Supporting Information

Trichostatin A Relieves Growth Suppression and Restores Histone Acetylation at Specific Sites in a FUS ALS/FTD Yeast Model.

*Seth A. Bennett§,£, Samantha N. Cobos§,#, Melagras Mirzakandova§, Michel Fallah§, Elizaveta Son§, George Angelakakis§, Navin Rana§, Muna Hugais§ and Mariana P. Torrente§,£,#**

§Department of Chemistry, Brooklyn College, Brooklyn, New York, 11210, United States

£Ph.D. program in Biochemistry, The Graduate Center of the City University of New York, New York, New York, 10016, United States

#Ph.D. program in Chemistry, The Graduate Center of the City University of New York, New York, New York, 10016, United States

*Corresponding Author

Corresponding Author

Mariana Torrente – Email: mariana.torrente@brooklyn.cuny.edu

Table of Contents

1. Materials and Methods

Materials.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Yeast Strains, Media and Plasmids.

All yeast were W303a (*MATa, can1-100,his3-11, 15,leu2,3,11,12,trp1-1,ura3-1,ade2-1).*(1) Yeast were grown in synthetic dropout medium (Clonetech Laboratories, Mountain View, CA) supplemented with 2% glucose, raffinose or galactose. The FUS plasmid (pAG303GAL-FUS) was a gift from A. Gitler (Addgene plasmid no. 29614).(2) A control ccdB plasmid, pAG3030GAL-ccdB, was a gift from Susan Lindquist (Addgene plasmid no. 14133).(1) The TDP-43 plasmid (pAG303GAL-TDP-43) was a gift from A. Gitler (Addgene plasmid no. 27468).(*3*) Yeast were transformed using standard poly(ethylene glycol) and lithium acetate protocols.(*4*)

Solid Media Growth Assays.

Yeast were grown to saturation overnight in raffinose-supplemented dropout media at 30°C. Overnight cultures were diluted 2-fold, then serially diluted 5-fold. A volume of 2 μL for each dilution was pipetted onto synthetic dropout medium glucose or galactose containing no treatment, DMSO, 0.625 μM TSA, 1.25 μM TSA or 2.50 μM TSA (except for TDP-43, where only 2.50 μM TSA was used). Plates were analyzed after 3-4 days of growth at RT. Images of the plates were imported into ImageJ (Fiji),(*5*) and the density of the middle spot of each plate was measured using the oval tool. All experiments were repeated a minimum of three times with three independently transformed yeast strains.

Protein Overexpression.

Yeast strains were grown to saturation overnight in raffinose-supplemented dropout media in a shaking incubator at 30 °C and 200 rpm. Overnight cultures were then diluted to an OD_{600} of 0.30 in 50 mL of raffinose-supplemented synthetic dropout media and grown for another 4 hours at 30 °C. Cells were then pelleted at 850 rcf at 4 °C and washed 3x with sterile distilled water. The pellet was then resuspended in 50 mL of galactose-supplemented media with DMSO, 2.5 μM TSA or untreated and grown for 5 hours at 30 °C. After induction, cultures were normalized by cell count and harvested at 850 rcf at 4 °C for 5 minutes and washed 3X with sterile distilled water. Yeast were then resuspended in 500 μL sterile distilled water, evenly split into five microcentrifuge tubes and harvested at 850 rcf at 4 °C for 5 minutes. The supernatant was removed and the pellets were flash frozen in liquid nitrogen and stored at -80 °C. All experiments were repeated a minimum of three times with three independently transformed yeast strains.

Western Blotting.

Frozen yeast cell pellets were thawed and treated with 0.2 M NaOH for 10 minutes on ice, pelleted again, and subsequently resuspended in 100 μL of 1x SDS sample buffer and boiled for 10 minutes. Cell lysates were separated using SDS-PAGE (15%) and then transferred to a PVDF membrane (EMD Millipore). Membranes were blocked using LI-COR blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour at RT. Membranes were incubated with primary antibodies at 4 °C overnight. Primary antibodies used were: rabbit anti-FUS polyclonal (Bethyl Laboratories, Montgomery, TX; cat. no. A300-302A, 1:1,000 dilution), mouse anti-PGK monoclonal (Novex, Frederick, MD; cat. no. 459250, 1:2,000 dilution), mouse anti-H3 total (Abcam, Cambridge, MA; cat. no. ab24834, 1:2,000 dilution), rabbit anti-H3S10ph (Abcam, Cambridge, MA; cat. no.

ab5176, 1:1,000 dilution), rabbit anti-H3K14ac (Millipore, cat. no. 07-353, 1:2,000 dilution), rabbit anti-H3K56ac (ActiveMotif, Carlsbad, CA; cat. no. 39281, 1:5,000 dilution) and rabbit anti-H4R3me2asym (Abcam, Cambridge, MA; cat. no. ab194683, 1:1,000 dilution). Blots were processed using goat anti-mouse and anti-rabbit secondary antibodies from LI-COR Biosciences (both at 1:20,000 dilution) and imaged using an Odyssey Fc imaging system (LI-COR Biosciences). All immunoblotting experiments were independently repeated a minimum of three times. Densitometric analysis of Western blots was preformed using Image Studio (LI-COR Biosciences). The signals obtained for histone modifications were normalized to their respective total H3 signals (modification/total H3). These values were then compared with untreated control-sample values to obtain fold change values (sample/control), which were used for statistical analysis. Similarly, FUS and TDP-43 bands were compared to Phosphoglycerate Kinase-1 signal values for normalization.

Filter Retention Assay.

Filter retention assay procedures were modified from Alberti et al., 2010.(*6*) Briefly, cells were incubated in spheroblasting solution [1. M D-Sorbitol, 0.5 mM $MgCl_2$, 20 mM Tris - pH 7.5, 50 mM β-mercaptoethanol, 0.5 mg/mL zymolase (Nacalai Tesque, Kyoto, Japan; cat. no. 07663-91)] for 1 hours at 30 °C while rotating. The samples were then centrifuged at 800 rcf for 5 min and the supernatant was removed. The pellets were then resuspended with in 180 µL of lysis buffer [20 mM Tris – pH 7.5, 10 mM β-mercaptoethanol, 0.5% Triton X-100, 2X HALT Protease Inhibitor (Thermo Scientific, Waltham, MA; cat. no. 78425)] and incubated at room temperature for 10 minutes. The sample was then centrifuged at 21,130 rcf for 2 minutes and 60 µL of the supernatant was added to the well of 96-well plate, in triplicate. 80 µL of sterile distilled water was added to

the well of each plate. The Bio-Dot vacuum manifold (Bio Rad, Hercules, CA) was assembled according to manufacturer's instructions after the cellulose acetate membrane was soaked in PBS. Any unused wells were covered with tape to increase vacuum pressure. 100 μL of sample were loaded into wells of the apparatus and allowed to gravity filter, followed by the addition of 200 μL PBS and gravity filtration. 200 μL of blocking buffer (LI-COR Biosciences) were added and allowed to gravity filter and then washed twice with 200 μL PBS, which were vacuum filtered. 100 μL of FUS primary antibody (Bethyl Laboratories, 1:1,000 dilution) was then added and allowed to gravity filter, followed by three PBS washes which were vacuum filtered. Finally, 100 μL of secondary antibody was added (anti-rabbit, LI-COR Biosciences, 1:20,000 dilution) and allowed to gravity filter, followed by two PBS washes which were vacuum filtered. The blot was imaged using an Odyssey Fc imaging system (LI-COR Biosciences).

Sedimentation Assay.

The sedimentation assay was adapted from Krynduskin et al., 2011.(*7*) Briefly, pellets were lysed using a bead beater in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 1 mM dithiothreitol and 1x HALT protease inhibitor. Cell debris was removed by centrifugation at 200 g for 10 min and the protein concentration was measured by a Bradford assay. The lysate was then spun down at 16,000 g for 10 min and the pellet and supernatant were separated. The samples were then mixed with 4X loading buffer and run through SDS-PAGE, followed by Western blotting with antibodies against FUS.

Immunocytochemistry.

FUS and control yeast were imaged using a standard protocol.(*8*) Briefly, cells were fixed for 15 minutes at constant rotation in 1 mL 4% paraformaldehyde solution (Ted Pella, Reeding, CA, cat. no. 18501; in 0.1 M sucrose), followed by 2 washes in 1 mL KPO_4 and 1 wash with 0.1 M KPO_4/1.2M sorbitol. Cells were the resuspended in 1 mL of 0.1 M KPO_4. Yeast were then spheroplasted for 12~13 minutes in 0.1 M KPO_4, 0.3 M β-mercaptoethanol and 0.1 mg/mL Zymolase-100T, followed by two washes with 0.1 M KPO_4, harvested by 1 minute centrifugation in a microcentrifuge. Cells were resuspended in 50 μL 0.1 M KPO_4. 15 μL of cells were then adhered to Teflon coated slides that were coated with 0.1% poly-lysine (Epredia, Portsmouth, NH, cat. no. 86-010) and the supernatant aspirated off. The slide was immediately submerged into ice cold methanol for 6 minutes, followed submersion into room temperature acetone, quickly air dried. The cells were then blocked for 30 minutes with 25 μL PBS-BSA (150 μM Bovine Serum Albumin, 0.05 M KPO_4, 0.15 M NaCl, 30 mM NaN_3). The cells were then incubated with primary antibody overnight (1:400 for FUS), followed by 5 washes with blocking buffer, 1.25 hours incubation with secondary antibody (anti-rabbit AlexaFluor-488), 5 washes with blocking buffer and finally two washed sterile filtered PBS. All volumes were 25 μL/ well and all steps after addition of secondary antibody took place in the dark. The cells were mounted with 5 μL Fluoromount-G Mounting Medium with DAPI (Invitrogen, Waltham, MA, cat. no. 00-4959-52). The slides were images on a Zeiss LSM 800 confocal microscope at 63X magnification using the DAPI and AF488 lasers. Laser intensity was kept constant between control and FUS samples. The resulting images were processed using ImageJ.(*9*) The threshold was set to include the whole cell without background in the green channel and the average intensity was measured. Five images

were acquired from three independent biological replicates. Each image contained at least five cells.

Statistical Analysis.

Statistical analysis of data was performed in RStudio 1.2.5001 using the built-in stats package (R-Project, Vienna, Austria). Significant differences between drug treatment groups were determined using one-way ANOVA followed by Tukey's test for pairwise comparison of the group means with $p = 0.05$ as the cutoff for significance. Error bars on the graphs represent standard deviation (SD) calculated from values obtained in the data analysis steps described above. All graphs were constructed with ggplot2 in RStudio (R-Project, Vienna, Austria).(*10*)

2. Supplementary Figures

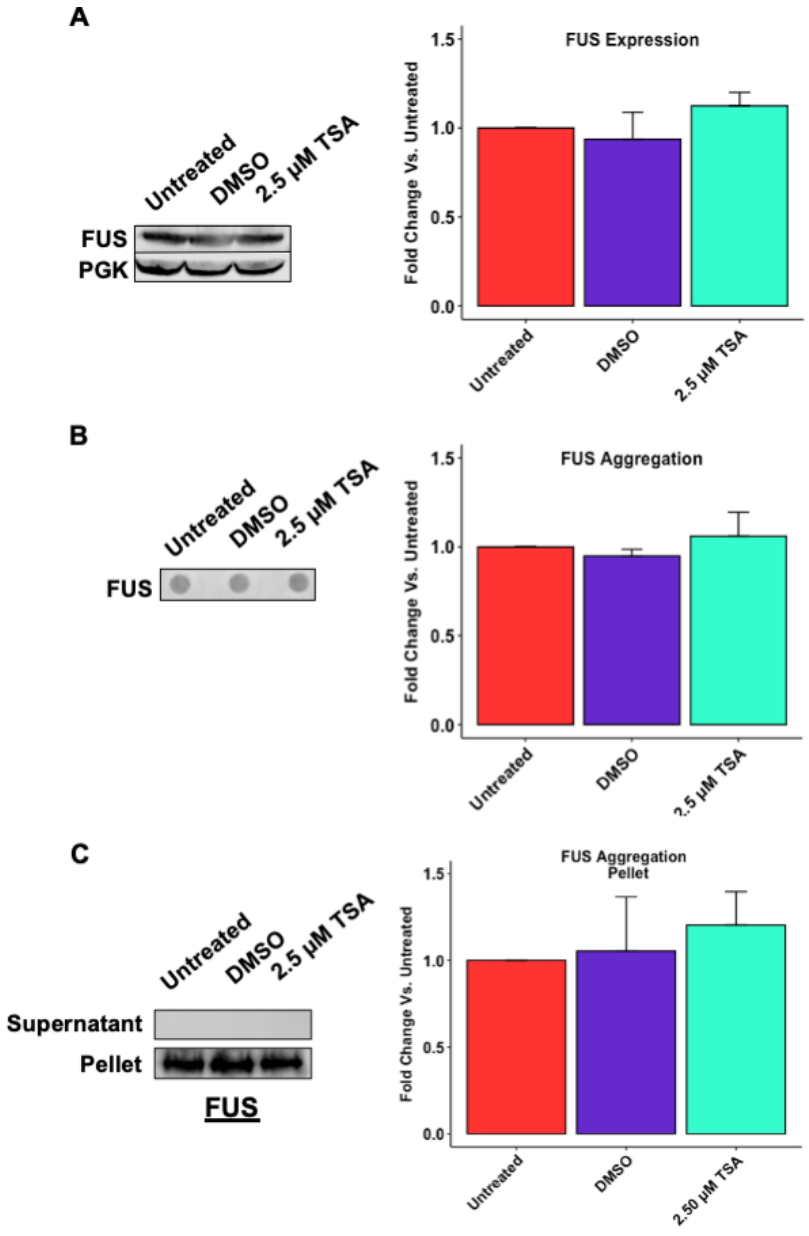

Figure S1. Trichostatin A has no effect on FUS expression or aggregation. FUS expression was measured by Western blotting (A, n = 4), while FUS aggregation was measured with a filter retention assay (B, n = 6) and a sedimentation assay (C, n = 3). Histograms compiling multiple biological replicates display the mean fold change in FUS expression or aggregation based on densitometric analysis. Error bars represent +SD.

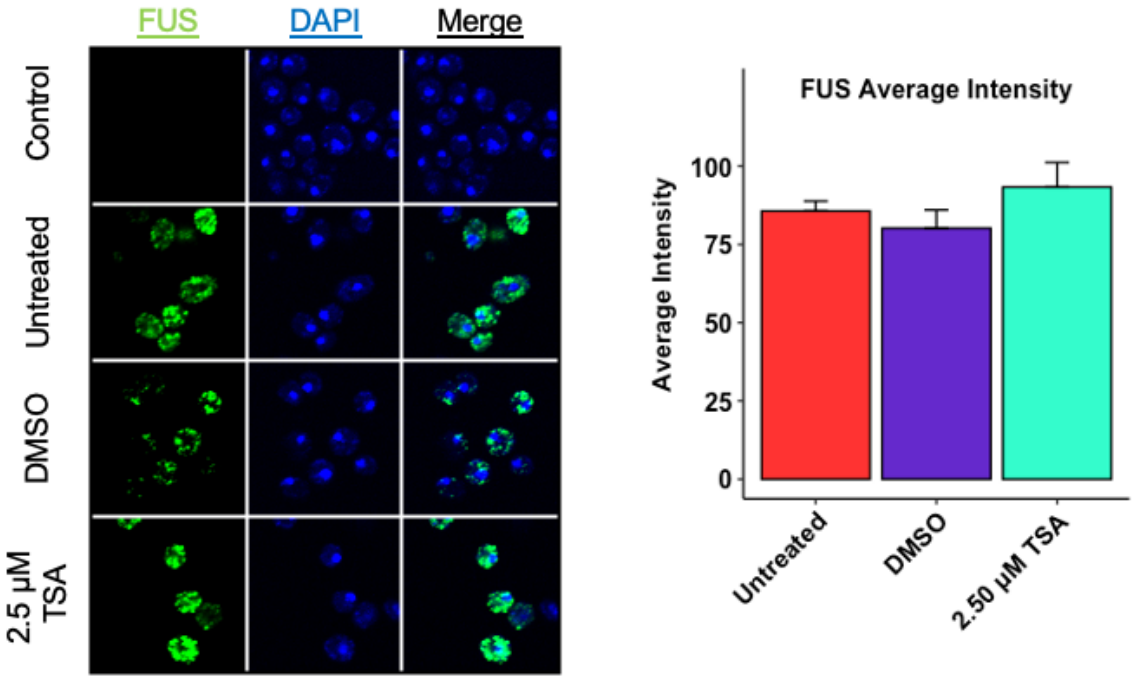

Figure S2. FUS aggregates in yeast are not reduced by Trichostatin A Treatment. Control and FUS yeast untreated, treated with DMSO or 2.50 µM TSA were probed with a FUS antibody and counterstained with DAPI. The raw average intensity for the FUS signal was measured for each image. n = 3. Error bars represent +SD.

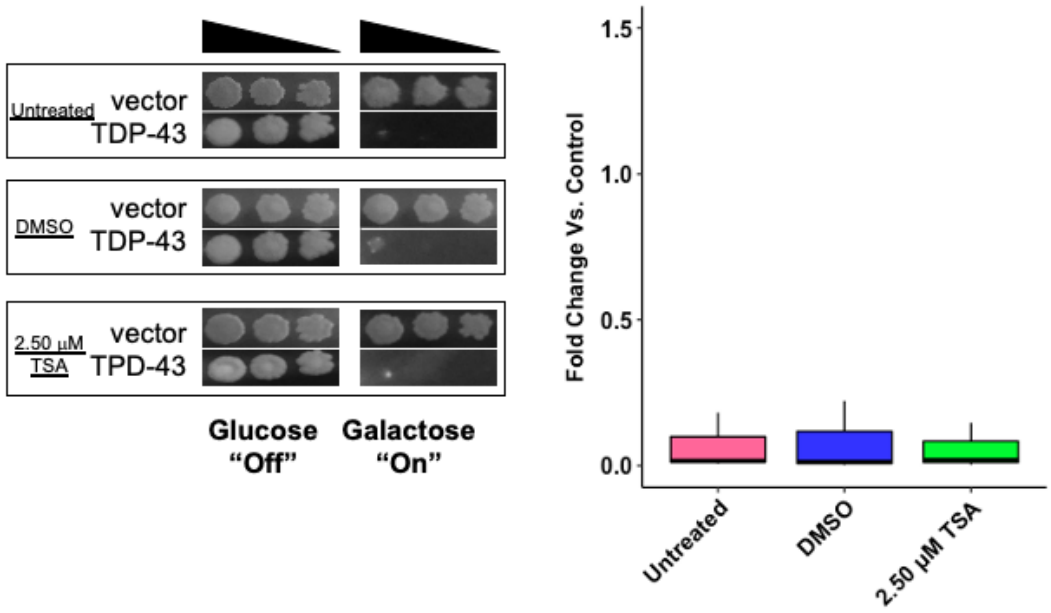

Figure S3. Trichostatin A does not relieve growth suppression in yeast overexpressing TDP-43. Yeast integrated with a gene encoding TDP-43 or an empty vector were serially diluted 5-fold and spotted on glucose (off) or galactose (on) medium in the absence (untreated, DMSO) or presence of 2.50 µM TSA. Densitometric measurement of growth compared to the untreated control is depicted. Box plot whiskers represent upper and lower quartiles. n = 3.

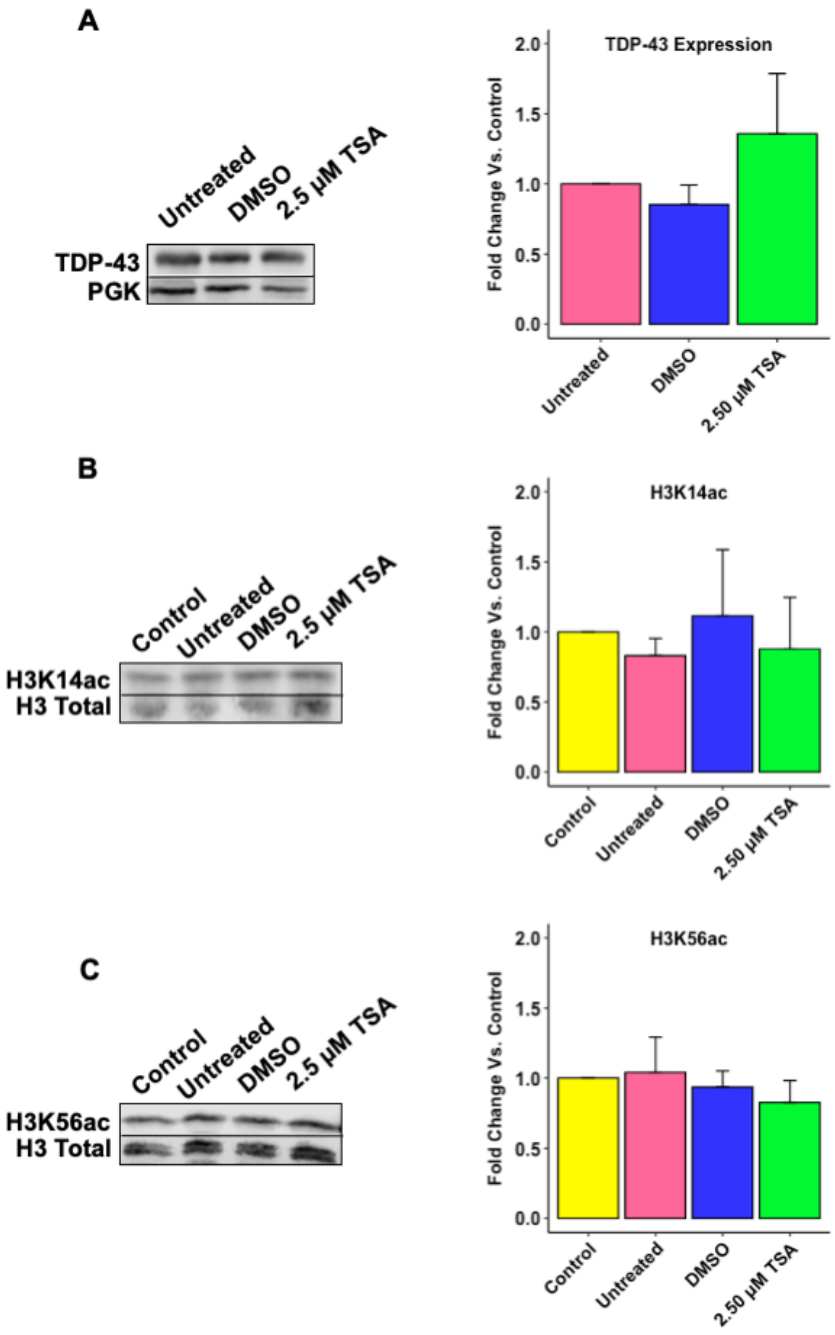

Figure S4. Trichostatin A has no effect on TDP-43 expression or acetylation levels on Histone H3K14 and K56. Representative Western blots displaying the levels of TDP-43 expression (A, n =3), H3K14ac (B, n =4) and H3K56ac (C, n =4) are shown. Histograms compiling multiple biological replicates are represented alongside blots. Error bars represent +SD.

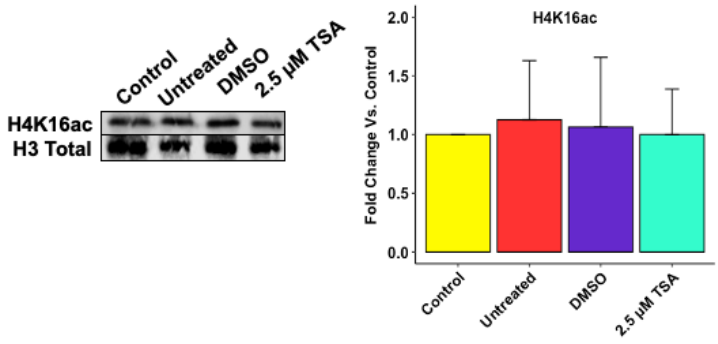

Figure S5. Trichostatin A treatment has no effect on H4K16ac levels in yeast FUS ALS/FTD models. A representative Western blot displaying the levels of H4K16ac (n = 6) is shown. A histogram compiling multiple biological replicates is also shown. Error bars represent +SD

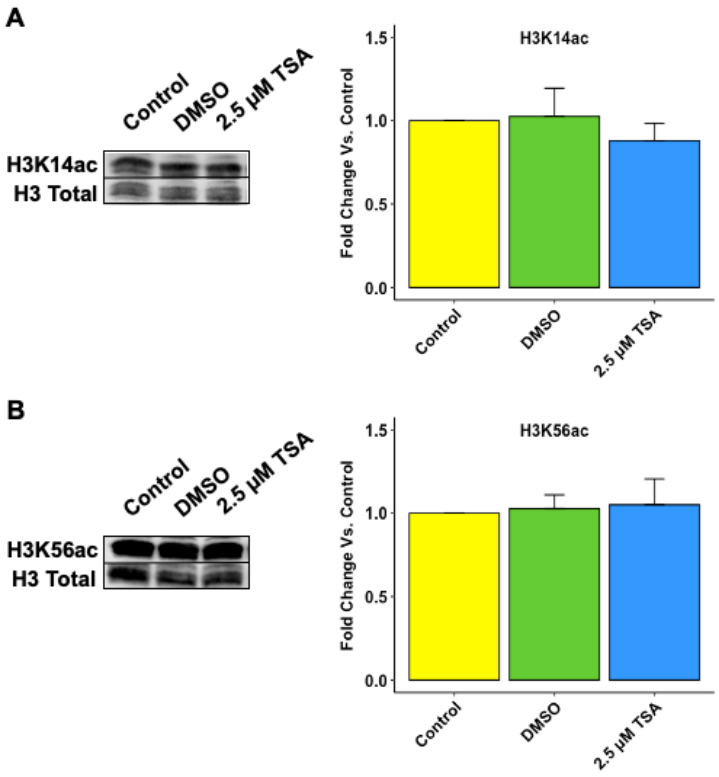

Figure S6. Trichostatin A has no effect on histone acetylation levels on Histone H3 K14 and K56 in vector control yeast. Representative Western blots displaying the levels of H3S10ph (a, n = 3) and H3K56ac (b, n = 4) are shown. Histograms compiling multiple biological replicates are represented alongside blots. Error bars represent +SD.

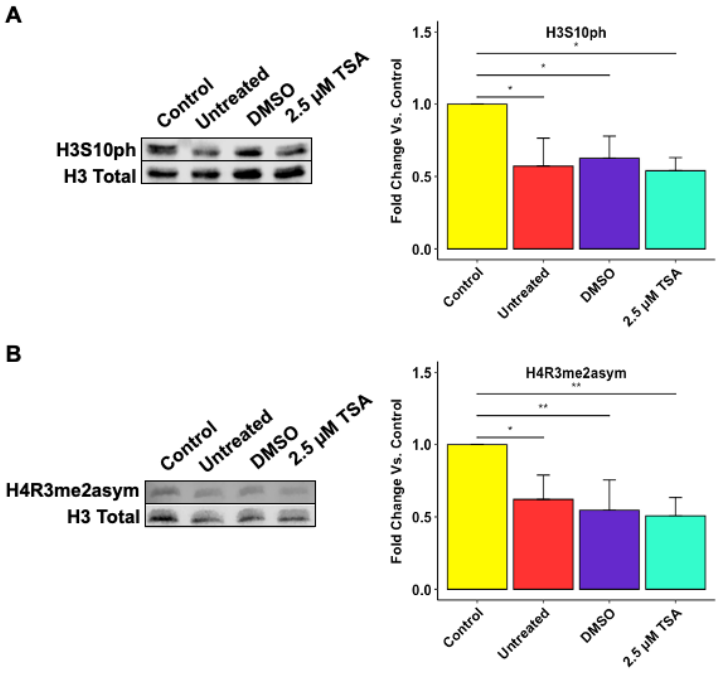

Figure S7. Trichostatin A has no effect on H3S10ph or H4R3me2asym levels in yeast FUS ALS/FTD models. Representative Western blots displaying the levels of H3S10ph (a, n = 3) and H3K56ac (b, n = 4) are shown. Histograms compiling multiple biological replicates are represented alongside blots. Error bars represent +SD.* = $p < 0.05$, ** = $p < 0.01$.

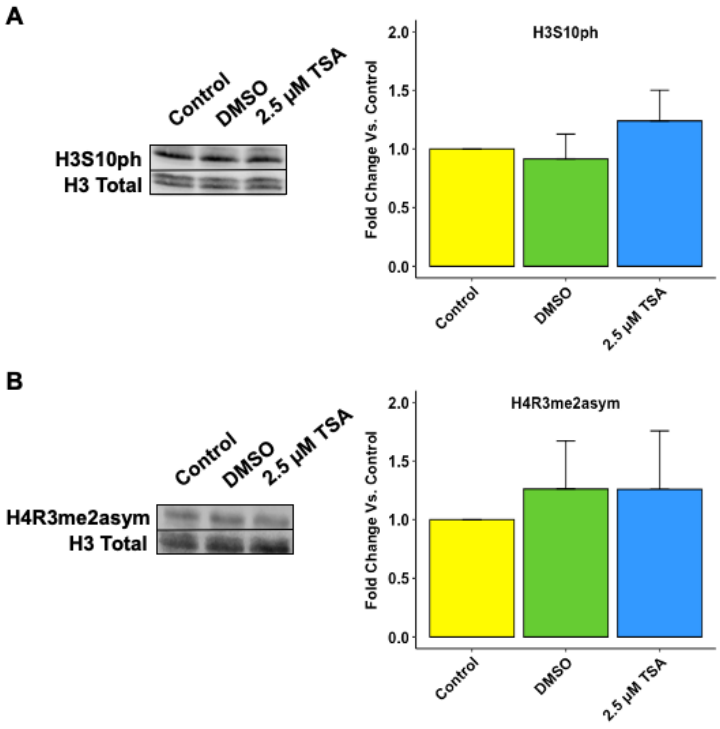

Figure S8. Trichostatin A has no effect on H3S10ph or H4R3me2asym levels in vector control yeast. Representative Western blots displaying the levels of H3S10ph (a, n = 3) and H4R3me2asym (b, n = 3) are shown. Histograms compiling multiple biological replicates are represented alongside blots. Error bars represent +SD.

3. Supplementary Information References

[1] Sanchez, Y., and Lindquist, S. L. (1990) HSP104 required for induced thermotolerance, *Science 248*, 1112.

[2] Sun, Z., Diaz, Z., Fang, X., Hart, M. P., Chesi, A., Shorter, J., and Gitler, A. D. (2011) Molecular Determinants and Genetic Modifiers of Aggregation and Toxicity for the ALS Disease Protein FUS/TLS, *PLoS biology 9*, e1000614.

[3] Johnson, B. S., Snead, D., Lee, J. J., McCaffery, J. M., Shorter, J., and Gitler, A. D. (2009) TDP-43 Is Intrinsically Aggregation-prone, and Amyotrophic Lateral Sclerosis-linked Mutations Accelerate Aggregation and Increase Toxicity, *Journal of Biological Chemistry 284*, 20329-20339.

[4] Au - Bennett, S. A., Au - Cobos, S. N., Au - Meykler, M., Au - Fallah, M., Au - Rana, N., Au - Chen, K., and Au - Torrente, M. P. (2019) Characterizing Histone Post-translational Modification Alterations in Yeast Neurodegenerative Proteinopathy Models, *JoVE*, e59104.

[5] Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis, *Nature Methods 9*, 671-675.

[6] Alberti, S., Halfmann, R., and Lindquist, S. (2010) Chapter 30 - Biochemical, Cell Biological, and Genetic Assays to Analyze Amyloid and Prion Aggregation in Yeast, In *Methods in Enzymology*, pp 709-734, Academic Press.

[7] Kryndushkin, D., Wickner, R. B., and Shewmaker, F. (2011) FUS/TLS forms cytoplasmic aggregates, inhibits cell growth and interacts with TDP-43 in a yeast model of amyotrophic lateral sclerosis, *Protein & Cell 2*, 223-236.

[8] Amberg, D. C., Burke, D. J., and Strathern, J. N. (2006) Yeast Immunofluorescence, *Cold Spring Harbor Protocols 2006*, pdb.prot4167.

[9] Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis, *Nature Methods 9*, 676-682.

[10] Wickham, H. (2009) Elegant graphics for data analysis, *Media 35*, 10.1007.