

Title: Mutations in *ANXA11* cause familial and sporadic amyotrophic lateral sclerosis

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One sentence Summary: A stringent filtering strategy applied to 751 ALS exomes identified p.D40G and other mutations in *ANXA11* that produced cytoplasmic aggregates in patient spinal cord and *in-vitro* and disrupted calyculin binding.

Abstract:

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder. We screened 751 familial ALS exomes and identified 6 novel, or extremely rare, *ANXA11* mutations in 13 individuals. The novel p.D40G mutation which was absent from 70,000 control exomes; segregated with disease in two kindreds, was present in another two unrelated cases ($p=0.0102$), and all carriers shared a common haplotype. Annexin A11-positive aggregates were abundant in spinal motor neurons and hippocampal axons in a p.D40G patient. Transfected cells expressing p.D40G and other N-terminal mutations had altered calyculin binding while the p.R235Q mutant formed insoluble aggregates. *ANXA11* mutations accounted for ~1% of familial and ~1.7% of apparent sporadic cases. Mutations in *ANXA11* highlight the role of defective cellular trafficking in the pathogenesis of ALS.

Introduction

Gene hunting in rare Mendelian disorders has been transformed by exome sequencing. This approach is particularly attractive for late-onset autosomal dominant syndromes with short disease durations, such as Amyotrophic Lateral Sclerosis (ALS) in which DNA from multiple affected individuals in the same kindred are rare. ALS has a life-time risk of 1 in 400 and is characterized by degeneration of brain and spinal cord motor neurons resulting in progressive paralysis and death within an average of 3 years [1]. 10% of cases are familial (FALS) and a causative gene mutation can be identified in ~60% of European kindreds [2]. Mutations in the same genes account for ~10% of sporadic ALS cases (SALS) reflecting incomplete penetrance. Non-synonymous mutations in *SOD1*, *TARDBP*, *FUS* and an intronic hexanucleotide repeat expansion in *C9orf72* account for ~20% of ALS cases and the other genes for ~1-3% [3]. Exome sequencing has identified seven ALS genes by either a shared variant segregation analysis in ALS kindreds (*VCP*, *PFN1*, *MATR3* and *CHCHD10*) or a rare variant burden analysis (*TUBA4A*, *TBK1*, *NEK1*) [4-12]. Here we analysed FALS exomes and identified a novel non-synonymous founder mutation in *ANXA11* that is present in all affected family members tested and is also found in multiple unrelated index cases. Annexin A11 is a widely expressed, calcium-dependent phospholipid-binding protein (505AA, 56kDa) that belongs to the larger human Annexin family of 12 members [13]. Each family member has four highly

conserved annexin domains many of which can complex a calcium ion facilitating binding to anionic cell membranes. Unique to the annexin family, Annexin A11 has the longest N-terminus (~196 amino acids) that is hydrophobic and disordered that binds several interacting partners: the most characterized being calcyclin (encoded by *S100A6*) [14]. In regard to involvement with disease, Annexin A11 is associated with autoimmune disorders such as systemic lupus erythematosus and case control studies have found a genetic association of the R230C single nucleotide polymorphism (SNP) with sarcoidosis [15, 16]. Additionally, enhanced Annexin A11 expression is also found in breast cancer and other acquired malignancies [15]. For the first time, we present a novel role of Annexin A11 in a rare, neurodegenerative Mendelian disorder as evidenced by novel mutations that are associated with Amyotrophic Lateral Sclerosis (ALS).

Results

(i) Exome sequencing, filtering and gene identification

From our cohort of 751 European FALS exomes (negative for *C9orf72* GGGGCC expansions) we obtained exome data for two or more affected relatives from only 50 families (average=2.14 individuals per family), highlighting the difficulty in accessing DNA from extended kindreds for this late onset disorder. Families ranged from a simple pair of siblings (sharing an estimated 50% of their variants) to an index case, parent and a second cousin (sharing an estimated 1.5% of their variants). On average 84% (range=58-97%) of the protein coding bases contained in Refseq transcripts were sequenced in all family members to a depth of ≥ 10 reads. Our filtering strategy was to detect novel, high quality, coding and splicing variants that are absent from 1000genomes, UK10K, EVS and ExAC ($n > 72,000$). This produced an average of ~10 candidates per family (range=0-27) (Supplementary Table 1). As proof of principle the analysis identified mutations shared in single kindreds from several known ALS genes, including *SOD1*, *TARDBP*, *FUS*, *DCTN1* and *TUBA4A* [8, 17-20]. It was immediately apparent that only two variants appeared in the list of candidates for more than one family; the well characterized pathogenic M337V mutation in *TARDBP* [21] was found in two North American families, and a novel p.D40G variant in *ANXA11* (Refseq NM_145869). Variants in this novel gene were found in two British families (an uncle/niece pair, and two cousins). The full list of candidate variants found in both UK *ANXA11* p.D40G families are listed in Supplementary Table 2. One additional Italian proband from the exome cohort also carried the same *ANXA11* D40G variant.

We then extended the analysis to include 694 unrelated European FALS probands (including only the 50 probands from our multiplex families) and sought novel protein-changing variants that were shared by three or more probands. This approach also identified *ANXA11* D40G, and the following well characterised pathogenic ALS mutations: 10x *SOD1* I114T [22], 6x *TARDBP* A382T [18], 5x *SOD1* A5V [23] 4x *FUS* R521C [19], 3x *TARDBP* M337V, 3x *SOD1* G94D [24], 3x *FUS* P525L [25].

We then Sanger sequenced *ANXA11* in a separate set of 180 British apparent SALS cases and identified one further heterozygous p.D40G carrier, bringing the total number to four out of a combined cohort of 874 probands.

As the two p.D40G UK families were underpowered to conduct a linkage analysis (Simulated LOD score of 0.63 using MERLIN [26]) we ascertained the significance of the p.D40G mutation. It is possible that a variant could be present 4 times in a sample of 874 cases and absent from 72,000 other individuals yet still be unrelated to disease. We therefore tested the null hypothesis: that any equal sized cohort of Europeans could also contain a novel variant shared by at least 4 people. We achieved this by running simulation studies using the aggregated variant call counts from the Non-Finnish European (NFE) subset of the ExAC database (n=33,370). In brief, all ExAC NFE variants were randomly distributed across 33,370 simulated individuals and random cohorts of 874 people extracted. The cohort was deemed to have 'passed' if it contained at least one protein-changing variant found 4 or more times within the cohort, but absent from the remainder of ExAC, UK10K, EVS, and 1000genomes. After 250,000 iterations, only 2,550 simulated cohorts contained such a variant, which demonstrates that the detection of the D40G variant is statistically significant ($p=0.0102$). Although not population-matched to our cohort, we consider ExAC to be suitable for this purpose as it contains a high proportion of Swedes who are on average more genetically homogeneous than UK individuals (Supplementary Table 3). Therefore ExAC would be expected to produce more shared non-pathogenic variants than our cohort, and so we expect this estimate of significance to be a conservative one. In conjunction with the family-based study the simulation analysis provides additional evidence that the *ANXA11* D40G mutation is associated with ALS.

(ii) The D40G mutation has a common European founder

Sanger sequencing of DNA from 17 family members across the two multi-generational British kindreds confirmed the presence of the p.D40G mutation in all four affected individuals identified from the exome capture data (Figs 1A-B). Four unaffected individuals also carried the mutation, but incomplete penetrance of ALS

mutations is well recognized and three of these were in their forties whilst the average age of disease onset in *ANXA11* p.D40G affected carriers is 72 years of age. DNA was available from five British p.D40G carriers who share a common haplotype on the disease allele defined by 4 exonic SNPs and two polymorphic microsatellites spanning the locus with phase determined by a cluster of carriers in UK Family 2, confirming a common founder (Supplementary Figure 1) (primers Supplementary Table 4). The minimal haplotype is defined by 4 SNPs, and a physical stretch of 2.5MB of DNA common to all p.D40G carriers spanning the *ANXA11* locus. The 4 SNP haplotype is present in ~5% of our extended FALS cohort (n=787, including *C9orf72* expansion positive cases) and ~5% of Europeans from 1000genomes (n=514) which suggests that the mutation arose on a European background. The maximal recombination region defining the limits of the p.D40G locus is 7.1MB and contains 23 genes (Supplementary Figure 2). Interrogation of exome sequencing data found that no p.D40G carriers shared any additional protein-changing variants in the 23 genes within this region. 70.3–97.2% of coding bases were covered to a read depth ≥ 10 for each sample, and 97.9% of bases were covered by ≥ 10 reads in at least one sample (Supplementary Table 5). This evidence indicates that p.D40G is the sole causal variant within this locus.

(iii) ANXA11 mutations in FALS and SALS cluster in the N-terminus

Additional *ANXA11* variants identified in the FALS cohort were two unrelated individuals carrying a c.112G>A, p.G38R variant (rs81930608) that was absent from local UK and Italian exome controls (n=4505). Although the p.G38R variant is present in 5/31804 NFE ExAC exomes it is still significantly associated with FALS in our cohort (p=0.004, 2-tailed Fishers exact test). Novel p.G175R (c.523G>A) and p.R346C (c.1036C>T) variants were also detected in two index cases. The p.G175R was also present in an affected sibling with ALS (Fig 1C) confirming segregation with disease. All FALS cases possessing *ANXA11* variants were negative for exonic coding mutations in all known ALS genes including *SOD1*, *TARDBP*, *FUS*, *PFN1*, *UBQLN2*, *MATR3*, *CHCHD10*, *TBK1*, *OPTN*, *VAPB*, *ANG*, *SQSTM1*, *NEK1* and *VCP*.

All coding exons of *ANXA11* were Sanger sequenced in an additional 180 British SALS cases (primers listed in Supplementary Table 4). As previously mentioned, an additional D40G case was identified as well as an individual carrying a p.G189E (c.566G>A) variant, present once in local controls (n=4505) and 14 times in the ExAC NFE sub-population (n=33,140). Interestingly p.G189E is also present once in ALSdb (<http://chgv.org/alsdb/index.jsp>). The last variant, located in the first annexin

domain, was a p.R235Q (c.704G>A) change absent in local controls (n=4505) but present once as a low quality variant call in ExAC. Of note, ALS cases carrying *ANXA11* mutations did not carry the R230C risk allele (T/A, rs1049550) that is associated with Sarcoidosis [16]. All variants found in FALS and SALS cases are listed in Supplementary Table 6 with control database frequencies and prediction of pathogenicity following guidelines laid out by the American College of Genetics [27].

Four of the six mutations in Annexin A11 cluster with p.G38R and p.D40G within the long N-Terminus implying that this region has functional importance. All mutation positions are completely conserved in mammals and those in the annexin domains are also conserved in all currently sequenced birds, amphibians and reptiles (Fig 1D).

(iv) ANXA11 D40G cases have late disease onset and cytoplasmic immuno-reactive inclusions in post mortem tissue

All patients with *ANXA11* mutations had late disease onset (average 67 yrs) with a classical ALS phenotype without features of dementia (Supplementary Table 7). Five of six patients carrying the p.D40G variant had bulbar onset. Post mortem tissue was available from a SALS case carrying the p.D40G variant showing classical pathological features of ALS with a unique feature of large Annexin A11 immunoreactive inclusions that were absent from other ALS cases or controls (Fig 2). Spinal cord sections revealed marked neuronal loss within the anterior horns. There was marked myelin pallor and astrogliosis in the anterior and lateral corticospinal tracts. Many surviving motor neurons contained cytoplasmic inclusions positive for p62 and phospho-TDP-43 (Fig 2A). Phospho-TDP-43 inclusions were also found in the medulla, temporal neocortex and hippocampus (data not shown). Annexin A11 staining of spinal cord sections showed numerous neuronal cytoplasmic inclusions (NCIs) in the perikaryon and proximal axon (Figs 2B-E). These included skein-like (Fig 2B), large-caliber tubular-shaped structures (Figs 2C-D) as well as filamentous and more complex basket-like inclusions (Fig 2E). Annexin A11 positive NCIs were also evident in small numbers in the motor cortex, dentate gyrus of the hippocampus and temporal neocortex sometimes accompanied by abundant torpedo-like neuritic structures in the neuropil (Fig 2F). Occasional, sparse Annexin A11 positive NCIs and neurites were also seen in the occipital lobe but no staining was observed in cerebellum. Staining for the amyloid precursor protein, Neurofilament (light, medium and heavy) and β 3 tubulin was negative. SALS (n=13) devoid of known mutations were negative for Annexin A11 NCIs in spinal cord (Figure 2G), as were 2 *C9orf72*

expansion positive cases and a *SOD1* positive case with a p.D101G mutation (Figs 2H-J). Additionally, tissue from 3 ALS/FTD cases (Fig 2K), 3 Alzheimer's (Fig 2L) and 3 Parkinson's were negative for Annexin A11 positive NCIs in the cortex (n=2) and spinal cord (n=1) for ALS/FTD and in cortical and brainstem tissue for AD and Parkinson's respectively. Spinal cord Annexin A11 staining was negative in 13 controls (Figure 2M) (including frontal and temporal cortex and medulla regions in 3 controls). Double fluorescent labelling of phospho-TDP-43 and annexin A11 in spinal cord neurons showed no evidence of co-localization (Fig 2N and O). Some Annexin A11 positive aggregates were ubiquitinated (Fig P). The cytoplasmic aggregation seen in post-mortem tissue from the D40G patient may be due to enhanced Annexin A11 expression due to an additional promoter mutation. However, screening of the *ANXA11* promoter identified no novel, rare or common variants (MAF<0.05) in any of the cases carrying *ANXA11* mutations. Furthermore, protein expression of lysates made from frontal cortex tissue of the SALS case possessing the p.D40G change did not show any changes in expression due to the mutant D40G allele compared to 3 age and sex matched controls (Supplementary Figure 3).

(v) Over-expressed Annexin-A11^{R235Q} forms foci in mouse Primary Motor Neurons that are insoluble

In order to understand the functional significance of these variants we undertook a series of cellular studies of mutations identified in our exome set. We generated Annexin-A11^{WT}, Annexin-A11^{G38R}, Annexin-A11^{D40G} and Annexin-A11^{R235Q} tagged with hemagglutinin (HA) or GFP at the C-terminus. Constructs were expressed in mouse primary motor neurons (PMNs) and human embryonic kidney 293 (HEK) cells. PMNs transfected with AnnexinA11-HA^{WT} constructs showed nuclear and cytoplasmic localization. In the cytoplasm Annexin A11 was present in larger vesicle-like structures, smaller foci structures and diffusely distributed throughout the soma, axons and dendrites (Fig 3A). Apart from Annexin A11-HA^{R235Q}, which was elevated in the cytoplasm, there was no evidence that the other mutations affected the distribution between the nucleus and cytoplasm (Fig 3B) [28]. The Annexin A11-HA^{R235Q} mutant appeared to aggregate into foci, was never associated with vesicle-like structures and diffuse staining was absent (p<0.001) (Fig 3C). Annexin A11-HA^{D40G} had a similar pattern to WT but Annexin A11-HA^{G38R} displayed significantly less association with the vesicle-like structures (p=0.005). Annexin A11-GFP^{R235Q} in HEK cells showed marked aggregation into ubiquitin and p62 positive inclusions that were also specific for endogenous Annexin A11 antibody and confirmed by western

blot (Supplementary Figure 4). Annexin A11-GFP^{R235Q} also produced high molecular weight species on western blot that were insoluble (Figs 3D-E) (p=0.007).

(vi) Annexin-A11^{R235Q} sequesters wild-type Annexin-A11

We also investigated whether the aggregation prone R235Q mutation recruits WT Annexin A11. We did this by co-transfecting SH-SY5Y cells with either Annexin A11-GFP^{WT} and Annexin A11-HA^{WT} or Annexin A11-GFP^{R235Q} and Annexin A11-HA^{WT} constructs (Figure 4a). The merge of Annexin A11-HA^{WT} and Annexin A11-GFP^{WT} showed nuclear and diffuse cytoplasmic staining as expected (top panel), whereas Annexin A11-GFP^{R235Q} cytoplasmic puncta and WT-HA showed co-localization (bottom panel). This suggests that insoluble, mutant Annexin A11 recruits and sequesters wild-type Annexin A11. This was confirmed by firstly conducting a solubility assay on HEK cells co-transfected with Annexin A11-HA^{WT} and either Annexin A11-GFP^{WT} or Annexin A11-GFP^{R235Q} and secondly by immunoprecipitation (IP) and analyzed by Western blot. For the solubility assay, only the insoluble urea fraction of the Annexin A11-HA^{WT} / Annexin A11-GFP^{R235Q} co-transfection contained Annexin A11-HA^{WT} compared to the WT-Annexin A11^{HA/GFP} co-transfection (Figure 4b), demonstrating that the insoluble R235Q mutant can sequester wild-type Annexin A11. Furthermore, co-transfection of HEK cells with Annexin A11-HA^{WT} and Annexin A11-GFP^{R235Q}, and pulldown with rabbit-GFP antibody and blotting with mouse-HA, demonstrated co-precipitation of Annexin A11-HA^{WT} with clear banding of higher molecular weight insoluble species Figure 4c). This provides evidence that mutant Annexin A11 species recruits WT Annexin A11 and thus may act in a dominant negative manner.

(vii) Annexin A11 mutants abolish binding to Calcyclin

Residues 50-62 in Annexin A11 bind calcyclin (encoded by *S100A6*) [29] and are close to the mutants p.G38R and p.D40G, and so we sought evidence for any effect on their interaction. Calcyclin is a 10kDa protein that contains 2 EF-hand calcium-binding motifs [30]. Jpred4 secondary structure predictions [31] on an alignment of all mammalian Annexin A11 orthologue sequences predicted that residues 40-44 and 51-59 form dual amphipathic helices (Supplementary Figure 5A). These helices mirror those seen in Annexin A1 which in the absence of calcium are embedded within the annexin core but are released on calcium activation facilitating its binding to S100A11 and also mediating calcium-dependent protein-membrane interactions [32, 33] (Supplementary Figure 5B-C). Whilst the N-terminus of Annexin 11 is much longer than, and highly divergent from the N-terminus of Annexin 1, the high

conservation of the first 4 residues in the second helix (QEYV in Annexin 1 versus QDYL in Annexin 11) imply a possible conserved binding site in the annexin core and is additional evidence for a similar mechanism of action. Both the Annexin A11 p.G38R and p.D40G variants are predicted to prevent or severely reduce formation of the first amphipathic helix (Supplementary Fig 5D). Extending this analysis to 22 rare and common polymorphisms listed in ExAC, spanning the D40 residue, predicted no abolition of the first or second N-terminal amphipathic helices (Supplementary Figure 6) suggesting p.G38R and p.D40G to be disease specific.

In order to assess the effects of mutations in Annexin A11 on calcyclin binding we performed binding assays of Annexin A11-GFP^{WT} and the Annexin A11^{G38R/D40G/G189E/R235Q} mutants using a previously published *in-vitro* IP assay [34] (also methods). As expected, Annexin A11-GFP^{WT} co-immunoprecipitated with calcyclin, confirming calcyclin–Annexin A11 binding. Annexin A11-GFP^{D40G} severely disrupted calcyclin binding, as did Annexin A11-GFP^{G189E} and Annexin A11-GFP^{R235Q} mutants (Figure 5A). Annexin A11-GFP^{G38R} binding however was markedly increased compared to WT. To assess the specificity of the loss of calcyclin binding due to ANXA11 mutants, we conducted binding assays with four ANXA11 polymorphisms present in ExAC. These were a rare p.D40H variant (once in ExAC, rs368751524) not detected in our ALS cases and 2 common NFE N-terminal polymorphisms flanking p.D40G ie. p.P8L (rs147334030, MAF 0.011) and p.R191Q (rs2229554, MAF 0.07) and the p.R230C SNP associated with Sarcoidosis (rs1049550, MAF 0.44). These polymorphisms behaved in the same manner at wild-type and did not abolish calcyclin binding (Supplementary Figure 7) when compared to the p.D40G and p.R235Q mutations. Therefore, disruption to calcyclin binding in ALS cases harbouring ANXA11 mutations is disease specific. No differences were observed in Annexin A11 binding to other known-binding partners ALG2 and Sorcin (Supplementary Figure 8).

(viii) Calcyclin is over-expressed in astrocytes of the corticospinal tract in ALS patients

Spinal cord sections from the ALS patient carrying the p.D40G mutation were stained with anti-calcyclin antibody. Although there was no observable difference in neuronal staining between the ALS case and controls, very high levels of calcyclin expression were detected in the cytoplasm of astrocytes in the lateral corticospinal tracts compared to controls (Figure 5Bi-ii). This is not a mutation-specific event as elevated calcyclin expression was also seen in apparent sporadic ALS cases without known

ALS gene mutations (Figure 5Biii). Increased expression of calcyclin in astrocytes of the corticospinal tract has previously been reported in sporadic ALS cases as well as SOD1^{G93A} transgenic mice, but the functional significance of this observation is unknown [35, 36].

(ix) Calcyclin co-overexpression in HEK cells clears R235Q aggregates

We also explored the impact of calcyclin interaction on Annexin A11 solubility. FLAG-Calcyclin^{WT} was co-transfected with Annexin A11-GFP^{WT} or Annexin A11-GFP^{R235Q} in HEK cells and assessed by NP40 insolubility assay and western blot. Remarkably, the increase in calcyclin expression reduced levels of soluble WT and p.R235Q but it completely cleared insoluble Annexin A11-GFP^{R235Q} (Figure 5C). This suggests that increasing calcyclin may inhibit Annexin A11 expression or enhance Annexin A11 clearance via a degradation pathway. Treating the cells with the ubiquitin proteasome inhibitor MG132 treatment for 24hrs significantly restored Annexin A11-GFP^{R235Q} insolubility (Figure 5D, $p < 0.001$).

Discussion

Using a stringent filtering strategy on a large cohort of FALS exomes (n=694) in conjunction with a shared variant analysis of 50 Caucasian kindreds we identified a non-synonymous mutation p.D40G in *ANXA11*, shared in two UK families but absent from >70,000 control exomes. The mutation segregated with disease in the two UK families and an additional Italian FALS proband also carried the p.D40G variant. Sequencing of 180 unrelated UK SALS identified a fourth p.D40G individual, providing further evidence that p.D40G is causative for ALS. An independent simulation strategy designed to ascertain the significance of mutations identified multiple times in our ALS cohort (n=874) found the D40G mutation (n=4) to be associated with ALS, $p = 0.0102$. All p.D40G individuals had a common founder p.D40G haplotype, which adds to the growing list of published founder mutations that are abundant in ALS [37-39]. In addition a novel p.G175R mutation segregated with disease in one kindred and several other *ANXA11* mutations clustered in the N-terminus of the molecule were also associated with ALS.

Mutations in *ANXA11* present a distinct clinical phenotype characterized by late-onset classical ALS with 4 of 5 p.D40G cases having bulbar onset disease. Post mortem tissue from a p.D40G ALS case displayed abundant Annexin A11-positive aggregates within motor neurons in spinal cord and neurons and neuropil in the

neocortex and hippocampus (Figure 2) in addition to the classical features of neuronal loss, phospho-TDP-43 inclusions and astrogliosis.

Annexin A11 is a phospholipid binding protein, which may participate in vesicle trafficking as it has been reported to play a role in apoptosis, exocytosis and cytokinesis and forms phospholipid vesicles *in-vitro* in a calcium dependent manner [15, 40]. Annexin A11 co-localises with calyculin in the nuclear envelope during prophase of mitosis [41], however little is known about its role in post-mitotic neurons and its trafficking within the cell. From our study Annexin A11 does form vesicle-like structures in PMNs (Figure 3), which suggests a role for Annexin A11 in vesicular transport. Recent evidence has found that Annexin A11 plays an important structural role in regulating the delivery of vesicular cargoes from the endoplasmic reticulum to the Golgi apparatus which is mediated through its binding to ALG2 to stabilize Sec31A [42]. In our study, Annexin A11 mutations did not directly affect ALG2 binding it is therefore more likely that the mutant alleles disrupt vesicular trafficking by altered binding to calyculin.

Annexin A11 mutations may behave in a dominant negative manner and interfere with the normal function of Annexin A11 in neurons; which is currently unknown. The sequestering of wild-type Annexin A11 by the R235Q mutation, located in the first annexin domain offers this plausible mechanism (Figure 4). However most *ANXA11* mutations are N-terminal and do not aggregate *in-vitro* in our functional studies or form insoluble aggregates. In spite of this, the N-terminal p.D40G mutation under endogenous, long-lived conditions can aggregate as evidenced by abundant immuno-reactive inclusions seen in post mortem tissue from the p.D40G SALS case (Figure 2). Although the R235Q mutant failed to bind to calyculin, over-expression of calyculin completely cleared insoluble p.R235Q aggregates by facilitating proteasomal degradation (Figure 5). Calyculin is known to play a role in proteostasis as it forms a functional complex with calyculin binding protein (CACYPB) and the RING type E3 Ubiquitin Ligase SIAH-1 that regulates ubiquitination and degradation of synaptophysin and binds with alpha-synuclein [43, 44]. More subtle differences in calyculin binding were also observed for other Annexin A11 mutants. Calyculin binding was inhibited by p.D40G and p.G189E mutations and consistently increased above wild-type for the p.G38R mutant. Therefore loss of calyculin binding may result in an accumulation of cytoplasmic Annexin A11 promoting its aggregation. As most N-terminal mutations abolish Calyculin binding, this suggests Calyculin may also be necessary for Annexin A11 structure and/or function and N-terminal mutations may

therefore act by a loss of function mechanism. Whether aggregating D40G Annexin A11 sequesters wild-type Annexin or disrupts the function of the WT Annexin A11 protein is unknown, but is fuel for future investigation.

We and others have shown that the levels of calcyclin are greatly increased in astrocytes in apparent sporadic ALS cases as well as the one carrying the p.D40G mutation [35, 36]. Even though this phenomenon is not solely linked to *ANXA11* mutations, it raises the possibility that increased astrocytic calcyclin expression is a response to defective proteostasis. Interestingly, cells in which TDP43 is knocked-down demonstrate a significant increase in the expression of calcyclin transcripts [45, 46]. Additionally, elevated calcyclin concentrations can form oligomers that have amyloidogenic properties that have been shown to seed SOD1 aggregation [47].

In summary, we have identified 6 novel, or extremely rare, *ANXA11* mutations in 13 individuals, which account for ~1% of familial and 1.7% of apparent sporadic ALS cases. The mutations alter binding to calcyclin, which can lead to cytoplasmic aggregation in transfected cells and neuronal cytoplasmic inclusions in a patient carrying the D40G mutation at post mortem. The identification of *ANXA11* mutations presents a new field of ALS investigation and highlights the significance of calcium binding proteins and intracellular trafficking in ALS pathobiology [48]. Further work investigating how mutations affect the folding of Annexin A11, its binding to calcyclin and the effect on vesicular transport and TDP-43 aggregation will clarify the underlying disease mechanisms.

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Conflicts of Interest

None.

Author Contributions

B.N.S, S.T, C.F, H.S, H.C, C.T, A.K, S.A.S, J.L, M.W, V.S and C.E.S designed the experiments. B.N.S, S.T, C.F, H.S, H.C, C.T, A.K N.T, K.K, C.V, D.M.D, A.S, A.S.G, J.M, C.H.W, M.M, E.S, W.K, P.S, C.M, N.P and M.N performed the experimental procedures and analyzed the data. P.N, F.B, V.J, A.A, A.R, J.B, J.P, C.T, F.V, S.D, N.L, H.P, K.M, A.A.C, P.J, J.K, M.T, K.T, O.H, J.G, J.D.B, C.G, A.R, R.B, S.A.S, V.S, J.L and C.E.S collected and/or contributed DNA samples or exome sequences. B.N.S, S.D.T and C.E.S wrote the manuscript with contributions from the authors.

Figure 1. *Annexin A11* mutations identified in ALS patients following stringent filtering of exome sequencing data.

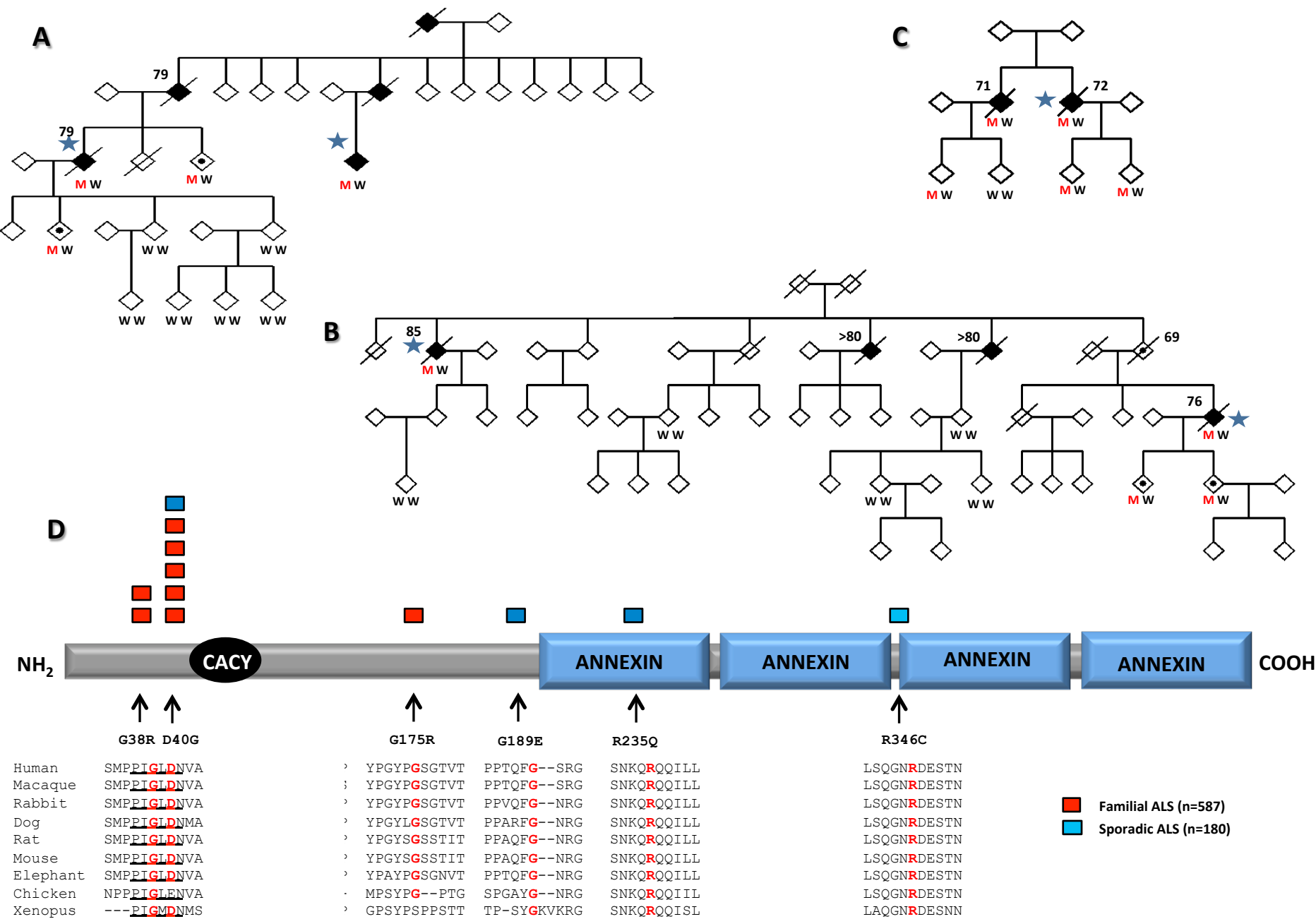


Figure 2. ANXA11 Immunohistochemistry of post mortem tissue from D40G sporadic patient

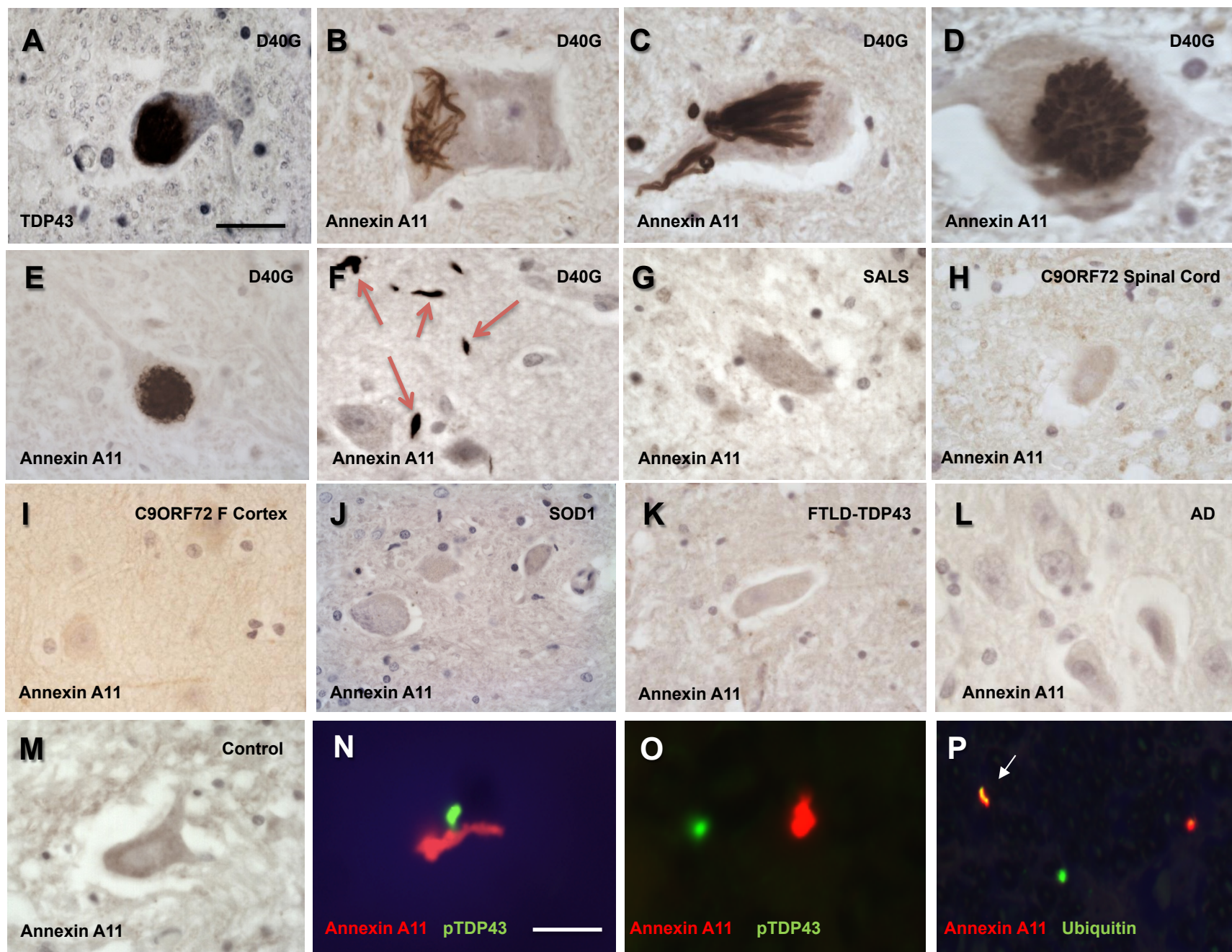


Figure 3. Functional Annexin A11 studies in mouse Primary Motor Neurons and HEK cells

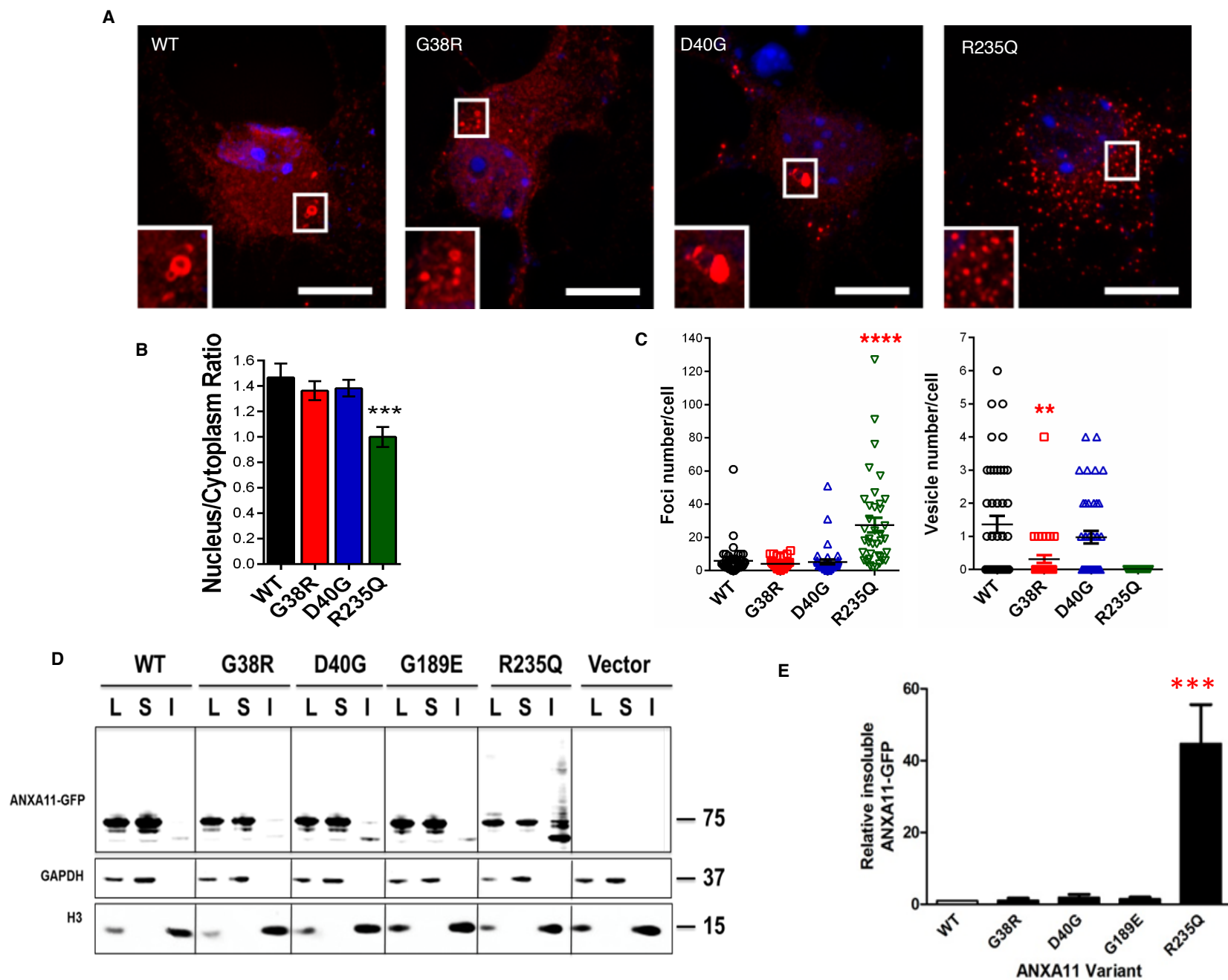


Figure 4. R235Q aggregates sequester WT Annexin A11

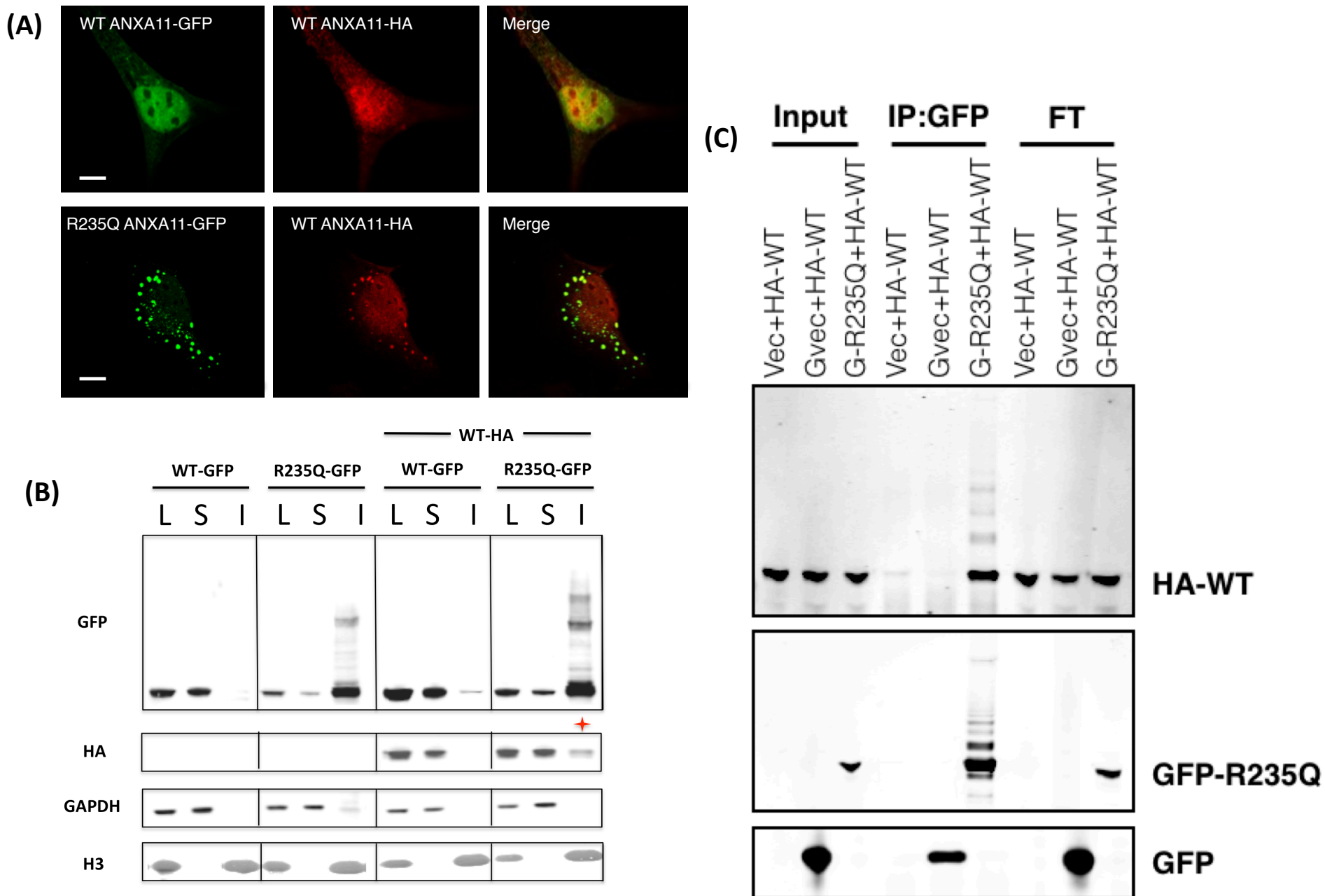
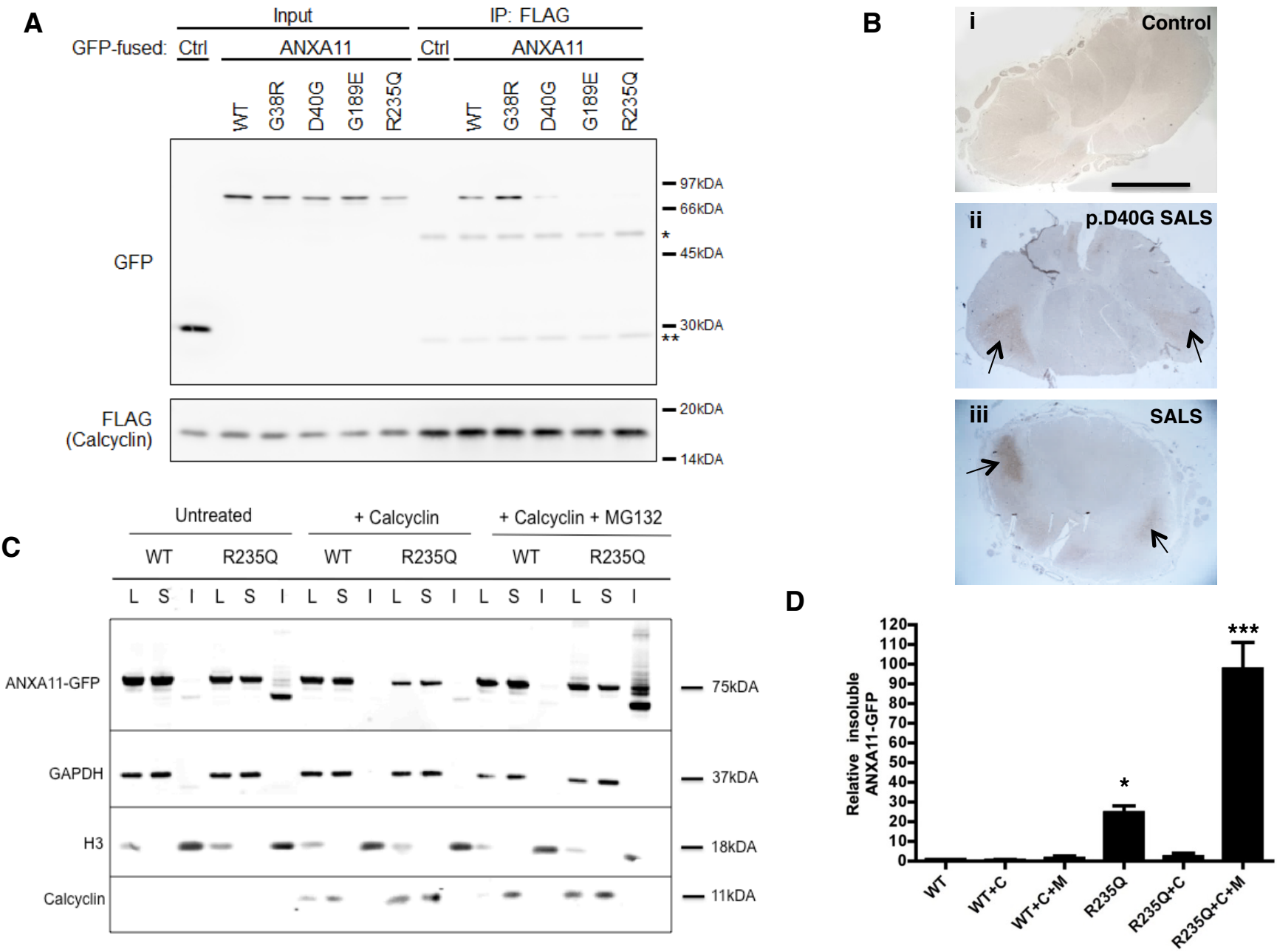


Figure 5. Calcyclin analysis: *in-vitro* binding assays with mutant ANXA11 constructs and staining of post-mortem tissue.



Materials and Methods

DNA Samples

Full patient consent was provided by FALS and SALS index patients and control individuals for research purposes. All patients had a diagnosis of definite or probable ALS based on revised El Escorial criteria (Brooks, 2000) with at least one relative known to have ALS+/- FTD. All FALS were pre-screened for the C9ORF72 intronic hexanucleotide expansion, and any positive samples excluded from this analysis. The discovery cohort was comprised of 694 FALS probands sourced from the United States (266), UK (193), Italy (138), Spain (33), Germany (25), Ireland (17), Canada (9), the Netherlands (9), Belgium (3) and New Zealand (1). An additional 60 affected relatives of these probands, representing 50 families, were also exome sequenced in tandem for segregation analysis. Sequence data for 102 of the cases in the cohort were obtained, with permission, from the dbGAP repository (NIH Exome Sequencing of Familial Amyotrophic Lateral Sclerosis, NINDS, phs000101.v4.p1, Traynor).

Exome Sequencing and Bio-informatic Analysis

Paired-end FASTQ files from Illumina exome sequencing were aligned to the hg19 human reference using NovoCraft Novoalign and variants called with samtools v1.1 mpileup. VCF files were filtered at DP \geq 10, GQ \geq 20, MQ \geq 50, and indels normalized with bcftools v1.1 norm. Functional annotation, pathogenicity predictions and matches to 1000 genomes were added with table_annoar.pl (Wang K et.al), whilst all other annotation was added via custom perl scripts. Variants were filtered out if they were not novel (defined as being present in either 1000 genomes (<http://www.1000genomes.org>), ExAC (<http://exac.broadinstitute.org/>), ESP (<http://evs.gs.washington.edu/EVS/>), UK10K (<http://www.uk10k.org/>) or 672 In-house exome controls), or if they were located at positions with a read depth <10 in >25% of ExAC samples. Insertions and deletions were excluded due to the increased error rate in matching variants to control databases assembled and called by alternative pipelines. Synonymous and intronic variants were assessed by Netgene2 and Genesplicer, and excluded if no changes in scores compared to the reference allele were observed at locations matching to known Refseq acceptor or donor splice sites. Common ancestry between samples was taken from existing familial annotation where available, and also deduced from IBD analysis in Plink v1.07 (Purcell, 2007).

Two parallel approaches were then taken to narrow down this set of high quality, novel, protein-changing variants to those most likely to be pathogenic in ALS. Firstly, we identified the variants that were shared between all exome sequenced cases of the 50 families for which data was available for more than one family member, an approach used successfully in many Mendelian disorders (Yu et al, 2014). Secondly, we identified all the variants that were shared by three or more probands, selected as the sample from each of the 694 families with the highest proportion of the exome covered at a depth \geq 10x.

Simulation studies using ExAC variant calls

In order to assess the null hypothesis, that finding a novel protein-changing variant ≥ 4 times would also be likely to occur in a non-disease cohort, a simulation strategy was chosen based on variant calls from the ExAC database. The VCF files for the r.0.3 release of ExAC were annotated and filtered by the identical pipelines described above, with the exception that only variants present within the NFE (Non-Finnish European) population of ExAC samples were considered. ExAC NFE heterozygote and homozygote counts were added together to give NFE carrier counts. The data was then further filtered to only retain variants shared by ≥ 4 carriers ($n=67,154$). These variants were then randomly distributed across 33,370 simulated ExAC European individuals. The FALS + SALS cohort was then simulated by randomly selecting 874 of these individuals (694 FALS probands + 180 SALS) from the total pool. To replicate the variant being novel across the remainder of the dataset, each variant in the filtered ExAC dataset was rejected if all NFE carriers were not within the simulated cohort. A cohort was deemed to have passed if at least one variant met the above criteria. The p-value was calculated as the proportion of cohorts that passed, after sufficient permutations of random cohorts had been generated to achieve a stable result to 2 significant figures. All random integers were generated using the perl CPAN Math::Random::Secure irand function.

Genetics Screening

All coding exons of *ANXA11* (Refseq ID NM_145869) were amplified using standard polymerase chain reaction procedures. At least 100bp of flanking intronic sequence was included to detect splice site mutations (Primers listed in Supplementary Table 4). Amplicons were directly sequenced with Big-Dye Terminator v1.1 on an ABI3130 genetic analyzer (Applied Biosystems Pty Ltd, Warrington, UK) and sequence chromatograms analyzed for mutations using Sequencher 4.10 directly by eye (Gene Codes Corporation, Ann Arbor, Michigan, USA). Reconfirmation of novel mutations was conducted by re-dilution of stock DNA and re-PCR and direct sequencing. Rare variant positions in *ANXA11* were also filtered against 3596 Italian exome controls. Nine hundred and nine local control UK samples matched for sex and age were assayed for *ANXA11* genomic SNP positions for the G38R, D40G, G189E and R235Q mutations using KASPar genotyping methods by LGC Genomics (LGC Genomics, Teddington, UK).

Haplotype Study

Microsatellites surrounding the *ANXA11* locus were identified using Hg19 build of the UCSC Genome Browser (<http://www.genome.ucsc.edu/>) and amplified by standard PCR with the forward primer incorporating a fluorescent FAM label. 1ul of PCR product was run using Fragment Analysis on an ABI3130 genetic analyzer. Allele sizing was conducted using GeneMapper V4. SNP markers rs41291392, rs17617713, rs72821609 and rs72805713 were genotyped using standard PCR and direct sequencing (primers listed in Supplementary Table

4). The haplotype figure (Supplementary Figure 2) was drawn using the 'Dalliance' program (Down TA et.al 2011).

Plasmids and Cloning

A cDNA encoding ANXA11 was amplified from pEGFP-C3/ANXA11 (Tomas and Moss, 2003) with the following pair of oligonucleotides with a *Bam*HI site (underlined): 5'-TAGGATCCACCATGAGCTACCCTGGCTATCC-3' (sense) and 5'-GCGGATCCGAGTCATTGCCACCACAGATCTT-3' (antisense). The DNA fragment obtained was subcloned into the pCR-BluntII-TOPO (Invitrogen). To construct pANXA11-GFP, a *Bam*HI fragment from pCR-Blunt II-TOPO/ANXA11 was inserted into the *Bam*HI site of pGFP-N-SGG (Shibata et al. 2010). Single amino acid changes (G38R, D40G and R235Q) were introduced into pANXA11-GFP by PCR-based site-directed mutagenesis. To construct pFLAG-S100A6, an *Eco*RI fragment of pCR2.1 TOPO/S100A6 (kindly provided by Dr. Kiyotaka Hitomi, Nagoya University, Japan) was inserted into the *Eco*RI site of pCMV3xFLAG-B (Kato et al. 2003). A cDNA encoding sorcin was amplified from human fetus cDNA library (Clontech) and subcloned into the pCMV-Tag 2A (Stratagene) to construct pFLAG-sorcin. An expression vector encoding ALG-2 N-terminally tagged with FLAG (pFLAG-ALG-2 RNAi^R) was described previously (Okumura et al. 2009).

ANXA11 expression vectors (Gateway pcDNA3.1/nV5-DEST, Invitrogen) encoding c-terminal HA tagged ANXA1 wild type (WT), G38R, D40G and R235Q mutants were used in this study. Site-directed mutagenesis was performed according to the manufacturer's protocol (Quickchange II Site-Directed Mutagenesis Kit, Stratagene) using a ANXA11-HA tagged pDONR221 entry clone plasmid to produce constructs harboring the novel mutants identified in this study. The pDONR221-ANXA11 mutant constructs were then recombined with pcDNA3.1/nV5-DEST to create the final mutant expression constructs. All constructs were verified by sequencing.

Antibodies

Mouse monoclonal anti-GFP at 1/2000 (cat.no sc-9996, Santa Cruz, Dallas, USA), mouse monoclonal anti-GAPDH (cat.no G8795, Gillingham, UK) and mouse monoclonal Histone H3 (cat.no 96C10, New England Biolabs Hitchin, UK) were used for detecting lysate, soluble and insoluble fractions on Western blot from NP40 insolubility assays. In the Annexin A11:EF-hand proteins (calcyclin, ALG-2 and sorcin) binding assays, mouse monoclonal anti-FLAG (cat.no. F3165, Sigma-Aldrich) was used to immunoprecipitate FLAG-tagged EF-hand proteins, and mouse monoclonal anti-GFP (cat.no. sc-9996, Santa Cruz) and mouse monoclonal anti-FLAG or rabbit polyclonal anti-FLAG (cat.no. F7425, Sigma-Aldrich) were used to detect GFP-fused proteins and FLAG-tagged proteins, respectively. Polyclonal Rabbit Anti-ANXA11 (cat.no. 10479-2-AP, Proteintech, Manchester, UK) was used for ANXA11^{R235Q} staining of HEK cells (Supplementary Figure 4) and spinal cord of the D40G SALS patient, SALS patient devoid of known ALS causing mutation, other neurodegenerative disorders and

controls. Polyclonal rabbit Anti-calcyclin (cat.no. 10245-1-AP, Proteintech) was utilized for IHC of patient and control post mortem tissue and detection of FLAG-tagged calcyclin by Western blot in HEK cells.

Transfection of HEK and SH-SY5Y cells

HEK293T and SH-SY5Y cells were maintained in DMEM with high glucose plus Glutamax and DMEM/F12 respectively (Life Technologies, Paisley, UK) with 10% fetal bovine serum, 100U/mL penicillin, and 100 mg/mL streptomycin in a water jacketed incubator at 5% CO₂. For solubility fractionation, transfections were performed in 12-well plates with 500 ng of plasmid DNA and 1.5ml Fugene HD (Promega, Southampton, UK) per well, as per the manufacturer's protocol. For immunofluorescence, HEK293T cells were plated at 25,000 cells/cm² on 13 mm diameter, 1.5-mm-thickness coverslips coated with poly-D-lysine (Sigma-Aldrich, Dorset, UK) in 24-well plates and transfected with 250ng of DNA and 0.75ml of Fugene HD. SH-SY5Y cells were transfected in the same manner except the transfection reagent used was Lipofectamine (Fisher Scientific, Loughborough, UK).

FLAG-S100A6 and ANXA11-GFP Binding Assays

HEK293 YS14 cells (a sub-cloned HEK293 cell line) (Shibata et al. 2007) which had been plated on the previous day at a density of 2×10^6 cells per 10 cm dish were transfected with expression plasmids using Polyethyleneimine (150 µg) (Polysciences) and then cultured for 24 hr. To attain equivalent expression level, the following amounts of plasmid DNA for GFP-fused proteins were used: pGFP-N-SGG, 3 µg; pANXA11^{WT}-GFP, 5 µg; P8L, 5µg; G38R, 6 µg; D40G, 7 µg; D40H, 5 µg, G189E, 6 µg; R191Q, 5µg; R230C, 5 µg; R235Q, 10 µg. The cells were washed and harvested with PBS, suspended in 470 µL per 10 cm dish (for cells expressing SGFP2-fused proteins) or in 350 µL per 10 cm dish (for cells expressing FLAG-tagged proteins) of lysis buffer (20 mM HEPES-KOH, pH 7.2, 142.5 mM KCl, 2.5 mM MgCl₂) containing 0.2% Triton X-100, 10 µM EGTA, protease inhibitors (1 µM E64, 3 µg/mL leupeptin, 0.1 mM pefabloc, 2 µM pepstatin A, 0.2 mM PMSF) and phosphatase inhibitors (50 mM NaF, 10 mM β-glycerophosphate, 1 mM Na₃VO₄). After 30 min on ice, the cell lysates were centrifuged at 15,000 g for 10 min at 4°C. The cleared lysates of the cell expressing GFP-fused proteins were divided into aliquots of 200 µL and mixed with 100 µL of the cleared lysates of the cells expressing FLAG-tagged proteins. Twenty µL of the mixture was taken as input. The samples were rotated at 4°C for 90 min in the presence of 100 µM of CaCl₂, mixed with 0.8 µg of mouse antibody against FLAG (M2) (cat.no F7425, Sigma, Poole, UK) and rotated at 4°C for further 60 min. Then the samples were incubated overnight at 4°C with 10 µL of Dynabeads Protein G (Novex, Invitrogen). The beads were collected using a magnet and washed twice with 500 µL of lysis buffer containing 0.1% Triton X-100 and 100 µM CaCl₂. Washed beads were boiled for 5 min in 56 µL of 1x SDS-PAGE sample buffer. Four µL of input and 8 µL of IP (for detection of FLAG-tagged proteins) or 1.2 µL of input and 12 µL of IP (for detection of GFP-fused proteins) were run on a western blot.

FLAG-Calcyclin and ANXA11-GFP over-expression in HEK cells

500ng of ANXA11-GFP^{WT} and ANXA11-GFP^{R235Q} constructs were co-transfected with 500ng of FLAG-Calcyclin in a 6 well dish of HEK cells plated at 25,000 cells/cm². Untreated ANXA11-GFP^{WT} and ANXA11-GFP^{R235Q} were co-transfected with empty pEGFP-C1 vector (Clontech Pty Ltd, Mountain View, California, USA). Duplicate wells of ANXA11-GFP^{WT} and ANXA11-GFP^{R235Q} transfected cells were also treated with 0.5µM MG132 24hrs before harvesting (48 hours post transfection). Cells were harvested and processed as per the NP40 insolubility assay below.

Primary Motor Neuron (PMN) Cultures

Motor neurons were isolated from mouse embryos, cultured and transfected as described (Fallini et al, Mol. Neurodegen., 2010). Cells were fixed 1 or 4 days after transfection and processed for immunofluorescence. After antigen retrieval in citrate buffer for 20 minutes and blocking in 5% bovine serum albumin for 1 hour, cells were hybridized overnight at 4°C with HA primary antibody (Covance, 1:1000) Alexa488 or Alexa594-conjugated secondary antibodies (Jackson ImmunoResearch) were incubated for 1 hour at room temperature. Vesicles were defined as structures with a diameter between 0.5µm to 1.9 µm (mean 1 µm) and foci 0.16µm to 0.5 µm (mean 0.3 µm).

Protein Fractionation, Insolubility Assay and Western Blot

ANXA11-GFP WT and mutant construct transfected HEK cells were harvested at 48 wells post-transfection and fractionated using a NP40 solubility assay as described previously (Smith et.al Neurobiology of Aging 2015). Lysate and soluble fractions were quantified using a BSA standard protein assay and 5ug of lysate and soluble fractions analysed by Western blotting. The same volume of insoluble fraction was loaded as per cell lysate. Nitrocellulose membranes were probed with mouse monoclonal anti-GFP at 1/2000 dilution, mouse monoclonal anti-GAPDH 1/2000 dilution and mouse monoclonal Histone H3 1/1000 dilution in 1% Skim Milk and PBS-Tween. Band intensities were quantified using Image J (<http://imagej.nih.gov/ij/>). Membrane imaging was conducted with fluorescent secondary antibodies, Goat Anti-rabbit and Anti-mouse IgG (H+L) Dylight 680 Conjugate (Thermolifesciences cat #35568 and #35521) on a Licor Odyssey Imager. 15µg of frontal cortex lysate was run on PVDF membranes and probed with 1/400 Proteintech Rabbit ANXA11 and developed using ECL and visualized using anti-rabbit chemi-luminescent horse raddish peroxidase (HRP) (Millipore Immobilon cat# WBKLS0500).

Co-transfection Immuno-precipitation

Individual wells of a 6 well plate were seeded with HEK cells and transfected at 70% confluence with either Annexin A11-GFP^{R235Q} or Annexin A11-GFP^{R235Q} plasmid. A duplicate set of wells with Annexin A11-GFP^{WT} or Annexin A11-GFP^{R235Q} were co-transfected with

Annexin A11-HA^{WT}. At 48hrs cells were harvested in immunopurification buffer (50mM Tris pH7.4, 150mM NaCl, 1% TritonTM X-100, 100uM CaCl₂ with protease and phosphatase inhibitor). The cell lysate was collected and incubated with immunopurification antibody (rabbit anti-GFP, Abcam) and Dynabead protein G (Life Technologies) overnight at 4°C. The Dynabead protein Antibody- protein complex was purified using magnetic separation and washed with immunopurification buffer before elution in loading buffer (variation on protocol described in Chen et.al Brain 2016). Lysates, IP and flow through fractions were run on a nitrocellulose membrane and membrane imaging was conducted with fluorescent secondary antibodies and a Licor Odyssey.

Statistical Analysis

Statistical analysis of PMN immunofluorescence data, NP40 insolubility data and FACs Survival Data was performed with Graphpad Prism software. Normality of the datasets was assessed with the D'Agostino-Pearson test. One-way ANOVA with Dunnett's post *hoc* test or Kruskal-Wallis with Dunn's post *hoc* test were performed depending on normality.

Mutation modelling and Jpred Analysis

The Refseq protein database was searched with human ANXA11 (NP_001148.1) via BlastP at the NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The highest matching sequence per vertebrate species from the first 250 results was aligned with all others using Muscle (<http://www.ebi.ac.uk/Tools/msa/muscle/>). Sequences were removed if they had a better reciprocal match to a different human annexin gene or if they contained significant numbers of gaps, mismatching regions or consecutive "X"s, implying incomplete or mistranslated sequence. Fish were also excluded, as they consistently showed significant divergence along the majority of the N-terminus, and with the exception of the first 20 amino acids a satisfactory alignment to other Classes could not be obtained. The resulting multiple alignment was hand edited in GeneDoc and submitted to JPred4 (<http://www.compbio.dundee.ac.uk/jpred4/>) for secondary structure prediction. Predictions were also made for the ALS-associated variants G38R and D40G, and 22 non-ALS variants from the ExAC database flanking these residues, by replacing every amino acid in the multiple alignment with the variant residue. Possible amphipathicity of any resulting alpha helices was tested by submitting the sequences to Helixator (http://www.tcdb.org/progs/helical_wheel.php). The procedure was then repeated for human ANXA1, but with this gene it was not necessary to exclude fish species from the alignment.

Neuropathology

Brain tissue samples in 10% formalin-fixed, paraffin-embedded tissue blocks were available from the London Neurodegenerative Diseases Brain Bank (King's College London, UK). Consent for autopsy, neuropathological assessment and research were obtained from all subjects and all studies were carried out under the ethical approval of the tissue bank. Block

taking for histological and immunohistochemical studies and neuropathological assessment for neurodegenerative diseases was performed in accordance with standard criteria.

Immunohistochemistry:

Immunohistochemistry was carried out as per previously published protocols [35]. In brief, sections of 7µm thickness were cut from the paraffin-embedded tissue blocks, deparaffinised in xylene, endogenous peroxidase was blocked by 2.5% H₂O₂ in methanol and immunohistochemistry performed. To enhance antigen retrieval, sections were kept in citrate buffer for 10 minutes following microwave treatment. After blocking in normal serum primary antibody was applied overnight at 4°C.

Following washes, sections were incubated with biotinylated secondary antibody (DAKO), followed by avidin:biotinylated enzyme complex (Vectastain Elite ABC kit, Vector Laboratories, Peterborough, UK). Finally sections were incubated for 10–15 min with 0.5 mg/mL 3,3'-diaminobenzidine chromogen (Sigma-Aldrich Company Ltd, Dorset UK) in Tris-buffered saline (pH 7.6) containing 0.05% H₂O₂. Sections were counterstained with Harris' haematoxylin and immunostaining analysed using a Leica microscope (Leica, Wetzlar, Germany).

Double Immunofluorescence:

7µm sections were cut from formalin fixed paraffin embedded blocks, dewaxed in xylene and dehydrated in 99% industrial methylated spirit. Sections were then pretreated by microwaving in citrate buffer and blocked using normal goat serum (1:10 for 45min). Primary antibodies were then applied and sections incubated at 4°C overnight. Sections were washed and secondary Alexa Fluor antibody (Invitrogen, Paisley, UK) applied for 45 min (in dark). Autofluorescence was quenched by incubating the sections in Sudan black for 10min followed by numerous washes in phosphate buffered saline before coverslip mounting using hard set media with DAPI. Sections were visualised using a fluorescent microscope (Zeiss Axiovert S 100, Gottingen, Germany) and images captured using ImagePro Express (V6).

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