

# 1        **Distinct TDP-43 aggregate features and toxicity stratify FTD patients**

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42 **Abstract**

43 Accumulation of abnormally phosphorylated TDP-43 (pTDP-43) is the main pathology in affected  
44 neurons of patients with amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).  
45 Morphological diversity and neuroanatomical distribution of pTDP-43 accumulations allowed  
46 classification of FTD cases into at least four different subtypes, which correlate with clinical  
47 presentations and genetic causes. To understand the molecular basis of this heterogeneity, we  
48 developed SarkoSpin, a new method for extremely pure biochemical isolation of pathological TDP-43.  
49 Combining SarkoSpin with mass spectrometry, we revealed proteins beyond TDP-43, which become  
50 abnormally insoluble in a disease subtype-specific manner. We show that pTDP-43 extracted from  
51 disease brain forms large and stable assemblies of distinct densities and morphologies that correlate  
52 with disease subtypes. Importantly, biochemically extracted pTDP-43 assemblies displayed  
53 differential neurotoxicity and seeding that correlated with disease duration of FTLD patients. Our data  
54 indicate that disease heterogeneity may originate from alternate pathological TDP-43 conformations,  
55 reminiscent of prion strains.

56

57 **Introduction**

58 Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are neurodegenerative  
59 disorders thought to represent the two ends of a disease spectrum, due to their genetic, pathological  
60 and clinical overlap<sup>1</sup>. ALS is the most common form of motor neuron disease (MND), affecting upper  
61 and lower motor neurons and ultimately leading to progressive paralysis. FTD is the second most  
62 common form of dementia after Alzheimer's disease<sup>2</sup> and is characterized by severe atrophy of frontal  
63 and temporal lobes and is, therefore, also referred to as frontotemporal lobar degeneration (FTLD).  
64 Approximately 10% of ALS and 40% of FTD cases are familial and are associated with mutations in a  
65 large number of genes<sup>1,3</sup>, the most common of which are hexanucleotide repeat expansions on  
66 chromosome 9 open reading frame 72 gene (*C9ORF72*)<sup>4,5</sup>. The remaining cases are sporadic with  
67 apparent lack of family history.

68 The major common hallmark of these two diseases is the presence of pathological, ubiquitinated  
69 protein inclusions in affected neurons, which in almost all ALS and about 45% of FTD patients  
70 primarily contain the RNA/DNA-binding protein TDP-43 (Transactivation Response DNA Binding  
71 Protein of 43 kDa)<sup>6,7</sup>. TDP-43 is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP)  
72 family<sup>8</sup> and it plays essential roles in splicing regulation<sup>9,10</sup> which are dependent on the formation of  
73 physiological TDP-43 oligomers<sup>11,12,13</sup>. TDP-43 is predominantly nuclear, but it can shuttle to the  
74 cytoplasm<sup>14</sup>, where it incorporates into stress<sup>15</sup> and transporting RNA<sup>16,17</sup> granules via its low  
75 complexity domain (LCD), which mediates phase separation<sup>18,19</sup>.

76 FTLD- and ALS-associated pathological TDP-43 was shown to undergo several abnormal post-  
77 translational modifications including poly-ubiquitination, hyper-phosphorylation and proteolytic  
78 cleavage<sup>6,7</sup>. The latter leads to the formation of a highly aggregation-prone C-terminal fragment of 25  
79 kDa that is deposited in the inclusions along with the full-length TDP-43<sup>20,21</sup>. Notably, the majority of  
80 ALS-linked mutations are localized within this C-terminal LCD<sup>22,1</sup> and were shown to increase TDP-43  
81 aggregation propensity<sup>23,24</sup> without directly affecting its function in RNA splicing<sup>25</sup>. Rather, aggregate

82 formation and mislocalization of mutant or, most frequently, the wild type protein are thought to trigger  
83 two non-exclusive toxic mechanisms: loss of its normal function and gain of aggregate toxicity<sup>26</sup>.  
84 While ALS presents with rather unified TDP-43 neuropathology, FTLD is more heterogeneous and  
85 manifests with diverse histological patterns. The latter correlate with various clinical presentations,  
86 including behavioral variant FTD (bvFTD), semantic dementia (SD) and primary non-fluent aphasia  
87 (PNFA), as well as genetic mutations<sup>27</sup>. Based on the morphology and neuroanatomical distribution of  
88 pathological TDP-43 accumulations, FTLD-TDP can be classified into the following distinct  
89 subtypes<sup>28,29</sup>. FTLD-TDP type A (FTLD-TDP-A) is characterized by neuronal cytoplasmic inclusions  
90 (NCI), dystrophic neurites (DN) and neuronal intranuclear inclusions (NII) in upper cortical layers and  
91 typically manifests with bvFTD but no MND. Some FTLD-TDP-A patients carry *GRN* or *C9ORF72*  
92 mutations, which are non-distinguishable from sporadic cases based on TDP-43 pathology. FTLD-  
93 TDP type B cases (FTLD-TDP-B), which can also be linked to *C9ORF72* mutations, show primarily  
94 granular NCI expanding across all cortical layers, and present with a combination of FTD and MND.  
95 Finally, FTLD-TDP type C (FTLD-TDP-C) comprises long and thick DN and few NCI primarily in the  
96 superficial cortical layers. These cases are sporadic and present with SD without MND. Recently,  
97 other subtypes, which do not fully adhere to these pathological classification criteria were  
98 described<sup>28,29</sup>, suggesting that we are only beginning to identify the full spectrum of disease  
99 heterogeneity. The origin of this pathological diversity, which is associated with specific clinical  
100 presentations<sup>30</sup> remains unknown, but was proposed to correlate with specific molecular  
101 signatures<sup>31,32</sup>.

102 In order to address the molecular underpinnings of disease heterogeneity, we sought to analyze the  
103 biochemical and physical characteristics of TDP-43 aggregates such as size, shape, density and  
104 composition. The first and mandatory step in this characterization is the isolation of pathological  
105 assemblies and their physical separation from physiological TDP-43 and similar proteins. Currently,  
106 there is no reliable method for native extraction of TDP-43 aggregates for the analysis of pathological  
107 TDP-43 conformation, aggregation status, neurotoxicity and seeding potential. To achieve this, we

108 developed SarkoSpin, a novel and simple extraction method for physical separation of pathological  
109 TDP-43 from more than 99% of total protein mass including the extreme bulk of physiological,  
110 monomeric and oligomeric<sup>11</sup> TDP-43. Using SarkoSpin on brain cortical samples from over 80  
111 patients, we found that TDP-43 forms large and buoyant assemblies of distinct densities,  
112 polyubiquitination levels and morphologies that correlate with specific neuropathological  
113 classifications. Importantly, coupling SarkoSpin with mass spectrometry, we illustrate that a specific  
114 subset of proteins, beyond TDP-43, become insoluble in each disease subtype. These proteins are  
115 rarely co-aggregated with pTDP-43 and most likely represent a downstream effect of TDP-43  
116 pathology. One of these proteins depicts a distinct astrocytic reaction discriminating FTLD-TDP-A  
117 from FTLD-TDP-C patients, illustrating divergent pathogenic mechanisms within these two disease  
118 subtypes. Most importantly, we show evidence that SarkoSpin extracted pTDP-43 assemblies exhibit  
119 cytotoxicity and protein seeding ability. Remarkably, pathological aggregates extracted from FTLD-  
120 TDP-A were significantly more cyto- and neurotoxic than those extracted from FTLD-TDP-C, thereby  
121 correlating with the significant difference in disease duration between these two subtypes.  
122 Collectively, our data demonstrate that ALS and FTLD heterogeneity is consistently reflected in the  
123 biochemical, neurotoxic and seeding properties of TDP-43 and the associated insoluble proteome.  
124 We propose that alternative TDP-43 pathological conformations may underlie the diversity of TDP-43  
125 proteinopathies, reminiscent of prion strains<sup>33,34</sup>.

126

## 127 **Results**

### 128 **Summary of patient cohort and characterization of FTLD-TDP-A and FTLD-TDP-C cases**

129 Brain cortical samples from over 80 patients, including control patients with no apparent CNS  
130 pathology or with non-TDP-43-linked neurodegeneration, as well as ALS and FTLD patients of  
131 different subtypes (from both sporadic and genetic origins) were obtained from the University of  
132 California in San Diego (USA), the Queen Square Brain Bank for Neurological Disorders, University  
133 College London (UK), and the Netherlands Brain Bank (**Supplementary Table 1**). The morphology

134 and neuroanatomical distribution of pathological TDP-43 accumulations of cases used in this study  
135 adhered to the current classifications<sup>28,29</sup>. FTLD-TDP-A cases were primarily characterized by NCI  
136 with few, short DN and NCI in upper cortical layers, a pattern that was indistinguishable in sporadic or  
137 patients with *C9ORF72* mutations (**Supplementary Fig. 1a**). In contrast, FTLD-TDP-C cases, which  
138 were exclusively sporadic and did not develop MND, harbored long DN and few NCI, primarily in the  
139 superficial cortical layers (**Supplementary Fig. 1a**). Furthermore, it was recently reported that FTLD-  
140 TDP-C patients show significantly longer disease duration compared to all other subtypes<sup>29</sup>, which we  
141 could also confirm in our cohort (**Supplementary Fig. 1b-c**).

142

#### 143 **SarkoSpin: a new method for the isolation of pathological TDP-43 from human brain**

144 Although pathological TDP-43 is resistant to detergents, such as sarkosyl<sup>35,36,31,37</sup>, there is currently no  
145 method that allows the physical separation of pathological TDP-43 from the pool of other proteins in  
146 complex tissues. In fact, physiological TDP-43, as well as other proteins containing low complexity  
147 domains, typically co-purify with pathological TDP-43 in the sarkosyl-insoluble fractions from patient  
148 brain tissue<sup>36,37</sup> (**Supplementary Fig. 2a-b**). This contamination is likely due to the formation of large  
149 complexes with nucleic acids, including physiological nuclear TDP-43 oligomers<sup>11</sup>, which become  
150 partially resistant to detergent solubilization (**Supplementary Fig. 2b**).

151 To overcome this limitation and to purify pathological TDP-43 from its normal counterpart bound to  
152 nucleic acids, we combined harsh solubilization with nuclease treatment and a single centrifugation  
153 step, in a new method we termed SarkoSpin (**Fig. 1a-b**). In order to assess the efficiency of our  
154 technique, we analyzed motor and frontal cortex homogenates from six controls, six ALS, six FTLD-  
155 TDP-A, three FTLD-TDP-B and six FTLD-TDP-C (**Supplementary Table 1**). Total protein amounts  
156 partitioning in the SarkoSpin supernatant and pellet fractions were estimated by silver staining of  
157 SDS-PAGE analyzed samples (**Fig. 1c** and **Supplementary Fig. 2c**) and by measuring protein  
158 concentrations. On average, 99.8% of the total protein mass resided in the supernatant with no  
159 significant difference between disease and control, or between disease types (**Fig. 1d**). 99.9% of total

160 TDP-43 was found in the supernatants of control samples (**Fig. 1e-f** and **Supplementary Fig. 3**). The  
161 monoclonal anti-TDP-43 antibody used (#60019-2-Ig), also non-specifically binds to a faster migrating  
162 band (<40 kDa), which was not detected by a panel of anti-TDP-43-specific antibodies  
163 (**Supplementary Fig. 4**). Interestingly, in disease cases, only 88% of total TDP-43 was found in  
164 supernatants, indicating that the rest of TDP-43 was present in the sarkosyl-insoluble pellet and  
165 corresponds to the pathological species. Indeed, the pellet fraction in these cases presented a clear  
166 signature characteristic for pathological TDP-43, namely poly-ubiquitination, C-terminal cleavage and  
167 hyperphosphorylation, detected as a higher molecular weight band (~50 kDa) seen by a total TDP-43  
168 antibody (**Fig. 1b, e, f** and **Supplementary Fig. 3**). The latter, detected strongly by a specific antibody  
169 against phosphorylated TDP-43 (pS409/410), shows great enrichment of pathological TDP-43  
170 exclusively in disease pellets (**Fig. 1g**) at an average of 64% with no significant difference between  
171 disease types (**Fig. 1h**). Based on this analysis, we estimate that the average percentage of total  
172 TDP-43 that is phosphorylated on serine residues 409/410 in patient samples is 12% with wide inter-  
173 individual variation and no significant difference between disease types (**Supplementary Fig. 5a**).

174 Proteins with low-complexity domains or RNA/DNA binding ability, such as other members of the  
175 hnRNP family, as well as other ALS/FTD-associated proteins (FUS, hnRNPA1, hnRNPH, SOD1) and  
176 normal proteins partitioning in complex macromolecular assemblies (actin, histones), were virtually  
177 absent in SarkoSpin pellets (**Fig. 1i** and **Supplementary Fig. 3**). More than 99.8% of each of these  
178 proteins was found in SarkoSpin supernatants of all samples tested (**Fig. 1j**). Taken together, these  
179 data demonstrate that SarkoSpin allows the strict isolation of pathological TDP-43 and its physical  
180 separation from the vast majority of physiological TDP-43 and other proteins, including those with  
181 nucleic acid binding ability, low complexity domains and/or high aggregation propensity.

182

### 183 **SarkoSpin captures native pathological TDP-43 assemblies**

184 To further characterize the properties of these assemblies, we next performed native PAGE  
185 electrophoresis of SarkoSpin supernatants and pellets from control and disease cases. Total TDP-43

186 native blots on SarkoSpin supernatants of all cases including controls showed a wide smear, ranging  
187 from the stacking gel (>10 MDa) to the size of monomeric TDP-43, where a strong band was  
188 observed (**Supplementary Fig. 6**, top left panel). TDP-43 is therefore present in different oligomeric  
189 states in all samples, in agreement with our recent report<sup>11</sup>. Notably, total protein concentrations were  
190 equalized before SarkoSpin, explaining the overall lower signal observed in the control lane, which  
191 lack accumulated TDP-43 aggregates.

192 Strikingly, a strong TDP-43 signal was detected in the stacking gel in the patient but not in the control  
193 samples. When immunoblotted with pTDP-43-specific antibody, only this particular high molecular  
194 weight signal was detected (**Supplementary Fig. 6** top right panel), indicating that it corresponds to  
195 the pathological species. Importantly, in contrast to control samples, SarkoSpin-pelleted patient  
196 samples immunoblotted with total TDP-43- or pTDP-43-specific antibodies revealed solely the  
197 presence of high molecular weight assemblies retained in the stacking gels and complete absence of  
198 monomeric or oligomeric TDP-43 (**Supplementary Fig. 6**, lower panels), further confirming the  
199 efficient separation of pathological from physiological assemblies. If exclusively composed of TDP-43,  
200 such high ordered aggregates would consist of at least 200 monomers, which may represent the  
201 minimal unit comprising the large inclusions found in patient-affected neurons. No differences were  
202 observed among the various disease types. However, it cannot be excluded that subtype-specific  
203 differences occur above the resolved sizes, undetectable by the resolution of this assay.

204

## 205 **Insoluble proteome and levels of pathological TDP-43 ubiquitination distinguishes disease** 206 **subtypes**

207 To explore which other proteins, beyond TDP-43, become insoluble in patient samples, we analyzed  
208 SarkoSpin preparations from brain homogenates of eleven controls, three FTLD-Tau, six ALS or FTD-  
209 ALS, nine FTLD-TDP-A, three FTLD-TDP-B and six FTLD-TDP-C by liquid chromatography-mass  
210 spectrometry (LC-MS). SarkoSpin pellets were denatured with 8 M Urea and protein concentration  
211 was equalized across the samples prior to enzymatic digestion and LC-MS analysis. Samples were



212 measured on a high-resolution mass spectrometer and the acquired tandem mass spectra were  
213 searched against human protein database. Spectral counting was used for quantifying differences in  
214 protein abundance between the different samples. The most significantly enriched protein ( $p = 10^{-3}$ )  
215 and most consistently correlating ( $r = 0.65$ ) with patient, but not control or FTLD-Tau, SarkoSpin  
216 pellets was TDP-43 (**Supplementary Fig. 7**), in accordance with previous studies<sup>7,6</sup>. All remaining  
217 proteins had weaker correlation values ( $r < 0.5$ ) and were less consistently enriched in the patient  
218 group, illustrating that indeed TDP-43 is the main component of insoluble protein aggregates and  
219 further validating SarkoSpin as a reliable method for their isolation.

220 Importantly, when we compared the insoluble proteins enriched in each disease group separately  
221 (**Supplementary Fig. 8-11**), we found that a distinct set of proteins characterized each subtype (**Fig.**  
222 **2a**). This suggests that while TDP-43 is the only protein consistently converting into an aggregated  
223 form in all disease cases tested, a specific subclass of proteins accompany TDP-43 within the  
224 insoluble fraction of each individual disease group. A few of these proteins such as LAMA1 and  
225 HepaCAM belong to the extracellular matrix and most probably associate with TDP-43 after tissue  
226 lysis due to their high abundance and adhesive nature. Conversely, all other proteins found in patient  
227 SarkoSpin pellets are localized intracellularly and might be interacting with aggregating TDP-43 within  
228 affected neurons.

229 To confirm these findings, we first tested a number of commercially available antibodies for sensitivity  
230 and specificity to several of the protein targets, predicted to correlate with pTDP-43 levels in specific  
231 disease subtypes by our analysis (**Fig. 2a**). Based on their performance, we selected one protein per  
232 disease group, namely: profilin 1 (PFN1), which we found enriched in ALS cases and was previously  
233 reported to be mutated in rare familial ALS cases<sup>38,39</sup>, N-acylsphingosine amidohydrolase (ASAH1),  
234 enriched in FTLD-TDP-A cases and reported to be mutated in some cases of spinal muscular  
235 atrophy<sup>40</sup> and Thioredoxin-like protein 1 (TXNL1), enriched in FTLD-TDP-C in our dataset. We then  
236 tested their presence in SarkoSpin fractions of individual patients by immunoblot and found that, while  
237 their levels were comparable on SarkoSpin supernatants (**Supplementary Fig. 12a-b**), they were

238 enriched in the pellets of samples from TDP-43 proteinopathies (**Fig. 2b**). Notably, this enrichment  
239 best correlated with pTDP-43 levels in the respective disease subtype (**Fig. 2c**), in agreement with our  
240 mass spectrometry results. Despite the large variability in pTDP-43 levels of individual patient  
241 samples, we confirmed the validity of these correlations, by the almost perfect correlation for all  
242 groups with total TDP-43 in these pellets (**Supplementary Fig. 12c-d**), as expected.

243 A similar approach was used to correlate ubiquitin and pTDP-43 levels in SarkoSpin pellets, which  
244 revealed an interesting and unexpected observation. Ubiquitin blots of SarkoSpin pellets and  
245 supernatants showed a completely different pattern, with ubiquitin-positive smears characteristic for  
246 pathological polyubiquitination present only in the pellets (**Fig. 2d**). This confirmed that SarkoSpin  
247 specifically concentrated aggregated and misfolded proteins that escape the proteasome degradation  
248 pathway in neurodegeneration. Surprisingly, however, the ubiquitin levels of pTDP-43 extracted from  
249 FTLD-TDP-C patients were consistently lower than those derived from FTLD-TDP-A and ALS patients  
250 (**Fig. 2d-e**). Comparison of co-immunofluorescence analysis of pTDP-43 and ubiquitin on postmortem  
251 brain sections of FTLD-TDP-A and FTLD-TDP-C patients confirmed the differential levels of pTDP-43  
252 polyubiquitination (**Fig. 2f-g** and **Supplementary Fig. 12e**), consistent with distinct molecular  
253 mechanisms generating pathology in these two disease subtypes.

254 To investigate if the increased insolubility of ASAH1 and TXNL1 resulted from direct co-aggregation  
255 with pTDP-43, or if these proteins become insoluble as a downstream event of TDP-43 aggregation,  
256 similar to what has been shown in other protein aggregation diseases<sup>41</sup>, we performed co-  
257 immunofluorescence analysis with pTDP-43 on control, FTLD-TDP-A and FTLD-TDP-C brain  
258 sections. ASAH1 and TXNL1 both demonstrated cytoplasmic staining, which was increased in levels  
259 and displayed a punctate pattern in both patient subtypes (**Supplementary Fig. 13**). This pattern  
260 appeared largely independent of pTDP-43 inclusions, with rare co-localization observable in both  
261 disease subtypes. This suggested that increased insolubility of these proteins in disease does not  
262 result from direct interaction and co-aggregation with pTDP-43 and that SarkoSpin likely dissociated

263 other proteins previously shown to co-aggregate with pTDP-43 on patient tissues<sup>42</sup>, thereby releasing  
264 the pTDP-43 “core” of these pathological assemblies.  
265 Importantly, one of the proteins enriched in the FTLD-TDP-A group in our mass spectroscopy analysis  
266 was the F-Box Protein 2 (FBXO2), which during our initial screen by conventional  
267 immunohistochemistry stained neuronal cytoplasm in control and FTLD-TDP-C brain sections, while  
268 in the FTLD-TDP-A subtype a characteristic astrocytic-like pattern was observed (**Supplementary**  
269 **Fig. 14a**). To further explore this finding, we performed co-immunofluorescence analysis of FBXO2  
270 and the astrocytic marker glial fibrillary acidic protein (GFAP). Excitingly, while in both disease  
271 subtypes astrogliosis was evident by the increased GFAP staining, FBXO2 marked FTLD-TDP type A,  
272 but not type C astrocytes (**Fig. 2h** and **Supplementary Fig. 14b**). These data indicate a previously  
273 unrecognized distinct astrocytic reaction characterizing FTLD-TDP-A patients, once again illustrating  
274 divergent pathogenic mechanisms within these two disease subtypes. In conclusion, the individual  
275 signatures of insoluble protein ensembles within each disease subtype, as well as the differential level  
276 of pTDP-43 polyubiquitination likely reflect distinct pathological processes, despite the coinciding  
277 TDP-43 aggregation.

278

### 279 **Diverse size distributions of TDP-43 aggregates isolated from different types of TDP-43** 280 **proteinopathies**

281 In order to further understand the differences in protein composition of TDP-43 aggregates and to  
282 explore the molecular basis underlying disease subtypes, we assessed the sedimentation profiles of  
283 TDP-43 aggregates to uncover specific size or shape differences. Brain cortex homogenates of eight  
284 controls, two ALS and two FTD-ALS (pooled in a single ALS group), eleven FTLD-TDP-A, three  
285 FTLD-TDP-B and nine FTLD-TDP-C were SarkoSpin-solubilized and layered on top of a linearized  
286 10-25% iodixanol continuous gradient before ultracentrifugation (310,000 g) for 45 minutes at 4°C  
287 (**Fig. 3a**). The gradients were then fractionated in 16 fractions of equal volume and TDP-43 or pTDP-  
288 43 distribution was assessed by immunoblot or dot blot using the respective antibodies. In both

289 control and disease samples monomeric, as well as oligomeric TDP-43, were retained in the top five  
290 fractions, peaking in the first three (**Fig. 3b-d** and **Supplementary Fig. 15-16**). In addition, in disease,  
291 but not in control samples, populations of larger sizes were identified, carrying the specific post-  
292 translational modifications of pathological TDP-43. In ALS samples, sedimentation profiles revealed  
293 one population of aggregated and phosphorylated TDP-43 in fractions 8 to 13 and peaking between  
294 10-11. FTLD-TDP-A sedimentations revealed a wide diversity of profiles, with TDP-43 aggregates  
295 sedimenting in fractions 5 to 14, and two apparent populations peaking in fractions 6-7 and in fraction  
296 11. The presence of a second subpopulation could indicate a difference in size or shape of the  
297 aggregates. Type A cases used in this study were separated in three different subgroups according to  
298 their genetic background: sporadic (with no familial history and no known mutations identified), *GRN*  
299 and *C9ORF72*, while all FTLD-TDP-B cases carried *C9ORF72* mutations. Despite slight differences in  
300 the relative distribution of aggregates, with the *GRN* patients favoring the larger TDP-43 populations,  
301 no striking patterns separating the three groups of patients classified as type A (**Supplementary Fig.**  
302 **17**), Importantly, neuropathological type rather than *C9ORF72* mutation better correlated with the size  
303 distribution of TDP-43 aggregates, as they followed the pattern observed for the respective disease  
304 type (peaks in fractions 6-7 and 11 for type A and fractions 10-12 for type B) and clearly separated in  
305 different groups.

306 Moreover, FTLD-TDP-C sedimentation profiles also revealed one broad population of TDP-43  
307 aggregates (**Fig. 3b-c** and **Supplementary Fig. 15-16**). However, in contrast to the other types, these  
308 assemblies were sedimenting further, in fractions 10-16, which may indicate that they were of bigger  
309 size and/or that their intrinsic density is higher. Purified recombinant human TDP-43 protein  
310 expressed in *E. coli* and aggregated *in vitro* concentrated in fractions 11-13, showing distinct  
311 properties from all patient-derived aggregates (**Supplementary Fig. 18a-b**). Importantly, hnRNPA1,  
312 FUS and SOD1 remained in the first three fractions in all disease types, indistinguishable from  
313 controls (**Supplementary Fig. 19**), indicating that the solubility of these proteins is not significantly  
314 altered in TDP-43 proteinopathies.

315 Furthermore, taking into account that the top five fractions correspond to the physiological TDP-43,  
316 while fractions 6 to 16 contain only the pathological assemblies, the percentage of aggregated TDP-  
317 43 could be calculated for each individual patient (**Supplementary Fig. 5b**) and was found between  
318 20-30% depending on the disease group. Levels of aggregated TDP-43 followed a similar trend as  
319 phosphorylated TDP-43 (**Supplementary Fig. 5a**), with FTLD-TDP-C being the highest, followed by  
320 FTLD-TDP-A and then ALS, albeit these differences did not reach statistical significance. The  
321 observation that there is more aggregated than phosphorylated TDP-43 (**Supplementary Fig. 5a-b**)  
322 indicates that not all aggregated TDP-43 was phosphorylated, and potentially also that not all  
323 pathological phosphorylation occurred at amino acids S409/410.

324

### 325 **Distinct density profiles of TDP-43 aggregates isolated from different types of TDP-43** 326 **proteinopathies**

327 To determine whether the observed differences in velocity sedimentation are due to distinct aggregate  
328 size, shape or density, we sought out to measure the intrinsic density of TDP-43 aggregates by  
329 combining our SarkoSpin solubilization conditions with density floatation gradients<sup>33</sup>. To this end, we  
330 submitted the same patient samples to SarkoSpin solubilization, followed by ultracentrifugation  
331 (125,000 g) in a 10-60% iodixanol discontinuous gradient for 17 hours at 4°C to reach the isopycnic  
332 equilibrium (**Fig. 4a**). The gradients were then fractionated and TDP-43 and pTDP-43 density profiles  
333 were determined as described above (**Fig. 4b-d** and **Supplementary Fig. 20-21**).

334 In all samples, the majority of normal TDP-43 was present in one peak spanning fractions 8 to 12, i.e.  
335 at an average density of 1.22 g/mL, as calculated by refractometry and iodixanol concentration  
336 (**Supplementary Fig. 22**). Remarkably, all density gradients from ALS, FTLD-TDP-A (**Fig. 4b-c**) as  
337 well as FTLD-TDP-B cases (**Supplementary Fig. 20a**) showed an additional population of TDP-43,  
338 present in one sharp peak in fraction 7, corresponding to density of 1.16 g/mL. This consistent  
339 population demonstrates all known TDP-43 pathological features such as ubiquitination, C-terminal  
340 cleavage, and phosphorylation (**Supplementary Fig. 21a-b**). Notably, pTDP-43 was not detected on

341 dot blots or immunoblots of all control density gradients, showing the specificity of the S409/410  
342 phosphorylation site as a marker of pathological TDP-43. The lower density of pathological TDP-43  
343 compared to the main population of its physiological counterpart is likely determined by the three-  
344 dimensional packing of these aggregates, rather than their direct association with lipids, as the  
345 purified human recombinant TDP-43 aggregates reconstituted *in vitro*, showed exactly the same  
346 density (**Supplementary Fig. 18c-d**).

347 Surprisingly, the density profiles of all nine FTLD-TDP-C samples were significantly different from all  
348 other types of TDP-43 proteinopathies tested. The FTLD-TDP-C pathological TDP-43 population was  
349 intermingled with the normal TDP-43 peak in denser fractions 8 to 10, i.e. 1.20 g/mL (**Fig. 4b-c**,  
350 **Supplementary Fig. 20b**, **Supplementary Fig. 21b**). These results strikingly show that pathological  
351 TDP-43 aggregates extracted from FTLD-TDP-C were ordered in a different fashion than those found  
352 in other FTLD-TDP subtypes or ALS. Therefore, the distinct histopathological profiles and clinical  
353 symptoms described for this subtype were directly correlated with this specific molecular signature.

354

#### 355 **Alternate morphologies of TDP-43 aggregates isolated from different disease subtypes**

356 To further characterize the properties and structural differences of these assemblies, we performed  
357 native PAGE immunoblots on density floatation gradient fractions from control and patient samples.  
358 Control density gradients were totally devoid of pTDP-43 signal, whereas the high molecular weight  
359 pTDP-43 aggregates described previously were present in fraction 7 and 8-10 for FTLD-TDP-A and  
360 FTLD-TDP-C, respectively (**Supplementary Fig. 23** green and blue arrows), in accordance with  
361 previous findings (**Fig. 4**). In addition to the pathological TDP-43 aggregates, two major populations of  
362 assemblies were distinguishable in all cases, including controls: one in fractions 8 to 12 under 66 kDa,  
363 and one in fractions 5 to 7 smearing from 66 kDa to 10 MDa. The latter was composed of a wide  
364 diversity of TDP-43 oligomers<sup>11</sup>, which were more buoyant than monomeric species. We estimated  
365 that under these conditions approximately 25-35% of total TDP-43 appears to be oligomeric in  
366 controls (**Supplementary Fig. 5c**). This further demonstrated that in diseased brains, TDP-43 is

367 present in different distinguishable arrangements: monomers, oligomers of different sizes and larger  
368 aggregates.

369 As the resolution of native PAGE electrophoresis did not allow for a precise definition of their sizes,  
370 extracted aggregates were subjected to filtration, to define whether these particles were smaller than  
371 220 nm, and if so, to further characterize them using electron microscopy (see below). SarkoSpin  
372 supernatants and pellets of all samples were subjected to syringe low-retention 0.22  $\mu\text{m}$  filtration, prior  
373 to dot blotting with TDP-43, pTDP-43 and hnRNPA1-specific antibodies (**Supplementary Fig. 24a**),  
374 without altering the relative partition of different proteins in supernatant or pellet fractions. Surprisingly,  
375 the majority of TDP-43 aggregates were recovered after filtration, as confirmed by positive pTDP-43  
376 signal on dot blots of filtered SarkoSpin pellets. Filtration resulted in an average reduction of pTDP-43  
377 signal of 33% and 36% in FTLD-TDP-A and FTLD-TDP-C SarkoSpin pellets, respectively  
378 (**Supplementary Fig. 24b**). The reduction of total TDP-43 due to filtration of pellets was lower, with  
379 20% and 30% respectively. Normal TDP-43 and hnRNPA1 were not retained in the filter as they  
380 showed signal reduction of less than 10% for all cases with no significant difference between control  
381 and disease samples. These results demonstrate that around 70% of TDP-43 pathological aggregates  
382 are smaller than 220 nm for all disease groups. Combined with the velocity sedimentation profiles, in  
383 which 70% of sedimenting TDP-43 showed an estimated size of 220 nm, the median size separating  
384 the Gaussian-like repartition of aggregated TDP-43 in two equal populations would be on average  
385  $\sim$ 150 nm.

386 In order to visualize TDP-43 aggregates, anti-TDP-43 immunogold labeling followed by transmission  
387 electron microscopy was performed on filtered SarkoSpin pellets of control, FTLD-TDP-A and FTLD-  
388 TDP-C cases (**Fig. 5a**). To avoid drying artifacts common in standard negative-stain EM, samples  
389 were embedded in methylcellulose. No immunoreactivity or large protein assemblies could be  
390 detected in control samples. In contrast, FTLD-TDP-A and FTLD-TDP-C SarkoSpin pellets contained  
391 numerous protein structures of various shapes and sizes, ranging from 50 to 150 nm, which were  
392 decorated by gold bead-labeled anti-TDP-43 antibody (**Fig. 5a**, red arrows). Interestingly, although the

393 same monoclonal antibody recognizing a short epitope spanning amino acids 203-209 under  
394 denaturing conditions did not show consistent differences between the two disease groups,  
395 immunogold-EM revealed differential immunoreactivity between the two disease subtypes.  
396 Assemblies isolated from FTLD-TDP-A patients were strongly immuno-positive whereas those  
397 extracted from FTLD-TDP-C cases were only sparsely labeled by the same antibody. This lower  
398 antibody detection points to distinct epitope accessibility, and thus different packaging of pathological  
399 TDP-43 type A versus type C aggregates. It is conceivable that the antibody epitope, which lies within  
400 the second RNA recognition motif (RRM2), is buried inside the core of FTLD-TDP-C aggregates.  
401 These results corroborate the higher density of type C aggregates and further confirm their  
402 discriminating three-dimensional shapes compared to type A.

403 Notably, we did not detect any long amyloid fibrils in any disease case, in agreement with the  
404 apparent lack of amyloidophilic staining in the majority of ALS/FTD patient brains<sup>43,44</sup>. Nevertheless,  
405 careful observation of the immunogold-EM images suggested the presence of short proteinaceous  
406 filaments of a thickness of 5 to 8 nm, and a length of 30 to 50 nm, which were frequently decorated by  
407 the anti-TDP-43 antibody in FTLD-TDP-A cases (**Fig. 5a**, orange arrows). Similar structures were  
408 detected in FTLD-TDP-C samples, albeit mostly independent from antibody labeling (**Fig. 5b**, orange  
409 arrows). These structures potentially originated from filamentous material in FTLD brains as  
410 previously reported<sup>45</sup>, which would be in agreement with a recent study showing high-resolution  
411 structure of a short TDP-43 fragment residing in its LCD<sup>46</sup>.

412 As the use of methylcellulose lowers the image resolution, immunolabelled SarkoSpin pellets  
413 prepared without this preservation step were also analyzed by transmission electron microscopy. This  
414 allowed us to obtain higher resolution images of type A TDP-43 aggregates. These showed  
415 heterogenous spheroid or elongated and coiled protein assemblies (**Fig. 5c**). Due to lower  
416 immunoreactivity, such structures could not be identified in type C samples. Importantly, both  
417 methods for immunogold-labeled sample preparation, with or without methylcellulose, showed no  
418 unspecific binding of the gold-conjugated secondary antibody in the absence of TDP-43 primary



419 antibody (**Supplementary Fig. 25a**). The presence of lipids that sometimes appeared adjacent to the  
420 TDP-43 aggregate structures (**Fig. 5a**, yellow arrows) could be efficiently removed by a slight  
421 adaptation of our SarkoSpin method with centrifugation over a sucrose cushion (**Supplementary Fig.**  
422 **25b**).

423 To obtain an independent, albeit indirect, measure of the different conformational features of TDP-43  
424 aggregates we used partial proteolysis. The latter has extensively been used as a surrogate for  
425 probing different pathological conformations of the prion protein, since the size of the proteolytic  
426 fragments is directly related to the specific site that is accessible to the chosen protease<sup>34,47</sup>. In  
427 contrast to the prion protein, TDP-43 is largely proteinase K-sensitive (**Supplementary Fig. 25c**), but  
428 has previously been reported to be partially resistant to chymotrypsin and trypsin<sup>31,32</sup>. Here, we  
429 systematically analyzed the proteolytic profile of TDP-43 species extracted from different disease  
430 subtypes (**Fig. 5d**). Indeed, chymotrypsin and trypsin-treated SarkoSpin pellets showed specific and  
431 consistent proteolytic profiles in each disease subtype, suggesting that the arrangement of these  
432 aggregates and, by extension, the borders of the protease-resistant cores are distinctive. Additionally,  
433 probing with antibodies raised against defined TDP-43 domains revealed resistance of the most C-  
434 terminal fragment within the low-complexity domain, suggesting that the cleavage site is hidden within  
435 the core of the aggregates. Interestingly, these were found to be significantly more resistant to  
436 proteolysis by trypsin in FTLD-TDP-C compared to type A (**Fig. 5e**). Lastly, we explored this  
437 proteolytic resistance of TDP-43 species to visualize the resistant cores of the aggregates, which we  
438 identified using immunogold labeling with an antibody against the TDP-43 phosphorylation sites at  
439 amino acids 409/410 (**Fig. 5f**). This treatment resulted in protein structures with high anti-pTDP-43  
440 immunopositivity for both FTLD-TDP-A and FTLD-TDP-C, further confirming that the lack of anti-  
441 RRM2 immunogold labeling in FTLD-TDP-C samples (**Fig. 5a**) was due to epitope accessibility within  
442 these TDP-43 assemblies. In conclusion, the observed differential resistance to proteolysis indicates  
443 distinct aggregate structures in different neuropathological subtypes, in agreement with immunogold-  
444 EM analysis.

445

446 **Differential cyto- and neurotoxicity of FTLD-TDP type A and type C pathological assemblies**

447 In order to determine whether these distinct TDP-43 conformations were associated with different  
448 toxic properties, we tested their effect on the viability of cell lines and primary neurons. To this end,  
449 we used the Flp-In T-Rex technology to generate a stable cell line with inducible expression of C-  
450 terminally tagged TDP-43, to obtain a homogeneous population of cells expressing traceable TDP-43  
451 at similar levels (**Supplementary Fig. 26a-c**), which is unfeasible by transient transfection. We used  
452 the HA tag, which comprises of only 9 amino acids and we placed it at the very end of the structurally  
453 disordered region to minimize any interference with protein structure, which might hinder protein  
454 seeding. We first used our TDP-43-HA-expressing cells to optimize the internalization of SarkoSpin-  
455 extracted GFP-labeled pTDP-43 aggregates produced in cells, further demonstrating that our novel  
456 extraction method can also be applied to cellular models of disease (**Supplementary Fig. 26d-f**).

457 To test if SarkoSpin specifically concentrated potentially toxic TDP-43 seeds, we subsequently  
458 introduced pellets from six control, nine FTLD-TDP-A and seven FTLD-TDP-C patients into mitotically  
459 arrested cells, since the continuous proliferation and high metabolic activity of these cells could mask  
460 the toxicity of exogenous aggregates. We reasoned that if toxicity of pTDP-43 assemblies was  
461 dependent on seeding of endogenous TDP-43, the effect would be more profound upon increased  
462 expression of the substrate, i.e. TDP-43-HA. Therefore, after induction of TDP-43-HA expression with  
463 doxycycline, we calculated the ratio of viability between both conditions (+DOX/-DOX). This analysis  
464 revealed that upon a 3-fold increase in TDP-43 protein levels (**Supplementary Fig. 26g**), the viability  
465 of cells inoculated with FTLD-TDP-A extracts was significantly decreased in comparison to that of  
466 cells inoculated with control or FTLD-TDP-C aggregates (**Fig. 6a-b**).

467 To directly test whether the observed toxicity is dependent on aggregation of endogenous TDP-43  
468 triggered by the exogenous TDP-43 seeds, we subsequently performed SarkoSpin on mitotically  
469 arrested TDP-43-HA-expressing cells inoculated with SarkoSpin pellet pools from three control, three  
470 FTLD-TDP-A and three FTLD-TDP-C patients. Immunoblot with anti-HA antibody against the

471 endogenously expressed TDP-43-HA fusion protein allowed for the unambiguous distinction of newly  
472 induced aggregates from exogenous patient-derived seeds. Interestingly, while levels of TDP-43-HA  
473 were equal in all conditions (**Supplementary Fig. 26g**), cells inoculated with FTLD-TDP-A pellets  
474 presented increased TDP-43-HA aggregation, compared to cells treated with control or FTLD-TDP-C  
475 seeds (**Fig. 6c**). Collectively, these data indicated that pTDP-43 assemblies extracted from FTLD-  
476 TDP-A, but not control or FTLD-TDP-C patients were cytotoxic and induced endogenous TDP-43  
477 aggregation, potentially via protein seeding. These findings are in line with the notion that FTLD-TDP-  
478 A aggregate species were more potent, thereby causing a more aggressive clinical phenotype with  
479 significantly shorter disease span than FTLD-TDP-C patients (**Supplementary Fig. 1e**).

480 To confirm the increased potency of FTLD-TDP-A aggregates in a disease-relevant system, we  
481 quantified their effect on the neurite length of primary cortical neurons, prepared from E18 mouse  
482 embryos and plated in 96-well plates, using high content screening<sup>48</sup>. Neurite length was previously  
483 found to be decreased by treatment with artificial oligomers of  $\alpha$ -synuclein, which are capable of  
484 entering cells and acting as seeds of an autonucleation process<sup>49</sup>. Neuronal cultures were exposed  
485 to four different doses of SarkoSpin pellet pools from control, FTLD-TDP-A or FTLD-TDP-C patients  
486 (**Fig. 6d**). In order to estimate the protein content of these pellets, all inocula were submitted to  
487 immunoblotting against TDP-43, pTDP-43 and actin (**Supplementary Fig. 27**). TDP-43 and pTDP-43  
488 were absent from control SarkoSpin pellets as expected, while their quantity in FTLD-TDP-C inocula  
489 was approximately half of FTLD-TDP-A for all doses (**Supplementary Fig. 27a-b**). Importantly, pTDP-  
490 43 levels were equivalent between FTLD-TDP-A at dose of 0.3  $\mu$ g and FTLD-TDP-C at 1  $\mu$ g total  
491 protein, which allowed the direct comparison of the two types of pathological pTDP-43 assemblies.  
492 Total protein amounts were normalized against actin for all doses between control, FTLD-TDP-A and  
493 FTLD-TDP-C (**Supplementary Fig. 27c**). The effect of exogenous pTDP-43 SarkoSpin extracts on  
494 neurite growth was then monitored by automated live cell imaging with combined phase-contrast and  
495 epifluorescence imaging over 10 days (**Fig. 6e,f**). In agreement with our observations in immortalized  
496 cells, TDP-43 aggregates extracted from FTLD-TDP-A drastically reduced neurite length (**Fig. 6h**),

497 which reached a plateau immediately after inoculation (after 5 days *in vitro* (DIV) at 55 nm length),  
498 while control or FTLD-TDP-C SarkoSpin pellets had no effect (plateau reached after 10 DIV at 80 nm  
499 neurite length). At 10 DIV, all primary neuronal cultures were submitted to calcein fluorescence  
500 imaging allowing counting of cell bodies and measuring neurite length for all conditions (**Fig. 6g-h**). As  
501 seen in phase contrast, calcein staining of live cells decreased drastically and proportionally to the  
502 dose of FTLD-TDP-A-extracted TDP-43 aggregates, while control and FTLD-TDP-C SarkoSpin pellet  
503 inoculations had no effect. This specific neurotoxic effect of FTLD-TDP-A aggregates was measured  
504 both by the decrease of cell body number (**Fig. 6g**) and neurite length (**Fig. 6h**) in a dose-dependent  
505 manner. Since levels of pTDP-43 in FTLD-TDP-C were identical to those of FTLD-TDP-A at one dose  
506 lower (i.e. pTDP-43 levels of FTLD-TDP-C at 0.3  $\mu\text{g}$  dose equals that of FTLD-TDP-A at 0.1  $\mu\text{g}$  dose),  
507 indicating that even at equivalent pTDP-43 inoculated amounts, FTLD-TDP-A SarkoSpin pellets  
508 inhibited significantly more neurite outgrowth and decreased neuronal viability compared to FTLD-  
509 TDP-C ones. Using this approach, automated analysis of the phase contrast and fluorescence images  
510 indicated that FTLD-TDP-A extracted TDP-43 pathological aggregates exhibit severe neurotoxicity in  
511 a dose-dependent manner (**Fig. 6f-h**).

512 Collectively, our data demonstrate that pathological TDP-43 adopts stable configurations with specific  
513 biophysical properties, which allow its physical separation from its physiological counterpart. Notably,  
514 the biochemical properties of pathological TDP-43 and a subset of insoluble proteins can discriminate  
515 between specific neuropathological subtypes, indicating that they may underlie specific disease  
516 characteristics. Most importantly, we show evidence that pTDP-43 aggregates extracted by our new  
517 purification method confer cyto-/neurotoxicity accompanied by induced aggregation of endogenous  
518 TDP-43. Remarkably, the most toxic aggregates are those found in the brains of FTLD-TDP-A  
519 patients, who show significantly shorter disease duration (**Supplementary Fig. 1e**), indicating a direct  
520 link between the properties of TDP-43 aggregates and clinical presentation.

521

## 522 **Discussion**

523 TDP-43 pathology characterizes most instances of ALS and FTD and its spatiotemporal manifestation  
524 coincides with neurodegeneration, supporting its central pathogenic role in these diseases<sup>2</sup>. Despite  
525 this unifying TDP-43 pathology, ALS and FTD patients display large heterogeneity in terms of clinical  
526 disease presentation, progression and neuropathological findings, a phenomenon that remains  
527 mechanistically unexplained. Recent discoveries indicate that several protein aggregation diseases  
528 follow a prion-like paradigm<sup>50</sup> through amplification of pathological conformers. These findings provide  
529 a plausible explanation of the origin of disease heterogeneity. Indeed, differential quaternary  
530 structures of the prion protein are responsible for different subtypes of infectious prion diseases and  
531 are associated with distinct neuropathological profiles and clinical presentations<sup>47</sup>. The notion of prion  
532 strains was recently expanded to other protein aggregation diseases with compelling data for tau<sup>51</sup>,  $\alpha$ -  
533 synuclein<sup>52</sup> and A $\beta$ ,<sup>53</sup> suggesting that this may indeed be a general phenomenon.

534 Here, we unambiguously show that human patient-derived TDP-43 adopts stable protein assemblies  
535 with specific biophysical properties, which allow for its physical separation from its physiological  
536 counterpart. We exploited these features to develop a reliable method for the isolation of TDP-43  
537 aggregates from the majority of normal proteins. Following our recent discovery that physiological  
538 TDP-43 forms oligomers that bind RNA<sup>11</sup>, we combined nuclease-dissociation with detergent  
539 solubilization without denaturation to accomplish an unprecedented level of separation of pathological  
540 TDP-43 assemblies from physiological complexes. We foresee that our new method, SarkoSpin, will  
541 become a valuable tool for any application requiring a source of faithful, disease-associated TDP-43  
542 aggregates.

543 We used SarkoSpin for the native extraction of pathological TDP-43 from autopsy brain tissues of >80  
544 patients from different types of TDP-43 proteinopathies and controls. An unbiased proteomic  
545 approach of isolated pathological assemblies revealed that TDP-43 was the most significantly  
546 enriched protein and the only one found insoluble in all disease cases, indicating that we isolated the  
547 pTDP-43 core of pathological assemblies. Comparison of other proteins that become insoluble in ALS  
548 and FTLD human brains revealed specific subclasses of proteins characterizing each disease

549 subtype. These proteins did not co-localize with pTDP-43 in patient brains and sometimes even  
550 occurred in different cell types – i.e. FBXO2 in astrocytes – suggesting that they most likely represent  
551 a downstream molecular signature of the divergent pathogenic mechanisms characterizing the  
552 different disease subtypes.

553 Combining SarkoSpin with a number of biochemical, molecular and imaging methodologies, we  
554 provide evidence that TDP-43 extracted from different disease subtypes exhibits distinct biochemical  
555 and morphological features. In particular, the intrinsic density of the isolated particles is remarkably  
556 consistent within each disease subtype, while it is a distinguishing feature of pathological TDP-43 in  
557 different FTLD types. Our results indicate that FTLD-TDP-C, an exclusively sporadic type of dementia  
558 with significantly longer disease duration than other types<sup>29</sup>, is associated with very dense TDP-43  
559 aggregates showing lower levels of polyubiquitination and a highly protease resistant C-terminal core,  
560 compared to TDP-43 assemblies extracted from FTLD-TDP-A or ALS. While no long fibrillary  
561 structures were detected within TDP-43 aggregates of either disease subtype, short proteinaceous  
562 filaments were present in both, albeit with differential accessibility to specific antibodies, indicative of  
563 alternate aggregate packaging. Surprisingly, the neuropathological type, rather than the genetic cause  
564 of the disease, better predicted the features of TDP-43 aggregates, indicating that the same genetic  
565 trigger may give rise to variable pathological assemblies, perhaps determined by other, still unknown  
566 factors.

567 Most importantly, these biochemical and morphological differences in the pTDP-43 assemblies  
568 extracted from FTLD-TDP-A or FTLD-TDP-C patients were associated with differential seeding and  
569 neurotoxic potential. Indeed, FTLD-TDP-A aggregates caused toxicity and induced endogenous TDP-  
570 43 aggregation in cellular models, while FTLD-TDP-C seemed inert under the same conditions and  
571 equivalent pTDP-43 doses. This finding indicated that FTLD-TDP-A aggregates extracted from  
572 patients with fast progressing disease possessed high seeding and neurotoxic potential, while FTLD-  
573 TDP-C aggregates from slow progressing disease were inert. Moving forward and following the  
574 paradigm now established for several other protein aggregates<sup>51,52,53</sup>, it would be exciting to find out

575 whether these patient-derived TDP-43 pathological conformations can faithfully propagate *in vivo*,  
576 reproducing their distinctive features and disease phenotypes in animal models. Collectively, our work  
577 suggests that disease heterogeneity may originate from alternate pathological TDP-43 conformations,  
578 reminiscent of prion strains and opens new avenues for creating cellular and animal models.

579

## 580 **Materials and methods**

### 581 **Cases**

582 Brains were donated to the Queen Square Brain Bank for Neurological disorders, Department of  
583 Movement Disorders, UCL Institute of Neurology, London, UK, or the Department of Neurology,  
584 University of California, San Diego, CA, USA, or the Netherlands Brain Bank, Netherlands Institute for  
585 Neuroscience, Amsterdam, Netherlands (open access: [www.brainbank.nl](http://www.brainbank.nl)). All patient material has  
586 been collected from donors for or from whom a written informed consent for a brain autopsy and the  
587 use of the material and clinical information for research purposes has been obtained by the respective  
588 institution.

589

### 590 **Immunohistochemistry**

591 Fresh tissue from frontal cortex of FTLD-TDP-A cases, either sporadic or with *C9ORF72* mutation, as  
592 well as a FTLD-TDP-C cases were fixed with phosphate-buffered 3.65% formaldehyde, embedded in  
593 paraffin, cut into 6 µm serial sections and mounted on glass slides. Sections were deparaffinized and  
594 rehydrated using a gradient of alcohol. Antigen retrieval was done by boiling the sections in 10 mM  
595 citrate buffer (pH 6.0) in a microwave oven. Immunohistochemistry was performed using the avidin-  
596 biotin complex (ABC) detection system (Vector Laboratories) and 3,3-diaminobenzidine. Endogenous  
597 peroxidases were first quenched with 5% H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes, and sections were blocked  
598 in 0.1 mol/L Tris with 2% fetal bovine serum for 5 minutes. Primary antibodies were incubated for 2  
599 hours at room temperature. After washing, sections were sequentially incubated with biotinylated  
600 secondary antibodies for 1 hour and avidin-biotin complex for 1 hour. Bound antibody complexes were

601 visualized by incubating sections in a solution containing 100 mM Tris, pH 7.6, 0.1% Triton X-100, 1.4  
602 mM di-aminobenzidine, 10 mM imidazole, and 8.8 mM H<sub>2</sub>O<sub>2</sub>. Sections were then lightly  
603 counterstained with hematoxylin, dehydrated, and coverslipped. Digital images were obtained using  
604 Life Technologies EVOS FL Auto imaging system (Life Technologies).

605

606 All antibodies used for immunofluorescence and western blot are listed in **Supplementary Table 2**  
607 with the respective catalogue numbers and working dilutions indicated for each assay.

608

### 609 **Homogenization of brain tissue**

610 Frozen tissue from motor or frontal cortex of all groups of patients were homogenized at 20 % w/v in  
611 homogenization - solubilization buffer (HS) without detergent: 10 mM Tris pH 7.5, 150 mM NaCl, 0.1  
612 mM EDTA, 1 mM DTT, Complete EDTA-free protease inhibitors (Roche) and PhosSTOP  
613 phosphatase inhibitors (Roche). Samples were subjected to three rounds of 30 seconds  
614 homogenization in tubes containing a mixture of ceramic beads of 1.4 and 2.8 mm of diameter on a  
615 Minilys device (Bertin) at full speed with cooling on ice between each round. Samples were aliquoted,  
616 snap frozen in liquid nitrogen and kept in -80°C before use.

617

### 618 **SarkoSpin on patient tissues**

619 All SarkoSpin steps were done in 1.5 mL Protein Low Binding tubes (Eppendorf). Brain homogenate  
620 aliquots of 150 µL were thawed on ice and equalized for protein concentration in final volume of 200  
621 µL using HS buffer. Samples were then diluted 1:1 in HS buffer with 4% (w/v) N-lauroyl-sarcosine  
622 (sarkosyl, Sigma), 2 U/µL Benzonase (Novagen) and 4 mM MgCl<sub>2</sub>, reaching final volume of 400 µL  
623 with 2% Sarkosyl, 1 U/µL Benzonase and 2 mM MgCl<sub>2</sub> final concentrations. SarkoSpin solubilization  
624 was then performed by incubating at 37°C under constant shaking at 600 rpm (Thermomixer,  
625 Eppendorf) for 45 minutes. Solubilized brain homogenates (400 µL) were then further diluted by  
626 adding 200 µL of ice-cold HS buffer containing 0.5% w/v sarkosyl prior to centrifugation at 21,200 g on



627 a benchtop centrifuge (Eppendorf) for 30 minutes at room temperature. Alternatively, for EM sample  
628 preparation, to remove lipids from precipitating along with protein aggregates, solubilized material was  
629 overlaid on top of 400  $\mu$ l sucrose cushion (40% w/v sucrose (Sigma) in HS buffer containing 0.5% w/v  
630 sarkosyl) and centrifuged at 21,200 g for 1 hour at room temperature. For the analysis of proteolytic  
631 profiles, after the initial 15 minutes of SarkoSpin solubilization, the respective protease was added for  
632 the remaining 30 minutes of incubation in the following final concentrations: 100  $\mu$ g/mL for Proteinase  
633 K, 10  $\mu$ g/mL for Chymotrypsin or 100  $\mu$ g/mL for Trypsin (from stock solutions of 2 mg/mL, 0.2  
634 mg/mL or 2 mg/mL, respectively). Supernatants (approx. 600  $\mu$ L) were collected in a new tube and  
635 pellets were resuspended in desired volume of HS buffer containing 0.5 % w/v sarkosyl for further  
636 analysis, including dot blot, SDS-PAGE and immunoblot or native PAGE. Whenever specified, the  
637 fractions were passed through low protein binding 0.22  $\mu$ m filters (Millex-GV) to remove large non-  
638 solubilized particles.

639

#### 640 **SarkoSpin on cells**

641 Transfected NSC-34 cells in 10 cm dishes (see below) were lysed in 400  $\mu$ L of ice-cold 1X HS buffer  
642 with 0.5% sarkosyl, 2 mM  $MgCl_2$  and ~100 U Benzonase, and 60  $\mu$ L of the lysate was put aside for  
643 protein concentration measurements and immunoblots. For SarkoSpin of inoculated HEK cells, cells  
644 plated in a 6-well plate were lysed in 170  $\mu$ l of ice-cold 1X HS buffer with 0.5% sarkosyl, 2 mM  $MgCl_2$   
645 and ~25 U Benzonase per well. For sample solubilization, to aliquots of 170  $\mu$ L of lysate, 52  $\mu$ L 1X HS  
646 and 178  $\mu$ L 2X HS with 4% sarkosyl were added, for a final concentration of 2% sarkosyl in 400  $\mu$ L of  
647 sample. After a 30-minute incubation on ice with vortexing every 10 minutes, the samples were diluted  
648 by adding 200  $\mu$ L of ice-cold HS buffer and centrifuged as described before for SarkoSpin on brain  
649 homogenates. Supernatants were collected in a new tube and pellets were resuspended in 1X HSI  
650 buffer with 0.5% sarkosyl for immunoblot samples or in PBS for inoculation experiments in HEK cells.  
651 SarkoSpin fractions from cell line experiments were processed for immunoblot as described before for  
652 brain homogenates.

653

654 **Mass Spectrometry**

655 After clean-up with C18 cartridges, the peptide samples were measured on a TripleTOF 5600  
656 instrument (AB Sciex, Concord ON) equipped with a nano-electrospray ion source and an Eksigent  
657 1D-plus liquid chromatography system (Eksigent, Dublin, CA) or a Q Exactive Plus instrument  
658 (Thermo Fisher) equipped with an EASY-nLC 1000 (Thermo Fisher). For the TripleTOF  
659 measurements, peptides were loaded onto a 20-cm long, 75  $\mu\text{m}$  i.d. PicoFrit Column (New Objective,  
660 Woburn, MA) packed in-house with Magic C18 AQ 3  $\mu\text{m}$  beads (Michrom Bioresources, Auburn, CA)  
661 and separated with a linear gradient from 5% to 35% acetonitrile in 90 min at a flowrate of 300 nL/min.  
662 Survey scans were acquired in 300 ms and up to 20 product ion scans were collected in 75 ms if  
663 exceeding a threshold 150 counts per second with a charge state from 2+ to 5+. For the Q Exactive  
664 Plus measurements, peptides were loaded on a 40 cm long, 75  $\mu\text{m}$  i.d. column packed in-house with  
665 ReproSil-Pur C18-AQ resin (1.9  $\mu\text{m}$ , Dr. Maisch). Peptides were eluted for 50 min using a segmented  
666 linear gradient of 5% to 40% acetonitrile at a flow-rate of 300 nL/min. Survey full-scan mass spectra  
667 were acquired with mass range 350-1500 m/z, at a resolution of 70,000 at 200 m/z and the 20 most  
668 intense ions above an intensity of  $3.6 \times 10^4$  were sequentially isolated, fragmented (HCD at 25 NCE) and  
669 measured at a resolution of 17,500 at 200 m/z. Peptides with a charge of +1 or with unassigned  
670 charge state were excluded from fragmentation for MS2, and a dynamic exclusion of 30 s was  
671 applied. Ions were accumulated to a target value of  $3 \times 10^6$  for MS1 and of  $1 \times 10^5$  for MS2.  
672 MGF-peak lists generated from the acquired spectra by ProteinPilot from the TripleTOF runs and  
673 .RAW files from the Q Exactive Plus runs were searched against a human Uniprot protein database  
674 (68978 sequence) by Sequest HT in Proteome Discoverer 1.4 (Thermo Scientific, San Jose, CA) with  
675 enzymatic specificity trypsin, precursor mass tolerance 20 ppm and fragment tolerance 0.1 Da and  
676 0.02 Da for TripleTOF and Q Exactive Plus, respectively. Methionine oxidation and  
677 carbamidomethylation of cysteines were set as respectively variable and static modifications.

678 Percolator was used to filter the results to a 1% peptide false discovery rate. Spectral counting was  
679 used for quantifying protein differences between samples.

680

### 681 **Statistical Analysis of Mass Spectrometry Data**

682 Unless stated otherwise the higher-level data analysis was carried out using R/Bioconductor (R Core  
683 Team, 2017). The count values for each sample were normalized by dividing the values by the  
684 respective trimmed mean (removing the highest and lowest 5% values). As the samples were run in  
685 two separate batches, the ComBat method<sup>54</sup> was applied to correct for the batch effect. To identify  
686 proteins that were most consistently enriched in patient samples, we established an idealized vector  
687 correlation method: a vector of the same number of 0s as control samples and the same number of 1s  
688 patient samples was created. For each protein, we measured the correlation of this idealized vector to  
689 the vector of normalized and batch-corrected protein abundance values of control and patient  
690 samples. We then selected the 50 best-correlated proteins and established a heatmap of the log<sub>2</sub>  
691 abundance values. For the heatmap, we applied a median per protein normalization, by dividing the  
692 values of each protein by the respective median value of the control samples.

693

### 694 **Validation of Mass Spectrometry hits by immunofluorescence**

695 Formalin fixed paraffin embedded tissue was sectioned at 7  $\mu\text{m}$ . Sections were deparaffinized in  
696 xylene and rehydrated using a gradient of alcohol. Endogenous peroxidases were first quenched with  
697 0.3%  $\text{H}_2\text{O}_2$  in methanol for 10 minutes. Antigen retrieval was undertaken in a pressure cooker for 10  
698 minutes at maximum pressure in 10 mM citrate buffer (pH 6.0) and sections were blocked in 10%  
699 non-fat milk for 30 minutes. Sections were incubated with phosphorylation-specific rabbit polyclonal  
700 pAb anti-pTDP (pS409 1:20,000 Cosmobio). After washing, sections were sequentially incubated with  
701 biotinylated swine anti-rabbit secondary antibody for 30 minutes and avidin-biotin complex for 30  
702 minutes. pTDP-43 was visualized using TSA Rhodamine amplification (Perkin-Elmer) at 1:500 for 30  
703 minutes. Sections were then incubated with antibodies raised against either TLXN1, FBXO2, ASAH1

704 followed by incubation in AlexaFluor 468 at 1:1000 for 1 hour at room temperature. Sections were  
705 viewed and imaged with a Leica TCS4D using a 3-channel scan head fluorescent microscope and  
706 image deconvoluted using Leica software.

707

#### 708 **Velocity sedimentation and density floatation gradients**

709 Brain homogenates were thawed on ice and solubilized as described previously (SarkoSpin). For  
710 velocity sedimentations, a volume of 200  $\mu$ L was loaded on top of a 4 mL continuous 10-25%  
711 iodixanol gradient (Optiprep, Sigma) in HS buffer with 0.5% w/v sarkosyl linearized directly in  
712 ultracentrifuge 4.2 mL tubes (Seton) with a Gradient Master (Biocomp). For density floatation  
713 gradients, 220  $\mu$ L of HS buffer with 40% iodixanol and 0.5% w/v sarkosyl was loaded within a 4 mL  
714 10-60% discontinuous iodixanol gradient in HS buffer with 0.5% w/v sarkosyl. The gradients were  
715 centrifuged at 310,000 g for 45 min (velocity) or at 125,000 g for 17 hours (density) in a swinging-  
716 bucket SW-60 Ti rotor using an Optima XPN-100 ultracentrifuge (Beckman Coulter). Gradients were  
717 then segregated into 16 equal fractions from the top using a piston fractionator (Biocomp) and a  
718 fraction collector (Gilson). Fractions were aliquoted for further analysis of their content by dot blot,  
719 immunoblot on SDS-PAGE or native-PAGE. Gradient linearity was verified by refractometry.

720

#### 721 **Analysis of protein content of SarkoSpin, velocity and density fractions**

722 Aliquots of the collected fractions were mixed with SDS-PAGE loading buffer with reducing agent (Life  
723 Technologies) and denatured at 95°C for 5 minutes, prior to migration on Bolt 12% Bis-Tris gels (Life  
724 Technologies). For visualizing total proteins, gels were stained with silver nitrate. For immunoblots,  
725 gels were electro-transferred onto nitrocellulose membranes with iBlot 2 (Life Technologies). For dot  
726 blotting, native fractions were spotted onto nitrocellulose 0.2  $\mu$ m membranes using a dot blot vacuum  
727 device (Whatman). Nitrocellulose membranes were blocked with 5 % w/v skimmed powder milk (or 5  
728 % w/v BSA for pS409/410 antibody) in PBS-Tween and probed with primary antibodies  
729 (**Supplementary Table 2**). Immunoreactivity was visualized by chemiluminescence (GE Healthcare).

730 The amount of the respective protein in each fraction was determined by the Image Studio Lite  
731 software, after acquisition of chemiluminescent signals with a Fuji LAS 4000 digital imager (GE  
732 Healthcare). Profiles obtained by immunoblot were normalized and plotted with SD, with all respective  
733 student test and ANOVA using the Prism software. Fractions were also subjected to native-PAGE  
734 immunoblotting, for which aliquots were mixed with native sample buffer and G250 additive (Life  
735 Technologies) and resolved using 3-12% Bis-Tris Native PAGE gels (Life Technologies) and  
736 NativeMark protein ladder (Life Technologies). Gels were incubated with 2x transfer buffer and electro-  
737 transferred with iBlot 2 on PVDF membranes. After fixation with 8% acetic acid, air-drying and  
738 reactivation with methanol, membranes were immunoblotted and probed as described above.

739

#### 740 **Transmission Electron Microscopy**

741 SarkoSpin pellets from different disease types and control patients were resuspended in water and  
742 passed through protein low binding 0.22  $\mu\text{m}$  syringe filters. The resulting filtrates were fixed with  
743 glutaraldehyde water solution (EMS #16220) (final concentration of 0.05 %) and subjected to immuno-  
744 gold labeling. A 300 mesh copper grid (Plano #G2300C), formvar coated and glow discharged, was  
745 put on a 3  $\mu\text{L}$  drop of fixed sample for 3 minutes. The grid was then washed 3 times 20 seconds in  
746 aldehyde blocking solution with 0.15 % Glycine (Sigma #G7126) in PBS and 3 times 20 seconds in  
747 PBS, followed by 5 minute incubation in blocking solution (PBG): PBS with 0.2 % gelatin (Sigma  
748 #G6650) and 0.5 % BSA (Applichem #A6588). The grid was probed with TDP-43 60019-2-Ig (mouse  
749 1:50 in PBG solution, Proteintech) for 20 minutes (the negative control without primary antibody was  
750 incubated instead in PBG solution). The grid was washed in PBG solution 3 times 20 seconds, and  
751 incubated for another 5 minutes in PBG, before probing with secondary antibody anti-mouse gold 12  
752 nm gold (1:20 in PBG solution, Jackson ImmunoResearch #115-205-146) for 20 minutes. The grid  
753 was washed 4 times 20 seconds in PBG and twice 20 seconds in PBS, followed by a second fixation  
754 step for 30 seconds in 0.1 % glutaraldehyde in PBS and 4 times 20 seconds washed in water. The  
755 grid was then counterstained with 1 % uranyl acetate (Fluka #73943) in water for 1 minute. Excess

756 solution was sucked off using filter paper and the grid was air-dried. Alternatively, to avoid drying  
757 artifacts, the counterstaining solution included 1.8 % methylcellulose (Sigma) in water and 0.3 %  
758 uranyl acetate in water (incubation for 3 minutes staining procedure on ice). Lastly, the grid was  
759 picked up with a loop, excess solution was sucked off with filter paper, air-dried, and the specimen  
760 imaged with a CM100 transmission electron microscope (Thermo Fisher Scientific, Eindhoven, The  
761 Netherlands) at an acceleration voltage of 80 kV using a Gatan Orius 1000 digital camera (Gatan,  
762 Munich, Germany).

763

#### 764 **Plasmid generation**

765 A multiply-tagged TDP-43-encoding pcDNA5 plasmid<sup>55</sup> was modified in order to obtain a sequence  
766 coding for a single C-terminally HA-tagged TDP-43. The N-terminal GFP, His6 and myc (2) tags and  
767 the residual C-terminal amino acids between the HA tag and the stop codon were deleted in two  
768 subsequent reactions using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, E0554S)  
769 according to the manufacturer's instructions, with the following primers: 5'-TCT GAA TAT ATT CGG  
770 GTA ACC GAA GAT GAG AAC GAT GAG-3', 5'- CAT CCG CGG GGC AGG GGT-3' (tags, Ta = 72  
771 °C), 5'-TAA ACC CGC TGA TCA GCC-3', 5'-AGC ATA ATC AGG GAC ATC ATA AG-3' (residual  
772 amino acids, Ta = 63 °C).

773

#### 774 **Cell culture, stable cell line generation and transient transfection**

775 HEK293 Flp-In T-REx cells (Invitrogen, R78007) were cultured in DMEM (Sigma, D5671)  
776 supplemented with 10% FBS (Gibco, 10270-106), 1X PenStrep (Gibco, 15140-122), 1X GlutaMAX  
777 (Gibco, 35050061), 15 µg/mL blasticidin S (Gibco, R21001; Invivogen, ant-bl-10p) and 100 µg/mL  
778 zeocin (Gibco, R25001). In order to generate a stable cell line expressing HA-tagged TDP-43, 3·10<sup>5</sup>  
779 cells were plated per well in a 6-well plate and transfected after 48 hours with 125 ng of pcDNA5-  
780 TDP43-HA plasmid and 1125 ng of pOG44 plasmid with 6.25 µL Lipofectamine 2000 (Invitrogen,  
781 11668019) according to the manufacturer's instructions. After a 48-hour recovery, cells were

782 passaged to a 10-cm dish with same media as before with the substitution of zeocin for 100 µg/mL  
783 hygromycin (Gibco, 10687-010) for the selection of cells that integrated the TDP-43-HA encoding  
784 sequence in the genome. Cells were selected for 2-3 weeks before expansion and banking, and  
785 afterwards cultured in the same medium.

786 For production of SarkoSpin-insoluble phosphorylated GFP-TDP-43 aggregates,  $2 \cdot 10^6$  motor neuron-  
787 like mouse NSC-34 cells were plated on a Matrigel-coated (Corning, 354234) 10 cm dish in  
788 differentiation media, consisting of DNEM/F-12 (Gibco, 21331-020) supplemented with 1X B27  
789 supplement (Gibco, 17504-044), 1% GlutaMAX, 1X N2 supplement (Gibco, 175020-01) and 0.1X  
790 PenStrep. After 48 hours, cells were transfected with 5 µg of GFP-TDP-43-HA and 20 µL  
791 Lipofectamine 2000 according to the manufacturer's instructions.

792

### 793 **Viability assay on HEK293 cells**

794 To study the toxicity of the SarkoSpin-insoluble pellets,  $6 \cdot 10^4$  HEK293 cells/well were plated on a  
795 Matrigel-coated 24-well plate and TDP-43-HA expression was induced after 24 hours with 1 µg/mL of  
796 doxycycline. 20 hours later, cells were mitotically arrested with 10 µM AraC (Sigma, C1768), and after  
797 another 6-8 hours, transfected with freshly-prepared SarkoSpin pellet fractions. To prepare the  
798 inocula, SarkoSpin pellets were washed with 300 µl of sterile PBS to remove traces of sarkosyl and  
799 remove the vast majority of lipid contaminants. Pellets were subsequently resuspended in 200 µl cell-  
800 culture grade PBS and sonicated at 60% amplitude for 2 minutes of sonication time (1 s ON /1 s OFF  
801 pulses) in water-bath sonicator (Q500, QSonica). 20 µl of sonicated material was transfected with 2.5  
802 µL Lipofectamine in a total volume of 290 µL OptiMEM per well. Additional 210 µL of media was  
803 added 4 hours after protein transfection, followed by whole media change after 24 hours, with fresh  
804 doxycycline and AraC. For the viability measurements, on day 3 after lipofection, 55 µL of 5 mg/mL  
805 Thiazolyl Blue Tetrazolium Bromide (Sigma, D5655) was added per well and incubated at 37 °C for 1  
806 hour. The reaction was stopped and the formazan solubilized with 100 µL of a solution containing  
807 16% sodium dodecyl sulfate (SDS), 40% dimethylformamide (DMF) and 2% glacial acetic acid.

808 Absorption of the solubilized formazan at 570 nm was subtracted the absorption from cell debris and  
809 other contaminants at 630 nm. The values of absorption in cells expressing TDP-43-HA were divided  
810 by the those of non-induced cells, and the value of the ratio of cells inoculated with SarkoSpin pellets  
811 from control patients set to one by dividing every value by the average ratio of all control conditions.  
812 Every condition was performed in triplicates.

813

#### 814 **Immunocytochemistry**

815 TDP-43-HA-expressing HEK293 on microscopy 24-well plates (Ibidi, 82406) inoculated with GFP-  
816 TDP-43-HA aggregates were wash once with PBS before fixation in 4% formaldehyde (brand) for 10  
817 minutes at room temperature. After washing twice with PBS, cells were permeabilized and blocked in  
818 blocking buffer (10% donkey serum (Millipore) with 0.3% Triton X-100 (Sigma) in PBS) for at least 30  
819 minutes at room temperature. Primary antibodies against HA-tag (1:500, Cell Signalling Technologies)  
820 and GFP (1:1000, Rockland Immunochemicals) were incubated in blocking buffer overnight at 4 °C.  
821 After three washes with PBS, secondary antibody Alexa-647 donkey anti-rabbit (Invitrogen, A-31573)  
822 was applied in PBS for 1 hour at room temperature, followed by three additional washes in PBS. Cells  
823 were covered with coverslips using ProLong Gold Antifade Mountant with DAPI (Invitrogen, P36931).

824

#### 825 **Primary mouse cortical neuronal culture in 96-well plate**

826 Timed pregnant C57/BL6J female mice were received from Charles River Labs 2 days before  
827 initiation of the primary culture. Cortices were harvested from E18 mouse embryos and dissociated  
828 enzymatically and mechanically (using the neuronal tissue dissociation kit, C-Tubes, and an  
829 Octodissociator with heaters, Miltenyi Biotech, Germany) to yield a homogenous cell suspension. The  
830 cells were then plated at 20000 per well in 96-well plates in neuronal medium (Neuronal Macs  
831 medium, Miltenyi Biotech, Germany) containing 0.5% Penicillin/Streptomycin, 0.5 mM alanyl-  
832 glutamine, and 2% Neurobrew supplement (Miltenyi Biotech, Germany). In such cultures, and in  
833 control conditions, neurons represented approximately 85-95% of the cell population, with astrocytes



834 and oligodendrocytes reaching 5-15% (GFAP+ or CNPase+), while the microglia (Iba1+) were  
835 undetectable (not shown). Thus, in the text, and for the sake of simplicity, they are referred to as  
836 “neurons”. After 4 days *in vitro* (DIV), Sarkosyl pellet pools from 3 Control, 3 FTLD-TDP-A or 3  
837 FTLD-TDP-C patients were resuspended in sterile PBS and sonicated with a cup horn sonicator  
838 (Q500, QSonica) at 100% set amplitude for 4 minutes of 1 second pulse / 1 second pause, and added  
839 to the medium at four different dilutions after normalization based on BCA-measured protein  
840 concentrations (1, 0.3, 0.1, 0.03 µg of total protein amount). For controlling total protein amounts, as  
841 well as levels of TDP-43 and pTDP-43 in the inocula, all treatment medium were submitted to dot  
842 blotting with antibodies against actin, TDP-43 and pTDP-43.

843

#### 844 **High content imaging of neurite outgrowth**

845 Neuritic network development was followed by phase contrast<sup>48</sup>. The signal was acquired using an  
846 Incucyte S3 platform (Essen Biosciences) with a 20x objective. White light was used for phase  
847 contrast, green and red leds for fluorescence. Neurite outgrowth was computed from both the phase  
848 contrast (time lapse) and fluorescence images (endpoint) using the Neurotrack software (Essen  
849 Biosciences). At 4 DIV, cultures were then challenged with the four concentrations of SarkoSpin  
850 pellets or medium for untreated wells. At 6 days post inoculations, effects of the treatments were  
851 evaluated by live cell staining with Calcein-AM to visualize the viable neurons and their processes.

852

853

#### 854 **Figure legends** (max 250 words/legend)

855 **Figure 1:** SarkoSpin physically separates pathological TDP-43 from the majority of normal proteins in  
856 FTLD and ALS brains

857 **a.** Schematic representation of SarkoSpin protocol, including the number of autopsy samples from  
858 each disease type used in this figure. Briefly, samples were homogenized and solubilized with  
859 sarkosyl at 37°C in presence of Benzonase prior to a single centrifugation step to separate

860 supernatant and pellet fractions. **b-j.** Biochemical analysis of SarkoSpin fractions extracted from  
861 human brain samples. Supernatants and pellets were subjected to SDS-PAGE followed by silver  
862 staining of total protein (**c**), or immunoblotted against total TDP-43 (**b, e**), phosphorylated TDP-43 at  
863 position S409/410 (pTDP-43) (**g**), or hnRNPA1, hnRNPH, FUS, SOD1, Histone H3 and actin  
864 antibodies (**i**). Physiological TDP-43 was found exclusively in supernatants for all patients, while the  
865 pathological species marked by phosphorylation were pelleted for all disease types, but are absent in  
866 controls. Pathological posttranslational modifications (PTMs) of TDP-43 were observed specifically in  
867 SarkoSpin pellets of disease cases: poly-ubiquitination smear (\*\*\*) , hyper-phosphorylation of TDP-43  
868 detected by both anti-pTDP-43 (S409/410) and anti-TDP-43 (total) as a slower migrating band at 45  
869 kDa (\*\* ) and the C-terminal fragment of 25 kDa (\*). **d,f,h,j.** Quantifications of the percentage of each  
870 protein residing in SarkoSpin supernatant or pellet for all patients used in this experiment. Error bars  
871 represent standard deviations. S – supernatant, P – pellet.

872

873 **Figure 2:** SarkoSpin extracts from different disease subtypes have distinct protein compositions  
874 beyond TDP-43

875 **a.** Overlap of proteins with the highest values of idealized vector correlation when comparing each  
876 ALS, FTL-D-TDP-A, and FTL-D-TDP-C to controls. Only proteins with strong correlation values >0.5  
877 were taken into account. The only common protein among the three disease subtypes is TDP-43. The  
878 proteins of interest, which were further validated by immunoblot and immunohistochemistry analysis  
879 of the SarkoSpin pellets, are highlighted in bold. **b.** Representative immunoblots on SarkoSpin pellet  
880 probed against several mass spectrometry hits – PFN1, ASAH1 and TXNL1, showing their  
881 enrichment in the insoluble fraction in respective disease subtypes. **c.** Correlation dot plots of pTDP-  
882 43 levels and insolubility of selected mass spectrometry candidates analyzed in (**b**).. **d.**  
883 Representative immunoblots on SarkoSpin pellet and supernatant fractions probed with ubiquitin  
884 antibody, indicating enrichment of ubiquitinated pathological assemblies in the SarkoSpin pellets  
885 fractions. **e.** Correlation dot plot of pTDP-43 and ubiquitin levels in SarkoSpin pellets across disease

886 subtypes. **f.** Co-immunofluorescence of pTDP-43 with ubiquitin in FTLD-TDP-A and FTLD-TDP-C  
887 post mortem brain sections. **g.** Quantification of aggregates double-positive for pTDP-43 and ubiquitin  
888 in FTLD-TDP-A and FTLD-TDP-C, showing significantly lower ubiquitination levels in type C (dots  
889 represent single % values quantified per each image; the error bars show mean with SD). **h.**  
890 Representative images of co-immunofluorescence of GFAP marking astrocytes with a mass  
891 spectrometry hit FBXO1, showing its upregulation in astroglia specifically in FTLD-TDP-A, but not  
892 FTLD-TDP-C. In panels **f** and **h** the images are overlaid with DAPI nuclear staining in blue.

893

894 **Figure 3:** Diverse size distributions of TDP-43 aggregates isolated from different types of TDP-43  
895 proteinopathies

896 **a.** Schematic representation of the velocity sedimentation protocol. After SarkoSpin solubilization,  
897 brain homogenates were loaded on top of iodixanol gradients and fractionated by sedimentation  
898 velocity upon ultracentrifugation. **b.** Representative immunoblots of the distribution of TDP-43 (top  
899 panels, immunoblots) and pTDP-43 (lower panels, dot blots) on velocity sedimentation fractions  
900 (numbered from top to bottom of the gradient). **c-d.** The relative amounts of TDP-43 (**c**), and pTDP-43  
901 (**d**) per fraction were quantified from control (black, n=6), ALS (red, n=4), FTLD-TDP A (green, n=10),  
902 FTLD-TDP C (blue, n=8) velocity sedimentation fractionations. Data presented are the mean curves  
903 (bold lines) with standard deviations (lighter shade areas) for each patient group. Panels below the  
904 graphs show the statistical significance of the difference in TDP-43 partition in each fraction, based on  
905 student t-test comparisons of the different groups.

906

907 **Figure 4:** Distinct density profiles of TDP-43 aggregates isolated from different types of TDP-43  
908 proteinopathies

909 **a.** Schematic representation of the density floatation protocol. Briefly, after SarkoSpin solubilization,  
910 brain homogenates were loaded in the middle of iodixanol gradients and fractionated by density upon  
911 isopycnic equilibrium ultracentrifugation. **b.** Representative immunoblots of the density sedimentation

912 profiles of physiological and pathological TDP-43 in various disease types. SarkoSpin-solubilized  
913 brain homogenates from frontal and motor cortex of control (black), ALS (red), FTLN-TDP-A (green),  
914 and FTLN-TDP-C (blue) were fractionated by density and the distribution of total TDP-43 (top panels,  
915 immunoblots) and of pTDP-43 (bottom panels, dot blots) was analyzed in the collected fractions  
916 (numbered from top to bottom of the gradient). Density values calculated by refractometry are  
917 indicated on the top of the graph. **c-d.** The relative amounts of TDP-43 (**c**), and pTDP-43 (**d**) per  
918 fraction were quantified from all immunoblots of control (black, n=6), ALS (red, n=4), FTLN-TDP-A  
919 (green, n=10), FTLN-TDP-C (blue, n=8) density fractionations. Data presented are the mean curves  
920 (bold lines), with standard deviations (lighter shade areas) for each group of patients. Panels below  
921 the graphs show the statistical significance of the difference in TDP-43 partition in each fraction,  
922 based on student t-test comparisons of the different groups.

923

924 **Figure 5:** Distinct structure of pathological TDP-43 isolated from different disease subtypes

925 **a.** Transmission Electron Microscopy (TEM) images of SarkoSpin-extracted aggregates from control  
926 (black frame), FTLN-TDP type A (green frame) and FTLN-TDP type C (blue frame) immunolabeled  
927 with monoclonal TDP-43 antibody (epitope: 203-209). All the EM grid samples were embedded in  
928 methylcellulose to avoid drying artifacts. The red arrows point to examples of gold-positive structures,  
929 while the yellow arrows point to lipid contaminants, which are pulled down in SarkoSpin together with  
930 the pathological protein assemblies. The orange arrows to proteinaceous filamentous structures. **b.**  
931 To better visualize these filamentous structures, part of the FTLN-TDP-C image shown in panel **a** was  
932 band-pass filtered. The scale bar is 100 nm **c.** TEM images of SarkoSpin-extracted aggregates from  
933 FTLN-TDP-A patient immunogold labeled against TDP-43, without the additional step of embedding in  
934 methylcellulose (MC), in order to obtain higher imaging resolution. In panels **a** and **b.** **c.** Proteolytic  
935 profiles of pathological TDP-43 from different disease subtypes, as annotated. Two control and four of  
936 each ALS, FTLN-TDP-A and FTLN-TDP-C samples were incubated with either 10 µg/mL  
937 Chymotrypsin (upper blots) or 100 µg/mL Trypsin for 30 min at 37°C during SarkoSpin solubilization.

938 Pellets were immunoblotted with anti-TDP-43 antibodies, raised against defined protein regions,  
939 showing distinct proteolytic fragments for each disease group. As a reference to the antigens and  
940 epitopes of used antibodies, TDP-43 secondary structure is represented below. NTD – N-terminal  
941 domain; NLS – nuclear localization signal; RRM1 and 2 – RNA-recognition motifs; LCD – low-  
942 complexity domain; Phosphorylation is marked by added phospho-groups at the very C-terminus of  
943 TDP-43. **d.** Dot plot representing the levels of pTDP-43 Chymotrypsin and Trypsin-resistant fragments  
944 normalized to total level of undigested TDP-43 species across disease subtypes, showing significantly  
945 higher Trypsin-resistance of aggregates extracted from FTLD-TDP-C or ALS compared to the ones in  
946 FTLD-TDP-A (dots represent single analyzed patients per disease subtype; the error bars show mean  
947 with SD). **e.** Representative TEM images of trypsinized SarkoSpin-extracted aggregates from FTLD-  
948 TDP type A and type C immunogold-labeled against pTDP-43 pS409/410. Red arrows point to  
949 examples of gold-positive structures.

950

951 **Figure 6:** Differential cyto- and neurotoxicity of FTLD-TDP type A and type C pathological assemblies  
952 **a.** Schematic representation of SarkoSpin inoculations on arrested HEK293 cells expressing TDP-43-  
953 HA. Briefly, expression of TDP-43-HA was induced with doxycycline 24hrs after plating the cells and  
954 their growth was arrested with AraC before lipofection of SarkoSpin pellets from control (n=6), FTLD-  
955 TDP-A (n=9) and FTLD-TDP-C (n=7) postmortem brain samples. At 3 days after inoculation, viability  
956 was measured by MTT assay or cells were collected for SarkoSpin analysis. **b.** Viability ratio of cells  
957 inoculated with SarkoSpin pellets from different patients with induced expression of TDP-43-HA  
958 (+DOX) and without it (-DOX). Each point represents the viability ratio of cells inoculated with the  
959 SarkoSpin pellet of a single patient; bars show mean  $\pm$  SD. **c.** Immunoblots of SarkoSpin supernatant  
960 and pellet fractions from TDP-43-HA-expressing cells inoculated with SarkoSpin pellet pools extracted  
961 from control (n=3), FTLD-TDP-A (n=3) or FTLD-TDP-C (n=3) patients. Full-size blots are shown in  
962 **Supplementary Fig. 26h.** **d.** Schematic representation of neurite outgrowth and toxicity assay on  
963 mouse primary cortical neuronal cultures upon inoculations of SarkoSpin pellets from different disease

964 subtypes. Briefly, cortical neurons from E18 mouse embryos were dissociated and plated in 96-well  
965 plates, challenged at 4 DIV with SarkoSpin pellet pools extracted from control (n=3), FTLD-TDP-A  
966 (n=3) and FTLD-TDP-C (n=3) patients and monitored for neurite outgrowth by phase contrast and  
967 fluorescence using an IncuCyte S3. **e.** Representative fluorescence calcein (green, upper rows)  
968 images and corresponding neurite and cell body segments (blue and yellow respectively, lower rows)  
969 for control (upper panel), FTLD-TDP-A (middle panel) and FTLD-TDP-C (lower panel) SarkoSpin  
970 pellet inoculations at indicated total protein amounts at 10 DIV endpoint. **f.** Dynamics of neurite  
971 outgrowth revealed by quantification of the phase contrast images for control (black), FTLD-TDP-A  
972 (green) and FTLD-TDP-C (blue) SarkoSpin pellets inoculated neurons at 0.3 µg total protein dose per  
973 well. **g.** Quantification of the endpoint calcein fluorescence images using a cell body segmentation  
974 algorithm for different doses of SarkoSpin extracts derived from control (black), FTLD-TDP-A (green)  
975 and FTLD-TDP-C (blue). **h.** Quantification of the endpoint calcein fluorescence images using a cell  
976 body segmentation algorithm for different doses of SarkoSpin extracts derived from control (black),  
977 FTLD-TDP-A (green) and FTLD-TDP-C (blue). Both **g** and **h** confirm the dose response of FTLD-  
978 TDP-A aggregates visually apparent in **e**.

979

## 980 **Supplementary Figure Legends**

981 **Supplementary Table 1:** Summary of patient information of the autopsy material used in this study  
982 Summary table of the different groups of patient autopsy material used in this study, with number of  
983 patients and identified genetic causes. All samples were used for development and standardization of  
984 the SarkoSpin method. FTLD: Frontotemporal Lobar Degeneration. ALS: Amyotrophic Lateral  
985 Sclerosis. FTLD-TDP: FTLD with TDP-43 pathology. FTLD-Tau: FTLD with Tau pathology. Sporadic:  
986 No identified genetic cause and absence of familial history. *C9ORF72*: Hexanucleotide repeat  
987 expansions in chromosome 9 open reading frame 72. *GRN*: Granulin mutation. *MAPT*: Microtubule-  
988 Associated Protein Tau mutation.

989

990 **Supplementary Figure 1:** Characteristics of disease subtypes and correlation to disease duration  
991 **a.** Phosphorylated TDP-43 immunohistochemical staining of temporal cortex from FTLD-TDP  
992 subtypes, highlighting differences in their pathological hallmarks. Neuronal cytoplasmic inclusions  
993 (NCI) (arrows) and short dystrophic neurites (DN) (arrowhead) were observed in FTLD-TDP-A without  
994 (toppanel ) or with (middle panel) *C9ORF72* repeat expansions. In FTLD-TDP-C, long twisted neurites  
995 were observed (lower panel, arrowhead). Scale bar 25  $\mu$ m. **b-c.** Age at death (**b**) and disease duration  
996 (**c**) of patients from the different disease groups used in the study. FTLD-TDP-C patients showed  
997 significantly longer disease duration.

998

999 **Supplementary Figure 2:** Benzonase treatment releases physiological TDP-43 complexes from  
1000 SarkoSpin pellets

1001 **a.** Schematic representation of sequential insolubility assay. Brain homogenates are solubilized with  
1002 indicated detergents (without Benzonase) and centrifuged after each solubilization step. Each time,  
1003 pellets are resuspended in buffer with increasing detergent stringency until the SDS-insoluble fraction.  
1004 **b.** Sequential insolubility fractions from control (black, left panels) and ALS (red, right panels) were  
1005 subjected to SDS-PAGE followed by immunolabeling of TDP-43 (top) and hnRNP A1 (bottom). Both  
1006 of these proteins are present in SDS fraction, in any health or disease case, showing the importance  
1007 of using Benzonase for assessing the insolubility of nucleic acid binding proteins. **c.** Biochemical  
1008 analysis of sarkosyl-soluble and insoluble fractions extracted from human brain samples, with or  
1009 without Benzonase treatment as noted. Supernatants and pellets were subjected to SDS-PAGE  
1010 followed by silver staining of total protein (upper panel), or immunoblotted against total TDP-43  
1011 (middle panel) or phosphorylated at position S409/410 TDP-43 (pTDP-43) (lower panel). Physiological  
1012 TDP-43 in the pellet of the control sample (black arrow) is released upon Benzonase treatment (white  
1013 arrow).

1014

1015 **Supplementary Figure 3:** Immunoblots of SarkoSpin fractions from all groups of patients show  
1016 consistent isolation of pTDP-43  
1017 Brain from control (black, C), ALS (red), FTLD-TDP type A (green, F-A), type B (yellow, F-B) and type  
1018 C (blue, F-C) motor cortex (**a, b**) and frontal cortex (**c**) were subjected to SarkoSpin. The supernatant  
1019 and pellet fractions were analyzed by SDS-PAGE and immunoblotted against TDP-43, pTDP-43,  
1020 hnRNPA1 and SOD1 antibodies. Posttranslational modifications related to pathological TDP-43 are  
1021 marked with asterisks: poly-ubiquitination smear (\*\*\*), hyper-phosphorylation (\*\*), and C-terminal  
1022 fragmentation (\*). S- supernatant, P – pellet, L – ladder.

1023

1024 **Supplementary Figure 4:** Immunoblots against TDP-43 with different mono- and polyclonal  
1025 antibodies

1026 Total homogenates from frontal and motor cortex of control (black), ALS (red), FTLD-TDP A (green),  
1027 B (yellow) or C (blue) were subjected to SDS-PAGE after equilibration of protein amounts, and were  
1028 immunoblotted with monoclonal (two left panels), polyclonal (two right panels) anti-TDP-43 or actin  
1029 antibodies. The monoclonal 60019-2-Ig antibody detected an additional unspecific band just below 40  
1030 kDa. This band is not observed with any other anti-TDP-43 antibody used.

1031

1032 **Supplementary Figure 5:** Estimated percentages of pathological and physiological TDP-43  
1033 assemblies in human brain

1034 **a.** Percentages of phosphorylated TDP-43 in control and patient brain SarkoSpin preparations were  
1035 calculated considering that only phosphorylated TDP-43 is present in SarkoSpin pellets, and knowing  
1036 the fraction of total TDP-43 in this pellet. Each dot represents a value from a single patient and the  
1037 line is the average for each group. **b.** Calculated percentages of aggregated TDP-43 from velocity  
1038 sedimentations. The sum of relative amount of TDP-43 in fractions 6 to 16 (aggregated TDP-43) of  
1039 velocity sedimentations from control (black, n=6), ALS (red, n=4), FTLD-TDP-A (green, n=7), and  
1040 FTLD-TDP-C (blue, n=7) was divided by the sum of relative amount in fractions 1 to 5 (normal TDP-



1041 43) in order to estimate the ratios between these two forms of the protein, which are represented as a  
1042 percentage of the total. Each dot represents a value from a single patient and the line is the average  
1043 for each group. **c.** Percentage of oligomeric TDP-43 was estimated in control samples, based on  
1044 Native PAGE TDP-43 immunoblots, where oligomeric TDP-43 appears in fractions 5 to 7 as a broad  
1045 smear, while monomeric TDP-43 concentrates in fractions 8-12 as a condensed band of apparent ~66  
1046 kDa. Each dot represents a value from a single patient and the line is the average.

1047

1048 **Supplementary Figure 6: SarkoSpin captures native pathological TDP-43 assemblies**

1049 Native PAGE immunoblots of SarkoSpin supernatants (top panels) and pellets (lower panels)  
1050 extracted from different disease types were immunoblotted against total TDP-43 (left panels) or  
1051 pTDP-43 (pS409/410, right panels). The indicative molecular weights were established by NativeMark  
1052 protein ladder, and material retained in the stacking gels was considered of more than 10 MDa.

1053

1054 **Supplementary Figure 7: SarkoSpin extracts from different ALS and FTLD samples have distinct  
1055 protein compositions beyond TDP-43**

1056 Abundance levels of selected proteins that were most consistently different in patient samples of  
1057 human TDP-43 proteinopathies, FTLD-Tau (FTLD-T) and controls. We applied the idealized vector  
1058 method for control and pooled TDP-43 proteinopathy samples (ALS, FTLD-TDP-A, FTLD-TDP-B and  
1059 FTLD-TDP-C). The top 50 correlating proteins were sorted based on these correlation values, which  
1060 are shown bar plot on the right. Strong correlation values ( $r > 0.5$ ) were separated from medium and  
1061 weak correlation values by the red dashed line.

1062

1063 **Supplementary Figure 8: Proteins enriched in SarkoSpin pellets of ALS brains**

1064 Abundance levels of selected proteins that were most consistently different in ALS brain samples  
1065 compared to controls. We applied the idealized vector method for control and ALS samples. The top  
1066 50 correlating proteins were sorted based on these correlation values, which are given as a bar plot

1067 on the right. Strong correlation values ( $r > 0.5$ ) were separated from medium and weak correlation  
1068 values by the red dashed line.

1069

1070 **Supplementary Figure 9:** Proteins enriched in SarkoSpin pellets of FTLD-TDP-A brains

1071 Abundance levels of selected proteins that were most consistently different in FTLD-TDP-A brain  
1072 samples compared to controls. We applied the idealized vector method for control and FTLD-TDP-A  
1073 samples. The top 50 correlating proteins were sorted based on these correlation values, which are  
1074 given as a bar plot on the right. Strong correlation values ( $r > 0.5$ ) were separated from medium and  
1075 weak correlation values by the red dashed line.

1076

1077 **Supplementary Figure 10:** Proteins enriched in SarkoSpin pellets of FTLD-TDP-B brains

1078 Abundance levels of selected proteins that were most consistently different in FTLD-TDP-B brain  
1079 samples compared to controls. We applied the idealized vector method for control and FTLD-TDP-B  
1080 samples. The top 50 correlating proteins were sorted based on these correlation values, which are  
1081 given as a bar plot on the right. Strong correlation values ( $r > 0.5$ ) were separated from medium and  
1082 weak correlation values by the red dashed line.

1083

1084 **Supplementary Figure 11:** Proteins enriched in SarkoSpin pellets of FTLD-TDP-C brains

1085 Abundance levels of selected proteins that were most consistently different in FTLD-TDP-C brain  
1086 samples compared to controls. We applied the idealized vector method for control and FTLD-TDP-C  
1087 samples. The top 50 correlating proteins were sorted based on these correlation values, which are  
1088 given as a bar plot on the right. Strong correlation values ( $r > 0.5$ ) were separated from medium and  
1089 weak correlation values by the red dashed line.

1090

1091 **Supplementary Figure 12:** Further characterization on the protein content in SarkoSpin soluble and  
1092 insoluble fractions in different neuropathological subtypes

1093 **a.** Representative immunoblots on SarkoSpin soluble fractions corresponding to immunoblots on  
1094 insoluble material in **Fig. 2b**. **b.** Full-size immunoblots on SarkoSpin supernatant and pellet fractions  
1095 probed against PFN1, showing unspecific bands above 30 kDa (\*\*) in addition to the specific Profilin  
1096 band at 15 kDa (\*). **c.** Immunoblot on SarkoSpin pellets probed against total TDP-43 **d.** Correlation  
1097 dot plot of pTDP-43 and total TDP-43 quantified from (**c**). **e.** Representative co-immunofluorescence  
1098 of pTDP-43 and ubiquitin in control, FTL-D-TDP-A and FTL-D-TDP-C brain sections corresponding to  
1099 merged images in **Fig. 2f**.

1100

1101 **Supplementary Figure 13:** Co-immunofluorescence of pTDP-43 with mass spectrometry hits,  
1102 ASAH1 and TXNL1

1103 Representative images of co-immunofluorescence of pTDP-43 with selected mass spectrometry hits  
1104 ASAH1 (**a**) and TXNL1 (**b**) in control, FTL-D-TDP-A and FTL-D-TDP-C post mortem brain sections. On  
1105 the right side, high magnification images are shown, with examples of partial co-localization of  
1106 pathological pTDP-43 aggregates with ASAH (1) and TXNL1 (2) in FTL-D-TDP-A and with TXNL1 (3)  
1107 in FTL-D-TDP-C. All images are overlaid with DAPI nuclear staining in blue.

1108

1109 **Supplementary Figure 14:** FBXO1 upregulation in astrocytes in FTL-D-TDP-A patients, but not  
1110 FTL-D-TDP-C

1111 **a.** Representative immunohistochemistry images showing FBXO1 immunoreactivity in control, FTL-D-  
1112 TDP-A and FTL-D-TDP-C brain sections. **b.** Representative co-immunofluorescence of GFAP and  
1113 FBXO1 in control, FTL-D-TDP-A and FTL-D-TDP-C brain sections corresponding to merged images in  
1114 **Fig. 2h**. All images are overlaid with DAPI nuclear staining in blue.

1115

1116 **Supplementary Figure 15:** Distinct size distributions of TDP-43 aggregates isolated from different  
1117 types of TDP-43 proteinopathies

1118 Brain homogenates from control (black, n=6), FTLN-Tau (grey, n=2), ALS (red, n=4), FTLN-TDP-A  
1119 (green, n=11), FTLN-TDP-B (yellow, n=3) and FTLN-TDP-C (blue, n=9) patients were subjected to  
1120 SarkoSpin solubilization and velocity sedimentation by ultracentrifugation on a continuous iodixanol  
1121 gradient. The collected fractions (numbered from top to bottom of the gradient) were analyzed for  
1122 TDP-43 (left panel) and pTDP-43 (right panel) content by dot blot.

1123

1124 **Supplementary Figure 16:** Immunoblots representing the distribution of TDP-43 and pTDP-43 on  
1125 velocity sedimentation gradients

1126 **a-b.** Representative immunoblots of the velocity sedimentation profiles of TDP-43 (**a**) and pTDP-43  
1127 (**b**). SarkoSpin solubilized brain homogenates from frontal and motor cortex of control (black), FTLN-  
1128 TDP-A (green), and FTLN-TDP-C (blue), were fractionated by velocity and the collected fractions  
1129 (numbered from top to bottom of the gradient) were analyzed for TDP-43 (**a**) and pTDP-43 (**b**) by  
1130 western blotting.

1131

1132 **Supplementary Figure 17:** Neuropathological profile rather than genotype correlates with the size  
1133 distribution of pathological TDP-43

1134 **a-b.** Relative distribution of TDP-43 and pTDP-43 (lower graph) on sedimentation velocity  
1135 fractionations of FTLN-TDP type A (green) and B (yellow) patient brain samples. SarkoSpin  
1136 solubilized brain homogenates from frontal and motor cortex of sporadic, *GRN* and *C9ORF72* patient  
1137 brain samples were fractionated by velocity. The relative amounts of TDP-43 (**a**), and pTDP-43 (**b**) per  
1138 fraction were quantified from immunoblots. Data presented are the mean curves (bold lines) with  
1139 standard deviations (lighter shade areas) for each group of patients. Panels below the graphs show  
1140 the statistical significance of the difference in TDP-43 partition in each fraction, based on student t-test  
1141 comparisons of the different groups.

1142

1143 **Supplementary Figure 18:** Velocity sedimentation and density floatation profiles of human  
1144 recombinant TDP-43 aggregated *in vitro*  
1145 Representative silver stainings of the distribution of human recombinant full-length TDP-43,  
1146 aggregates reconstituted *in vitro* on velocity sedimentation (a) and density floatation (c) gradients.  
1147 TDP-43 velocity (b) and density (d) profiles of recombinant TDP-43 aggregates are represented as  
1148 the mean curves (bold lines), with standard deviations (lighter shade areas). d. Representative native  
1149 PAGE silver staining of recombinant TDP-43 fractionated by density. Only fractions 1 to 14 were  
1150 loaded due to available wells on native PAGE gels. Note the presence of high molecular weight  
1151 assemblies retained in the stacking gel (increased exposure on top panel).

1152

1153 **Supplementary Figure 19:** Sedimentation profiles of FUS, SOD1 and hnRNPA1 in human brains  
1154 Representative immunoblots of the distribution of FUS, hnRNPA1 and SOD1 on velocity  
1155 sedimentation gradients. SarkoSpin-solubilized brain homogenates from frontal and motor cortex of  
1156 control (black), ALS (red), FTLN-TDP-A (green), and FTLN-TDP-C (blue) were fractionated by velocity  
1157 and the collected fractions (numbered from top to bottom of the gradient) were analyzed for FUS  
1158 (top), hnRNPA1 (middle) and SOD1 (bottom) content by western blotting.

1159

1160 **Supplementary Figure 20:** Distinct density profiles of TDP-43 aggregates isolated from different  
1161 types of TDP-43 proteinopathies  
1162 SarkoSpin-solubilized brain homogenates from control (black, n=6), FTLN-Tau (grey, n=2), ALS (red,  
1163 n=4), FTLN-TDP type A (green, n=11), B (yellow, n=3) and C (blue, n=9) patients were fractionated by  
1164 density upon isopycnic equilibrium floatation gradients. The collected fractions (numbered from top to  
1165 bottom of the gradient) were analyzed for TDP-43 (a) and pTDP-43 (b) content by dot blot. The  
1166 density values calculated by refractometry are indicated on the top of the gradients.

1167

1168 **Supplementary Figure 21:** Immunoblots representing the distribution of TDP-43 and pTDP-43 on  
1169 density floatation

1170 **a-b.** Representative immunoblots of the distribution of TDP-43 (**a**) and pTDP-43 (**b**) on velocity  
1171 gradients. SarkoSpin-solubilized brain homogenates from frontal and motor cortex of control (black),  
1172 FTLD-TDP-A (green), and FTLD-TDP-C (blue) were fractionated by density and the collected  
1173 fractions (numbered from top to bottom of the gradient) were analyzed for pTDP-43 and TDP-43  
1174 content by western blotting.

1175

1176 **Supplementary Figure 22:** Density estimation and linearity of iodixanol gradient fractions

1177 **a.** Fractions of density floatation gradients were loaded on an optical refractometer (Zeiss). Their  
1178 density was calculated from tables of correlations between refractive index (RI density) or iodixanol  
1179 concentration (% iodixanol density) and density, respectively, provided by the Optiprep medium  
1180 manufacturer. The average density of each fraction was determined as a mean of the measured and  
1181 calculated densities. **b.** The linearity of density in iodixanol gradients was assessed by plotting the  
1182 average of measured and calculated density of each fraction as described above, with a linear  
1183 correlation  $R^2$  of 0.99964.

1184

1185 **Supplementary Figure 23:** Verification of the size of TDP-43 aggregates segregated by density  
1186 floatation gradients

1187 Representative Native PAGE immunoblots of TDP-43 (left panels), and pTDP-43 (right panels) on  
1188 density fractionations from control (black), FTLD-TDP-A (green), and FTLD-TDP-C (blue) patient brain  
1189 samples. Only fractions 1 to 14 were loaded due to available wells on native PAGE gels. Density  
1190 values calculated by refractometry are indicated on the top of the graph. pTDP-43-positive  
1191 pathological aggregates are seen at >10 MDa for FTLD-TDP-A and FTLD-TDP-C (green and blue  
1192 arrowheads respectively), whereas oligomeric TDP-43 assemblies are observed in all samples  
1193 between 66 kDa and 10 MDa (black brackets).

1194

1195 **Supplementary Figure 24:** Relative retention of different proteins by filtration of SarkoSpin fractions

1196 **a.** SarkoSpin supernatants and pellets from different disease types and control patients were passed  
1197 through 0.22  $\mu\text{m}$  syringe filters. The resulting filtrates were captured on a nitrocellulose membrane,  
1198 along with the corresponding unfiltered samples for a direct comparison and immunoblotted for pTDP-  
1199 43, TDP-43, and hnRNPA1. **b.** The relative reduction of signal between filtered and non-filtered  
1200 fractions was calculated for each protein on four independent SarkoSpin filtration experiments.

1201

1202 **Supplementary Figure 25:** Further structural analysis of pathological TDP-43 isolated from different  
1203 disease subtypes

1204 **a.** TEM images of negative control, without the use of primary antibody, of immunolabeled FTLD-TDP-  
1205 A insoluble material corresponding to TEM images from **Fig. 5a-b**, showing no unspecific binding of  
1206 the gold-conjugated secondary antibody. Sample in panel on the left was prepared including the  
1207 methylcellulose (MC) preservation step, and the sample represented on the right without the MC step.

1208 **b.** Upper panel: TEM image of immunolabeled FTLD-TDP-A aggregates with lipid contaminants  
1209 surrounding the protein assemblies. Lower panel: TEM image of immunolabeled FTLD-TDP-A sample  
1210 purified over sucrose cushion during SarkoSpin extraction, which efficiently removes the lipid  
1211 contaminants. **c.** Representative immunoblots of SarkoSpin pellets extracted from control, ALS,  
1212 FTLD-TDP-A and FTLD-TDP-C brains after proteolytic digestion. Brain homogenates were digested  
1213 by different proteases for 30 minutes at 37  $^{\circ}\text{C}$  during SarkoSpin solubilization step. SarkoSpin pellets  
1214 were subjected to SDS-PAGE and immunoblotted against total TDP-43 (top panels) and pTDP-43  
1215 (bottom panels). N – not treated; P – treated with 0.5 mg/ml Proteinase K; T – treated with 0.1 mg/ml  
1216 Trypsin; C – treated with 0.01 mg/ml Chymotrypsin. **d.** Full size representation of the immunoblots on  
1217 Chymotrypsinized and Trypsinized SarkoSpin pellets shown in **Fig. 5c**.

1218

1219 **Supplementary Figure 26:** Inoculation of phospho-GFP-TDP-43-HA SarkoSpin pellets in a  
1220 doxycycline-inducible TDP-43-HA HEK293 cell line

1221 **a.** Schematic representation of the pcDNA5 constructs used to generate the TDP-43-HA-expressing  
1222 stable HEK293 cell line (top) and to produce SarkoSpin pellets containing phosphorylated GFP-TDP-  
1223 43-HA (bottom) employed to test the inoculation conditions in the stable cell line. **b-c.** Sensitivity of the  
1224 inducible system was tested by induction of TDP-43-HA expression with 1µg/ml of doxycycline for 48  
1225 hours and analyzed by Western blot (**b**) and immunofluorescence (**c**) with an HA-tag specific  
1226 antibody. **d.** SarkoSpin-isolated TDP-43 aggregates in NSC-34 cells transiently transfected with the  
1227 GFP-TDP-43-HA construct for 48 hours (loading: homogenate 20µg of protein, 3.3% of SarkoSpin  
1228 supernatant, 20% of SarkoSpin pellet). Note that the pellet is enriched in TDP-43 and devoid from  
1229 other soluble or RNA-binding proteins, with similar purity to that achieved in human brain samples. **e.**  
1230 TDP-43-HA-expressing HEK293 cells internalized lipofected SarkoSpin-insoluble GFP-TDP-43-HA. 4  
1231 hours after inoculation, cells were trypsinized and collected by centrifugation before lysis in order to  
1232 remove all GFP-TDP-43-HA aggregates adhered to the cell surface and only visualize those that have  
1233 truly entered the cells. L2000: Lipofectamine 2000. **f.** Immunocytochemistry of TDP-43-HA-expressing  
1234 cells lipofected with GFP-TDP-43-HA aggregates. Side views of the z-stack (yz plane) are depicted in  
1235 1 and 2, show examples of internalized GFP-TDP-43-HA aggregates. **g.** Immunoblots on  
1236 homogenates for SarkoSpin from TDP-43-HA HEK cells inoculated with SarkoSpin pellets from  
1237 control, FTLD-TDP-A or FTLD-TDP-C patients. **h.** Full-size immunoblots of SarkoSpin supernatant  
1238 and pellet fractions from TDP-43-HA expressing cells inoculated with SarkoSpin pellets from control,  
1239 FTLD-TDP-A or FTLD-TDP-C patients, corresponding to Figure 6c.

1240

1241 **Supplementary Figure 27:** Verification of TDP-43, pTDP-43 and total protein amounts inoculated to  
1242 primary cortical neurons at 4 DIV

1243 Normalization of total protein amounts in Sarkospin pellets inocula was achieved by BCA prior to  
1244 dilution for reaching doses of 1, 0.3, 0.1 and 0.03 µg of protein per well. In parallel of the cell



1245 treatments, an aliquot of each inoculum type and dose was loaded on dot blot and immunolabeled  
1246 with antibodies against TDP-43 (**a.**), pTDP-43 pS409/410 (**b.**) and actin (**c.**). **a.** and **b.** show that TDP-  
1247 43 and pTDP-43 are only present in Sarkosyl pellets from FTLD patients, and at approximately  
1248 double dose in FTLD-TDP-A compared to FTLD-TDP-C subtypes inocula.

1249

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1268

## 1269 **Author contributions**

1270 F.L. developed and performed the biochemical experiments with the help of Z.M. and M.P. conceived  
1271 and directed the study. M.P-B. generated and characterized the stable cell line with inducible TDP-43-  
1272 HA, in which she performed cell toxicity experiments. M.H-P., L.G., E-M.H., and T.A. helped with  
1273 performing gradients and developing the SarkoSpin method. P.B. and P.P. performed mass  
1274 spectrometry, which was bioinformatically analysed by U.W.. G.B. performed electron microscopy  
1275 experiments. A.A-A., A.L. and H.S. gave critical input on the structural analysis of extracted  
1276 aggregates by electron microscopy and performed preliminary cryo-EM and CLEM experiments.  
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1278 and A.I., while S.C.F. did the pTDP-43 immunohistochemistry and immunofluorescence and Y.T.A.  
1279 sampled the cases from the Queen Square Brain Bank. F.DG., F.I. and E.B. performed and analyzed  
1280 inoculations on primary neurons. F.L., Z.M. and M.P wrote the manuscript. All authors read, edited  
1281 and approved the final manuscript.

1282

#### 1283 **Footnotes**

1284 Mass spectrometry raw files were uploaded to the ProteomeXchange Consortium via the PRIDE  
1285 partner repository. Dataset identifier: PXD007873.

1286

#### 1287 **Competing financial interests**

1288 The authors declare no competing financial interests.

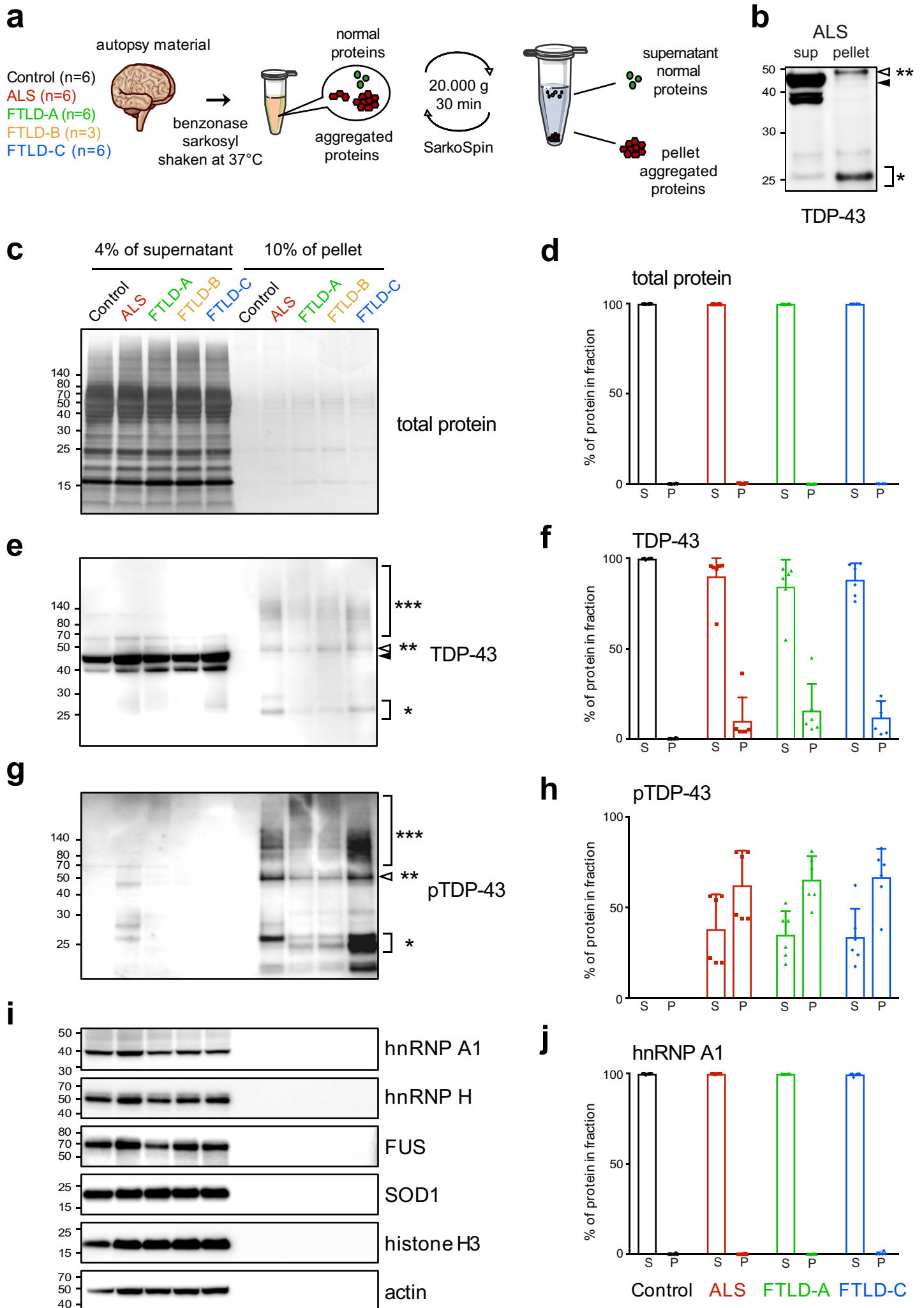
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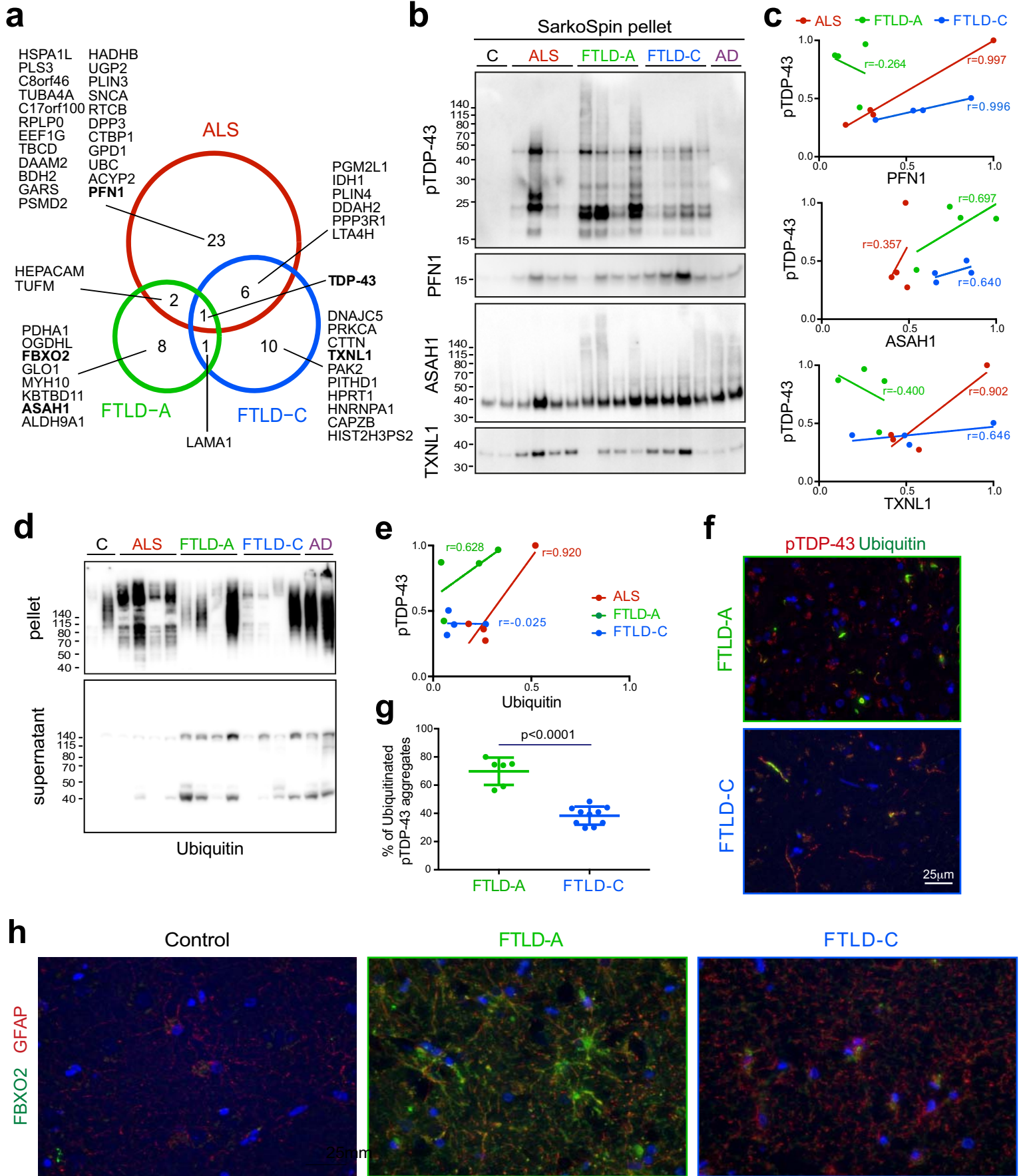
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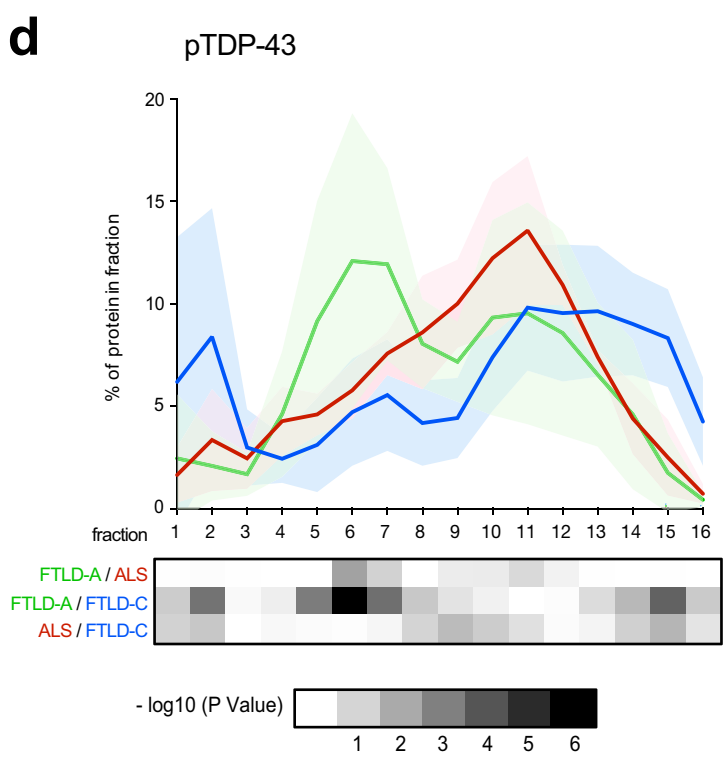
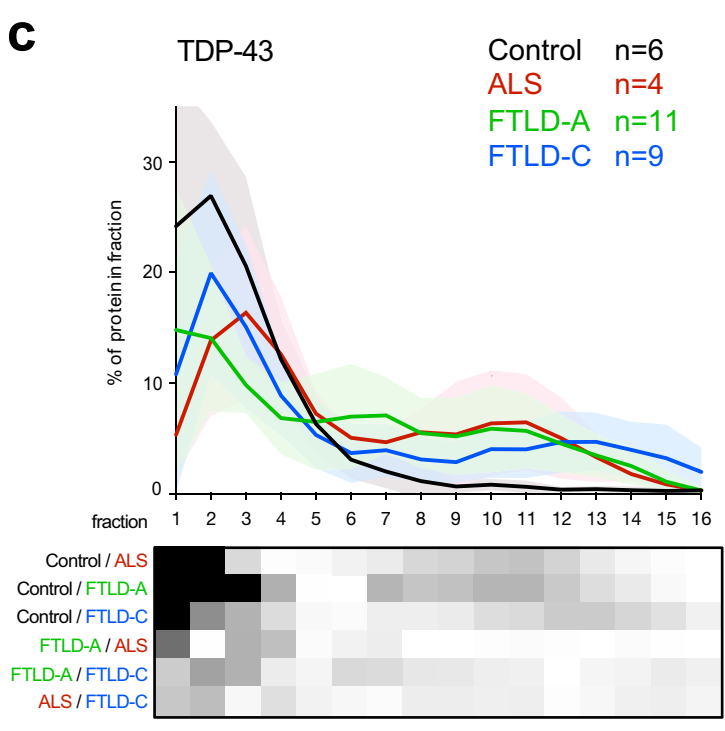
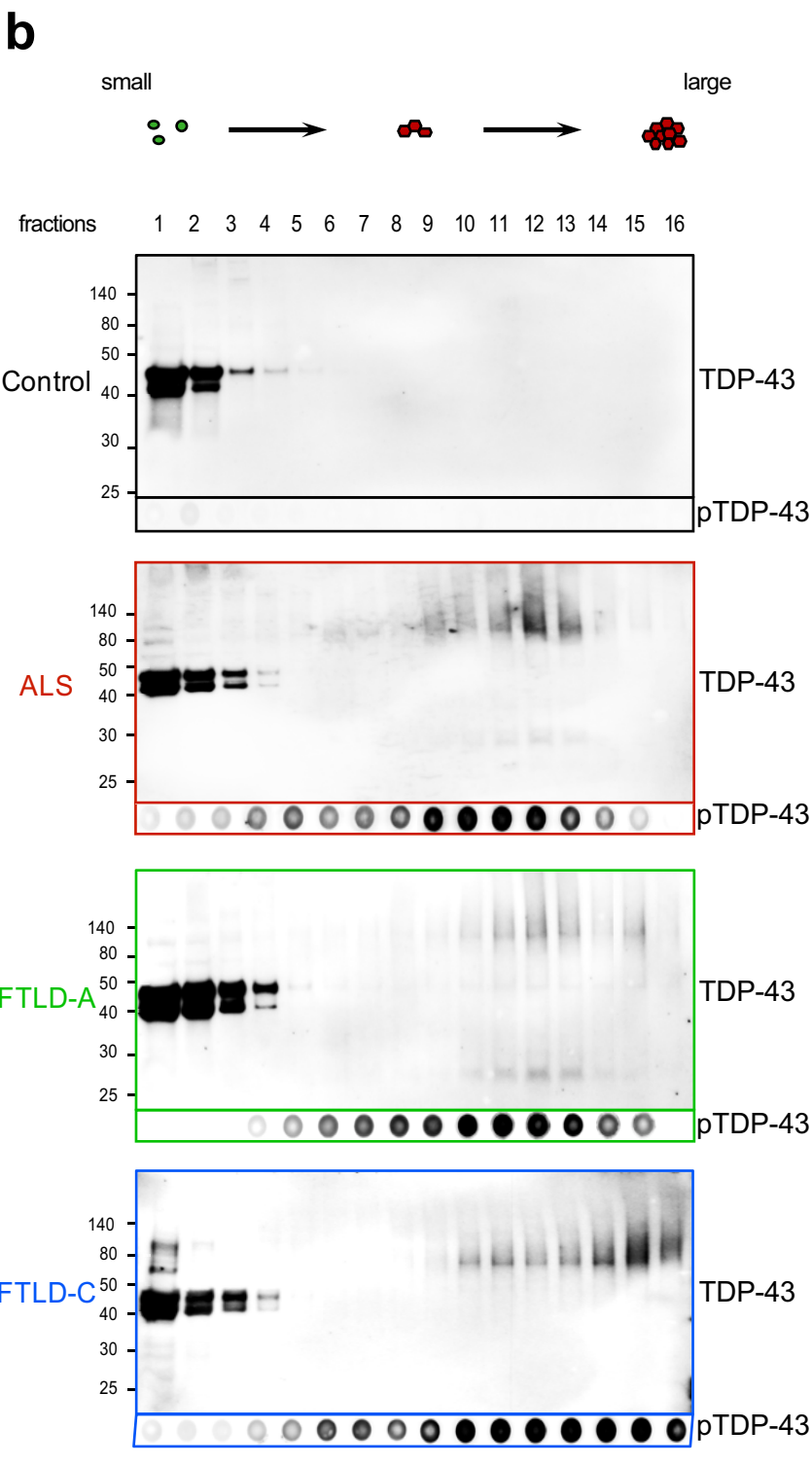
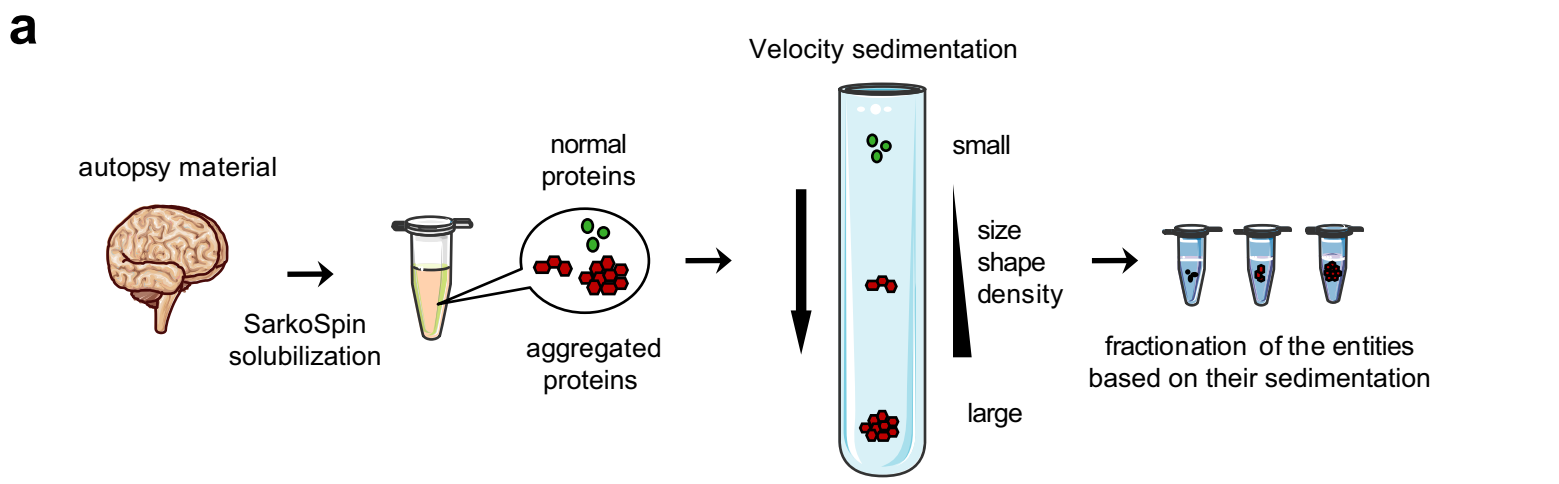
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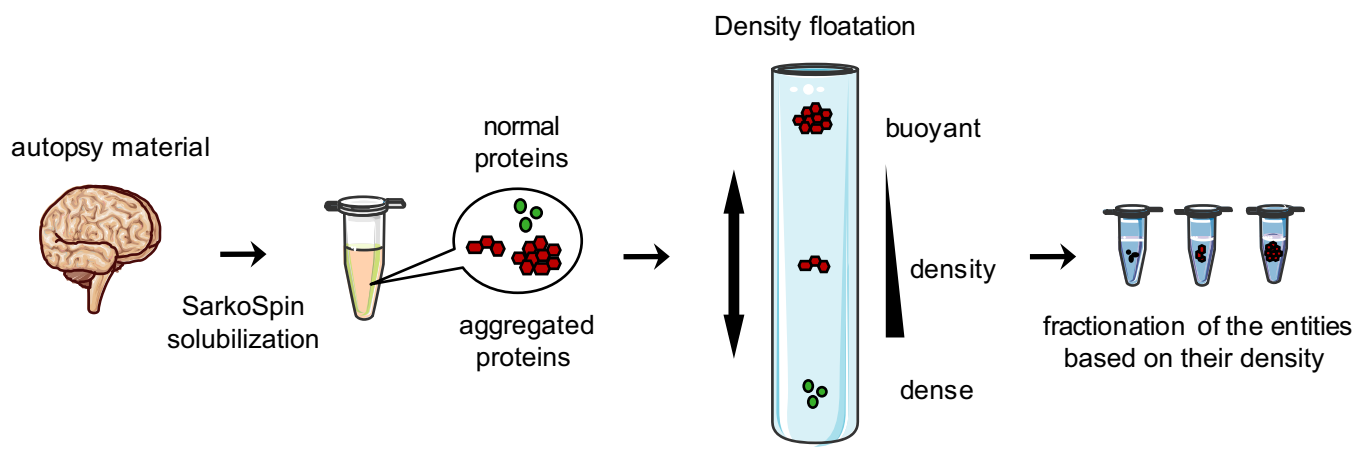
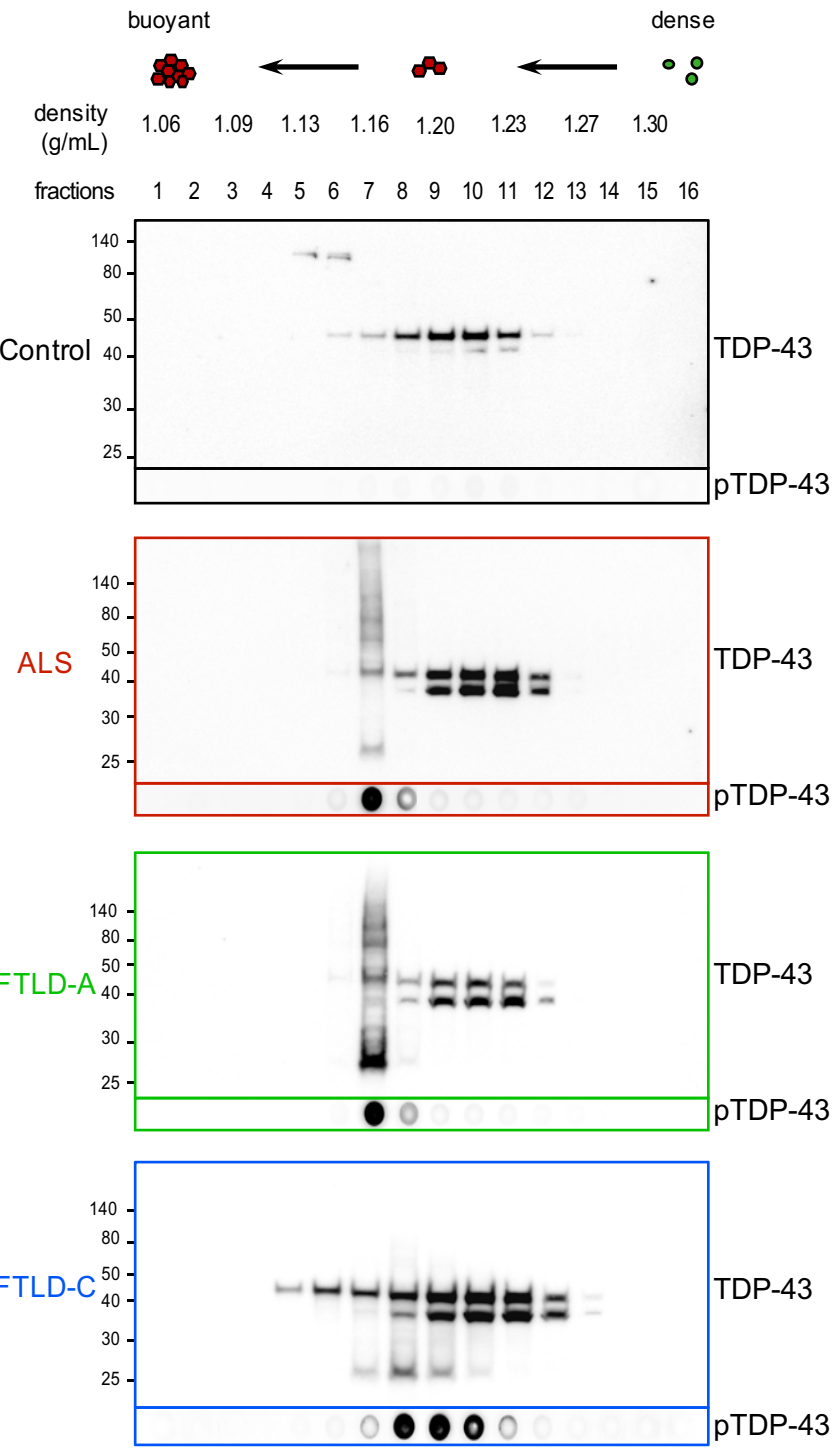
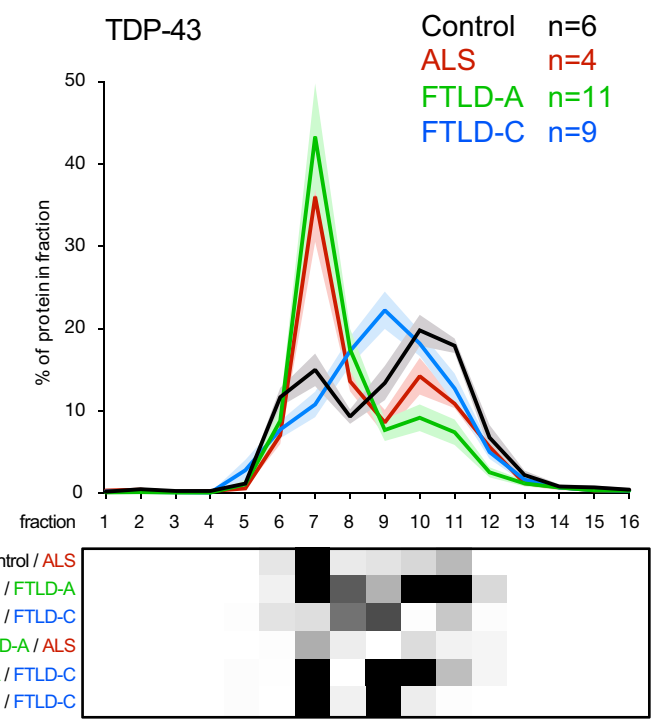
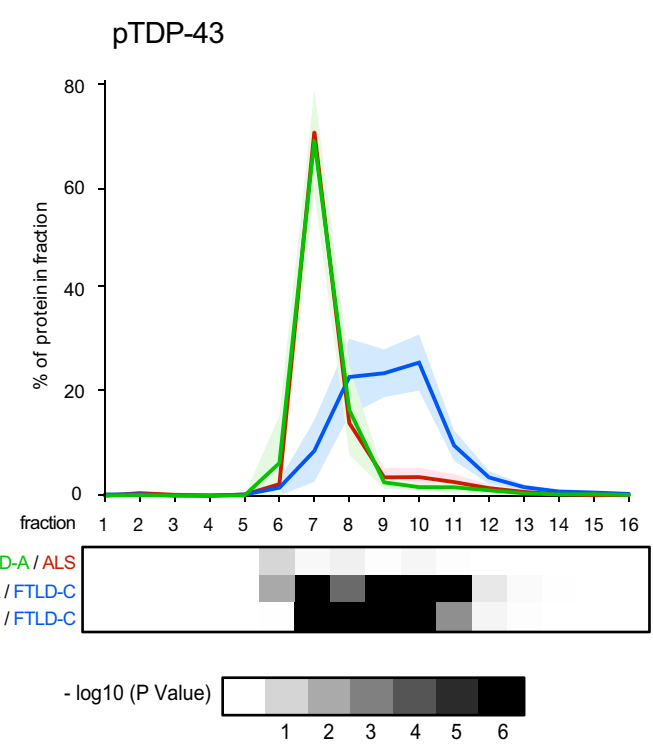
Laferriere et al. Figure 1



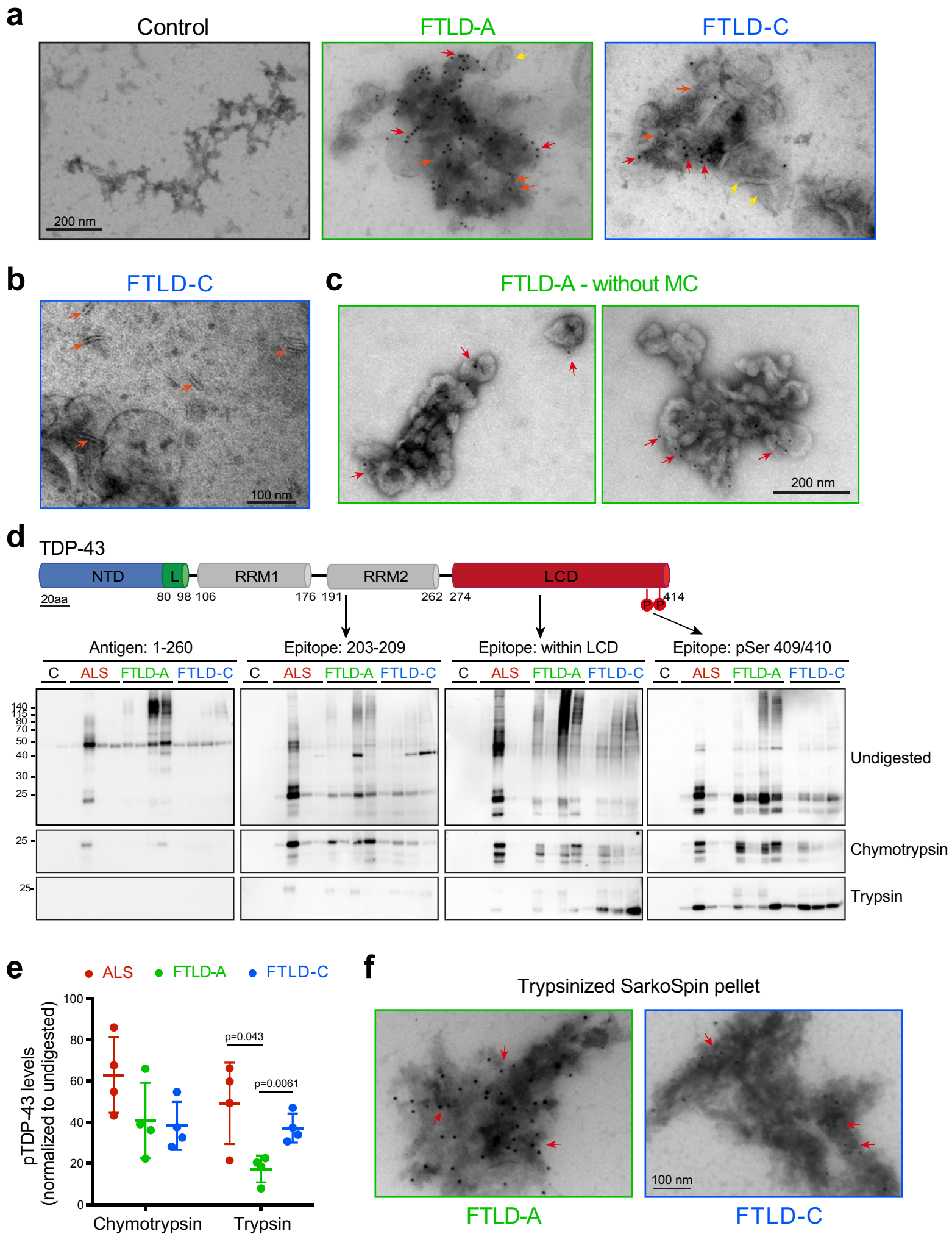




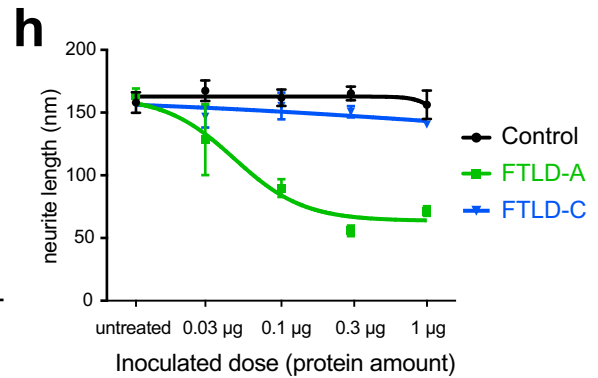
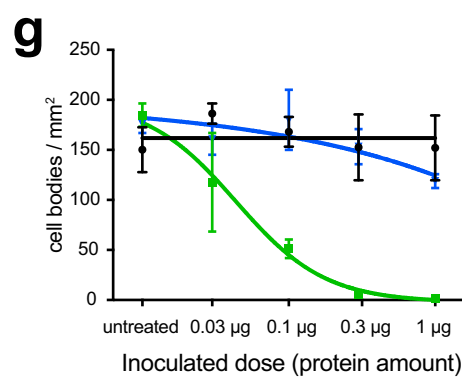
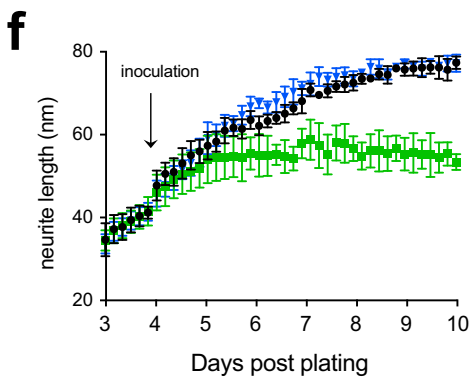
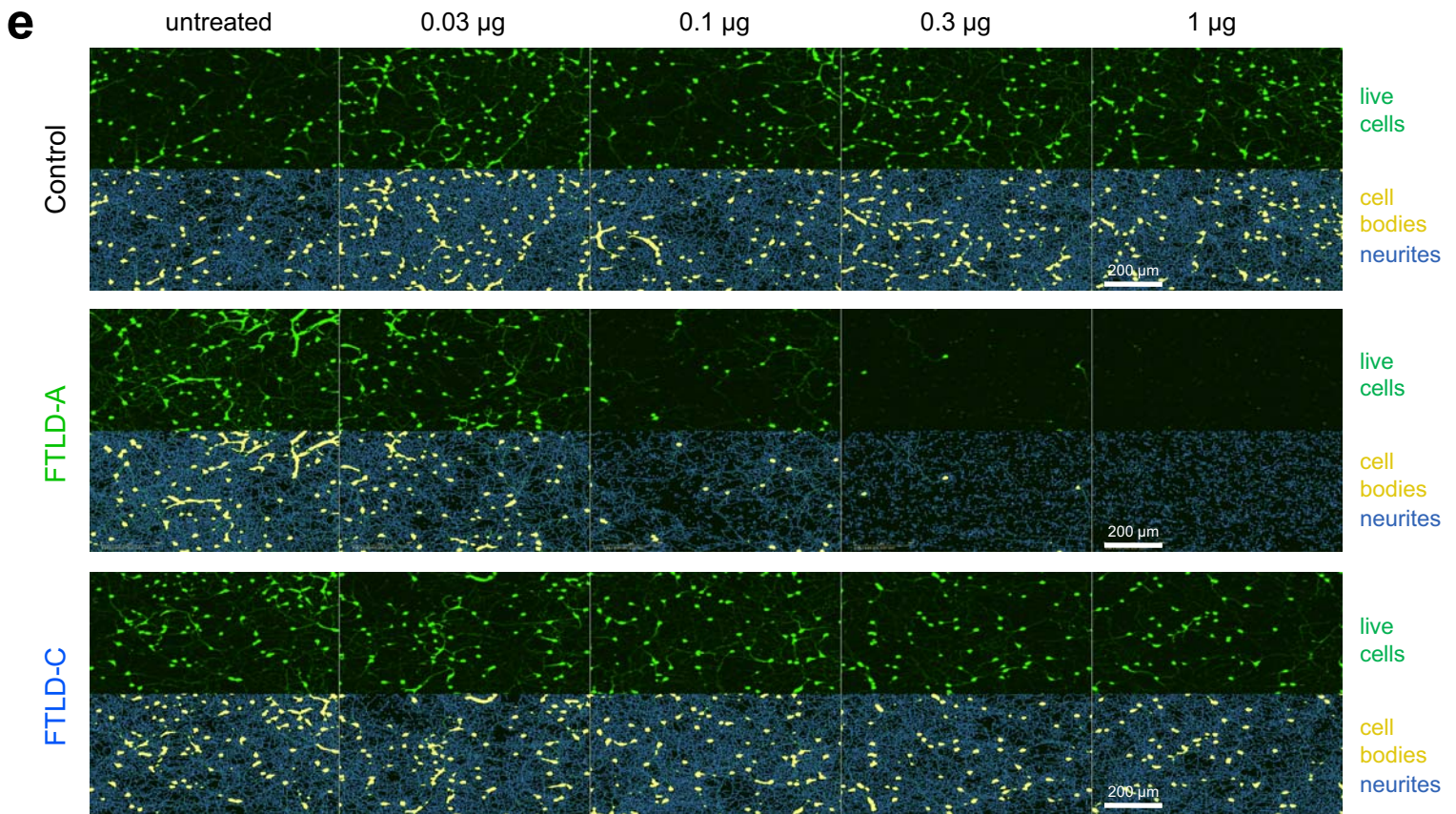
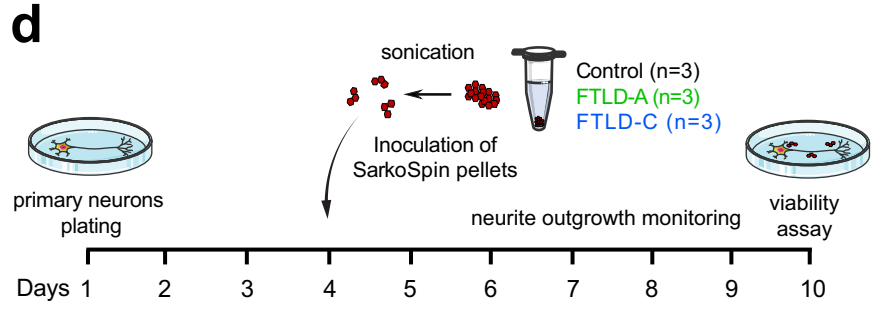
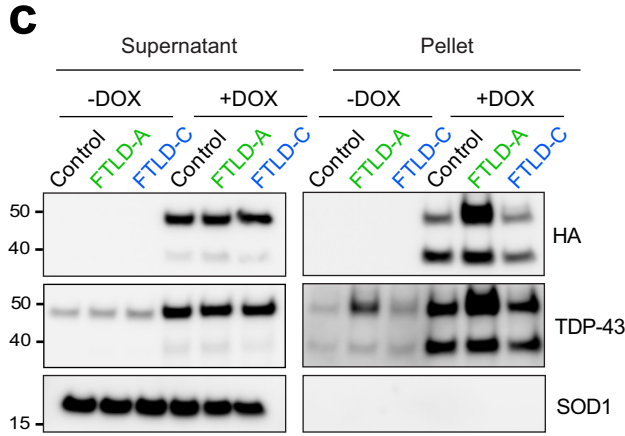
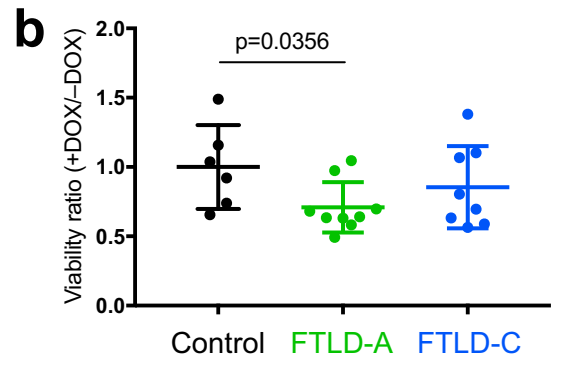
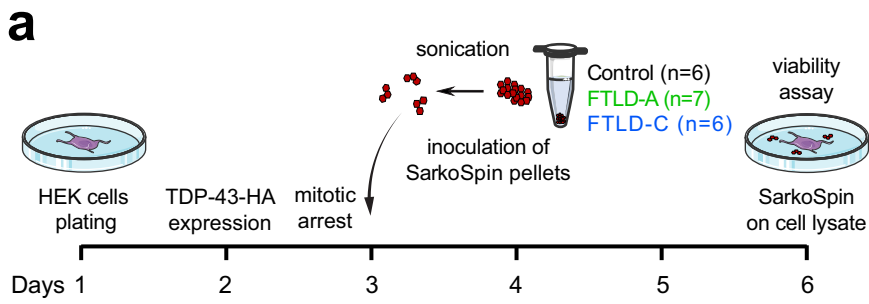
Laferriere et al. Figure 3

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Laferrriere et al. Figure 4



Laferriere et al. Figure 5



Laferriere et al. Figure 6