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Corresponding Author:	Mariana Torrente Brooklyn College Brooklyn, NY UNITED STATES
Corresponding Author's Institution:	Brooklyn College
Corresponding Author E-Mail:	mariana.torrente@brooklyn.cuny.edu
Order of Authors:	Seth Bennett
	Samantha Cobos
	Michel Fallah
	Marcella Meykler
	Navin Rana
	Karen Chen
	Mariana Torrente
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TITLE:

Characterizing Histone Post-translational Modification Alterations in Yeast Neurodegenerative Proteinopathy Models

AUTHORS AND AFFILIATIONS:

Seth A. Bennett[1,2], Samantha N. Cobos[1,3], Marcella Meykler[1], Michel Fallah[1], Navin Rana[1], Karen Chen[1], Mariana P. Torrente[1,4]

[1]Chemistry Department of Brooklyn College, Brooklyn, NY, United States
[2]Ph.D. Program in Biochemistry, The Graduate Center of the City University of New York, New York, NY, United States
[3]Ph.D. Program in Chemistry, The Graduate Center of the City University of New York, New York, NY, United States
[4]Ph.D. Programs in Chemistry, Biochemistry, and Biology, The Graduate Center of the City University of New York, New York, NY, United States

Corresponding Author:

Mariana P. Torrente (mariana.torrente@brooklyn.cuny.edu)

Email Addresses of Co-authors:

Seth A. Bennett (Seth.Bennett@brooklyn.cuny.edu)
Samantha N. Cobos (Samantha.Cobos@brooklyn.cuny.edu)
Marcella Meykler (mmeykler5732@gmail.com)
Michel Fallah (michelfallah112@gmail.com)
Navin Rana (navinrana13@gmail.com)
Karen Chen (kchen0033@gmail.com)

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neurodegeneration, amyotrophic lateral sclerosis, Parkinson's disease, α-synuclein, fused in sarcoma, TAR DNA-binding protein 43, histone post-translational modifications, epigenetics

SUMMARY:

This protocol outlines experimental procedures to characterize genome-wide changes in the levels of histone post-translational modifications (PTM) occurring in connection with the overexpression of proteins associated with ALS and Parkinson's disease in *Saccharomyces cerevisiae* models. After SDS-PAGE separation, individual histone PTM levels are detected with modification-specific antibodies via Western blotting.

ABSTRACT:

Neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD), cause the loss of hundreds of thousands of lives each year. Effective treatment options able to halt disease progression are lacking. Despite the extensive sequencing efforts in large patient populations, the majority of ALS and PD cases remain unexplained by genetic mutations alone. Epigenetics mechanisms, such as the post-translational modification of histone proteins,

may be involved in neurodegenerative disease etiology and progression and lead to new targets for pharmaceutical intervention. Mammalian in vivo and in vitro models of ALS and PD are costly and often require prolonged and laborious experimental protocols. Here, we outline a practical, fast, and cost-effective approach to determining genome-wide alterations in histone modification levels using *Saccharomyces cerevisiae* as a model system. This protocol allows for comprehensive investigations into epigenetic changes connected to neurodegenerative proteinopathies that corroborate previous findings in different model systems while significantly expanding our knowledge of the neurodegenerative disease epigenome.

INTRODUCTION:

Neurodegenerative diseases are devastating illnesses with little to no treatment options available. Among these, amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) are particularly dreadful. Approximately 90% of ALS and PD cases are considered sporadic, occurring without family history of the disease, while the remaining cases run in families and are generally linked to a specific gene mutation[1,2]. Interestingly, both of these diseases are associated with protein mislocalization and aggregation[3-6]. For instance, fused in sarcoma (FUS) and TAR DNA-binding protein 43 (TDP-43) are RNA binding proteins that mislocalize to the cytoplasm and aggregate in ALS[7-12], while α-synuclein is the principle component of proteinaceous aggregates termed Lewy bodies in PD[5,13-15].

Despite the extensive genome-wide association efforts in large patient populations, the overwhelming majority of ALS and PD cases remain unexplained genetically. Can epigenetics play a role in neurodegenerative disease? Epigenetics comprises changes in gene expression occurring without changes to underlying DNA sequence[16]. A main epigenetic mechanism involves the post translational modifications (PTMs) of histone proteins[16]. In eukaryotic cells, genetic material is tightly wrapped into chromatin. The base unit of chromatin is the nucleosome, consisting of 146 base pairs of DNA wrapped around a histone octamer, composed of four pairs of histones (two copies each of histones H2A, H2B, H3, and H4)[17]. Each histone has an N-terminal tail that protrudes out of the nucleosome and can be modified by the addition of various chemical moieties, usually on lysine and arginine residues[18]. These PTMs are dynamic, which means they can be easily added and removed, and include groups such as acetylation, methylation, and phosphorylation. PTMs control the accessibility of DNA to the transcriptional machinery, and thus help control gene expression[18]. For example, histone acetylation reduces the strength of the electrostatic interaction between the highly basic histone protein and the negatively charged DNA backbone, allowing the genes packed by acetylated histones to be more accessible and thus highly expressed[19]. More recently, the remarkable biological specificity of particular histone PTMs and their combinations has led to the histone code hypothesis[20,21] in which proteins that write, erase, and read histone PTMs all act in concert to modulate gene expression.

Yeast is a very useful model to study neurodegeneration. Importantly, many neuronal cellular pathways are conserved from yeast to humans[22-24]. Yeast recapitulate cytotoxicity phenotypes and protein inclusions upon overexpression of FUS, TDP-43, or α-synuclein[22-26]. In fact, *Saccharomyces cerevisiae* models of ALS have been used to identify genetic risk factors in

humans[27]. Furthermore, yeast overexpressing human α-synuclein allowed for the characterization of the Rsp5 network as a druggable target to ameliorate α-synuclein toxicity in neurons[28,29].

Here, we describe a protocol exploiting *Saccharomyces cerevisiae* to detect genome-wide histone PTM changes associated with neurodegenerative proteinopathies (**Figure 1**). The use of *S. cerevisiae* is highly attractive because of its ease of use, low cost, and speed compared to other in vitro and animal models of neurodegeneration. Harnessing previously developed ALS and PD models[22,23,25,26], we have overexpressed human FUS, TDP-43, and α-synuclein in yeast and uncovered distinct histone PTM changes occurring in connection with each proteinopathy[30]. The protocol that we describe here can be completed in less than two weeks from transformation to data analysis.

PROTOCOL:

1. Transforming *S. cerevisiae* with neurodegenerative proteinopathy-associated protein constructs

1.1 Grow wild type (WT) 303 yeast in yeast extract peptone dextrose (YPD) broth overnight with shaking (200 rpm) at 30 °C.

1.2 After 12−16 h of growth, dilute yeast to an optical density at 600 nm (OD_{600}) of 0.25 with YPD. As 10 mL of yeast liquid culture will be needed for each transformation, prepare 50 mL of yeast liquid culture for five transformations corresponding to FUS, TDP-43, α-synuclein, and vector only (ccdB) constructs, as well as a negative control transformation without DNA.

1.3 Grow yeast with agitation at 30 °C until an OD_{600} between 0.60 and 0.80 is reached. This generally takes 4−6 h.

1.4 Thirty minutes before yeast growth is complete, linearize plasmid constructs.

NOTE: The plasmid constructs used here must be integrated directly into the genome, and hence linearization is required prior to transformation.

1.4.1 Calculate the volume of plasmid stock that amounts to 1 µg. Subtract this volume from 44 µL to calculate the amount of nuclease free H_2O needed.

1.4.2 Add nuclease free H_2O, 5 µL of 10x restriction enzyme buffer (**Table of Materials**), 1 µL of NheI restriction enzyme (**Table of Materials**), and the appropriate amount of plasmid (calculated in step 1.4.1) to a microcentrifuge tube. Mix thoroughly by pipetting up and down and incubate at 37 °C for 15 min. The plasmid is now linearized and ready for transformation.

1.5 After yeast culture reaches an OD_{600} of 0.6-0.8, harvest cells in a 50 mL conical tube and spin down at 850 x *g* at 4 °C for 3 min. Discard supernatant.

1.6 Wash cell pellet in 10 mL of sterile H_2O and centrifuge at 850 x *g* at 4 °C for 3 min. Discard supernatant. Repeat 3x for a total of four washes.

1.7 At the start of the third wash, thaw and boil 10 µL of salmon sperm DNA per transformation reaction for 5 min on a heating block.

1.8 Resuspend cell pellet in 100 µL of dH_2O per transformation and split into appropriate number of microcentrifuge tubes. For five transformations, resuspend pellet into 500 µL of dH_2O and split evenly into five separate microcentrifuge tubes.

1.9 Centrifuge for 5 min at 850 x *g* at room temperature (RT) and remove all remaining water.

1.10 Add, in the following order, 50 µL of sterile H_2O, 240 µL of 50% polyethylene glycol (PEG), 36 µL of 1 M LiAc, 10 µL of salmon sperm DNA, 20 µL of linearized plasmid DNA (or nuclease free water for no DNA control transformation). Mix thoroughly after each addition and vortex briefly after all transformation components have been added.

1.11 Incubate transformation reactions at 42 °C for 20 min.

NOTE: Carry out this incubation in a water bath for more consistent temperature control.

1.12 Centrifuge at 470 x *g* at RT for 5 min. Discard supernatant and resuspend each cell pellet in 200 µL of sterile H_2O.

1.13 Plate yeast suspension on selective media plates and spread with rolling beads or spreader. Incubate plates for 2−3 days at 30 °C.

NOTE: Synthetic-defined (SD)-His plates are used for the plasmids described here.

2. Surveying colony growth suppression and storage in glycerol stocks

2.1 Inoculate single colonies from transformation plates in 5 mL of selective media supplemented with 2% raffinose. Grow on a shaker at 30 °C overnight. Select at least 12 colonies per transformation.

NOTE: No colonies are expected in the “no DNA” transformation control plate.

2.2 Sterilize pin-frogger with ethanol, followed by flaming.

2.3 For each culture, aliquot 100 µL of saturated cell suspension in the first row of a 96-well plate. Add 200 µL of sterile H_2O in all wells of the adjacent well columns. For 1:5 serial dilutions, add 50 µL from the first column to the adjacent column behind with multichannel pipet. Mix thoroughly by pipetting. Then add 50 µL from the second column to the third column and mix

by pipetting. Repeat this for the rest of the plate.

2.4 Plate yeast by placing frogger in a 96-well plate and then stamping by gently and evenly pressing down on selective media plates with and without galactose. Incubate at 30 °C for 2–3 days.

NOTE: SD-His and SGal-His plates are used for the plasmids described here.

2.5 For FUS, TDP-43, and α-synuclein transformations, identify the colony displaying the most growth suppression in the presence of galactose. Select this colony from the SD-His plate and inoculate into 10 mL of selective media supplemented with 2% raffinose for overnight growth. For the vector control, pick a colony displaying no growth suppression in the presence of galactose.

2.6 To prepare glycerol stocks, combine 0.5 mL of saturated liquid culture with 0.5 mL of 50% glycerol. Mix by pipetting up and down and freeze at -80 °C.

NOTE: Glycerol stocks can be stored for up to a year at -80 °C.

3. Overexpression of neurodegenerative proteinopathy associated proteins in *S. cerevisiae*

3.1 From frozen glycerol stocks, re-streak yeast on histidine, 2% glucose agar selective media plates and incubate at 30 °C for 2–3 days.

3.2 Inoculate single colonies for each of overexpression models and control in 5 mL of histidine selective liquid media supplemented with 2% raffinose. Grow with shaking (200 rpm) at 30 °C overnight.

3.3 Grow 100 mL of liquid culture for each of the overexpression models and controls (FUS, TDP-43, and vector control; α-synuclein and vector control).

3.3.1 Prepare 100 mL of culture in selective media supplemented with 2% galactose.

3.3.2 Measure the OD_{600} of overnight cultures and calculate the amount of overnight culture needed to render overexpression cultures at a starting OD_{600} of 0.3. Typically, overnight cultures will reach an OD_{600} of 0.9–10, requiring about 5 mL of overnight culture to start 100 mL of fresh culture.

3.3.3 Induce protein overexpression by growing yeast on galactose media (see step 3.3.1) for 5 h (FUS and TDP-43) or 8 h (α-synuclein) with shaking at 30 °C.

3.4 Measure OD_{600} of each culture at the end of the induction period. Standardize all cell counts to the lowest OD_{600} value. Harvest culture in 50 mL tubes by centrifuging at 850 x *g* for 5 min at 4 °C. Discard supernatant.

NOTE: If the OD_{600} values measured at the end of induction are 0.654 and 0.984, respectively, for 100 mL of α-synuclein culture and 100 mL of control culture, harvest 95 mL of the α-synuclein culture and 67.1 mL of the control culture.

3.5 Resuspend pellet in 1 mL of sterile dH_2O for every 10 mL of culture grown. Split pellet evenly by aliquoting 1 mL of cell resuspension into microcentrifuge tubes. For example, if starting with 100 mL of culture, resuspend pellet in 10 mL of sterile dH_2O and divide it into 10 microcentrifuge tubes.

3.6 Centrifuge at 850 x *g* for 5 min at 4 °C, then remove supernatant. Snap freeze cell pellets in liquid nitrogen and store at -80 °C.

NOTE: Cell pellets can be stored at -80 °C for up to one year.

4. Cell lysis and western blotting to detect histone post-translational modifications

4.1 Cell lysis

4.1.1 Thaw yeast cell pellets on ice and resuspend cell pellets in 100 µL of dH_2O.

4.1.2 To the cell resuspension, add 300 µL of 0.2 M NaOH and 20 µL of 2-mercaptoethanol. Resuspend by pipetting up and down.

4.1.3 Incubate cells on ice for 10 min and then centrifuge at 3,200 x *g* on a tabletop centrifuge for 30 s at RT. Discard supernatant.

4.1.4 Resuspend cell pellet in 100 µL of 1x loading dye and boil for 10 min on a heating block.

NOTE: The recipe for 6x loading dye can be found in the **Table of Materials**.

4.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis and membrane transfer

4.2.1 Prepare gel chamber by placing two gels in a gel holder and filling inner chamber to the top with running buffer and filling the outside chamber to the two-gel line mark.

4.2.2 Load 15 µL of sample from step 4.1.4 per well into a 10-well 12% polyacrylamide gel. For the protein ladder lane, load 5 µL.

4.2.3 Run gel for approximately 45 min at 150 V, or until loading dye front reaches the bottom of the gel.

4.2.4 While the gel is running, prepare for membrane transfer by soaking fiber pads (two per gel) in transfer buffer (**Table of Materials**) and soaking polyvinylidene fluoride (PVDF)

membrane in methanol. Rinse membrane in transfer buffer before transfer.

NOTE: The PVDF membrane should be cut to the size of the gel and use one PVDF membrane for every gel being transferred.

4.2.5 Assemble, on semi-dry transfer apparatus cell, a transfer 'sandwich': from bottom electrode, place (1) presoaked fiber pad, (2) presoaked and rinsed PVDF membrane, (3) gel from step 4.2.3, and (4) a second presoaked fiber pad.

NOTE: Make sure to avoid air bubbles in the transfer 'sandwich.' Gently roll 'sandwich' with serological pipet to force out bubbles. Once the gel has been placed on top of the PDVF membrane, make sure not to move it.

4.2.6 Carry out protein transfer by setting power pack to 150 mA for 1 h (for one 'sandwich').

4.3 Detection of histone PTMs with modification specific antibodies

4.3.1 Remove membrane from transfer apparatus. Rinse briefly with dH_2O.

4.3.1.1 Optionally, check transfer of proteins with Ponceau-S stain by pouring enough Ponceau-S stain to cover membrane in a small plastic box and incubating at RT with gentle shaking for 30–60 s. Remove Ponceau-S stain and rinse with dH_2O until background stain disappears and protein bands are visible to the naked eye. Continue to rinse with dH_2O until all stain is removed from membrane.

4.3.2 Block membrane by incubating blot for 1 h at RT with gentle rocking with tris buffered saline (TBS) blocking buffer (**Table of Materials**) in a small staining box. Use enough blocking buffer to cover blot.

NOTE: Be careful to place membrane upright in the staining box so that the membrane side on which proteins lie is not facing down.

4.3.3 Incubate blot in the staining box overnight with a histone modification-specific antibody reactive towards yeast at 4 °C. Dilute the antibody in TBS blocking buffer according to manufacturer specifications. Also include a proper nuclear loading control, such as anti-total H3 raised in a different host species from the modification-specific antibody. For instance, if probing for H3S10ph with an anti-H3S10ph antibody raised in rabbit, use an anti-total H3 antibody raised in mouse as a loading control. Repeat this for every blot as necessary.

NOTE: Antibody dilutions can be reused for a total of three times within a month. Store them at 4 °C.

4.3.4 Wash the membrane 4x in house-made TBS + 0.1% polysorbate 20 (TBST) for 5 min with rocking at RT.

4.3.5 Incubate blot with fluorescent secondary antibodies at manufacturer-specified dilutions (donkey anti-rabbit 680 and donkey anti-mouse) for 1 h at RT.

NOTE: Fluorescent secondary antibodies must be protected from light during both storage and usage. Carry out incubation in dark plastic boxes or cover with aluminum foil.

4.3.6 Wash membrane 4x with TBST for 5 min and wash with TBS for 5 min while rocking at RT.

4.3.7 Visualize blot on a fluorescent Western blot imaging system for 2 min. Visualize the anti-rabbit 680 and anti-mouse 800 secondary antibodies in the 800 nm and 700 nm channels, respectively.

NOTE: Replicates are independently conducted starting from section 1. It is necessary to verify that the antibody signal response is within the range over which the signal response is linear for appropriate data interpretation in these experiments.

5. Data analysis and statistics

5.1 Open image of blot in an imaging software (**Table of Materials**). Acquire the raw density of modification-specific bands in the vector control, TDP-43, and FUS (or vector control and α-synuclein) samples, by drawing a rectangle that frames the band in analysis mode.

5.2 Repeat step 5.1 for loading control bands.

NOTE: There will be a loading control band and a modification-specific band in each sample.

5.3 Calculate the relative density of the modification-specific bands by dividing each band by the density of corresponding band in the vector control sample. This normalizes the data to the vector control.

5.4 Calculate the relative density of the loading control band by dividing each band by the density of corresponding loading control band in the vector control sample.

5.5 Calculate the adjusted relative density of each band by dividing the relative density of the modification-specific band over the relative density of the loading control band for each sample. Now, the data can be visualized in histogram form.

NOTE: For ease of processing, steps 5.3–5.5 can be done in a spreadsheet (**Supplemental File**).

5.6 After multiple independent replicates, run Welch's T-tests between the two control samples and the appropriate proteinopathy model (control versus FUS, control versus TDP-43, and control versus α-synuclein) with $p = 0.05$ as the cutoff for significance.

6. Sucrose tuning of FUS overexpression

6.1. Overexpress FUS and harvest yeast as described in section 3, but use sucrose to galactose ratios corresponding to: all sucrose; 40 sucrose: 1 galactose; 20 sucrose: 1 galactose; 10 sucrose: 1 galactose; 1 sucrose: 2 galactose; all galactose. The total concentration of sugar in each sample will amount to 2% in all conditions. For example, to make a 100 mL of media containing a 40:1 ratio of sucrose to galactose, add 9.756 mL of 20% sucrose and 244 µL of 20% galactose to 90 mL of selective media.

6.2 For data analysis, compare the fold changes in the PTM levels, standardizing to the sucrose condition, and run a one-way ANOVA with a $p = 0.05$ as a cutoff.

REPRESENTATIVE RESULTS:

To illustrate this method, we will take advantage of recently published results[30]. WT human FUS and TDP-43 were overexpressed for 5 h, while WT α-synuclein was overexpressed for 8 h. A ccdB construct was used as a vector negative control. **Figure 2** shows growth suppression in solid and liquid cultures. Yeast was harvested as described and Western blotting with modification-specific antibodies was performed. Anti-total H3 was used as a loading control. A significant decrease in the levels of H3S10ph and H3K14ac is apparent in the FUS overexpression model (**Figure 3a,b**). There are significant increases in the levels of acetylation on H4K12 and H4K16 in the TDP-43 overexpression model that are not observed in either the FUS or α-synuclein overexpression model (**Figure 3c,d**). There are also significant decreases in the levels of H3K36me2 and H2BT129ph in the α-synuclein overexpression model (**Figure 3e,f**).

To show that the change in histone PTMs correlates with the amount of expression of the neurodegenerative proteinopathy protein, the expression of FUS can be tuned by inducing protein expression in varying levels of galactose (**Figure 4a**). Galactose is mixed with sucrose in changing ratios such that the total concentration of sugar is constant at 2%, but the amount of galactose is modified resulting in a range of protein expression levels. In *S. cerevisiae,* sucrose is slowly metabolized to glucose, which suppresses galactose induction[31]. Hence, sucrose neither activates nor suppresses galactose induction. The lower the amount of galactose used to induce FUS overexpression, the less toxicity was observed in both solid and liquid culture (**Figure 4b,c**)**.** Importantly, the lower the level of FUS overexpression, the smaller the magnitude of the reduction in H3S10ph levels (**Figure 4d-f**).

FIGURE AND TABLE LEGENDS:

Figure 1: Method overview for the characterization of changes in histone post-translational modifications connected to neurodegenerative disease proteinopathies in yeast models.

Figure 2: Toxicity associated with overexpression of neurodegenerative proteinopathy-related proteins in yeast models. Spotting assays show cell viability for yeast overexpressing vector control, TDP-43, FUS, or α-synuclein in the presence of glucose (**a**) or galactose (**b**). (**c**) Growth curve illustrating cell viability in liquid culture under galactose induction. Error bars indicate the ± standard deviation. n = 3 for each strain. Replicates result from completely

independent experiments. Data adapted with permission from Chen, K. et al. Neurodegenerative Disease Proteinopathies Are Connected to Distinct Histone Post-translational Modification Landscapes. *ACS Chemical Neuroscience.* **9** (4), 838-848 (2018). Copyright 2018 American Chemical Society.

Figure 3: Changes in histone post-translational modifications associated with overexpression of neurodegenerative proteinopathy-related proteins in yeast models. An FUS proteinopathy model shows decreased levels of (**a**) H3S10ph, n = 6, and (**b**) H3K14ac, n = 3. Conversely, a TDP-43 proteinopathy model shows increased levels of (**c**) H4K12ac, n = 3, and (**d**) H4K16ac, n = 6, while a α-synuclein model shows decreased levels of (**e**) H3K36me2, n = 3, and (**f**) H2BT129ph, n= 7. Error bars show + standard deviation. *, $p < 0.05$, ***, $p < 0.001$. Replicates result from completely independent experiments. Data adapted with permission from Chen, K. et al. Neurodegenerative Disease Proteinopathies Are Connected to Distinct Histone Post-translational Modification Landscapes. *ACS Chemical Neuroscience.* **9** (4), 838-848 (2018). Copyright 2018 American Chemical Society.

Figure 4: Extent of decrease in H3S10ph levels is tied to the level of FUS overexpression. (**a**) Cartoon illustration of the use of sucrose and galactose ratios to tune the amount of FUS overexpression. (**b**) Spotting assays showing cell viability in the presence of varying levels of galactose. (**c**) Growth curve in liquid culture showing cell viability in the presence of varying levels of galactose. Error bars indicate ± standard deviation. (**d**) Representative immunoblots showing that the FUS protein levels rise as cells are exposed to increasing ratios of galactose. Phosphoglycerate kinase (PGK) was used as a loading control. (**e**) Representative immunoblots showing corresponding decreases in H3S10ph levels with increasing ratios of galactose. (**f**) Quantitation histogram of (**e**). n = 3 for each condition. Error bars indicate + standard deviation for quantification chart. Replicates result from completely independent experiments. Data adapted with permission from Chen, K. et al. Neurodegenerative Disease Proteinopathies Are Connected to Distinct Histone Post-translational Modification Landscapes. *ACS Chemical Neuroscience.* **9** (4), 838-848 (2018). Copyright 2018 American Chemical Society.

DISCUSSION:

The protocol described here provides a straightforward, expedient, and cost-effective way of categorizing genome-wide histone PTM changes correlated with neurodegenerative proteinopathies. While there are other models of ALS and PD, such as *in vitro* human cell lines and murine models[32], *S. cerevisiae* remains attractive because of its ease of use. For instance, yeast models do not require use of a sterile hood, nor do they require the intensive training that goes along with cell culture work. Furthermore, the reagents for culturing yeast are also much more affordable than mammalian cell culture supplies. Yeast models have been exploited to not only reveal the domain determinants of protein aggregation[22-26], but also have led to uncovering genetic risk factors in ALS[27], as well as modifiers of protein aggregation toxicity[22]. Furthermore, the deletion of Set3, a member of the histone deacetylase complex, has been found to reduce TDP-43 toxicity in yeast[33]. Interestingly, our findings in yeast are in agreement with recent reports on histone modification alterations in both an FUS mouse model and in a human SH-SY5Y FUS overexpression model[34,35]. Furthermore, recently discovered alterations in

DNA methylation patterns around key PD genes, such as *SNCA* and *PARK2*, support a role for epigenetics in PD[36,37].

Here, we show that these yeast models can also be used to discover how neurodegenerative proteinopathies interact with the epigenome[30]. We find that distinct changes in histone modifications are associated with each proteinopathy model. The protocol outlined here allows us to corroborate previous findings in other model systems. Most remarkably, this method has heightened our understanding of the epigenomic panorama of neurodegenerative disease. The expanded set of modifications presented here could uncover novel histone writer and eraser targets for pharmacological intervention. These results highlight the possible contribution of histones PTMs and epigenetics in the pathology of ALS, PD, and other neurodegenerative diseases and may provide novel avenues for treatment of these diseases.

Particular care needs to be taken in steps 1.9 and 1.10 when transforming yeast to overexpress neurodegenerative proteinopathies. Specifically, adding the reagents in the proper order during step 1.10 is necessary to ensure high transformation efficiency. Furthermore, it is very important to pick the colony displaying the most growth suppression in galactose. Care should also be taken when assembling the Western sandwich. Accidental addition of bubbles between the gel and membrane will block transfer of the protein and provide block spots on the blot that can hinder data analysis.

One limitation of this protocol is that antibodies are restricted to only binding and detecting one or two histone modifications at a time. It is also crucial to properly standardize samples after protein overexpression (steps 3.4−3.6). Completely removing all growth media will ensure that each cell pellet winds up resuspended in the same volume. Similarly, it is important to carefully aliquot the same amount of cell suspension into the microcentrifuge tubes (step 3.4). If the samples are not properly standardized, it is difficult to draw any conclusions from any changes on histone PTM levels. Such a problem would become apparent in discrepancies on the levels of the loading control of the Western blot (section 4.2). To remedy this, an SDS-PAGE gel loaded with relevant samples can be Coomassie stained, allowing for protein band quantification of the samples (section 5) and subsequent re-standardization of samples based on the amount of protein present on each sample.

In conclusion, this protocol allows for the analysis of histone PTM changes associated with the overexpression of proteins related to neurodegenerative proteinopathies in just less than two weeks, from transformation to statistical analysis. Aside from enabling the study of proteinopathies associated with neurodegenerative diseases, this general protocol can be used to study histone PTM alterations in any yeast overexpression model.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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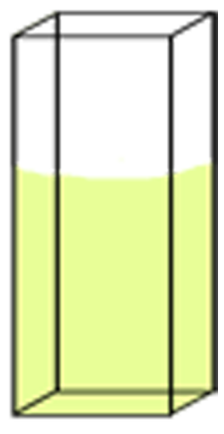
1.Grow Yeast

Streak cells out onto a YPD dish, allow colonies to grow 2-3 days

Inoculate colonies in liquid media



Measure OD_{600} ensuring it is between 0.6-0.8 using a spectrophotometer

2. Transform Yeast

Harvest yeast from media

Add transformation mix and neuro-degenerative disease plasmids. Heat shock

Plate yeast suspen-sion onto selective media

3. Spotting Assay

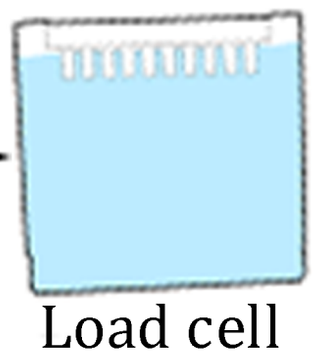
Grow trans-formed yeast in liquid media

Transfer media to 96-well plate to create serial dilutions with dH_2O

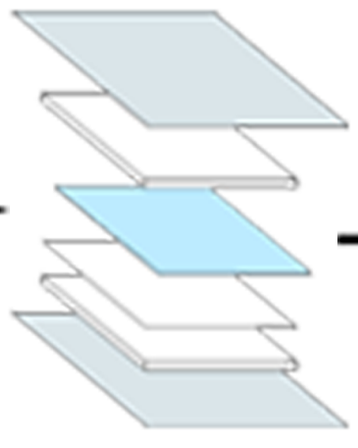
Spot onto selective media with galactose to select for toxicity

4. Western Blot

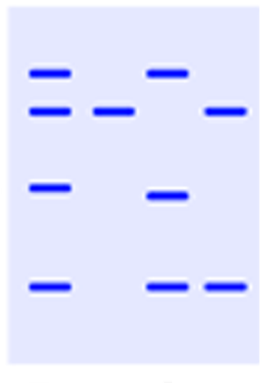
Lyse cell samples and add loading dye



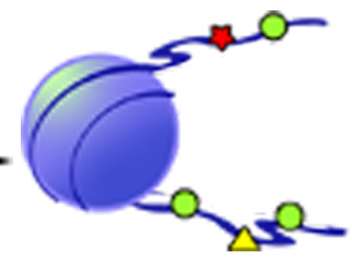
Load cell samples into polyacrylamide gel



Using a semi-wet transfer apparatus, lay filter pad, PVDF membrane, gel, and another filter pad to transfer bands onto membrane



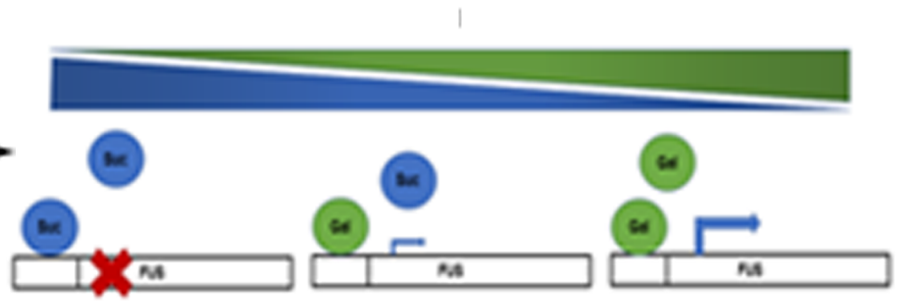
Develop blot with antibodies of choice



Histone PTM landscapes associated with neurodegenerative proteinopathies

5. Sucrose Tuning

Grow transformed yeast on selective media



Inoculate colonies in selective liquid media with varying ratios of sucrose to galactose

Freeze down harvested cells, and run Western blot

Figure 2

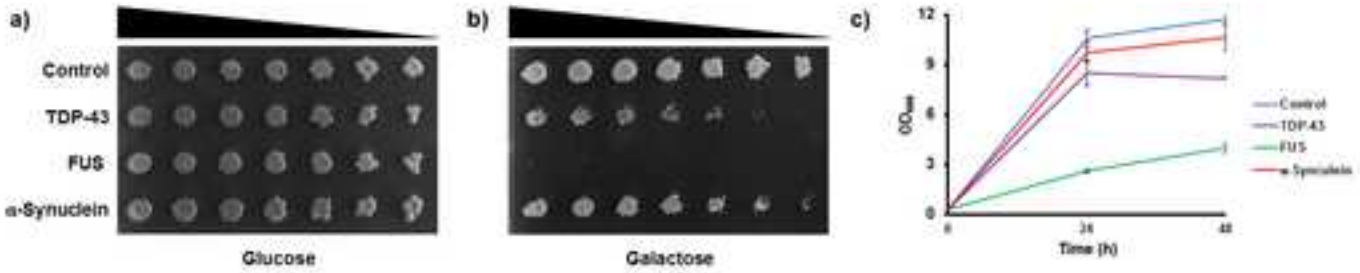


Figure 3

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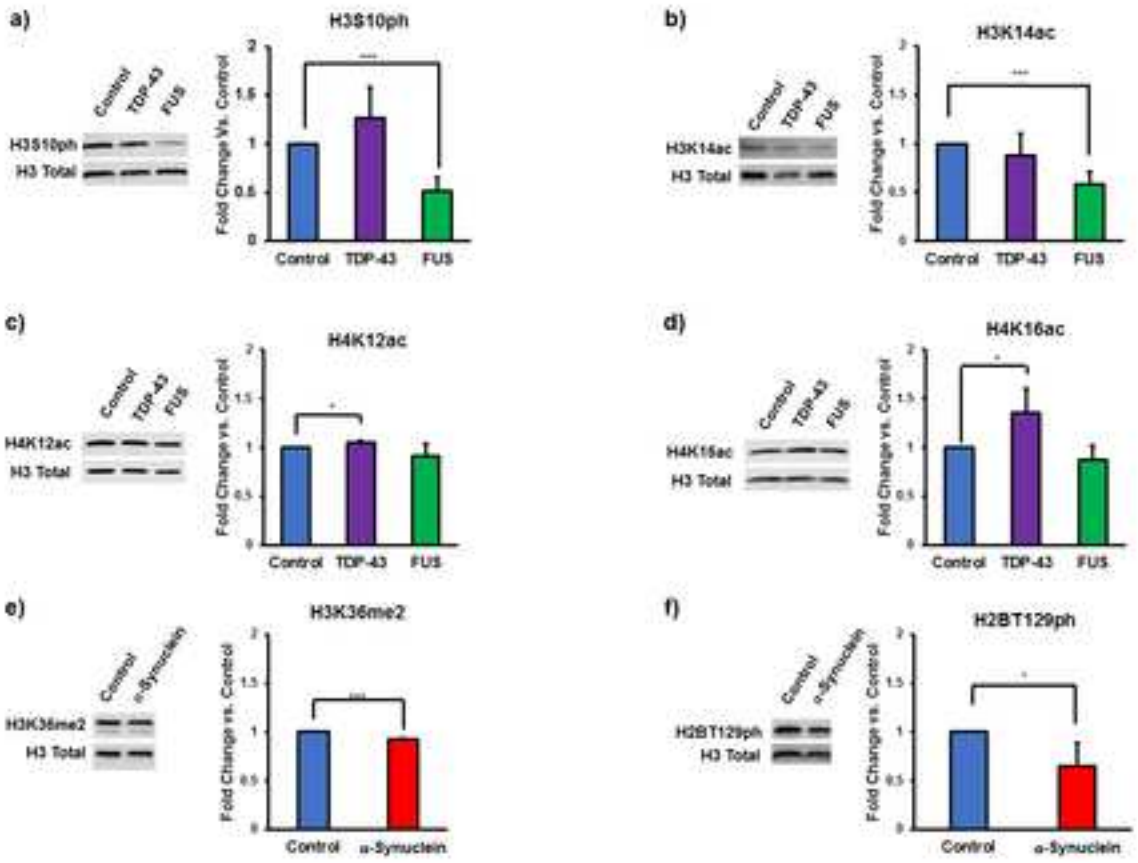

Figure 4

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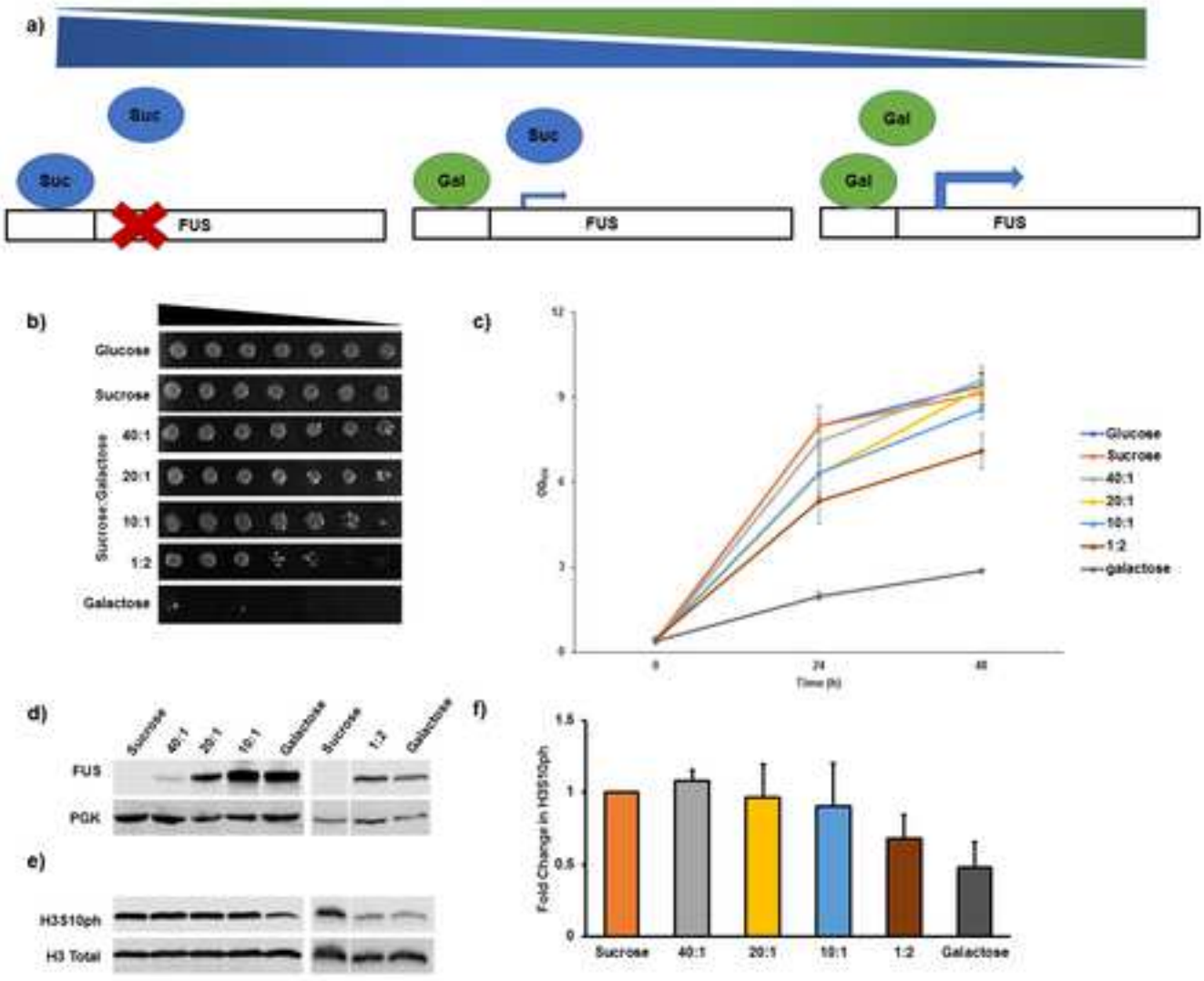

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
-His DO Supplement	Clontech	630415	
10x Running Buffer			Mix: 141.65 g glycine (ThermoFisher BP381-1), 30.3 g Tizma base (Sigma-Aldrich T6066), 10 g sodium dodecyl sulfate (Sigma-Aldrich L3771), and 1 L deionized water, pH 8.8.
12% Polyacrylamide Gels	BIO-RAD	456-1041	
2-mercaptoethanol	Sigma-Aldrich	M3148	
Anti-acetyl-Histone H3 (Lys14) Primary Antibody	MilliporeSigma	07-353 (Lot No. 2762291)	Dilution: 1/1000
Anti-acetyl-Histone H4 (Lys 16) Primary Antibody	Abcam	ab109463 (Lot No. GR187780)	Dilution: 1/2000
Anti-acetyl-Histone H4 (Lys12) Primary Antibody	Abcam	ab46983 (Lot No. GR71882)	Dilution: 1/5000
Anti-dimethyl-Histone H3 (Lys36) Primary Antibody	Abcam	ab9049 (Lot No. GR266894, GR3236147)	Dilution: 1/1000
Anti-Histone H3 Primary Antibody	Abcam	ab24834 (Lot No. GR236539, GR174196, GR3194335)	Nuclear Loading Control; Dilution: 1/2000
Anti-phospho-Histone H2B (Thr129) Primary Antibody	Abcam	ab188292 (Lot No. GR211874)	Dilution: 1/1000
Anti-phospho-Histone H3 (Ser10) Primary Antibody	Abcam	ab5176 (Lot No. GR264582, GR192662, GR3217296)	Dilution: 1/1000

BioPhotometer D30	Eppendorf	6133000010	
Cell Culture Dish (100 x 20 mm)	Eppendorf	30702118	
Cell Culture Plate, 96 well	Eppendorf	30730011	
Centrifuge 5804/5804 R/5810/5810 R	Eppendorf	22625501	
Donkey Anti-Mouse IRDye 800 CW	LI-COR	926-32212 (Lot No. C60301-05, C61116-02, C80108-05)	Dilution: 1/5000
Donkey Anti-Rabbit IRDye 860 RD	LI-COR	926-68073 (Lot No. C60217-06, C70323-06, C70601-05, C80116-07)	Dilution: 1/2500
Ethanol	Sigma-Aldrich	E7023	
Extra thick blot paper (filter paper)	BIO-RAD	1703968	
Galactose	Sigma-Aldrich	G0750	Prepare 20% w/v stock solution.
Glucose	Sigma-Aldrich	G8270	Prepare 20% w/v stock solution.
Glycerol	Sigma-Aldrich	G5516	Prepare 50 % w/v solution.
Immobilon-FL Transfer Membranes	MilliporeSigma	IPFL00010	
Lithium acetate dihydrate (LiAc)	Sigma-Aldrich	L4158	Prepare a 1 M solution.

Loading Dye			Mix: 1.2 g sodium dodecyl sulfate, 6 mg bromophenol blue (Sigma-Aldrich B8026), 4.7 mL glycerol, 1.2 mL 0.5M Trizma base pH 6.8, 0.93 g DL-Dithiothreitol (Sigma-Aldrich D0632), and 2.1 mL deionized water.
Methanol	ThermoFisher	A412-4	
Mini-PROTEAN Tetra Vertical Electrophoeresis Cell	BIO-RAD	1658004	
Multichannel pipet	Eppendorf	2231300045	
NEB Restriction Enzyme Buffer 2.1, 10x	New England Bio Labs	102855-152	
Nhe I Restriction Enzyme	New England Bio Labs	101228-710	
Nuclease Free Water	Qiagen	129114	
Odyssey Fc Imaging System	LI-COR Biosciences	2800-03	
OmniTray Cell Culture Treated w/Lid Sterile, PS (86 x 128 mm)	ThermoFisher	165218	
pAG303GAL-a-synuclein-GFP	Gift from A. Gitler		
pAG303GAL-ccdB	Addgene	14133	
pAG303Gal-FUS	Addgene	29614	
pAG303GAL-TDP-43	Gift from A. Gitler		
Poly(ethylene glycol) (PEG)	Sigma-Aldrich	P4338	Prepare a 50% w/v solution.

Ponceau S Stain	Sigma-Aldrich	P3504	Mix: 0.5 g 0.1% w/w Ponceau S dye, 5 mL 1% v/v acetic acid (Sigma-Aldrich 320099), and 500 mL deionized water.
PowerPac Basic Power Supply	BIO-RAD	164-5050	
Raffinose pentahydrate	Sigma-Aldrich	R7630	Prepare 10% w/v stock solution.
Salmon Sperm DNA	Agilent Tech	201190	
SD-His plates			Aldrich A1296), 0.77 g -His DO supplement, 6.7 g yeast Nitrogen Base w/o
SGal-His plates			
Buffer			1.2 g sodium dodecyl
Sodium hydroxide	Sigma-Aldrich	221465	Prepare 0.2 M solution.
Sucrose	Sigma-Aldrich	84097	Prepare 20% w/v stock solution.
TBS + 0.1% Tween 20 (TBST)			mL Tween 20 (Sigma-
TBS Blocking Buffer	LI-COR	927-5000	
Trans-Blot SD Semi-Dry	BIO-RAD	170-3940	
Transfer Buffer			Mix: 22.5 g glycine, 4.84 g
Tris-Buffered Saline (TBS)			10X, 7.6 pH, Solution: Mix
WT 303 *S. cerevisiae* yeast	Gift from J. Shorter		
Yeast Extract Peptone Dextrose	Sigma-Aldrich	Y1375	

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CORRESPONDING AUTHOR:

Name: MARIANA TORRENTE

Department: CHEMISTRY

Institution: BROOKLYN COLLEGE – CUNY

Article Title: CHARACTERIZING HISTONE POST-translational Modifications

Signature: [signature]

Date: 09/14/18

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Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please provide Figure 1 with higher resolution.

Figure 1 at 300dpi has been provided.

2. Please add more details to your protocol steps (in particular those highlighted for filming). There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See specific comments marked in the attached manuscript.

- **'Critical step' removed from step 1.9.**
- **'Critical step' removed from step 1.10.**
- **'Critical step' removed from step 2.5.**
- **Step 3.1 was clarified to say re-streak from frozen glycerol stock.**
- **The selective media plates are specified as –Histidine, 2% glucose agar plates in step 3.1.**
- **The selective media is specified as –Histidine in step 3.2.**
- **The number and type of overexpression model was clarified in step 3.3.**
- **The amount of culture to prepare in step 3.3.1. is now specified as 100 mL.**
- **Clarified that the amount of overnight culture needs to be calculated in step 3.3.2.**
- **Step 3.3.3. was clarified to explain how protein overexpression is induced.**
- **An example of how to standardize cultures for harvesting was added to step 3.4. Furthermore, step 3.4 was verified to contain all pertinent information after steps 3.4 and 3.5 were merged from a previous draft.**
- **Use of a heating block was specified in step 4.1.4.**
- **The number of gels to be prepared is now specified in step 4.2.1.**
- **The sample is identified in step 4.2.2.**

- **The size and number of PVDF membrane has been added to step 4.2.4.**
- **‘Critical step’ removed from step 4.2.5.**
- **The gel to be transferred in step 4.2.3 has been clarified.**
- **Added power pack to step 4.2.6 and clarified for each sandwich.**
- **Description of the type of box used for Ponceau S staining as well as how to observe the band has been added to step 4.3.1.1.**
- **Instructions for blocking membrane were clarified and TBS was spelled out in section 4.3.2.**
- **Incubating blot in staining box was clarified in step 4.3.3.**
- **The language in step 4.3.3. was cleaned up.**
- **Step 4.3.4 now reads 4x instead of repeat 3 times, accepted editor change.**
- **Step 4.3.6 now reads 4x instead of repeat 3 times, accepted editor change.**
- **Instructions for obtaining the density of each band have been added to step 5.1.**
- **Step 5.2 was revised to say ‘repeat step 5.1.’ and the number of bands has been clarified.**
- **Description of error bars was removed from step 5.5.**
- **The number of control samples was clarified in step 5.6.**
- **Critical steps are now addressed in the discussion. (Lines 547-553)**
- **Additional information added to reference #3.**
- **Full journal name added to references #22 and #34.**
- **Page numbers and additional information added to reference #30.**

3. As the authors indicated that copyright permission has been obtained, please upload this file to the editorial manager when submitting the revision.

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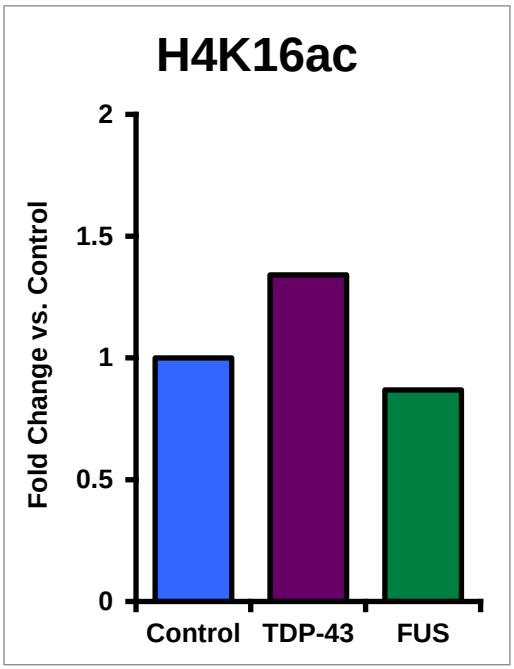
Sample Calculation of Adjusted Relative Density

	From Image Analysis H4K16ac Raw Density	Relative Density
Control	14.6	1
TDP-43	20	1.369863014
FUS	13.5	0.924657534



for H4K16ac

Adjusted Relative Density	From Image H3 Total Raw Density	Relative Density
1	1.86	1
1.341023792	1.9	1.021505376
0.868617684	1.98	1.064516129

*no error bars are included as this sample calculation represents data processing for a single expe

eriment

Title: Neurodegenerative Disease Proteinopathies Are Connected to Distinct Histone Post-translational Modification Landscapes

Author: Karen Chen, Seth A. Bennett, Navin Rana, et al

Publication: ACS Chemical Neuroscience

Publisher: American Chemical Society

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