A yeast functional screen predicts new ALS disease genes

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Introduction

ALS is a devastating adult-onset neurodegenerative disease that attacks upper and lower motor neurons (1). Symptoms of this disease are a progressive and fatal muscle paralysis, causing death within 2 to 5 years of disease onset.

Mutations in several genes have been linked to both sporadic or familial forms of ALS, including *SOD1*, *TARDBP*, *FUS/TLS* and others (2). Two of these genes, *TARDBP* (TDP-43) and *FUS/TLS* (FUS) both encode similar types of RNA-binding proteins (3) and have been identified as components of pathological inclusions in neurons of ALS patients (4-6). An emerging concept suggested by the discoveries of FUS and TDP-43 in ALS is that defects in RNA metabolism might contribute to the pathogenesis.

Could other human RNA-binding proteins with similar properties as TDP-43 and FUS also contribute to ALS?

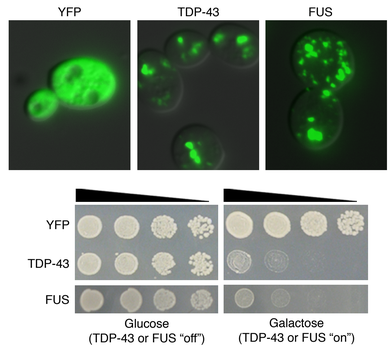

Figure 1. Yeast functional screen identifies human RNA binding proteins with similar properties as FUS and TDP-43. When expressed in yeast cells TDP-43 and FUS form multiple cytoplasmic aggregates (top) and both are also toxic when expressed in yeast (bottom).

Project

TDP-43 and FUS both contain RNA recognition motif (RRM) (3) and we found that they both formed cytoplasmic aggregates and were toxic when expressed in yeast (Fig. 1 and (7, 8)).
Including FUS and TDP-43, there are at least 226 RRM-containing proteins (PFAM ID PF00076) present in the human proteome. Are there other human RRM-containing proteins with similar properties as TDP-43 and FUS (e.g. aggregation-prone and toxic in yeast)?
If so, might these be potential ALS disease genes?

We therefore designed a simple yeast functional screen to identify such genes (Fig. 2). We cloned 133 different human RRM-containing open reading frames (ORFs) into yeast expression vectors as YFP fusions, under the control of a galactose-inducible promoter, and individually transformed them into yeast cells. Fluorescence microscopy and spotting assays were used to determine respectively the localization of each protein and to assess toxicity.

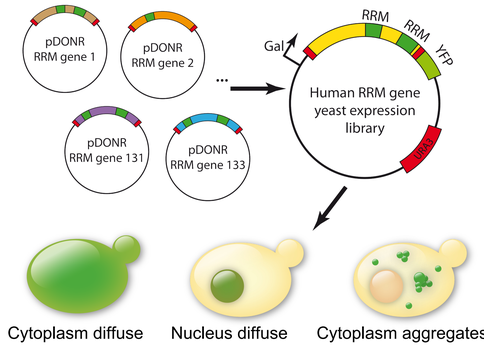

Figure 2. Yeast functional screen designed to identify additional human RNA-binding proteins that aggregate and are toxic in yeast. A library of 133 different human ORFs encoding RNA-binding proteins as YFP fusions was individually transformed into yeast cells.

Yeast screening for toxic and aggregating RRM RNA-binding proteins

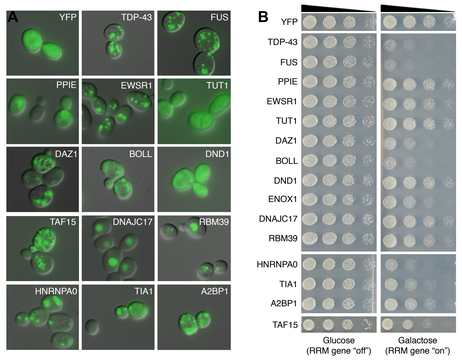

Figure 3. A) Examples of various localization patterns in yeast cells of human RRM RNA-binding proteins. Some proteins were localized diffusely throughout the cytoplasm and others were localized diffusely in the nucleus. Some formed multiple foci in the nucleus and several others resembled FUS and TDP-43, which formed multiple cytoplasmic foci. **B)** Spotting assays to assess the toxicity of human RRM proteins. Transformants were grown on synthetic media containing either glucose (control, RNA-binding protein "off") or galactose (to induce expression of candidate ORFs, RNA-binding protein "on"). Some proteins were very toxic when overexpressed, like FUS and TDP-43 while others were moderately toxic or not toxic.

Missense mutations found in ALS patients

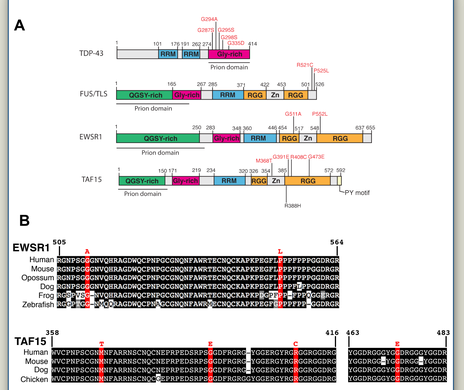

Figure 4. DNA was obtained from ALS (735) and control (1328) patients and sequenced for mutations. **A)** Comparison of FUS, TDP-43, and TAF15 demonstrates similar domain architecture. **B)** Sequence alignment of amino acids 505 to 564 of EWSR1, 358 to 416 of TAF15 and 463 to 483 of TAF15 from diverse vertebrate species indicates that the mutated residues in TAF15 are highly conserved.

EWSR1, TAF15 and its ALS-linked variants are aggregation-prone

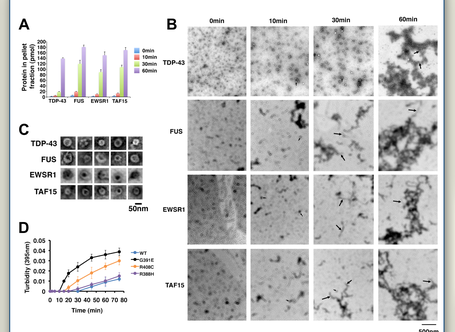

Figure 5. A) At the indicated times GST-tagged proteins (3μM, 25ºC, agitation, TEV) reactions were processed for sedimentation analysis. The amount of protein in the pellet fraction was determined by densitometry after SDS-PAGE and Coomassie Brilliant Blue staining. **B)** At various times GST-tagged proteins (3μM, 25ºC, agitation, TEV) were processed for EM. Small arrows denote small pore-shaped oligomers and large arrows denote linear polymers. **C)** Gallery of TDP-43, FUS, EWSR1 and TAF15 oligomers formed during aggregation reactions. **D)** ALS-linked TAF15 variants (G391E, R408C) displayed accelerated aggregation kinetics than variant also found in controls (R388H). Error bars = mean ± S.E.M. (N=3)

EWSR1 and TAF15 confer neurodegeneration in Drosophila

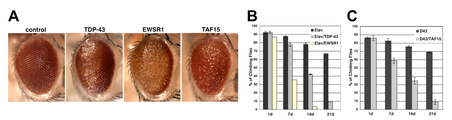

Figure 6. A) EWSR1 and TAF15 overexpression cause degeneration and disruption of the Drosophila retinal structure, akin to TDP-43. Control is driver line alone gmr-GAL4/+. TDP-43 is gmr-GAL4/UAS-TDP-43-YFP. EWSR1 is gmr-GAL4/UAS-EWSR1. TAF15 is gmr-GAL4/UAS-TAF15 (grown at 29ºC). **B,C)** Progressive loss of climbing behavior upon expression of TDP-43, EWSR1 and TAF15 in the nervous system (elav, **B**) or selectively in motor neurons (D42, **C**).

EWSR1 and TAF15 accumulate in ALS patient spinal cord neurons cytoplasm

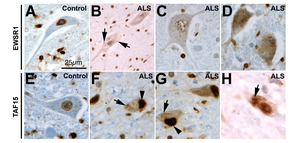

Figure 7. Immunohistochemistry to visualize localization of EWSR1 (**A-D**) and TAF15 (**E-H**) in spinal cord of control or ALS patients. In control spinal cord neurons EWSR1 (**A**) and TAF15 (**E**) are localized predominantly to the nucleus. In ALS spinal cord neurons, EWSR1 was present in cytoplasmic punctate granular structures (**B-C**, arrows) or in a diffuse pattern throughout the cytoplasm (**D**). In addition to nuclear localization (arrows) TAF15 accumulated in cytoplasmic puncta (**F-H**).

ALS-linked TAF15 variants promote cytoplasmic localization in motor neurons

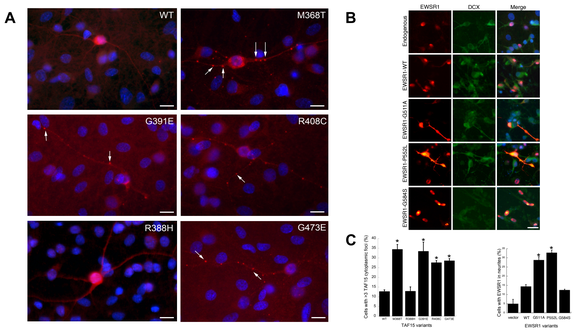

Figure 8. A functional assay to distinguish potentially damaging from benign variants. **(A)** We performed an unbiased assessment of all TAF15 missense variants identified from sequencing of ALS cases (M368T, G391E, R408C, and G473E) and controls (R388H). Primary rat embryonic neuron cultures were transfected 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 μm. **B)** Primary mouse neuron cultures were transfected with wild-type or mutant EWSR1, stained with α–EWSR1 (red) and α–doublecortin (green). Endogenous EWSR1 is almost exclusively localized within the nucleus of neurons. Overexpression of wild-type EWSR1 shows localization within the nucleus or cytoplasm of neurons, with rare neurites containing EWSR1. The ALS-linked mutant forms of EWSR1 showed increased mislocalization into the neurites, including dendrites and axons. **(C)** Quantitation of mislocalization of transfected WT or mutant TAF15 or EWSR1. Variants found in ALS cases (TAF15: M368T, G391E, R408C, G473E ; EWSR1: G511A, P552L) showed mislocalization whereas variants found in both cases and controls (TAF15: R388H, EWSR1: G584S) behaved like WT. *, $P < 0.01$ (localization of TAF15 variants compared to WT, Student's t test). Error bars = mean ± S.E.M.

Conclusion

In an effort to discover new ALS disease genes, we devised a simple yeast functional screen to identify additional RNA-binding proteins with properties similar to FUS and TDP-43. This screen resulted in the identification of 35 RNA binding proteins that behave like FUS and TDP-43 in yeast (cytoplasmic aggregation and toxicity), 9 containing a predicted prion domain. As evidence of the usefulness of this approach, we identified missense mutations in two of these genes, EWSR1 and TAF15. They now join FUS and TDP-43 as RNA-binding proteins linked to ALS, further underscoring a central role for perturbations in RNA metabolism in ALS pathogenesis. In a broader sense, there is probably a very delicate balance in RNA processing within motor neurons and slight perturbations from any one of several different aggregation-prone RNA-binding proteins could be disastrous. Indeed, some ALS-linked mutations in TDP-43 increase aggregation of the protein (8) and just as the mutations we found in TAF15 and EWSR1 also increase aggregation. We also showed that overexpression of TAF15 and EWSR1 confer neurodegeneration in Drosophila, as for FUS and TDP-43. Finally, we observed mislocalization of these proteins in ALS patient spinal cord, and show that mutations in TAF15 and EWSR1 lead to a cytoplasmic localization in neurons. We anticipate that we have merely scratched the surface and many other aggregation-prone RNA binding proteins could also contribute to ALS. Meanwhile, our list of ALS candidate genes from this yeast screen will hopefully jumpstart efforts to identify new ALS disease genes and spur innovative new diagnostic and therapeutic approaches.

Acknowledgments

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