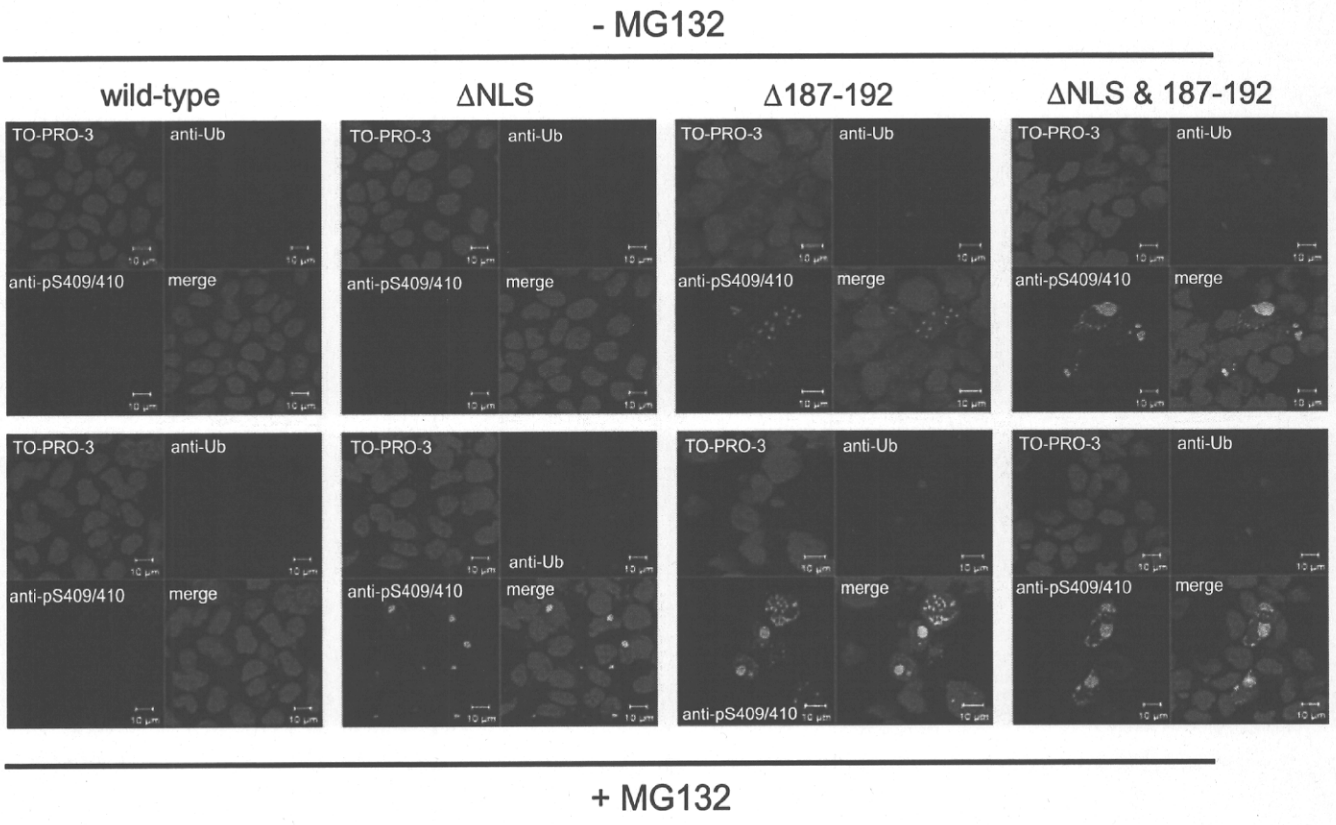

Fig. 4 The formation of inclusion-like structures in cells transfected with deletion mutants of transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43). When pcDNA3-TDP-43 wild-type was expressed in SH-SY5Y cells, no staining was observed by the phosphorylation-specific anti-TDP-43 antibody (pS409/410), indicating that transfected wild-type TDP-43 and endogenous TDP-43 are not phosphorylated at Ser409/410. The deletion mutant lacking nuclear localization signal (ΔNLS: 78–84 residues) was not recognized by pS409/410 without MG132 treatment, while round cytoplasmic inclusion-like structures were stained by both pS409/410 and anti-ubiquitin antibodies in those cells treated with MG132. In cells expressing another deletion mutant lacking 187–192 residues (Δ187–192), pS409/410-positive but ubiquitin-negative intranuclear dot-like structures were observed without treatment. With MG132, round intranuclear inclusions positive for pS409/410 and ubiquitin were formed. In cells expressing the double-deletion mutant (ΔNLS and 187–192), cytoplasmic inclusions positive for pS409/410 and ubiquitin were formed even in the absence of MG132.

Based on these findings so far, we would like to propose that TDP-43 proteinopathy can be divided into two groups (Table 1). One is "pure" TDP-43 proteinopathy, in which only TDP-43 accumulates in brains as a pathological protein. The other is "combined" TDP-43 proteinopathy, which shows multiple protein aggregates. TDP-43 pathology is always found in all cases of pure TDP-43 proteinopathy and familial and endemic cases of combined TDP-43 proteinopathy, while it is found in a subpopulation of cases with sporadic combined TDP-43 proteinopathy.

ESTABLISHMENT AND ANALYSES OF CELLULAR MODELS OF TDP-43 PROTEINOPATHY

To establish the cellular models for intracellular aggregates of TDP-43, we first examined two candidate sequences for the nuclear localization signal (NLS) (Fig. 4).[57] Deletion of residues 78–84 resulted in cytoplasmic localization of TDP-43 in SH-SY5Y cells, proving that this sequence indeed functions as NLS. This result is largely consistent with the previous report by Winton *et al.* which showed that residues 82–98 were required for TDP-43 entry into the nucleus.[58] On the other hand, the mutant lacking residues 187–192 localized in nuclei, forming unique dot-like structures. Proteasome inhibition caused these to assemble into aggregates. Furthermore, double-deletion mutant of these sequences caused cytoplasmic inclusion formation without proteasomal inhibition. Immunohistochemical and immunoblot analyses showed that these inclusions consisted of phosphorylated and ubiquitinated TDP-43, suggesting that these cellular models recapitulate the phenotypes of TDP-43 proteinopathies both pathologically and biochemically.

Then, we tried to generate and analyze the cellular models by expressing C-terminal fragments of TDP-43 in

SH-SY5Y cells, since 18–26 kDa C-terminal fragments of TDP-43 are major constituents of inclusions in FTLD-TDP and ALS brains.[37] The results showed that expression of several TDP-43 C-terminal fragments as green fluorescent protein (GFP), including 162–414, 218–414, 219–414 and 247–414, led to the formation of cytoplasmic inclusions positive for pTDP-43 and ubiquitin (Fig. 5).[59] The N-termini of the latter two peptides, 219–414 and 247–414, correspond to the cleavage sites of TDP-43 C-terminal fragments accumulated in FTLD-TDP brains identified by our mass spectra analyses. Igaz *et al.* reported another cleavage site at Arg 208 in a pathological TDP-43 C-terminal fragment from FTLD-TDP brains and inclusion formation in cultured cells expressing resultant C-terminal fragment (residues 208–414).[60] Our immunoblot analysis showed that these aggregated pTDP-43 C-terminal fragments were recovered in sarkosyl-insoluble fraction as those in brains of FTLD-TDP and ALS.

Several groups have recently reported increased accumulation of TDP-43 fragments in the brain homogenates[13] and cultured cells[15,16] in some of the pathogenic mutations of TARDBP identified in ALS. However, in our cellular models, immunoblot analyses failed to show any significant differences in the generation of fragments of TDP-43 with or without various mutations. Alternatively, pathogenic mutations consistently enhanced aggregation of TDP-43 if they are present in the C-terminal fragment, GFP-TDP 162–414 (Fig. 6). These results suggest that pathogenic mutations and N-terminal truncation synergistically promote abnormal accumulation of TDP-43.

METHYLENE BLUE AND DIMEBON INHIBIT AGGREGATION OF TDP-43 IN CELLULAR MODELS

Inhibition of the aggregation of TDP-43 and promotion of its clearance are considered to be major therapeutic avenues for ALS and FTLD-TDP. As for other neurodegenerative diseases, current tools include antibodies, synthetic peptides, molecular chaperones and chemical compounds. Of the latter, methylene blue (MB) and dimebon have recently been reported to have significant beneficial effects in phase II clinical trials of AD.[61,62] MB is a phenothiazine compound that has been used for treating methemoglobinemia,[63,64] inhibiting nitric oxide synthase,[65] reducing nGMP,[66] enhancing β-oxidation in mitochondria,[67] inhibiting of noradrenalin re-uptake[68] and enhancing brain mitochondrial cytochrome oxidase activity.[69,70] It has also been shown to inhibit AD-like Aβ and tau aggregation *in vitro*.[71,72] Dimebon is a non-selective antihistaminergic compound that was in clinical use for many years before more selective agents became available.[73] It has been reported to inhibit butyrylcholinesterase, acetylcholinesterase, NMDA receptors, voltage-gated calcium channels, adrenergic receptors, histamine H1 receptors, histamine H2 receptors and serotonin receptors, as well as to stabilize glutamate-induced Ca2+ signals.[74–76] The effects of dimebon on pathological protein aggregation have not been studied in detail.

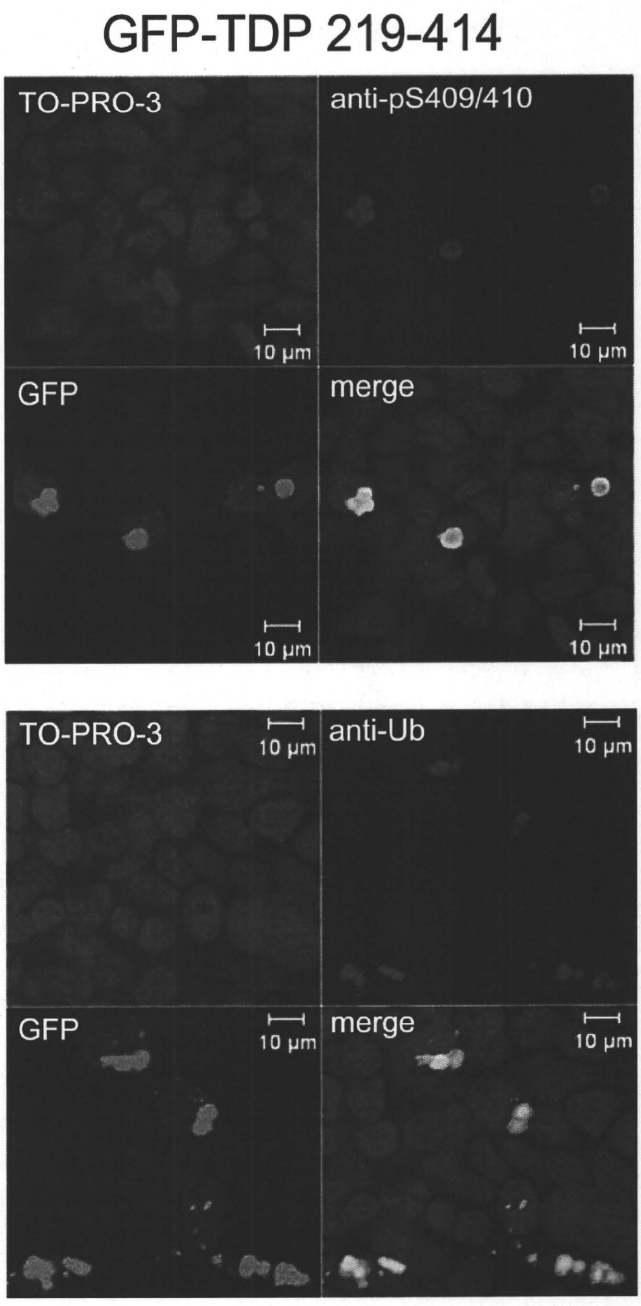

Fig. 5 Transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43) C-terminal fragments identified in diseased brains form cytoplasmic inclusions in cells. Round cytoplasmic inclusions with strong green fluorescent protein (GFP) intensities were observed in SH-SY5Y cells expressing GFP-TDP 219–414. These were positive for pS409/410 and ubiquitin Ub).

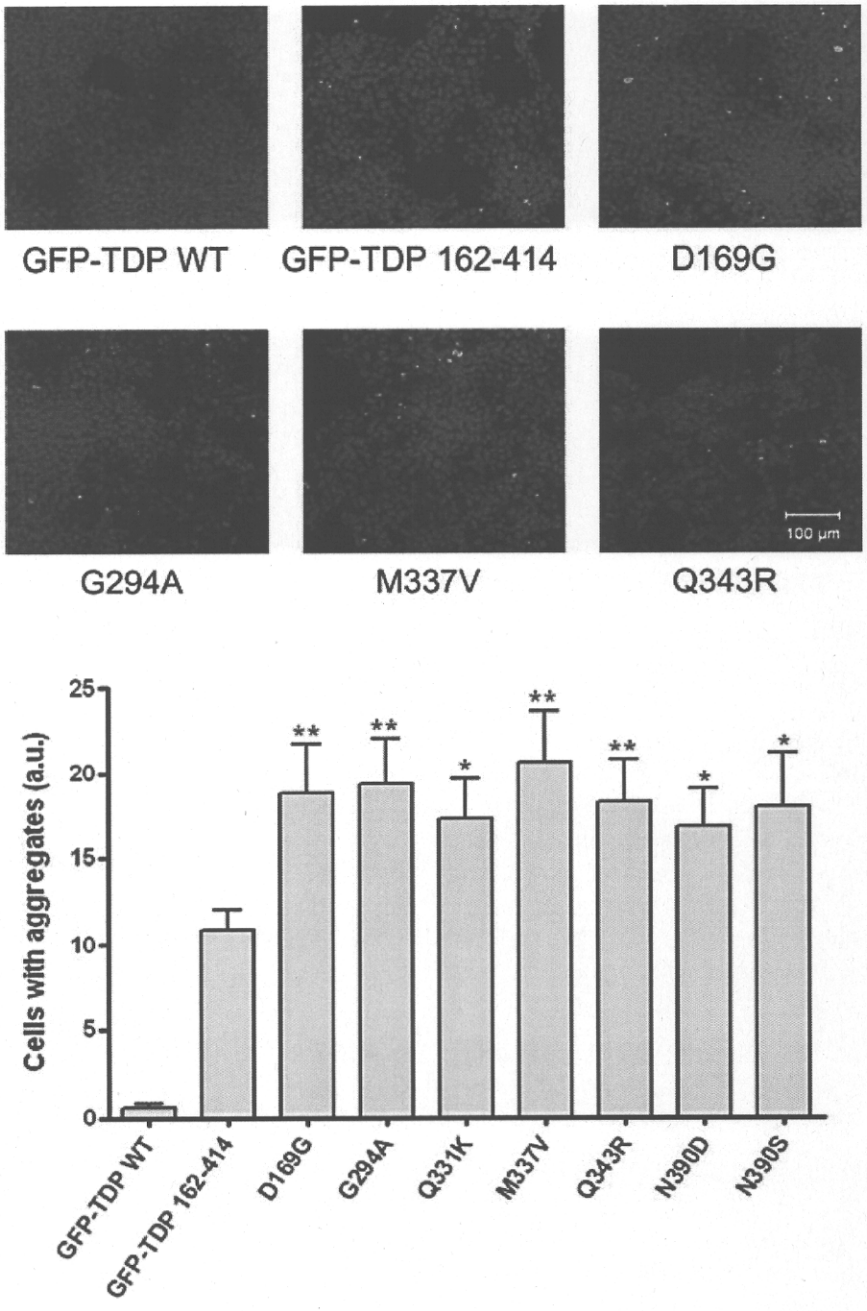

Fig. 6 The effect of transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43) mutations on aggregates formation of the C-terminal fragment of TDP-43. All seven mutations significantly facilitated the formation of intracellular aggregates of green fluorescent protein (GFP)-TDP 162–414, as compared with those of wild-type GFP-TDP 162–414.

Using our cellular models of TDP-43 proteinopathy described above, we investigated the effects of MB and dimebon on the formation of TDP-43 aggregates.[77] Following treatment with 0.05 μM MB or 5 μM dimebon, the number of TDP-43 aggregates was reduced by 50% and 45%, respectively (Fig. 7A–C,I). The combined use of MB and dimebon resulted in an 80% reduction in the number of aggregates (Fig. 7D,I), and in the significant reduction of phosphorylated TDP-43 in insoluble fraction of the cell lysate (Fig. 7E–H). These results suggest that MB and dimebon may be useful for the treatment of ALS, FTLD-TDP and other TDP-43 proteinopathies.

CONCLUSION

Intracellular aggregation of TDP-43 takes place in brains of patients with ALS, FTLD and a variety of other neurodegenerative diseases, suggesting the possibility that TDP-43 has wide influence on neuronal dysfunction and neurodegeneration. Phosphorylated and truncated forms of TDP-43 are major species accumulated in diseased brains, and the proteolytic cleavage of TDP-43 may play an important role for the pathological process of TDP-43 proteinopathy. In cultured cells, expression of the TDP-43 C-terminal fragments results in accelerated aggregate formation and in

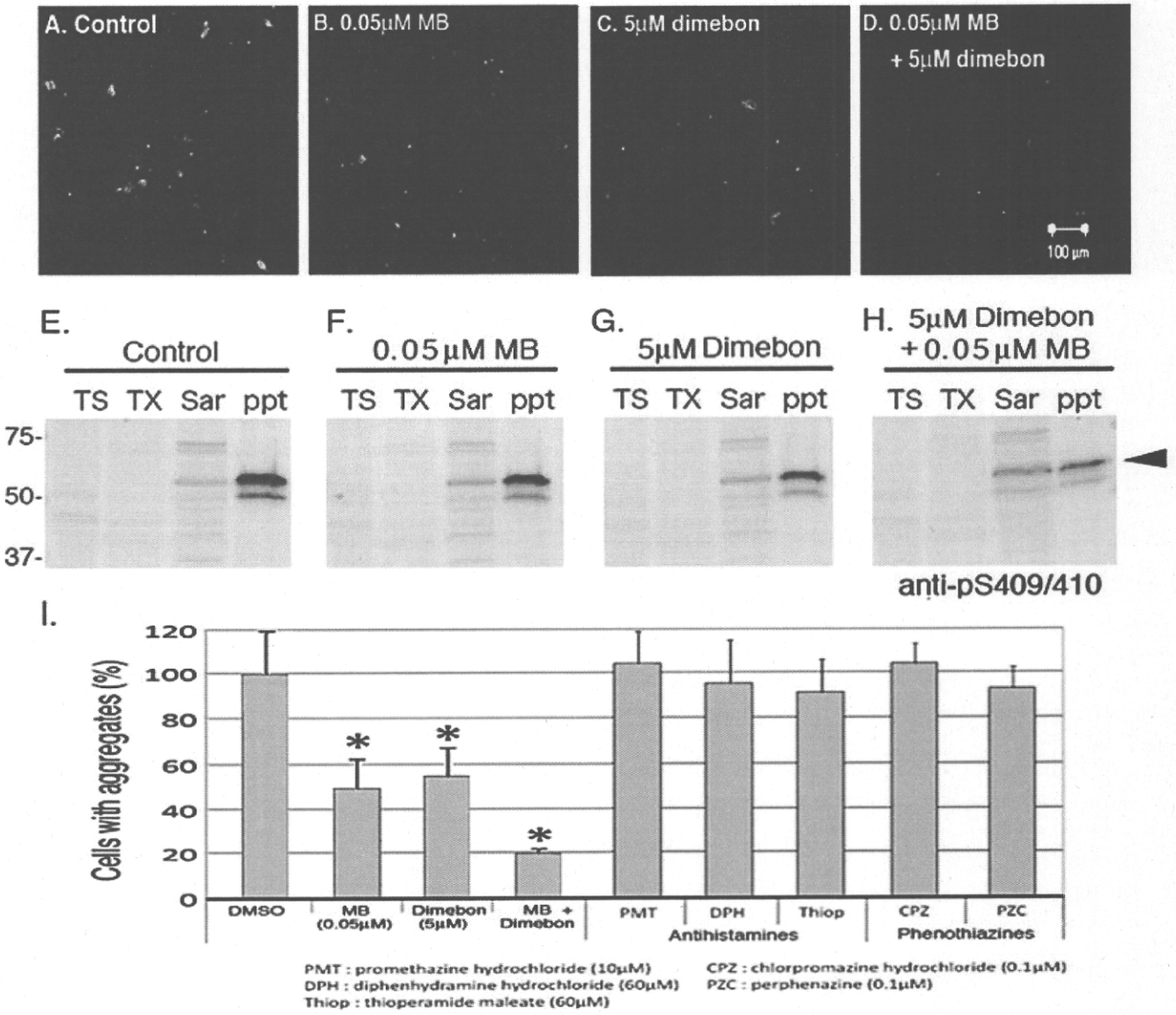

Fig. 7 Inhibition of aggregates formation of transactivation response (TAR) DNA-binding protein of Mr 43 kDa (TDP-43) in cellular models by methylene blue (MB) and dimebon. (A–D) Immunohistochemical analysis of the effects of MB and dimebon on the aggregation of TDP-43 in SH-SY5Y cells expressing TDP-43 (ΔNLS and 187–192). TDP-43 inclusions were stained with anti-pS409/410 antibody and detected with Alexa Fluor 488-labeled secondary antibody. Representative confocal images from cells treated with control (dimethyl sulfoxide + distilled water) (A), 0.05 μM MB (B), 5 μM dimebon (C) and 0.05 μM MB + 5 μM dimebon (D) are shown. (E–H): Immunoblot analysis of the effects of MB and dimebon on the aggregation of TDP-43 in SH-SY5Y cells expressing green fluorescent protein (GFP)-tagged TDP-43 C-terminal fragment (162–414). Tris saline (TS)-soluble material, Triton X-100 (TX)-soluble material, Sarkosyl (Sar)-soluble material and the remaining pellet (ppt) were prepared from control cells (E) and from cells treated with 0.05 μM MB (F), 5 μM dimebon (G), and 0.05 μM MB + 5 μM dimebon (H), run on SDS-PAGE and immunoblotted with anti-pS409/410 antibody. (I) Quantitation of cells with TDP-43 aggregates. The number of cells with intracellular TDP-43 aggregates was counted and expressed as the percentage of cells with aggregates in the absence of compound (taken as 100%). Data are means ± SEM $^*P < 0.01$ by Student's *t*-test.

failure of nuclear localization of endogenous TDP-43. At present, it is unknown whether loss of function, toxic gain of function, or a combination of both mechanisms contributes to neurodegeneration. Cultured cells or animal models expressing those abnormal TDP-43 species are expected to be useful tools to investigate the pathogenesis of TDP-43 proteinopathy and to develop effective diagnostics and therapeutics.

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CONSENSUS PAPER

Nomenclature and nosology for neuropathologic subtypes of frontotemporal lobar degeneration: an update

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One year ago, in this journal, we published a recommended nomenclature for the neuropathologic subtypes of frontotemporal lobar degeneration (FTLD) [7]. A major impetus behind this was to resolve the confusion that had arisen around the use of the term "FTLD with ubiquitinated inclusions" (FTLD-U), following the discovery that the molecular pathology of these cases was heterogeneous, with most, but not all, being characterized by pathological TDP-43 [6, 11]. In addition, a system of nosology was introduced that grouped the FTLD subtypes into broad categories, based on the molecular defect that is most characteristic, according to current evidence. This system provided a concise and consistent terminology that has now been widely adopted in the literature. Another anticipated advantage was the ability to readily accommodate new discoveries. At the time, we did not anticipate how quickly this attribute would be put to use.

Although most FTLDs are characterized by cellular inclusion bodies composed of either tau (FTLD-tau) or TDP-43 (FTLD-TDP), approximately 10–15% of cases

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remain, that include a number of uncommon FTLD subtypes, in which the pathologic protein is unknown. Recently, two studies identified mutations in the gene encoding the *fused in sarcoma* (FUS) protein (also known as *translocated in liposarcoma*, TLS), as the cause of familial amyotrophic lateral sclerosis (ALS) type 6 [5, 14]. The recognized clinical, genetic and pathological overlap between ALS and FTD, and the high degree of functional homology between FUS and TDP-43, prompted a number of subsequent studies that demonstrated that the inclusions of several of the tau/TDP-43-negative FTLDs are immunoreactive (ir) for FUS [8–10]. One such group are those cases with TDP-43-negative FTLD-U pathology, originally referred to as atypical FTLD-U (aFTLD-U) [6, 11]. According to the previous nomenclature recommendations, the neuropathology of these cases was designated as FTLD-UPS because the inclusions were only detectable with immunohistochemistry against proteins of the ubiquitin proteasome system (UPS) [7]. However, based on the discovery that all the ubiquitin-positive pathology in these cases is immunoreactive for FUS, we now recommend that they should be reclassified as FTLD-FUS [9]. In addition, the characteristic neuronal cytoplasmic inclusions of basophilic inclusions body disease (BIBD), previously of unknown biochemical composition, have also been shown to be consistently FUS-ir [8]. Perhaps most surprising has been the identification of abundant FUS-positive pathology in cases of neuronal intermediate filament inclusion disease (NIFID) [10]. The diagnostic criterion for NIFID is the presence of neuronal inclusions that are negative for tau, α-synuclein and TDP-43 but immunoreactive for class IV intermediate filaments (IF) [1] and therefore the term FTLD-IF was designated in the previous nomenclature recommendations [7]. However, the finding that only a minority of the inclusions in NIFID are IF-ir, the absence of any identifiable genetic or molecular abnormality of IF in these cases and the recognition that immunohistochemistry for IF is not specific for this condition, is consistent with the possibility that another protein may be more central to the pathogenesis. The recent demonstration that a much larger proportion of the inclusions in NIFID are FUS-ir, that all the cells with IF-ir inclusions also contain pathological FUS, and that there are widespread FUS-ir glial inclusions, suggests that the abnormal accumulation of FUS may be more fundamental in the disease process and that IF pathology probably develops as a secondary process [10].

Taking these studies together, we now recommend that aFTLD-U, BIBD and NIFID should be grouped together under the designation of FTLD-FUS (Table 1). It is important to recognize, however, that this does not imply that a defect in FUS metabolism is known to be causal in any of these conditions. Rather, it simply indicates that they share FUS accumulation as the most prominent molecular pathology. Whether or not this indicates that aFTLD-U, BIBD and NIFID are actually all part of a continuous spectrum of disease must await detailed comparative clinicopathological studies of larger numbers of cases. Nonetheless, the presence of FUS pathology sets these cases apart and should aid in their neuropathological diagnosis and classification.

Although it now appears that most, if not all, cases of sporadic FTLD-UPS (i.e. aFTLD-U) have FUS-immunoreactive pathology [9], the designation FTLD-UPS remains appropriate for at least one condition: familial FTD linked to chromosome 3 (FTD-3), caused by mutations in the *CHMP2B* gene. In addition to being negative for tau and TDP-43 [2], a recent study has shown that the ubiquitin/p62-immunoreactive neuronal inclusions in these cases do not label with antibodies against FUS [3]. Although these inclusions may eventually be discovered to contain a single major pathologic protein, it is also possible they have more heterogeneous composition that results from a primary defect of endosomal function [13]. Until this is determined, FTLD-UPS remains an appropriate designation for the neuropathology of FTD-3 and possibly for some FUS-negative sporadic cases.

With these recent advances, virtually all cases of FTLD can now be assigned to one of the three major molecular subgroups (FTLD-tau, FTLD-TDP or FTLD-FUS). This classification does not presuppose a primary role of the signature protein in pathogenesis (although in FTLD-tau and FTLD-TDP there is growing evidence to support this), but provides a logical way of grouping neuropathologic subtypes that is likely to have relevance regarding common disease mechanisms, diagnostic tests and possibly treatments. The specific role of the pathologic proteins and their relationship to causal gene defects is crucial information

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Table 1 Updated nomenclature for neuropathologic subtypes of frontotemporal lobar degeneration

2009 recommendation		2010 recommendation		Associated genes
Major molecular class	Recognized subtypes[a]	Major molecular class	Recognized subtypes[a]	
FTLD-tau	PiD	FTLD-tau	PiD	*MAPT*
	CBD		CBD	
	PSP		PSP	
	AGD		AGD	
	MSTD		MSTD	
	NFT-dementia		NFT-dementia	
	WMT-GGI		WMT-GGI	
	Unclassifiable		Unclassifiable	
FTLD-TDP	Types 1–4	FTLD-TDP	Types 1–4	*GRN*
	Unclassifiable		Unclassifiable	*VCP*
				9p
				(*TARDBP*)[b]
FTLD-UPS	**FTD-3**	**FTLD-UPS**	**FTD-3**	*CHMP2B*
	aFTLD-U			
FTLD-IF	**NIFID**	**FTLD-FUS**	**aFTLD-U**	(*FUS*)[c]
BIBD			**NIFID**	
			BIBD	
FTLD-ni		FTLD-ni		

Entries in bold indicate major revisions

aFTLD-U, atypical frontotemporal lobar degeneration with ubiquitinated inclusions; AGD, argyrophilic grain disease; BIBD, basophilic inclusion body disease; CBD, corticobasal degeneration; CHMP2B, charged multivescicular body protein 2B; FTD-3, frontotemporal dementia linked to chromosome 3; FTLD, frontotemporal lobar degeneration; FUS, fused in sarcoma; *GRN*, progranulin gene; IF, intermediate filaments; MAPT, microtubule associated protein tau; MSTD, multiple system tauopathy with dementia; NFT-dementia, neurofibrillary tangle predominant dementia; ni, no inclusions; NIFID, neuronal intermediate filament inclusion disease; PiD, Pick's disease; PSP, progressive supranuclear palsy; TARDBP, transactive response DNA binding protein; TDP, TDP-43; UPS, ubiquitin proteasome system; VCP, valosin containing protein; WMT-GGI, white matter tauopathy with globular glial inclusions; 9p, genetic locus on chromosome 9p linked to familial amyotrophic lateral sclerosis and frontotemporal dementia

[a] Indicates the characteristic pattern of pathology, not the clinical syndrome. Note that FTDP-17 is not listed as a pathological subtype because cases with different *MAPT* mutations do not have a consistent pattern of pathology. These cases would all be FTLD-tau, but further subtyping would vary

[b] Rare case reports of patients with clinical FTD and TDP-43 pathology associated with *TARDBP* genetic variants [4]

[c] One patient reported with a *FUS* mutation and FTD/ALS clinical phenotype but no description of pathology [12]

that requires further neuropathological and experimental investigations.

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