these cells (Fig. 2c). To determine whether inhibiting Dbr1 rescues TDP-43 toxicity, we transfected these cell lines with a small interfering RNA (siRNA) against human DBR1 or a non-targeting control siRNA (Fig. 2d). Neither siRNA affected TDP-43 expression in these cells (data not shown). Whereas the non-targeting siRNA had

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Effect on TDP-43 toxicity</th>
<th>Function</th>
<th>Human homolog</th>
</tr>
</thead>
<tbody>
<tr>
<td>fld1Δ</td>
<td>Enhancer</td>
<td>Involved in lipid droplet morphology, number and size; proposed to be involved in lipid metabolism</td>
<td>BSCL2 (Berardinelli-Seip congenital lipodystrophy); seipin</td>
</tr>
<tr>
<td>mrt40Δ</td>
<td>Enhancer</td>
<td>Mitochondrial ribosomal protein of the large subunit</td>
<td>RPL13A</td>
</tr>
<tr>
<td>msn2Δ</td>
<td>Enhancer</td>
<td>Stress-induced transcriptional activator</td>
<td>None</td>
</tr>
<tr>
<td>nxh1Δ</td>
<td>Enhancer</td>
<td>Vacuolar and endosomal Na+/H+ exchanger involved in pH regulation</td>
<td>SLC9A6</td>
</tr>
<tr>
<td>rpl16Δ</td>
<td>Enhancer</td>
<td>Component of the large (60S) ribosomal subunit</td>
<td>RPL13A</td>
</tr>
<tr>
<td>tif4631Δ</td>
<td>Enhancer</td>
<td>Translation initiation factor</td>
<td>EIF4G1</td>
</tr>
<tr>
<td>cce1Δ</td>
<td>Suppressor</td>
<td>Mitochondrial cruciform cutting endonuclease, cleaves Holliday junctions formed during recombination of mitochondrial DNA</td>
<td>None</td>
</tr>
<tr>
<td>dbr1Δ</td>
<td>Suppressor</td>
<td>RNA lariat debranching enzyme, involved in intron turnover</td>
<td>DBR1</td>
</tr>
<tr>
<td>dom34Δ</td>
<td>Suppressor</td>
<td>EndoRNase involved in no-go mRNA decay</td>
<td>PELO</td>
</tr>
<tr>
<td>pbp1Δ</td>
<td>Suppressor</td>
<td>Involved in P body–dependent granule assembly; interacts with Pab1p to regulate mRNA polyadenylation</td>
<td>ATXN2</td>
</tr>
<tr>
<td>rpl16Δ</td>
<td>Suppressor</td>
<td>Component of the large (60S) ribosomal subunit</td>
<td>RPL13A</td>
</tr>
<tr>
<td>set3Δ</td>
<td>Suppressor</td>
<td>Member of histone deacetylase complex</td>
<td>ASH1</td>
</tr>
<tr>
<td>siw14Δ</td>
<td>Suppressor</td>
<td>Tyrosine phosphatase involved in actin filament organization and endocytosis</td>
<td>None</td>
</tr>
<tr>
<td>ydr067cΔ</td>
<td>Suppressor</td>
<td>Cytoplasmic protein required for replication of Brome mosaic virus in S. cerevisiae, which is a model system for studying replication of positive-stranded RNA viruses in their natural hosts</td>
<td>None</td>
</tr>
</tbody>
</table>
no effect on toxicity, transfection with the DBR1-specific siRNA significantly reduced TDP-43 toxicity (Fig. 2d).

**Inhibiting Dbr1 suppresses TDP-43 toxicity in primary neurons**

We next asked whether knockdown of Dbr1 prevents TDP-43 toxicity in primary neurons. We showed previously that overexpression of TDP-43 in primary cortical neurons induces neurodegeneration with cytoplasmic mislocalization and aggregation of TDP-43, recapitulating several key pathological features of TDP-43 proteinopathies in vitro. To determine whether Dbr1 knockdown protects neurons from TDP-43 cytotoxicity, we used automated microscopy and longitudinal analysis, a technique that identifies and characterizes variables that significantly contribute to neuronal survival. We isolated and cultured rat primary cortical neurons and transfected them with three constructs expressing (i) mApple, (ii) enhanced green fluorescent protein (EGFP) or TDP-43 fused to EGFP (TDP-43–EGFP) and (iii) siRNA directed against Dbr1 or scrambled siRNA. With automated microscopy, hundreds of individual neurons were imaged from each cohort at regular 24-h intervals for 8 d (Fig. 3a). In this experiment, mApple serves as a sensitive survival marker, as the loss of mApple fluorescence, cell blebbing or disruption of the cell membrane signifies cell death. These criteria are at least as specific as those used with traditional markers of particular cell death pathways but have the advantage of detecting all forms of cell death in a single assay, thereby rendering them more sensitive. Because each neuron in a population can be followed longitudinally for an extended period of time, the analysis is analogous to that used in a prospective clinical trial. Powerful statistical tools developed for human epidemiological studies, including Kaplan-Meier survival analysis and Cox proportional hazards analysis, are used to describe the data that are generated from these experiments.

We initially performed a small pilot experiment to determine the optimal amount of Dbr1 siRNA (Supplementary Fig. 3) and found that the most effective dose was 30 nM. On the basis of the effect size that we noted in the pilot experiment, the population sizes of subsequent experiments were scaled to achieve the power necessary to...
Figure 3 Dbr1 knockdown reduces TDP-43 toxicity in primary rat neurons. (a) Automated fluorescent microscopy of primary rat cortical neurons transfected with constructs encoding mApple (top) and TDP-43–EGFP (bottom). Two neurons are depicted here at repeated intervals. While one of the neurons lives for the entire experiment (arrowheads), the other has died by 144 h (arrows). Scale bar, 10 μm. (b) Cumulative hazard plot showing the cumulative risk of death for neurons in each cohort as a function of time. Expression of TDP-43–EGFP (n = 380 neurons counted) significantly increases the risk of death over that of neurons expressing EGFP alone (n = 245 neurons counted; hazard ratio = 2.22). Knockdown of Dbr1 using 30 nM Dbr1 siRNA in neurons expressing TDP-43–EGFP (n = 300 neurons counted) decreases the risk of death by 19% compared to neurons expressing TDP-43–EGFP that received scrambled siRNA (n = 380 neurons counted). Data were pooled from two independent experiments, and statistical significance was determined using Cox proportional hazards analysis. ***, hazards ratio = 2.23 and P < 1 × 10−9; **, hazards ratio = 1.83 and P < 1 × 10−4; *, hazards ratio = 0.81 and P = 0.04. (c) Kaplan-Meier survival curve of the same data. (d) Dbr1 knockdown results in increased percentage of neurons containing cytoplasmic TDP-43 compared to neurons treated with control scrambled siRNA. Data are shown as mean ± s.e.m. *P < 0.0005, two-tailed t test.

Depletion of Dbr1 reduced TDP-43 toxicity in yeast (Fig. 1), mammalian cells (Fig. 2) and primary neurons (Fig. 3), but it was unclear whether the rescue was because of loss of debranching enzymatic activity or was potentially related to another Dbr1 function. To test this directly, we used ‘debranching activity–dead’ mutants of Dbr1. We cotransformed dbr1Δ yeast cells with expression plasmids for human TDP-43 and either wild-type yeast Dbr1 or two independent Dbr1 point mutants (Asp40Ala or Asn85Ala) that lack debranching enzymatic activity in vitro and in vivo15. Immunoblotting confirmed that the wild-type and mutant Dbr1 proteins were expressed at equivalent levels (Fig. 4a). Whereas expressing wild-type Dbr1 restored TDP-43 toxicity to dbr1Δ cells, expressing either of the debranching-inactive Dbr1 point mutants did not (Fig. 4b). Moreover, we tested a panel of 16 Dbr1 mutants, including some that retain full debranching activity, some that have lost all activity and some with partial debranching activity16. There was an exact correlation between debranching activity and the ability to restore TDP-43 toxicity to dbr1Δ cells: wild-type Dbr1 fully restored TDP-43 toxicity, whereas completely inactive Dbr1 had no effect, and partially active Dbr1 partially restored TDP-43 toxicity (Supplementary Table 1). Thus, inhibiting Dbr1 debranching enzymatic activity is sufficient to rescue TDP-43 toxicity in yeast cells. Finally, expressing mouse Dbr1 in dbr1Δ cells was also sufficient to restore TDP-43 toxicity (Fig. 4b), indicating that the debranching function of Dbr1 is conserved from yeast to mammals.

Lariat intron accumulation suppresses TDP-43 toxicity

In the absence of Dbr1, steady-state levels of lariat introns are greatly increased17. This suggested the intriguing possibility that the accumulating lariat introns in dbr1Δ cells could act as a kind of decoy for TDP-43, sequestering it away from important cellular RNAs and other RNA-binding proteins. To test this, we first determined whether the rescue of TDP-43 toxicity was specific to lariat intron accumulation or if accumulation of any nonspecific RNA species rescued toxicity. We expressed TDP-43 in three other yeast deletion strains (upf1Δ, upf2Δ and xrn1Δ) that each result in accumulation of RNA species within the cell, owing to alterations in the nonsense-mediated RNA decay (NMD) pathway18–22. Whereas deleting DBR1 rescued
TDP-43 toxicity, TDP-43 toxicity was not suppressed in the nucleus in dbr1Δ cells. Expressing wild-type yeast Dbr1 in dbr1Δ cells restored toxicity. Two Dbr1 mutants (D40A and N85A) that lack debranching activity were unable to restore TDP-43 toxicity to dbr1Δ cells. Expressing mouse Dbr1 in dbr1Δ yeast cells restored TDP-43 toxicity, indicating that the function of Dbr1 is conserved from yeast to mammals. (c) Yeast spotting assay showing that TDP-43 toxicity is suppressed in dbr1Δ cells but not in three other deletion strains (upf1Δ, upf2Δ and xrn1Δ), which each accumulate non-specific RNA species, owing to defects in the cellular NMD pathway. Schematic of yeast cells accumulating no excess RNA species (WT), lariat RNAs (dbr1Δ) or nonspecific linear RNAs (upf1Δ, upf2Δ or xrn1Δ) are shown next to the spotting assays.

Intronic lariats suppress TDP-43 toxicity in the cytoplasm

We next sought to determine the mechanism by which intronic lariats suppress TDP-43 toxicity in dbr1Δ cells. We and others have shown that TDP-43 is toxic in the cytoplasm in multiple cellular and animal model systems. Thus, we reasoned that intronic lariats might accumulate in the nucleus of dbr1Δ cells, sequester TDP-43 there and prevent it from aggregating in the cytoplasm, thereby suppressing toxicity. In this model, sequestering TDP-43 in the cytoplasm should overcome the toxicity suppression by dbr1Δ. To test whether this was the case, we retained TDP-43 in the cytoplasm by mutating its nuclear localization signal (NLS). We reasoned that, if dbr1Δ suppresses TDP-43 toxicity in the nucleus, the TDP-43 mutant lacking an NLS would still be toxic. Unexpectedly, DBR1 deletion still suppressed toxicity when TDP-43 was sequestered in the cytoplasm (Fig. 5a), suggesting that the site of action of intronic lariats is the cytoplasm.

The above genetic result suggested that intronic lariats act in the cytoplasm to suppress TDP-43 toxicity. To test this idea directly, we developed a method to visualize in living cells the endogenous intronic lariats that accumulate in dbr1Δ cells. We reasoned that, if we could visualize the intronic lariats, we could determine (i) whether they localized to the cytoplasm or nucleus and (ii) whether they colocalized with TDP-43 inclusions. A technique to visualize the localization of endogenous mRNAs in living yeast cells was recently developed. m-TAG uses homologous recombination to insert binding sites for the RNA-binding MS2 coat protein (MS2-CP) between the coding region and 3′ UTR of any yeast gene. Expression of MS2-CP fused to GFP enables the visualization of any yeast mRNA that has been tagged with MS2-binding sites. We modified m-TAG to visualize intronic lariats. Instead of tagging the 3′ UTR of a target yeast gene, we used homologous recombination to integrate MS2-binding sites in the intron of the ACT1 gene (Fig. 5b), which has previously been shown by RNA blot analysis to accumulate in the absence of Dbr1 activity.

We transformed wild-type and dbr1Δ yeast cells, each harboring MS2-binding sites integrated in the intron of the ACT1 gene (Fig. 5c), with a plasmid containing a 3× GFP–tagged MS2-CP RNA-binding protein under control of the inducible MET25 promoter. We analyzed the localization of MS2-CP–GFP by fluorescence microscopy. As reported, because the MET25 promoter is somewhat leaky, we detected the GFP fusion protein even without induction (by growth in the presence of methionine). In wild-type cells, in which no intronic lariats should accumulate, we detected faint MS2-CP–GFP expression in a diffuse pattern throughout the cell (Fig. 5c and Supplementary Fig. 4). In contrast, in dbr1Δ cells, MS2-CP–GFP accumulated in one or two bright foci per cell, and these were always located in the cytoplasm (Fig. 5c). We observed similar results by tagging an independent intron from another yeast gene, CYH2 (data not shown). These foci could represent the excised lariat introns that accumulate in dbr1Δ cells or a splicing intermediate.

Intronic lariats colocalize with TDP-43 cytoplasmic foci in yeast

Having established that intronic lariats accumulate in the cytoplasm of dbr1Δ cells, we next tested our hypothesis that the accumulating lariats in dbr1Δ cells act as a decoy for TDP-43 by preventing it from interacting with other important cellular RNAs and RNA-binding proteins. We expressed untagged TDP-43 in wild-type and dbr1Δ cells and visualized its localization by immunocytochemistry with a TDP-43–specific antibody (Fig. 5d,e). We visualized tagged intronic lariats by fluorescence microscopy. Notably, in every dbr1Δ cell analyzed, TDP-43 colocalized with intronic lariats (Fig. 5e). Consistent with our genetic results (Fig. 5a) and fluorescence microscopy experiments (Fig. 5c), TDP-43 was not retained in the nucleus in dbr1Δ cells and accumulated in the cytoplasm (Fig. 5e). However, the size and shape of TDP-43 inclusions were profoundly different in wild-type and dbr1Δ cells. In wild-type cells, TDP-43 formed multiple small, irregularly shaped cytoplasmic foci (Fig. 5d), whereas in dbr1Δ cells, TDP-43 always formed at least one large, perfectly round focus (Fig. 5d), which always colocalized with intronic lariats (Fig. 5e).
Intronic lariats compete with RNAs for binding to TDP-43

We also assessed whether the TDP-43 ribonucleoprotein complex contains lariats. We expressed Flag-tagged TDP-43 in wild-type and dbr1Δ cells and immunoprecipitated TDP-43 from cell lysates with an antibody to Flag. The associated RNA was separated by two-dimensional electrophoresis. Linear RNA molecules migrate in a diagonal on two-dimensional gels, but lariat and lariat breakdown products migrate in a separate arc, owing to the reduced mobility conferred by their altered structures (Supplementary Fig. 6a).

As expected, in wild-type and dbr1Δ cells, TDP-43 associated with RNA. However, in dbr1Δ cells, the TDP-43 ribonucleoprotein complex contained additional RNA species, consistent with intronic lariats.

Finally, to directly test our hypothesis that the intronic lariats that form in dbr1Δ cells act as decoys for TDP-43 by competing with it for binding to other cellular RNAs, we performed in vitro electrophoretic mobility shift assays. Incubation of a radioactively labeled RNA probe known to bind TDP-43 (ref. 46) with recombinant TDP-43 results in a gel shift (Supplementary Fig. 6b,c). We isolated total RNA from wild-type and dbr1Δ cells and tested the ability of these RNAs to compete for binding to TDP-43. As expected, RNAs from wild-type and dbr1Δ cells competed for TDP-43 binding (Supplementary Fig. 6b), consistent with TDP-43 binding a large number of RNA targets47. To specifically test the ability of intronic lariats to compete for binding to TDP-43 (to act as decoys), we treated RNAs isolated from wild-type and dbr1Δ cells with RNase R, which degrades all RNAs except for branched lariats48. When these RNase R–treated samples were incubated with the TDP-43 binding reaction, only the RNase R–treated RNAs isolated from dbr1Δ cells competed TDP-43 away from its binding site (Supplementary Fig. 6c). Thus, dbr1Δ cells contain an RNase R–resistant species, likely intronic lariats, that compete for binding to TDP-43.

DISCUSSION

Using two unbiased genetic screens in yeast, we discovered Dbr1 as a potent modifier of TDP-43 toxicity. We provide evidence that, in the absence of Dbr1, intronic lariats accumulate in the cytoplasm, and we propose that these act as decoys to sequester TDP-43, preventing it from interfering with essential RNAs and RNA-binding proteins. Thus, we suggest that the debranching enzymatic activity of Dbr1 could be a novel therapeutic target to combat toxic effects from cytoplasmic TDP-43 aggregation in ALS. The additional genetic modifiers of TDP-43 toxicity uncovered from these two yeast screens (Table 1 and Supplementary Data) will hopefully provide even more insight into disease mechanisms and open new avenues for therapeutic intervention.

Recent studies indicate that RNA binding is an important component of TDP-43 toxicity in cellular and animal models19,22,49,50. One potential mechanism of neurotoxicity is that TDP-43 aggregates in the cytoplasm, sequestering essential cellular RNAs and RNA-binding proteins.
proteins away from their normal functions. This could lead to catastrophic changes in RNA metabolism, owing to defects in the stability, splicing, transport and/or translation of essential RNAs. This effect might be more deleterious to motor neurons if motor neuron–specific transcripts were preferentially sequestered by TDP-43 or even if motor neurons were simply more sensitive to subtle alterations in any of these RNA metabolic pathways. TDP-43 loss of function likely also contributes to disease. TDP-43 is depleted from the nuclei of affected neuronal populations in individuals with ALS, and downregulation of TDP-43 in mouse leads to widespread dysregulation of splicing. Finally, TDP-43 missense variants might act in a dominant-negative manner, interfering with the function of the wild-type protein. These three pathogenic mechanisms (gain of toxic properties in the cytoplasm, loss of function in the nucleus and dominant-negative effects) are not mutually exclusive and likely all contribute to ALS pathogenesis.

Inhibiting Dbr1 function might overcome or prevent cytoplasmic TDP-43 (and FUS) aggregates from disrupting critical RNAs and RNA-binding proteins. We propose that the accumulated intracellular lariat species in dbr1Δ cells suppress TDP-43 toxicity by acting as decoys, causing TDP-43 to bind to them rather than to important cellular RNA targets (Fig. 5f). The catalog of genome-wide RNA targets for TDP-43 is emerging. Notably, TDP-43 seems to preferentially bind to long intronic pre-mRNA sites rather than coding exonic regions or UTRs. This might explain why the intronic RNA sequences (lariats) that accumulate in dbr1Δ cells sequester TDP-43 away from other RNA species in the cell and suppress TDP-43 toxicity. TDP-43 might also preferentially recognize the lariat branch-point structure, or binding to the lariat might promote nucleation or other alterations to TDP-43 conformation.

For Dbr1 to be a therapeutic target, several questions must be addressed. First, could inhibition of Dbr1 debranching activity be toxic itself? Indeed, the highest concentration of Dbr1 siRNA (50 nM) in primary rat neurons increased the risk of death (Supplementary Fig. 3), suggesting that too much Dbr1 inhibition could be deleterious. However, in budding yeast, DBR1 deletion is tolerated (Fig. 1a), and siRNA knockdown in M17 cells does not result in growth inhibition (ref. 56 and M.A. and A.D.G., unpublished data). Nevertheless, it will be important to determine what level of Dbr1 inhibition is tolerated and whether an appropriate therapeutic index can be achieved. An additional consideration for this approach in mammalian cells is the non-coding RNAs in intronic regions that depend on Dbr1 activity. Expression of these non-coding RNAs might be dysregulated by Dbr1 inhibition, and the consequences of this must be assessed. Furthermore, as abnormal accumulation of RNA is hypothesized to trigger autoimmunity and there would likely be a large pool of lariats in human cells, the effects of Dbr1 inhibition on autoimmunity should be analyzed. Although, to our knowledge, Dbr1 inhibitors are not available, our new method to visualize intronic lariats in living cells (Fig. 4) will facilitate screening of chemical compound libraries for small molecule Dbr1 inhibitors.

There are currently no TDP-43–directed therapies for ALS or related TDP-43 proteinopathies. Antisense oligonucleotides and RNA interference approaches are emerging as attractive therapeutic strategies in neurodegenerative diseases in which decreasing levels of a toxic mutant protein might be efficacious. Indeed, treating a mouse model of inherited ALS (caused by a mutation in SOD1) with antisense oligonucleotides to SOD1 significantly slowed disease progression. This approach offers tremendous promise for treating patients with ALS with SOD1 mutations, but, as mutations in SOD1 account for only ~2–5% of all ALS cases, additional therapeutic strategies are needed. Because TDP-43 appears to contribute to ALS pathogenesis broadly, targeting TDP-43 with antisense approaches could be effective. However, it is unknown whether TDP-43 contributes to disease via loss-of- or gain-of-function mechanisms, mandating caution in pursuing TDP-43–lowering approaches. We present an alternative strategy: by targeting Dbr1 using small molecule inhibitors or antisense oligonucleotides, it might be possible to antagonize the toxic effects of TDP-43 in the cytoplasm without interfering with potentially critical functions in the nucleus.

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

ACKNOWLEDGMENTS
We thank K. Lynch and S. Smith for helpful suggestions and discussions about RNA and splicing; T. Nakaya for advice and assistance with lentivirus transduction experiments and analysis; C. Kurischko for advice and assistance with visualizing P bodies and stress granules; Q. Mitrovich and A. Plocik for advice with running the two-dimensional nucleic acid gels; S. Collins and D. Cameron for useful advice and assistance in performing the data analysis for screen 2; B. Hodges, D. Hosangadi, P. Patel, P. Nathanson and C. Mrejen for advice with yeast experiments; J. Epstein and A. Raphael for critical comments on the manuscript and helpful suggestions; and G. Howard for editorial assistance. A. Elden helped with initial stages of this project. We are grateful to J. Gerst (Weizmann Institute) for providing the yeast m-TAG plasmids, R. Parker (University of Arizona) for sharing the P-body and stress granule marker plasmids and J. Weibezahn (University of California, San Francisco) for providing temperature-sensitive CDC48 (cdc48-3, SM 4783) and wild-type CDC48 isogenic (SM 5124) yeast strains. We thank B. Schwer (Weill Cornell Medical College) for providing the yeast mutant Dbr1 expression plasmids. We thank C. Boone (University of Toronto) for the MATα strain Y7092. This work was supported by US National Institutes of Health (NIH) Director’s New Innovator Awards 1DP2OD004417 (to A.D.G.) and 1DP2OD002177 (to J.S.), NIH grants NS065317 and NS065317 (to A.D.G.), NS067534 (to J.S.), GM084448, GM084279, GM081879 and GM098101 (to N.J.K.), NS39974 and NS045491 (to S.F.) and NS072233 (to S.F.), a New Scholar in Aging Award from the Ellison Medical Foundation (to J.S.), a grant from the Packard Center for ALS Research at Johns Hopkins (A.D.G. and J.S.), a grant from the Consortium for Frontotemporal Research (to R.V.F.), NIH grant Z1P1A02074 (to S.F.), a grant from the ALS Association (to S.F.) and the Taube-Koret Center and Hellman Family Foundation (to S.F.). A.D.G. is a Pew Scholar in the Biomedical Sciences, a Searle Scholar and a Keck Young Investigator. R.V.F. is an Investigator of the Gladstone Institutes. The D. David Gladstone Institutes received support from National Center for Research Resources Grant RR18928.

AUTHOR CONTRIBUTIONS

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

URLs
Study protocols, http://www.gladstone.ucsf.edu/gladstone/site/finkbeiner/.


Yang, C. et al. The C-terminal TDP-43 fragments have a high aggregation propensity and harm neurons by a dominant-negative mechanism. PLoS ONE 5, e15878 (2010).


ONLINE METHODS

Yeast strains, media and plasmids. The dbr1Δ strain was obtained by replacing the DBR1 coding region with a KanMX4 cassette in the BY4741 or W303 strain and verified by colony PCR. CEN and 2-micron galactose-inducible TDP-43 (ref. 28), 2-micron ΔNLS-TDP-43-YFP, PUS1, α-synuclein40 and Htt103Q expression plasmids were as described63. The yeast Dbr1 mutant expression plasmids33 and m-TAG plasmids pLOXHISMS2L, pSH147 and pMS2-CP-GFP (×3) were as described44.

Yeast TDP-43 toxicity modifier screens. We used the SGA technique to screen the collection of nonessential-only yeast knockout strains (screen 1, performed in the laboratory of A.D.G.) or a collection of nonessential yeast knockout strains combined with essential genes reduced in expression by DAmP67 (screen 2, performed in the laboratories of R.V.F. and N.K.) for modifiers of TDP-43 toxicity. These screens were performed as described26,66,68, with some modifications66, using a Singer RoToR HDA (Singer Instruments). For screen 1, the galactose-inducible TDP-43 expression construct (pAG416Gal–TDP-43) was introduced into MATα strain Y7092 to generate the query strain. This query strain was mated to the yeast haploid deletion collection of nonessential genes (MATα, each gene deleted by KanMX cassette; Fig. 1a). Haploid mutants harboring the TDP-43 expression plasmid were grown in the presence of glucose (TDP-43 expression off) or galactose (TDP-43 expression on). After growth at 30 °C for 2 d, plates were photographed, and colony sizes were measured by ImageJ image analysis software as described47. The entire screen was repeated three times, and only hits that were reproduced all three times were selected for further validation by random spore analysis on DNA sequencing of deletion strain barcodes.

For screen 2, a control query strain containing the empty vector was generated by transforming the yeast strain BY5563 (Matα) with p415-Gal-GFP, and the experimental query strain was generated by transforming BY5563 (Matα) with p415-Gal–TDP-43–GFP. Both strains were mated with the combined yeast knockout DAmP collection (Matα). Haploid yeast containing the deletion/DAmP yeast gene with the control or experiment plasmid were selected as described45. Three biological replicates were selected for further validation. For each screen, six replicates (three biological and two technical replicates) were performed. Plates were imaged at seven time points after final inoculation on glucose or galactose. Colony size was assessed as described47.

Isolation and culturing of primary cortical neurons. Rat cortical neurons were isolated from embryonic day 20–21 rat (Sprague Dawley) pups, cultured in serum-free medium in 96-well plates for 4 d in vitro and then transfected using Lipofectamine 2000 (Invitrogen; see URLs for full protocols). Neurons were transfected with transplasmids (pGW1 backbone) encoding mApple and either TDP-43–EGFP or EGFP and with siRNA directed against Dbr1 (SMARTpool, Dharmacon) or scrambled siRNA (Dharmacon).

Live-cell imaging for longitudinal analysis. For neuronal survival analysis, we used a robotic imaging system as described28,40. Briefly, approximately 24–48 h after transfection, images were acquired with an inverted Nikon microscope (TE2000) equipped with PerfectFocus, an extra-long working distance (ELWD) 20× objective lens and an Andor Clara 16-bit, ultra-cooled camera. Illumination was provided by an adjustable, high-intensity and long-lasting xenon lamp, and a liquid light guide was used to maximize the signal-to-noise ratio. The stage and shutter movements, fluorescence excitation and emission filters, focusing and image acquisition were fully automated and were controlled through a 64-bit host computer running a combination of proprietary software (ImagePro, Media Cybernetics) and custom-designed algorithms.

Survival analysis. Digitized images from the red fluorescent protein (RFP; mApple) and green fluorescent protein (GFP; TDP-43–EGFP) channels were assembled into montages with ImagePro, and cells were prospectively counted in the RFP channel by original code developed in Matlab. Cell death was marked by loss of fluorescence, membrane rupture or neurite retraction. The time of death for each neuron was defined as the last time the neuron was seen. Kaplan-Meier and cumulative risk of death curves were generated using the survival package in R. Statistical significance of survival differences between cohorts of neurons was determined by the log-rank test, and Cox proportional hazards analysis was used to measure the relative change in the risk of death, the hazard ratio.

Two-dimensional nucleic acid gels. Nucleic acid pellets from immunoprecipitation were resuspended in 5 μl of nuclease-free water and 5 μl of 2× nucleic acid loading dye (0.025 g xylene cyanol and 0.025 g bromophenol blue in 100% formamide) and were heated at 85 °C for 5 min. Nucleic acids were separated in the first dimension by loading samples into a prerun (300 V for 10 min) 5% polyacrylamide gel (19:1 acrylamide-bis-acrylamide) containing 7 M urea and 1× TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.0) and electrophoresing them at 300 V. Nucleic acids were separated in the second dimension by placing the entire lane from the first-dimension gel above the second-dimension gel. The second-dimension gel was exactly the same as the first-dimension gel, except that it contained 10% polyacrylamide. Samples were electrophoresed at 300 V until xylene cyanol dye reached the bottom of the second-dimension gel. Two-dimensional nucleic acid gels were washed briefly in water and then placed in 1× SYBR Gold (Invitrogen) solution prepared in water. Gels were protected from light and gently agitated at 23–25 °C in 1× SYBR Gold solution for 45 min. Images of the gels were captured on an ultraviolet light box at an excitation wavelength of 302 nm with a 4 s exposure time.

Genomic tagging strategy to visualize intron lariats. To visualize intronic lariats in dbr1Δ cells, we adapted the m-TAG protocol42 for use with introns. We used PCR-based chromosomal gene tagging, using homologous recombination to integrate thelox::Spit5::lox::MS2 cassette into the intron of either the ACT1 gene or the CYH2 gene in wild-type or dbr1Δ cells. For ACT1, we inserted the MS2 cassette at nucleotide 159 of the ACT1 genomic DNA sequence (ACT1/YFL039C on chromosome VI from coordinates 54,696 to 53,260). For CYH2, we inserted the MS2 cassette at nucleotide 303 of the CYH2 genomic DNA sequence (RPL28/VYG103W on chromosome VII from coordinates 310,967 to 311,927). To excise thelox::Flanked Spit5 cassette, we transformed cells with plasmid pSH47, which expresses Cre recombinase under control of a galactose–regulated promoter. Proper integration of MS2 sequences was verified by colony PCR and DNA sequencing.

Visualizing tagged intronic lariats by fluorescence microscopy. We visualized MS2-tagged intronic lariats in wild-type and dbr1Δ cells as described42. We transformed wild-type and dbr1Δ cells harboring an MS2-tagged ACT1 intron with pMS2–CP-GFP (×3), which expresses MS2-CFP fused to three copies of GFP under the control of the MET25 promoter. Yeast cells were fixed with 70% ethanol and stained with DAPI in Vectashield mounting medium. Colocalization of an MS2-tagged intronic lariat with TDP-43 was determined using fluorescence microscopy to detect lariats (GFP) and immunocytochemistry with a TDP-43–specific antibody to detect untagged TDP-43.

TDP-43 lentiviruses. To generate a lentiviral vector for the conditional expression of TDP-43 (Gln331Lys), a Gateway entry clone encoding N-terminally Flag-tagged and C-terminally Myc-tagged TDP-43 (Gln331Lys) was used in a Gateway LR reaction (Invitrogen) to transfer it to the pSLIK-Neo lentiviral destination vector. Recombinant lentiviruses were produced as described39. For lentiviral transduction, human M17 neuroblastoma cells (ATCC) were incubated with lentiviral supernatants for 6 h at 37 °C. Stable cell lines were selected by culturing transfected cells for 5 d in growth medium (minimum essential medium (MEM) alpha/F-12 (Invitrogen) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium) with 100 μg/ml Geneticin). Individual clones were selected and analyzed for inducible expression by immunoblotting and immunofluorescence analyses.

TDP-43 toxicity assays in mammalian cells. We assessed the effect of TDP-43 on cell proliferation in the MTT assay. M17 cells stably transduced with either an empty vector or with the vector encoding TDP-43 (Gln331Lys) were seeded in 96-well plates. To induce TDP-43 (Gln331Lys) expression, doxycycline (0.1, 1 or 2 μg/ml) was added to the growth medium, and cells were incubated

doi:10.1038/ng.2434
for 3 d. To measure proliferation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well, and cells were incubated for 4 h at 37 °C. Acidic isopropanol (40 mM HCl) was then added to each well to solubilize the blue formazan crystals. Absorbance at 570 nm was read with a Tecan Safire II plate reader. Absorbance measurements were normalized to the absorbance of cells not treated with doxycycline and used to calculate the percent viability under each condition. Two-way ANOVA was used to evaluate differences in means between two groups, and P values of <0.05 were considered statistically significant. Each condition was replicated at least four times, and each experiment was independently repeated at least three times.

**siRNA knockdown of **DBR1** in M17 cells.** siRNA targeting human **DBR1** and non-targeting control siRNA were purchased from Thermo-Scientific (DBR1, J-008290-08-0005; non-targeting, D-001810-01-05). siRNA knockdown was validated by immunoblotting with a Dbr1-specific antibody (Proteintech, rabbit polyclonal antibody, 16019-1-AP). The M17 cell line with inducible TDP-43, and toxicity was then assessed by MTT assay 2 d, cells were transferred to medium containing doxycycline (0 and 1 µg/ml) and incubated for 15 min at 30 °C in the presence of various dilutions of competitor RNA (0–12.9 μg). Binding reactions were then transferred to ice, and heparin was added to a final concentration of 0.5 μg/μl. Reactions were analyzed on a 4.5% native gel (acylamide:bi-acrylamide (29:1), Bio-Rad).

**TDP-43 in vitro RNA-binding assay.** Glutathione S-transferase (GST)–TDP-43 was purified from *Escherichia coli* as described. Briefly, competitor RNA was purified from wild-type or dbr1Δ W303 yeast using the RNeasy MiniElute Cleanup kit (Qiagen). A radioactively labeled RNA probe, UG₆₇, which is known to bind TDP-43 (ref. 46), was generated by T7 polymerase–catalyzed transcription of a DNA template in the presence of [³²P]-labeled UTP. Standard binding reactions were carried out in a 10-µl volume containing 4 mM MgCl₂, 25 mM phosphocreatine, 1.25 mM ATP, 1.3% polyvinyl alcohol, 25 ng of yeast tRNA, 0.8 mg of BSA, 1 mM DTT, 0.1 µl Rnasin (Promega, 40 U/ml), 75 mM KCl, 10 mM Tris, pH 7.5, 0.1 mM EDTA and 10% glycerol. Binding reactions containing GST–TDP-43 (0.5 µM) and [³²P]-labeled UG₆₇ (1 µl) were incubated for 15 min at 30 °C in the presence of various dilutions of competitor RNA (0–12.9 μg). Binding reactions were then transferred to ice, and heparin was added to a final concentration of 0.5 μg/μl. Reactions were analyzed on a 4.5% native gel (acylamide:bi-acrylamide (29:1), Bio-Rad).