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Haploinsufficiency of *TBK1* causes familial amyotrophic lateral sclerosis and fronto-temporal dementia

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Abstract

Amyotrophic lateral sclerosis (ALS) is a genetically heterogeneous neurodegenerative syndrome hallmarked by adult-onset loss of motor neurons. We performed exome sequencing of 252 familial ALS and 827 control individuals. Gene-based rare variant analysis identified an exome-wide significant enrichment of 8 loss-of-function (LoF) mutations in the gene TANK-binding kinase 1 (*TBK1*) in 13 fALS pedigrees. No enrichment of LoF mutations was observed in a targeted mutation screen of 1,010 sporadic ALS and 650 additional control individuals. Linkage analysis in 4 families gave an aggregate LOD score of 4.6. *In vitro* experiments confirmed loss of expression of *TBK1* LoF mutant alleles, or loss of interaction of the c-terminal TBK1 coiled-coil domain (CCD2) with the TBK1 adaptor protein optineurin, an established ALS gene. We conclude that haploinsufficiency of *TBK1* causes ALS and fronto-temporal dementia (FTD).

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive disorder ultimately resulting in the death of motor neurons and fatal paralysis typically three to five years after disease onset. Mutations in 29 genes have been linked to pathogenesis of ALS and several of them also associated with fronto-temporal dementia (FTD). However, mutations in these genes explain less than 1/3 of ALS cases ¹.

In recent years, availability of next-generation sequencing techniques lead to the discovery of several new ALS genes greatly improving our knowledge about basic mechanisms involved in ALS pathogenesis. In the present study, we identified 8 loss-of-function (LoF) mutations in *TBK1* to be associated with familial ALS at a genome-wide significance level. Analysis of extended pedigrees with *TBK1* LoF mutations confirmed *TBK1* as an ALS disease gene. *TBK1* is a multifunctional kinase active in autophagosome-mediated degradation of ubiquitinated cargo and inflammatory signaling ². Both optineurin and p62 are substrates of *TBK1* ³⁻⁵ and mutations in the respective genes have been reported in ALS families ^{6,7}. Thus, *TBK1* links three ALS-associated proteins in a common pathway. Furthermore, our combined genetic and biochemical data indicate that haploinsufficiency of *TBK1* or loss of interaction of the C-terminal *TBK1* coiled-coil domain with its adaptor proteins can cause ALS/FTD.

Results

Genome-wide significant association of loss-of-function mutations in *TBK1* with ALS/FTD

To evaluate the contribution of low-frequency variants in protein-coding genes to ALS, we performed whole exome sequencing in a discovery set of index cases from 252 familial ALS (fALS) pedigrees. They were selected from families with two or more affected individuals per family from European countries (**Supplementary Table 1**) following a negative screen for *SOD1* and *C9orf72* mutations. Control exomes (n=827) from Germany were used to compare the variant burden. We used a gene-based test (CMC test, ⁸) to analyze missense variants at two allele frequency thresholds (minor allele frequency (MAF) between 1% and 5%, and MAF less than 1%). In addition, LoF variants including frameshift variants were investigated separately (MAF less than 5%). To account for multiple testing we used Bonferroni correction and set the significance-threshold at $8.3e-7$ (corresponding to 60,000 tests). While analysis of missense variants resulted in no hits, the test for LoF variants revealed a single outlier, *TBK1*, with a *p*-value of 1.67×10^{-7} (**Supplementary Fig. 1**). The fALS patients carried 7 different dominant *TBK1* LoF mutations in 9 patients, whereas LoF variants were absent in controls (**Fig. 1a, Table 1 and Table 2**).

We assessed the frequency of *TBK1* LoF variants in additional controls representing a variety of disease states from diverse populations. Whereas we did not find any *TBK1* LoF variant in 3,101 in-house exomes, the ExAC dataset ⁹ contained 8 LoF variants (**Table 1, Supplementary Table 2**). We expected 0 and 1 LoF variant in the two control exome datasets respectively making three conservative assumptions about the lifetime risk for ALS (1 in 450), the proportion of familial ALS (10%) and the estimated contribution of *TBK1* mutants to fALS (4%) ^{10,11}. The most likely explanation for the over-representation of LoF variants within the ExAC dataset are disease-specific samples including those from patients with a diagnosis of schizophrenia, which has been clinically linked to ALS ¹².

The search in the ExAC dataset confirmed the high conservation of *TBK1* which carried only a single missense variant with a MAF larger than 1% in approximately 65,000 control individuals. Also inter-species conservation is high for *TBK1* ¹³.

A targeted mutation screen was used to evaluate *TBK1* variants in sporadic ALS (sALS) individuals using high-resolution melting curve analysis (HRMA). Screening of 500 Swedish and 510 German sALS patients as well as 650 Swedish control subjects revealed a splice site variant p.Ala417X in three Swedish patients and one Swedish control suggesting that *TBK1* LoF variants are highly enriched for familial compared with sporadic ALS cases (**Table 2, Supplementary Table 2**). The p.Ala417X variant had been identified in the discovery set in one Swedish fALS pedigree with reduced disease

penetrance. Also, genealogical investigations revealed that the p.Ala417X-carrying control subject had two distant relatives who had both died with a diagnosis of ALS.

***TBK1* LoF variants co-segregate with ALS/FTD**

Next, we reanalyzed and extended families from index patients with *TBK1* LoF mutations to evaluate co-segregation with disease phenotypes. This included an additional ALS family from France (fam13) with a further *TBK1* LoF mutation, which had not been part of the initial burden test (**Table 2**). Thus, adding this French family to the results from the exome sequencing of the discovery cohort and the subsequent screening of sALS patients using HRMA, we identified a total of 8 different LoF mutations in 13 families (**Fig. 1a**). From these 7 families (fam02, fam03, fam05, fam06, fam11-fam13) lent themselves to evaluate penetrance of the disease (**Supplementary Fig. 2** and **Fig. 2**). Thirty-three affected individuals in these 7 families had a mean age of onset of 60 years and 50% (14 out of 28) showed cognitive impairment often progressing to fulminant FTD. Bulbar symptoms were reported in 87% (20 out of 23) during the disease course, with bulbar onset observed in 15% (4/27) (**Supplementary Table 3**). The oldest age of onset observed was 77 years in fam12 with a standard deviation of all cases of 12 years. Seven currently unaffected mutation carriers are aged between 60 and 74 years. This clinical evaluation suggested a high proportion of patients with cognitive impairment for the mutation carriers and a high penetrance for disease in these 7 pedigrees. In an ALS family from Germany (fam05) (**Supplementary Fig. 2**) mutation analysis revealed an independent segregation of both LoF *TBK1* and a pathogenic *FUS* missense variant. Detailed clinical data were only available from three affected members of this family but we note that this is the pedigree with the lowest onset age of the studied 13 *TBK1* pedigrees.

The four most extended families (fam02, fam11, fam12, fam13) were included in a linkage analysis using the genotype at the mutation site as marker. Based on two patients with an identical 24 amino acid (aa) in-frame deletion (p.690-713del) in the C-terminal domain of *TBK1*, we succeeded in linking two previously unrelated families now living in Denmark (fam11) and Sweden (fam12) to a single ancestral couple 5 generations back in Sweden (**Fig. 2**). Autopsy brain samples were available from a member of this family with rapidly progressing ALS-FTD. Staining revealed massive Type B TDP-43 positive perinuclear inclusion in neurons in lamina III of the temporal lobe and p62 positive perinuclear inclusions in lamina III in the right para-hippocampal gyrus, but not in other parts of the brain (**Supplementary Fig. 3**). The spinal cord was not available for examination. Linkage analysis in this extended pedigree resulted in a LOD score of 2.6. Analysis of families 2 and 13 led to a LOD score of 1.4 and 0.6, respectively for an aggregate LOD score of 4.6 thus exceeded the genome-wide significance threshold.

Haploinsufficiency as the most likely mechanism of *TBK1*-linked ALS/FTD

The observed enrichment of LoF variants suggested haploinsufficiency as the most likely pathogenic mechanism for *TBK1*-associated neurodegeneration. We therefore investigated 2 truncating and 3 splice site mutations by quantitative real-time PCR (qRT-PCR) in patient-derived cell lines. The 2 truncating (c.555T>A, c.1343_1346delAATT) and two of the splice site mutations (c.358+2T>C, c.1340+1G>A) leading to a premature STOP codon showed the predicted reduction of *TBK1* expression to approximately 50% at the mRNA level which was also reflected at the protein level (**Supplementary Fig. 4; Supplementary Table 4**). A protein product was also virtually absent when expressing 3 frameshift mutations (c.958delA, c.1434_1435delTG, c.1343_1346delAATT) in HEK293T cells (**Fig. 3a**). The third splice site mutation, c.2138+2T>C, resulted into the expression of a shortened RT-PCR product carrying an in-frame 72 bp deletion (r.2067_2138del, p.690_713del) in exon 20 (**Supplementary Fig. 4a,c**). The expression of the corresponding protein was confirmed by Western blotting (**Supplementary Fig. 4e**).

Loss of *TBK1*/*OPTN* binding may be sufficient to cause ALS/FTD

The latter mutation (p.690-713del) results in a 24 aa deletion in the C-terminal coiled-coil domain (CCD2) of *TBK1* (**Fig. 1a**). CCD2 mediates interactions with multiple adaptor protein complexes regulating the cellular distribution of *TBK1* and activation of downstream signaling pathways^{14,15}. Among these adaptor proteins is optineurin that controls selective autophagy of pathogens³, damaged mitochondria¹⁶ and cytosolic protein aggregates¹⁷. *OPTN* has been found mutated in rare cases of fALS with both dominant and recessive inheritance^{6,18}). Here we show that the mutations in the CCD2 domain (p.690-713del and p.Glu696Lys) are unable to bind *OPTN* in immunoprecipitation (**Fig. 3a**) and GST-pull down assays (**Fig. 3b, Supplementary Fig. 5a,b, Supplementary Table 5**). Nevertheless, both mutants are still able to bind the transcription factor and *TBK1* substrate IRF3, which binds to the more N-terminal ubiquitin-like domain of *TBK1* (**Fig. 3b** and **Supplementary Fig. 5c**). The kinase activity of overexpressed *TBK1* p.690-713del and p.Glu696Lys was preserved as assessed by the phosphorylation of IRF3 (pSer396) (**Fig. 3c**) and by measuring the increase in activation of Interferon-beta promoter activities using luciferase assays (**Fig. 3d**), further pointing to the relevance of mutations in the *TBK1* CCD2 domain for disease pathogenesis.

Functional testing of *TBK1* missense variants

By exome sequencing of fALS patients in the discovery set and sALS patients in the subsequent targeted *TBK1* screening, we identified 9 missense variants with a minor allele frequency <1:10,000 of unknown significance (**Fig. 1b**). Five of these variants were selected and functionally investigated due to their non-conservative amino acid exchanges, high evolutionary conservation, their predicted

disturbance of the protein structure and location in exemplary functional domains of TBK1. The latter include the kinase domain (p.Arg47His), the linker between the kinase domain and the ubiquitin-like domain necessary for substrate binding (p.Arg308Gln), the ubiquitin-like domain required for IRF3 binding (p.Arg357Gln), the dimer interface required for TBK1 trans-activation (p.Met559Arg), and the CCD2 domain (p.Glu696Lys) (**Supplementary Fig. 6**). Four of these 5 variants revealed the predicted functional impairment (**Fig. 3; Supplementary Fig. 5, Supplementary Table 5 for overview**).

Discussion

We provide parallel evidence from association testing and linkage analysis that LoF *TBK1* variants cause a dominant form of ALS/FTD. The enrichment of LoF mutations in the ALS cohort strongly argues in favor of *TBK1* haploinsufficiency as the molecular genetic mechanism. No significant enrichment of missense variants was observed in ALS/FTD cases, which, however, does not exclude the existence of pathogenic missense mutations. Four out of 5 selected *TBK1* missense mutants that were tested *in vitro* indeed revealed the predicted functional deficits. Such missense mutations could be the basis for further characterization of the multifunctional kinase *TBK1*. While this manuscript was under revision, another study based on all-exome sequencing of mostly sporadic ALS patients showed a significant enrichment of *TBK1* missense variants in ALS patients¹⁹.

Our genetic and functional data also reveal a critical role of the C-terminal adaptor protein-binding coiled-coil domain 2 (CCD2)^{14,15} of *TBK1* in the pathogenesis of ALS/FTD. While most of the LoF mutations resulted in a loss of expression of the mutant *TBK1* allele, the p.690-713del mutant, which results in a 24 aa deletion in the CCD2 of *TBK1*, was expressed at the protein level in patient-derived cell lines. Moreover, the rare missense variant p.Glu696Lys is located in the highly conserved CCD2 and was found in two independent ALS patients with a similar disease phenotype. Both CCD2 mutations abrogated optineurin binding but not phosphorylation and activation of IRF3, which binds to the more N-terminal ubiquitin-like domain of *TBK1*²⁰. Dysregulation of optineurin causes ALS and normal pressure glaucoma^{6,14,18}. Overall, our findings point to the relevance of mutations in the *TBK1* CCD2 domain for disease pathogenesis.

Clinical evaluation of *TBK1* LoF mutation carriers suggests an incomplete but high penetrance of the disease, at least in the pedigrees available for examination. Moreover, the patients with *TBK1* LoF mutations displayed a high proportion of cognitive impairment or overt FTD (50%), similar figures to what we observe in ALS-FTD families with *C9orf72* mutation²¹. In this context the striking similarity between the neuropathology of the here presented autopsied *TBK1*-ALS-FTD patient and the pathology of *C9orf72* patients should be noted. The observation of a *TBK1* and a *FUS* mutation co-segregating in the same family supports the notion of an oligogenic basis of ALS in some cases, which has previously been reported for other ALS mutations²².

In summary, we demonstrate that *TBK1* haploinsufficiency is associated with ALS and FTD. As the frequency of dominant *TBK1* LoF mutations was 4% in our exome-sequenced cohort of 252 genetically unexplained fALS index patients, *TBK1* mutations may be one of the more common genetic causes of ALS among Caucasians. The present study greatly expands the phenotype of *TBK1* LoF mutation carriers to including also FTD patients, and demonstrates co-segregation of *TBK1* mutations in several ALS pedigrees. It furthermore shows that the mutations co-segregating with disease result in impairment of the optineurin-binding CCD2 domain of *TBK1*. Earlier studies reported

mutations in optineurin ⁶ and p62 ⁷, which are both autophagy regulating adaptor proteins and substrates of TBK1 ^{3,4}, as possible causes of ALS. Our results provide a common link between these two previously known ALS/FTD associated genes and stress the importance of TBK1 and possibly autophagy in maintaining lifelong neuronal homeostasis.

Table 1. *TBK1* variants with the respective allele frequencies in case and control datasets.

	variants	fALS cases (exomes) European	controls (exomes) German	sALS cases (HRMA ^a) Swedish/German	controls (HRMA ^a) Swedish	in-house exomes International	ExAC exomes International
n	202	252	827	1,010	650	3,101	60,706
LoF	13	9 (1.8%)	0	3 (0.1%)	1 (0.1%)	0	8 (0.0%)
Inframe indel	4	0	0	2 (0.1%)	0	0	47 (0.0%)
missense MAF<=5% and >1%	1	16 (3.2%)	38 (2.3%)	66 (3.3 %)	34 (5.2 %)	97 (1.6%)	1,561 (1.3%)
missense MAF<=1.0%	184	5 (0.8%)	6 (0.4%)	13 (0.6%)	4 (0.6%)	25 (0.4%)	780 (0.6%)

^aHigh resolution melting analysis.

Table 2. *TBK1* loss-of-function and rare missense variants (MAF < 0.0001) detected in ALS patients. Included are all LoF mutations found by whole exome sequencing of the discovery cohort as well as in the French family which had not been part of the initial burden test.

Type of variant	Variant	Predicted consequence at protein level	Index patient (if pedigree is displayed in Fig. 2 or Supp. Fig. 2)	Family	fALS/sALS	Country of origin	Detection method: exome seq or HRMA
loss-of-function mutation	c.958_958delA	p.Thr320GlnfsX40	-	family 1	fALS	Sweden	ES
	c.1343_1346delAATT	p.Ile450LysfsX15	III/1 (Supp. Fig 2)	family 2	fALS	Denmark	ES
	c.1343_1346delAATT	p.Ile450LysfsX15	III/2 (Supp. Fig 2)	family 3	fALS	Sweden	ES
	c.1434_1435delTG	p.Val479GlufsX4	-	family 4	fALS	Sweden	ES
	c.555T>A	p.Tyr185X	III/5 (Supp. Fig 2)	family 5	fALS	Germany	ES
	c.358+2T>C	p.Thr77TrpfsX4	III/2 (Supp. Fig 2)	family 6	fALS	Germany	ES
	c.1340+1G>A	p.Ala417X	-	family 7	fALS	Sweden	ES
	c.1340+1G>A	p.Ala417X	-	family 8	sALS	Sweden	HRMA
	c.1340+1G>A	p.Ala417X	-	family 9	sALS	Sweden	HRMA
	c.1340+1G>A	p.Ala417X	-	family 10	sALS	Sweden	HRMA
	c.2138+2T>C	p.690-713del	III/2 (Fig. 2)	family 11	fALS	Denmark	ES
	c.2138+2T>C	p.690-713del	IV/2 (Fig. 2)	family 12	fALS	Sweden	ES
	c.1318C>T	p.Arg440X	III/2 (Supp. Fig 2)	family 13	fALS	French	ES (not part of the initial whole exome sequencing screen)
in-frame deletion	c.1928_1930delAAG	p.Glu643del	-	family 13	sALS	Germany	HRMA
	c.1928_1930delAAG	p.Glu643del	-	family 14	sALS	Germany	HRMA
missense	c.140G>A	p.Arg47His	-	family 15	fALS	Germany	ES
	c.314A>G	p.Tyr105Cys	-	family 16	sALS	Germany	HRMA
	c.914T>C	p.Ile305Thr	-	family 17	sALS	Sweden	HRMA
	c.923G>A	p.Arg308Gln	-	family 18	sALS	Sweden	HRMA
	c.1070G>A	p.Arg357Gln	-	family 19	fALS	Sweden	ES
	c.1676T>G	p.Met559Arg	-	family 20	fALS	Portugal	ES
	c.1712C>T	p.Ala571Val	-	family 21	sALS	Sweden	HRMA
	c.1792A>G	p.Met598Val	-	family 22	sALS	Sweden	HRMA
	c.2086G>A	p.Glu696Lys	-	family 23	sALS	Sweden	HRMA
	c.2086G>A	p.Glu696Lys	-	family 24	fALS	Sweden	ES

Abbreviations: Exome sequencing (ES); High resolution melting curve analysis (HRMA); familial ALS (fALS); sporadic ALS (sALS)

Main figure Legends

Figure 1. Summary of *TBK1* mutations found in ALS patients. (a) Predicted consequences of all *TBK1* loss-of-function (LoF) mutations found in ALS patients on *TBK1* protein. Included are all LoF mutations found by whole exome sequencing of the discovery cohort as well as in the French family which had not been part of the initial burden test (p.Arg440x). See (b) for the color-coding of domains. Nonsense amino acids at the C-termini caused by frameshifts are indicated in white. In patient-derived cell lines, only expression of the p.690-713del variant could be shown at the protein level. The majority of other LoF variants was neither expressed on mRNA nor on protein level (see **Supplementary Fig. 4** and **Supplementary Table 4** for details). (b) Schematic drawing of the *TBK1* protein showing the N-terminal Serine/Threonine (S/T) protein kinase domain, the Ubiquitin (Ub)-like domain and the two C-terminal coiled-coil domains. The positions of *TBK1* missense variants with a MAF < 1:10,000 as well as the in-frame deletion (c.1928_1930delAAG; p.Glu643del) identified in ALS patients in this study are indicated. Mutations predicted to impair *TBK1* functions (see main text and **Supplementary figure 5**) are highlighted in red.

Figure 2. Pedigrees of the Danish/Swedish ALS families 11 and 12 heterozygous for the p.690-713del *TBK1* mutation. The families could be linked to a common founder 5 generations earlier. Numbering of families refers to the numbering in **Table 2**. Haplotype analysis was in agreement with a common founder for families 11 and 12 in Sweden. Information below patient symbols is given in the format patient ID, age of onset/disease duration or current age (for unaffected mutation carriers) (in years), genotype, phenotype.

Figure 3. Adaptor protein binding and kinase activity of ALS-associated mutated *TBK1*. (a) Interaction of *TBK1* with its adapter protein and autophagy receptor optineurin (OPTN). Co-immunoprecipitation (Co-IP) of EGFP-OPTN and Flag-*TBK1*-WT or the indicated mutants from HEK293T cells using anti-Flag agarose. (Co-)Immunoprecipitated proteins were analyzed by immunoblotting (IB) with the indicated antibodies. Arrowheads point to Flag-*TBK1*-WT or truncated mutant proteins. Asterisk points to heavy IgG bands. (b) Mutations in the C-terminal coiled-coil domain impair binding to OPTN but not IRF3. GST pull-down assay: Lysates from HEK293T cells expressing Flag-*TBK1* WT, p.Glu696Lys or p.690-713del were incubated with recombinant, immobilized GST-OPTN and GST-IRF3. Co-precipitated Flag-*TBK1* proteins were analyzed by IB with the indicated antibodies. Equal GST-protein levels were confirmed by Ponceau S staining. GST-IRF3 was used as a positive control. Asterisk points to an unspecific band. (c) Kinase domain activity of different *TBK1* mutants assessed by IRF3 phosphorylation. Flag-*TBK1*-WT or the indicated mutants were transiently overexpressed in HEK293T cells for 48h (top panel). Wild type and mutant *TBK1*-kinase activity was assessed by phosphorylation

of the substrate IRF3, which was detected with a phospho-specific pSer396 antibody (c) and by luciferase reporter activity (d). Arrowheads point to Flag-TBK1-WT or truncated mutant proteins. (d) Luciferase reporter assay for TBK1-induced promoter activities of IFN-inducible genes. HEK293T cells expressing Flag-TBK1-WT or the indicated mutants were harvested for Western blot analysis (c) and luciferase assay (d). The internal control was measured by β GAL activity. Error bars represent mean \pm S.D. of n=3 independent experiments. *** $p < 0.001$; ns: not significant. Unpaired t -test. Note: Western blot signals for IRF3 phosphorylation (c) correspond to the luciferase activities measured in (d). WT (wild type); KD (kinase-dead TBK1 mutation; p. Lys38Ala). Western blots were repeated at least three times with no limits in reproducibility.

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Author contributions

TMS, PMA, ACL, JHW, TM, ID, TW, AF, BR, WR, VS, TB, PK, PL, AEV, DRT designed experiments, analysed and interpreted data. KM, NM, EG, UN, MSF, KMD, DRT, TB, AEV, PL performed and analysed experiments. FN, AH, PW, SP, RP, SM, NM, EB, CD, MHS, JD, TM, ASW, JW, MdC, MO, ACL, PMA and JHW clinically characterized patients. JHW, PMA, TMS, TM, ID, BR, and ACL wrote the manuscript. SP and TB Provided patient-derived cell lines.

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Material and Methods

Patients and Ethics statement. All ALS patients were diagnosed according to the EFNS Consensus criteria²³. With informed written consent and approved by the national medical ethical review boards in accordance with the Declaration of Helsinki (WMA, 1964), EDTA blood samples were drawn from control individuals and ALS patients as well as their healthy relatives. Anonymous genotyping of healthy risk carriers (relatives of fALS patients) was performed after informed written consent and approved by the local medical ethical review boards. Patients were not informed about genetic results that were obtained for scientific purposes only, according to the written informed consent. DNA was extracted from EDTA blood samples according to standard procedures²⁴.

Genotyping for *SOD1* and *C9orf72* mutations. Mutations in *SOD1* and *C9orf72* were excluded before exome sequencing of familial ALS cases or high resolution melting curve analysis of Swedish ALS patients was performed. *SOD1* mutations were excluded by Sanger sequencing of all exons²⁵. Genotyping for extended *C9orf72* hexanucleotide repeat expansions was performed by repeat-primer PCR and fragment length analysis and all repeat-positive cases were excluded from the study. Southern blot was performed in the *TBK1*-mutant cases to absolutely exclude the presence of a GGGGCC-repeat expansion in *C9orf72* as described²⁶.

Whole exome sequencing. We sequenced exomes of 252 cases and 827 German controls. Controls were comprised of healthy parents of children with various diseases, healthy control tissues of individuals with tumor diseases and 200 individuals of the KORA study. Exomes were enriched in solution and indexed with SureSelect XT Human All Exon 50Mb kits, version 3, 4 and 5 (Agilent Technologies) (**Supplementary Table 6**). Sequencing was performed as 75 or 100 bp paired-end reads on HiSeq2000/2500 systems (Illumina). For exomes, we generated on average 10.1 (SD ± 1.94) gigabases of sequence resulting in an average depth of coverage of 123 (SD ± 24.0) with 94.4% (SD ± 3.4) of the target regions covered at least 20 times. Minimal requirements for inclusion were 7 gigabases of mapped sequence and 90% of target regions covered at least 20 times. Image analysis and base calling was performed using Illumina Real Time Analysis.

Read mapping and variant calling. Reads were aligned against the human assembly hg19 (GRCh37) using Burrows-Wheeler Aligner. Multi-sample calling and filtering of all 252 cases and 827 controls was performed with GATK (version 3.3) HaplotypeCaller and VariantRecalibrator as described in the GATK best practices guide (<https://www.broadinstitute.org/gatk/guide/best-practices>). Variants were called in the merged target regions of the three used enrichment kit versions, extended by a 50 bp margin. Subsequently, variants overlapping low-complexity regions were filtered²⁷. Initially, we called on average 25,249 variants per exome. The VariantRecalibrator removed on average 3295 variants, the low complexity filter 214 variants leaving 21,740 variants per exome.

Rare variant association analysis. The CMC burden test was used as implemented in the EPACTS software, version 3-2-6 (<http://genome.sph.umich.edu/wiki/EPACTS>). Genotype calls at sites with a read depth below 10 or a genotype quality below 30 were set to missing. To adjust for the use of different enrichment kit versions, variants with a call rate below 90% were removed. We performed principal component analysis as implemented in the PLINK software package (version 1.07)²⁸. After PCA, we removed 7 outliers. The CMC test of missense variants with MAF between 1% and 5% was

performed on 6,080 genes, the test with MAF less than 1% on 17,919 genes, and the test of LoF variants with MAF less than 5% on 6,904 genes.

Screening for *TBK1* variants by high resolution melting curve analysis. We used Idaho LightScanner® high-resolution melting curve analysis (Biofire Inc.) to screen the coding regions and exon/intron boundaries of *TBK1* in 1,660 individuals (510 German sALS, 500 Swedish sALS, 650 Swedish controls). DNA was analyzed in doublets to prevent false positive signals. Samples with aberrant melting patterns were Sanger sequenced to identify the underlying variant. Oligonucleotide sequences for 20 amplicons are available on request.

Linkage analysis. We assumed an autosomal dominant model for the analysis. Penetrance was set at 0.7. The frequency of the deleterious allele was set at 0.0001, the phenocopy rate at 0.003, and the marker allele frequency to 0.001. Linkage analysis was performed using Merlin (version 1.1.2) (29). We set the phenotype to unknown if an unaffected individual was either younger than 60 years or died before the age of 60 years.

Haplotype analysis. Haplotype blocks were generated using the default algorithm in the program Haploview³⁰. Block definitions are based on Illumina HumanOmni2.5 array data from 500 control samples. The haplotype block spanning the *TBK1* gene comprises 36 SNP markers (from rs115725023 to rs7970355) covering a genomic region of 112.7 kb. All variant carriers were genotyped with the same Illumina HumanOmni2.5 array and the haplotypes were assigned to the specific haplotypes from the *TBK1* block definition.

Molecular modeling. The possible effects of the missense variants on *TBK1* structure and function were assessed based on the *TBK1* crystal structure³¹. Structural analysis was carried out using COOT³², and figures were prepared in PyMOL (www.pymol.org). Based on the analysis, conservative amino acid replacements on the protein surface were not studied further.

Neuropathological evaluation and immunohistochemistry. Formalin fixed and paraffin embedded sections from the following areas were studied: Left frontal lobe; Right occipital lobe; Left cingulate gyrus; Right temporal lobe; Left corpus striatum; Right Thalamus; Right uncus with hippocampus; Mesencephalon; Pons; Medulla oblongata; Cervical spinal cord; and Cerebellum. All were investigated by routine staining (hematoxylin/eosin, luxol fast blue/cresyl violet, Van Gieson) and by immunohistochemistry. The immunohistochemistry were done using the Ventana® BenchMark ULTRA system. The following primary antibodies were used: anti-TDP-43 (1:100, CCD2 pretreatment, Proteintech™ 10782-2-AP); anti-p62 (1:50, CCD2 pretreatment, BD Biosciences 610832).

Plasmids and constructs. **Supplementary Table 7** summarizes all plasmids used in this study. *TBK1* frameshift mutations and amino acid substitutions were introduced into pEF-BOS Flag-*TBK1* wt using site directed mutagenesis (**Supplementary Table 8**). The expression vector for Flag-*TBK1* Δ690-713 mutant was generated by amplifying bp 1-2066 of wt *TBK1* from pEF-BOS *TBK1* wt using oligonucleotides FW and RV(1) (**Supplementary Table 9**) adding a BamHI restriction site to the 5'-end. In two subsequent PCRs with oligonucleotides FW and RV(2) or RV(3), respectively, bp 2139-2190 of wt *TBK1* as well as a Sall restriction site were added to the 3'-end of the first PCR product. The resulting *TBK1* fragment missing bp 2067-2138 was cloned into BamHI and Sall restriction sites of pEF-BOS Flag. The correctness of all DNA sequences was verified by sequencing.

Protein expression and purification. GST-fusion proteins were expressed in *Escherichia coli* BL21 (DE3) cells in LB medium. Expression was induced by addition of 0.2 mM IPTG and cells were incubated at 16°C overnight. Harvested cells were lysed using sonication in a lysis buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA, 5 mM EGTA, 150 mM NaCl) and the supernatant was subsequently applied to glutathione sepharose 4B beads (GE Healthcare). After several washes, fusion protein-bound beads were used directly in GST pulldown assays or GST-proteins were eluted from beads with elution buffer (10 mM glutathione, 50 mM Tris-HCl pH 8.0).

Immunoprecipitation and GST pulldown. HEK293T cells were transfected with expression constructs encoding the protein of interest using GeneJuice transfection reagent (Merck Millipore) according to the manufacturer's instructions. 36-48 h post-transfection cells were lysed in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM NaF) supplemented with Phosphatase Inhibitor Cocktail 2 (Sigma) and protease inhibitors and supernatants (10 min, 10.000 x g) were incubated at 4°C for 6 h with GST-fusion protein-bound beads (GST pull down) or for 1 h with Flag- or GFP-beads (Immunoprecipitation). For immunoprecipitation GFP-Trap beads were purchased from ChromoTek and M2 anti-Flag affinity gel from Sigma. After 4 washes with lysis buffer, the beads and precipitated proteins were eluted with 2x SDS-PAGE loading buffer, boiled and loaded onto SDS-PAGE gels for analysis using chemiluminescence based detection.

Luciferase reporter assay. 5×10^5 HEK293T cells were transfected with (each 0.25 µg) pEF-BOS Flag-TBK1 constructs and pGL3-IFN-beta as luciferase reporter and pUT651 as β-galactosidase reporter using GeneJuice transfection reagent (Merck Millipore). At 48 h post-transfection the cells were washed with 1XPBS and lysates were subjected to luciferase assays following the manufacturer's protocol (Roche). Internal control was measured by β-galactosidase activity (Roche). Three independent experiments were performed using triplicate samples in each experiment.

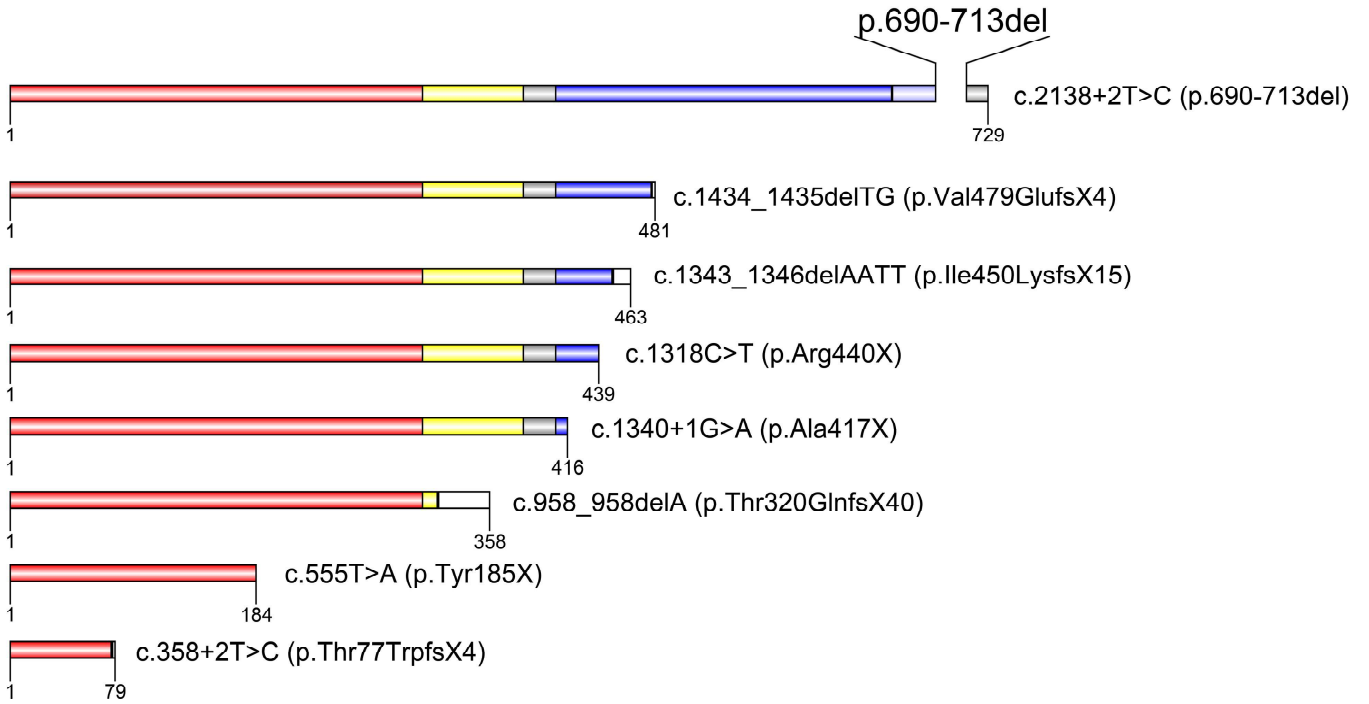
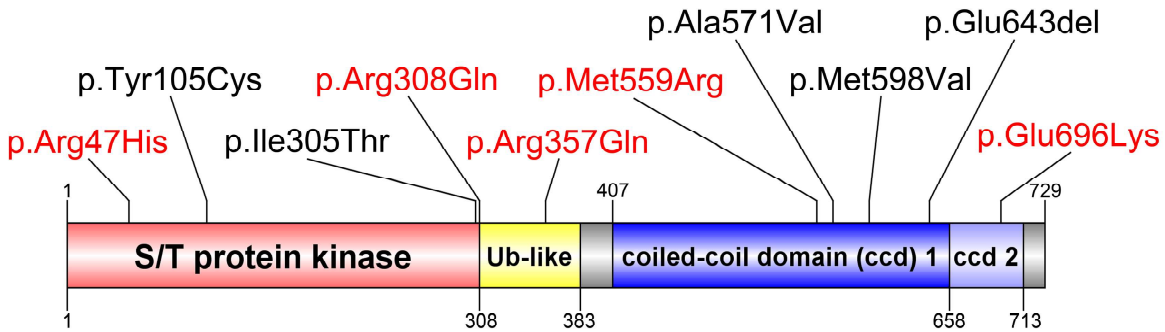
Analysis of patient-derived cell lines. Epstein-Barr virus transformed lymphoblastoid cell lines (LCLs), fibroblasts and keratinocytes were obtained from healthy controls and 5 *TBK1* mutant ALS patients. Total RNA from cell lines was isolated with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and reverse transcription reactions were carried out using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) or the iScript cDNA Synthesis Kit (Bio-rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Resulting cDNA was used for subsequent PCR reactions. Quantitative real-time PCR (qRT-PCR) measurement of *TBK1* mRNA was performed with a CFX96 Real-Time System (Bio-Rad) using the EXPRESS SYBR GreenER qPCR Supermix (life technologies) and oligonucleotides 5'-CGAGATGTGGTGGGTGGAATG-3' and 5'-CACAGACTGTCCATCTTCCCC-3'. *TBK1* mRNA levels were normalized relative to TBP (TATA Box Binding Protein) mRNA using oligonucleotides 5'-CCCATGACTCCCATGACC-3' and 5'-TTTACAACCAAGATTCCTGTGG-3' for amplification and $2^{-\Delta\Delta Ct}$ -method for normalization³³.

In two instances, regions of interest of the *TBK1* mRNA were amplified by PCR and visualized on a 1% Agarose-Gel by staining with Ethidium –bromide. Oligonucleotides 5'-CCTTCGTCCAGTGGATGTTCA-3' and 5'-CACAGACTGTCCATCTTCCCC-3' spanning Exons 3 and 5 (322 bp fragment) were used to amplify a region potentially altered by the *TBK1* c.358+2T>C mutation (skipping of Exon 4). Oligonucleotides 5'-GTCAGAAGAATGGATAAGAAAG-3' and 5'-CCATCCATGGTTAAAGAGCC-3' spanning Exons 17/18 and 21 (318 bp fragment) were used to amplify a region potentially altered by the *TBK1* c. 2138+2T>C mutation (skipping of Exon 20).

To determine the abundance of TBK1 protein, lysates of LCLs, fibroblasts and keratinocytes were subjected to Western blotting.

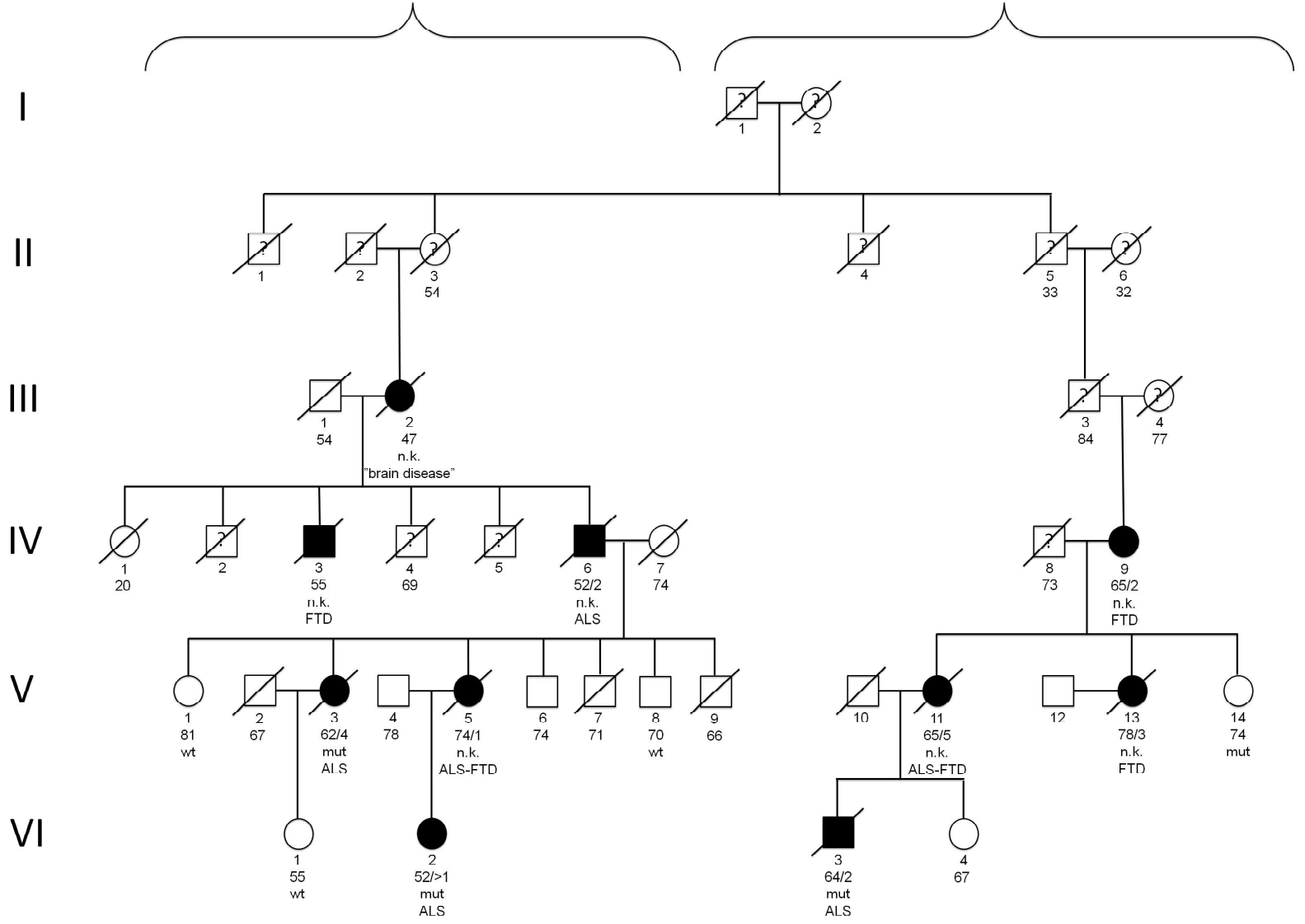
Antibodies used in this study. Antibodies used in this study. Anti-OPTN (Abcam, ab23666), anti-myc (Santa Cruz, #sc-40), anti-GFP (Living Colors; Clontech; # 632592), anti-IRF3 (Cell signaling, #4962), anti-pIRF3 (Ser396) (Cell signaling, 4D4G, #4947), anti-FLAG M2 (Sigma, F3165), anti-Vinculin (Sigma, V9131), anti-TBK1 (Pierce, PA5-17478), anti- β -actin (Cell signaling, #4970), anti-TDP-43 Proteintech™, 10782-2-AP), anti-p62 (BD Biosciences, 610832). The pS177 OPTN antibody was generated by immunoGlobe (as described in ³). Secondary HRP conjugated antibodies, goat anti-mouse and goat anti-rabbit IgGs were used for immunoblotting.

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a**b**

Family 11 (Denmark)

Family 12 (Sweden)



+ GFP-OPTN WT

