Supplemental Material

Truncated TDP-43 proteoforms diagnostic of frontotemporal dementia with TDP-43 pathology

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1 Supplemental methods

1.1 Materials

The following materials were obtained from the indicated commercial sources: ammonium persulfate [A3678], bicinchoninic acid protein assay kit [BCA1-1KT], Calcium chloride [10043-52-4], dithiothreitol (DTT) [D9779], ethanol [362808], ethylenediaminetetraacetic acid (EDTA) [E4884], formic acid [399388], iodoacetamide [I5161], N-lauroylsarcosine (sarkosyl) [61745], N,N,N0,N0-tetramethylethylenediamine [T9281], sodium chloride (NaCl) [S7653], sodium dodecyl sulfate (SDS) [L3771], Tween 20 [P1379] and urea [U5378] were obtained from Sigma-Aldrich (Canada). Acetonitrile (ACN) [BDH83640], acrylamide/bis-acrylamide solution [J63279], ammonium hydrogen carbonate (AHC) [A18566], bovine serum albumin (BSA) [0332], Coomassie G250 [0615], Eppendorf 1.5 mL Protein LoBind tubes [022431081], and Laemmli SDS sample buffer [J61337], Roche protease inhibitors [4693159001], tris [0826], and tris buffered saline (TBS) [97063-680] were obtained from VWR (Canada). Tosyl phenylalanyl chloromethyl ketone-treated (TPCK) trypsin [LS003744] was obtained from Worthington (USA). Clarity Max ECL substrate [1705062], filter paper [09-802-1A], and nitrocellulose membrane [1620115] were obtained from Bio-Rad. Chymotrypsin, TLCK-treated, mass spectrometry grade [90056], C18 extraction disks [2215] C18 silica [711025-100], methanol [A456-4], and molecular weight protein ladder [26616] were obtained from Fisher Scientific (Canada). Anti-phosphorylated-TDP-43 rat monoclonal antibody clone 1D3 [829901] was obtained from Biolegend (USA). Horseradish peroxidase (HRP)-conjugated rabbit anti-rat IgG antibody [ab-6734] was obtained from Abcam (Canada). Oasis MCX uElution plates [186001830BA] were purchased from Waters (Canada). Heavy labelled peptides $TDP_{252-263}$ [arginine ^{13}C and ^{15}N-labeled, synthesized by New England Peptide] and $TDP_{276-293}$ lysine ^{13}C and ^{15}N-labeled, synthesized by Vivitide].

Equipment used in this analysis included centrifugal vacuum (Vacufuge plus, Eppendorf), gel imager (G:BOX Chemi XRQ, Syngene, USA), microvolume spectrophotometry (ND-8000, NanoDrop Technologies, USA), and ultrasonication probe (FB120110, Fisher Scientific). For discovery proteomics experiments, a nanoflow LC-quadrupole high resolution time of flight mass spectrometer (Easy nano LC 1000 LC, ThermoFisher Scientific; Bruker Impact II QTOF, Canada) was used. For biomarker verification experiments, a µLC-triple quadrupole mass spectrometer (SCIEX M5 µLC and QTRAP 6500, Canada), with an in-line 5 µm C18, 20 × 0.3 mm trap column (Luna, Phenomenex, USA), and 2.6 µm XB-C18, 50 × 0.3 mm microflow analytical column (Kintex, Phenomenex) were used.

1.2 Sample preparation

Brain tissue was homogenized using a pestle for 2 min in tris-EDTA (TE) buffer (10 mM tris-HCL and 1 mM EDTA, pH 7.5, and protease inhibitor cocktail) containing 10% sucrose, 0.8 M NaCl, and 2% Tween-20 (5 mL buffer/g tissue), incubated for 30 min at 24°C, and centrifuged at 100,000 x g for 30 min at 20°C; the remaining pellet was homogenized (following the same steps as above) in TE buffer containing 1% sarkosyl. The sarkosyl-insoluble pellet (i.e., the pathological TDP-43 fraction) was reconstituted in urea buffer (50 mM tris, pH 8.5, 8 M urea and 2% SDS), ultrasonicated (five 1 s pulses), and stored at -70°C before further use.

The brain tissue fraction enriched for TDP-43 pathology, both from individual brain tissue samples and pools (i.e., FTLD type A, B and C, FTLD-tau, AD, and unaffected controls), was normalized

by protein concentration using the bicinchoninic assay as per the manufacturer's instructions, then reduced (30 min incubation at 37°C with 1 µg DTT) and alkylated (20 min incubation with 5 µg of iodoacetamide in the dark). Subsequently, samples were separated via electrophoresis using 10% polyacrylamide gels under denaturing conditions, in duplicate, with one gel transferred to a nitrocellulose membrane for Western blot analysis, as previously described.[1,2]

For gel electrophoresis, 40 µg for individual cases or 100 µg for pooled cases of total protein were loaded on the gel. Gels were fixed for 1 h (40% ethanol; 10% acetic acid); stained overnight (0.12% Coomassie G250, 10% ammonium sulphate, 10% phosphoric acid, 20% methanol); and de-stained in water overnight prior to imaging.

To prepare gel pieces for digestion, sequential incubations were performed at room temperature in 50% 25 mM AHC, 50% ACN, until gel pieces were transparent (overnight), and then the gel pieces were dehydrated in 100% ACN for 20 min. Gel pieces were then rehydrated in 50 mM AHC, digested overnight at 37°C using either trypsin or chymotrypsin. For the analysis of pooled samples, digestion was performed using 1 µg of TCPK-chymotrypsin (2 mM calcium chloride and 100 mM tris-HCl, pH 8) or 1 µg of TPCK-trypsin. For analysis of individual cases, 1 µg of TPCK-trypsin was used. Digestion was halted by adding 50 µL of 1% formic acid. The supernatant was removed and saved for analysis. Peptides were extracted by incubating for 20 min (with shaking at 1000 rpm) in 0.5% formic acid, then 40% ACN containing 0.1% formic acid, and then 100% ACN. Supernatants were combined and dried by vacuum centrifugation. Dried peptide extracts were reconstituted in 0.5% formic acid and desalted using C18 tips (prepared and used as previously described).[3] Eluates for each sample were collected in a single tube and dried by vacuum centrifugation.

1.3 High resolution mass spectrometry

The following search parameters were used; semi-specific enzymatic cutting, maximum of 1 missed cleavage site, variable modifications of deamidation (+0.984016 @ N, Q), acetylation (@ protein N-term), and oxidation (+15.994915 @ M), and a mass tolerance of 20 ppm. Peptide FDR was calculated manually by sorting the data by the |log prob| parameter and calculating the percent of reverse-hits.

Quantitative analyses of TDP-43 proteoform concentration were based on the peptide $TDP_{276-293}$, as this was the most frequently observed peptide and of the commonly observed TDP-43 peptides had the highest signal intensity. For total truncated TDP-43 concentration, mid- and low-TDP peptide concentrations were combined via logistic regression. Proteoform correlations were done through comparing $TDP_{276-293}$ peptide concentrations; an outlier for the mid:low-TDP comparison was removed as it was 10-fold higher than all other data-points, limiting the interpretation of these data.

To rule-out in-source fragmentation of peptides with non-tryptic/chymotryptic cleavage sites, peptide retention time was compared between the fully-tryptic or fully-chymotryptic peptides and the respective non-fully-tryptic/chymotryptic peptide. If the fully-tryptic/chymotryptic and non-fully-tryptic/chymotryptic peptides have a similar retention time then in-source fragmentation is suspected as both peptides would have had to have similar structures to migrate similarly through the analytical column. If retention times are different, then in-source fragmentation is ruled-out. Peptides with one non-tryptic/chymotryptic cleavage site were further vetted by investigating the

literature for previous reports and enzymes that could create this TDP-43 cleavage site. Peptides with non-tryptic/chymotryptic cleavage sites with which in-source fragmentation could be ruled out and experimental evidence shows human enzymes could cleave at the respective site are referred to herein as "confident" *in vivo* proteolytic fragments, whereas sites with less evidence are referred to "potential" *in vivo* proteolytic cleavage sites.

1.4 Targeted mass spectrometry

1.4.1 Method development

Mass spectrometer parameters were optimized using tryptic digests of recombinant TDP-43 in buffer. Collision energy (CE) and declustering potential (DP) were optimized for each transition. The observed m/z of peptide precursor and product ions were verified using Skyline. Chromatography conditions were as follows; mobile phases A and B consisted of 0.1% formic acid in nano-pure grade water and 0.1% formic acid in LC-MS grade acetonitrile, respectively. A flow rate of 0.4 µL/min and an analytical column temperature of 40 °C was used with the following gradient: ramp from 5–13% B from 0 to 1 min to 15% B by 3 min, to 30% B by 3.5 min, to 32% B by 5.5 min, to 45% B by 6 min, to 90% by 8.9 min, and to 5% from 9 to 11 min for re-equilibration.

1.4.2 Peptide quantification

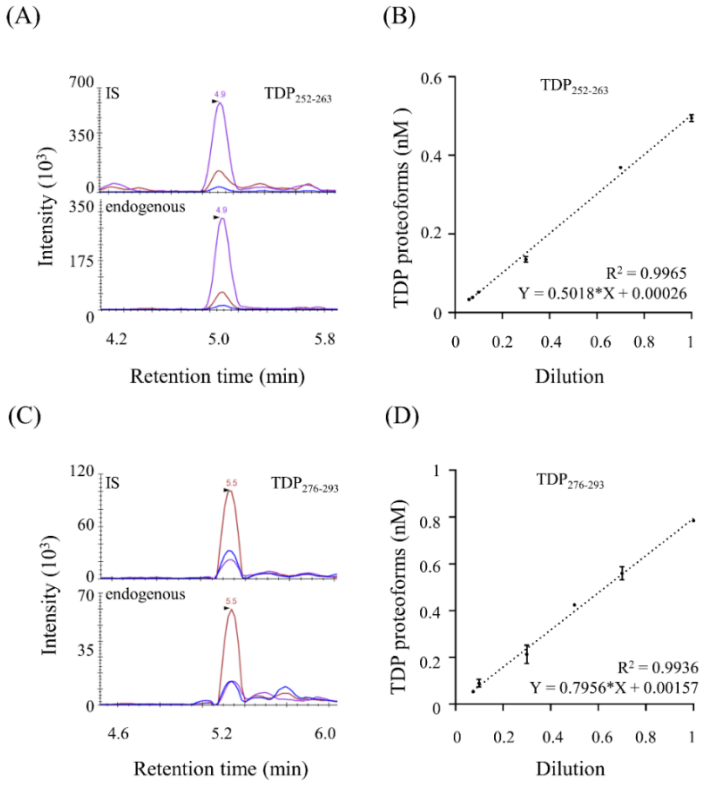
Quantification was performed via single-point calibration using a heavy-isotope labeled synthetic version of $TDP_{252–263}$ and $TDP_{276–293}$ as the IS; lyophilized IS peptides were solubilized with deionized water, and 5% ACN + 0.1% FA, respectively. IS was then aliquoted and stored at − 80 °C. The IS was spiked into each sample post-digestion to a final concentration of 0.464 nmol/L $TDP_{252–263}$ and 1.38 nmol/L $TDP_{276–293}$. Quantification of each monitored TDP-43 peptide was performed using single-point calibration of the respective IS peptide. Pooled human FTLD-TDP brain tissue spiked with IS was used for quality control (QC) material.

1.4.3 Method validation

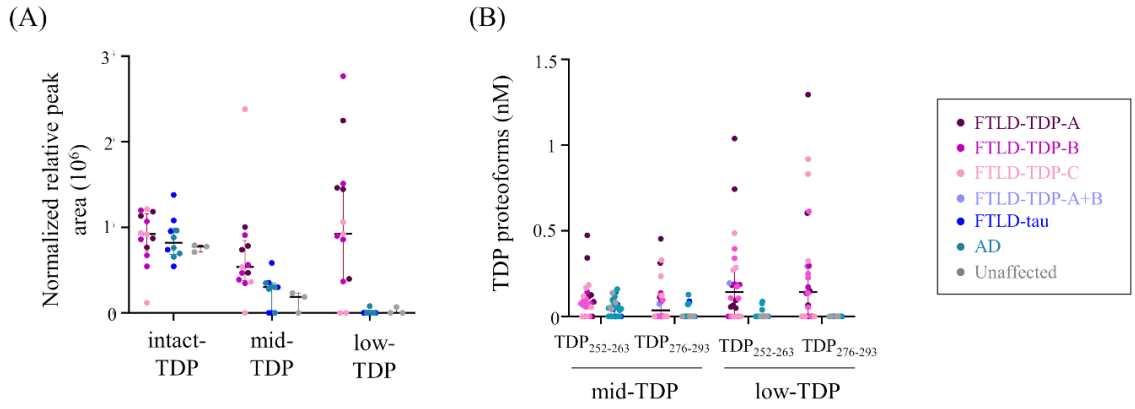
The Clinical and Laboratory Standards Institute Liquid Chromatography-Mass Spectrometry Methods C62-A guideline was used as a guide for the method validation experiments.[4] Precision studies were performed by doing triplicate measurements of the QC, repeated on three days. Imprecision was shown as % coefficient of variation (CV). The criteria for the lower limit of the measuring interval (LLMI) was a S/N ratio ≥ 3 for the observed quantifier ion and a transition peak area ratio (PAR) within 15% of the expected peak area ratio (ePAR), where the ePAR was determined from previous analysis of TDP-43 standards.[1,2] S/N was calculated as peak-to-peak within a 0.2 min retention time window of the signal peak. Linearity was assessed via a mixing study using brain tissue homogenate pools with high and low FTLD-TDP TDP-43 concentration (**Supplemental Figure 1**).

Imprecision was less than 10% for all concentrations tested for both peptides in both inter- and intra-assay comparisons (**Table 3**). This assay was linear from 0.033-0.494 nM for $TDP_{252-263}$ and 0.053-0.633 nM for $TDP_{276-293}$ with a lower limit of measuring interval of 0.033 nM (±0.002) and 0.053 nM (±0.003), respectively (**Supplemental Figure 1**).

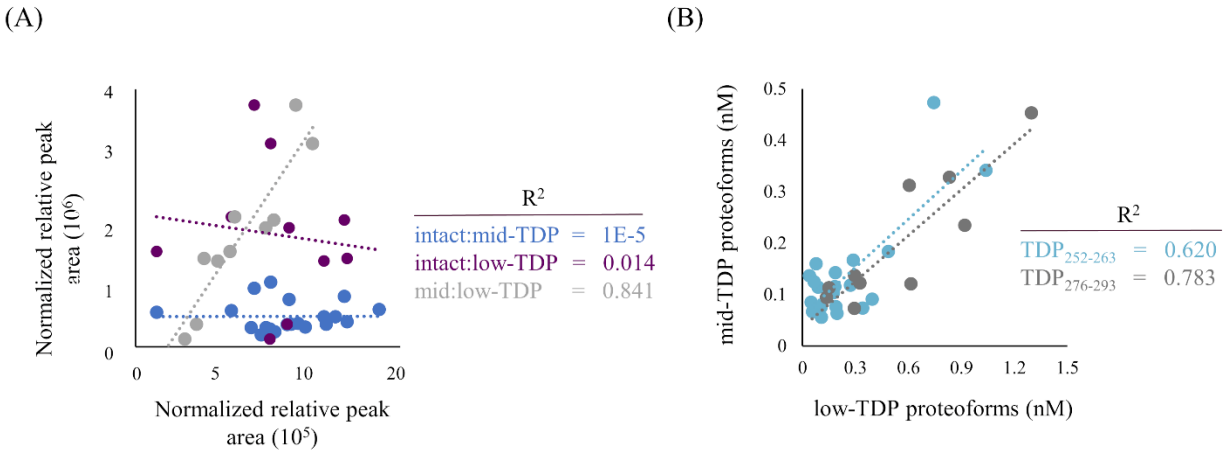
2 Supplemental figures



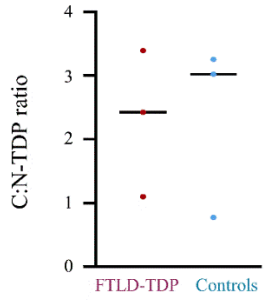
Supplemental Figure 1: Representative chromatograms and linearity of our targeted MRM method for absolute quantification of TDP-43 proteoforms. (A & C) Representative chromatograms from the analysis of human brain tissue with internal standards (IS). (B & D) Linearity of (B) $TDP_{252-263}$ and (D) $TDP_{276-293}$, mean and standard deviation shown.



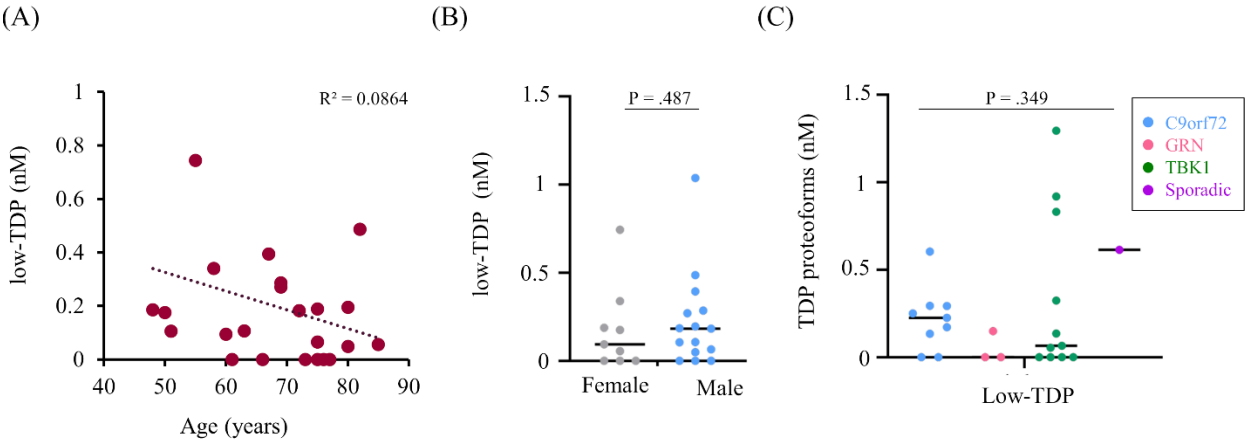
Supplemental Figure 2: TDP-43 proteoform concentration by neuropathological designation including FTLD-TDP subtype from the (A) the discovery (high resolution mass spectrometry) and (B) verification (targeted MRM) experiments demonstrating no clear trends by subtype.



Supplemental Figure 3: The concentration of truncated proteoforms did correlate with each other but not the concentration of intact TDP-43 as demonstrated in the regression analysis of data from (A) the discovery (high resolution mass spectrometry) and (B) verification (targeted MRM) experiments.



Supplemental Figure 4: The ratio of C-to-N-terminal TDP-43 peptides did not discriminate cases with and without TDP-43 pathology (data from analysis of pooled FTLD-TDP and control tissues).



Supplemental Figure 5: Investigations of the correlation/effects of low-TDP-43 concentration with (A) age at death, (B) sex, and (C) genetic status.

3 Supplemental tables

Supplemental Table 1: Characterization of cases analyzed in the discovery and verification phases.

Case	Sample type	Sex	Age at death	Genetic variant	IHC in the frontal cortex					TDP-43 in hippocampus	Discovery study		Verification study
					TDP	Ubiq.	pTau	Aβ	α-synuclein		pools	individual	
1	FTLD-TDP-A	M	67	GRN	+	+	-	-	+	+	✓	✓	-
2	FTLD-TDP-A	M	72	GRN	+	+	-	-	-	+	✓	✓	✓
3	FTLD-TDP-A	M	60	no	+	+	-	-	-	+	✓	✓	✓
4	FTLD-TDP-A	F	85	GRN	+	+	-	+	-	+	✓	✓	✓
5	FTLD-TDP-B	F	50	C9orf72	+	+	-	-	-	+	✓	✓	✓
6	FTLD-TDP-B	M	77	no	+	+	-	-	-	+	✓	✓	✓
7	FTLD-TDP-B	M	63	no	+	+	-	-	-	+	✓	✓	✓
8	FTLD-TDP-B	M	51	no	+	+	-	-	-	+	✓	✓	✓
9	FTLD-TDP-B	F	58	C9orf72	+	+	-	-	-	+	✓	✓	✓
10	FTLD-TDP-C	M	73	no	+	+	-	-	-	+	✓	✓	✓
11	FTLD-TDP-C	M	82	no	+	+	-	-	-	+	✓	✓	✓
12	FTLD-TDP-C	F	76	no	+	+	-	+	-	+	✓	✓	✓
13	FTLD-TDP-C	M	69	no	+	+	-	-	-	+	✓	✓	✓
19	CBD	M	53	na	−	+	+	-	-	-	✓	✓	✓
20	CBD	M	75	na	−	+	+	-	-	-	✓	✓	-
21	PiD	F	66	na	−	+	+	-	-	-	✓	✓	✓
22	PSP	F	61	na	−	+	"	-	-	-	✓	✓	✓
23	PSP	M	81	na	−	+	+	+	-	+	✓	✓	✓
14	AD	M	88	na	−	+	+	+	-	-	✓	✓	✓
15	AD	F	70	na	−	+	+	+	-	-	✓	✓	✓
16	AD	M	62	na	−	+	+	+	-	-	✓	✓	✓
17	AD	F	84	na	−	+	+	+	-	-	✓	✓	✓
18	AD	F	75	na	−	+	+	+	-	-	✓	✓	✓
24	non-neurological	F	62	na	−	-	-	-	-	-	✓	✓	✓
25	non-neurological	F	91	na	−	-	-	-	-	-	✓	✓	✓
26	non-neurological	M	70	na	−	-	-	-	-	-	✓	✓	✓
27	FTLD-TDP-A	M	80	no	+	+	+	+	-	+	✓	-	✓

28	FTLD-TDP-C	F	61	no	+	+	-	-	-	+	✓	-	✓
29	non-neurological	F	73	na	−	+	-	+	-	-	✓	-	✓
30	FTLD-TDP-A	F	60	GRN	+	+	-	-	-	+	-	-	✓
31	FTLD-TDP-A	F	55	C9orf72	+	+	-	-	-	+	-	-	✓
32	FTLD-TDP-A+B	M	48	C9orf72	+	+	-	-	-	+	-	-	✓
33	FTLD-TDP-A	M	75	C9orf72	+	+	-	-	-	+	-	-	✓
34	FTLD-TDP-A + mod AD	M	80	C9orf72	+	+	-	+	-	+	-	-	✓
35	FTLD-TDP-A+B	M	77	C9orf72	+	+	mild	+	-	+	-	-	✓
36	mild FTLD-TDP-B	F	66	C9orf72	+	+	-	-	-	+	-	-	✓
37	FTLD-TDP-B	M	67	TBK1	+	+	-	+	-	+	-	-	✓
38	FTLD-TDP-A+B	F	75	C9orf72	+	+	+	+	-	+	-	-	✓
39	FTLD-TDP-C	M	69	no	+	+	-	-	-	+	-	-	✓
40	PSP	F	59	na	-	+	+	+	-	-	-	-	✓
41	PSP	M	75	na	-	+	+	-	-	-	-	-	✓
42	AD	F	79	na	-	+	+	+	-	-	-	-	✓
43	AD + CVD	F	79	na	-	+	+	+	-	-	-	-	✓
44	AD + DLB	M	82	na	-	+	+	+	+	-	-	-	✓
45	LBD, moderate AD	F	75	na	-	+	+	+	+	-	-	-	✓
46	PSP	M	84	na	-	+	+	-	-	-	-	-	✓
47	AD + DLB	M	92	na	-	+	+	+	+	-	-	-	✓
48	AD	M	65	na	-	+	+	+	-	-	-	-	✓
49	AD	F	73	na	-	+	+	+	-	-	-	-	✓
50	AD	M	79	na	-	+	+	+	-	-	-	-	✓
51	PSP + DLB	M	74	na	-	+	+	-	+	-	-	-	✓
52	AD + DLB + TDP	M	82	na	-	+	+	+	+	+	-	-	✓
53	AD + DLB + TDP	F	72	na	-	+	+	+	+	+	-	-	✓

Supplemental Table 2: Transitions monitored in the quantitative MRM method.

Peptide	Q1 ion (m/z)	Q1 charge state	Q3 ion (m/z)	Fragment ion	DP (V)	CE (V)
$TDP_{252-263}$	417.90	3+	541.29	y_{10}	80	18
			448.24	y_8	80	25
$TDP_{252-263}$	420.57	3+	545.29	y_{10}	80	18
			452.24	y_8	80	23
$TDP_{276-293}$	863.89	2+	676.31	y_{14}	60	42
			993.45	y_{10}	60	45
$TDP_{276-293}$	868.89	2+	681.32	y_{14}	60	42
			1003.46	y_{10}	60	45

Supplemental Table 3: Upper and lower limit of the measuring interval (ULMI and LLMI, respectively) and precision of the quantitative MRM method.

Peptide	Point	Concentration (nM)	CV (%)
$TDP_{252-263}$	ULMI	0.49	1.77
	LLMI	0.04	3.34
$TDP_{276-293}$	ULMI	0.78	0.60
	LLMI	0.05	9.10

Supplemental Table 4: TDP-43 peptides identified in published discovery proteomics studies that investigated human central nervous system tissue.

TDP-43 residues	C or N term*	TDP-43 specific peptide	ISF[1]	known enzyme cleavage‡		MW gel fraction	Maximal predicted MW¶	Gel fraction matches predicted MW§	Disease type	ALS and/or FTLD specific#	Ref.
				predicted	empirical						
208-nd	C	✓	nd	✓	✗	22	21-40	✓	FTLD-U	nd	[5]
276-291	N	✓	nd	✗	✓	39-43	33-155	✓	FTLD-U	✓	[6]
230-256	N	✓	nd	✓	✗	25-26	29-141	✓	ALS	nd	[7]
257-276	C	✓	nd	✓	✗	25-26	16-18	✗	ALS, AD	✗	[7]
175-187	C	✓	nd	✗	✗	Not reported	25-79	N/A	ALS, AD	✗	[7]
219-227	C	✓	nd	✓	✓	23	20-39	✓	FTLD-U	nd	[8]
247-251	C	✗	nd	✓	✗	23	17-19	✓	FTLD-U	nd	[8]

* *In vivo* cleavage site (i.e., non-tryptic/chymotryptic site) on the N- or C-terminus of the peptide

[1] In source fragmentation (ISF) ruled out (✓) or in (✗) using retention time differences and relative alterations in hydrophobicity

‡ Predicted or empirical evidence of human enzymatic cleavage site based on Expasy data and previously published experiments, respectively

¶ Peptide maximal molecular weight (without – with maximal predicted PTMs) and including empirical evidence

§ Molecular weight of the protein sequence based on the in vivo truncation site is consistent with the gel electrophoresis band region from which the peptide was found, including consideration of other additive potential post-translational modifications (e.g., phosphorylation)

Compared to relevant controls and found only in FTLD-TDP or ALS cases

4 References

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