Lariat RNAs sequester TDP-43:
a possible treatment for amyotrophic lateral sclerosis

A review by Leigh Henderson

A critical discussion of the article:

> Armakola, M., Higgins, M. J., Figley, M. D., Barmada, S. J., Scarborough, E. a, Diaz, Z., Fang, X., et al. (2012). Inhibition of RNA lariat debranching enzyme suppresses TDP-43 toxicity in ALS disease models. *Nature genetics*, *44*(12), 1302–9. doi:10.1038/ng.2434

Background

Amyotrophic lateral sclerosis (ALS) is a disease of progressive motor neuron loss. Diagnosis is typically made in adults on the basis of muscle weakness: slurring of speech, difficulty swallowing, loose grasp, or trouble climbing stairs, for example. Life expectancy after diagnosis is only 3-5 years. One tenth of ALS cases are inherited; the others are sporadic with no identified cause. Hyper-excitation of motor neurons and free radical damage are hypothesized to lead to ALS. In support of this hypothesis, inhibiting motor neuron excitation with the drug riluzole inhibits motor neuron degeneration. Riluzole is currently the only treatment that slows disease course[1]. However, newly discovered drug targets, such as TDP-43, guide the search for other treatments.

TDP-43 is an RNA-protein complex belonging to the heterogeneous ribonucleoprotein (hnRNP) family. Like other hnRNPs, TDP-43 is involved in mRNA splicing. The complex also functions in transcriptional regulation, mRNA degradation & translation, and microRNA processing[2]. Cytoplasmic inclusions of TDP-43 are observed in motor neurons from most ALS patients (Fig. 1). In experimental models, overexpression and mutation of TDP-43 can cause the toxicity observed in ALS. Mouse & rat models show progressive, dose-dependent motor neuron degeneration similar to human cases[3]. Overexpression in yeast results in cell death. Therefore, it is hypothesized that reduction in TDP-43 levels will slow motor neuron degeneration in ALS. Armakola et al. use the yeast overexpression model to identify protein mediators of TDP-43 toxicity.

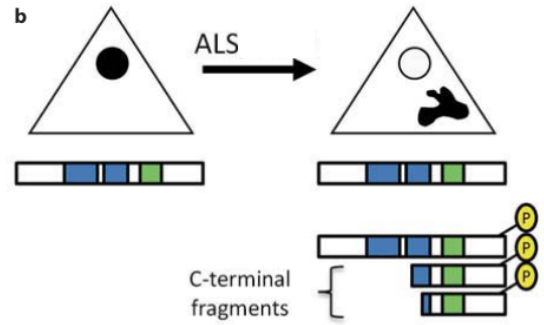

Figure 1: In ALS, post-transcriptionally modified TDP-43 accumulates in the nucleus of motor neurons. In this diagram, motor neurons are stylized as a triangle with a circular nucleus. TDP-43 is represented by the black color in the nucleus (healthy cell) or in the cytoplasm (ALS cell). Types of modifications are also illustrated: phosphorylation and cleavage.
Figure adapted from Wegorzewska and Baloh, 2011[3].

RNA lariat debranching enzyme (dbr1) is one of the proteins identified as a mediator of TDP-43 toxicity. The first splicing enzyme to be identified, dbr1 cleaves the 2'-5' phosphodiester linkage of excised intron lariats[4]. Monomeric dbr1 debranches with manganese as a cofactor[5]. The absence of dbr1 causes non-lethal accumulation of lariats RNAs in yeast[4]. Because dbr1 knockout is not lethal, knockdown is a potential therapy. Knockdown in human osteosarcoma cells inhibits human immunodeficiency virus (HIV) replication without reports of adverse effects on the cells[6]. Similarly, Armakola et al. test whether knockdown of dbr1 will prevent TDP-43 accumulation, inhibiting motor neuron degeneration without adverse effects.

Objectives

Overexpressed TDP-43 toxicity leads to motor neuron degeneration in ALS. Therefore, Armakola et al. seek to identify genes that modify overexpressed TDP-43 toxicity. From the list of genes that suppresses TDP-43 toxicity, one gene (dbr1) is chosen for further study. In ALS, mutant forms of TDP-43 or the related protein FAS can also cause disease, so Armakola et al. determine whether mutant TDP-43 or FAS toxicity are suppressed by dbr1 deletion. To be sure that dbr1 is a suitable therapeutic target in humans, inhibition of dbr1 is tested in mammalian cells, in addition to yeast, for TDP-43 toxicity suppression. Next, the authors search for the mechanism by which dbr1 deletion/knockdown suppresses TDP-43 toxicity. Mechanistic studies may inform rational drug design and shed light on the pathogenesis of ALS.

Special Methods

Yeast screen for modifiers of TDP-43 toxicity

Two yeast libraries are utilized. The first contains yeast with single-gene knockout of non-essential genes. The second contains single-gene knockdown of essential genes. Each strain of yeast is transfected with a plasmid encoding TDP-43 under a galctose-activated promoter. Transfected cells overexpress TDP-43 only when grown in a galactose-containing media. In wild type yeast, overexpression of TDP-43 causes reduction in cell survival inversely proportional to the amount of galactose in the media (which controls the level of TDP-43). Mutant yeast strains with increased/decreased cell survival over wild type have a mutation which suppresses/enhances TDP-43 toxicity. Candidate suppressor/enhancer genes are knocked down/out in an independent strain of yeast and tested for TDP-43 toxicity for validation.

Visualization of intronic lariat localization in live yeast cells

The MS2 loop genomic-tagging strategy (m-TAG) fluorescently labels a specific RNA sequence in live cells (see Fig. 2)[7]. The following m-TAG DNA sequence is created: loxp site, selectable marker, loxp site, MS2 loops. Sequences homogous to the targeted insertion site are added to the either end of the m-TAG sequence. The m-TAG sequence is then PCR amplified and transformed into yeast. During replication, the yeast chromosomes undergo homologous recombination, during which some cells incorporate the sequence into their genome at the target site. Cells with incorporation are selected for. These cells are transfected with a vector that expresses cre, causing recombination between the loxp sites and removal of the selectable marker and one loxp site. When the targeted sequence is expressed, the RNA contains MS2 loops. MS2 coat protein fused with GFP (MS2-CP-GFP) expressed from a vector binds MS2 loops in RNA. The GFP can be imaged in live cells.

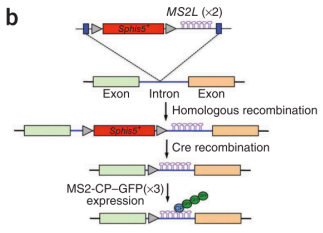

Figure 2: The MS2 loop genomic tagging stragegy (m-TAG). The floxed selectable marker Sphis5 + MS2L sites are inserted into an intron by homologous recombination. Cells with the insert are selected, then Sphis5+ is removed by cre-mediated recombination. MS2-CF-GFP binds to the intronic RNA's MS2 loops so that the intron can be located by fluorescent imaging. Figure from Armakola et al. 2012[9].

Models of TDP-43 toxicity in neurons

Human neuroblastoma cell line M17 cells are infected with a lentivirus encoding Q331K mutant TDP-43 (with a flag tag) expressed only when doxycycline is present. Small interefering RNA (siRNA) against DBR1 are transfected into the M17 cells to knockdown DBR1. A non-targeting siRNA is transfected as a control. M17 cells are immortalized and have been grown in vitro for many passages. Primary neurons may behave differently. Therefore, primary rat cortical neurons are also tested for TDP-43 toxicity. Primary cells are transfected with TDP-43 fused with enhanced green fluorescent protein (EGFP) as well as mApple (a red fluorescent protein). During live-cell fluorescent microscope imaging, GFP shows the

location of TDP-43 while mApple shows the location of live cells. Control cells are transfected with GFP and mApple. Dbr1 is knocked down with siRNA; a non-targeting siRNA is used as a control.

Automated fluorescence microscopy of live cells

Primary rat cortical neurons transfected with the fluorescent proteins described above are imaged by an automated microscope system. Individual cells are imaged once a day for 8 days. Blebbing of cell membranes detected under the red fluorescent channel indicates cell death.

Results

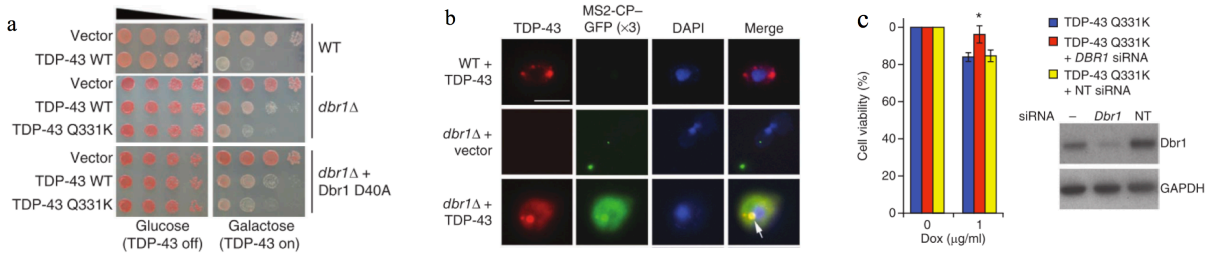

Figure 3: Key figures demonstrating the mechanism of dbr deletion on reduction of TDP-43 toxicity and relevance to human disease. (a) Wild type yeast overexpressing TDP-43 from a galactose-controlled promoter exhibit reduced survival in media containing galactose. Survival is rescued in dbr1Δ cells even when an enzymatically inactive form of dbr1 (D40A) is expressed. (b) Immunostaining for TDP-43 (red), in vivo expression of GFP fused to MS2-CP (green), and nuclear staining (blue) show colocalization of TDP-43 cytoplasmic inclusions with GFP-labeled intronic RNA larits in dbr1Δ but not WT yeast. (c) Human neuroblastoma line M17 cells express Q331K TDP-43 when induced by doxycycline. Similar to yeast, wild type cells show toxicity that is rescued by DBR1 knockdown. Adapted from Armakola et al., 2012, figures 4b (a), 5e (b), and 2d (c).

Identification of gene modifiers of TDP-43 toxicity by yeast screen

Two yeast libraries (one of non-essential single-gene knockout and one of essential single-gene knockdown strains) undergo screening. Each yeast strain is transfected with a galactose-induced copy of TDP-43. Overexpression of TDP-43 induced by plating on galactose-containing media reduces survival of wild type (WT) yeast. Some strains, however, survive better/worse than WT. These strains have a gene knockout/knockdown which suppresses/enhances TDP-43 toxicity. Suppressor and enhancer genes are listed in Armakola et al. Table 1 and Supplementary Data. Knockout of dbr1 suppressed toxicity most effectively, so it was chosen for further study.

While TDP-43 overexpression is a good model for ALS, the disease can also be caused by mutation in TDP-43 (such as Q331K) or by overexpression of the gene FUS. Q331K TDP-43 or FUS are transfected into yeast on a galactose-induced vector and yeast are assayed for survival. Deletion of dbr1 rescues survival just as it did during TDP-43 overexpression (Armakola et al. Figure 1a). Therefore, targeting DBR1 in humans has potential to treat ALS caused by TDP-43 overexpression/mutation and FUS overexpression.

Interactions between TDP-43 and dbr1 in yeast

Armakola et al. test multiple hypotheses regarding the interaction between dbr1 and TDP-43:

Hypothesis 1) Deletion of dbr1 may reduce TDP-43 expression.

Not supported. An immunoblot for TDP-43 shows TDP-43 expression is not changed in dbr1Δ yeast relative to WT (Armakola et al. Figure 1b).

Hypothesis 2) Lariat debranching activity by dbr1 is separate from TDP-43-affecting activity.

Not supported. When dbr1 is expressed from a vector in dbr1Δ yeast, TDP-43 toxicity returns. But when mutant forms of dbr1 lacking debranching activity (D40A and N85A) are expressed in dbr1Δ yeast, TDP-43 toxicity remains suppressed. A control experiment shows similar TDP-43 toxicity in dbr1Δ + WT dbr1 and WT yeast. Another control shows that WT, D40A, and N85A dbr1 vectors are expressed at similar levels (Armakola et al. Figure 4a). These results show that dbr1 debranching activity is essential to the maintenance of TDP-43 toxicity in WT cells.

Hypothesis 3) Accumulation of lariats in the absence of dbr1 could reduce TDP-43 toxicity.
Supported. The deletion of other RNA-processing genes and subsequent accumulation of linear, non-lariat RNA did not reduce TDP-43 toxicity (Armakola et al. Figure 4c).

Hypothesis 4) Intronic lariats could interact with TDP-43.
Supported. Three assays show association between TDP-43 and lariat RNA.

An intronic lariat sequence expressed from a vector is labeled with GFP and imaged using the m-TAG strategy. In WT yeast, the labeled intronic lariats diffuse evenly throughout the cell because intronic lariats do not accumulate. In dbr1Δ yeast, intronic lariats localize to foci (Armakola et al. Figure 5c). Immunostaining reveals TDP-43 co-localization with intronic lariat foci (Figure 3b).

When TDP-43 is flag-tagged and pulled down from dbr1Δ cell lysate, nucleic acid lariats are bound. Lariats run in an arc through 2D electrophoresis gels due to their bulky shape and uneven travel, whereas linear RNA/DNA runs on the diagonal (Armakola et al. Supplementary figure 6a).

GST-pulled down TDP-43 binds to radio-labeled linear RNA, visualized by an electrophoretic mobility shift assay (EMSA). Because TDP-43 binds RNA, addition of RNA isolated from WT and dbr1Δ cells competes for TDP-43 binding and reduces the size of the gel-shifted band. Treatment of RNA isolated from dbr1Δ yeast with RNase R (which degrades linear RNA) results in the isolation of lariat/non-linear RNA. This lariat/non-linear RNA showed competition for binding to TDP-43 (Armakola et al. Supplementary figure 6b,c). Competition for binding is evidence of an interaction between lariat RNA and TDP-43

Hypothesis 5) Intronic lariats sequester TDP-43 in the nucleus, away from cytoplasmic targets.
Not supported. As previously discussed, immunostaining reveals co-localization of TDP-43 with GFP-tagged intronic lariats in the cytoplasm (Fig. 3b). Furthermore, removal of the nuclear localization signal on TDP-43 does not affect toxicity in WT or lack thereof in dbr1Δ cells (Armakola et al. figure 5a).

<u>Dbr1 knockdown reduces mutant TDP-43 toxicity in human and rat neurons</u>

As shown so far, deletion of dbr1 reduces TDP-43 toxicity in yeast. Further proof is needed to determine whether reducing dbr1 levels will be useful to treat TDP-43 toxicity in people with ALS. Armakola provide evidence in two neuronal models: human and rat.

The immortalized human M17 neuroblastoma cell line was transfected with a doxycycline-induced, flag-tagged, Q331K TDP-43 coding sequence. Control experiments show that the mutant TDP-43 is expressed only upon doxycycline induction and that expression of mutant TDP-43 reduces cell viability more than doxycycline alone (Armakola et al. Figure 2b and 2c). Importantly, siRNA knockdown of DBR1 rescues cell viability when mutant TDP-43 is expressed (Fig. 3c). Non-targeting siRNA and no siRNA controls do not show rescued viability. DBR1 knockdown therefore reduces TDP-43 toxicity in human neurons.

Primary neurons isolated from rat brains were transfected with mApple and TDP-43 fused with EGFP or EGFP alone. Loss of mApple expression indicated cell death. Eight-day-long experiments show that cells overexpressing TDP-43 had a lower survival rate and higher risk of death than cells expressing EGFP alone (Armakola et al. Figure 3b and 3c). DBR1 knockdown with siRNA improves survival rate

and lowers risk of death in cells expressing TDP-43, although not to the level of cells expressing EGFP alone. Targeting DBR1 therefore reduces TDP-43 toxicity in rat neurons.

Conclusions

The overexpression or mutation of the protein TDP-43 leads to motor neuron decay and, accordingly, amyotrophic lateral sclerosis. Armakola et al. identify genetic targets for the treatment of ALS. One target is explored in depth: dbr1, a lariat debranching enzyme. In yeast, deletion of dbr1 reduces the toxicity of ALS-associated TDP-43 overexpression or mutation. Similarly, knockdown of dbr1 in rat and human neurons reduces cell death when TDP-43 is overexpressed or mutated. This reproducibility from yeast, to rat, to human is evidence of the therapeutic potential of DBR1 knockdown for the treatment of ALS.

In yeast, dbr1 deletion does not simply reduce TDP-43 expression. Rather, lack of debranching activity results in the accumulation of [branched] lariat RNA. Lariat RNA bind TDP-43 and co-localize in the cytoplasm, suggesting that the lariats sequester TDP-43 (Fig. 4). Perhaps TDP-43 sequestration prevents the protein from inhibiting translation of essential mRNAs and prevents TDP-43 depletion from the nucleus, where it may have a role in cell survival.

Although reduction of DBR1 expression is shown to reduce TDP-43 toxicity, knockout or knockdown of a protein is not currently feasible in humans. Other strategies will need to be devised, such as DBR1 inhibition. The yeast model, assays, and mechanism of dbr1 effect on TDP-43 reported by Armakola et al. will be useful in designing new strategies.

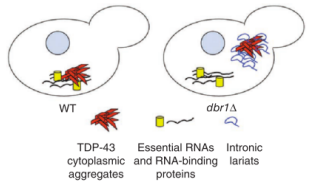

Figure 4: Hypothesis on the mechanism of TDP-43 toxicity and the suppression of toxicity by dbr1 deletion. TDP-43 binds to essential RNAs and interferes with translation. Intronic lariats accumulated in dbr1Δ cells compete for binding to TDP-43, leaving essential RNAs available for transcription.

Critique

Overall, a strong case is presented for DBR1 as a potential therapeutic target in the treatment of ALS. However, several experiments would provide further support:

- Immunostaining showed TDP-43 foci in the cytoplasm. However, it is important to know which cytoplasmic structures, if any, TDP-43 foci associate with. It would be particularly interesting to co-stain for ribosomes (is translation occurring near TDP-43 inclusions?) or the cytoskeleton (are the TDP-43 inclusions near the cell membrane?). Note that Armakola et al. supplementary figure 5 does show co-localization with P-bodies and stress-granules.
- To more decisively show that intronic lariats, not dbr1, sequester TDP-43, sequestration should occur in the presence of accumulated lariats *and* WT dbr1. Perhaps a lariat that can't be debranched by dbr1 could be expressed from a vector.

Further research is needed before therapeutics targeting DBR1 are used to treat ALS. Armakola et al. do not discuss this important future research direction:

- TDP-43 binds both linear and lariat/non-linear RNA (Armakola et al. Supplementary figure 6b and c). However, the accumulation of linear, nonsense mutation containing mRNA did not suppress TDP-43 toxicity (Armakola et al. Figure 4c). Why isn't TDP-43 sequestered by linear RNA? Future kinetic studies should measure the strength of binding between TDP-43 and different RNA structures. RNAs of different 3D structure, length, and bonds could be tested. In addition to lariat and linear RNA, circular RNA could be tested (circular RNAs can act as regulatory molecules[5]). Such studies will also reveal whether TDP-43 binds RNA directly.

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