Hippocampal aggregation signatures of pathogenic *UBQLN2* in amyotrophic lateral sclerosis and frontotemporal dementia

Supplementary methods, tables, and figures

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Supplementary methods

Clinical information for *UBQLN2* variant cases

MN17 – T487I

Reproduced from Williams *et al.*[1]: "Diagnosis of ALS was at 58 years in this patient. Due to the strong family history of ALS, she suspected she might have been affected when her left hand begun to waste and she could not carry objects. She also had had a "croaky" voice for 6 years. Initial examination of the patient by a neurologist showed hoarse speech, can't rapidly repeat "cc", heel to toe walking unsteady, and she could not heel walk. Both hands were wasted, with more wasting in the left hand. She could not oppose her thumb and finger, and wrist extension is 3/5. She had increased jaw jerk, generalised hyper reflexia, plantars flexor and increased tone in her lower limbs. She had no fasciculations and her sensory was normal. A frontotemporal dementia test conducted 12 months after her ALS diagnosis showed no frontotemporal impairment. Three years after diagnosis, her voice had gotten more hoarse and she could no longer elevate her left arm. Her legs were stiff but she had no footdrop, she had increased tone in her legs and hyperreflexia. Nerve conduction studies showed decreased motor amplitudes and prolonged distal latencies. An EMG showed chronic neurogenic changes in upper limb which were predominantly distal with fibrillation, and only showed minimal lower limb changes. She is still alive 3 years after diagnosis." The patient subsequently died at age 61.

V:7 – T487I

The patient had a complex neuropsychiatric history prior to diagnosis at age 37 of ALS with personality and behavioural changes. His presenting complaint at age 30 was speech symptoms. He did not engage with neurology services and died at age 39.

P497H

The patient first presented with respiratory symptoms at age 46 (reported as age 41 in Deng *et al.*[2] but amended here) and was diagnosed with ALS. She lived another 72 months, having gone on invasive ventilation 36 months after onset.

P506S

Reproduced from Gkazi *et al.*[3]: The patient "was diagnosed with FTD at age 54 years with gradual behavioral and personality changes over a period of 2 years. A year after her initial diagnosis, she clearly showed signs of ALS with muscle wasting, fasciculation, and exaggerated reflexes in the cranial nerve, upper limb territories, and lower limbs alone being the first symptoms. The duration of disease was 3 years."

S222G

The patient was noted as having mild cognitive impairment at age 88 in the context of an Alzheimer's Disease (AD) research study. He developed sudden onset confusion after a heart attack at age 90 and was diagnosed with probable AD. Prior to this, his family reported a history of slowness of speech and understanding, and apathy. In his last year of life, he was incontinent and wheelchair bound. He died at age 93 and subsequent neuropathological analysis excluded Alzheimer's disease, finding instead progressive supranuclear palsy (PSP).

II:3 – P497L

Modified from Fahed *et al.*[4]: The patient first presented with left lower extremity weakness at 63 years of age. Over the course of disease, her clinical features involved dysarthria, dysphagia, and diffuse muscle weakness. The patient did not present with stiffness, involuntary movements, or dementia until her death at 67 years.

III:3 – P497L

Modified from Fahed *et al.*[4]: The patient presented with slurred speech at 20 years of age. Spastic paralysis in all extremities, dysarthria, dysphagia, spastic gait, and behavioural dementia were noted as clinical features throughout her disease. The duration of disease was 17 years.

IV:2 – P497L

Modified from Fahed *et al.*[4]: The patient presented with dysarthria at 20 years of age. Spastic paralysis in all extremities, dysarthria, dysphagia, spastic gait, and behavioural dementia were noted as clinical features. She died at age 24, 4 years after symptom onset.

Supplementary Table 1 FALS 5 and FALS 14 relatedness analysis sample information

Family ID	Pedigree ID	Sex	Disease Status	*UBQLN2* p.T487I
FALS5	III:8	Female	Affected	Yes
FALS5	IV:9	Female	Affected	Yes
FALS5	IV:18	Female	Affected	Yes
FALS14	II:1	Female	Unaffected	No
FALS14	III:2	Female	Affected	Yes

Supplementary Table 2 Demographics and clinical features of study cohort

Case type	Case code	Diagnosis	Sex	Genetic mutation	AAO (y)	AAD (y)	Site of Onset	Brain weight (g)	PMD (h)
Control	H211	NNDC	M	N/A	N/A	41	N/A	1513	8
	H215	NNDC	F	N/A	N/A	67	N/A	1232	23.5
	H230	NNDC	F	N/A	N/A	57	N/A	1243	32
	H238	NNDC	F	N/A	N/A	63	N/A	1323.5	16
	H239	NNDC	M	N/A	N/A	64	N/A	1529.1	15.5
	H247	NNDC	M	N/A	N/A	51	N/A	1671	31
Sporadic ALS	MN4	MND-ALS	M	None found[a]	39	41	Bulbar	1485	7
	MN5	MND-ALS	F	None found[a]	54	55	Bulbar	1296	5
	MN6	MND-ALS	M	None found[a]	53	58	Spinal	1289	8.5
	MN8	MND-ALS	M	None found[a]	83	84	Spinal	1252	3
	MN9	MND-ALS	M	None found[a]	87	88	Spinal	1241	36
	MN10	MND-ALS	M	None found[a]	42	46	Spinal – LL	-	9
	MN12	MND-ALS	M	None found[a]	46	49	Respiratory	1433.4	34
	MN13	MND-ALS	M	None found[a]	55	55	Spinal – UL	1384	10
	MN15	MND-ALS+FTD	F	None found[a]	52	54	-	-	18
	MN16	MND-ALS	M	None found[a]	63	69	Spinal – UL	1387.2	16.5
	MN19	MND-ALS	M	None found[a]	72	75	Spinal – LL	-	20.5
	MN20	MND-ALS	M	None found[a]	-	85	Spinal – LL	1328.9	15
	MN22	MND-ALS	F	ND	58	65	Spinal – UL	1137.6	9
	MN25	MND-ALS	M	ND	-	71	-	-	23.5
	MN26	MND-ALS	M	ND	75	77	Spinal – UL	1183	3
	MN27	MND-ALS+LBD-bs	F	ND	86	87	-	-	-
	MN29	MND-ALS	M	ND	-	72	-	1610.9	24
	MN30	MND-ALS	F	ND	83	84	Bulbar	1227	17.5
	MN31	MND-ALS	F	ND	58	58	-	1251	19
	MN32	MND-ALS	M	ND	57	59	-	1657.7	6
Familial ALS of unknown genotype	MN11	MND-ALS	F	None found[a]	-	77	Spinal – LL	1206	18
	MN14	Probable-ALS	F	None found[a]	57	59	Bulbar	1180.2	19
	MN21	MND-ALS	F	None found[a]	56	59	Spinal – LL	-	17.5
***SOD1*-linked**	MN24	MND-ALS	F	*SOD1* p.E101G	-	54	-	1328	-
***FUS*-linked**	BBN_10244	MND-ALS	F	*FUS* p.P525L	22.3	23	Spinal	-	37
***UBQLN2* VOUS or *UBQLN2*-linked**	BBN_16359	PSP	M	*UBQLN2* p.S222G	-	93	-	-	19.5
	MN17 – T487I	MND-ALS+FTD	F	*UBQLN2* p.T487I	58	61	Spinal	-	69
	V:7 – T487I	MND-ALS+FTD	M	*UBQLN2* p.T487I	30	39	-	-	25.5
	P497H	ALS	F	*UBQLN2* p.P497H	46	57	Respiratory	-	-
	II:3 – P497L	ALS	F	*UBQLN2* p.P497L	63	67	Spinal – LL	-	-
	III:3 – P497L	ALS+FTD	F	*UBQLN2* p.P497L	20	37	Bulbar	-	-
	IV:2 – P497L	ALS+FTD	F	*UBQLN2* p.P497L	20	24	Bulbar	-	-
	BBN_15292	MND-ALS+FTD	F	*UBQLN2* p.P506S	54	56	Cognitive	-	35
***C9orf72*-linked**	MN2	MND-ALS	F	*C9orf72* expansion[b]	52	53	Spinal	-	>24
	MN18	MND-ALS	F	*C9orf72* expansion[c]	47	53	Spinal – LL	1269	12
	MN23	MND-ALS	F	*C9orf72* expansion[d]	77	79	Spinal – LL	1232	27
	MN28	MND-ALS	F	*C9orf72* expansion[e]	50	62	Bulbar and Spinal – UL	1127	14
	MN33	MND-ALS	M	*C9orf72* expansion[d]	63	65	Spinal – LL	1304	46

[a] No mutations detected in *C9orf72, TARDBP, FUS,* or *SOD1,* as previously described in Scotter *et al.*[5].
[b] Obligate carrier, affected offspring genotyped.
[c] Genotyped in Scotter *et al.*[5].
[d] Genotyped during life.
[e] Genotype inferred from neuropathology.
Cases MN17 – T487I, V:7 – T487I, P497H, and P506S were previously published in references [1], [2], and [3], respectively. Cases II:3 – P497L, III:3 – P497L, and IV:2 – P497L were previously published in reference [4]. Abbreviations: -, unknown; AAD, age at death; AAO, age at

onset; LBD-bs, LL, lower limb; Lewy body disease- brainstem predominant; N/A, not applicable; ND, not done; NNDC, non-neurologically diseased control; PMD, post-mortem delay; PSP, progressive supranuclear palsy; UL, upper limb.

Supplementary Table 3 Primary antibodies

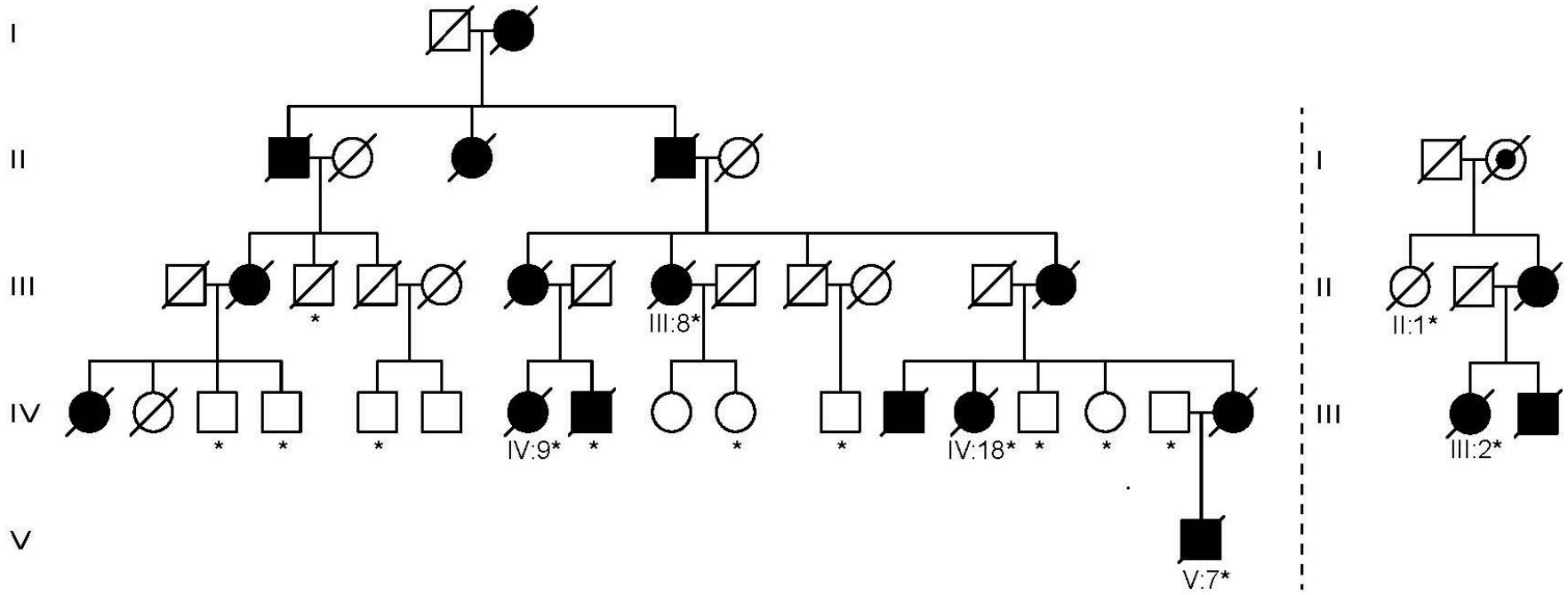
Primary antibody	Species (isotype)	Manufacturer	Catalogue #	RRID	Clonality	Dilution
Multiplex Fluorescent Immunohistochemistry panel						
C9RANT (PolyGA)	Mouse (IgG1κ)	Merck Millipore	MABN889	AB_2728663	Monoclonal	1:2000
Ubiquilin 2	Mouse (IgG_{2a})	Santa Cruz Biotechnology	SC-100612	AB_2272422	Monoclonal	1:1000
C9RANT (PolyGP)	Rabbit (IgG)	Novus Biologicals	NBP2-25018	AB_2893239	Polyclonal	1:3000
pTDP-43 (Ser409/410)	Rat (IgG_{2a})	BioLegend	BL829901, Clone 1D3	AB_2564934	Monoclonal	1:3000
p62	Guinea Pig (IgG)	Progen	GP62-C	AB_2687531	Polyclonal	1:500
Double-label Fluorescent Immunohistochemistry for STED imaging panel						
C9RANT (PolyGA)	Mouse (IgG1κ)	Merck Millipore	MABN889	AB_2728663	Monoclonal	1:2000
Ubiquilin 2	Mouse (IgG2a)	Santa Cruz Biotechnology	SC-100612	AB_2272422	Monoclonal	1:1000

Abbreviations: RRID, Research resource identifier; pTDP-43, phosphorylated (Ser409/410) TDP-43.

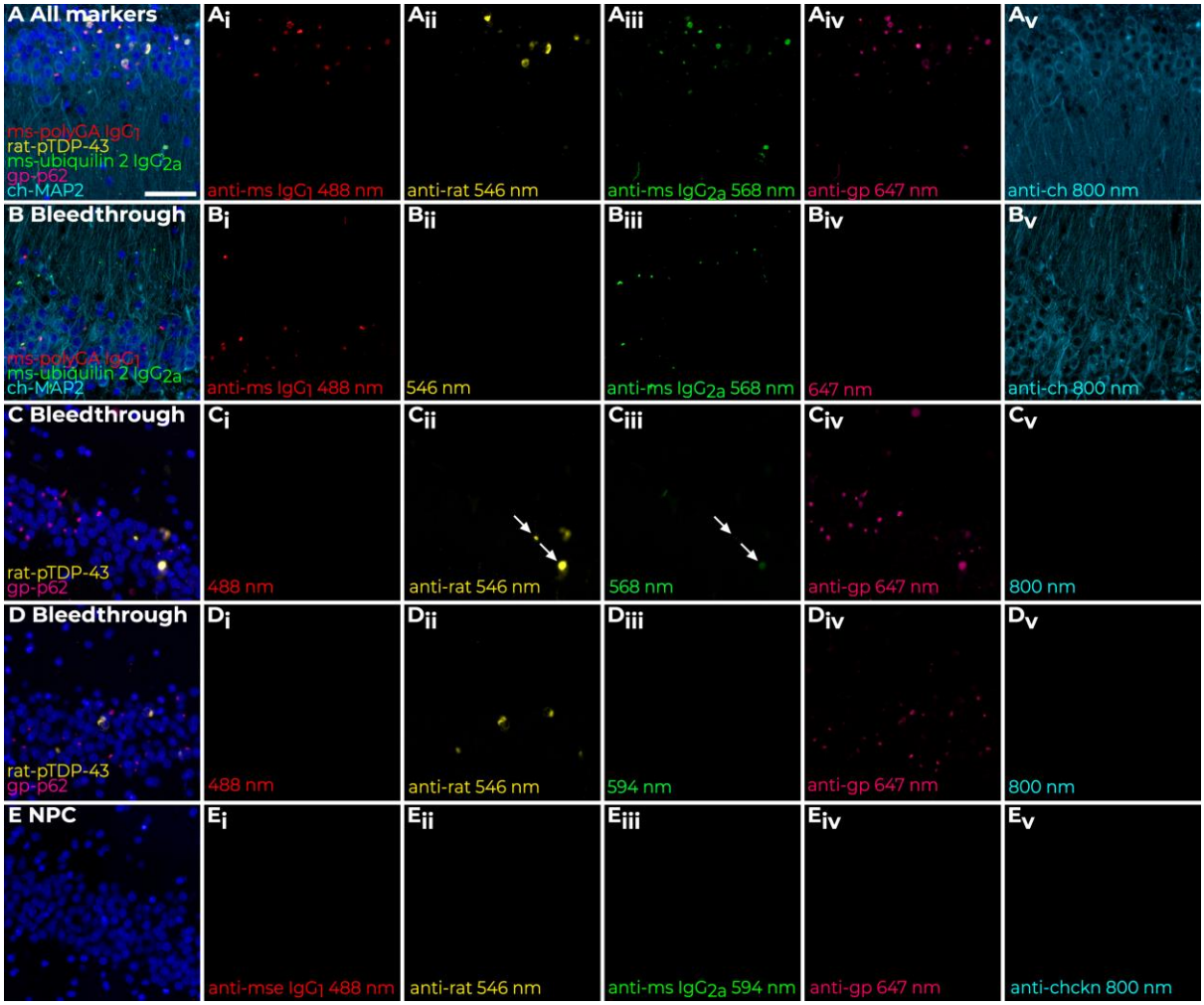
Supplementary Table 4 Secondary antibodies and stains

Secondary antibody	Conjugate	Manufacturer	Catalogue #	RRID	Clonality	Dilution
Multiplex Fluorescent Immunohistochemistry panel						
Goat anti-mouse IgG1$_K$	Alexa Fluor® 488	Thermo Fisher Scientific	A-21121	AB_2535764	Monoclonal	1:500
Goat anti-mouse IgG$_{2a}$	Alexa Fluor® 594	Thermo Fisher Scientific	A-21135	AB_2535774	Monoclonal	1:500
Goat anti-rabbit IgG	IRDye 800CW	LI-COR Biosciences	926-32211	AB_2651127	Polyclonal	1:500
Goat anti-rat IgG$_{2a}$	Alexa Fluor® 546	Thermo Fisher Scientific	A-11081	AB_141738	Polyclonal	1:500
Goat anti-guinea pig IgG	Alexa Fluor® 647	Thermo Fisher Scientific	A-21450	AB_141882	Polyclonal	1:500
Stain	**Species (isotype)**	**Company**	**Catalogue #**	**RRID**	**Clonality**	**Dilution**
Hoechst 33342 nuclear stain	-	Thermo Fisher Scientific	H3750		-	1:2000
Double-label Fluorescent Immunohistochemistry for STED imaging panel						
Goat anti-mouse IgG1k	Biotin	Thermo Fisher Scientific	A10519	AB_1500809	Monoclonal	1:500
Abberior Star Red	Neutravidin	Abberior	STRED-0121		-	1:500
Goat anti-mouse IgG$_{2a}$	Alexa Fluor® 594	Thermo Fisher Scientific	A-21135	AB_2535774	Monoclonal	1:500

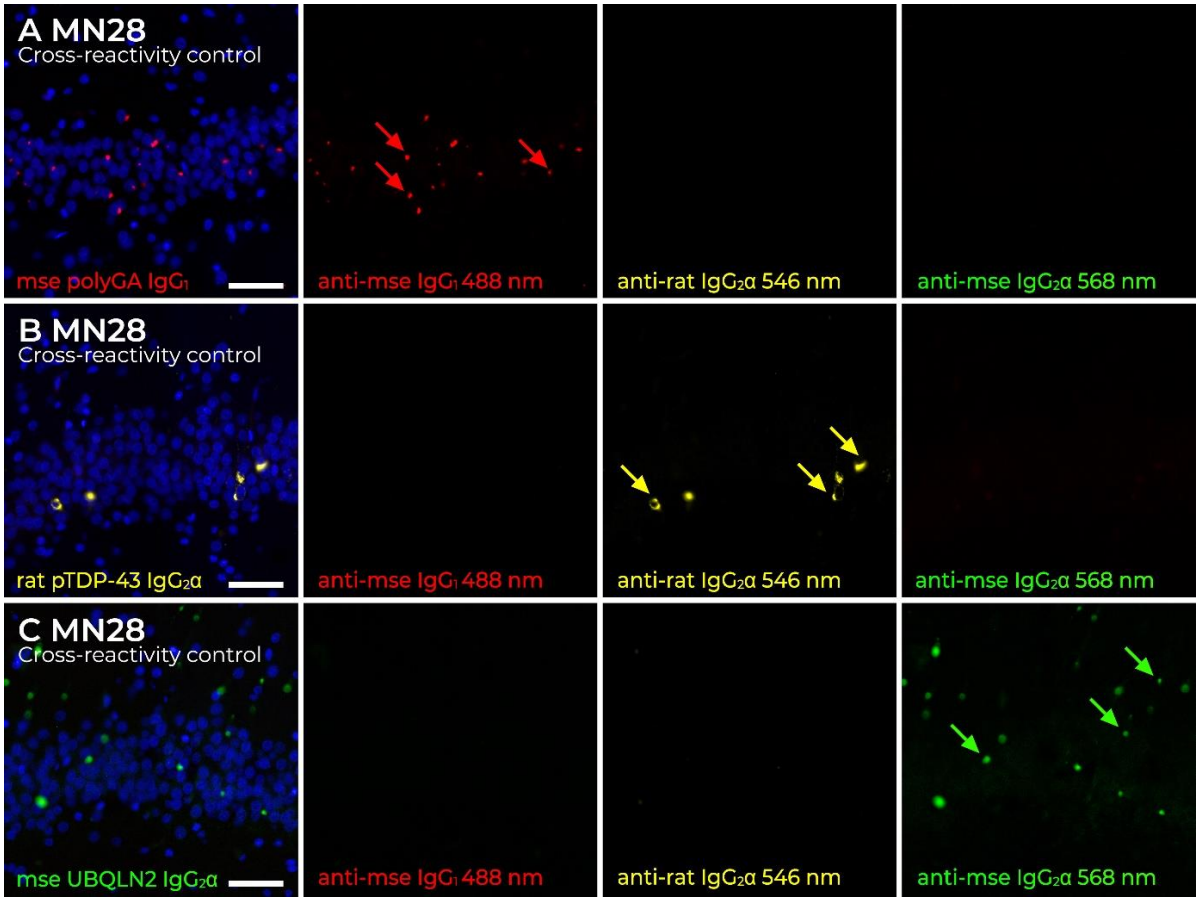
Abbreviations: RRID, Research resource identifier; pTDP-43, phosphorylated (Ser409/410) TDP-43.



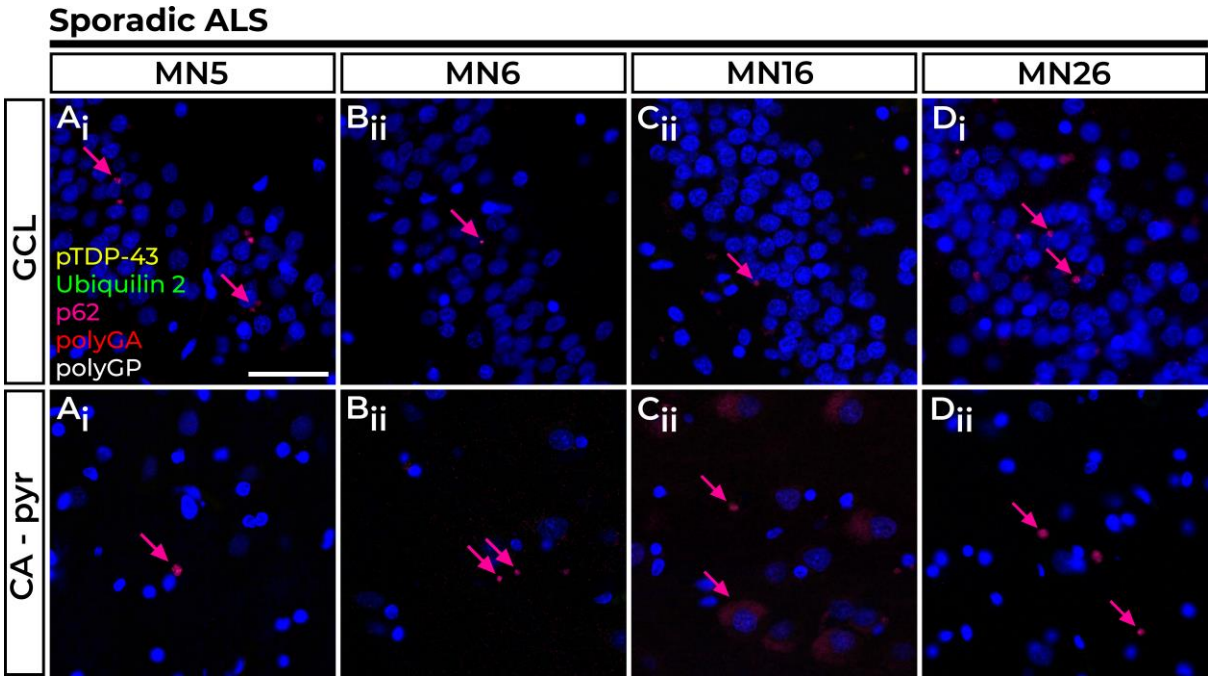
Supplementary Figure 1 Pedigrees of families FALS5 and FALS14. Pedigrees of family FALS5 (left) and FALS14 (right) showing segregation of the *UBQLN2* c.1460C>T (p.T487I) mutation. DNA from individuals III:8, IV:9, and IV:18 from FALS5; and individuals II:1 and III:2 from FALS14 were analysed for identity-by-descent. Brain tissue from individuals IV:18 and V:7 from FALS5 was analysed for neuropathology. Filled symbols, affected by ALS and/or FTD; Open symbols, unaffected; Asterisks, genotyped for *UBQLN2* p.T487I. Original pedigrees were published in [1].



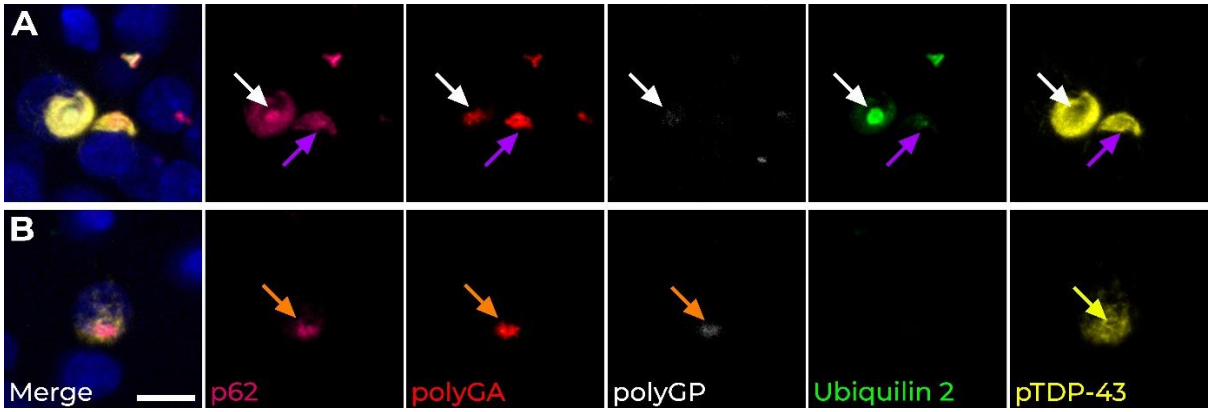
Supplementary Figure 2 Multiplex immunohistochemistry secondary antibody bleed-through, bleed-back, and secondary-only controls. Aggregate staining was first optimised in *C9orf72*-linked ALS case MN28, as it was immunopositive for all markers (**A-A$_v$**). DPR protein polyGA staining in the 488 nm channel (**B$_i$**) demonstrated no bleed-through into the next channel, 546 nm (**B$_{ii}$**). Additionally, ubiquilin 2 staining in 568 nm (B3) showed no bleed-back into 546 nm (**B$_{ii}$**), nor bleed-through into the 647 nm channel (**B$_{iv}$**). pTDP-43 staining in the 546 nm channel (**C$_{ii}$**) showed trace amounts of bleed-through into the 568 nm channel (**C$_{iii}$**, white arrowheads). As a result, a 594 nm secondary antibody was used to detect ubiquilin 2 instead and when imaged, showed no bleed-through of pTDP-43 (**D$_{iii}$**). Secondary antibodies were specific to primary antibodies directed against polyGA, pTDP-43, ubiquilin 2, p62, and MAP2 as no staining was observed when primary antibodies were omitted (**E-E$_v$**). Scale bar for all images, 50 µm.



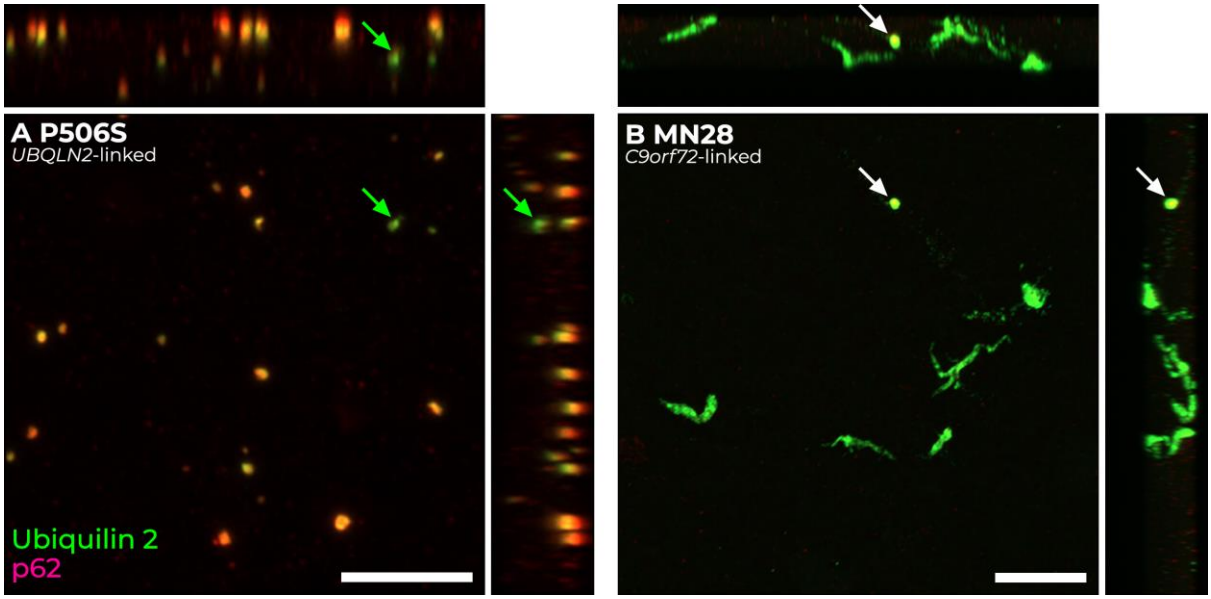
Supplementary Figure 3 Multiplex immunohistochemistry secondary antibody cross-reactivity controls. Aggregate staining was first optimised in *C9orf72*-linked ALS case MN28, as it stains for all markers. DPR protein polyGA staining using a mouse IgG1κ primary (**A**, red arrows) was not detected by secondaries against rat IgG2a (546 nm) or mouse IgG2a (568 nm) when all secondaries were added. Additionally, pTDP-43 staining using a rat IgG2a primary (**B**, yellow arrows) was not detected by secondaries against mouse IgG1 (488 nm) or IgG2a (568 nm). Finally, a mouse IgG2a secondary (568 nm) specifically detected ubiquilin 2 aggregates stained using a mouse IgG2a primary (**C**, green arrows), as no staining was observed in the 488 nm or 546 nm channels. Note: In the final immunohistochemical runs for all cohort cases, a mouse IgG2a 594 nm secondary was used to visualise ubiquilin 2 staining. The 568 nm secondary antibody shown in this optimisation and the 594 nm secondary antibody are equivalent as per manufacturer's information, but with different conjugated fluorophores. Scale bar for all images, 50 μm.



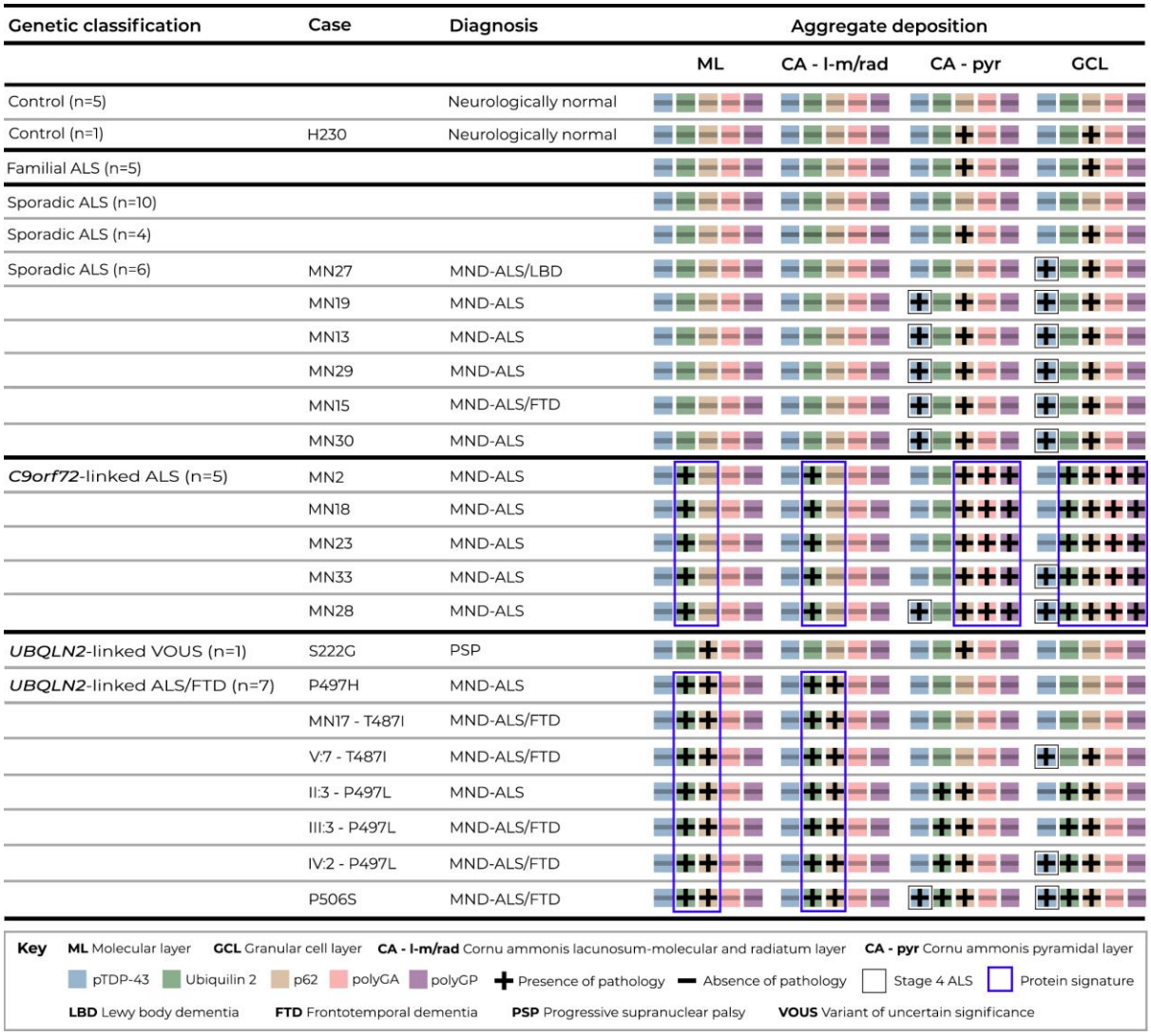
Supplementary Figure 1 P62 pathology in the hippocampal dentate gyrus and cornu ammonis regions of sporadic ALS cases. Punctate p62-positive inclusions were found in the GCL (**A_i-D_i**), and CA-pyr layers (**A_{ii}-D_{ii}**) in MN5, MN6, MN16, and MN26, all ALS cases of unknown genotypic cause. No hippocampal ubiquilin 2, pTDP-43, or DPR (polyGA and polyGP) protein pathology was observed in any of these cases. Scale bar, 50 μm.



Supplementary Figure 2 Hippocampal pTDP-43 rarely co-localises with p62-positive ubiquilin 2 and polyGA and/or polyGP in *C9orf72*-linked case MN28. In the hippocampal granule cell layer, two types of aggregates were observed to be positive for pTDP-43; one was immunopositive for all markers (**A**, white arrows), while the other was immunopositive for p62, polyGA, and ubiquilin 2, and pTDP-43 without polyGP (**A**, purple arrows). Another rare form of dipeptide repeat aggregate was immunopositive for p62, polyGA and polyGP (**B**, orange arrows) with a pTDP-43 shell (**B**, yellow arrow). Scale bar, 10 μm.



Supplementary Figure 3 Mutant ubiquilin 2 was p62 positive in hippocampal molecular layer. Maximum intensity Z-projections with orthogonal planes of the molecular layer confirmed that almost all mutant ubiquilin 2 aggregates in *UBQLN2*-linked ALS/FTD case p.P506S were compact and p62-labelled (**A**, z= 22.25 µm) with very few ubiquilin 2 aggregates that were p62-negative (**A**, green arrow), while the majority of wildtype ubiquilin 2 in *C9orf72*-linked case MN28 were wispy and p62-negative (**B**, z= 13 µm). A rare p62 co-labelled ubiquilin 2 aggregate is indicated with a white arrow in **B**. Scale bar, 10 µm.



Supplementary Figure 7 Combined pathological signatures discriminated between sporadic, *C9orf72*-linked, and *UBQLN2*-linked ALS/FTD. Key shown within figure. P62 aggregates ('+' symbol on brown box) without other co-labelling were observed in CA – pyr and GCL cells in a subset of neurologically normal controls (n=1 of 6), unrelated familial ALS (n=5 of 5), and sporadic ALS cases (n=4 of 20). In contrast, pTDP-43 pathology (boxed '+' symbol on blue box) was present in these layers in a separate subset of sALS cases (n=6 of 20), co-localising with p62. Ubiquilin 2 pathology ('+' symbol on green box) was present in the hippocampus when ubiquilin 2 was wildtype (*C9orf72*-linked ALS) or mutant (*UBQLN2*-linked ALS/FTD). Wildtype ubiquilin 2 in *C9orf72*-linked ALS ML and CA – l-m/rad regions was p62-negative, and in the CA – pyr layer was associated with polyGA, polyGP, and p62 aggregates. PolyGA and polyGP aggregates were found in the GCL with or without ubiquilin 2, all colocalising with p62. CA – pyr cell and GCL aggregates had additional pTDP-43 pathology in only one case, and even then, only rarely. Mutant ubiquilin 2 aggregates in *UBQLN2*-linked ALS/FTD ML, and CA – l-m/rad regions were p62-positive and associated with GCL pTDP-43 aggregates only in some cases and either with or without ubiquilin 2 co-labelling. Blue outlines indicate unique aggregation features of *C9orf72*-linked ALS and *UBQLN2*-linked ALS/FTD hippocampal pathology. Abbreviations: CA – l-m/rad, cornu ammonis – lacunosum-molecular and radiatum layers; CA – pyr, cornu ammonis – pyramidal cells; GCL, granule cell layer.

References for Supplementary materials

1. Williams KL, Warraich ST, Yang S, et al. UBQLN2/ubiquilin 2 mutation and pathology in familial amyotrophic lateral sclerosis. *Neurobiol Aging*. 2012;33(10):2527.e3-2527.e10. doi:10.1016/j.neurobiolaging.2012.05.008

2. Deng HXX, Chen W, Hong STT, et al. Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia. *Nature*. 2011;477(7363):211-215. doi:doi:10.1038/nature10353

3. Gkazi SA, Troakes C, Topp S, et al. Striking phenotypic variation in a family with the P506S UBQLN2 mutation including amyotrophic lateral sclerosis, spastic paraplegia, and frontotemporal dementia. *Neurobiol Aging*. 2019;73:229.e5-229.e9. doi:10.1016/j.neurobiolaging.2018.08.015

4. Fahed AC, McDonough B, Gouvion CM, et al. UBQLN2 mutation causing heterogeneous X-linked dominant neurodegeneration. *Ann Neurol*. 2014;75(5):793-798. doi:10.1002/ana.24164

5. Scotter EL, Smyth L, Bailey JWT, et al. C9ORF72 and UBQLN2 mutations are causes of amyotrophic lateral sclerosis in New Zealand: a genetic and pathologic study using banked human brain tissue. *Neurobiol Aging*. 2017;49:214.e1-214.e5. doi:10.1016/j.neurobiolaging.2016.06.019