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Quantitative analysis of the detergent-insoluble brain proteome in frontotemporal lobar degeneration using SILAC internal standards

Nicholas T. Seyfried^{1,3,7,†}, Yair M. Gozal^{3,†}, Laura E. Donovan³, Jeremy H. Herskowitz³, Eric B. Dammer², Qiangwei Xia², Li Ku⁶, Jianjun Chang⁴, Duc M. Duong², Howard D. Rees³, Deborah S. Cooper⁵, Jonathan D. Glass³, Marla Gearing⁵, Malú G. Tansey⁴, James J. Lah³, Yue Feng⁶, Allan I. Levey³, and Junmin Peng^{2,7}

¹Department of Biochemistry, Center for Neurodegenerative Disease, School of Medicine, Emory University, Atlanta, GA, 30322

²Department of Human Genetics, Center for Neurodegenerative Disease, School of Medicine, Emory University, Atlanta, GA, 30322

³Department of Neurology, Center for Neurodegenerative Disease, School of Medicine, Emory University, Atlanta, GA, 30322

⁴Department of Physiology, Center for Neurodegenerative Disease, School of Medicine, Emory University, Atlanta, GA, 30322

⁵Department of Pathology and Laboratory Medicine, Center for Neurodegenerative Disease, School of Medicine, Emory University, Atlanta, GA, 30322

⁶Department of Pharmacology, Center for Neurodegenerative Disease, School of Medicine, Emory University, Atlanta, GA, 30322

Summary

A hallmark of neurodegeneration is the aggregation of disease related proteins that are resistant to detergent extraction. In the major pathological subtype of frontotemporal lobar degeneration (FTLD), modified TAR-DNA binding protein 43 (TDP-43), including phosphorylated, ubiquitinated and proteolytically cleaved forms, is enriched in detergent-insoluble fractions from post-mortem brain tissue. Additional proteins that accumulate in the detergent-insoluble FTLD brain proteome remain largely unknown. In this study, we used proteins from stable isotope-labeled (SILAC) human embryonic kidney 293 cells (HEK293) as internal standards for peptide quantitation across control and FTLD insoluble brain proteomes. Proteins were identified and quantified by liquid-chromatography coupled with tandem mass spectrometry (LC-MS/MS) and twenty-one proteins were determined to be enriched in FTLD using SILAC internal standards. In parallel, label free quantification of only the unlabeled brain derived peptides by spectral counts (SC) and G-test analysis identified additional brain-specific proteins significantly enriched in disease. Several proteins determined to be enriched in FTLD using SILAC internal standards were not considered significant by G-test due to their low total number of SC. However immunoblotting of FTLD and control samples confirmed enrichment of these proteins, highlighting the utility of SILAC internal standard to quantify low abundance proteins in brain. Of these, the RNA binding protein PTB-associated splicing factor (PSF) was further characterized because of structural and functional similarities to TDP-43. Full-length PSF and shorter molecular weight fragments, likely

⁷Corresponding Authors: Nicholas T. Seyfried, Tel. 404.712.8589, nseyfri@emory.edu and Junmin Peng Tel. 404.712.8510, jpeng@genetics.emory.edu.

[†]These authors contributed equally to this work

resulting from proteolytic cleavage, were enriched in FTLD cases. Immunohistochemical analysis of PSF revealed predominately nuclear localization in control and FTLD brain tissue and was not associated with phosphorylated pathologic TDP-43 neuronal inclusions. However, in a subset of FTLD cases, PSF was aberrantly localized to the cytoplasm of oligodendrocytes. These data raise the possibility that PSF directed RNA processes in oligodendrocytes are altered in neurodegenerative disease.

Introduction

The accumulation of detergent-insoluble protein inclusions is observed in a wide variety of neurodegenerative diseases [1, 2]. For example, the neuropathological hallmarks of Alzheimer's disease (AD) are characterized by the presence of senile plaques and neurofibrillary tangles composed of detergent-insoluble amyloid-beta ($A\beta$) and phosphorylated-tau (pTau), respectively. The most common pathological subtype of frontotemporal lobar degeneration (FTLD) is defined by the presence of TAR DNA binding protein 43 (TDP-43) inclusions [3]. In healthy individuals, TDP-43 resides in the nucleus, but in disease, the protein redistributes to the cytoplasm where it is found phosphorylated and ubiquitinated [3–5]. TDP-43 pathology is also observed in the spinal cord of individuals with amyotrophic lateral sclerosis (ALS), a severe form of motor neuron disease (MND) that can occasionally co-occur with FTLD [6]. To date, over 30 mutations in TDP-43 (*TARDBP*) have been reported in ALS [5]. However, with the exception of two rare *TARDBP* mutations and those individuals harboring mutations in progranulin (*PGRN*), valosin-containing protein (*VCP*), or within chromosome 9p [7], the majority of FTLD cases with TDP-43 pathology are idiopathic with no genetic cause identified [4, 5]. Thus, TDP-43 is a prototypical protein with biochemical signatures occurring in the detergent-insoluble proteome, but other proteins yet to be identified, may very well be important markers of neurodegenerative disease processes. To this end, we employed a quantitative proteomic approach to assess relative changes in the FTLD detergent-insoluble proteome extract from post-mortem human brain.

Protein levels can be determined by mass spectrometry (MS) using a variety of peptide labeling strategies and label-free approaches. Label-free quantification can be performed by peptide spectral counts (SCs) or extracted ion intensity and labeling approaches can include chemical derivitization strategies using isobaric stable isotope tagging reagents, such as tandem mass tags (TMT) or isobaric tags for relative and absolute quantification (iTRAQ) [8]. Alternatively, metabolic labeling strategies with heavy isotopes (e.g. ^{15}N) or stable isotope labeling with amino acids in cell culture (SILAC) are being increasingly utilized [9]. Recently, *in vivo* SILAC labeled mice [10] and *Drosophila* [11] have been reported for comparative proteomic studies after implementing a selective diet highly enriched in heavy isotopic forms of lysine. Although SILAC has worked in cells and higher eukaryotes, the approach is incompatible for the direct analysis of post-mortem human tissues (e.g. control versus disease). Thus, label-free or chemical derivitization strategies have been preferred for quantitative proteomic studies using clinical tissue. However, these approaches suffer from certain limitations. For example, labeling peptides post-digestion cannot account for experimental error during earlier protein and peptide purification steps [8]. Additionally, iTRAQ and TMT quantitation is based on the intensities derived from reporter ions with low m/z values (e.g. 114, 115, 116 and 117 m/z) that are observed only after precursor MS/MS fragmentation. A limitation of linear ion-trap mass spectrometers is the “one third rule”, i.e., that fragment ions with m/z values less than 30% of the precursor m/z display decreased stability and are less reliably detected [12]. For example, fragment ions from a precursor ion at m/z 900 will not be reliably detected below m/z 300. Therefore, measurements of low m/z reporter ions in linear ion-trap mass spectrometers often require optimization employing

pulsed Q collision induced dissociation (PQD) to sufficiently balance backbone precursor ion fragmentation and maintain reporter ion intensity for accurate quantitative measurements [12, 13].

Taking the above factors into consideration, we chose to use cell derived isotopically labeled protein standards for quantitative proteomic analysis of post-mortem brain tissue. This approach was successfully employed for the relative and absolute quantification of proteins from mouse brain tissues [14]; however, utilizing this strategy for analyses of human brain tissues has been largely unexplored. Here, whole lysate from SILAC labeled human embryonic kidney (HEK293) cells was equally mixed with detergent-insoluble fractions from pools of four FTLN and four control post-mortem cases. SILAC labeled peptides served as internal standards between FTLN and control detergent-insoluble fractions. HEK293 cells efficiently incorporate heavy isotopic amino acids in culture [15] and express neurofilaments, G-protein coupled receptors, vimentin and α -internexin as well as many other proteins typically found in neurons [16]. Thus, HEK293 cells were deemed a reasonable source for quantitation of brain proteins. In total, over 1,500 proteins were identified and the SILAC standard provided accurate quantification of approximately 62% of the brain proteome. Twenty-one proteins were determined to be enriched in the detergent-insoluble FTLN proteome based on quantification of light and heavy peptide pairs. These included the splicing factor polypyrimidine tract binding (PTB) protein-associated splicing factor (PSF), cytosolic non-specific dipeptidase isoform 2 (CNDP2), triosephosphate isomerase 1 (TPI1), cofilin-1, cathepsin D and Rho GDP dissociation inhibitor (GDI)- α (RHOGDI- α). Based on the structural similarities among PSF, TDP-43, and fused in sarcoma (FUS), another RNA binding protein found in ALS [17] and some FTLN cases [18], we chose to further characterize brain PSF protein in a panel of control and FTLN cases by immunoblotting and immunohistochemistry. Immunoblot analyses validated that PSF was significantly enriched in the FTLN detergent-insoluble fraction, both the full-length form and truncated fragments of lower molecular weight. In parallel, immunohistochemical examination indicated that the subcellular localization of PSF in brain was predominantly nuclear and not associated with phosphorylated pathologic TDP-43 neuronal inclusions in FTLN. However, in a subset of FTLN cases, PSF accumulated in the cytoplasm and distal processes of Olig2 immunoreactive oligodendrocytes. Together these data highlight the utility of SILAC internal standards for the quantification of low abundant detergent-insoluble proteins in human post-mortem brain tissue.

Experimental Procedures

Case selection and protein extraction

Cases included in this study all had a diagnosis of FTLN, as determined by extensive clinical assessment and neuropathological characterization based on well established consensus criteria at the Emory Alzheimer's Disease Research Center [19, 20]. The cases characterized as FTLN exhibited phosphorylated TDP-43 positive, tau- and α -synuclein-negative, cytoplasmic inclusions in neurons of both the superficial frontal cortex and dentate gyrus of the hippocampus. Additionally, these cases did not meet criteria for the neuropathological diagnosis of AD (CERAD [21] or NIA-Reagan [22]) or Lewy Body disease [23], nor did they show any tau pathology consistent with a tauopathy [24, 25]. In total, frozen frontal cortex from twelve FTLN and eleven control cases (Supplemental Table S1) were homogenized and detergent-insoluble protein extraction were performed by differential ultracentrifugation as described previously [26].

Stable isotope labeling with amino acids in cell culture (SILAC) and LC-MS/MS

HEK293 cells ($\sim 1 \times 10^8$) were cultured in DMEM (deficient in L-Lysine and L-Arginine) supplemented with 2% dialyzed fetal calf serum (Invitrogen) as described [27]. For stable isotopic labeling, heavy forms L-Arginine (Arg10; $^{13}\text{C}_6^{15}\text{N}_4$) and L-Lysine (Lys8; $^{13}\text{C}_6^{15}\text{N}_2$) were added (Cambridge Isotope Laboratories) to a final concentration of 0.26 mM. Excess proline was added at 200 mg/L to block arginine to proline conversion [28]. Cells were washed twice and collected in ice cold phosphate buffered saline (PBS) and then lysed in ice cold cell lysis buffer (50mM Tris-HCl pH 7.4, 1mM EDTA, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% w/v SDS). Complete protease cocktail inhibitor (Roche, Indianapolis, IN) was added to all buffers immediately prior to use. Before SDS-PAGE, 10 μg of SILAC labeled HEK293 whole cell lysate was added to 10 μg of detergent-insoluble fractions from four pooled control and four pooled FTLN extracts, respectively, matched as closely as possible by age and post-mortem interval (Supplemental Table S1). The mixed (light and heavy) samples were reduced with 10 mM dithiothreitol (DTT), and resolved on a 10% polyacrylamide SDS gel. After staining with Coomassie G-250, each gel lane was cut into five gel pieces according to molecular weight (A-E; 25, 25–40, 40–60, 60–100 and 100 kDa, respectively) and subjected to in-gel digestion (12.5 ng/ μl trypsin). Extracted peptides were loaded onto a C₁₈ column (75 μm i.d., 10 cm long, ~ 300 nl/min flow rate, 3 μM resin from Michrom Bioresources, Auburn, CA) and eluted during a 10–30% gradient (Buffer A: 0.4% acetic acid, 0.005% heptafluorobutyric acid, and 5% AcN; Buffer B: 0.4% acetic acid, 0.005% heptafluorobutyric acid, and 95% AcN). The eluted peptides were detected by Orbitrap (300–1600 m/z, 1,000,000 automatic gating control (AGC) target, 1,000 ms maximum ion time, resolution 30,000). MS/MS scans in an LTQ linear-ion trap mass spectrometer (2 m/z isolation width, 35% collision energy, 5,000 AGC target, 150 ms maximum ion time) (Thermo Finnigan, San Jose, CA) were acquired by data-dependent acquisition. All data were converted from raw files to the .dta format using ExtractMS version 2.0 (Thermo Electron) and searched against human reference database downloaded from the National Center for Biotechnology Information (November 19, 2008) using the SEQUEST Sorcerer algorithm (version 3.11, SAGE-N). Searching parameters included mass tolerance of precursor ions (± 50 ppm) and product ion (± 0.5 m/z), semi-tryptic restriction, with a dynamic mass shift for oxidized Met (+15.9949), Lys (+8.01420 for $^{13}\text{C}_6^{15}\text{N}_2$) and Arg (+10.00827 for $^{13}\text{C}_6^{15}\text{N}_4$), four maximal modification sites and a maximum of two missed cleavages. Only b and y ions were considered during the database match. To evaluate false discovery rate (FDR), all original protein sequences were reversed to generate a decoy database that was concatenated to the original database (77,764 entries) [29]. The FDR was estimated by the number of decoy matches (nd) and total number of assigned matches (nt). $\text{FDR} = 2 \cdot \text{nd}/\text{nt}$, assuming mismatches in the original database were the same as in the decoy database. To remove false positive matches, assigned peptides were grouped by a combination of trypticity (fully and semi) and precursor ion-charge state. Each group was first filtered by mass accuracy (10 ppm for high-resolution MS), and by dynamically increasing correlation coefficient (Xcorr, minimum 1.0) and ΔCn (minimum 0.05) values to reduce protein FDR to less than 1 percent. All MS/MS spectra for proteins identified by a single peptide were manually inspected as described previously [30]. If peptides were shared by multiple members of a protein family, the matched members were clustered into a single group. On the basis of the principle of parsimony, the group was represented by the protein with greatest number of assigned peptides.

Peptide quantification and bioinformatics

Quantitative pair-wise comparison of control and FTLN samples was performed in technical replicate according to previously reported methods using in-house software [15, 31, 32]. (i) Ion extraction from MS scans: The ion currents for identified peptides were extracted in MS

survey scans of high-resolution (30,000), based on the isotopic ion selected for MS/MS sequencing. A number of features were derived, including precursor m/z, charge state, retention time, ion peak width, height and noise level. The global noise level (GNL) was derived by averaging signal intensity of all ions in the MS scan after removing signal ion outliers that were at least two standard deviations away from the mean. The intensity of ions was presented by the peak height and normalized according to the noise intensity under the assumption that the noise level of MS scans reflects, at least partially, variable ionization efficiency. (ii) Ion matching among light, and heavy peptide pairs was allowed with a mass tolerance of 10 ppm. If a sequenced peptide could not be matched to a signal above GNL, we estimated that the maximum ion current for undetected signal was equal to the local noise level (LNL) of ions within the 10 ppm window ($LNL = 0.5$). (iii) Data integration: The ratio of every peptide pair (light/heavy) was transformed into logarithmic ($\log_2$) values and the detection of outliers determined using Dixon's Q test [33]. If a peptide was identified in multiple MW fractions the intensities for the peptide pair (light and heavy) were summed to give a single cumulative peptide ratio (light/heavy). The averaged $\log_2$ ratio over all peptides for a particular protein was used to determine the protein ratio and a standard error (S.E.). The results are summarized in Supplementary Tables S2 and S3 with the number of peptides quantified for every protein and the individual peptide ratios for control and FTLN (Supplementary Tables S5-S8) from each technical replicate. (iv) Data normalization: According to the null hypothesis, the histogram of the difference (FTLN-Control) between all shared protein $\log_2$ ratios was fitted to a Gaussian distribution to evaluate systematic bias according to the mean and biological variation based on standard deviation using Graphpad Prism (ver. 5.0, GraphPad Software, La Jolla, CA). The data were then normalized by subtracting the mean from every protein ratio. Proteins were considered enriched if the brain/SILAC $\log_2$ values for control and FTLN peptide mixtures (within a replicate) had signal/noise (S/N) greater than 10 and were quantified by two or more peptides. Analysis of bimodal distribution in Control- and FTLN- SILAC (HEK293) mixtures was performed using multi-peak fit package on Igor (version 6.0, WaveMetrics, Inc., Lake Oswego, OR). Finally, the quantified proteins were manually examined with respect to MS/MS assignment, ion peak matching and ion intensity. To confirm that brain-specific proteins are a major component of the unlabeled and unpaired proteins present in the brain/HEK293 SILAC protein mixture, we performed Ingenuity Pathway Analysis IPA (Ingenuity® Systems, www.ingenuity.com). NCBI RefSeq identifiers for proteins identified with a SILAC light/heavy $\log_2$ ratio above 3 were submitted to IPA. The identifiers mapped to 604 unique genes, and the top disease-associated pathway was noted as neurological disease, involving 50 percent (301) of the genes. In addition, the top physiological function represented was nervous system development and function, involving 30 percent (179) of the genes. All nervous system pathway lists of genes represented were exported with p-values and genes in each list were counted, to produce a chart of the number of proteins identified as brain enriched and involved in the top physiological function represented.

Label free protein quantification by spectral counting

Label-free spectral counting was used to determine differences between the control (CTL) and FTLN detergent-insoluble proteomes using SC and G-test as previously described [34, 35]. In this analysis only brain derived peptides were considered (light peptides only). Several of the proteins identified in this study were found exclusively in FTLN or control samples. For those proteins only identified in one sample (FTLN or control), a spectral count of 1 was applied. The spectral counts were normalized to ensure that average spectral count ratio per protein was the same in the two datasets [36]. G-test was used to judge statistical significance of protein abundance difference as previously described [37–40]. The G-value of each protein was calculated as shown in equation 1, where f_{FTLN} and f_{CTL} are

the detected SC of a given protein in the FTLD and control samples, respectively, and “ln” is the natural logarithm.

$$G=2 \left[f_{FTLD} \bullet \ln \left(\frac{f_{FTLD}}{f_{FTLD}+f_{CTL}} \right) + f_{CTL} \bullet \ln \left(\frac{f_{CTL}}{f_{FTLD}+f_{CTL}} \right) \right] \quad (1)$$

The p -value of each protein was calculated as the probability of observing a random variable larger than G from the Chi-square distribution (one degree of freedom). The frequency histogram of the p -values was created and 0.01 was set as the cutoff to detect significant changes. The R statistical package was used for this analysis. Proteins, normalized spectral count ratios, and p values can be viewed in Supplementary Table S4.

Immunoblotting

Immunoblotting was performed according to standard procedures as reported previously [15]. Briefly, samples in Laemmli sample buffer were resolved by SDS-PAGE (12% w/v acrylamide) before an overnight transfer to Immobilon-P membranes (Millipore, Bedford, MA). These were blocked with TBS (Tris-buffered saline) plus blocking buffer (USB Corporation, Cleveland, OH) at room temperature for 90 min and probed with primary antibodies in TBS with 0.1% Tween-20 plus blocking buffer overnight at 4°C. The following day, membranes were rinsed and incubated with secondary antibodies conjugated to fluorophores (Molecular Probes/Invitrogen) for one hour at room temperature. Images were captured using an Odyssey Image Station (LiCor, Lincoln, NE) and band intensities were quantified using Scion Image. Statistical analysis was performed using Student's t test for independent samples. Antibodies included: Mouse anti-CNDP2 (R&D Systems, MAB3560), rabbit anti-PSF (Abcam, ab38148), rabbit anti-TDP-43 (ProteinTech, 10782-1-AP), rabbit anti-cofilin-1 (Santa Cruz), rabbit anti-RHOGDI-alpha (ProteinTech, 10509-1-Ig), rabbit anti-PGP9.5 (*i.e.*, UCHL1) Chemicon International, Temecula CA), mouse anti-CNP (Chemicon International, Temecula CA), rabbit anti-PTB (Invitrogen) and goat anti-TPI1 (Abcam, ab28760).

Immunocytochemistry

The GFP-PSF wild-type construct was kindly provided by James G. Patton (Vanderbilt University) and characterized as previously described [41]. HEK293 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Cambrex, Walkersville MD), and supplemented with 10% fetal bovine serum (Gibco, Grand Island NY) and 1% penicillin-streptomycin (Cambrex). Cells were grown in a humidified 5% CO₂ environment at 37°C, and prepared for transfection (Lipofectamine 2000) by plating on Matrigel-coated coverslips. Immunocytochemistry was performed 48 hours after transfection as previously described [42]. Images were captured with a 1µm optical thickness for subsequent analysis on a Zeiss LSM 510 confocal microscope (Zeiss, Thornwood NY).

Primary cell culture

Primary cultured rat cortical neurons were raised using a previously described procedure [43]. Briefly, cortices of E16 Sprague-Dawley pups were minced and gently agitated to obtain dissociated cells in DMEM containing 5% fetal calf serum and 5% horse serum. After washing, cells were resuspended and seeded onto poly-L-Lysine-coated plates and incubated at 37°C in 5% CO₂. After 1 hr, cells were re-fed with Neurobasal medium containing B-27 supplement (Invitrogen) and incubated for 5 days before harvest. Primary cultures of oligodendroglia were raised using a well-established procedure [44]. Briefly, mixed glial cultures were prepared from the brains of neonatal Sprague-Dawley rats (P2), plated on 75

cm² flasks and allowed to reach confluence in DMEM containing 12% fetal bovine serum (FBS). Oligodendroglia progenitor cells (OPCs) were removed by shaking at 180 rpm over night and further purified from microglia and astrocytes that quickly attach to uncoated Falcon dishes. The floating OPCs were isolated and plated onto poly-lysine-coated dishes and maintained in a defined serum-free medium with platelet-derived growth factor and fibroblast growth factor. Differentiation was induced by removing growth factors and mature oligodendrocytes were obtained after 5 days in the differentiation medium lacking growth factors. After the shaking step defined above, the confluent glia layer contained more than 90% pure astrocytes. The cells were re-plated into medium containing 10% serum and harvested 24 hrs later. Primary cultures of microglia were raised as previously described [45]. Briefly, the brains of 1-day-old neonatal Sprague-Dawley rats were minced and cells dissociated by agitation before passing through a nylon membrane (pore size 75µm). Mixed glia culture was raised in RPMI-1640 containing 10% FBS, 2 mM glutamine, and 1% penicillin-streptomycin on poly-L-lysine coated flasks and allowed to reach confluence after 14 days. Microglia were isolated by gently shaking the mixed glia culture for 2 hr at 150 rpm at 37°C and plated on uncoated Costar culture dishes to remove non-adherent astrocytes. The adherent microglia was further cultured for 24 hr before harvest.

Immunohistochemistry

Paraffin-embedded sections from post-mortem human frontal cortex (8 µm thick) were deparaffinized and microwaved in citrate buffer (10 mM, pH 6) for 5 minutes. After cooling to room temperature, sections were rinsed and endogenous peroxidase activity was blocked with 3% hydrogen peroxide at 40°C. Sections were then incubated with normal horse serum for 15 minutes at 40°C, followed by rabbit anti-PSF primary antibody (Abcam; 1:200), rabbit anti-TDP-43 (1:1000, ProteinTech), mouse anti-Olig2 (1:200 Millipore) or mouse anti-phosphorylated TDP-43 (Ser409/410) antibody (1:8000, CosmoBio) diluted in 1% BSA for 3,3'-diaminobenzoic acid (DAB) development or 0.25% (v/v) Brij-35, 100 mM NaCl, 50 mM MgCl₂, 100 mM Tris-HCl, pH 7.5 for immunofluorescence, overnight at 4°C. The following day, sections were incubated with biotinylated secondary antibody for 30 minutes at 37°C, followed by avidin-biotin peroxidase complex (Vector Laboratories) for 60 minutes at 37°C. DAB was used as the chromogen (for color development) and was followed with hematoxylin nuclear counterstain. For pre-adsorption studies of PSF, primary antibody was pre-incubated with 100x molar excess of the immunizing peptide (Abcam) overnight at 4°C before incubation with the tissue. For immunofluorescence in paraffin embedded tissue, Alexa-488 and Alexa-fluor 546 were used as secondary antibodies (Jackson ImmunoResearch, Suffolk, UK). Free floating brain sections (50 µM) were prepared with a freezing microtome (Microm, Heidelberg, Germany) from FTLN brain blocks of the frontal cortex. Sections were subsequently incubated with serum followed by rabbit anti-PSF and mouse anti-GFAP (Millipore) primary antibodies overnight at 4°C. After washes with Tris-buffered saline, sections were incubated with fluorescent secondary antibodies (Alexa-488 and Alexa-fluor 546) for 1 hour at 4°C. Staining was visualized using an Olympus BX51 fluorescent/bright field microscope or confocal microscopy as described above.

Results

Analysis of the detergent-insoluble proteome from FTLN and control brain

To identify and quantify detergent-insoluble proteins in FTLN cases (Fig. 1A), we examined post-mortem brain tissue samples using SILAC internal standards. In this strategy, four cases of control and four cases of FTLN frontal cortex homogenates were pooled by diagnosis and matched for age and sex (Supplemental Table 1). Samples were serially extracted with buffers of increasing stringency: First by sarkosyl detergent-containing buffer and then by urea to enrich for detergent-insoluble proteins [26, 46]. By pooling the urea

solubilized proteins from our cases prior to discovery-mode proteomic experiments, the between-subject variability and pathophysiological heterogeneity as well as the variability in the efficiency of detergent extraction can be diluted [26, 47]. For quantitative proteomics, whole cell lysate from SILAC labeled HEK293 cells was mixed equally with detergent-insoluble fraction pools derived from either control or FTLN cases (Figs. 1B and 2A). Shared SILAC-labeled HEK293 proteins served as internal standards for software based measurement of protein abundance between control and FTLN samples (Fig. 2B). Each protein fraction was resolved by SDS-PAGE and the brain/SILAC (light/heavy) mixtures of control and FTLN samples were excised into five gel slices, trypsin digested, and analyzed via liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) on an Orbitrap mass spectrometer (Fig. 2A and C).

Quantitative analysis of shared proteins in the brain/SILAC mixed proteome versus label-free quantitation of brain-derived proteins

Quantitative pair-wise comparison in the brain/SILAC mixed samples was carried out in technical replicate as described in the materials and methods section. In control/SILAC sample, protein $\log_2$ values (light/heavy) were plotted as a histogram and a bimodal distribution was observed (Fig. 3A). A similar bimodal pattern of distribution was observed in the FTLN/SILAC mixture (Supplemental Figure 1). Analysis of all pooled (FTLN/SILAC and control/SILAC) technical replicate LC-MS/MS data showed higher variation at protein $\log_2$ values greater than 3.0 and less than -2.4 (Fig. 4A and B). Thus, the heavy labeled HEK293 internal standard greatly reduced the standard error in the proteomic quantification within this range ($\log_2$ -2.4 to 3.0) when compared to those proteins quantified outside this range ($\log_2$ <-2.4 or >3.0). It should be noted that we included both semi-tryptic and tryptic peptides in protein quantification. As expected, the control brain sample had a significant level of proteolysis compared to HEK293 cell standard, which may be a result of both brain specific *in vivo* cleavage events as well as post-mortem interval (Supplemental Figure 2). Importantly, the inclusion of semi-tryptic peptides did not overall negatively impact the accuracy of quantification of most proteins reported because they represent a small proportion (<8%) of total peptides quantified. Proteins with $\log_2$ values below -2.4 mainly represented HEK293-specific or -enriched proteins (i.e. having much higher levels in the SILAC internal standard compared to brain). For example, peptides from SET translocation protein (myeloid leukemia-associated) had an average $\log_2$ value of -5.17 and were more intense in the heavy labeled HEK293 cells than in unlabeled (light) native brain (Fig. 3B). Conversely, those proteins having $\log_2$ values greater than 3.0 mainly represented brain specific or enriched proteins (i.e. having a much higher level in brain compared to the SILAC internal standard). These included the neuron specific protein microtubule-associated protein-2 (MAP2; $\log_2$ = 4.25) (Fig. 3D). For proteins with $\log_2$ values between the lower and upper limits (-2.4 to 3.0), such as ubiquitin activating enzyme 1 (UBA1), the intensity of matching peptides was sufficient for calculating the light/heavy ratio (Figs. 3C and 4). In the second replicate control brain proteome 34.0% (1032/3032) and 10.7% (323/3032) can be considered proteins outside the upper and lower limits (-2.4 to 3.0) of quantification. This highlights an important limitation of the HEK293 generated SILAC internal standard in that of all brain proteins identified only approximately 62% (1677/2709) are shared with brain, likely proteins which are found across most cell types, rather than proteins specific to brain. We confirmed this by performing Ingenuity Pathway Analysis using the 604 gene products identified specifically as unlabeled peptides ($\log_2$ brain/SILAC ratio > 3). This analysis confirmed that 50 percent of these proteins can be linked to neurological disease. Further, 30 percent of the gene products identified, but not well quantified by SILAC standards are involved in nervous system development and function (Supplemental Figure 3). Nonetheless, we hypothesized that we could validate changes and

brain-specific function of one or more of the shared or “universal,” SILAC-quantified, changing proteins as relevant to FTLN pathogenesis, as described in below results sections.

Here we define the criteria for consideration of brain/HEK293 shared proteins, and briefly extend analysis to rescue identifications of changing brain-specific proteins in FTLN. A histogram of the difference (FTLN-Control) between all shared protein $\log_2$ values was fitted to a Gaussian distribution to evaluate systematic bias according to the mean and variation based on the standard deviation (SD) for the replicates (R1 and R2) individually (Fig. 5B). The SD in R1 and R2 was calculated to be 1.0 and 0.90, respectively. The difference in $\log_2$ protein values (FTLN-Control) in both replicates were normalized by subtracting the experimental mean, 0.32 in R1 and 0.40 in R2. As a filtering criterion, FTLN proteins with potentially increased abundance that fell outside the 90% confidence interval (1.64 SD away from the mean, $p < 0.05$ in a one-tailed analysis) were considered as a subgroup of interest. In order to reduce the variability on protein measurements largely based on non-paired peptides, we required that the $\log_2$ ratio in at least one of the two samples (control and FTLN) for either replicate fall within the $\log_2$ range of -2.4 to 3.0 . Two more criteria were required of the proteins appearing on the final table: i) the average $\log_2$ value from R1 and R2 must indicate at least 2 fold enrichment (average $\log_2 > 1.0$) and ii) S.E between replicates < 0.5 . In total, twenty-one proteins met these criteria and were considered enriched in FTLN using the SILAC approach. These proteins are classified according to their cellular location and function (Table 1). The difference (FTLN-Control) between $\log_2$ values for all proteins in R1 and R2 quantified is provided in Supplementary Tables S2 and S3.

To assess whether the protein fold changes we observed using SILAC internal standard were also reflected in a label-free quantitation approach via spectral counts (SC), and to potentially rescue the identification of FTLN changed proteins that are brain-specific, we performed G-test analysis of SC only derived from light labeled peptides in the FTLN and control samples (Replicate 1 and 2). Of the enriched proteins reported in Table 1 (quantified using SILAC standards), 11 of the 21 (52%) were also found significantly enriched by SC ($p < 0.01$). Spectral counting is based on the observation that more abundant peptides will be selected for fragmentation and will produce a higher number of MS/MS spectra, and therefore, SC should be proportional to protein amount in data-dependent acquisition [48]. However, it is not surprising that proteins identified by only a few unlabeled peptides with low total SCs were not considered significant by G-test. For example RHOGDI- α , calreticulin and cathepsin D, which were considered enriched using SILAC internal standards, were sequenced with too low a frequency in the mass spectrometer (< 15 total SCs in both control and FTLN) to be considered significant by G-test. Importantly, SC analysis did reveal several brain-specific proteins as being significantly enriched in FTLN that were outside our upper limit of detection ($\log_2 > 3$) using SILAC internal standard (Table 2). These proteins included brain creatine kinase (CKB), glial fibrillary acidic protein (GFAP), secernin (SCRN) and peptidylprolyl isomerase A (PPIA), which lacked significant signal from internal peptide standards for accurate quantification. All proteins, normalized spectral count ratios, and p values can be viewed in Supplementary Table S4. Thus, complementary quantitative approaches such as SILAC internal standards and spectral counting, respectively, enhance the ability to quantify low abundant proteins with shared brain peptides, or brain specific and brain-enriched proteins albeit with lower sensitivity. The remainder of this study focuses on the proteins identified as changing via SILAC quantitation.

Immunoblotting to confirm enrichment of insoluble proteins in FTLN brain tissue

Our primary goal was to employ an unbiased approach to identify disease-associated components in the detergent-insoluble FTLN proteome, which can include inclusion

proteins among other insoluble proteome cellular components. To validate our SILAC approach for proteome quantification results, we re-purified the detergent-insoluble proteome (starting from frozen tissue) from the same control and FTLD cases pooled for proteomics. Immunoblot analysis using commercially available antibodies for CNBP2, TPI1, RHOGDI- α , cofilin-1 and cathepsin D showed strong immunoreactivity in all FTLD cases (Fig. 5C and Table 1), however, there was more heterogeneity in the cases which were used for the control pool in the brain/SILAC proteomics experiment. For example, in control case 5, the signal intensities for TPI1, cofilin-1 and RHOGDI- α were equivalent to FTLD cases, but were significantly weaker in other control cases. In comparison, CNBP2, which had the greatest $\log_2$ difference (average normalized $\log_2 = 2.47 \pm 0.48$) was barely detected in any of the control insoluble samples. It should be noted that while SILAC comparison identified significant changes in cathepsin D and RHOGDI- α which we validated here, these proteins were not considered significantly enriched by SC. Thus, the SILAC internal standard aided in the accurate quantification of these low abundance proteins in the FTLD detergent-insoluble proteome. In contrast to the absolute differences we saw for the above proteins we did not observe significant enrichment of TDP-43 (averaged normalized $\log_2 = -0.03 \pm 0.02$) in the pooled FTLD samples by proteomics. It is important to note that native unmodified TDP-43 (~43 kDa) is intrinsically detergent-insoluble in control cases as previously demonstrated, whereas the biochemical signature of TDP-43 in FTLD is comprised of lower stoichiometry species (relative to the native 43 kDa species), which are cleaved, phosphorylated or resolve at high molecular weight [3, 49] (Supplemental Figure 4). This largely explains why we did not observe TDP-43 peptides outside of the gel region for the molecular weight range containing the vast majority of TDP-43. Only one TDP-43 peptide was sequenced in a MW region above 60 kDa and none below 25 kDa (see Supplemental Tables S5-8). Thus, lower stoichiometry TDP-43 species (i.e. ubiquitinated or cleaved) in FTLD frontal cortex were below the limit of detection in this experiment. Future studies may require enhanced separation of the insoluble protein extracts by SDS-PAGE and analysis of an increased number of gel fractions, which will reduce the protein complexity prior to in-gel digestion and LC-MS/MS. Those proteins that we did find more enriched represent a class of potential disease makers defined by their detergent insolubility even more than TDP-43, and may have important roles in the processes underlying pathogenesis.

Biochemical and immunohistochemical analysis of PSF in control and FTLD frontal cortex

Two RNA binding proteins, TDP-43 and FUS, are signature proteins of FTLD and ALS [5]. Of the 21 proteins enriched in FTLD by proteomics, one RNA binding protein found was PSF, a nuclear protein and a core component of paraspeckles, ribonucleoprotein bodies with reported roles in transcription and splicing [50]. Similar to TDP-43 and FUS, PSF contains two RNA recognition motifs (RRM) and a low complexity N-terminal glutamine and proline-rich region (Supplemental Figure 5). We examined PSF protein level and localization in FTLD. Native PSF (707 amino acids) is highly basic, and runs anomalously on SDS-PAGE at approximately 100 kDa [51]. Densitometry analysis of the native species in a panel of 20 detergent-insoluble fractions, including an additional number of control and FTLD cases not used for discovery proteomics, shows a significant enrichment in FTLD ($p < 0.05$) (Fig. 6A and B). Only certain FTLD cases showed enrichment, which may represent a unique subtype or variability in regional sampling of the FTLD brain tissue. No correlation between TDP-43 levels and PSF levels were observed in this analysis of detergent-insoluble fraction (data not shown). Smaller PSF species (~25–27 kDa) were also observed in six FTLD cases and one control. The immunoreactivity of the lower molecular weight (MW) species correlated with the intensity of native full-length PSF. Unique PSF peptides were not identified in lower MW regions (bands C-E), indicating that truncated PSF species were below the limit of detection in this experiment.

In healthy individuals, both TDP-43 and FUS reside in the nucleus and become sequestered in the cytoplasm in disease [5]. To assess PSF subcellular localization in frontal cortex tissue, we performed immunohistochemistry in FTLD and control cases. PSF protein was predominantly nuclear (Fig. 6C), which is consistent with previous reports in mouse brain [52]. Fluorescent labeling of PSF and phosphorylated TDP-43 (pTDP-43) was performed to determine if PSF displays altered distribution in neurons concurrent with cytoplasmic TDP-43 inclusions occurring in FTLD brain (Fig. 6D, upper panel). In layer II neurons of the frontal cortex, PSF (red) was exclusively localized to the nucleus and did not overlap with cytoplasmic pTDP-43 inclusions (green). Consistently, in hippocampal neurons in the dentate gyrus, colocalization between pTDP43 and PSF was not observed (Fig. 6D, lower panel). Importantly, compared to controls, three FTLD cases (5, 7 and 10), displayed a population of cells with profoundly abnormal cytoplasmic PSF distribution including distal processes at the interface of gray and white matter that were morphologically consistent with reactive glia. Although aberrant cytoplasmic PSF was most dramatic in this population of non-neuronal cells, we observed enhanced PSF immunoreactivity in a limited number of neuronal nuclei. Therefore, it remains possible that the increased levels of detergent-insoluble PSF identified by proteomics and validated by immunoblot originate from multiple cell-types, including neuronal and also non-neuronal populations. PSF immunoreactivity was abolished after pre-absorption with a molar excess of immunizing peptide, demonstrating the specificity of PSF antibody (Supplemental Figure 6). Since PSF isoforms that lack the C-terminal nuclear localization sequence (NLS; comprised of amino acids 703–707) due to alternative splicing or proteolytic cleavage display nearly exclusive cytoplasmic localization [41, 51], it is possible that cytoplasmic PSF observed in FTLD could similarly lack the C-terminal NLS. However, the rabbit polyclonal PSF antibody used in this study was raised against a C-terminus epitope (amino acids 650–707). To further characterize this antibody and to determine if the PSF antibody used in this study can detect PSF isoforms lacking the C-terminal NLS, recombinant green fluorescent protein (GFP) tagged, wild-type PSF-WT and PSF-ΔNLS (C-terminal NLS is deleted) were examined in HEK293 cells by immunocytochemistry and immunoblotting (Supplemental Figure 7). As expected, PSF-WT had primarily punctate nuclear localization, whereas PSF-ΔNLS was exclusive to the cytoplasm and in some cells formed aggregates, mimicking the aberrant PSF localization observed in FTLD [41]. Importantly, the polyclonal PSF antibody detected both PSF-ΔNLS and WT-PSF by immunoblotting. These results are consistent with the possibility that the cytoplasmic PSF observed in FTLD may be an isoform of PSF lacking the extreme C-terminal NLS. Alternatively, phosphorylation of full-length PSF by *BRK* tumor Kinase (BRK) has also been shown to promote PSF nuclear to cytoplasmic translocation [53]. Thus, there remain a number of putative causes for why cytoplasmic PSF is observed in FTLD.

PSF is expressed in neurons, astrocytes and oligodendrocytes

Since it was unclear what non-neuronal cell-type harbored cytoplasmic PSF in brain, we sought to profile PSF protein levels by immunoblotting PSF from total cell lysate from primary cultured rat neurons, astrocytes, oligodendrocytes and microglia (Fig. 7A). Consistent with previous reports, we detected full-length PSF protein in neurons and oligodendrocytes [52], and additionally, PSF was detected in rat and human oligodendrocyte cell lines (CG4 and HOG, respectively) (Fig. 7B). However, contrary to previous findings [52], we observed abundant PSF immunoreactivity in GFAP-expressing astrocytes, with low levels of PSF also detected in primary microglia cells (Fig. 7A). Protein levels for polypyrimidine tract binding protein (PTB) and TDP-43 were also analyzed by immunoblot in primary cultured brain cells. PTB, an hnRNP with roles in splicing, was previously identified in non-neuronal cells and characterized to complex with PSF [51]. In contrast to PSF, TDP-43 showed expression in all cell types including microglia, whereas PTB immunoreactivity was only observed in astrocytes and oligodendrocytes. Together, these

data indicate that structurally homologous RNA-binding proteins display cell-specific patterns of expression and that astrocytes and oligodendrocytes are the major non-neuronal cell types that express detectable levels of PSF.

Oligodendrocytes harbor cytoplasmic PSF in FTLD

To determine if astrocytes or oligodendrocytes display the cytoplasmic and branched process-specific PSF in FTLD, we performed double-labeled immunofluorescent studies with antibodies against PSF and the astrocyte marker GFAP or oligodendrocyte marker Olig2. In astrocytes, PSF was found predominantly in the nucleus (Fig. 8), and only a small percentage (~11%; 5/44 total cells captured in our analysis) displayed cytoplasmic PSF. These observations indicate that astrocytes are not the major glial cell type harboring cytoplasmic PSF in FTLD. Olig2 is normally located in the nucleus of some resting glial progenitors and the entire lineage of oligodendrocytes, but is not expressed in mature astrocytes [54]. In control brain, Olig2 and PSF localize in the nuclei of oligodendrocytes in discrete puncta (Fig. 9A, top panel). Of cells displaying prominent cytoplasmic PSF localization in FTLD cases, approximately 74% (31/42 cells captured in our analysis) co-localized with cytoplasmic Olig2 (Fig. 9A, bottom panel). Moreover, immunohistochemistry for PSF and Olig2 on adjacent sections revealed a morphologically consistent pattern of cytoplasmic subcellular redistribution (Fig. 9B). This analysis indicates that the aberrant cytoplasmic PSF accumulation in FTLD is predominantly observed in oligodendrocytes and can co-occur with cytoplasmic Olig2 in addition to nuclear Olig2 puncta in many of the same cells.

PSF and PTB levels are dynamic in the optic nerve during early myelin development

Oligodendrocytes are a type of glial cell, constituting approximately 50% of all cells around neurons in the frontal cortex and produce myelin in the white matter, which insulates and enhances the speed by which action potentials are propagated on axons [55]. Severe white matter changes often ensue after acute neurological damage (e.g. stroke), but are also a common feature in chronic progressive neurodegenerative diseases including FTLD [56–58]. Indeed, we noted that myelin basic protein (MBP) and myelin associated glycoprotein (MAG), observed in the brain specific proteome and quantified by SC, were significantly decreased ($p < 0.01$) in FTLD compared to control (Supplementary Table 4). Currently, the function of PSF in oligodendrocytes during normal myelin development is unclear. To assess whether PSF levels change during myelin development, we performed immunoblots for PSF in the mouse optic nerve (Fig. 7C). At birth, the entire optic nerve is unmyelinated and as mice age, the axons in the optic nerve become sheathed by the myelin produced by oligodendrocytes [59]. We observed a concomitant reduction of PSF and PTB levels after postnatal day 23 (p23) and more dramatically at p38, which correlated with an increase in 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP); a marker for mature myelin-producing oligodendrocytes. In contrast, TDP-43 levels remain largely unchanged, with only a slight reduction at p38 suggesting that both PSF and PTB act in oligodendrocytes and show a developmentally programmed decline during early myelination.

Discussion

Proteomic studies of human post-mortem brain tissue have helped shed light on pathogenic mechanisms of neurodegenerative disease [47]. In this study, we identified and validated several proteins that are selectively enriched in the FTLD detergent-insoluble proteome. Among these, the RNA-binding protein PSF was significantly enriched in both the full-length form and displayed significantly higher levels of truncated molecular weight fragments in FTLD compared to control cases. Immunohistochemical analysis indicated that PSF in control brain and FTLD cases was predominately nuclear and not associated with

phosphorylated TDP-43 inclusions in FTLD. However, in a subset of FTLD cases used in this study, cytoplasmic accumulation of PSF was observed in Olig2 immunoreactive oligodendrocytes, suggesting that the subcellular localization of PSF is altered in these cells in FTLD.

Understanding the biological consequences of alterations in detergent-insoluble proteins in the brain requires developing tools to quantify relative protein differences from individuals with neurodegenerative disease relative to healthy controls. In this study, we employed a SILAC based quantification method to assess relative changes across the control and FTLD detergent-insoluble proteome. Heavy labeled SILAC internal standards were utilized for the quantification of proteins with shared peptides in brain and HEK293 cells. The brain is a highly complex organ composed of glia and neurons that express discrete proteins. Therefore, the major limitation of SILAC internal standards for tissue quantification is that an individual cell line cultured *in vitro* inherently does not have the same intra- or extra-cellular protein expression as tissue. In this study, a bimodal distribution of $\log_2$ (Brain/SILAC) protein values was observed after quantitative analysis despite adding an equal amount of SILAC labeled HEK293 protein to the detergent-insoluble brain protein fractions (1:1 w/w). Proteins that were exclusively expressed or highly enriched in either brain or HEK293 cells could only be considered by the measurement of peptide signal-to-noise ratio, which reduces accurate quantitative measurement. A similar observation (i.e. a bimodal $\log_2$ protein distribution) was recently reported when an individual SILAC labeled tumor cell line serving as an internal standard was mixed equally with human tumor tissue [60]. In this case, the bimodal distribution was alleviated by mixing multiple SILAC labeled tumor cell lines into a single mixture, a method termed “super-SILAC”. Therefore, a mixture of SILAC labeled astrocytes, neurons, oligodendrocytes and microglia, at the same relative abundance found in brain, would potentially serve as an ideal brain-specific “super-SILAC” internal standard. Future studies incorporating a heavy labeled human neuroblastoma cell line (e.g. SH-SH5Y) would also likely aid in the quantification of neuron-specific proteins that were missed in this study employing HEK293 cells alone.

Despite the inherent limitations of HEK293 cells acting alone as a SILAC labeled internal standard in this study, the difference in $\log_2$ ratios between shared FTLD and control proteins was normally distributed and used to target proteins that were selectively enriched in FTLD. Proteins that shared common peptides between brain and the SILAC labeled HEK293 cells included CNDP2, TPI1, RHODGI- α , cofilin-1, cathepsin D and PSF, which were validated by immunoblotting. Immunohistochemical screening of TPI1, RHODGI- α , cofilin-1, cathepsin D and CNDP2 in several control and FTLD cases did not reveal any apparent differences (data not shown). However, this does not preclude deleterious consequences in the brain from the biochemical insolubility of these proteins or the potential utility of these proteins as biomarkers. For example, protein disulfide isomerase, which was significantly enriched in the FTLD sample, was recently shown to stain TDP-43 positive inclusions in ALS spinal cord [61].

Ubiquitinated and phosphorylated TDP-43 aggregates are the diagnostic hallmark of FTLD and although we did not observe specific pTDP-43 and PSF colocalization in neuronal inclusions we were intrigued that PSF accumulated in the cytoplasm of Olig2 immunoreactive oligodendrocytes in a subset of FTLD cases. PSF is a nuclear RNA-binding protein with roles in splicing and transcriptional regulation [62]. RNA-binding proteins interact with coding, untranslated and non-protein-coding RNAs in functional units called ribonucleoprotein (RNP) complexes [63, 64]. TDP-43, FUS and PSF are components of RNP complexes [65, 66], and may have roles in RNA homeostasis and transport [5, 67]. Recently, other RNA-binding proteins such as TAF15 and EWSR1 have also been implicated in neurodegenerative disease [68] and it has been proposed that the aggregation

of RRM containing RNA-binding proteins (TDP-43, FUS, TAF15 and EWSR1) is promoted by their shared prion-like domains. Consistently, PSF has a predicted prion-like domain (amino acids 41–104) and using an algorithm that ranks proteins based on prion-domain score PSF ranked 14th out of 209 RRM containing proteins and 79th out of all human proteins scored in a recent study [69]. Our observation that PSF accumulates in the cytoplasm of Olig2 immunoreactive glia in FTLD raises the question of whether oligodendrocyte-specific RNA processing is altered in neurodegenerative disease, and whether this occurs downstream of a dysfunction in protein folding specifically in oligodendrocytes in FTLD brain.

In healthy brain, Olig2 is a transcription factor located in the nucleus of resting glial progenitors and mature oligodendrocytes, but is not expressed in mature astrocytes [70]. In this study we used Olig2 as a marker for oligodendrocytes, but also observed Olig2 cytoplasmic colocalization with PSF in FTLD. Upon injury or neuroinflammation, Olig2 can be detected in the cytoplasm of cells that are activated [70–72]. Neuroinflammation, which coincides with severe white matter changes, ensues after acute neurological damage (e.g. stroke) and is also a common feature in neurodegenerative diseases [56, 57]. Thus, the nuclear to cytoplasmic mislocalization of PSF and Olig2 that we observed in FTLD may be representative of white matter degeneration. For example, one FTLD case with prominent PSF mislocalization (case 7) also had a tertiary neuropathological diagnosis of severe white matter degeneration. This suggests that accumulation of detergent-insoluble PSF may be associated with a specific subset of FTLD cases that exhibit severe white matter degeneration. In this study, we further showed that PSF exhibits a developmentally programmed decline during early myelin development (Fig. 7C), indicating that down-regulation of PSF in oligodendrocytes may be important for maintaining myelin levels in brain. Currently, the role of PSF in oligodendrocytes in neurodegeneration remains unclear, however, a recent study showed NG2 positive oligodendrocyte precursor cells exhibited enhanced proliferation and accelerated differentiation into oligodendrocytes in the spinal cord of a mouse ALS model [73]. Given the shared pathologies observed between FTLD and ALS future studies, aimed to assess the role of PSF in oligodendrocytes and NG2 oligodendrocyte precursor cells in human tissue and mouse models of ALS would seem to be warranted.

In summary, our data support the use of exogenous SILAC-labeled internal standards to quantify low abundance proteins in post-mortem human brain. The proteins identified in this study by this approach display a biochemical signature reflective of neurodegenerative processes such as protein misfolding, although they do not rule out an absolute increase in intrinsically detergent insoluble proteins. In this regard, our data highlight an emerging appreciation of the fact that even proteins shared with HEK293 cells, such as PSF, are capable of being altered in specific central nervous system cell types (i.e., oligodendrocytes) during neurodegeneration. It remains for future studies to unravel the likely complex mechanisms by which such proteins escape from normal processing, localization, and/or controlled expression in such specific cell types of the central nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALS	amyotrophic lateral sclerosis
FTLD	frontotemporal lobar degeneration
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
RRM	RNA recognition motif
PSF	polypyrimidine tract binding protein-associated splicing factor
SILAC	stable isotope labeling with amino acids in culture
TDP-43	HIV tat activating regulatory (TAR) DNA binding protein, 43 kDa
CNDP2	cytosolic non-specific dipeptidase isoform 2
TPI1	triosephosphate isomerase 1
RHOGDI-alpha	Rho GDP dissociation inhibitor (GDI) alpha
GFAP	glial fibrillary acidic protein
OLIG2	oligodendrocyte lineage transcription factor 2
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Figure 1. Proteomic Analysis of TDP-43 positive FTLN cases

(A) Immunohistochemical analysis of a control frontal cortex using a panTDP-43 antibody (upper panel) shows almost exclusively nuclear staining, whereas a phosphorylated TDP-43 specific (pTDP-43, Ser409/410) antibody detects cytoplasmic inclusions in FTLN frontal cortex (lower panel). Control cases do not stain positive for pTDP-43 (middle panel). Hematoxylin nuclear counterstain shown in blue. (B) Workflow for the SILAC quantitative proteomic approach in human brain tissue. IHC, immunohistochemistry.

Figure 2. Use of SILAC internal standards to quantify the detergent insoluble brain proteome (A) SDS-PAGE analysis of Brain/SILAC mixtures. Molecular weight marker (M; lane 1), whole cell lysate of SILAC labeled HEK293 cells (lane 2), FTLD brain insoluble-proteome alone (lane 3), FTLD/SILAC mixture (lane 4), control brain insoluble-proteome alone (lane 6), Control/SILAC mixture (lane 7). 20 μ g of protein loaded in each lane. The brain/SILAC samples (lanes 4 and 7) were, excised into gel slices based on MW (regions A-E), digested with trypsin, and analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) on a hybrid linear ion-trap/Orbitrap mass spectrometer. (B) The SILAC labeled peptides from the HEK293 cells are chemically identical to their native/unlabeled counterparts and serve as internal standards for the measurement of protein abundance across control and FTLD samples. (C) Representative base peak elution profiles (MW region A) of control:HEK293 and FTLD:HEK293 peptide mixtures, respectively.

Figure 3. Histogram analysis of the brain/SILAC mixed proteome

(A) Log₂ ratios (light/heavy) from a representative control brain/SILAC mixture (replicate 2) shows a bimodal distribution of proteins that can be fitted to two populations (HEK293 and brain). Upper panel is the residual associated with the fitted bimodal distribution using Igor software. (B-D) Full MS scans (top) and extracted ion-chromatograms (bottom) using a ± 20 ppm isolation window of brain and SILAC labeled HEK293 specific peptide ion pairs. In B, a SET specific peptide ion (m/z 608.818 corresponding to amino acid sequence VEVTEFEDIK) was observed exclusively in HEK293 cells (light peptide expected m/z 604.809 was at or below noise). In C, light/heavy peptide ion pair for UBA1 (m/z 955.009 (light) and 955.059 (heavy), respectively corresponding to amino acid sequence SLVASLAEPDFVVTDFAK) were almost equally intense in both brain and SILAC labeled HEK293 cells. In D, a MAP2 specific peptide ion (m/z 766.903 (light) corresponding to amino acid sequence SDTLQITDLGVSGAR) was observed exclusively in brain (heavy peptide expected m/z 771.910 was at or below noise). Red and blue circles represent heavy (SILAC labeled HEK293) and light peptides (brain), respectively. Triangles represent expected heavy (red) or light (blue) peptide signals.

Figure 4. The SILAC labeled internal standards increase precision of protein quantitation within a discrete range

(A) scatter plot of raw (brain/SILAC) protein ratios ($\log_2$ transformed) measured for one or more peptides in both technical replicate LC-MS/MS runs. This data includes protein ratios derived from both control/SILAC and FTLD/SILAC peptide mixtures. (B) The mean of the replicate measurements represented by x, y pairs in A were plotted (x-axis) versus the standard error of the pair (y-axis). Median standard error for bins of width 0.4 beginning at bin midpoint -3.8 and ending at midpoint 5.8 are overlaid as transparent bars.

Figure 5. Relative changes in the FTLN detergent-insoluble proteome
(A) MS spectra of detected peptide ion pairs (light and heavy) from control (upper panel) and FTLN (lower panel) detergent-insoluble samples. Presented are RHOGLI-alpha, triosephosphate isomerase 1 (TPI1), annexin I (ANXA1) and SAM68 (a negative control). White and black circles represent light (brain) and heavy (SILAC labeled HEK293 cells) peptide ion pairs, respectively. **(B)** Histogram analysis of the $\log_2$ difference (FTLN-control) for shared proteins in replicate two. **(C)** Immunoblots (IB) for protein targets (see Table 1) in individual control and FTLN cases are shown. Ponceau S staining of total protein is provided to show equal loading in all lanes.

Figure 6. Biochemical and immunohistochemical characterization of PSF in control and FTLN brain

(A) Detergent-insoluble fractions of frontal cortex samples from individual FTLN and control cases immunoblotted for PSF. Native (~100 kDa) and truncated PSF species (~25 kDa) are shown (arrows). Ponceau S staining of membrane is provided to show equal protein loading. (B) Quantification of native PSF (~100 kDa) by densitometry shows significant enrichment in FTLN cases ($p=0.035$). The line represent the mean value in control and FLTD. The four FTLN cases at or above the mean are labeled. (C) Immunohistochemical analysis for PSF in frontal cortex from a representative control and FTLN case (case 1 and 10, respectively). PSF expression was mostly nuclear in control and FTLN. PSF was observed accumulated in the cytoplasm in frontal cortex of a subset of FTLN cases (5, 7 and 10), and in these cases the stained cell morphology was consistent with glia. Lower panels represent zoomed magnification of boxed regions in upper panels. Scale bars = 20 μ M. (D) In neurons of the frontal cortex or hippocampus, PSF (red) was exclusively localized to the nucleus and did not colocalize with cytoplasmic pTDP-43 (green). Upper (frontal cortex) and lower (hippocampus dentate gyrus) panel images were captured on an Olympus BX51 florescent microscope (Scale bar = 20 μ M) and Zeiss LSM 510 confocal microscope (scale bar represents 10 μ M), respectively.

Figure 7. PSF is expressed in neurons, oligodendrocytes and astrocytes and shows developmentally programmed decline during myelin development

(A) Immunoblots for PSF, PTB and TDP-43 in primary cultured mouse neurons (Neu), astrocytes (Ast), oligodendrocytes (OL) and microglia (MG). Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1), GFAP and Olig2 were used as neuron, astrocyte and oligodendrocyte specific markers, respectively. (B) PSF, PTB and TDP-43 are expressed in human (HOG) and mouse (CG4) oligodendrocyte cell lines. (C) Optic nerve samples from mice at post-natal day 10, 23 and 38 were immunoblotted for PSF, PTB and TDP-43 in biological replicate. 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP) is an oligodendrocyte specific marker. Actin was used as a loading control in all immunoblots (A-C).

Figure 8. PSF is predominately localized to the nucleus of mature astrocytes and only rarely observed in the cytoplasm

Double-labeling immunofluorescence in free-floating sections in FTLD (case 7) frontal cortex for PSF (red) and GFAP (green) show PSF nuclear colocalization in the GFAP expressing astrocytes. (white arrows, left panel). Although the majority of cytoplasmic PSF does not co-localize with GFAP (center panel), a small percentage (~11 %) of GFAP positive astrocytes cells captured in our analysis did display some level of cytoplasmic PSF (right panel). All scale bars represent 50 μ M

Figure 9. PSF and Olig2 show a similar cytoplasmic distribution in oligodendrocytes in FTLD
(A) Double-labeling immunofluorescence staining in paraffin embedded sections (frontal cortex) for PSF (red) and Olig2 (green) in a control case 2 shows PSF and Olig2 nuclear localization in oligodendrocytes (top panel). In a subpopulation of oligodendrocytes in FTLD (case 10), PSF and Olig2 both display cytoplasmic colocalization (middle and bottom panel). Scale bar represents 25 μ M. **(B)** Immunohistochemistry in FTLD (case 10) on adjacent sections probed with either PSF or Olig2 antibodies showed cells with a comparable pattern of cytoplasmic distribution (scale bar represents 20 μ M).

Table 1

Proteins determined enriched in FTLD detergent insoluble proteome using SILAC internal standards

Reference	Gene Symbol	Description	Replicate 1 (R1)						Replicate 2 (R2)						R1				R2				Total SC (R1 + R2)				p value	Significant by SC
			Control			FTLD			Control			FTLD			Difference			Difference			FTLD			FTLD				
			log2 (L/H)	S.E.	N	log2 (L/H)	S.E.	N	log2 (L/H)	S.E.	N	log2 (L/H)	S.E.	N	log2 (L/H)	S.E.	N	Raw	S.E.S [†]	Norm	Raw	S.E.S [†]	Norm	Avg. Diff (R1+R2)	FTLD	Control		
			Cytosol/Metabolic																									
NP_004300.1	ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha (RHOGDI-alpha)	-0.81	0.06	2	1.2	0.13	3	-0.71	0.16	2	1.25	0.06	3	2.01	0.14	1.69	1.96	0.17	1.56	1.63	10	4	2.22E-01	No			
NP_478059.1	PSAT1	phosphoserine aminotransferase isoform 1	ND	ND	ND	2.38	0.30	3	0.19	0.06	6	2.95	0.14	7	2.38	ND	2.06	2.76	0.16	2.36	2.21	16	3	7.14E-03	Yes			
NP_000166.2	GPI	glucose phosphate isomerase	0.51	0.19	8	3.56	0.36	17	0.57	0.06	7	3.14	0.19	16	3.05	0.41	2.73	2.57	0.19	2.17	2.45	165	24	<2.2E-16 [*]	Yes			
NP_060705.1	CNDP2	CNDP dipeptidase 2	ND	ND	ND	3.26	0.32	5	1.3	0.18	2	3.69	0.29	10	3.26	ND	2.94	2.39	0.34	1.99	2.47	38	4	1.99E-07	Yes			
NP_001055.1	TKT	transketolase	-1.73	0.19	8	0.42	0.04	8	-1.91	0.09	10	0.33	0.05	17	2.15	0.20	1.83	2.24	0.10	1.84	1.84	40	3	1.04E-08	Yes			
NP_695012.1	UBA1	ubiquitin-activating enzyme E1	-0.29	0.14	17	1.65	0.17	17	0.01	0.17	17	2.01	0.19	15	1.94	0.22	1.62	2.00	0.26	1.60	1.61	138	68	1.49E-04	Yes			
NP_000356.1	TPH1	triophosphate isomerase 1	-1.39	0.08	4	0.58	0.14	9	-1.43	0.15	8	0.56	0.09	10	1.97	0.16	1.65	1.99	0.18	1.59	1.62	39	14	3.45E-03	Yes			
NP_001900.1	CTSD	cathepsin D preproprotein	-0.31	0.23	2	1.67	0.88	2	0.24	0.00	1	1.66	0.24	4	1.98	0.91	1.66	1.42	0.24	1.02	1.34	8	1	4.37E-02	No			
NP_002564.1	PAFAH1B3	platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit 29kDa	1.07	0.00	1	3.1	0.00	1	0.77	0.03	3	2.67	0.05	4	2.03	0.00	1.71	1.90	0.05	1.50	1.61	9	7	9.28E-01	No			
Cytoskeleton/Filaments																												
NP_005498.1	CFL1	cofilin 1	-0.17	0.06	4	1.85	0.04	3	-0.07	0.08	4	1.46	0.17	5	2.02	0.07	1.7	1.53	0.19	1.13	1.42	39	15	6.30E-03	Yes			
NP_075408.1	DCTN1	dynactin 1	1.43	0.57	3	2.57	0.85	2	1.94	0.05	4	3.88	0.29	9	1.14	1.02	0.82	1.94	0.30	1.54	1.18	15	10	6.04E-01	No			
Endoplasmic Reticulum/Chaperone																												
NP_004095.4	FASN	fatty acid synthase	-1.08	0.22	28	0.92	0.39	12	-1	0.24	35	0.60	0.25	29	2	0.45	1.68	1.60	0.35	1.20	1.44	60	50	9.85E-01	No			
NP_000933.1	PP1B	peptidylprolyl isomerase B	-3.19	0.25	4	-1.06	0.27	3	-3.34	0.20	9	-1.11	0.04	5	2.13	0.36	1.81	2.23	0.20	1.83	1.82	3	0	2.07E-01	No			
NP_004334.1	CALR	calreticulin	-2.33	0.26	4	-0.58	0.08	5	-2.55	0.09	8	-0.41	0.04	8	1.75	0.27	1.43	2.14	0.10	1.74	1.59	9	1	2.53E-02	No			
NP_005304.3	PDIA3	protein disulfide isomerase-associated 3	-1.15	0.13	6	0.42	0.11	8	-1.51	0.05	11	0.39	0.06	14	1.57	0.18	1.25	1.90	0.08	1.50	1.38	29	2	1.13E-06	Yes			
Mitochondria/Metabolic																												
NP_005521.1	IDH3A	isocitrate dehydrogenase 3 (NAD+)-alpha	1.44	0.08	4	3.48	0.17	11	1.28	0.07	9	2.72	0.21	11	2.04	0.19	1.72	1.44	0.22	1.04	1.38	80	27	6.98E-06	Yes			
NP_777280.1	IDH3B	isocitrate dehydrogenase 3, beta subunit isoform b	1.14	0.00	1	3.35	0.18	8	1.25	0.06	7	3.32	0.15	11	2.21	0.18	1.89	2.07	0.17	1.67	1.78	39	10	1.70E-04	Yes			
Nuclear/Splicing/Transcription																												

Reference	Gene Symbol	Description	Replicate 1 (R1)				Replicate 2 (R2)				R1		R2		Total SC (R1 + R2)										
			Control		FTLD		Control		FTLD		Difference		Difference		FTLD	Control	p value	Significant by SC							
			log2 (L/H)	S.E.	N	log2 (L/H)	S.E.	N	log2 (L/H)	S.E.	N	log2 (L/H)	S.E.	N					Raw	S.E.S [†]	Norm	Avg. Diff (R1+R2)			
NP_005057.1	SFPQ	polypyrimidine tract binding (PTB) protein associated splicing factor (PSF)	-1.97	0.17	8	-0.89	0.36	4	-2.36	0.23	7	-0.47	0.53	6	1.08	0.40	0.76	1.89	0.58	1.49	1.13	5	4	1.00E+00	No
Membrane/Transport																									
NP_000691.1	ANXA1	annexin I	-1.54	0.17	5	1.39	0.26	6	-1.84	0.06	9	1.22	0.02	8	2.93	0.31	2.61	3.06	0.06	2.66	2.64	26	6	1.35E-03	Yes
NP_000692.2	ATP1A1	Na ⁺ /K ⁺ -ATPase alpha 1 subunit isoform a	0.61	0.56	5	2.41	0.04	2	-0.24	0.49	12	1.64	0.30	8	1.8	0.57	1.48	1.88	0.58	1.48	1.48	13	16	2.74E-01	No

* p values are below the smallest floating point number in the statistical package R (2.2E-16).

† Standard error of sums

Table 2

Brain abundant proteins significantly enriched in FTLD detergent-insoluble proteome determined by spectral count (SC)

NCBI Reference	Gene Symbol	Protein	Spectral Counts (SC)			SC ratio		p value*
			% Coverage	TP	FTLD	Control	Control/FTLD	
NP_001814.2	CKB	brain creatine kinase	79	65	986	536	-0.615	< 2.2E-16*
NP_002046.1	GFAP	glial fibrillary acidic protein	73	78	1123	263	-1.827	< 2.2E-16*
NP_055581.2	SCRN1	secernin 1	21	13	59	1	-4.644	4.44E-16
NP_958780.1	PLEC	plectin 1	41	168	494	230	-0.836	3.00E-15
NP_066953.1	PPIA	peptidylprolyl isomerase A	36	10	42	0	-5.163	1.35E-12
NP_003156.1	STXBP1	synixin binding protein 1	60	68	597	335	-0.568	6.69E-10
NP_001377.1	DPYSL2	dihydropyrimidinase-like 2	57	32	208	84	-1.035	8.87E-10
NP_004172.2	UCHL1	ubiquitin carboxyl-terminal esterase L1	45	22	149	51	-1.265	1.06E-09
NP_008881.2	SYN1	synapsin I isoform Ia	64	37	260	118	-0.870	3.15E-09
NP_005264.2	GNB2	guanine nucleotide-binding protein, beta-2 subunit	25	12	125	40	-1.356	3.61E-09
NP_036226.1	PRDX5	peroxiredoxin 5 precursor, isoform a	37	7	35	3	-2.907	2.03E-07
NP_066268.1	GNAO1	guanine nucleotide binding protein, alpha activating polypeptide O isoform a	35	22	136	57	-0.977	2.23E-06
NP_002159.2	IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial precursor	39	23	216	110	-0.704	6.85E-06
NP_001748.1	CBR1	carbonyl reductase 1	60	16	111	47	-0.959	2.52E-05
NP_003119.2	SPTBN1	spectrin, beta, non-erythrocytic 1 isoform 1	52	127	514	324	-0.401	3.20E-05
NP_002620.1	PGAM1	phosphoglycerate mutase 1 (brain)	59	24	99	43	-0.921	1.19E-04
NP_006079.1	TUBB4B	tubulin, beta, 2	74	100	1510	1095	-0.200	2.34E-04
NP_003017.1	SH3GL2	SH3-domain GRB2-like 2	32	13	34	9	-1.544	6.08E-04

* p values are below the smallest floating point number in the statistical package R (2.2E-16).