Exploring the Mitigation of TDP-43 Toxicity by Sis1 in Yeast

Senior Thesis in the Biochemistry and Molecular Biology Major

by

Jackie Ham

Mentor: Dr. Sei-Kyoung Park

PI: Dr. Susan Liebman

Abstract:

Amyotrophic Lateral Sclerosis is a progressive neurodegenerative disease with a 100% fatality rate. Most cases have no known cause, with 5-10% of cases having a familial link. Currently there is no cure. Research has revealed that Tar DNA-binding Protein 43 cytoplasmic aggregates are involved in many cases of Amyotrophic Lateral Sclerosis. One possible mechanism for TDP-43 induced toxicity is that these aggregates are titrating away a molecular chaperone protein, Sis1, from the nucleus of the cell. Sis1 is important in the degradation of proteins, and without it, the cell would become non-viable. In order to examine the relationship between the two proteins, they were tagged with fluorescent proteins and examined for colocalization under fluorescent microscopy. However, while TDP-43 does appear to titrate Sis1 out of the nucleus, there was no evidence that the two proteins colocalized.

Introduction:

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that affects roughly 20,000 Americans each year. There are two types of ALS, familial and sporadic. Familial ALS means that the disease has been inherited, while sporadic ALS has no known cause. Only 5-10% of ALS cases are familial, meaning that science has no explanation for 90-95% of ALS instances. This is troubling, as ALS is a 100% fatal disease—the patient loses control of voluntary movements until they can no longer move, speak, or breathe.

We now know that there is some correlation between the presence of the mutated form of the protein TDP-43 and a diagnosis of ALS[1,8]. TAR DNA-binding protein 43 (TDP-43) is a nuclear protein whose normal function in the cell is not well known[5]. TDP-43 has been found to form protein aggregates outside of the nucleus, in many patients with ALS[4].

Modeling ALS, the overexpression of TDP-43 leads to toxicity of yeast cells and aggregation of TDP-43. This makes yeast a good model for looking for TDP-43 modifiers that may lead to ALS treatments. For example, the Gitler lab found the absence of the gene Pbp1 in yeast to reduce the TDP-43 induced toxicity. This led to the discovery of Pbp1's human analog, ATXN2, which was also found to be a modifier of TDP-43[3]. When TDP-43 is overexpressed in yeast cells, TDP-43 aggregates have been found to form in a similar manner to human cells[3]. The aggregates of TDP-43 are thought to be related to the toxicity of yeast and human cells[2]. However, little is known about the mechanism involved in the formation of the aggregates.

Research done by the Liebman lab has shown that overexpression of the molecular chaperone protein Sis1 reduces the toxicity produced by overexpression of TDP-43 in yeast cells. Sis1 is a molecular chaperone shuttling between the nucleus and the cytoplasm involved in protein degradation, and without it, the cell would become non-viable[7]. One possible hypothesis was that Sis1 is titrated away from the yeast nucleus by TDP-43 aggregates which prevents Sis1 from fulfilling its important role in the degradation of proteins. Since Sis1 is needed for the degradation of misfolded cytosolic proteins, if it is titrated away from the nucleus misfolded proteins would build up

resulting in the death of the cell. When Sis1 is overexpressed, the TDP-43 induced toxicity is relieved. Therefore, in this paper, the interaction between Sis1 and TDP-43 will be examined in order to find a mechanism for reduced TDP-43 toxicity through a colocalization study between fluorescently tagged TDP-43 and Sis1 in live yeast. If the two proteins colocalized under fluorescence microscopy, this would provide evidence to support our hypothesis.

The Liebman lab had already constructed a plasmid containing TDP-43 tagged with EYFP (Enhanced Yellow Fluorescent Protein). However, it was determined that the fluorescent proteins exhibited too much bleed-through to be useful in a colocalization experiment. The lab also had TDP-43 tagged with dsRed, but this tag caused the TDP-43 to form uncharacteristic, large aggregates. The TDP-43-dsRed tag also did not produce consistent signal, regardless of how many transformations were attempted. Therefore, EGFP (Enhanced Green Fluorescent Protein) and mCherry was chosen as a tag for TDP-43 and Sis, respectively. This allowed a colocalization study between Sis1 and TDP-43 to be done.

Methods:

Construction of Sis1-mCherry Chromosomal Tag

To tag Sis1 with mCherry fluorescence on the *Saccharomyces cerevisiae* chromosome, a PCR (polymerase chain reaction) was set up to amplify mCherry on pFA6a-mCherry-SpHis5 (p2268) with flanking sequence of C-terminus of Sis1. The reaction contained: 6 µL of 10µM dNTPs, 6 µL of 10µM forward primer, 6 µL of 10µM reverse primer, 6 µl of template plasmid (p2268), 3 µL of DNA Taq Polymerase, and 30

µL of 10x buffer. For the 1.5 kb mCherry-SpHis5 PCR, one cycle was run at 94 ºC for 10 minutes, 35 cycles at 95 ºC for 40 seconds of denaturation, 55 ºC for 1 minute on annealing, and 72 ºC for 2 minutes for extension. The sample was then held at 72 ºC for 10 minutes, and cooled to 4 ºC for an infinity hold.

The 1.5 kb PCR product was purified using the Qiagen PCR Purification kit. 1.5 mL of Buffer PB was added to the PCR sample and mixed, then the sample was applied to a QIAquick column inside of a 2 mL collection tube and centrifuged for 60 seconds at 15000 rpm, after which the flow through was discarded. 0.75 mL of Buffer PE was added to the column, which was centrifuged for 60 seconds at 15000 rpm, again discarding the flow through. The column was centrifuged again for 60 seconds to remove residual ethanol and placed in a clean Eppendorf tube. 50 µL of water was added to the column, allowed to stand for 1 minute, and centrifuged for 1 minute to elute the purified DNA, and the DNA was used to transform yeast, L1749 (*MATa ade1-14 ura3-52 leu2-3, 112 trp1-289 his3-200 [psi−] [PIN+]*).

For the transformation, L1749 was grown overnight at 30 ºC with shaking until the early log stage. The cells were spun down for 6 minutes at 8000 rpm, discarding the supernatant. The cells were resuspended in 10 mL of 0.1 M LiAce buffer, and incubated at 30 ºC for 60 minutes. The cells were spun down for 6 minutes at 8000 rpm, discarding the supernatant. The cells were resuspended in 100 µL of 0.1 M LiAce buffer, to which 10 µL of purified mCherry-SpHis5 PCR product was added, along with 5 µL of salmon sperm DNA in order to increase the efficiency of the transformation. The cells were incubated at 30 ºC for 30 minutes. 200 µL of 50 % PEG was added and mixed, and the

cells were incubated at room temperature for 60 minutes. The cells were heat shocked at 42 ºC for 10 minutes, spun down, and resuspended in 100 µL of water. Then the cells were plated on dextrose (SD)–His plates and grown at 30 ºC until colonies were formed. The transformants were confirmed using a Nikon Eclipse E600 Microscope with Y-FL EPI fluorescence attachment.

Construction of pRS416Gal-TDP-43-EGFP

In order to create a plasmid with TDP-43-EGFP under a *Gal* promotor in a low copy plasmid (pRS416 Gal-TDP-43-EGFP), pRS416 Gal-TDP-43-EYFP (p2042) and pRS426 Gal-TDP-43-EGFP (p2041) were used. Two restriction enzyme sites (HindIII and SpeI) were found to switch two fluorescence tags without disrupting the open reading frames. Two double digests were set up, using 17 µL of each plasmid, 2 µL of 10x Tango buffer, 0.3 µL SpeI enzyme, 0.6 µL HindIII enzyme and 0.1 µL water in each tube. The digest was incubated for 60 minutes at 37 ºC. The digests were run on a 1 % agarose gel along with a 1kB ladder. The gel was analyzed to determine if the samples were cut correctly.

To purify the DNA fragments, DNA bands were excised from the agarose gel and purified using QIAquick spin columns. The slices of gel were placed into clean Eppendorf tubes and weighed. Three volumes of Buffer QG was added to the gel slices, and incubated for ten minutes at 50 ºC, until the gels were completely melted. Then the samples were placed into columns and centrifuged for one minute. The flow through was discarded, and 0.5 mL of Buffer QG was added to the samples, which were then centrifuged again for one minute. The columns were washed with 0.75 mL of Buffer PE,

and centrifuged again for one minute. Each column was transferred to a clean Eppendorf tube to collect the purified DNA. In order to elute the DNA, 50 µL of water was added to the columns, allowed to stand for one minute, then both columns were centrifuged for one minute. The resulting DNA was analyzed using a 1 % agarose gel and purified, ligated, and transformed into *E. coli* in order to transform into L1749 and the previously created L1749 Sis1-mCherry (L3480).

The *E. coli* transformants were suspended in 250 µL of buffer P1 and mixed. 250 µL of Buffer P2 was added to the solution, which was gently mixed. 350 µL of Buffer P3 was added to the plasmid and mixed gently. The sample was then centrifuged for 10 minutes at 15000 rpm in a tabletop centrifuge. The supernatant was transferred to a QIAprep spin column and centrifuged for 60 seconds and the flow through was discarded. The QIAprep spin column was washed with 0.5 mL of Buffer PB and centrifuged for 60 seconds, discarding the flow through. The sample was washed again using 0.75mL of Buffer PE, centrifuged for 60 seconds and the flow through discarded. The sample was centrifuged for 60 seconds again to remove residual wash and buffer. The sample was moved to a clean 1.5 mL microcentrifuge tube. 50 µL of water was added to the column membrane. The sample was allowed to stand for 1 minute, then centrifuged for 60 seconds to elute the DNA. To confirm the insert was correct, the plasmid was digested with SpeI and run on a 1 % agarose gel. The correct clones were used to transform yeast L1749 and L3480.

L1749 and L3480 were transformed with the newly constructed pAG416 Gal-TDP-43-EGFP (p2288) as described above. Transformants were selected on SD-Ura

plates and confirmed the TDP-43-EGFP phenotype for its toxicity and fluorescence phenotype.

Stress test

L3480 cells were grown overnight at 30 ºC with shaking. Cells in the early log phase were divided into five time intervals at 42 °C to stress the cells—0 minutes, 10 minutes, 30 minutes, 10 minutes with recovery at 30 °C, and 30 minutes with recovery.

Fixing cells to view Sis1-mCherry and TDP-43-GFP

L1749 and L3480 with or without TDP-43-EGFP (p2288) were grown overnight in Raffinose (SR)-Ura at 30ºC with shaking. The next morning, TDP-43-EGFP was overexpressed by adding 2 % galactose. 450 μL of cells were placed into each Eppendorf tube, and heated for their allotted time. For better fluorescent analysis, the cells were fixed. 50 μL of 36 % formaldehyde was added, and the sample was kept at room temperature for 15 minutes. The sample was centrifuged for 5 minutes at 8,000 rpm, and the supernatant was removed. The cells were resuspended in 500 μL of 100 μM potassium phosphate buffer (KPO_4), centrifuged for 5 minutes at 8,000 rpm, again discarding the supernatant. The cells were resuspended in 30 μL of KPO_4 and kept at 4 ºC until they were examined using a Nikon Eclipse E600 Microscope with Y-FL EPI fluorescence attachment.

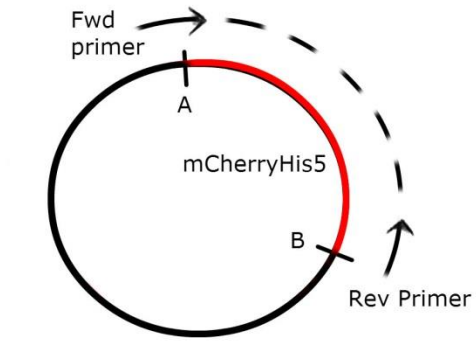
For the stress test, L3480 cells were grown overnight at 30 ˚C in SD-Ura. Cells in the early log phase were divided and heat stressed at 42˚C for 0, 10, and 30 minutes followed by a recovery at 30 ˚C. Cells were then fixed and imaged using fluorescence microscopy.

Results:

Sis1-mCherry localized mostly in nucleus and is diffused in cytoplasm

Figure 1 shows a strategy for construction of Sis1-mCherry on yeast chromosome. To tag mCherry on C-terminus of Sis-1 mCherry-SpHis5 DNA amplified by PCR using p2268 as a template and transformed into L1749.

The Sis1-mCherry signal was mostly localized to the nucleus where Sis1 resides, indicating that Sis1 was tagged correctly (Figure 2a). In addition, cell growth was compared between L1749 and L1749 Sis1-mCherry (L3480) containing p1752, which does not contain TDP-43 (Figure 2b). As expected, there was no difference in the amount of L1749 and L3480 cells grown on SD-Ura and SGal-Ura plates. This indicates that adding the Sis1-mCherry tag did not interfere with cell growth.



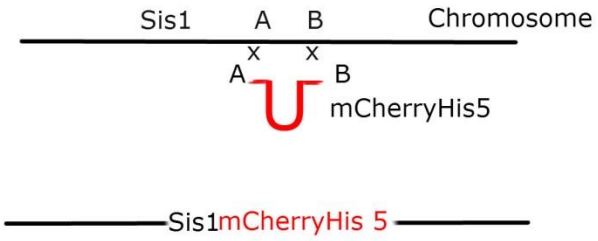


Figure 1. Creating a Sis1-mCherry tag. Using a forward and reverse primer (described in material and method) containing part of the Sis1 sequence (A and B), a PCR was used to amplify mCherry-His5. His5 is a selection marked for transformants. The mCherry was recombined at the C-terminus the chromosomal Sis1, resulting in a Sis1-mCherry tag.

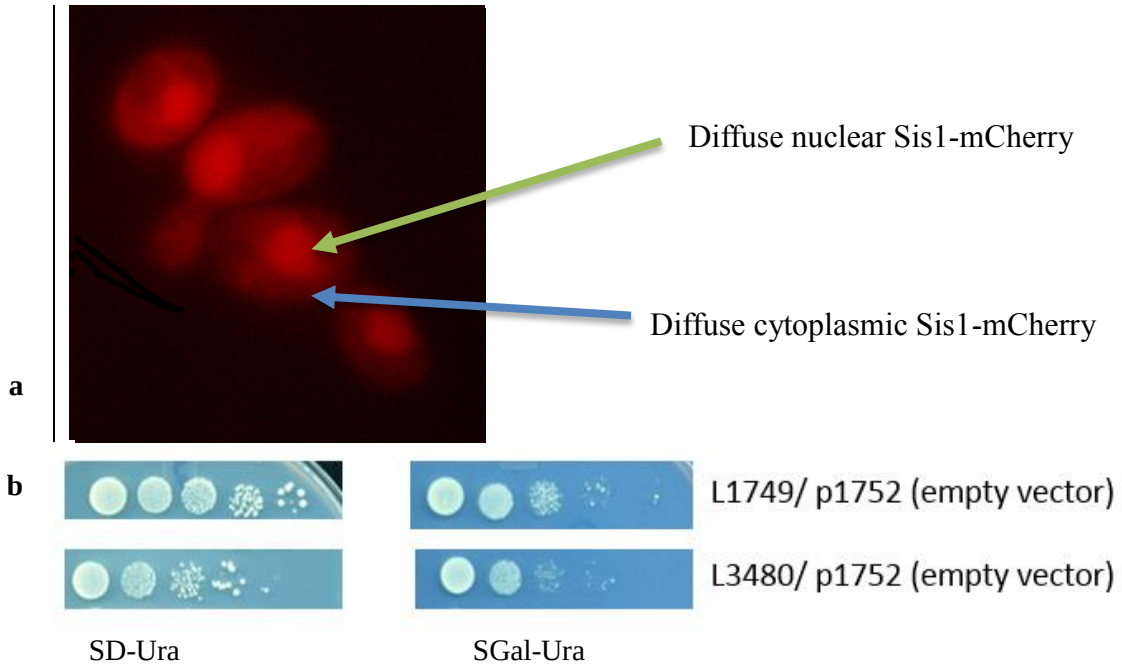
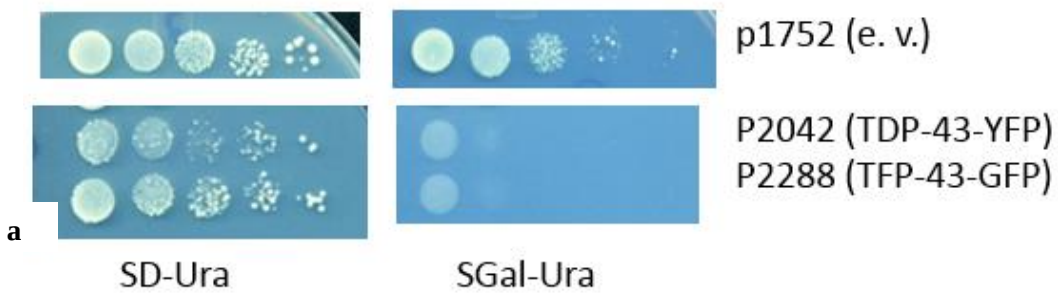
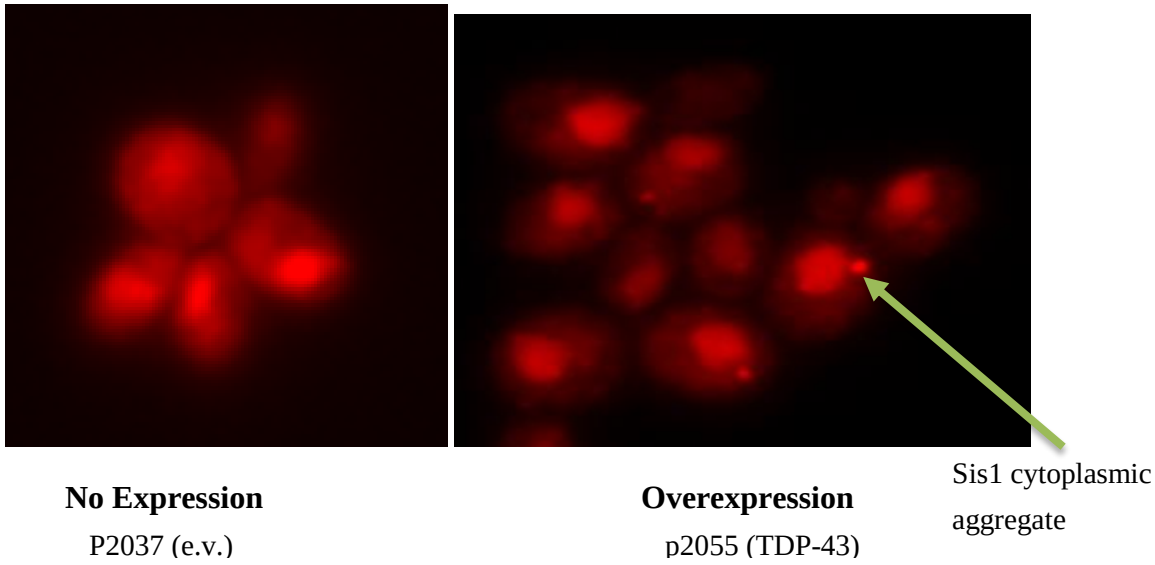


Figure 2. Sis1-mCherry. a. In order to confirm L1749 SiS1-mCherry transformants (L3480), the cells were examined using a Nikon Eclipse E600 Microscope with Y-FL EPI fluorescence attachment. **b.** A toxicity test comparing the cell growth of L1749 and L3480 transformed with p1552 (a control vector) was also performed to insure that the mCherry tag did nothing to alter growth phenotype. Samples were standardized to OD_{600}=2, then serially diluted 1:10 four times. All five dilutions of each strain were plated on SD-Ura and SGal-Ura plates and their growth was monitored.

TDP-43 overexpression causes Sis1-mCherry to form cytoplasmic aggregates



a



b

No Expression
P2037 (e.v.)

Overexpression
p2055 (TDP-43)

c

	Cells with cytoplasmic aggregates	Total cells	%
No TDP-43 overexpression	39	824	4.7
TDP-43 overexpression	194	835	23

Figure 3. Localization of Sis1 with and without TDP-43 overexpression. a. L3480 was transformed with a control plasmid p2037, or with TDP-43 (p2055). Growth was tested by spotting serially diluted cells grown in SR-Ura. **b.** For fluorescence imaging, each transformant was grown in SR-Ura overnight and 2 % galactose was added for TDP-43 overexpression. The images were taken from fixed cells. **c** Comparison of cells with Sis1-mCHerry cytoplasmic dots with or without TDP-43 over expression. The number of cells with cytoplasmic Sis1 aggregates were recorded.

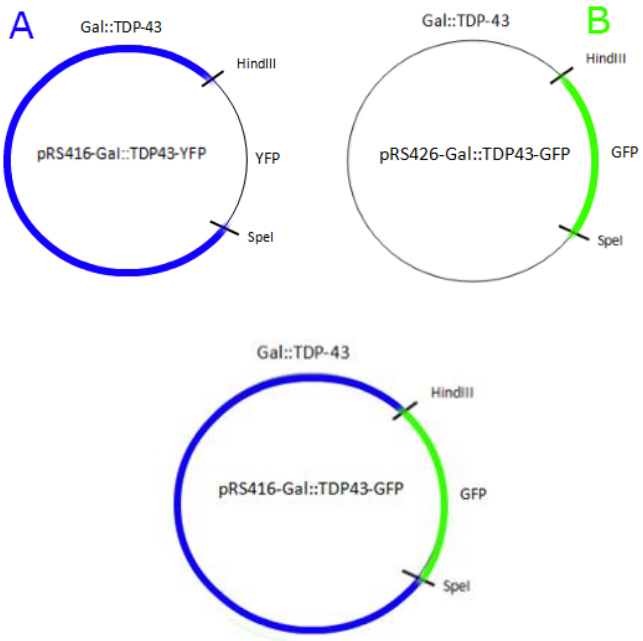
L3480 was transformed and growth was tested by spotting serially diluted cells grown in SR-Ura medium. Like TDP-43-EYFP overexpression, TDP-43-EGFP caused cells to grow less or not at all on SGal-Ura while cells with the control vector (p1752) formed robust patches (Figure 3a).

Sis1-mCherry was also examined under a fluorescence microscope before and after TDP-43 was overexpressed in SR-Ura and SGal-Ura medium, respectively (Figure 3b). This was done with untagged TDP-43 to avoid the problem of bleed through. The cells grown in SGal-Ura media exhibited TDP-43 overexpression, while those grown in SR-Ura did not. Cells from cultures with and without TDP-43 overexpression were examined for Sis1-mCherry cytoplasmic aggregates. As shown in Figure 3b, most of the Sis1-mCherry signal was localized to the nucleus, with diffuse signal in the cytoplasm. However, a few cytoplasmic Sis1-mCherry aggregates were recorded with TDP-43 overexpression. With overexpressed TDP-43 roughly 5 times more cells formed Sis1 cytoplasmic aggregates than did the cells grown with empty vector without TDP-43 overexpression (Figure 3c). This result indicates that overexpression of TDP-43 causes Sis1 to form cytoplasmic aggregates outside of the yeast nucleus.

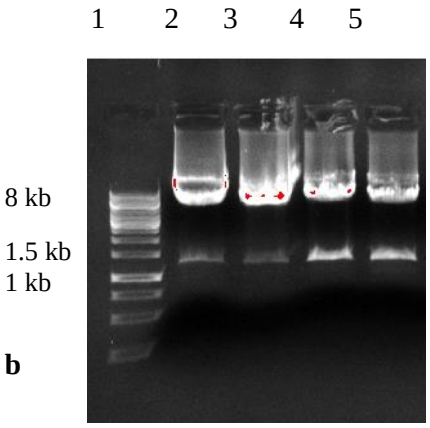
Sis1-mCherry does not show colocalization with TDP-43-EGFP

The Liebman lab already had constructed a plasmid with TDP-43 tagged with EGFP. However, a plasmid with a regulable *Gal* promoter was needed, as the other plasmid was not viable enough to use in colocalization studies. Figure 4a shows the cloning of pRS416 Gal::TDP-43-EGFP. As shown in Figure 4b, the cloning was successful. All four clones have EGFP insert (A) in pRS416 Gal::TDP-43 background

(B) when double-digested by HindIII and SpeI. After conformation of TDP-43-EGFP clone, the plasmid was used to transform L3480. As shown in Figure 4c, TDP-43-EGFP formed cytoplasmic aggregates, indicating no significant difference between TDP-43-EYFP and TDP-43-EGFP.



a



b

B ≈ 8 kb

A ≈ 1.5 kb

HindIII/SpeI cut

c

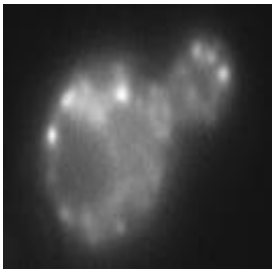
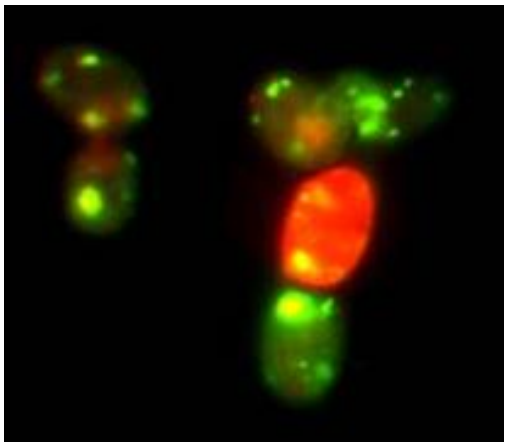


Figure 4. Construction of pRS416-Gal::TDP-43-EGFP plasmid. a In order to construct pRS416-Gal::TDP-43-EGFP, pRS416-Gal::TDP-43-EYFP and pRS426-Gal::TDP-43-EGFP were digested with HindIII and SpeI. Then regions A and B were ligated together. **b** Lane 1 contained a 1kB ladder. Lanes 2-5 contained pRS416-Gal::TDP-43-EGFP digested with HindIII and SpeI. After confirming that the plasmid contained the correct inserts, it was transformed into L3480. **c.** Fluorescence image of TDP-43-EGFP after TDP-43-EGFP was overexpressed in SGal-Ura.

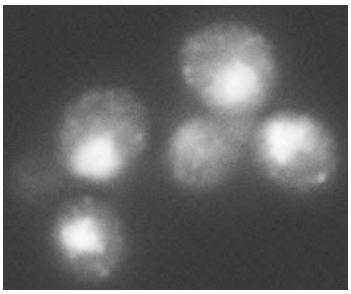
L3480 containing TDP-43-EGFP plasmid was examined after TDP-43-EGFP overexpression in 2 % galactose using fluorescence microscopy. Pictures were taken on the mCherry channel, then the EGFP channel, and the two images were merged (Figure 5). The yellow dot should represent the overlap between the two proteins. However, the TDP-43-EGFP was too strong to be sure that the apparent overlap was not simply due to bleed-through. As shown in Figure 5, Sis1-mCherry on the mCherry channel localized in the nucleus and formed cytoplasmic aggregates in some cells. A single Sis1-mChery dot might not colocalize with TDP-43-EGFP, as shown in the merged image, indicating that Sis1-mCherry titrated from the nucleus by TDP-43-EGFP might not colocalize with TDP-43-EGFP. In merged image, multiple strong dots in yellow in the cytoplasm were not actually the overlap between the two signals, rather were due to bleed-through as shown in control cells that lacked any Sis1-mcherry.



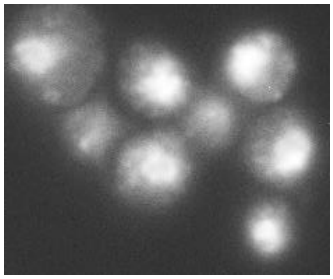
Merge of mCherry and GFP

Figure 5. Colocalization of Sis1 and TDP-43. Images were taken after TDP-43-EGFP overexpression in L3480 containing TDP-43-GFP plasmid using a Nikon Eclipse E600 Microscope. To prevent bleed-though of EGFP into mCherry, Sis1-mCherry was observed first, then TDP-43-EGFP, and the two pictures were merged. The overlapping area is colored yellow.

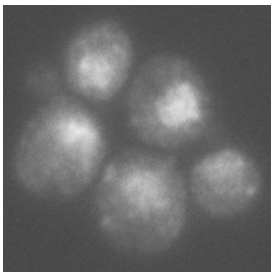
Thermal stress does not change Sis1-mCherry localization



at 30 ˚C



at 40 ˚C for
10 minutes



At 40 ˚C for 30 minutes
with recovery

Figure 6. Stress test. L3480 were grown overnight at 30 °C with shaking. Cells in log phase were incubated at 42 °C for 30 minutes to heat stress, and were fixed immediately using formaldehyde, or were allowed to incubate at 30 ºC for 30 minutes for recovery before fixing. Cells were then examined using florescence microscopy in order to determine change of Sis1-mCherry localization.

In order to determine whether the Sis1 cytoplasmic aggregates were caused by thermal stress, a stress test was performed on L3480. After incubating the cells at 42 ºC, half were allowed to recover for 30 minutes at 30 °C while the rest were immediately fixed and examined using a Nikon Eclipse E600 Microscope (Figure 6). However, Sis1-mCherry does not show any significant change before and after stress indicating that stress caused by TDP-43 expression and thermal stress resulted in different effects on Sis1-mCherry localization.

Discussion:

TDP-43 has been implicated in ALS[4]. An overexpression of TDP-43 in *Saccharomyces cerevisiae* has been shown to cause the cells to become non-viable. Other labs have found TDP-43 modifiers that may work as ALS treatments[3]. The Liebman lab has found that the overexpression of Sis1 relieved TDP-43 induced toxicity, indicating Sis1 is a possible modifier of TDP-43 induced toxicity. The hypothesis we were testing was that TDP-43 aggregates titrate Sis1 out of the nucleus, resulting in cell stress and death. In order to test this hypothesis, a colocalization study was performed on TDP-43 and Sis1. However, the colocalization study results were not supportive of this hypothesis due to Sis1-mCherry cytoplasmic aggregates not colocalizing with the TDP-43-EGFP aggregates.

The location of Sis1 was observed with and without TDP-43 overexpression under fluorescence microscopy. In this case, only the Sis1 was tagged. It was determined that 5 times more cells with TDP-43 overexpressed had cytoplasmic Sis1 aggregates. This would seem to indicate that Sis1 is moved out of the nucleus by TDP-43.

From this research, we found that Sis1 is moved out of the nucleus of the yeast cell by TDP-43 overexpression. However, it does not appear that Sis1 and TDP-43 colocalize in the cytoplasmic aggregates that both proteins form. In order to understand the interaction between Sis1 and TDP-43, more biochemical analysis, such as a pull down analysis, is needed to conclude that Sis1 and TDP-43 do not interact[7].

References:

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