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Original research

Value of systematic genetic screening of patients with amyotrophic lateral sclerosis

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ABSTRACT

Objective The clinical utility of routine genetic sequencing in amyotrophic lateral sclerosis (ALS) is uncertain. Our aim was to determine whether routine targeted sequencing of 44 ALS-relevant genes would have a significant impact on disease subclassification and clinical care.

Methods We performed targeted sequencing of a 44-gene panel in a prospective case series of 100 patients with ALS recruited consecutively from the Sheffield Motor Neuron Disorders Clinic, UK. All participants were diagnosed with ALS by a specialist Consultant Neurologist. 7/100 patients had familial ALS, but the majority were apparently sporadic cases.

Results 21% of patients with ALS carried a confirmed pathogenic or likely pathogenic mutation, of whom 93% had no family history of ALS. 15% met the inclusion criteria for a current ALS genetic-therapy trial. 5/21 patients with a pathogenic mutation had an additional variant of uncertain significance (VUS). An additional 21% of patients with ALS carried a VUS in an ALS-associated gene. Overall, 13% of patients carried more than one genetic variant (pathogenic or VUS). Patients with ALS carrying two variants developed disease at a significantly earlier age compared with patients with a single variant (median age of onset=56 vs 60 years, p=0.0074).

Conclusions Routine screening for ALS-associated pathogenic mutations in a specialised ALS referral clinic will impact clinical care in 21% of cases. An additional 21% of patients have variants in the ALS gene panel currently of unconfirmed significance after removing non-specific or predicted benign variants. Overall, variants within known ALS-linked genes are of potential clinical importance in 42% of patients.

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterised by progressive injury and cell death of upper and lower motor neurons in the motor cortex, brainstem and spinal cord. This leads to progressive failure of the neuromuscular system with death, usually from respiratory failure, within 2–5 years of symptom onset in most cases. Up to 50% of cases also show mild cognitive impairment, with approximately 5% progressing to clinically recognised fronto-temporal dementia (FTD).[1] While the majority of ALS cases are considered sporadic (sALS), 5%–10% have been shown to be familial, usually with autosomal dominant inheritance, and the genetic cause of approximately 60%–70% of familial ALS (fALS) cases has now been identified.[2] The most common genetic cause of ALS is due to expansion of a GGGGCC (G4C2) hexanucleotide repeat in the first intron of the *C9orf72* gene. This expansion has a frequency of 43% in fALS and 7% in sALS cases in our UK cohort,[3] which is comparable with worldwide figures of 39.3% for fALS and 7.0% for sALS.[4] Mutations in *SOD1*,[5 6] *TARDBP*,[5 7 8] and *FUS*[5 9] genes, the next most common genetic causes of ALS, have also been reported in both patients with fALS and those with sALS. Therefore, it is clear that apparently sporadic cases can also carry potentially pathogenic variants in known ALS genes. In a recent study which screened 17 ALS-related genes, 27.8% of apparently sporadic cases carried a potentially pathogenic or rare variant in a known ALS gene.[10] In addition, it was noted that 3.8% of patients also carried multiple variants, with these cases having a significantly earlier age of onset. Another recent report from an Australian sporadic ALS cohort found that one-third of patients carried a variant of interest and 7% carried two or more variants, which again was correlated with an earlier age of onset.[11] It has previously been reported that ALS is a six-step process, with genes, environment and time (in the form of ageing) contributing to disease development.[12] It was proposed that individuals with a genetic variant would require fewer steps than those without such variants. Using data from an ALS registry in Italy, this proved to be the case, with individuals carrying *C9orf72*, *TARDBP* or *SOD1* mutations showing a three-step, four-step and two-step process.[13]

Currently, only cases with a familial history of ALS, dementia or with a young age of disease onset tend to be routinely offered genetic screening in a clinical setting, at least in the UK.[14] However, with the advent of therapies targeting specific genetic forms of the disease associated with *SOD1* or *C9orf72* mutations (Biogen sponsored clinical trials

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Table 1 Cohort demographics and phenotypes

Total no of patients	100
Age (years), mean (range)	60.4 (22–87)
Male	54%
Female	46%
Site of first muscle weakness	
Upper limb	40%
Lower limb	39%
Bulbar	19%
Respiratory	1%
Trunk	1%
Disease subtype	
ALS	83%
LMN predominant	12%
UMN predominant	5%
Family history of ALS	7%
Time from onset of first muscle weakness (months), mean (SD, range)	20.8 (20.4, 1–134)
Time from onset of first muscle weakness until death (months), mean (SD, range)*	28.5 (21.0, 7–85)
ALSFRS-R, mean (range) at first assessment	36.9 (15–47)
Revised El-Escorial criteria at first assessment	
Clinically probable laboratory supported	36%
Clinically probable	30%
Clinically suspected	15%
Clinically definite	13%
Clinically possible	6%

ALS, amyotrophic lateral sclerosis; ALSFRS-R, revised ALS-Functional Rating Score; LMN, lower motor neuron; UMN, upper motor neuron.

of BIIB067 and BIIB078), this raises the question as to whether all patients with ALS should be offered genetic screening.

Currently, only two neuroprotective therapies are available for patients with ALS, riluzole[15] and edaravone[16], and these agents have only very modest effects on the course of the disease. New approaches are required to improve outcomes in ALS clinical trials and key to this is improved disease stratification, an approach which has been very successful in several areas of oncology.[17]

'*A Multi-Centre Biomarker Research Strategy in ALS*' (AMBRoSIA) is a longitudinal biosampling programme in which newly referred patients with ALS are approached for research participation. Consenting patients have genetic screening undertaken on a research basis and samples of their blood, urine and cerebrospinal fluid are collected longitudinally, together with a skin biopsy for fibroblast culture and reprogramming. Here, we report our findings from the prospective genetic screening of a highly representative and deeply phenotyped cohort of patients with sporadic and familial ALS.

MATERIALS AND METHODS

Patient cohort and biosamples

The first 100 patients with clinically identified ALS (7 familial and 93 apparently sporadic) recruited to the Sheffield site of the AMBRoSIA programme were analysed in this study (table 1). The patients were recruited between June 2017 and October 2019. Biosamples, including whole blood for DNA extraction, were obtained following informed written consent (REC 16/LO/2136). Other than a confirmed diagnosis of ALS, no strict inclusion or exclusion criteria were adopted (see online supplemental table S5 for AMBRoSIA inclusion/exclusion criteria), so patients were not prioritised for genetic screening by clinicians. The diagnosis of ALS was made by an experienced neuromuscular neurologist (PJS, CJM, TMJ, CH) following appropriate investigations to exclude alternative diagnoses, and detailed clinical and demographic features were recorded for all patients.

Illumina targeted panel sequencing

A panel of 44 ALS, motor system and FTD-linked genes (figure 1A) was screened to diagnostic standards using targeted next-generation sequencing by the UKAS-accredited Sheffield Diagnostic Genetics Service laboratory, as part of the AMBRoSIA project. The panel was approved for familial ALS with and without FTD by the UK Genetic Testing Network steering group. DNA for the panel was captured using SureSelectXT (Design ID: 0836801) automated library preparation and libraries were sequenced on an Illumina HiSeq 2500 in rapid run mode at 2×107 bp. A mean coverage of at least 100x was obtained (online supplemental figure S1).

C9ORF72 expansion testing

Hexanucleotide repeat expansions (G4C2) in *C9ORF72* were tested by flanking PCR and, if required, fluorescent repeat primed PCR (RP-PCR) (online supplemental table S1). Fragment size analysis was performed in GeneMapper (V.3.5). Expansions were reported as normal if <30 repeats were detected, and all expansions of >30 repeats plus the classical sawtooth pattern were reported as potentially pathogenic.

ATXN2 expansion testing

ATXN2 repeat expansions (CAG) were tested by standard PCR with one fluorescently labelled primer (online supplemental table S2), followed by analysis using GeneMapper (V.3.5). ATXN2 repeats were reported as normal between 14 and 28; 35 repeats and above were reported as consistent with spinocerebellar ataxia type 2 (SCA2) and repeat lengths of 29–34, which have been associated with an increased risk of ALS, were reported as intermediate repeat lengths.[18]

Sequencing and variant analysis

Sequencing data were analysed using a clinically validated bioinformatics pipeline. Samples were checked for contamination (online supplemental figure S2). Reads were aligned with bwa[19] (V.0.7.15) to a bespoke version of the human reference (hg19). Indels were realigned with GATK[20] (V.3.7). Variants were called with GATK Haplotype Caller (V.3.7) and decomposed and normalised with vt[21] (V.0.5) and uploaded to Fabric Genomics Opal (V.6.1.8). Variants with a quality score <1500 were excluded from further analysis. Protein coding variants, only, were retained.

The pathogenicity of a variant was determined using a multifaceted approach, including manual review in Opal, population frequency and in silico software algorithms, as well as the presence or absence of variants in our clinical reporting pipeline and the ALS literature.

Fabric Genomics Opal (V.6.1.8) provides population frequency from the 100,000 Genomes Project, Exome Variant Server of NHLBI GO Exome Sequencing Project, ExAC and gnomAD. We chose to report on overall population frequency using gnomAD, the largest dataset available.[22]

In silico analysis used the Omicia Score, which combines software algorithms MutationTaster,[23] Polymorphism Phenotyping v2 (PolyPhen2),[24] Sorting Intolerant from Tolerant (SIFT)[25] and phyloP[26]—placental, primate and vertebrate. Additional

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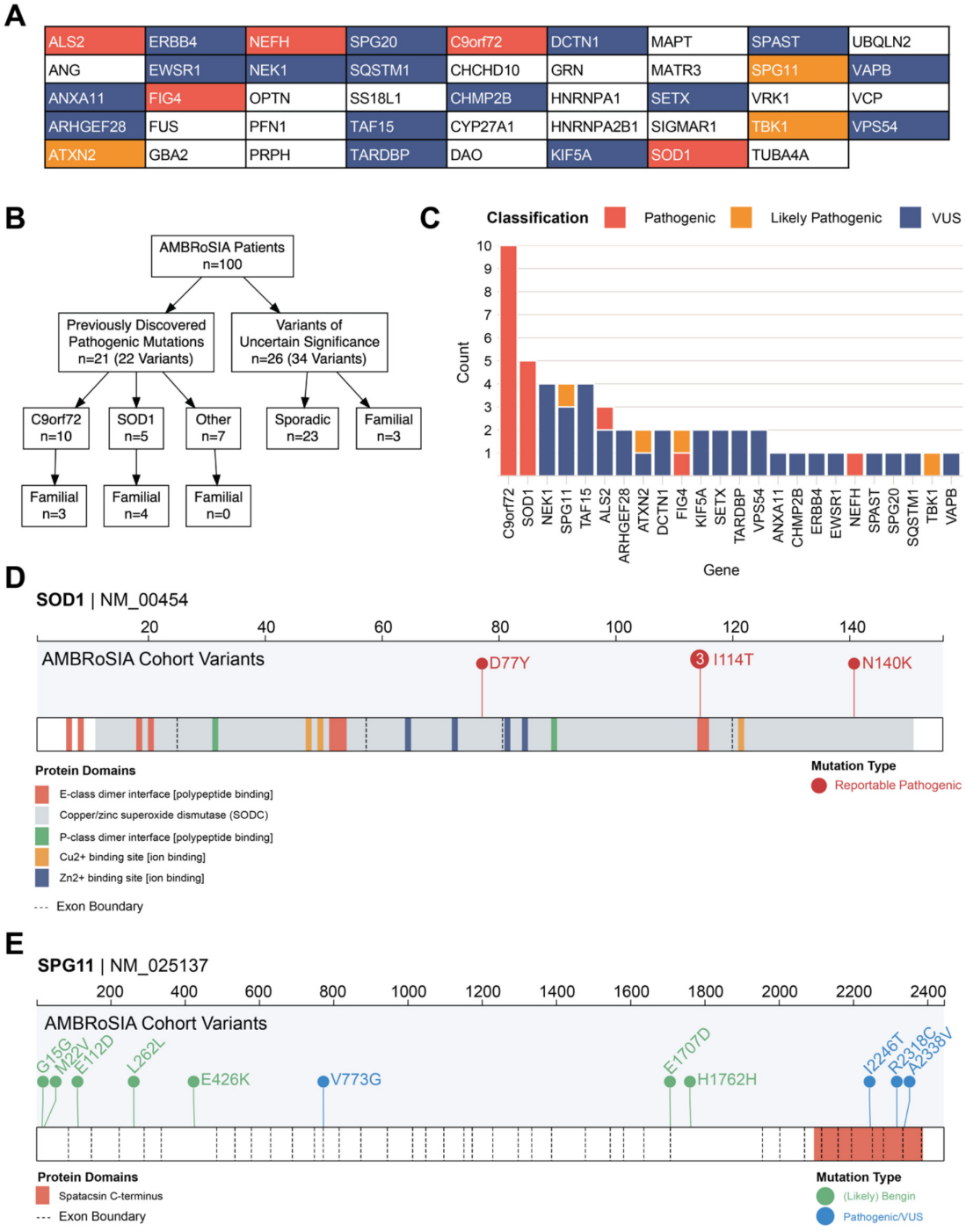

Figure 1 Clinical screening of all patients with ALS identifies pathogenic, likely pathogenic and variants of uncertain significance (VUS). (A) The 44 genes covered by the clinical ALS panel (42 profiled by next-generation sequencing and 2 by PCR). Those genes in which pathogenic (red), likely pathogenic variants (orange) or VUS (blue) were identified after filtering. No pathogenic variants or VUS were found in the genes depicted in the white boxes. Background colour of the box represents the most severe variant found in that gene. (B) Schematic of our variant analysis and filtering process. (C) Counts of reportable (red) and variants of unknown significance in ALS genes in the Sheffield AMBRoSIA cohort. (D) Five clinically reportable variants in *SOD1*. (E) 4 Variants of unknown significance were discovered in *SPG11*. Interestingly, 3 of these cluster in the C-terminal domain. Benign and likely benign mutations are conversely distributed throughout the protein. Protein domain figures created using ProteinPaint (https://pecan.stjude.cloud/proteinpaint).

algorithms used were VAAST Variant Prioritization (VVP) and Combined Annotation Dependent Depletion (CADD).[27] Classification of variant effects was based on transcripts detailed in online supplemental table S3. Potential splicing disruption was predicted using Neural Network Splice (NNSplice),[28] GeneSplicer[29] and MaxEntScan.[30]

The ClinVar database (2 January 2019[31]) was also used to determine if the variant had been previously assessed. Variants previously described as pathogenic or likely pathogenic in Opal, the genetics service clinical pipeline, or the literature (based ClinVar) were labelled as such. Variants with a lesser likelihood of pathogenicity were labelled as benign and excluded from reporting if

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Table 2 Clinically established and ACMG reportable variants in ALS-associated genes: 22 changes in 21 individuals

Sample	Gene	Genomic variant	Protein change (HGVSp canonical)	Population frequency (gnomAD)	Classification
SHF-094	ALS2	chr2:g.202625839G>A	p.Thr293Ile	1.00E−05	Pathogenic
SHF-014	C9orf72	–	–	NA	Pathogenic
SHF-017	C9orf72	–	–	NA	Pathogenic
SHF-018	C9orf72	–	–	NA	Pathogenic
SHF-043	C9orf72	–	–	NA	Pathogenic
SHF-081	C9orf72	–	–	NA	Pathogenic
SHF-083	C9orf72	–	–	NA	Pathogenic
SHF-103	C9orf72	–	–	NA	Pathogenic
SHF-138	C9orf72	–	–	NA	Pathogenic
SHF-152	C9orf72	–	–	NA	Pathogenic
SHF-162	C9orf72	–	–	NA	Pathogenic
SHF-021	ATXN2	–	–	NA	Likely pathogenic
SHF-065	FIG4	chr6:g.110107620T>TA	p.Thr689AsnfsTer12	None	Likely pathogenic
SHF-080	FIG4	chr6:g.110036336T>C	p.Ile41Thr	0.00102	Pathogenic
SHF-094	NEFH	chr22:g.29885959C>T	p.Pro777Leu	3.00E−05	Pathogenic
SHF-006	SOD1	chr21:g.33039672T>C	p.Ile114Thr	5.00E−05	Pathogenic
SHF-039	SOD1	chr21:g.33039672T>C	p.Ile114Thr	5.00E−05	Pathogenic
SHF-078	SOD1	chr21:g.33039672T>C	p.Ile114Thr	5.00E−05	Pathogenic
SHF-082	SOD1	chr21:g.33040846C>A	p.Asn140Lys	0	Pathogenic
SHF-007	SOD1	chr21:g.33038821G>T	p.Asp77Tyr	None	Pathogenic
SHF-085	SPG11	chr15:g.44856883G>A	p.Ala2338Val	1.00E−05	Likely pathogenic
SHF-084	TBK1	chr12:g.64891000TGAA>T	p.Glu643del	1.00E−05	Likely pathogenic

ACMG, American College of Medical Genetics and Genomics; ALS, amyotrophic lateral sclerosis; NA, not available.

they had one of the following properties: (1) intronic or synonymous variants with no predicted effect on splice sites; (2) an allele frequency >0.001 according to gnomAD[22]; (3) previously reported as benign in ClinVar; (4) a CADD score <15.[27] Variants of uncertain significance (VUS) were determined as those that did not fit the aforementioned criteria for pathogenic/likely pathogenic or benign. CADD score is not available for indels and therefore indels were denoted as VUS if minor allele frequency (MAF) <0.001 and they passed following QC criteria: Quality Depth (QD) <2, Fisher Strand (FS) >200, ReadPosRankSum <−20 (n=43) and if the frequency in the present cohort was <0.1 (n=23). Those that are >0.001 in gnomAD (n=20) and non-coding indels were removed (n=13). Finally, we removed those that were common in controls in ProjectMine (see online supplemental table S4 for details of the ProjectMine sequencing consortium), leaving 11 indels (online supplemental figures S3 and S4).

RESULTS

Prospective genetic testing leads to identification of clinically reportable pathogenic mutations

We profiled a panel of 44 relevant genes (figure 1A) in 100 prospectively identified patients with ALS using Best Practice NHS pipelines.[32] In 21 patients (21%), we identified 22 clinically reportable pathogenic and likely pathogenic variants (table 2 and figure 1B,C). Seven of the 21 patients reported a family history of ALS in a first-degree relative, but 14 patients with a clinically reportable pathogenic mutation had apparently sporadic disease.

As expected, the most frequently identified pathogenic mutation was in *C9ORF72* in 10 patients with ALS (10% of the cohort).[3] Three of these patients had reported a family history of ALS. We identified five patients (5% of the cohort) each with a clinically reportable *SOD1* mutation (table 2 and figure 1D), four of whom had a family history of ALS. Identification of *C9ORF72* and *SOD1* mutations is particularly important because these genes are associated with ongoing genetic-therapy trials (Biogen BIIB067 and BIIB078).

We identified two patients with sporadic ALS with clinically reportable changes in *FIG4*: p.Ile41Thr and p.Thr689AsnfsTer12 (table 2). The p.Ile41Thr mutation is reported as pathogenic in ClinVar, with no conflicts. The p.Thr689AsnfsTer12 variant has been reported twice before and only in sporadic ALS, suggesting variable penetrance. A frameshift mutation is likely to lead to haploinsufficiency (online supplemental figure S5). *FIG4* variants are thought to disrupt local folding of the protein leading to a reduction in stability which inhibits the normal function of the protein in lysosomal trafficking.[33]

Four additional mutations in three patients were identified which were classified as clinically reportable (table 2). A single patient with sALS carried a p.Pro777Leu *NEFH* mutation and a p.Thr293IIe *ALS2* mutation. The p.Thr293Ile change was heterozygous, whereas *ALS2* mutations are usually considered to be autosomal recessive.[34] As a result, the clinical significance of this finding is currently uncertain. However, it is possible that the two identified changes act synergistically. A single patient harboured a p.Ala2338Val *SPG11* mutation and a further single patient carried an inframe deletion within *TBK1* p.Glu643del. *SPG11* mutations are usually considered to be recessive and therefore the clinical significance of this finding is at present uncertain. The *TBK1* change is reported in ClinVar as pathogenic. We identified one patient with an expansion of 29 CAG repeats in the *ATXN2* gene which has been reported as a risk factor for ALS.[18] The majority of cases in this cohort had 22 or 23 repeats (range 15–29).

Panel screening leads to identification of VUS

Thirty-four variants in 26 patients with ALS (3 familial and 23 sporadic) (26%) were reported in ClinVar as of uncertain significance, or absent from ClinVar and met our criteria for predicted

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Table 3 Variants of unconfirmed significance with potential for pathogenicity in ALS-associated genes

Sample	Gene	Genomic variant	Protein change (HGVSp canonical)	Population frequency (gnomAD)	Deleterious score (CADD)
SHF-029	ALS2	chr2:g.202626478C>T	p.Ser80Asn	None	15.15
SHF-147	ALS2	chr2:g.202609025A>G	p.Leu709Pro	None	28.9
SHF-082	ANXA11	chr10:g.81930624G>C	p.Pro35Ala	0.00012	22.4
SHF-139	ARHGEF28	chr5:g.73181708C>T	p.Ala1030Val	7.00E−05	28.5
SHF-159	ARHGEF28	chr5:g.73200229A>T	p.Gln1387Leu	None	34
SHF-110	ATXN2	chr12:g.112036798TGCTGCTGCTGCTGCTGCTGC>T	p.Gln168AlafsTer75	2.00E−05	
SHF-129	CHMP2B	chr3:g.87289902A>G	p.Arg30Gly	0.00013	26
SHF-035	DCTN1	chr2:g.74597128C>G	p.Glu452Asp	None	25.9
SHF-127	DCTN1	chr2:g.74594023G>A	p.Arg785Trp	0.00018	32
SHF-095	ERBB4	chr2:g.212566786G>T	p.Asn465Lys	9.00E−05	17.1
SHF-159	EWSR1	chr22:g.29693883T>C	p.Met459Thr	1.00E−05	22.6
SHF-157	KIF5A	chr12:g.57970115C>T	p.Arg718Trp	None	27.3
SHF-062	KIF5A	chr12:g.57957406T>C	p.Val74Ala	None	26.2
SHF-097	NEK1	chr4:g.170398474A>C	p.Asn745Lys	0.00481	26.5
SHF-144	NEK1	chr4:g.170345826G>A	p.Gln1034Ter	None	35
SHF-138	NEK1	chr4:g.170458958A>G	c.1665+2T>C	1.00E−05	29.7
SHF-128	NEK1	chr4:g.170506573T>G	p.Asp245Ala	None	23
SHF-146	SETX	chr9:g.135205481G>A	p.Arg502Trp	0.00049	27.7
SHF-162	SETX	chr9:g.135211865C>T	p.Gly179Glu	0	26.6
SHF-009	SPAST	chr2:g.32289207T>TCGGCCC	p.Ala106_Pro107dup	3.00E−05	16.17
SHF-114	SPG11	chr15:g.44914544A>C	p.Val773Gly	0.00012	28.9
SHF-139	SPG11	chr15:g.44858099G>A	p.Arg2318Cys	3.00E−05	23.2
SHF-009	SPG11	chr15:g.44859639A>G	p.Ile2246Thr	3.00E−05	29.4
SHF-148	SPG20	chr13:g.36878530ACTTC>A	p.Glu657Ter	6.00E−05	23.5
SHF-057	SQSTM1	chr5:g.179250020G>A	p.Val90Met	4.00E−05	21.9
SHF-009	TAF15	chr17:g.34171988G>A	p.Arg562Gln	2.00E−05	18.24
SHF-029	TAF15	chr17:g.34171971CGGCTATGGAGGAGACCGAGGTGGG>C	p.Gly565_Gly572del	0.0001	20.9
SHF-092	TAF15	chr17:g.34171575GGGTGGCTATGGTGGAGACAGAAGCAGC>G	p.Tyr427_Gly435del	2.00E−05	21.6
SHF-103	TAF15	chr17:g.34149811G>A	p.Arg153Lys	1.00E−05	24.3
SHF-014	TARDBP	chr1:g.11082679A>G	p.Met405Val	0	21.9
SHF-062	TARDBP	chr1:g.11082430A>G	p.Met322Val	0	22.4
SHF-128	VAPB	chr20:g.56993275A>G	p.Thr23Ala	0	23.7
SHF-144	VPS54	chr2:g.64171737A>G	p.Val433Ala	None	18.2
SHF-141	VPS54	chr2:g.64189223G>C	p.Gln327Glu	1.00E−05	24.1

AMS, amyotrophic lateral sclerosis; CADD, Combined Annotation Dependent Depletion.

pathogenic variants (table 3). Also, 5/26 patients with a VUS had an additional pathogenic variant.

Without pedigree information or population-matched controls, it is difficult to conclusively demonstrate pathogenicity. ALS has a polygenic rare variant architecture and therefore we filtered for rare predicted pathogenic variants which we quantified as MAF <0.001 and CADD >15[27] (online supplemental figure S3). In addition, we used population-matched whole genome sequencing data from 5954 patients with ALS and 2238 controls included in Project MinE[35] to check for evidence of pathogenicity based on case:control ratio (Fisher exact test $p \leq 0.05$). VUS were excluded from further analysis if they were not significantly associated with ALS in population-matched data, unless low frequency within the whole genome sequencing dataset (<10 individuals) prevented conclusive testing.

For each identified VUS, we performed a literature search to explore the case that this was indeed a newly identified ALS-risk variant. In particular, we sought evidence that a discovered VUS was present within the same functional domain as described pathogenic variants: for *TBK1*, *NEK1* and *FIG4* this included nonsense and frameshift changes because pathogenic changes in these three genes are thought to act via loss of function.[33 36] Based on these criteria, we conclude that the following VUS are likely to be pathogenic: p.Met405Val and p.Met322Val within *TARDBP*; p.Met459Thr in *EWSR1*; p.Gln1034Ter and c.1665+2T>C in *NEK1*; p.Arg2318Cys and p.Ile2246Thr in *SPG11*. Our data are suggestive of pathogenicity, but confirmations will require further validation by future studies.

Indels have high rates of inaccurate sequencing. We removed all non-coding indels, those with low quality and those present in >10% of this cohort and present in Project MinE controls leaving two frameshift variants, seven inframe deletions and two splice region variants. *FIG4* has a frameshift in codon 689 which has not previously been reported in gnomAD (V.2.1.1—online supplemental figure S5) and *SPG20* has a frameshift close to the end of the protein at codon 1969 which has a very low population frequency (0.0000636—online supplemental figure S6). *TBK1* has an inframe deletion of a single codon, which has previously been reported in ClinVar as pathogenic (online supplemental figure S7).

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A

Sample	Age at Onset	Inheritance?	Gene	Variant	Gene	Variant	Gene	Variant
SHF-009	56	S	SPAST	p.Ala106_Pro107dup	SPG11	p.Ile2246Thr	TAF15	p.Arg562Gln
SHF-094	22	S	NEFH	p.Pro777Leu	ALS2	p.Thr293Ile		
SHF-159	68	S	ARHGEF28	p.Gln1387Leu	EWSR1	p.Met459Thr		
SHF-138	66	S	C9orf72	Expansion	NEK1	c.1665+2T>C		
SHF-162	63	S	C9orf72	Expansion	SETX	p.Gly179Glu		
SHF-082	53	F	ANXA11	p.Pro35Ala	SOD1	p.Asn140Lys		
SHF-139	49	S	ARHGEF28	p.Ala1030Val	SPG11	p.Arg2318Cys		
SHF-029	62	S	ALS2	p.Ser80Asn	TAF15	p.Gly565_Gly572del		
SHF-103	57	F	C9orf72	Expansion	TAF15	p.Arg153Lys		
SHF-014	52	F	C9orf72	Expansion	TARDBP	p.Met405Val		
SHF-062	51	S	KIF5A	p.Val74Ala	TARDBP	p.Met322Val		
SHF-128	56	S	NEK1	p.Asp245Ala	VAPB	p.Thr23Ala		
SHF-144	32	S	NEK1	p.Gln1034Ter	VPS54	p.Val433Ala		
			Variant 1		Variant 2		Variant 3	

S = Sporadic F = Familial

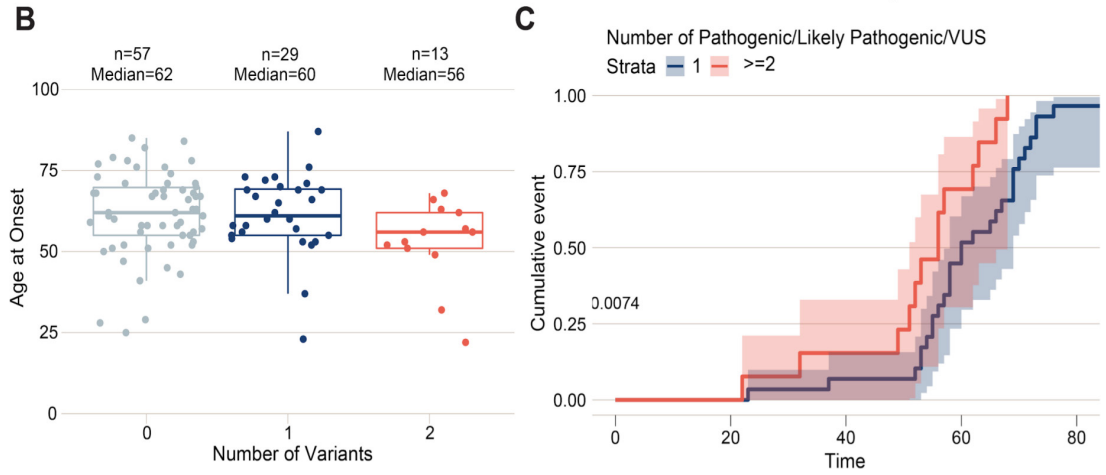

Figure 2 Increased mutation burden is associated with earlier age at onset. (A) Details of patients with two or more pathogenic, likely pathogenic or variants of uncertain significance (VUS) (n=13). (B) The number of pathogenic, likely pathogenic and VUS in each patient in the study. (C) Cumulative event plot showing those patients with two or more pathogenic, likely pathogenic or VUS have a significantly earlier age of onset.

Panel genes where no changes were found

For 20 (highlighted in figure 1A) of the 44 genes screened in our panel, no mutations (pathogenic or VUS) were identified in any member of our cohort. Many of these genetic changes are individually rare and our data are consistent with this. The absence of identified VUS in patients with sporadic ALS would be consistent with a model in which these genes harbour only high-effect, highly penetrant mutations.

Clinical correlation supports pathogenicity of VUS

In the absence of experimental validation, the biological effect of genetic variation can be inferred from correlation with clinical phenotype. The number of clinically reportable variants and VUS were combined to assign a 'mutation-load' to 42 patients with ALS (figure 2A). Patients with ALS without discovered variants were excluded because of absent information. To determine whether mutation load was clinically relevant, we compared age of onset between patients with one or two variants. Patients with ALS carrying two variants (n=13) compared with one variant developed disease at a significantly earlier age (log-rank test, p=0.0074, median age of onset=56 years vs 60 years, figure 2B,C). It is not yet possible to assess any potential effect on disease duration because 67% of this cohort are still living. Correlation with clinical phenotype is therefore consistent with a functional effect of both clinically reportable genetic variants and VUS.

DISCUSSION

An important strength of our study is its prospective nature. We performed targeted sequencing of ALS-relevant genes in 100 prospectively identified patients with ALS attending a large ALS centre in Northern England. This identified clinically reportable genetic changes in 21% of patients of whom 15 with *C9ORF72* or *SOD1* mutations would potentially be eligible for recruitment into an ongoing genetic-therapy trial. The number of clinically actionable results is likely to increase with the anticipated development of new genetic-therapy approaches for ALS. Previous genetic studies of ALS have been largely retrospective and were therefore unable to determine the utility of genetic screening in the clinic. In contrast, the present study strongly suggests that routine genetic testing should be offered in both patients with familial ALS and those with sporadic ALS, at least in our population.

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We identified 22 clinically reportable mutations and 34 VUS with potential for pathogenicity in 42 patients, including 35 patients with apparently sporadic ALS. Moreover, we identified a strong correlation between mutation load and age of onset, suggesting that the majority of the VUS may be functionally important. Evaluation of the pathogenicity of individual VUS requires further study, but comparison with previously described variants in the literature suggests that we have identified novel likely pathogenic changes in *TARDBP* (two variants), *EWSR1*, *SPG11* (two changes) and *NEK1* (two variants). Genetic changes were absent from 20 genes in the panel in this cohort (figure 1A).

Mutations in RNA binding proteins are significantly associated with ALS.[37] Most prominently, *TARDBP* encodes the protein TDP-43 which is the major component of the characteristic proteinopathy in ALS. Pathogenic mutations are clustered in the C-terminal glycine-rich domain which is important for interactions with other RNA-binding proteins.[38] We have identified two patients with rare and predicted deleterious mutations in *TARDBP* which are both located in the glycine-rich domain (table 3 and online supplemental figure S8). Both changes (p.Met322Val and p.Met405Val) are absent from 125 748 population controls in gnomAD and are predicted deleterious (CADD >15), although neither has been previously reported. FET proteins including *EWSR1*, *FUS* and *TAF15* are RNA-binding proteins which have been associated with fALS. We identified several VUS within FET proteins with some evidence for pathogenicity (table 3). One patient carried a p.Met459Thr *EWSR1* mutation within the RNA binding region which contains other ALS-associated mutations.

We identified four variants within *SPG11* (figure 1E): one clinically reportable change plus two of the VUS. All four variants cluster in the spatacsin C-terminal domain, suggesting that this could represent a region of functional importance. Conversely, benign mutations in this gene tend to be more dispersed and none are found in the spatacsin C-terminal domain. We conclude that the two p.Ile2246Thr and p.Arg2318Cys VUS we identified are highly likely to be pathogenic.

Mutations in *TBK1*,[36] *NEK1*[39] and *FIG4*[33] have conclusively been shown to be loss of function (LOF) changes. In our cohort, we identified two VUS within *NEK1* which significantly disrupt the translated sequence and are therefore predicted to be highly pathogenic via a LOF mechanism (online supplemental figure S8). This included p.Gln1034Ter and c.1665+2T>C which is a splice site mutation. Both mutations are rare or absent from gnomAD and are predicted pathogenic (table 3). Similarly, we identified a clinically reportable frameshift mutation in *FIG4* (p.Thr689AsnfsTer12-FIG4) and an in-frame deletion in *TBK1* (p.Glu643del-TBK1) which has previously been reported in a single case in ClinVar (online supplemental figure S7). Our data add to the weight of evidence that this change is pathogenic and that mutations in *TBK1* are an important contributor to the genetics of sporadic ALS.[39]

VUS were identified based on universal measures of pathogenicity [27] and population frequency.[22] However, we added an additional filtering step based on case:control ratio within a population-matched whole genome sequencing dataset.[35] When combined with clinically reportable changes, the resulting VUS were associated with age of onset which suggests that these are likely to represent pathogenic changes. We confirmed previous reports that *C9ORF72* expansions are frequently associated with additional likely pathogenic mutations in other ALS genes.[40] In the present cohort, 40% of patients with pathogenic *C9ORF72* expansions carried an additional VUS. The presence of such an additional genetic variant may be one factor influencing the penetrance of *C9ORF72* mutations.

The current model of ALS is considered a multistep process in which steps constitute genetic and/or environmental exposures.[12] A consistent finding in ALS genetics is that identification of a highly penetrant genetic risk factor correlates with earlier age of onset. It follows that such mutations might be associated with fewer steps and this has been supported.[13] Our work suggests that, when correctly prioritised, VUS can also contribute steps leading to earlier age of onset and potentially function as a prognostic biomarker. Similar findings have been reported previously[10] but not in a prospective cohort; moreover, the latter study was limited to a smaller number of genes. If an index case carries one or more VUS within ALS-linked genes, then screening for VUS within family members may inform risk and even age of onset counselling. In the present cohort, an additional VUS was associated with ~4 years earlier age of onset.

In conclusion, we have performed a prospective genetic study of 100 consecutive patients with ALS attending our clinic. Our results indicate that screening of known ALS genes can lead to clinically actionable results in ~21% of patients, and in a further ~21% of patients a VUS may be discovered with potential clinical implications. As future studies expand the number of verified genetic causes of ALS, these percentages are likely to increase. We developed a pipeline for prioritising VUS using whole genome case–control cohorts to predict clinical outcomes. Although this study took place in a large tertiary referral ALS centre and requires further validation in other settings, our data suggest that all patients with ALS should, with careful counselling, be offered genetic testing, especially in light of new personalised medicine treatments in development.

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Contributors PJS and JK conceived and designed the study. JC-K, MDP, MRT, AM and PF contributed to the design, theoretical analysis and implementation of the analysis pipeline. SRS, MDP, JC-K, NSV, LT, CH, TMJ, CJM, PJS, NB, EP and ESAS were responsible for data acquisition. SRS, MDP, JC-K, NSV, JK and PJS were responsible for analysis of data. JK, PJS, MDP and JC-K were responsible for interpretation of data. PJS, JK and MDP supervised the project. The Project MinE ALS Sequencing Consortium (online supplementary table 4) was involved in data acquisition and analysis. All authors meet the four ICMJE authorship criteria, and were responsible for revising the manuscript, approving the final version for publication, and for accuracy and integrity of the work.

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Supplementary Information

Name	Sequence
Sizing (standard) primers:	
C9ORF72-F	FAM-CAA GGA GGG AAA CAA CCG CAG CC
C9ORF72-R	GCA GGC ACC GCA ACC GCA G
Repeat primed-PCR primers:	
MRX-F	FAM-TGT AAA ACG ACG GCC AGT CAA GGA GGG AAA CAA CCG CAG CC
MRX-M13R	CAG GAA ACA GCT ATG ACC
MRX-R1	CAG GAA ACA GCT ATG ACC GGG CCC GCC CCG ACC ACG CCC CGG CCC CGG CCC CGG

Supplementary Table S1 Primers used for PCR of C9ORF72 hexanucleotide repeat expansions.

Name	Sequence
SCA2F-FAM	FAM-GGG CCC CTC ACC ATG TCG
SCA2R	CGG GCT TGC CCA CAT TGG

Supplementary Table S2 Primers used for standard PCR of *ATXN2* repeat expansions.

Gene	RefSeq Transcript	Gene	RefSeq Transcript
CHMP2B	NM_014043.3	MATR3	NM_199189.2
CYP27A1	NM_000784.3	NEFH	NM_021076.3
DCTN1	NM_004082.4	NEK1	NM_001199397.1
GRN	NM_002087.3	OPTN	NM_001008211.1
MAPT	NM_005910.5	PFN1	NM_005022.3
SPG11	NM_025137.1	PRPH	NM_006262.3
ALS2	NM_020919.3	SETX	NM_015046.5
ANG	NM_001145.4	SIGMAR1	NM_005866.3
ANXA11	NM_145869.1	SOD1	NM_000454.4
ARHGEF28	NM_001080479.2	SPAST	NM_014946.3
ATXN2	NM_002973.3	SPG20	NM_015087.4
C9orf72	NM_001256054.2	SQSTM1	NM_003900.4
CHCHD10	NM_213720.2	SS18L1	NM_198935.2
DAO	NM_001917.4	TAF15	NM_139215.2
ERBB4	NM_005235.2	TARDBP	NM_007375.3
EWSR1	NM_013986.3	TBK1	NM_013254.3
FIG4	NM_014845.5	TUBA4A	NM_006000.2
FUS	NM_004960.3	UBQLN2	NM_013444.3
GBA2	NM_020944.2	VAPB	NM_004738.4
HNRNPA1	NM_031157.2	VCP	NM_007126.3
HNRNPA2B1	NM_031243.2	VPS54	NM_016516.2
KIF5A	NM_004984.2	VRK1	NM_003384.2

Supplementary Table S3 Preferred Transcripts for Genes Included in Panel.

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Mamede	de Carvalho	mamedemg@gmail.com	Instituto de Fisiologia, Instituto de Medicina Molecular,Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal	Department of Neurosciences, Hospital de Santa Maria-CHLN, Lisbon, Portugal.
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Chris	Shaw	chris.shaw@kcl.ac.uk	Maurice Wohl Clinical Neuroscience Institute, King's College London, Department of Basic and Clinical Neuroscience, London, UK.	
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Karen E.	Morrison	k.morrison@qub.ac.uk	School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, UK.	
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Jonathan D.	Glass	jglas03@emory.edu	Department Neurology, Emory University School of Medicine, Atlanta, GA, USA.	Emory ALS Center, Emory University School of Medicine, Atlanta, GA, USA.
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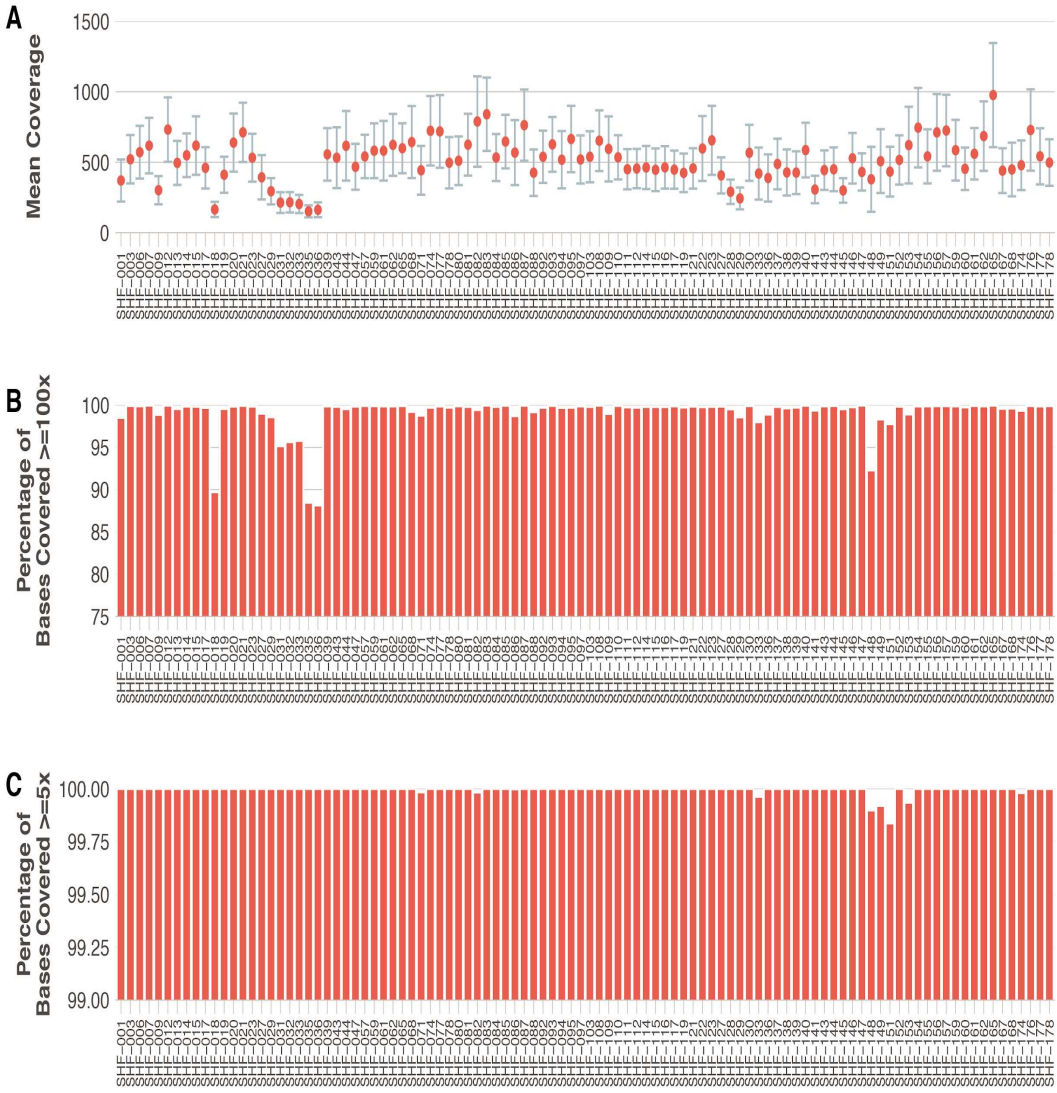

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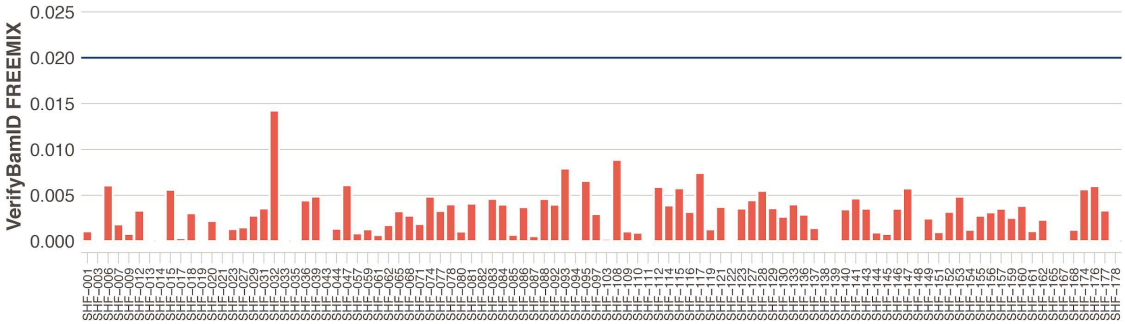

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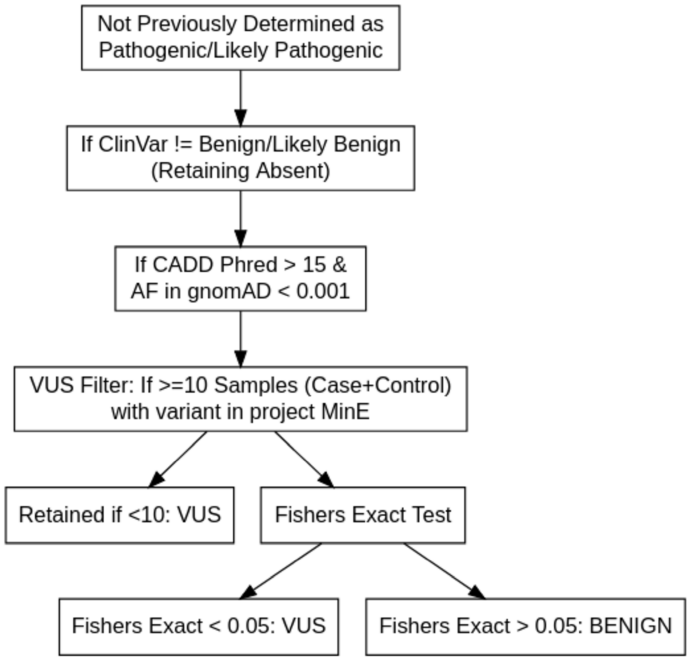
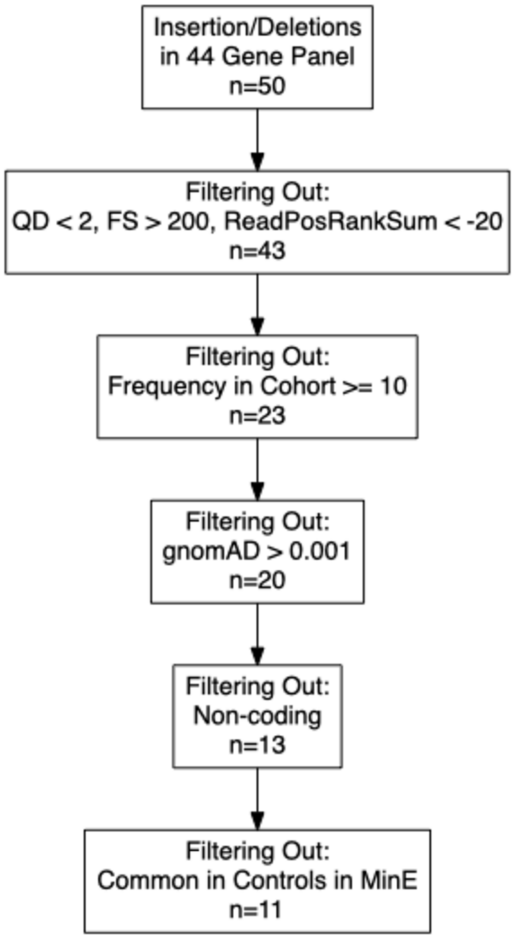


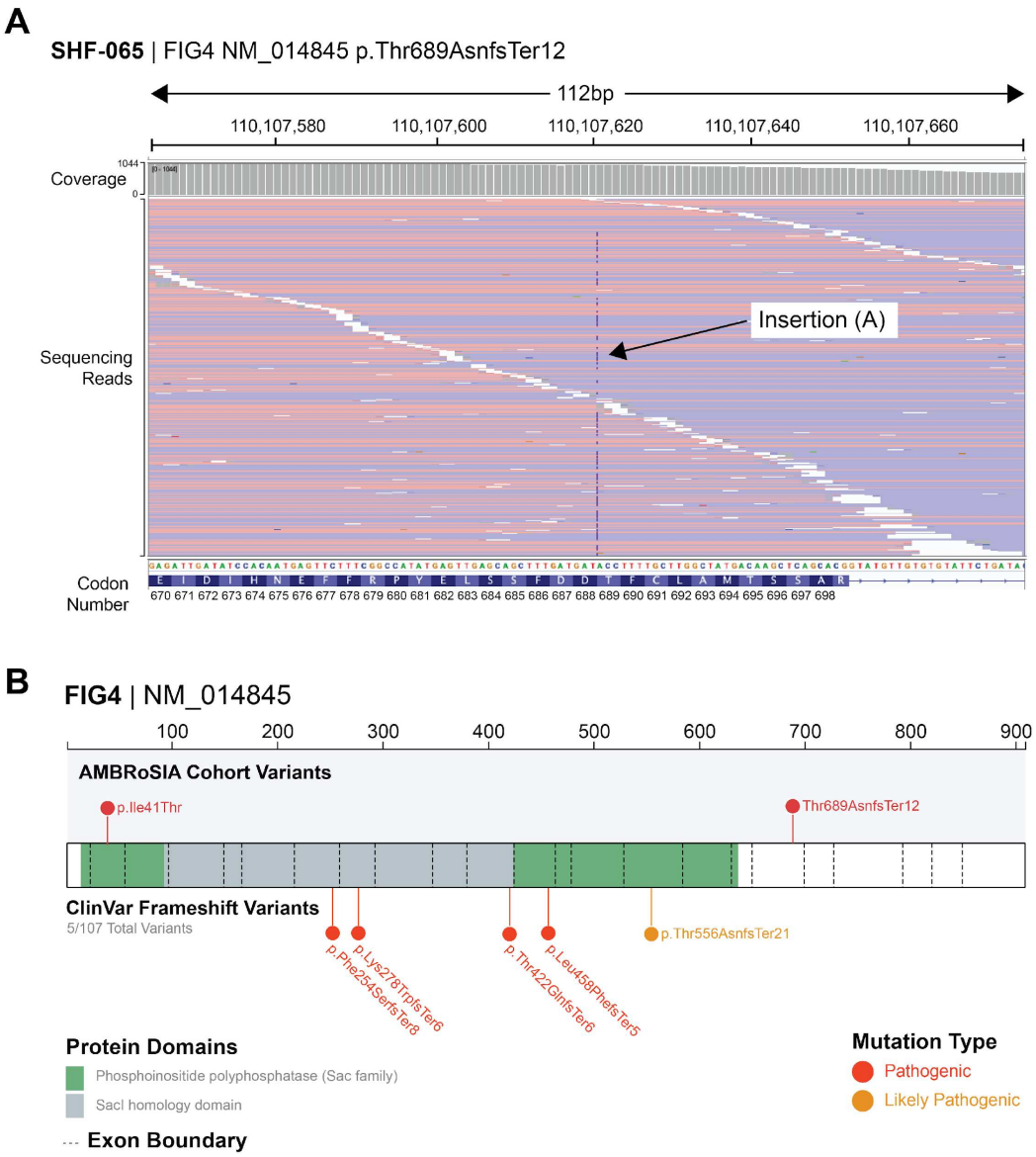
Supplementary Figure S3 Single Nucleotide Variant Filtering Scheme

Filtering scheme used for single nucleotide variants in this study.



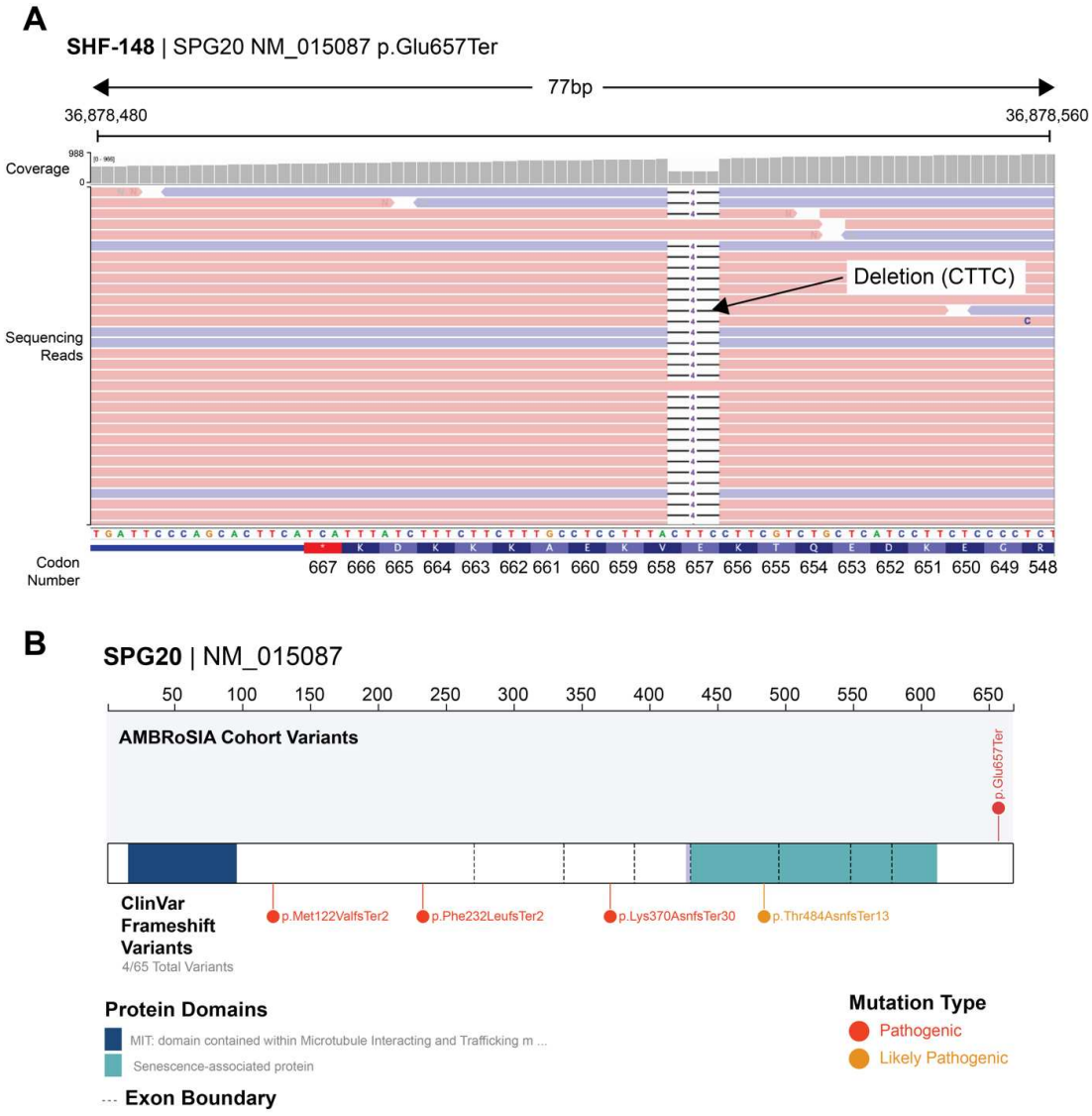
Supplementary Figure S4 Small Insertion Deletion (indel) Filtering Scheme

Small insertion and deletion filtering scheme used in this study.

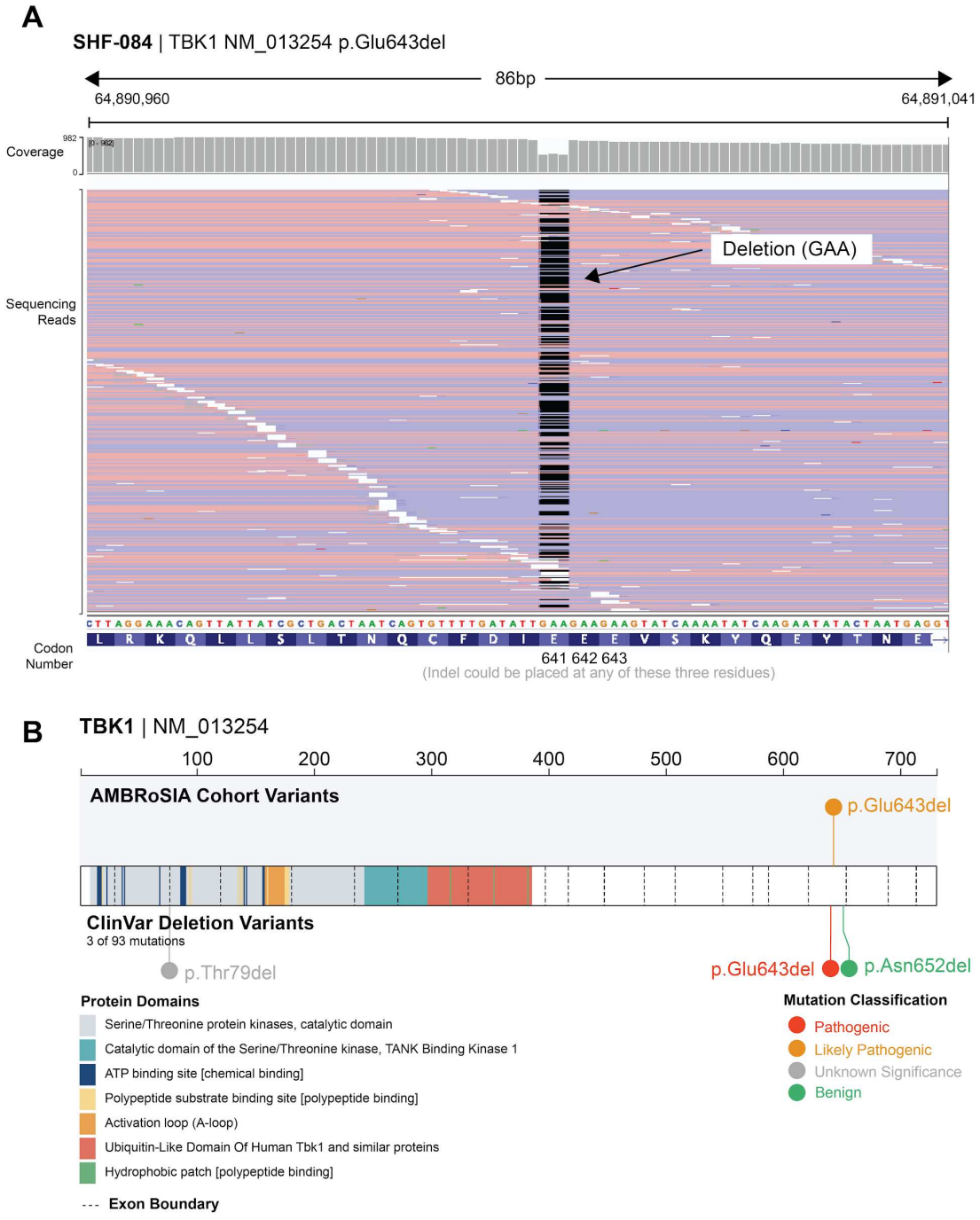


Supplementary Figure S5 *FIG4* Frameshift Variant

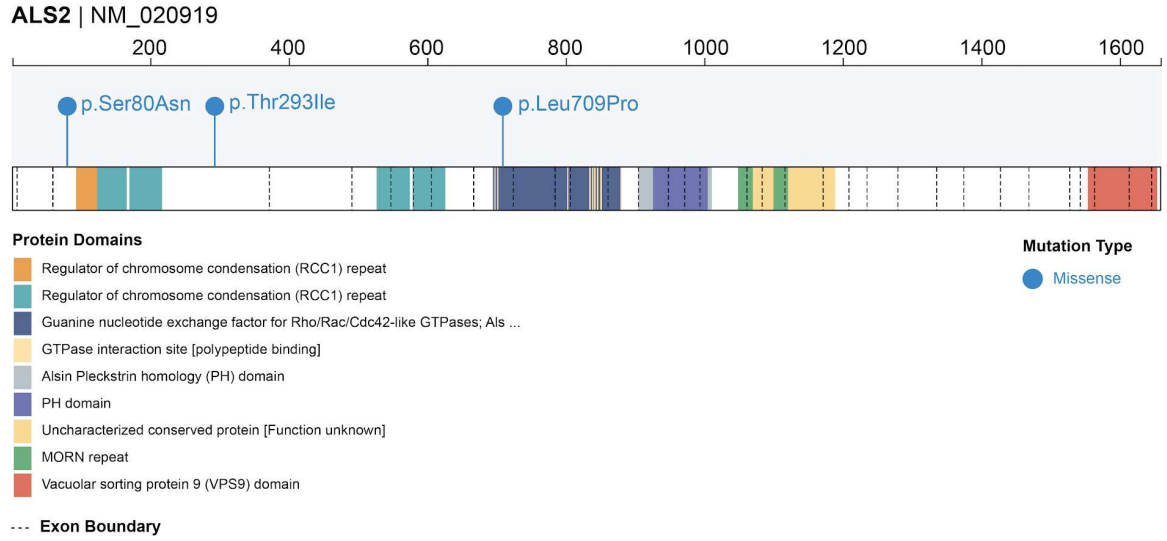
A. Raw sequencing reads from SHF-065 show the presence of a heterozygous insertion in codon 689 of *FIG4* (NM_014845). **B.** Position of the frameshift mutation detected in the AMBRoSIA cohort vs the 5 frameshift variants present in ClinVar at the time of writing, 4/5 are classed as pathogenic.

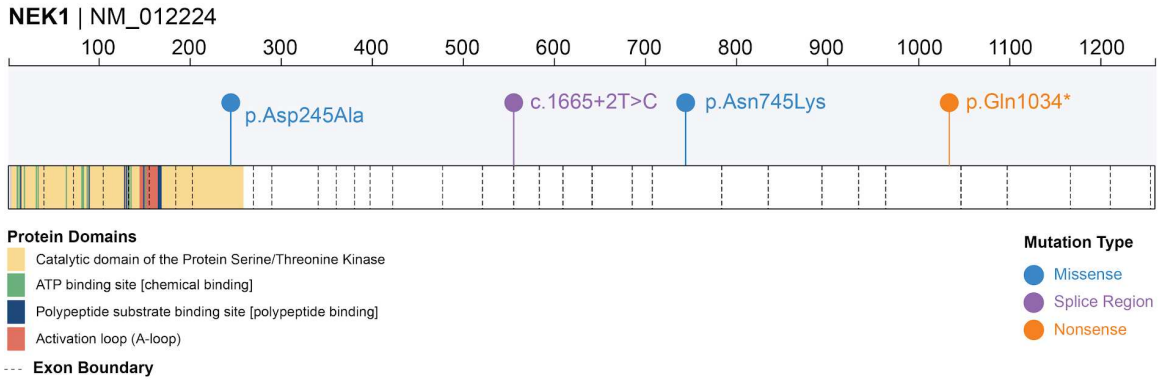


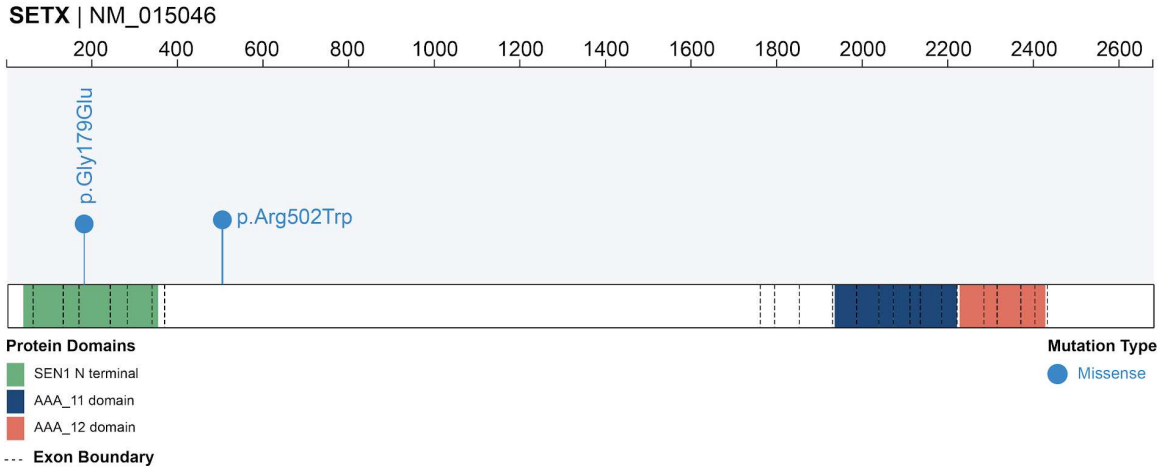
Supplementary Figure S6 *SPG20* Frameshift Variant
A. Raw sequencing reads from SHF-148 show the presence of a heterozygous deletion in codon 657 of *SPG20* (NM_015087). **B.** Position of the frameshift mutation detected in the AMBRoSIA cohort vs the 4 frameshift variants present in ClinVar at the time of writing, 3/4 are classed as pathogenic.

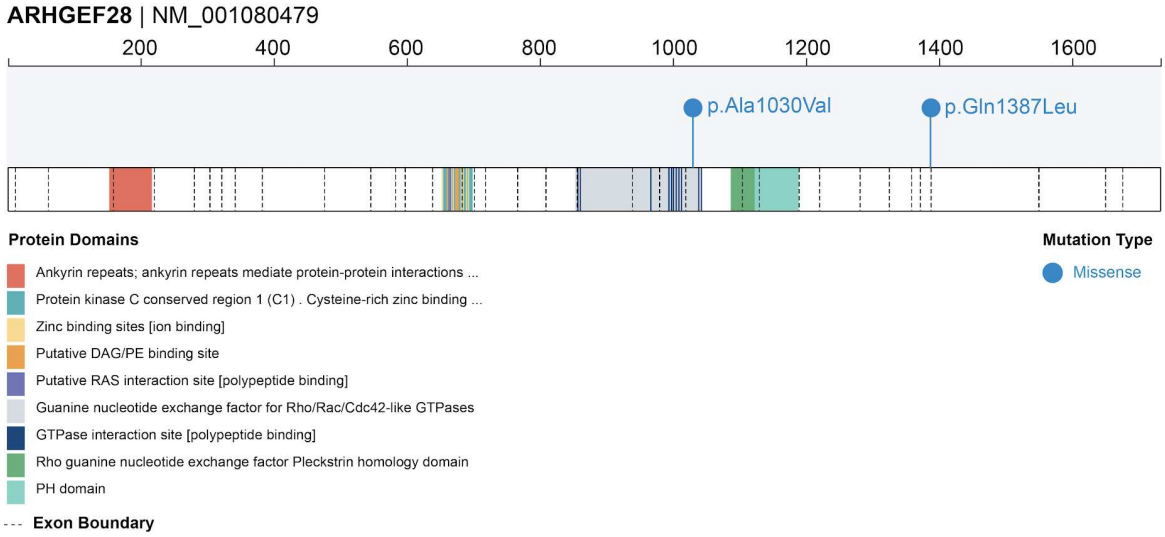


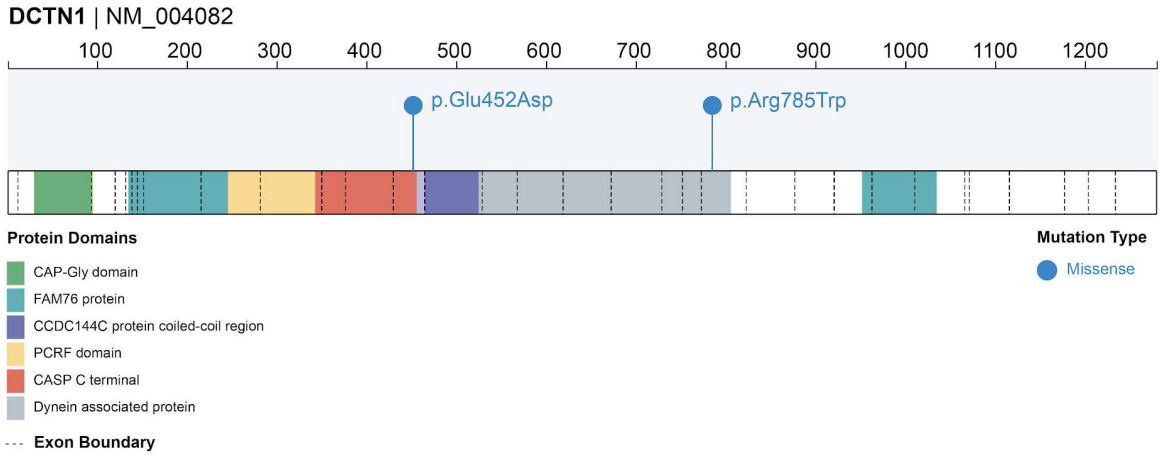
Supplementary Figure S7 *TBK1* Inframe Deletion Variant
A. IGV plot showing a heterozygous 3bp deletion in codon 641 of TBK1. **B.** ProteinPaint of the TBK1 protein showing the deletion detected in this dataset (top) compared to deletions detected in ClinVar (bottom). Deletion in AMBRoSIA cohort is at the same position as a reported pathogenic mutation in ClinVar.

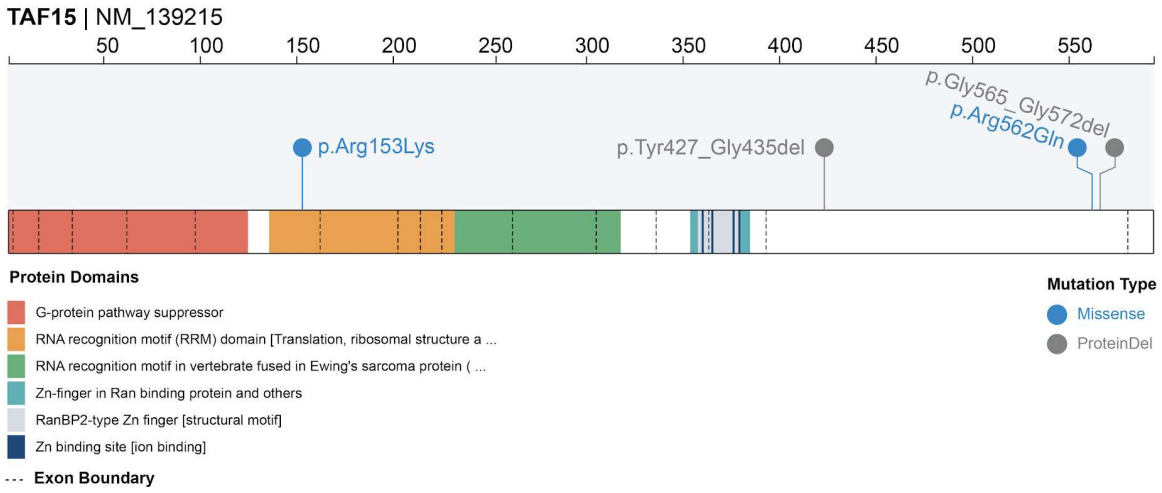

ALS2 | NM_020919
200
400
600
800
1000
1200
1400
1600
p.Ser80Asn
p.Thr293Ile
p.Leu709Pro
Protein Domains
Regulator of chromosome condensation (RCC1) repeat
Regulator of chromosome condensation (RCC1) repeat
Guanine nucleotide exchange factor for Rho/Rac/Cdc42-like GTPases; Als ...
GTPase interaction site [polypeptide binding]
Alsin Pleckstrin homology (PH) domain
PH domain
Uncharacterized conserved protein [Function unknown]
MORN repeat
Vacuolar sorting protein 9 (VPS9) domain
Exon Boundary
Mutation Type
Missense

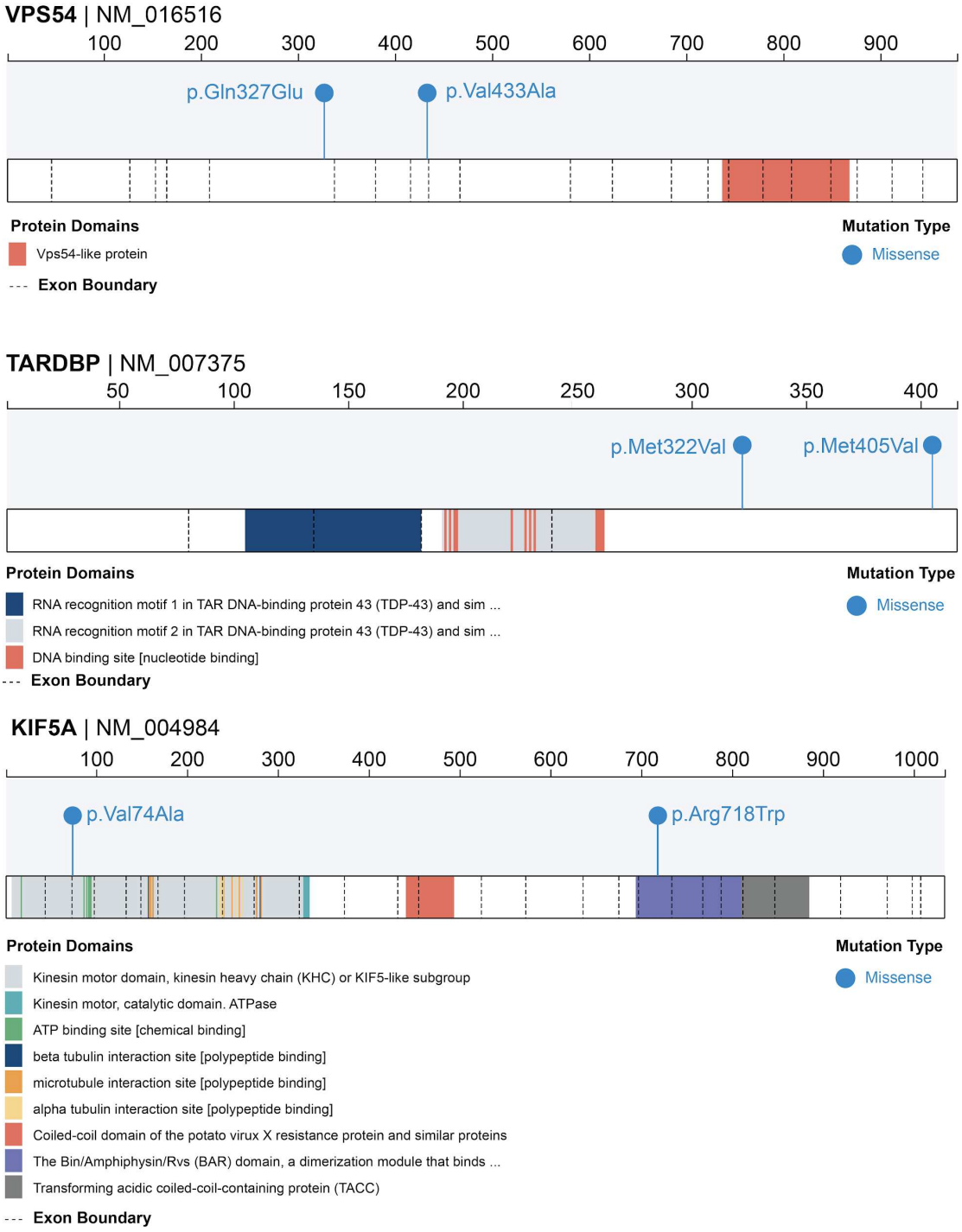

NEK1 | NM_012224
100
200
300
400
500
600
700
800
900
1000
1100
1200
p.Asp245Ala
c.1665+2T>C
p.Asn745Lys
p.Gln1034*
Protein Domains
Catalytic domain of the Protein Serine/Threonine Kinase
ATP binding site [chemical binding]
Polypeptide substrate binding site [polypeptide binding]
Activation loop (A-loop)
Exon Boundary
Mutation Type
Missense
Splice Region
Nonsense


SETX | NM_015046
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
p.Gly179Glu
p.Arg502Trp
Protein Domains
SEN1 N terminal
AAA_11 domain
AAA_12 domain
Exon Boundary
Mutation Type
Missense


ARHGEF28 | NM_001080479
200
400
600
800
1000
1200
1400
1600
p.Ala1030Val
p.Gln1387Leu
Protein Domains
Ankyrin repeats; ankyrin repeats mediate protein-protein interactions ...
Protein kinase C conserved region 1 (C1) . Cysteine-rich zinc binding ...
Zinc binding sites [ion binding]
Putative DAG/PE binding site
Putative RAS interaction site [polypeptide binding]
Guanine nucleotide exchange factor for Rho/Rac/Cdc42-like GTPases
GTPase interaction site [polypeptide binding]
Rho guanine nucleotide exchange factor Pleckstrin homology domain
PH domain
Exon Boundary
Mutation Type
Missense


DCTN1 | NM_004082
100
200
300
400
500
600
700
800
900
1000
1100
1200
p.Glu452Asp
p.Arg785Trp
Protein Domains
CAP-Gly domain
FAM76 protein
CCDC144C protein coiled-coil region
PCRF domain
CASP C terminal
Dynein associated protein
Exon Boundary
Mutation Type
Missense


TAF15 | NM_139215
50
100
150
200
250
300
350
400
450
500
550
p.Gly565_Gly572del
p.Arg562Gln
p.Arg153Lys
p.Tyr427_Gly435del
Protein Domains
G-protein pathway suppressor
RNA recognition motif (RRM) domain [Translation, ribosomal structure a ...
RNA recognition motif in vertebrate fused in Ewing's sarcoma protein (...
Zn-finger in Ran binding protein and others
RanBP2-type Zn finger [structural motif]
Zn binding site [ion binding]
Exon Boundary
Mutation Type
Missense
ProteinDel



Supplementary Figure S8 Additional ProteinPaints of genes implicated in ALS

Genes with at least 2 variants not already included in the manuscript and predicted to be Pathogenic/Likely Pathogenic or VUS were chosen for display here (excluding expansions). ProteinPaints were produced using https://pecan.stjude.cloud/proteinpaint and edited in Adobe Illustrator.

Supplementary Information

Name	Sequence
Sizing (standard) primers:	
C9ORF72-F	FAM-CAA GGA GGG AAA CAA CCG CAG CC
C9ORF72-R	GCA GGC ACC GCA ACC GCA G
Repeat primed-PCR primers:	
MRX-F	FAM-TGT AAA ACG ACG GCC AGT CAA GGA GGG AAA CAA CCG CAG CC
MRX-M13R	CAG GAA ACA GCT ATG ACC
MRX-R1	CAG GAA ACA GCT ATG ACC GGG CCC GCC CCG ACC ACG CCC CGG CCC CGG CCC CGG

Supplementary Table S1 Primers used for PCR of C9ORF72 hexanucleotide repeat expansions.

Name	Sequence
SCA2F-FAM	FAM-GGG CCC CTC ACC ATG TCG
SCA2R	CGG GCT TGC CCA CAT TGG

Supplementary Table S2 Primers used for standard PCR of *ATXN2* repeat expansions.

Gene	RefSeq Transcript	Gene	RefSeq Transcript
CHMP2B	NM_014043.3	MATR3	NM_199189.2
CYP27A1	NM_000784.3	NEFH	NM_021076.3
DCTN1	NM_004082.4	NEK1	NM_001199397.1
GRN	NM_002087.3	OPTN	NM_001008211.1
MAPT	NM_005910.5	PFN1	NM_005022.3
SPG11	NM_025137.1	PRPH	NM_006262.3
ALS2	NM_020919.3	SETX	NM_015046.5
ANG	NM_001145.4	SIGMAR1	NM_005866.3
ANXA11	NM_145869.1	SOD1	NM_000454.4
ARHGEF28	NM_001080479.2	SPAST	NM_014946.3
ATXN2	NM_002973.3	SPG20	NM_015087.4
C9orf72	NM_001256054.2	SQSTM1	NM_003900.4
CHCHD10	NM_213720.2	SS18L1	NM_198935.2
DAO	NM_001917.4	TAF15	NM_139215.2
ERBB4	NM_005235.2	TARDBP	NM_007375.3
EWSR1	NM_013986.3	TBK1	NM_013254.3
FIG4	NM_014845.5	TUBA4A	NM_006000.2
FUS	NM_004960.3	UBQLN2	NM_013444.3
GBA2	NM_020944.2	VAPB	NM_004738.4
HNRNPA1	NM_031157.2	VCP	NM_007126.3
HNRNPA2B1	NM_031243.2	VPS54	NM_016516.2
KIF5A	NM_004984.2	VRK1	NM_003384.2

Supplementary Table S3 Preferred Transcripts for Genes Included in Panel.

Shepheard SR, *et al. J Neurol Neurosurg Psychiatry* 2021;0:1–9. doi: 10.1136/jnnp-2020-325014

Supplementary Table S4 – PROJECT MinE Sequencing Consortium – Author Affiliations

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Miguel	Mitne Neto	mmitne@gmail.com	Universidade de São Paulo, Brazil	
Adriano	Chio	adriano.chio@unito.it	"Rita Levi Montalcini" Department of Neuroscience, ALS Centre, University of Torino, Turin, Italy.	Azienda Ospedaliera Città della Salute e della Scienza, Torino, Italy.
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Orla	Hardiman	orla@hardiman.net	Academic Unit of Neurology, Trinity College Dublin, Trinity Biomedical Sciences Institute, Dublin, Republic of Ireland.	Department of Neurology, Beaumont Hospital, Dublin, Republic of Ireland.
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Markus	Weber	markus.weber@kssg.ch	Neuromuscular Diseases Unit/ALS Clinic, Kantonsspital St. Gallen, 9007, St. Gallen, Switzerland.	
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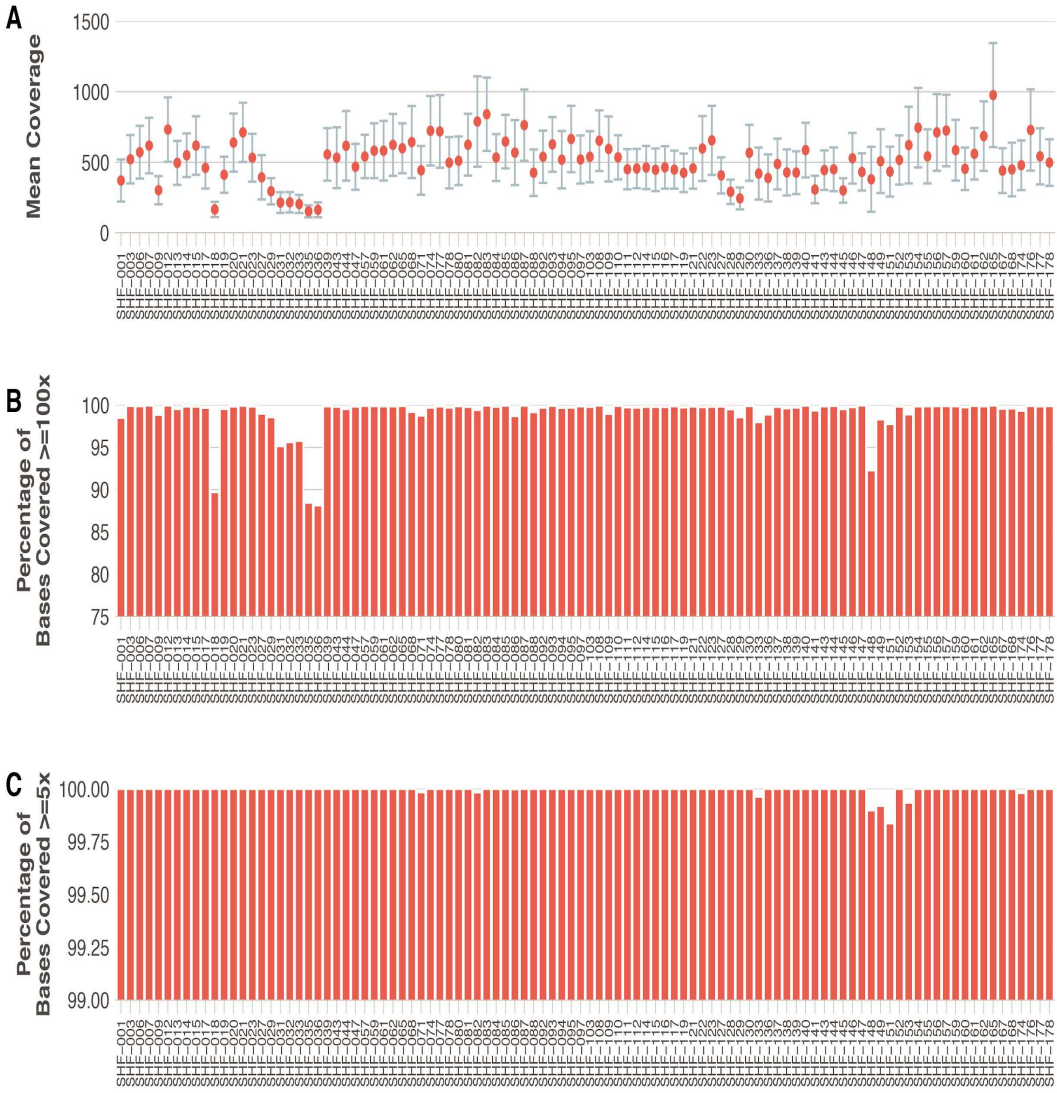

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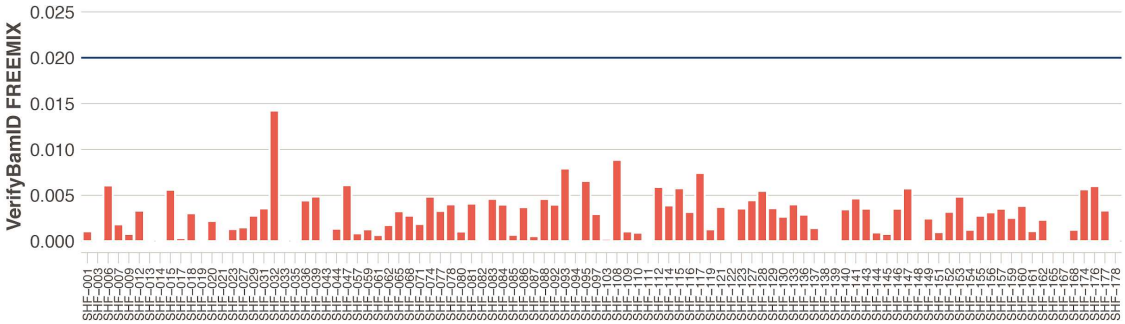

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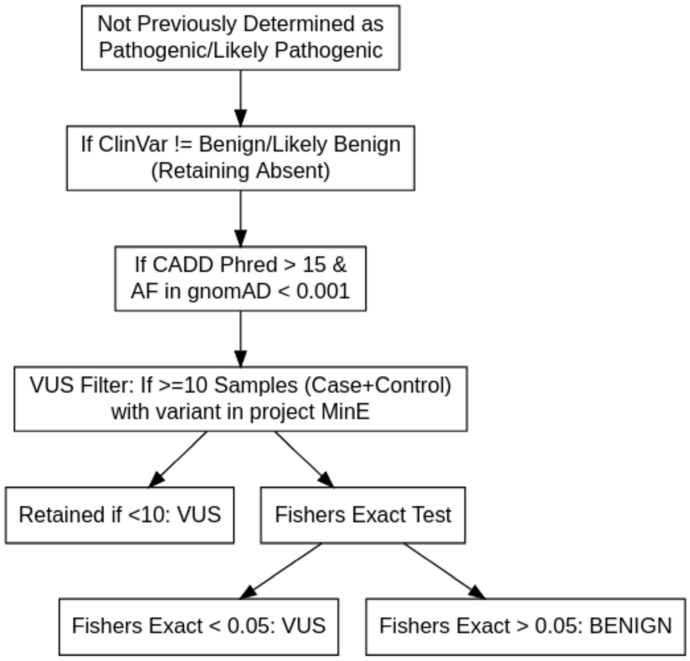

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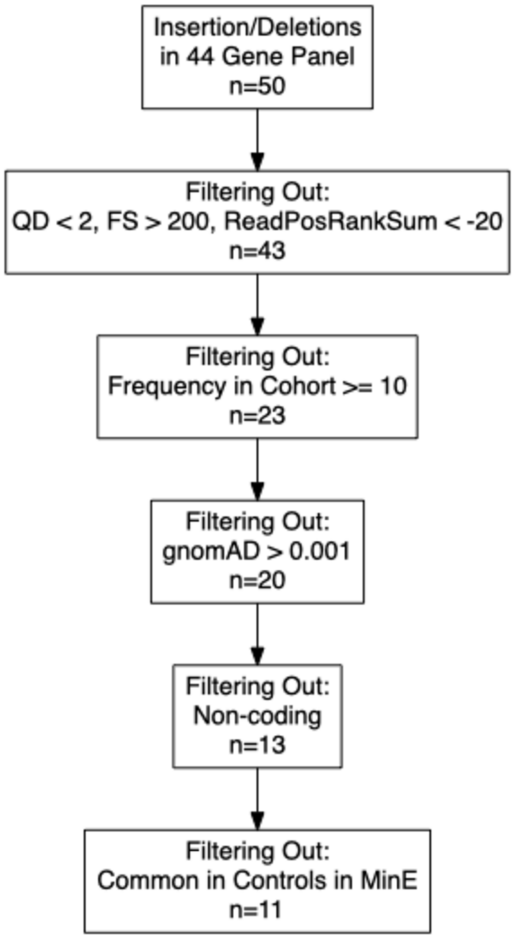
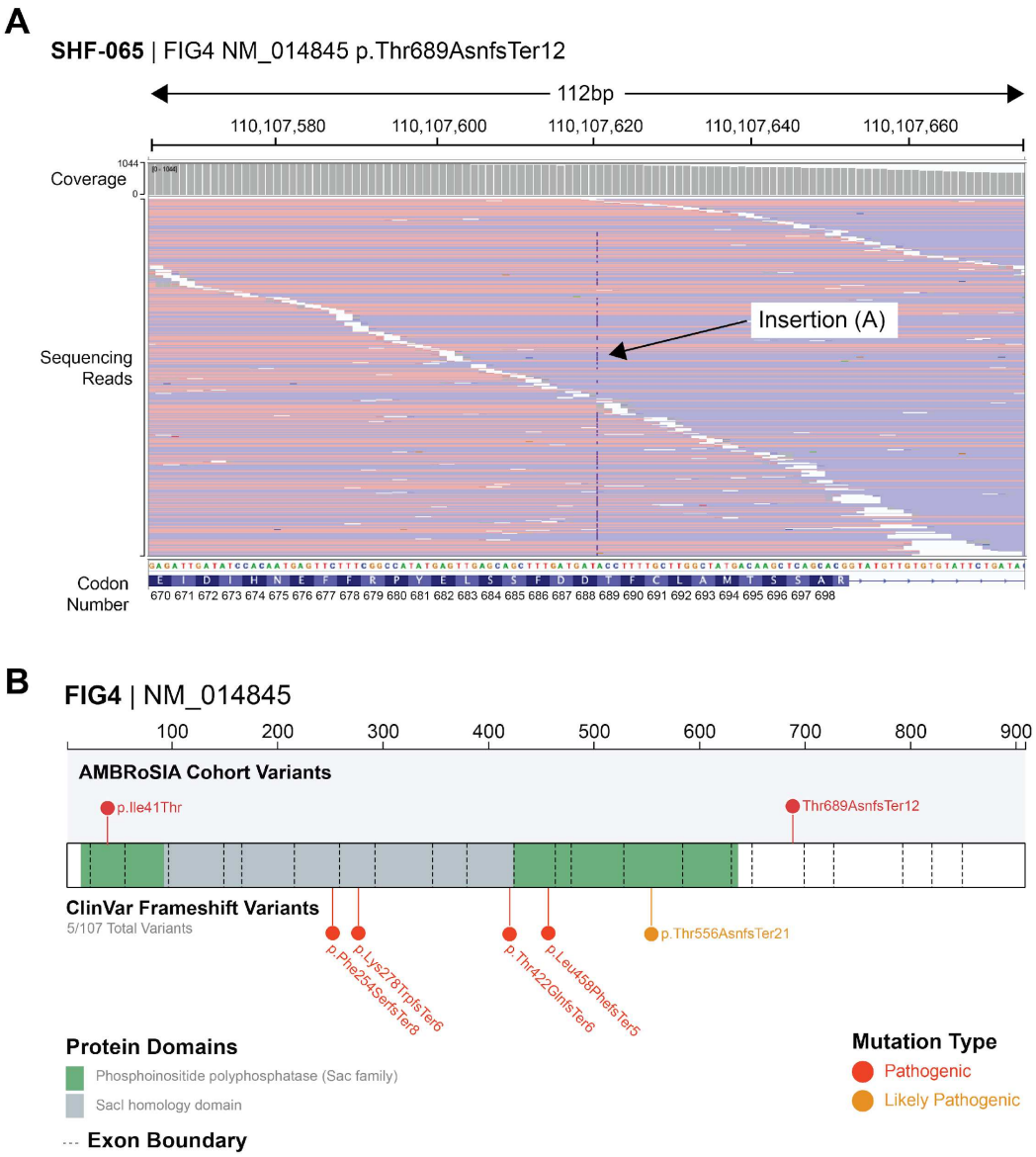


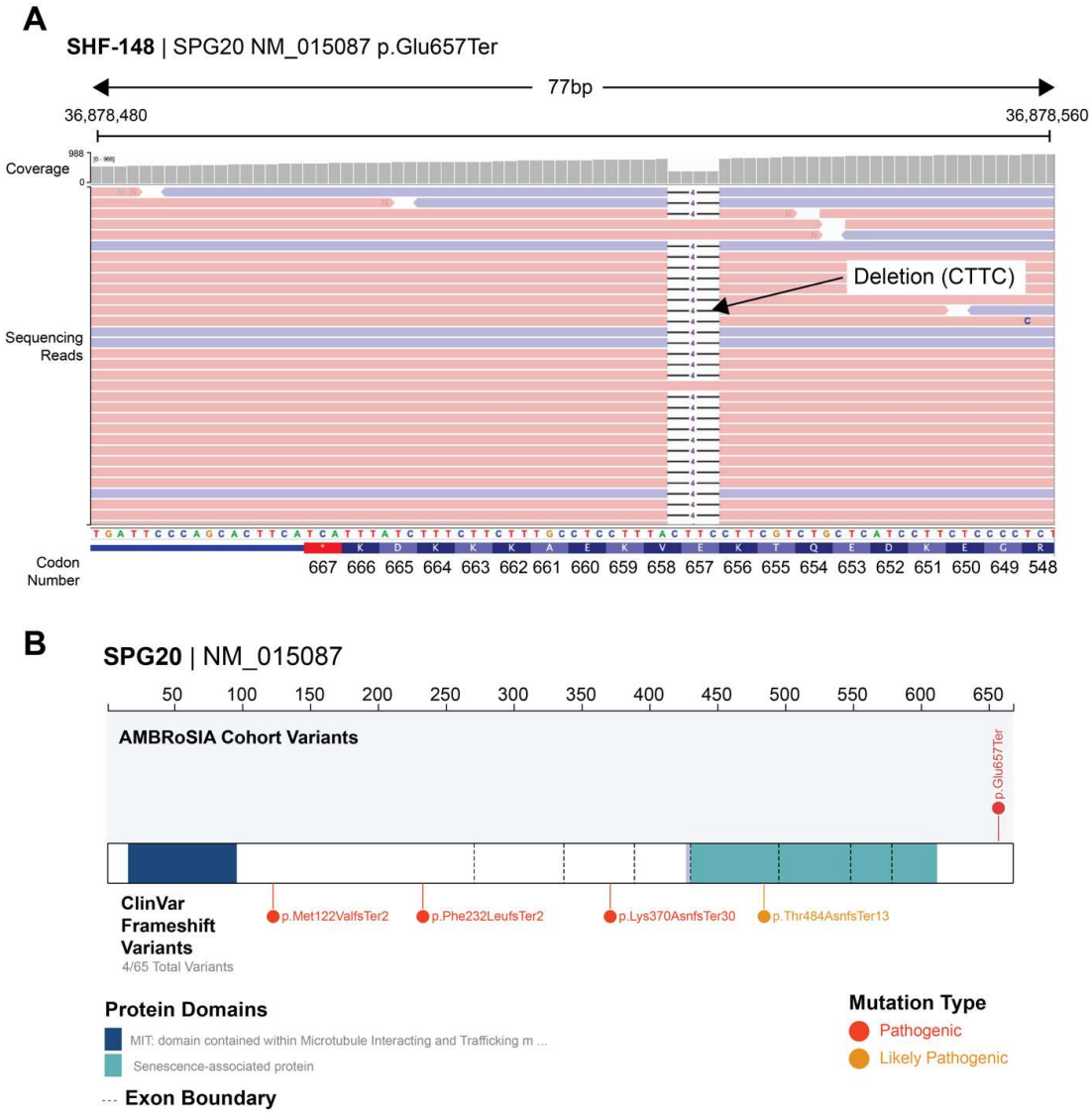
Supplementary Figure S4 Small Insertion Deletion (indel) Filtering Scheme

Small insertion and deletion filtering scheme used in this study.

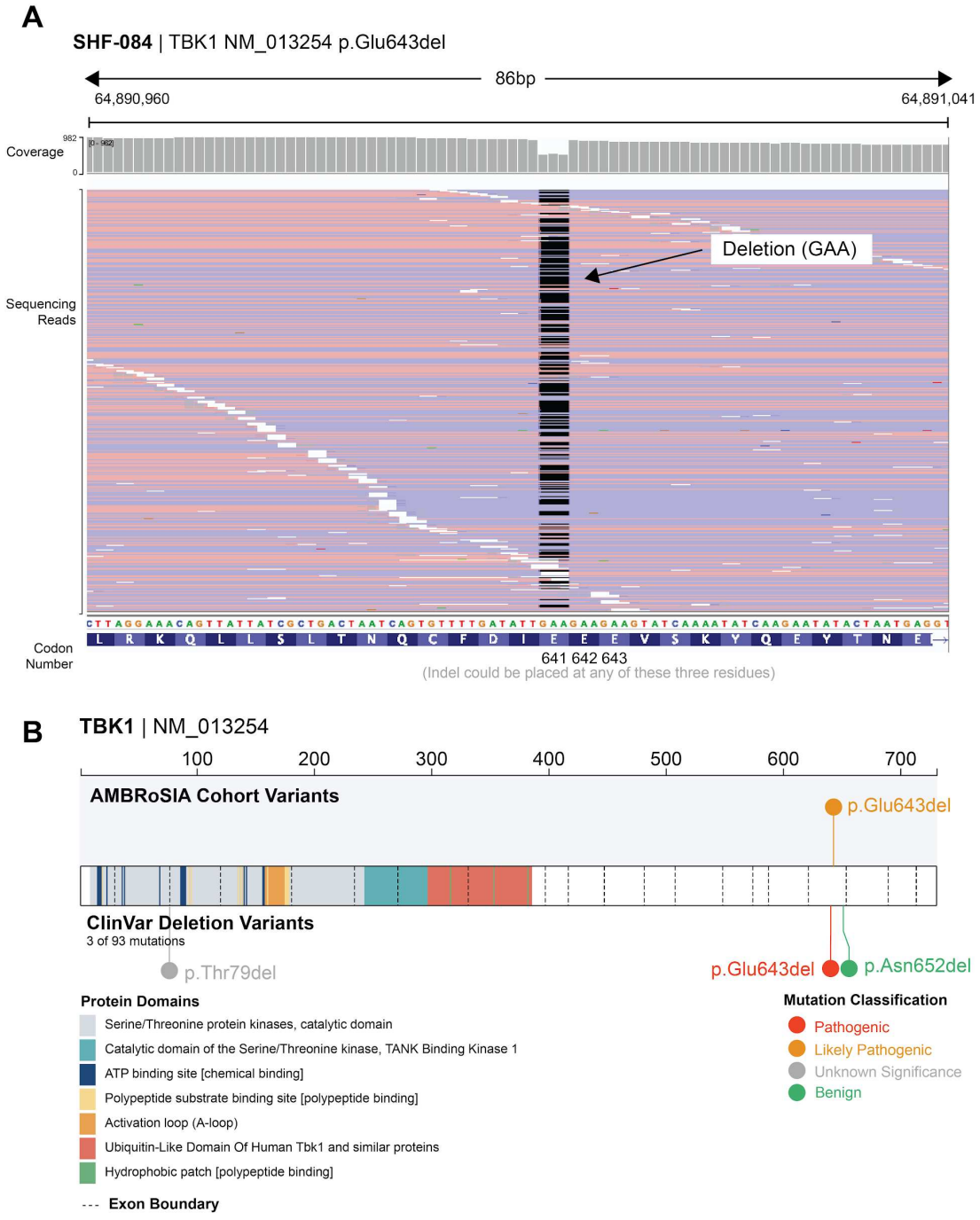


Supplementary Figure S5 *FIG4* Frameshift Variant

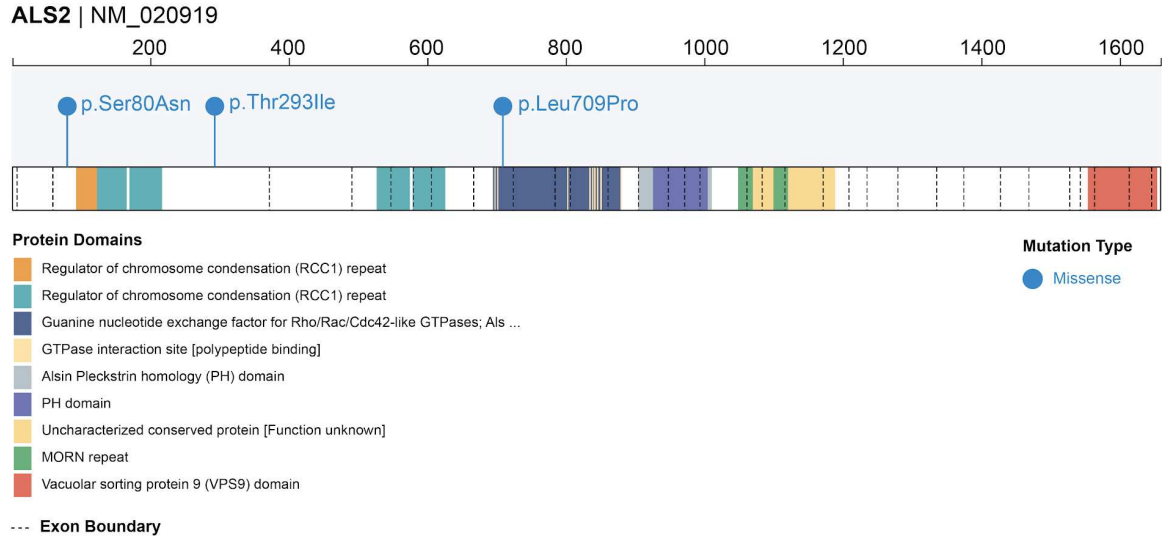
A. Raw sequencing reads from SHF-065 show the presence of a heterozygous insertion in codon 689 of *FIG4* (NM_014845). **B.** Position of the frameshift mutation detected in the AMBRoSIA cohort vs the 5 frameshift variants present in ClinVar at the time of writing, 4/5 are classed as pathogenic.

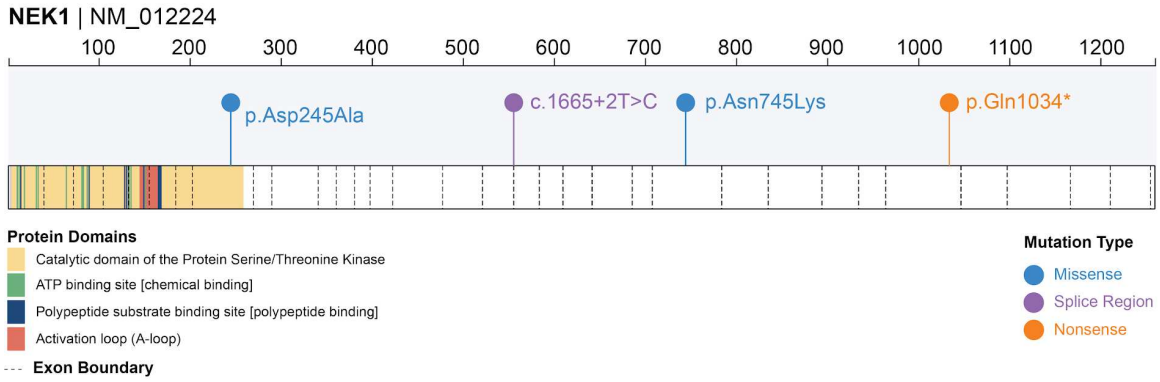


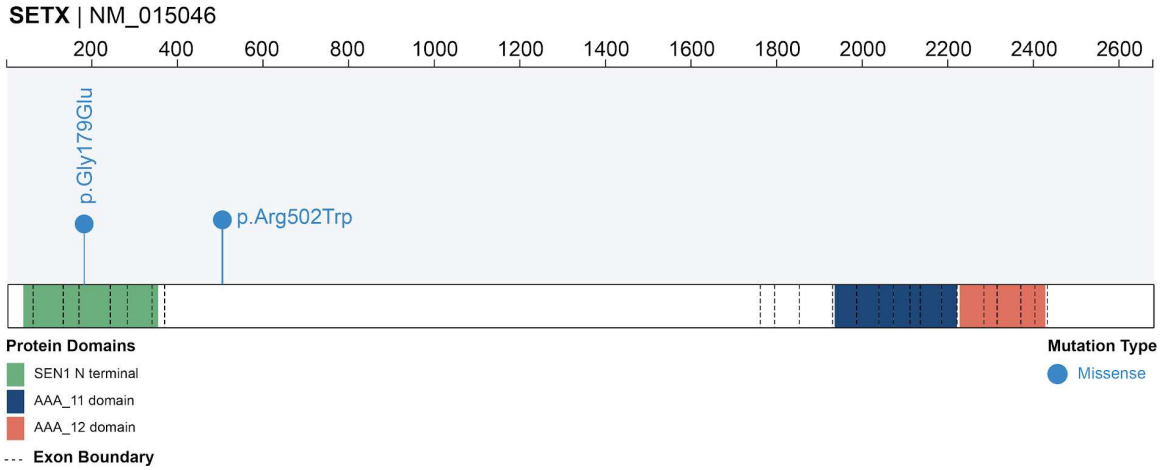
Supplementary Figure S6 *SPG20* Frameshift Variant
A. Raw sequencing reads from SHF-148 show the presence of a heterozygous deletion in codon 657 of *SPG20* (NM_015087). **B.** Position of the frameshift mutation detected in the AMBRoSIA cohort vs the 4 frameshift variants present in ClinVar at the time of writing, 3/4 are classed as pathogenic.

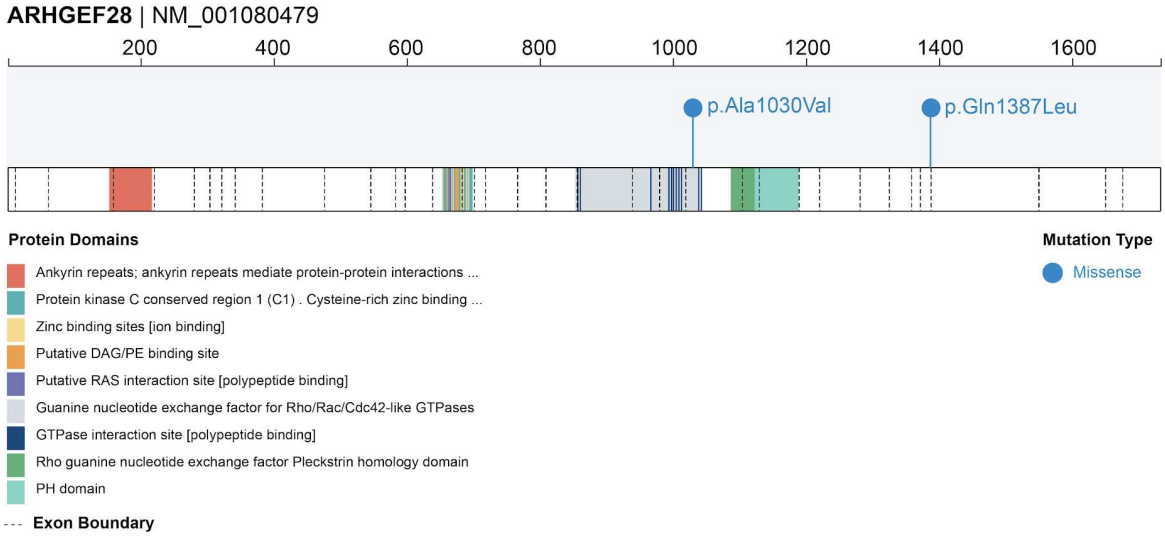


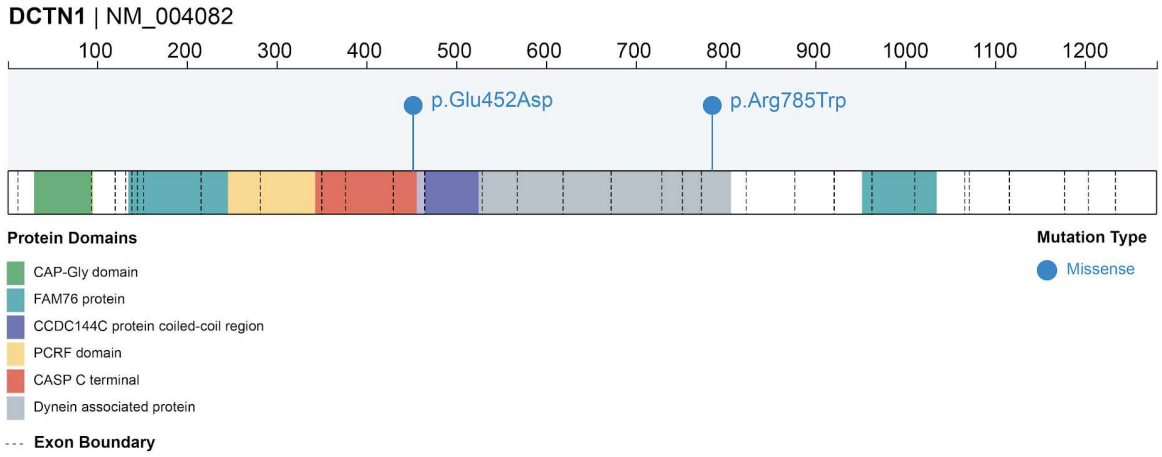
Supplementary Figure S7 *TBK1* Inframe Deletion Variant
A. IGV plot showing a heterozygous 3bp deletion in codon 641 of TBK1. **B.** ProteinPaint of the TBK1 protein showing the deletion detected in this dataset (top) compared to deletions detected in ClinVar (bottom). Deletion in AMBRoSIA cohort is at the same position as a reported pathogenic mutation in ClinVar.

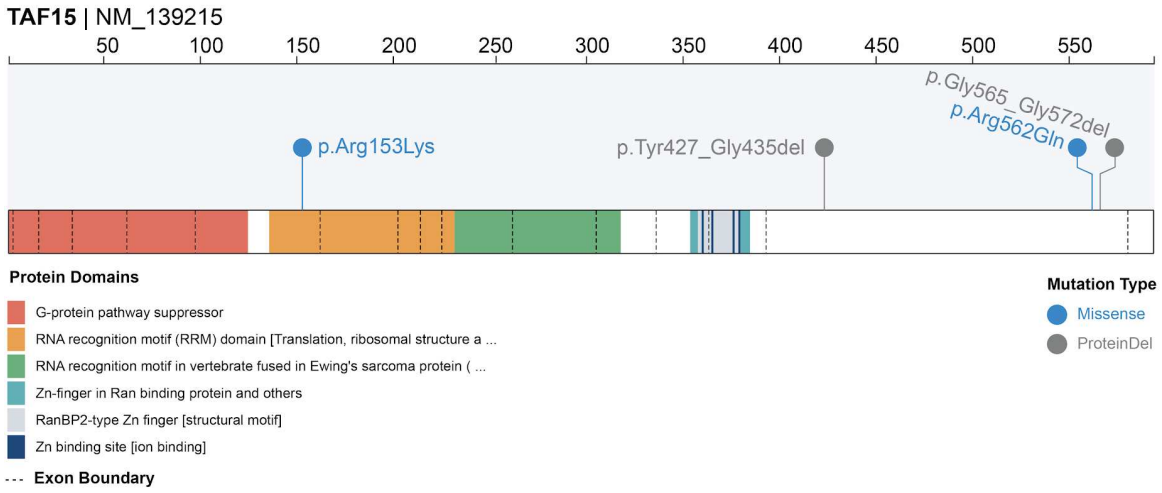

ALS2 | NM_020919
200
400
600
800
1000
1200
1400
1600
p.Ser80Asn
p.Thr293Ile
p.Leu709Pro
Protein Domains
Regulator of chromosome condensation (RCC1) repeat
Regulator of chromosome condensation (RCC1) repeat
Guanine nucleotide exchange factor for Rho/Rac/Cdc42-like GTPases; Als ...
GTPase interaction site [polypeptide binding]
Alsin Pleckstrin homology (PH) domain
PH domain
Uncharacterized conserved protein [Function unknown]
MORN repeat
Vacuolar sorting protein 9 (VPS9) domain
Exon Boundary
Mutation Type
Missense

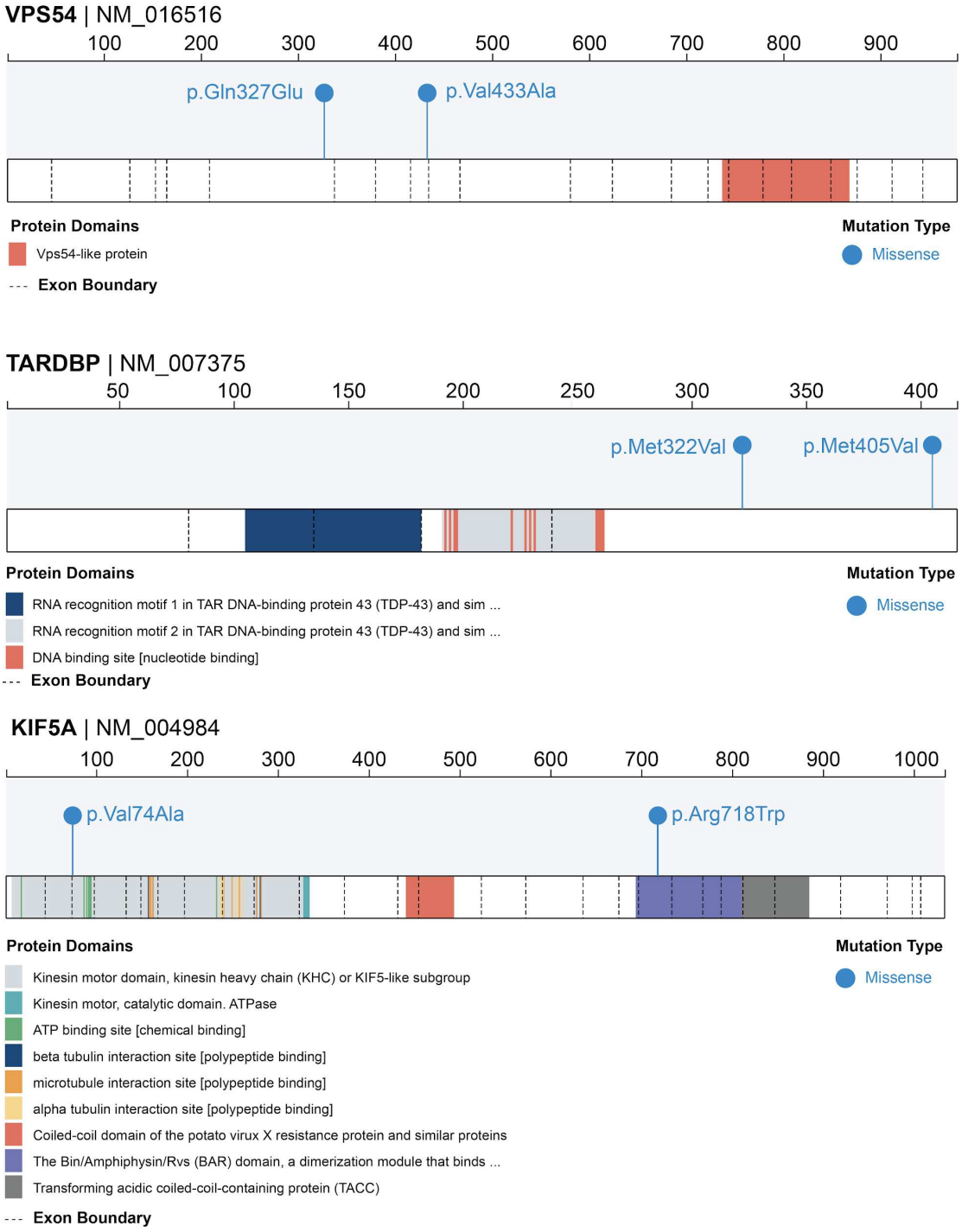

NEK1 | NM_012224
100
200
300
400
500
600
700
800
900
1000
1100
1200
p.Asp245Ala
c.1665+2T>C
p.Asn745Lys
p.Gln1034*
Protein Domains
Catalytic domain of the Protein Serine/Threonine Kinase
ATP binding site [chemical binding]
Polypeptide substrate binding site [polypeptide binding]
Activation loop (A-loop)
Exon Boundary
Mutation Type
Missense
Splice Region
Nonsense


SETX | NM_015046
200
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
p.Gly179Glu
p.Arg502Trp
Protein Domains
SEN1 N terminal
AAA_11 domain
AAA_12 domain
Exon Boundary
Mutation Type
Missense


ARHGEF28 | NM_001080479
200
400
600
800
1000
1200
1400
1600
p.Ala1030Val
p.Gln1387Leu
Protein Domains
Ankyrin repeats; ankyrin repeats mediate protein-protein interactions ...
Protein kinase C conserved region 1 (C1) . Cysteine-rich zinc binding ...
Zinc binding sites [ion binding]
Putative DAG/PE binding site
Putative RAS interaction site [polypeptide binding]
Guanine nucleotide exchange factor for Rho/Rac/Cdc42-like GTPases
GTPase interaction site [polypeptide binding]
Rho guanine nucleotide exchange factor Pleckstrin homology domain
PH domain
Exon Boundary
Mutation Type
Missense


DCTN1 | NM_004082
100
200
300
400
500
600
700
800
900
1000
1100
1200
p.Glu452Asp
p.Arg785Trp
Protein Domains
CAP-Gly domain
FAM76 protein
CCDC144C protein coiled-coil region
PCRF domain
CASP C terminal
Dynein associated protein
Exon Boundary
Mutation Type
Missense


TAF15 | NM_139215
50
100
150
200
250
300
350
400
450
500
550
p.Gly565_Gly572del
p.Arg562Gln
p.Arg153Lys
p.Tyr427_Gly435del
Protein Domains
G-protein pathway suppressor
RNA recognition motif (RRM) domain [Translation, ribosomal structure a ...
RNA recognition motif in vertebrate fused in Ewing's sarcoma protein (...
Zn-finger in Ran binding protein and others
RanBP2-type Zn finger [structural motif]
Zn binding site [ion binding]
Exon Boundary
Mutation Type
Missense
ProteinDel



Supplementary Figure S8 Additional ProteinPaints of genes implicated in ALS

Genes with at least 2 variants not already included in the manuscript and predicted to be Pathogenic/Likely Pathogenic or VUS were chosen for display here (excluding expansions). ProteinPaints were produced using https://pecan.stjude.cloud/proteinpaint and edited in Adobe Illustrator.