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ORIGINAL PAPER

Phosphorylation of S409/410 of TDP-43 is a consistent feature in all sporadic and familial forms of TDP-43 proteinopathies

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Abstract Accumulation of hyperphosphorylated, ubiquitinated and N-terminally truncated TAR DNA-binding protein (TDP-43) is the pathological hallmark lesion in most familial and sporadic forms of FTLN-U and ALS, which can be subsumed as TDP-43 proteinopathies. In order to get more insight into the role of abnormal phosphorylation in the disease process, the identification of specific phosphorylation sites and the generation of phosphorylation-specific antibodies are mandatory. Here, we developed and charac-

terized novel rat monoclonal antibodies (1D3 and 7A9) raised against phosphorylated S409/410 of TDP-43. These antibodies were used to study the presence of S409/410 phosphorylation by immunohistochemistry and biochemical analysis in a large series of 64 FTLN-U cases with or without motor neuron disease including familial cases with mutations in progranulin ($n = 5$), valosin-containing protein ($n = 4$) and linkage to chromosome 9p ($n = 4$), 18 ALS cases as well as other neurodegenerative diseases with concomitant TDP-43 pathology ($n = 5$). Our data demonstrate that phosphorylation of S409/410 of TDP-43 is a highly consistent feature in pathologic inclusions in the whole spectrum of sporadic and familial forms of TDP-43 proteinopathies. Physiological nuclear TDP-43 was not detectable with these mAbs by immunohistochemistry and by immunoblot analyses. While the accumulation of phosphorylated C-terminal fragments was a robust finding in the cortical brain regions of FTLN-U and ALS, usually being much more abundant than the phosphorylated full-length TDP-43 band, spinal cord samples revealed a predominance of full-length TDP-43 over C-terminal fragments. This argues for a distinct TDP-43 species composition in inclusions in cortical versus spinal cord cells. Overall, these mAbs are powerful tools for the highly specific detection of disease-associated abnormal TDP-43 species and will be extremely useful for the neuropathological routine diagnostics of TDP-43 proteinopathies and for the investigation of emerging cellular and animal models for TDP-43 proteinopathies.

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Introduction

After its initial identification in 2006 [28], numerous studies have now confirmed that abnormal neuronal and glial inclusions composed of the TAR DNA-binding protein 43

(TDP-43) are the neuropathological hallmark lesions in several neurodegenerative disorders, which can be subsumed under the term TDP-43 proteinopathies [20, 26]. This includes sporadic frontotemporal lobar degeneration with tau-negative and ubiquitin-positive inclusions (FTLD-U) with and without motor neuron disease (MND), familial forms of FTLD-U with mutations in the progranulin gene (GRN), valosin-containing protein (VCP) and linkage to chromosome 9p, as well as most forms of amyotrophic lateral sclerosis (ALS) with the exception of familial ALS with SOD-1 mutations [7, 9, 10, 23, 28]. In addition, concomitant TDP-43 pathology is present in a subset of other neurodegenerative diseases, such as Alzheimer's disease (AD) [1, 35], corticobasal degeneration (CBD) [35], Lewy-body dementia (LBD) [25] and parkinsonism-dementia complex of Guam [12, 14].

TDP-43 is a highly conserved 414-amino acid nuclear protein first cloned as a protein capable of binding to the transactive response DNA element of human immunodeficiency virus type 1 and later identified as part of a complex involved in splicing of the cystic fibrosis transmembrane conductance regulator gene [5, 30]. TDP-43 contains two RNA recognition motifs and a glycine-rich C-terminal region. Described functions include involvement in transcription regulation, exon skipping and a role as scaffold for nuclear bodies through an interaction with survival motor neuron protein [4, 39]. Pathological TDP-43 is abnormally phosphorylated, ubiquitinated, and cleaved to generate carboxy-terminal fragments (CTFs) in affected brain regions in TDP-43 proteinopathies [28]. Additionally, while TDP-43 is predominantly found in the nucleus under physiologic conditions, the nuclear staining is dramatically reduced in inclusion bearing cells [28] leading to the hypothesis that alterations of the nuclear import of TDP-43 might contribute to disease pathogenesis. A direct link between TDP-43 dysfunction and neurodegeneration was further provided recently by the identification of several mutations in the *TARDBP* gene encoding for TDP-43 in ALS [13, 18, 19, 32, 34, 36]. However, the underlying mechanisms leading to TDP-43 accumulation and the consequences of *TARDBP* mutations are still unclear. Due to the fact that TDP-43 is hyperphosphorylated in disease process and that several *TARDBP* mutations introduce either new potential phosphorylation sites or are predicted to increase phosphorylation of adjacent serine residues, alterations in phosphorylation/dephosphorylation of TDP-43 might play a crucial role in the pathogenesis of TDP-43 proteinopathies. Therefore, further insight of TDP-43 phosphorylation in physiological and disease conditions is essential and might help to elucidate the pathologic process in TDP-proteinopathies. TDP-43 has 41 serine, 15 threonine and 8 tyrosine residues, which might act as potential phosphorylation sites. To identify sites actually phosphorylated in TDP-43 we started to

generate antibodies raised against different phosphopeptides of TDP-43. While this work was in preparation, two publications using a similar approach have demonstrated that TDP-43 becomes abnormally phosphorylated at S379, 403, 404, 409, and 410 in small numbers of cases of sporadic FTLD-U, familial FTLD-U with *GRN* mutation and ALS [15, 17].

In this study, we describe the generation and characterization of novel rat monoclonal antibodies (mAbs) raised against phosphorylated S409/410 (pS409/410) of TDP-43, which allowed the highly sensitive and specific labeling of disease-associated TDP-43 species, but not physiologic TDP-43 by immunohistochemistry and immunoblot analysis. We extend our investigation of pS409/410 to a much larger series of cases covering the whole spectrum of TDP-43 proteinopathies ($n = 87$), including sporadic FTLD-U, familial FTLD-U with mutations in *GRN*, *VCP* and linkage to chromosome 9, ALS and tauopathies with concomitant TDP-43 pathology. Our findings demonstrate that phosphorylation of S409/410 of TDP-43 is a highly consistent feature of abnormal inclusions in the whole spectrum of TDP-43 proteinopathies and that these novel mAbs are powerful tools for the routine diagnostics of TDP-43 proteinopathies and for further analysis of cell-culture and animal models for TDP-43 proteinopathies.

Materials and methods

Generation of monoclonal antibodies

The phosphopeptide pS409/410 (SMDSKS(p)S(p)GWG), corresponding to amino acid residues 404–413 of human TDP-43 and phosphorylation of serine residues 409/410, was synthesised and coupled to bovine serum albumin or ovalbumin (OVA) by cysteine linkage at the amino-terminus (Peptide Specialty Laboratories GmbH, Heidelberg, Germany). Lou/c rats were immunized with subcutaneously and intraperitoneally with a mixture of 50 µg OVA-coupled pS409/410 peptide, 5 nmol CpG 2006 oligonucleotide (Tib Molbiol, Berlin, Germany), 500 µl PBS and 500 µl incomplete Freund's adjuvance. After a 6 weeks interval, rats were boosted with 50 µg OVA-coupled pS409/410 peptide in PBS. Hyperimmune spleen cells were fused with the mouse myeloma cell line P3X63Ag8.653 using standard procedures. Supernatants were first screened in a differential enzyme-linked immunosorbent assay (ELISA) with the phospho- and corresponding non-phosphopeptide to select for phosphorylation-specific mAbs. Identified and further characterized phosphospecific mAbs clone 1D3 and clone 7A9 are both rat IgG2a. To investigate whether both phosphorylation sites were necessary for antibody binding of 1D3 and 7A9, additional phosphopeptides with either pS409

(SMDSKS(p)SGWG) or pS410 (SMDSKSS(p)GWG) were synthesized, coupled to OVA and analyzed by ELISA.

Phosphopeptides for other potentially phosphorylated serine residues (S91/92, 183, 242, 254, 266, and 273) of TDP-43 used for antibody generation are listed in supplementary Table 1. These sites were chosen either due to published data as for pS91/92 [29] or due to a high predictive value for being phosphorylated by NetPhos2 search (<http://www.cbs.dtu.dk/services/NetPhos>). While phospho-specific supernatants were identified by ELISA assay for these peptides, none of them revealed any specific immunoreactivity by subsequent immunohistochemistry and immunoblot analysis of FTLD-U brains. However, these negative data do not necessarily indicate that these sites are not phosphorylated in TDP-43.

Case selection

The following cases from the Center for Neuropathology and Prion Research (Munich, Germany), Center for Neurodegenerative Disease Research (Philadelphia, USA) and Department of Neuropathology (Aalborg, Denmark) were included in the study: (1) familial FTLD-U with linkage to chrom 9p ($n = 4$), *GRN* ($n = 5$), and *VCP* ($n = 4$) mutations; (2) FTLD-U either sporadic or familial with unknown genetic defect (subtype 1 ($n = 14$); subtype 2 ($n = 19$); subtype 3 ($n = 18$) according to [33]); (3) ALS ($n = 18$); (4) Tauopathies with concomitant TDP-43 pathology [AD + TDP-43 ($n = 3$); CBD + TDP-43 ($n = 2$)]. Cases of FTLD-U had a clinical diagnosis of the FTD spectrum (behavioural variant of FTD, progressive non-fluent aphasia, semantic dementia) with or without concomitant MND. Cases of ALS had clinical signs of MND with some of them developing cognitive changes only late in disease process. In addition, healthy controls ($n = 5$), AD without TDP-43 pathology ($n = 5$), CBD without TDP-43 ($n = 4$) and TDP-43 negative FTLD-U cases ($n = 9$) [31] were included. Demographic, clinical and neuropathological data are summarized in Table 1.

Immunohistochemistry

Immunohistochemistry was performed with mAB 1D3 and 7A9 as well as phosphorylation-independent polyclonal TDP-43 (ProteinTech Group, Chicago, IL, USA; dilution 1:2000) and polyclonal C-tTDP-43 raised against amino acids 394–414 of human TDP-43 [16].

Tissue was either formalin-fixed with fixation-times ranging from 1 day up to 4 years or ethanol-fixed (1 day) and paraffin-embedded. Antigen retrieval was performed by boiling the sections in 10 mmol/L citrate buffer (pH 6.0) in a microwave oven. Immunohistochemistry was performed using biotinylated secondary antibodies and the avidin-biotin

complex detection system (Vector Laboratories, Burlingame, CA, USA) with 3,3'-diaminobenzidine as chromogen. Double-labeling immunofluorescence was performed using Alexa Fluor 488 and 594 conjugated secondary antibodies (anti-rat IgG and anti-rabbit IgG, Molecular Probes, Eugene, OR, USA). 4'-6-diamidino-2-phenylindol (DAPI) (Vector Laboratories, Burlingame, CA, USA) was used for nuclear counterstaining.

Biochemical fractionation and immunoblot analysis

Frozen brain tissue from different TDP-43 proteinopathies ($n = 66$) was used for the sequential extraction of proteins with buffers of increasing stringency, as described [28]. Briefly, gray matter was extracted at 5 mL/g (v/w) with low-salt buffer (10 mmol/L Tris, pH 7.5, 5 mmol/L EDTA, 1 mmol/L dithiothreitol, 10% sucrose, and a cocktail of protease inhibitors), high-salt-Triton X buffer (low-salt buffer + 1% Triton X-100 + 0.5 mol/L NaCl), myelin flotation buffer (Triton X buffer containing 30% sucrose), and Sarkosyl buffer (low-salt buffer + 1% *N*-lauroyl-sarcosine + 0.5 mol/L NaCl). The detergent-insoluble material was then extracted in 0.25 mL/g of urea buffer (7 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 30 mmol/L Tris, pH 8.5).

Where indicated, TDP-43 was dephosphorylated by dialysis (50 mmol/L Tris and 0.2 mmol/L EDTA, pH 8.0) and treated with *Escherichia coli* alkaline phosphatase (Sigma-Aldrich, St. Lois, MO) for 2 h at 56°C. For immunoblot analysis, equal volumes of urea fractions were resolved by either 10 or 15% Tris-glycine SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and probed with C-tTDP-43 and novel mAbs. Primary antibodies were either detected with alkaline phosphatase-conjugated anti-rabbit or anti-rat IgG (Dako), developed with CPD star (Roche Molecular Biochemicals, Mannheim, Germany) and visualized using the Chemolumineszenz Imager CHEMOCAM HR 16 (Intas, Goettingen, Germany) or with horseradish peroxidase-conjugated anti-rat or anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA), developed with Renaissance Enhanced Luminol Reagents (NEN Life Science Product, Inc., Boston, MA, USA), and visualized using a Fujifilm Intelligent Dark-box II (Fuji Systems USA, Stamford, CT, USA).

Results

Characterization of novel mAbs

ELISA data using serial dilutions of synthetic phospho- and non-phosphorylated peptides corresponding to amino acids

Table 1 Demographic, clinical, genetic and neuropathological data of cases

Case no.	Diagnosis	Disease duration	Age at death	Sex	Dementia (Y/N)	MND (Y/N)	Family history (Y/N)	Mutation/linkage
Familial FTLD-U with known genetic defect								
1	FTLD-U 2	2	61	m	N	Y	Y	chrom 9p
2	FTLD-U 2	3	66	f	N	Y	Y	chrom 9p
3	FTLD-U 2	2	74	m	Y	Y	Y	chrom 9p
4	FTLD-U 2	2	41	f	Y	Y	Y	chrom 9p
5	FTLD-U 3	8	69	f	Y	N	Y	GRN p.Q337X
6	FTLD-U 3	5	78	f	Y	N	Y	GRN p.W304X
7	FTLD-U 3	4	62	f	Y	N	Y	GRN p.R418X
8	FTLD-U 3	8	65	m	Y	N	Y	GRN p.R493X
9	FTLD-U 3	3	68	f	Y	N	NA	GRN p.R418X
10	FTLD-U 3	7	76	f	Y	N	Y	GRN p.A237TfsX5
11	FTLD-U 4	8	70	f	Y	N	Y	VCP R155C
12	FTLD-U 4	5	56	f	Y	N	Y	VCP R155H
13	FTLD-U 4	6	60	f	Y	N	Y	VCP R155H
14	FTLD-U 4	8	60	f	Y	N	Y	VCP R155H
Sporadic FTLD-U and familial FTLD-U with unknown genetic defect								
15	FTLD-U 1	8	73	m	Y	N	N	–
16	FTLD-U 1	NA	52	f	Y	N	N	–
17	FTLD-U 1	10	73	m	Y	N	N	–
18	FTLD-U 1	2	66	f	Y	N	N	–
19	FTLD-U 1	8	58	m	Y	N	N	–
20	FTLD-U 1	2	60	m	Y	N	N	–
21	FTLD-U 1	28	70	m	Y	N	N	–
22	FTLD-U 1	5	63	f	Y	N	NA	–
23	FTLD-U 1	7	54	m	Y	N	N	–
24	FTLD-U 1	3	92	m	Y	N	N	–
25	FTLD-U 1	12	77	m	Y	N	N	–
26	FTLD-U 1	5	69	f	Y	N	N	–
27	FTLD-U 1	NA	77	f	Y	N	N	–
28	FTLD-U 1	6	68	f	Y	N	N	–
29	FTLD-U 2	7	57	f	Y	Y	Y	–
30	FTLD-U 2	NA	72	m	Y	N	N	–
31	FTLD-U 2	1	59	m	Y	N	N	–
32	FTLD-U 2	3	42	f	Y	Y	N	–
33	FTLD-U 2	2	71	m	Y	N	N	–
34	FTLD-U 2	>3	51	m	Y	N	N	–
35	FTLD-U 2	9	49	m	Y	Y	N	–
36	FTLD-U 2	2	67	m	Y	Y	N	–
37	FTLD-U 2	NA	44	m	Y	Y	N	–
38	FTLD-U 2	2	47	f	Y	N	N	–
39	FTLD-U 2	3	57	m	Y	N	N	–
40	FTLD-U 2	6	41	m	Y	N	Y	–
41	FTLD-U 2	1	46	m	Y	N	Y	–
42	FTLD-U 2	10	76	m	Y	N	N	–
43	FTLD-U 2	13	76	f	Y	Y	Y	–
44	FTLD-U 2	7	54	f	Y	N	Y	–

Table 1 continued

Case no.	Diagnosis	Disease duration	Age at death	Sex	Dementia (Y/N)	MND (Y/N)	Family history (Y/N)	Mutation/linkage
45	FTLD-U 2	3	57	m	Y	Y	Y	–
46	FTLD-U 2	4	61	f	Y	N	Y	–
47	FTLD-U 2	10	67	m	Y	Y	Y	–
48	FTLD-U 3	NA	78	m	Y	N	Y	–
49	FTLD-U 3	2	60	f	Y	N	N	–
50	FTLD-U 3	2	37	m	Y	Y	N	–
51	FTLD-U 3	1	65	m	Y	Y	N	–
52	FTLD-U 3	3	58	f	Y	N	N	–
53	FTLD-U 3	3	64	m	Y	N	Y	–
54	FTLD-U 3	4	60	f	Y	N	Y	–
55	FTLD-U 3	3	72	m	Y	N	N	–
56	FTLD-U 3	3	49	f	Y	Y	N	–
57	FTLD-U 3	3	75	f	Y	N	N	–
58	FTLD-U 3	2	48	m	Y	Y	N	–
59	FTLD-U 3	2	53	f	Y	Y	N	–
60	FTLD-U 3	3	53	m	Y	Y	NA	–
61	FTLD-U 3	3	72	f	Y	N	N	–
62	FTLD-U 3	10	77	f	Y	N	NA	–
63	FTLD-U 3	4	63	f	Y	N	NA	–
64	FTLD-U 3	6	71	m	Y	N	NA	–
Amyotrophic lateral sclerosis								
65	ALS	2	81	f	N	Y	N	–
66	ALS	1	77	f	N	Y	N	–
67	ALS	2	51	m	N	Y	N	–
68	ALS	1–2	63	m	Y (mental retardation)	Y	N	–
69	ALS	3	68	m	N	Y	N	–
70	ALS	2	67	m	N	Y	N	–
71	ALS	1	76	f	N	Y	N	–
72	ALS	5	60	m	Y (late in disease course)	Y	N	–
73	ALS	2	68	m	N	Y	N	–
74	ALS	2	54	m	N	Y	N	–
75	ALS	2	56	f	N	Y	N	–
76	ALS	2	61	m	N	Y	N	–
77	ALS	1	55	f	Y	Y	N	–
78	ALS	2	51	m	N	Y	N	–
79	ALS	2	63	m	N	Y	N	–
80	ALS	1.5	51	f	Y	Y	N	–
81	ALS	1	65	m	Y	Y	N	–
82	ALS	5	66	f	N	Y	N	–
Other neurodegenerative disorders with concomitant TDP-43 pathology								
83	AD + neocortical TDP	15	81	f	Y	N	N	–
84	AD + neocortical TDP	4	80	f	Y	N	N	–
85	AD + limbic TDP	NA	93	f	Y	N	N	–
86	CBD + TDP-43	6	56	m	Y	N	N	–
87	CBD + TDP-43	3	61	f	Y	N	N	–

Table 1 continued

Case no.	Diagnosis	Disease duration	Age at death	Sex	Dementia (Y/N)	MND (Y/N)	Family history (Y/N)	Mutation/linkage
Neurodegenerative disorders without TDP-43 pathology								
88	aFTLD-U	5	33	m	Y	N	N	–
89	aFTLD-U	5	46	m	Y	N	N	–
90	aFTLD-U	NA	49	m	Y	N	N	–
91	aFTLD-U	9	48	f	Y	N	N	–
92	aFTLD-U	5	40	f	Y	N	N	–
93	aFTLD-U	4	50	f	Y	N	N	–
94	aFTLD-U	4	59	m	Y	N	N	–
95	aFTLD-U	15	54	f	Y	N	N	–
96	aFTLD-U	5	41	m	Y	N	N	–
97	AD	3	61	f	Y	N	N	–
98	AD	14	86	f	Y	N	N	–
99	AD	14	76	m	Y	N	N	–
100	AD/DLB	11	83	f	Y	N	N	–
101	AD	13	81	m	Y	N	N	–
102	AD	5	56	f	Y	N	N	–
103	CBD	3	76	f	Y	N	N	–
104	CBD	3	59	m	Y	N	N	–

FTLD-U Frontotemporal lobar degeneration with ubiquitin-positive inclusions, *ALS* amyotrophic lateral sclerosis, *aFTLD-U* atypical FTLD-U, *AD* Alzheimer's disease, *CBD* corticobasal degeneration, *m* male, *f* female, *NA* not available

404–413 of human TDP-43 revealed that mAbs 1D3 and 7A9 reacted strongly with the peptide phosphorylated at both serine residues 409 and 410 (pS409/410), but not with the non-phosphorylated peptide. A weaker signal was obtained for phosphopeptides with phosphorylation of either S409 (pS409) or 410 (pS410), suggesting that mAbs 1D3 and 7A9 have the highest binding affinity if both serine residues are phosphorylated.

As a next step, we performed immunoblot and immunohistochemical analysis on a selected FTLD-U case (#21, Table 1) to further characterize these mAbs and to determine their applicability and usefulness in these assays. Using the polyclonal phosphorylation-independent antibody C-tTDP-43 raised against amino acids 394–414 of human TDP-43 [16], sarcosyl-insoluble protein fractions isolated from FTLD-U brains shows a highly characteristic biochemical pattern in immunoblots with additional bands ~25 kDa, 45 kDa and a high molecular smear in addition to the physiological TDP-43 ~43 kDa (Fig. 1b), as described previously [28]. In contrast, mAbs 1D3 and 7A9 revealed a strong labeling of the abnormal TDP-43 species in the sarcosyl-insoluble protein fractions with no detection of the physiological TDP-43 band ~43 kDa (Fig. 1b). The phospho-specificity of the novel mAbs was further confirmed by immunoblot analysis of protein samples being dephosphorylated by alkaline phosphatase treatment, which

completely abolished the immunoreactivity of 1D3 and 7A9 (Fig. 1c). No labeling of TDP-43 was detectable in RIPA and urea fractions isolated from cell cultures or mouse brains with mAbs 1D3 and 7A9 (data not shown).

mAbs 1D3 and 7A9 strongly labeled pathological TDP-43 inclusions in paraffin-embedded tissue as demonstrated in the dentate granule cells of an FTLD-U case in Fig. 1d. In contrast to the phosphorylation-independent antibody C-tTDP-43, no physiological nuclear staining of non-inclusion bearing cells was detectable with 1D3 and 7A9 (Fig. 1d).

In combination with the immunoblot data, these data demonstrate that these antibodies are highly specific tools for the detection of abnormal pathological TDP-43 species and that phosphorylation of S409/410 is not present in a significant amount in the physiological state of TDP-43.

Immunohistochemistry of novel mAbs in wide spectrum of sporadic and familial forms of TDP-43 proteinopathies

Since the initial characterization of 1D3 and 7A9 by immunoblot and IHC showed similar results, detailed analyses of large numbers of TDP-43 proteinopathies were performed with mAb 1D3.

1D3 labels all types of abnormal inclusions previously described with phosphorylation-independent

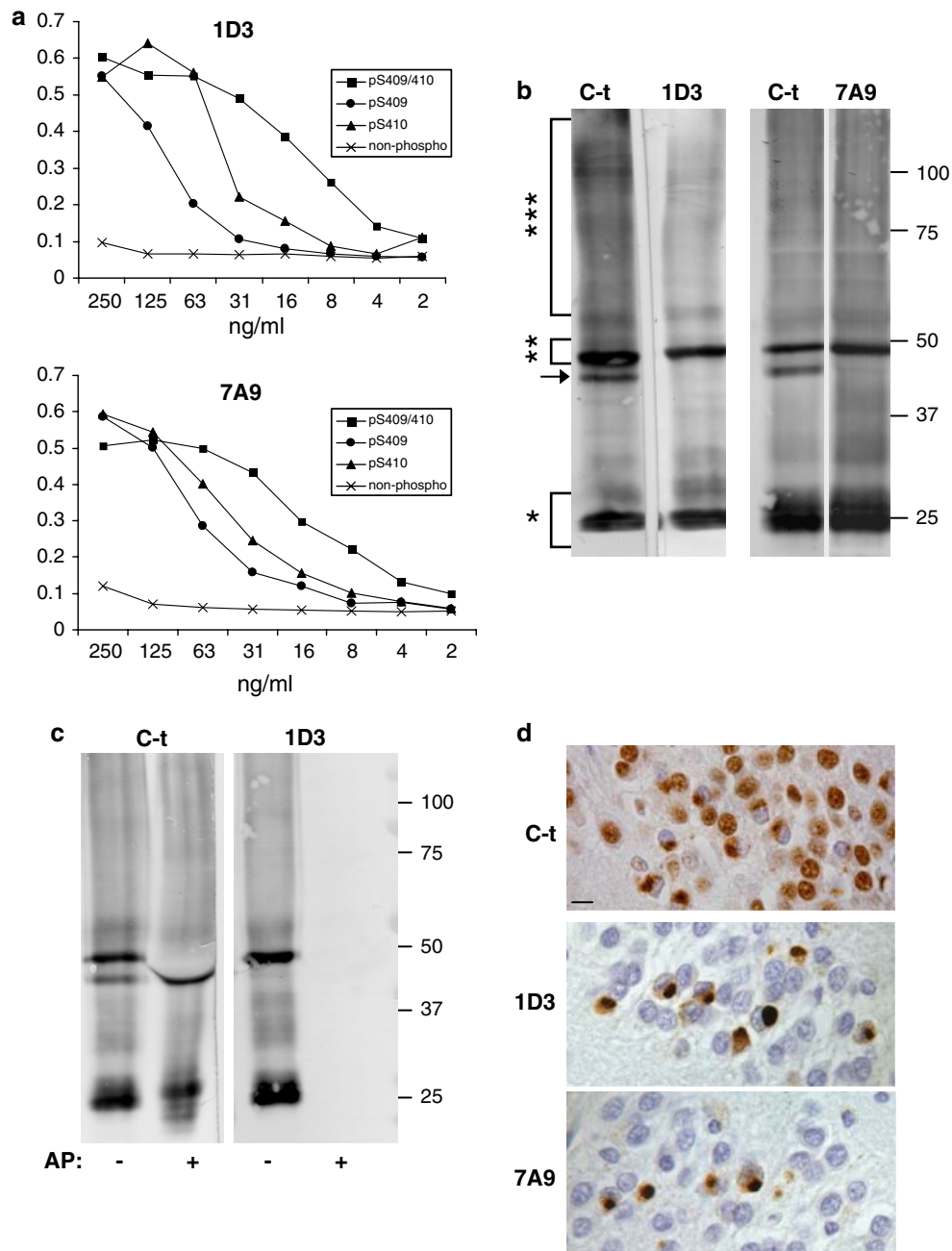


Fig. 1 Characterization of novel mAbs 1D3 and 7A9. **a** ELISA performed with serial dilutions of OVA-coupled phospho- and non-phosphopeptides corresponding to amino acids 404–413 of human TDP-43 (range 2–250 ng/ml) demonstrating the high specificity of mAbs 1D3 and 7A9 for the detection of peptides phosphorylated at S409/410. **b** Immunoblot analysis of urea fraction from FTLD-U brain separated by 10% SDS-PAGE with phosphorylation-independent polyclonal C-tTDP-43 antibody raised amino acids 394–414 of human TDP-43 and phosphorylation specific mAb 1D3. While C-tTDP-43 shows the physiological TDP-43 band ~43 kD (arrow) in addition to the pathologic TDP-43 species detected as ~25 kDa (*), 45 kDa (**), and high

molecular smear (***), mAb 1D3 and 7A9 specifically label only the abnormal TDP-43 species. **c** Immunoblot analysis of urea samples from FTLD-U dephosphorylated by alkaline phosphatase (AP) treatment show a complete lack of immunoreactivity confirming the specificity of mAb 1D3 against phosphorylated TDP-43 species. **d** Immunohistochemistry of hippocampus from FTLD-U brain shows numerous cytoplasmic inclusions as well as nuclear staining of non-inclusion bearing cells with C-tTDP-43 antibody. In contrast, mAb 1D3 and 7A9 only reveal the pathologic inclusions but do not show any nuclear immunoreactivity. Scale bar corresponds to 10 μ m

TDP-43 antibodies, such as dystrophic neurites (DNs), neuronal cytoplasmic inclusions (NCI), neuronal intranuclear inclusions (NII) and glial cytoplasmic inclusions

(GCI). Figure 2 shows representative images of 1D3 immunoreactivity in the investigated subgroups of TDP-43 proteinopathies, with strong labeling of long DN in FTLD-U

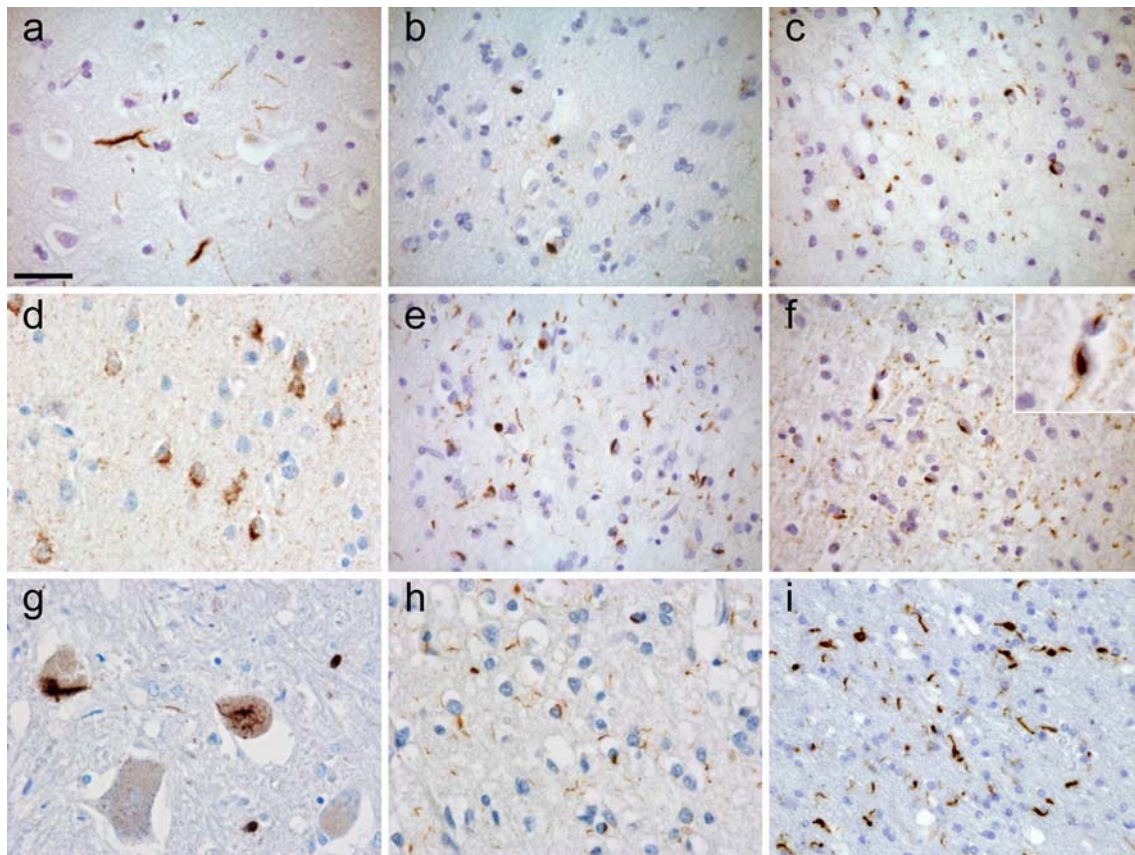


Fig. 2 TDP-43 is phosphorylated at S409/410 in the whole spectrum of sporadic and familial TDP-43 proteinopathies. Representative images from abnormal inclusions in the distinct subgroups of TDP-43 proteinopathies stained with 1D3 are shown. **a** sporadic FTL-D subtype 1: long neuritic profiles, **b** sporadic FTL-D subtype 2: cytoplasmic inclusions, **c** sporadic FTL-D subtype 3: cytoplasmic inclusions and small neuritic profiles, **d** familial FTL-D linked to chromosome

subtype 1 (Fig. 2a), NCIs in FTL-D subtype 2 (Fig. 2b), and NCIs, short DNs and NIIs in FTL-D subtype 3 (Fig. 2c). Chrom 9p linked FTL-D showed numerous “pre-inclusions” (Fig. 2d) in addition to compact NCIs. In *GRN* mutation cases, there was strong labeling of NCIs, short DNs and NIIs (Fig. 2e) and numerous NIIs and DNs were detectable in *VCP* mutation cases (Fig. 2f), while in ALS spinal cord motor neurons showed 1D3- positive round and skein-like inclusions (Fig. 2g). TDP-43 pathology can also occur in combination with other neurodegenerative diseases, such as AD and CBD, and, as demonstrated in Fig. 2h, i, the TDP-43 pathology in AD and CBD was also strongly labeled with 1D3. No staining for 1D3 was observed in healthy controls and TDP-43 negative FTL-D cases.

Finally, double-labeling immunofluorescence for 1D3 and phosphorylation-independent TDP-43 showed a complete overlap of labeling of pathologic inclusions (Fig. 3a–c), including the more granular, cytoplasmic “pre-inclusions” (Fig. 3d–f). While compact neuronal inclusions were consistently double-labeled by 1D3 and anti-ubiquitin

(Fig. 3g–i), the majority of “pre-inclusions” were only detectable with 1D3 and not by anti-ubiquitin (Fig. 3j–l). Overall, these data indicate that abnormal phosphorylation of S409/410 is present in inclusions in all familial and sporadic forms of TDP-proteinopathies and that hyperphosphorylation at S409/410 precedes ubiquitination.

Biochemical analysis of phosphorylated TDP-43 in wide spectrum of TDP-43 proteinopathies

To characterize the abnormal S409/410 phosphorylation of TDP-43 in more detail biochemically, immunoblots were performed on samples extracted from frozen brain tissue of cases from the distinct TDP-43 subgroups. A consistent finding in urea fractions extracted from frontal cortex of sporadic and familial FTL-D cases was the presence of abnormal TDP-43 species detectable with mAb 1D3 as a high molecular smear, a band ~45 kDa, and up to 4 bands ~20–25 kDa labeled as 1–4 (Fig. 4a). Since previous reports on small numbers of FTL-D cases claimed that

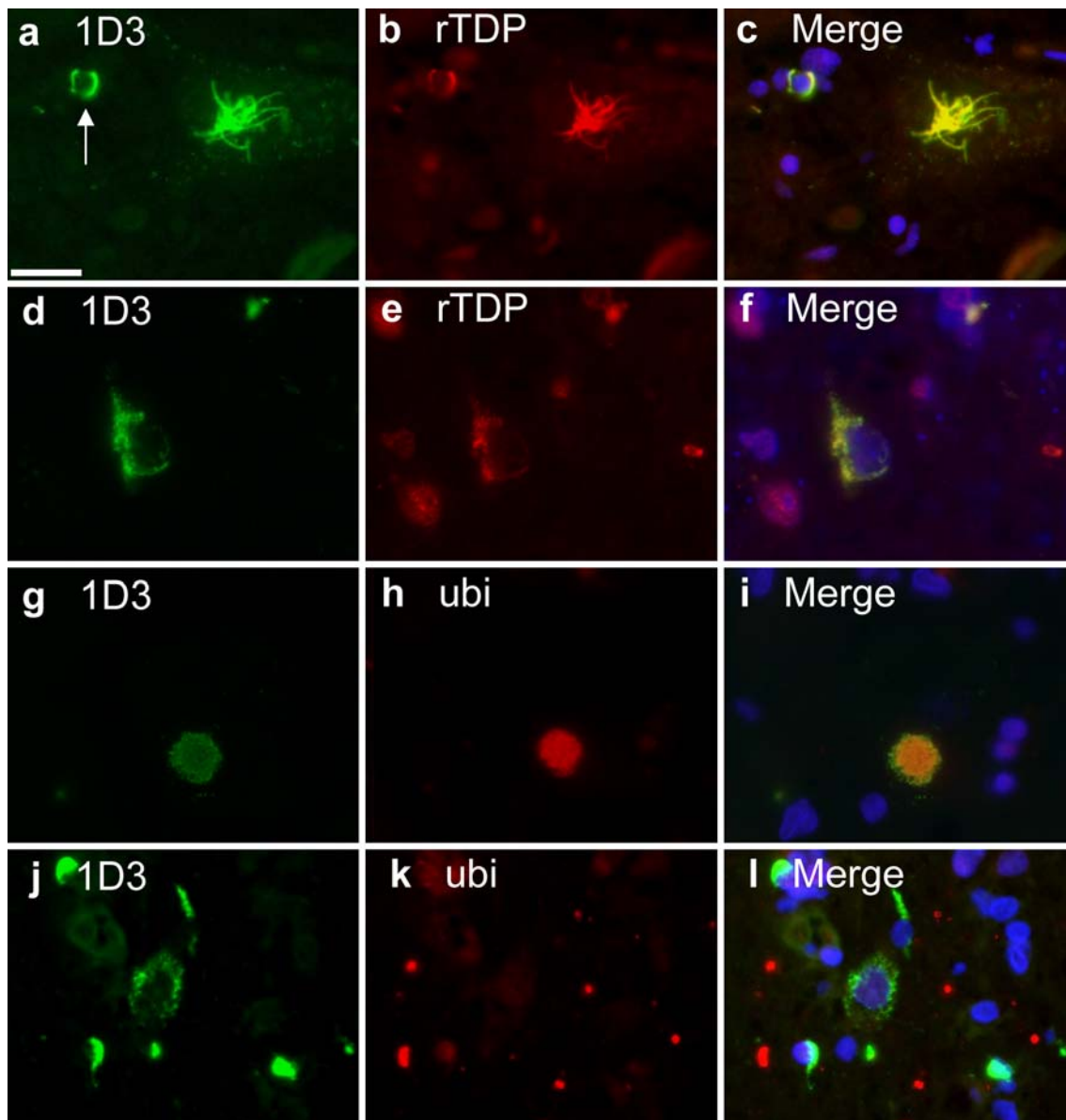


Fig. 3 Double-label immunofluorescence. Sections from spinal cord (**a–c**, **g–i**) and motor cortex (**d–f**, **j–l**) from an ALS case were either double-labeled for mAb 1D3 (**a**, **d**) and phosphorylation-independent rTDP (**b**, **e**) or 1D3 (**g**, **j**) and ubiquitin (**h**, **k**). Merged images are shown in **c**, **f**, **i** and **l**. 1D3 and rTDP-43 show a complete overlap of staining of pathologic inclusions, such as skein-like inclusion in motor neuron (**a–c**), glial cytoplasmic inclusions (*arrow* in **a–c**) as well as

more granular, diffuse cytoplasmic inclusions (“pre-inclusions”) in the motor cortex (**d–f**). Note the absence of nuclear staining with 1D3. In contrast with compact neuronal inclusions which are consistently double-labeled by 1D3 and anti-ubiquitin (**g–i**), 1D3-positive “pre-inclusions” are negative with anti-ubiquitin (**j–k**). Scale bar corresponds to 20 μ m

distinct patterns of C-terminal fragments (CTF) correlate with FTLD-U subtypes and clinical phenotypes [15, 17], we performed a detailed analysis of the distinct C-terminal fragments. The CTFs labeled as 1 and 2 in Fig. 4 were the most prominent bands present in all sporadic and familial FTLD-U cases although with variable amounts from case to case. While there was some similarity with previously described CTF patterns [15, 17], we were not able to delineate a distinct pattern of CTFs correlating with either histo-

logical, genetic or clinico-pathological (e.g. presence of MND) FTLD-U subtypes.

In ALS cases, the amount of pathological TDP-43 species detectable in motor cortex and spinal cord with 1D3 by immunoblot (Fig. 4b) was more variable and sometimes hard to detect most likely due to the lower number of inclusions in ALS compared to FTLD-U. While most protein samples extracted from the motor cortex of ALS showed a similar banding pattern as seen in FTLD-U, the spinal cord

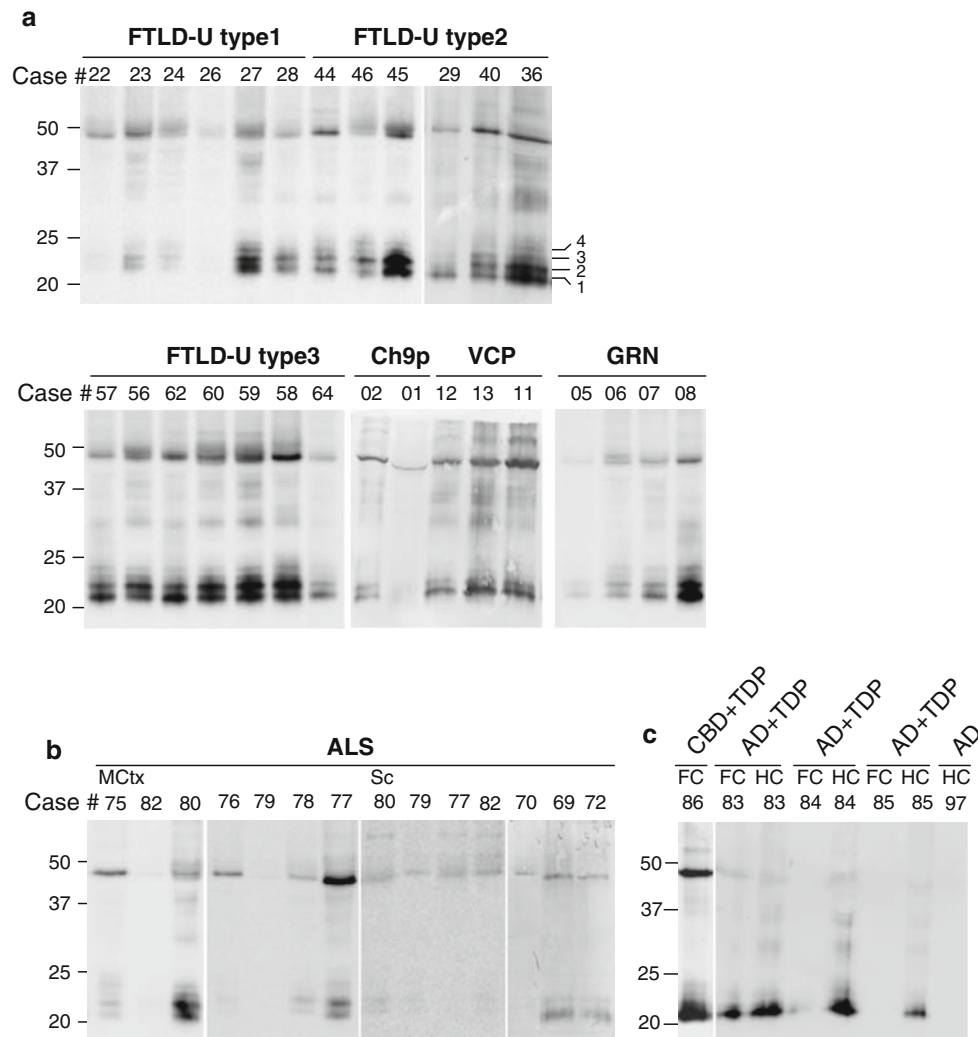


Fig. 4 Biochemical analysis of S409/410 phosphorylated TDP-43. **a** Sarcosyl-insoluble, urea-soluble protein fractions extracted from frontal cortex from distinct FTLD-U subgroups were separated by 15% SDS-PAGE and immunoblotted with mAb 1D3. The detection of C-terminal fragments (up to 4 distinct bands labeled as 1–4) between 20 and 25 kDa, phosphorylated full-length TDP-43 band (~45 kDa) and a high molecular smear was present in all tested sporadic and familial forms of FTLD-U. However, no clear correlation between distinct banding patterns of C-terminal fragments and FTLD-U subtypes

samples lacked the presence of C-terminal fragments in >50% of examined cases and only presented with a phosphorylated full-length TDP-43 band. This difference in phosphorylated full-length (FL) versus CTFs was further documented by quantitative analysis of band intensities of CTFs and FL TDP-43 and calculation of CTF:FL ratios (Fig. 5). The lowest ratio was obtained for the spinal cord samples (mean 0.5), while CTF:FL ratio for the examined cortical brains regions in FTLD-U and ALS ranged from 1.1 (ALS-MCtx) to 3.6 (FTLD-U type3).

Immunoblot analysis of examined cases with concomitant TDP-43 in the context of other neurodegenerative disease such as CBD and AD, revealed a labeling of abnormal

was detectable. **b** Urea-samples extracted from motor cortex (MCtx) and spinal cord (sc) samples of ALS cases and immunoblotted with 1D3. A similar biochemical profile as for FTLD-U brains was detectable in MCtx samples. However, in sc samples the predominant band detectable was phosphorylated full-length TDP-43. **c** Urea-samples extracted from frontal cortex (fc) or hippocampus (hp) from CBD and AD cases with or without TDP-43 pathology. Note the absence of immunoreactivity with 1D3 in AD case without TDP-43 pathology

FL and CTFs, with clear predominance of CTFs (Fig. 4c) especially in the AD cases. In contrast to FTLD-U cases, the smallest CTF band (labeled as 1 in Fig. 4a) seems to be the most intense.

No signal was obtained in AD cases without TDP-43 pathology (Fig. 4c), TDP-43 negative FTLD-U and healthy controls (data not shown).

Discussion

The abnormal accumulation of TDP-43 is the characteristic feature in a spectrum of neurodegenerative diseases, including

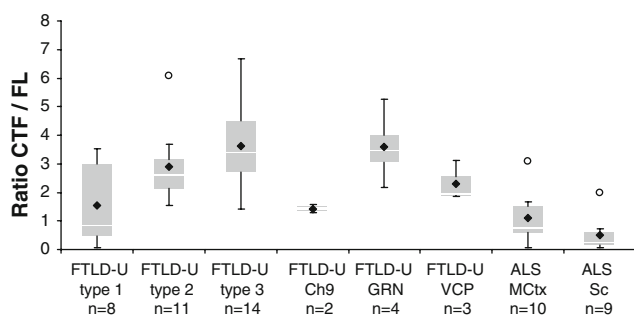


Fig. 5 Ratio of phosphorylated C-terminal fragments to full-length TDP-43. Immunoblots of FTLD-U and ALS cases probed with 1D3. Band intensities of full-length (FL) and C-terminal fragments (CTF) of TDP-43 were analyzed and the ratio between both values was calculated. CTF:FL ratios are depicted as a box and whiskers blot that gives the range of values, with the box being subdivided into the 25 and 75% quartiles by the median; circles represent outliers, filled rhombus represent the mean

most forms of familial and sporadic FTLD-U with or without MND and ALS. As is true for proteins accumulating in other neurodegenerative diseases, such as tau or α -synuclein [11, 21], abnormal phosphorylation of TDP-43 has also been demonstrated to occur in the disease process of TDP-43 proteinopathies [2, 28]. To learn more about the relevance of abnormal phosphorylation in the pathogenesis of these conditions, it is essential to identify potential phosphorylation sites and to study their functional consequences. Indeed very recently, several phosphorylation sites (S379, 403, 404, 409, and 410) of TDP-43 have been identified by generating antibodies against predicted phosphorylation sites and using these antibodies to analyze small numbers of sporadic cases of FTLD-U, familial FTLD-U with GRN mutations and ALS [15, 17]. We used a similar approach to generate mAbs against several potential phosphorylation sites of TDP-43, and here we describe the characterization of novel rat mAbs 1D3 and 7A9 raised against phosphorylated S409/410 of TDP-43 as well as the investigation of S409/410 phosphorylation by immunohistochemistry and biochemistry in a large series of TDP-43 proteinopathies.

Both, ELISA and immunoblot analysis of sarcosyl-insoluble protein fractions extracted from FTLD-U brains (with or without dephosphorylation by alkaline-phosphatase treatment) demonstrated that mAbs 1D3 and 7A9 have a high specificity to detect phosphorylated TDP-43 species. By immunohistochemistry using single and double label methods, we showed that mAbs 1D3 and 7A9 specifically labeled all types of pathologic inclusions, such as NCI, CGIs, NII, DN previously described with phosphorylation-independent TDP-43 antibodies [7, 27, 28]. Since FTLD-U is a highly heterogeneous group with respect to morphological subtypes and underlying genetic defects [6, 22, 33], we expanded our immunohistochemical analysis to a large

series of FTLD-U cases to see whether differences in phosphorylation might be part of the heterogeneity. However, we found that phosphorylation of TDP-43 at S409/410 was a highly consistent finding in all different subtypes of FTLD-U, including familial forms with mutations in GRN [3, 8], VCP [38] and linkage to chromosome 9p [24, 37]. Moreover, we also demonstrated that phosphorylation of TDP-43 at S409/410 is also consistently present in abnormal TDP-43 inclusions in ALS and in TDP-43 inclusions occurring in the setting of other neurodegenerative diseases such as AD and CBD. Therefore, our data indicate that phosphorylation at S409/410 occurs within the lesions in all subgroups of TDP-43 proteinopathies irrespective of the anatomical and topographical distribution, underlying genetic defect and clinical presentation, therefore making these phosphorylation-specific TDP-43 antibodies powerful tools in routine diagnostics of neurodegenerative diseases.

In contrast to phosphorylation-independent TDP-43 antibodies, 1D3 and 7A9 revealed no physiological nuclear TDP-43 staining by immunohistochemistry and no detection of the normal TDP-43 band of ~ 43 kDa by immunoblot analysis in cultured cells, mouse and human brain homogenates (data not shown). These findings are in accordance with previous results using different pS409/410 specific antibodies [15, 17] and thereby strongly argue that phosphorylation of S409/410 seems to be an abnormal event. However, it cannot be completely excluded that phosphorylation at S409/410 might occur physiologically to a minor extent in normal TDP-43 that is below current detection limits.

By detailed immunoblot analysis of sarcosyl-insoluble protein fractions extracted from cortical brain areas from different subgroups of TDP-43 proteinopathies, we consistently found phosphorylated CTFs and full-length TDP-43, running as bands between 20–25 kDa and bands of ~ 45 kDa, respectively. In contrast, protein fractions extracted from spinal cord samples predominantly showed full-length TDP-43 and weak or absent accumulation of CTFs, suggesting that protein composition in TDP-43 inclusions in brain and spinal cord neurons might be different. These findings are in accordance with a recent publication using N- and C-terminal specific TDP-43 antibodies [16], which showed that inclusions in cortical regions are strongly labeled with C-terminal antibodies but not with N-terminal antibodies, in contrast to inclusions in motor neurons in spinal cord where similar immunoreactivity was present with N- and C-terminally specific antibodies.

The mechanisms leading to CTF formation are still unclear. Hasegawa et al. [15] recently claimed that specific banding patterns of phosphorylated CTFs seem to correlate with underlying FTLD-U subtypes; however, this observation is based on results on very limited number of cases ($n = 1-2$) per FTLD-U subtypes. Nevertheless, in

combination with our initial description of subtype 1 and subtype 2 specific antibodies which detect CTFs in a highly selective manner in the respective subtypes [28, 33], it appears to be a tempting hypothesis and we therefore performed detailed analysis of the CTFs in our large series of FTL-D-U cases ($n = 42$). Up to four distinct CTF bands could be delineated with our phospho-specific antibody 1D3, however, the intensities of distinct bands varied considerably between the studied cases within similar FTL-D-U subtypes. While in general there was some overlap with the banding pattern described in previous studies [15, 17], we were not able to confirm or define distinct banding patterns reliably correlating with distinct FTL-D-U subtypes or clinical phenotypes in our studied cohort. In addition, the significance of the slightly different band intensities observed in AD and CBD cases with concomitant TDP-43 pathology remains unclear and needs to be examined in more detail in larger series of cases.

In summary, our data show that phosphorylation of S409/410 of TDP-43 is a highly consistent abnormal event occurring in all different types of TDP-43 inclusions in the whole spectrum of familial and sporadic TDP-43 proteinopathies. Therefore, phosphorylation specific antibodies, such as the described mAbs 1D3 and 7A9, will become powerful tools not only in the routine diagnostics of neurodegenerative diseases, but also in the evaluation of upcoming cellular and animal models for TDP-43 proteinopathies.

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