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Brief communication

A recessive S174X mutation in Optineurin causes amyotrophic lateral sclerosis through a loss of function via allele-specific nonsense-mediated decay

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ABSTRACT

Loss of function (LoF) mutations in *Optineurin* can cause recessive amyotrophic lateral sclerosis (ALS) with some heterozygous LoF mutations associated with dominant ALS. The molecular mechanisms underlying the variable inheritance pattern associated with *OPTN* mutations have remained elusive. We identified that affected members of a consanguineous Middle Eastern ALS kindred possessed a novel homozygous p.S174X *OPTN* mutation. Analysis of these primary fibroblast lines from family members identified that the p.S174X mutation reduces OPTN mRNA expression in an allele-dependent fashion by nonsense mediated decay. Western blotting correlated a reduced expression in heterozygote carriers but a complete absence of OPTN protein in the homozygous carrier. This data suggests that the p.S174X truncation mutation causes recessive ALS through LoF. However, functional analysis detected a significant increase in mitophagy markers TOM20 and COXIV, and higher rates of mitochondrial respiration and ATP levels in heterozygous carriers only. This suggests that heterozygous LoF *OPTN* mutations may not be causative in a Mendelian manner but may potentially behave as contributory ALS risk factors.

1. Introduction

Optineurin (*OPTN*) is involved in multiple cellular pathways including: vesicle trafficking, pathogen defense, and autophagy, particularly mitophagy (Markovinovic et al., 2017; Wong and Holzbaur, 2014; Ying and Yue, 2016). Mutations were first discovered in glaucoma (Rezaie et al., 2002) and later in a consanguineous Japanese ALS family suggesting loss of function (LoF) as the putative disease mechanism (Markovinovic et al., 2017; Maruyama et al., 2010). Subsequently, approximately 40 dominant and recessive variants have been found in ALS+/-FTD (frontotemporal dementia) (Markovinovic et al., 2017; Wild et al., 2011). Here we describe a novel *OPTN* nonsense mutation causing an

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autosomal recessive form of ALS in a consanguineous Palestinian family.

2. Materials and methods

The study was approved by the local Review Board. Informed consent was obtained by all participants. DNA from the proband (IV-1), one affected brother (IV-3), and 3 unaffected individuals (III-1, IV-5, and IV-6) were exome sequenced using the Nimblegen V3 probeset. After QC of exome data, variants were filtered using v2.1.1 and v3.1 of the gnomAD (http://gnomad.broadinstitute.org), and Greater Middle Eastern exome databases (http://igm.ucsd.edu/gme/) and a KCL in-house control exome database (n = 697) to identify extremely rare protein-altering mutations. While the pedigree was highly suggestive of a recessive mode of inheritance, segregating heterozygous variants were also considered to identify putative dominant acting genetic variants.

Homozygosity mapping was conducted on 2 of the affected individuals (IV-1 and IV-3) using HumanOmniExpress-12v1-1 microarray genotype data and runs of homozygosity across *OPTN* confirmed using PLINK v1.9 (Purcell et al., 2007). Detailed methods for functional experiments, including RNA extraction, RT-PCR, antibodies, fibroblast derivation and culture, qPCR, western blotting and immunocytohistochemistry (ICC) and in vitro assays including clinical and genetics details can be found in Supplementary Information.

3. Results

The proband's parents were first cousins. The father died age 81, with Alzheimer's disease diagnosed aged 78. The mother was 79 and neurologically intact. No other neurological diseases were diagnosed in previous generations. Four children developed ALS: the proband (IV-1), 2 brothers (IV-2 and IV-3) and a sister (IV-7) (Fig. 1A). The proband presented age 54 with lower-limb weakness spreading to upper-limbs within a year with no visual or cognitive symptoms. Examination revealed upper and lower motor-neuron dysfunction in all limbs and the bulbar region. Over the next 3 months she developed breathlessness, dysarthria, and dropped head. Electromyography confirmed diffuse LMN involvement. She declined assisted ventilation and died 6 months following diagnosis. Individuals IV-2 and IV-3 were diagnosed according to clinical records, with IV-2 reportedly becoming blind 6 months before death, however no ophthalmological assessment was performed. Due to the report of dementia in the proband's father, we analyzed exomes of both affected ALS cases (IV-1 and IV-3) for shared heterozygous variants, which could suggest an autosomal dominant genetic contribution (Supplementary Excel File). Both cases were devoid of mutations in MAPT but shared heterozygous variants in 44 other genes. However, none of these variants are known to be associated with dementia or other Mendelian or ALS risk loci. Given the absence of dementia in all the ALS cases in generation IV, coupled with the consanguinity, we concluded that the likely inheritance of ALS in this kindred was autosomal recessive.

DNA from individuals IV-1 and IV-3 were exome sequenced to a high coverage, with an average read depth of 108X and 112X, respectively, across all protein coding bases in the genome. Both cases were negative for candidate mutations in known ALS-linked genes, including *ALS2, ANG, ANXA11, ARPP21, ATXN2, C9orf72, CCNF, CHGB, CHMP2B, CYLD, DCTN1, DNAJC7, EPHA4, FIG4, FUS, HNRNPA1, KIF5A, MAPT, MATR3, NEFH, NEK1, PFN1, PRPH, SETX, SIGMAR1, SOD1, SPAST, SPG11, SPG7, SQSTM1, TARDBP, TBK1, TIA1, TUBA4A, UBQLN2, UNC13A, VAPB*, and *VCP*. After filtering for extremely rare and novel protein-changing variants IV-1 and IV-3 were found to share 4 homozygous variants (Supplementary Excel File and Supplementary Information). A missense homozygous p.E129K variant was identified in *C10ORF167* (NM_001010881:c.385G>A) that was common as a heterozygote in gnomAD controls (alleles = 27) and present once as a homozygote, so therefore was excluded. Novel homozygous mutations were found in *MASP2* (NM_006610:c.234+1G>T), *MYO9A* (NM_006901:c.2561A>C:p.K854T) and *OPTN* (NM_021980:c.521C>A:p.S174X). *MASP2* is a serine protease involved in the lectin pathway and mutations are not associated with neuromuscular disease. *MYO9A* is a member of the myosin superfamily, and recessive MYO9A mutations are associated with either congenital myasthenic syndrome or Bardet-Biedl Syndrome (BBS) Type 4 (pigmentary retinopathy, early-onset obesity, hypogenitalism, and mental retardation) (Mykytyn et al., 2001; O'Connor et al., 2016). Congenital myasthenic syndrome or BBS was not a phenotypic consideration in our kindred given the adult onset after normal childhood development, rapid progression, and prominent upper and lower motor neuron features. Thus, we are confident that the *Optineurin* p.S174X variant was the causative Mendelian mutation for this family.

The p.S174X mutation was confirmed by Sanger sequencing and found to segregate with disease in DNA from other family members. The unaffected mother (III-1) and 2 unaffected siblings (IV-8 and IV-9) were heterozygous for p.S174X whereas IV-1, IV-3, IV-5, and IV-7 were homozygous (Fig. 1B), however IV-5 remains asymptomatic to date. The p.S174X variant was absent from 114,680 gnomAD v2.1.1 non-neurological controls, 75,516 gnomAD v3.1 controls including 158 Middle Eastern individuals (gnomad.broadinstitute.org) and 993 Greater Middle Eastern Exomes (http://igm.ucsd.edu/gme/), but was present twice as a heterozygote in 2192 ethnically matched controls (0.09%). OPTN is considered tolerant to heterozygous LoF mutations in gnomAD (pLI = 0) (https://gnomad.broadinstitute.org/gene/ENSG00000123240). However, no *homozygous* LoF OPTN variants are present in this dataset. Furthermore, runs of Homozygosity (RoH) analysis of Illumina microarray genotyping data confirmed both IV-1 and IV-3 share a 10 Mb block of homozygosity spanning the *OPTN* gene compared to 8 ethnically matched controls (Fig. 1C). The p.S174X mutation truncates the OPTN protein eliminating the UBAN domain and TBK1 phosphorylation site (Ser177) (Wild et al., 2011) (Fig. 1D).

Primary fibroblast lines were obtained from 1 heterozygous healthy parent (III-1) and 3 healthy siblings: 2 heterozygous (IV-8 and IV-9), and 1 homozygous (IV-5) for the p.S174X mutation and genotype status confirmed by Sanger sequencing. *OPTN* expression in fibroblasts was investigated by quantitative PCR. When grouped by genotype, there was a significant decrease in *OPTN* expression in heterozygotes compared to controls (p = 0.0419, Fig. 1E). Furthermore, *OPTN* transcript levels in the mutant homozygous fibroblast line were significantly lower than in any other line (Fig. 1F).

We sought to determine whether mutant *OPTN* RNA is degraded by nonsense-mediated decay (NMD). cDNA derived from the heterozygous fibroblasts contained only a wild-type allele, indicating the mutated allele was not expressed (Fig. 1G, left column). Heterozygous fibroblasts treated with cyclohexamide (CHX) which inhibits NMD, revealed both wild-type and a mutated cDNA allele, suggesting p.S174X transcripts undergo NMD (Fig. 1G, right column and red stars). Interestingly, mRNA from the homozygous mutant was detected with and without CHX, indicating that NMD in homozygotes was incomplete.

Fibroblast lines were processed by western blotting and probed with an antibody targeting OPTN N-terminal fragments (1–14aa). No full-length OPTN was detected in homozygous p.S174X fibroblasts and no truncated fragment was detected in any fibroblast line (Fig. 2A). As shown in 2a, we observed a variable, but lower expression of OPTN in the heterozygous samples, which approached significance (Fig. 2B, p = 0.077).

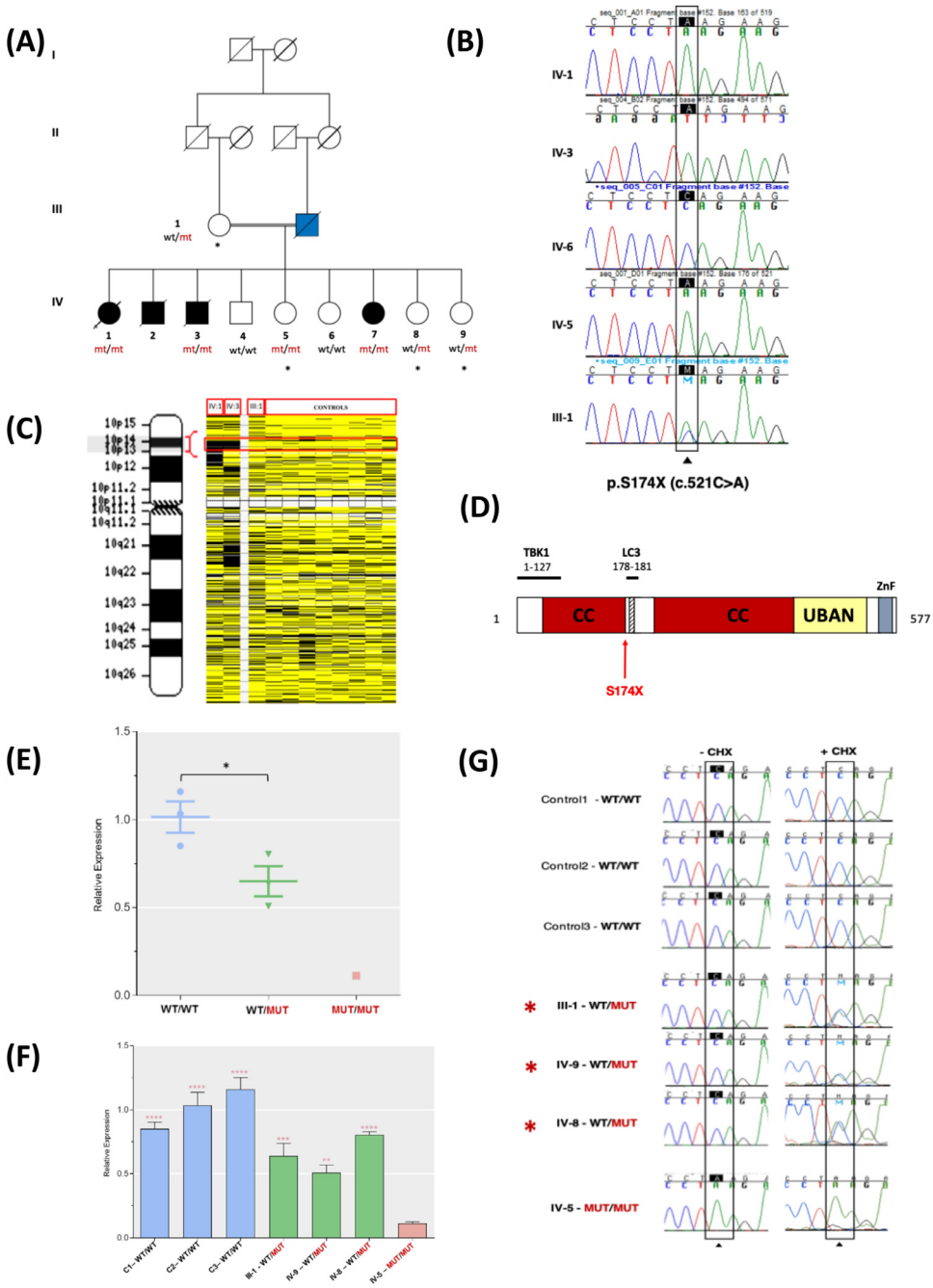

Fig. 1. Consanguineous family harboring the *OPTN* p.S174X variant. (A) Affected cases are indicated by filled black shapes with deceased individuals indicated by diagonal lines. Female and male individuals are marked with round and square shapes, respectively. The proband is IV:1 and homozygous mutant carriers indicated by mt/mt, heterozygous carriers mt/wt, and wild-type by wt/wt. The father of the proband affected by Alzheimer's disease is marked with blue square. (B) Sequencing chromatograms of WT (C allele) and OPTN mutant (C>A) carriers (C) Region of homozygosity spanning the *OPTN* locus in 2 affected members of the S174X family (IV:1 and IV:3) compared to the unaffected mother and 8 Middle Eastern controls devoid of a homozygous block across *OPTN*. The homozygous region shared by IV:1 and IV:3 spans a physical distance of ~3.7MB (chr10:12,097,530–15,838,060) that contains 111 genes within the region. (D) Schematic of OPTN indicating the position of the p.S174X mutation relative to the 5 OPTN domains, OPTN binding partners, and their binding regions. p.S174X truncates the LC3 binding domain and C-terminal ubiquitin binding domain. (E) qPCR of mRNA extracted from fibroblast lines derived from 3 WT controls, 3 heterozygous carriers of the p.S174X mutation and a single homozygous individual. OPTN expression grouped by genotype and analyzed by unpaired t-test showed a significant reduction in expression in heterozygous carriers (WT/WT vs. WT/MUT 2 tailed $p = 0.0419$). The single homozygous carrier displayed ~10% OPTN expression (F) A break-down of E, outlining individual expression from each fibroblast line, that is, WT (blue), heterozygotes (green), and the homozygous carrier (pink). The pink stars indicate the p value derived from the comparison of IV-5 with every other individual (one way ANOVA followed by Dunnett's correction **** is ≤0.0001, *** is ≤0.001, ** ≤0.01, * ≤0.05, error bars represent SEM, $n = 3$). Error bars represent the standard error of the mean (SEM). (G) Sanger sequencing chromatogram traces (left, -CHX) of cDNA from fibroblast controls and OPTN mutant individuals across the mutation site illustrate a loss of the mutant allele (A) in heterozygous carriers. However, addition of cycloheximide (a blocker of translation) reveals the mutant allele in heterozygous cases (right, +CHX) indicating that the mutant allele is degraded by nonsense mediated decay (NMD). Interestingly, the homozygous mutant is not affected. The red stars indicate the individuals expressing the mutant allele only after NMD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

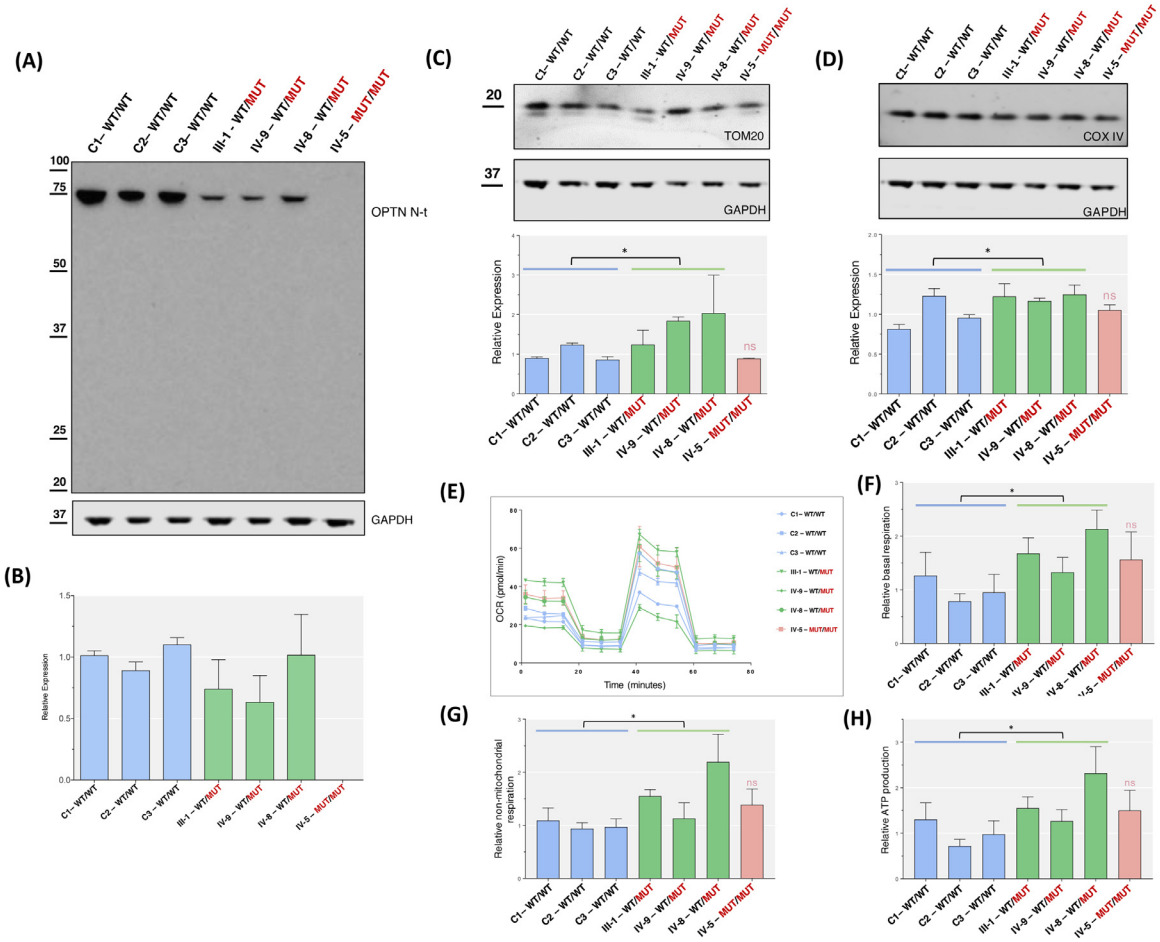

Fig. 2. Reduced OPTN protein expression due to heterozygous and homozygous S174X mutation. (A) Western blot showing the presence OPTN (~75 kDa) in all individuals with the exception of IV-5 (MUT/MUT). p.S174X expected molecular weight ~20 kDa. GAPDH shows an equal amount of protein loaded for each sample. $n = 3$. C = Control. (B) quantification of the amount of OPTN protein present in each sample normalized by GAPDH ($p = 3$) shows reduced expression that approached significance when analyzed by t-test (2 tailed $p = 0.077$). (C) Western blot of TOM20 expression (normalized against GAPDH) showed a significant increase of TOM20 expression in heterozygous carriers ($p = 0.0262$, 2 tailed unpaired t-test). The homozygous sample when compared with each individual control and heterozygous sample by one-way ANOVA followed by Dunnett's correction showed no significant difference in TOM20 expression ($p =$ ns). (D) Similarly, western blotting of fibroblast lysates of OPTN carriers revealed a significant upregulation of COXIV expression levels (2 tailed $p = 0.0339$). As per the approach for TOM20, the homozygous carrier did not show any significant difference in the expression of COXIV ($p =$ ns). Error bars represent SEM. (E) A representative example of the Agilent Seahorse XF Cell Mito Stress Test on control, heterozygous, and homozygous derived fibroblast lines. (F) A significantly higher basal respiration is observed in heterozygous samples only when compared to controls (unpaired t-test, 2 tailed $p = 0.0156$). (G) A significantly higher nonmitochondrial respiration is observed in heterozygous samples only when compared to controls (unpaired t-test, 2 tailed $p = 0.0245$), however the homozygous line trended toward significance ($p = 0.0755$, one-way ANOVA followed by Dunnett's correction). (H) A significantly higher level of ATP production is observed in heterozygous samples only when compared to controls (unpaired t-test, 2 tailed $p = 0.0310$).

As OPTN has a putative role in mitophagy, fibroblast lines were analyzed by western blot and probed for mitochondrial markers: mitochondrial-import receptor subunit TOM20 homolog (TOM20) and cytochrome-c oxidase subunit IV (COXIV). Following quantification, heterozygous lines showed modest increases in both COXIV ($p = 0.034$) and TOM20 ($p = 0.026$) levels when grouped by genotype and compared to controls. No increase was detected in homozygous lines when compared individually to every other sample by one-way ANOVA (Fig. 2 C–D).

Further mitochondria characterization was conducted using the Agilent Seahorse XF Cell Mito-Stress Test. Our analysis revealed heterozygous lines had higher basal respiration levels ($p = 0.0156$), nonmitochondrial respiration ($p = 0.0245$), and ATP production ($p = 0.0310$), when compared to controls (Fig. 2 E-H). However, no morphological differences were observed in mitochondria from these lines (Supplementary Fig. 1 A–E). Additionally, there was no disruption to mitochondria membrane potential when assessed by image cytometry (Supplementary Fig. 1 F–G).

4. Discussion

Here we describe the clinical, genetic, and functional characterization of a novel *OPTN* ALS mutation in a consanguineous recessive ALS family showing a likely recessive inheritance pattern. Similar to the p.Q398X mutation (Maruyama et al., 2010), we demonstrated that p.S174X OPTN mRNA undergoes NMD; as the mutant p.S174X transcript was undetectable in heterozygotes by Sanger sequencing of PCR amplified cDNA and reduced by ~50% using qPCR. Interestingly, cDNA from the homozygous p.S174X line was drastically reduced by qPCR but still retained ~10% of transcript. However, western blotting revealed that a truncated protein was absent in both heterozygous and homozygous p.S174X carriers. As we have demonstrated NMD and complete LoF of p.S174X in fibroblasts, we believe this would translate to a complete loss of OPTN expression in spinal cord and motor cortex, as evidenced by the absence of post-mortem tissue OPTN staining in p.Q398X homozygotes (Ayaki et al., 2018). Indeed, decreased OPTN transcripts, consistent with NMD, have been de-

scribed in compound heterozygous *OPTN* truncation mutations, the furthest located at residue 430 (L430Rfs*16, 3 quarters along the full OPTN protein, i.e., 577 residues) (Pottier et al., 2018). Therefore, it is plausible that most truncation *OPTN* mutations could potentially result in NMD. A recent investigation of motor cortex tissue from an ALS patient carrying a homozygous p.R217X mutation showed an absence of OPTN staining but a severe and widespread predominance of phosphorylated TDP43 inclusions compared to a heterozygous TDP43 M337V case (Nolan et al., 2020). This further demonstrates LoF due to homozygous OPTN truncation mutations, but also a clear mechanistic link to TDP43 pathology.

As expected, heterozygous carriers of *OPTN* mutations in which NMD has been proven (here, in p.S174X, and in p.Q398X (Maruyama et al., 2010), or implied by transcription studies (Pottier et al., 2018), do not develop disease, consistent with a LoF mechanism due to homozygous truncation mutations. By contrast, the role of missense *OPTN* mutations is unclear. It is noteworthy that OPTN function is mediated by a homo-multimeric complex, and that missense *OPTN* mutations are enriched within the ubiquitin binding domain. In particular, the p.E478G and p.D474H mutants form WT:mutant hybrids that result in compromised autophagosome maturation (Shen et al., 2015). This suggests that motif specific missense mutations may behave in a dominant-negative manner.

Data from this family suggests that heterozygous LoF *OPTN* carriers may not develop disease. This is supported by our screening of a large cohort of Arab controls ($n = 2192$) that identified 2 controls (0.09%) that were heterozygous for p.S174X. This implies that 50% levels of OPTN are sufficient for normal function. However, heterozygous *OPTN* mutations may potentially behave as low penetrant risk factors. Recent large burden analysis studies have shown a marginal association of *OPTN* mutations with ALS (Farhan et al., 2019; Kenna et al., 2016). Furthermore, the recently described *OPTN* 691_692insAG LoF ALS mutation is inherited recessively, however, the heterozygous form confers a significantly higher risk of ALS (Goldstein et al., 2016).Additionally, heterozygous *OPTN* mutations have been found in ALS patients who also harbor mutations in other ALS genes, such as *TBK1* and *C9ORF72,* supporting an oligogenic model of disease (Farhan et al., 2018; Pottier et al., 2015).

We speculate that our functional investigation of fibroblasts from *OPTN* p.S174X carriers may support this possibility, as heterozygous p.S174X fibroblasts had a mild, but significant impairment of mitochondrial function and evidence of impaired mitophagy. This manifested as an increased mitochondrial number and a higher rate of ATP-production and nonmitochondrial respiration. The 1 homozygous p.S174X carrier in our studies did not manifest mitochondrial dysfunction but displayed complete LoF by western blot. It is plausible that the lack of observed mitochondrial dysfunction in homozygotes may be a skewed artifact of genotype under-representation. Alternatively, nonsense mediated decay in heterozygotes may indicate that haploinsufficiency of OPTN is mildly disruptive to mitochondria, or that heterozygotes increase risk by a different mechanism. Whereas in contrast, Mendelian acting homozygous LoF OPTN mutations may cause disease by a general loss of autophagic clearance mechanisms.

One limitation of this study is the use of non-neuronal patient derived cell lines. Although they carry the p.S174X variant endogenously, they do not necessarily recapitulate the pathology present in the central nervous system (CNS). The reprogramming of these lines into induced pluripotent stem cells (iPSC) and subsequent differentiation into CNS-relevant cell types would shed light on CNS-specific pathogenic mechanisms.

In conclusion, we have demonstrated that OPTN LoF, mediated by NMD, and inherited recessively, causes ALS. Furthermore, heterozygous mutations may contribute to overall ALS risk, as evidenced by independent genetic studies and by our functional investigation in human S174X fibroblast lines.

Verification

The authors declare that this work has not been published previously in any journal.

However, the p.S174X OPTN mutation only, was previously reported in a review by Markovinovic et.al Progress in Neurobiology. 2017 Jul;154:1–20 that was sourced from a nonpeer reviewed poster of this submitted work that was presented at the International ALS Symposium 2016.

Authors' contributions

B.N.S., M.G., and C.E.S., Conceptualization, Methodology, Project Administration, Funding Acquisition. B.N.S., C.E.S., Supervision. M.d.M., C.W., S.T., B.N.S., C.V., M.K., A.N., and N.A. Investigation, Formal Analysis, Data Curation, Resources, Writing – Review & Editing. R.M.C., S.E.L., R.E., Y.L., and M.G., Resources. S.J.N., G.B., Investigation. S.T. and H.M.S., Data Curation.

Ethical approval and consent to participate

The study was approved by the local Institutional Review Board. Written informed consent for the use of primary material from family members in this study was obtained by all participating family members.

Consent for publication

All authors read and approved the final manuscript.

Availability of supporting data

All data generated or analyzed in this study are included in this published article and its supplementary information files.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2021.05.009.

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