Loss of VPS13C Function in Autosomal-Recessive Parkinsonism Causes Mitochondrial Dysfunction and Increases PINK1/Parkin-Dependent Mitophagy

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ABSTRACT

Autosomal recessive early-onset parkinsonism is clinically and genetically heterogeneous. The genetic causes of approximately 50% of autosomal recessive early-onset forms of Parkinson's disease (PD) remain to be elucidated. Homozygozity mapping and exome sequencing in 62 isolated individuals with early-onset parkinsonism and confirmed consanguinity followed by data mining in the exomes of 1,348 PD individuals identified, in three isolated cases, homozygous or compound heterozygous truncating mutations in the *vacuolar protein sorting 13C* gene (*VPS13C*). *VPS13C* mutations are associated with a distinct form of early-onset parkinsonism characterized by rapid and severe disease progression and early cognitive decline; the pathological features were striking and reminiscent of diffuse Lewy body disease. In cell models, VPS13C partly localized to the outer membrane of mitochondria. Silencing of *VPS13C* was associated with lower mitochondrial membrane potential, mitochondrial fragmentation, increased respiration rates, exacerbated PINK1/Parkin-dependent mitophagy and transcriptional upregulation of *PARK2* in response to mitochondrial damage. This work suggests that loss of function of VPS13C is a cause of autosomal recessive early-onset parkinsonism with a distinctive phenotype of rapid and severe progression.

Introduction

Parkinson's disease (PD [MIM 168600]) is a motor syndrome with variable combinations of akinesia, rigidity and rest tremor responding to levodopa. It is caused by degeneration of the dopaminergic neurons in the *substantia nigra pars compacta*, and is associated with Lewy bodies, intraneuronal inclusions enriched in α-synuclein. In recent years, our understanding of the pathophysiological mechanisms underlying molecular defects in familial forms of PD has greatly advanced. Three genes have been conclusively associated with autosomal dominant (AD) forms of PD (*SNCA* [MIM 163890], *LRRK2* [MIM 609007], and *VPS35* [MIM 601501], and eight (*PARK2* [MIM 602544], *PINK1* [MIM 608309], *DJ-1* [MIM 602533], *ATP13A2* [*MIM* 610513], *FBXO7* [MIM 605648], *PLA2G6* [MIM 603604], *SYNJ1* [MIM 604297], and *DNAJC6* [MIM 608375]) with early-onset (EO) autosomal recessive (AR) forms.[1] EO AR parkinsonism is clinically and genetically heterogeneous: mutations in *PARK2*, *PINK1* and *DJ-1* cause phenotypes similar to idiopathic PD with good and prolonged response to dopaminergic therapy. Other EO AR PD-associated genes cause more severe disease, a poor response to levodopa and additional clinical signs, such as dystonia and cognitive impairment.[1] Mutations in *PARK2* and *PINK1* are the most common cause of EO AR PD, accounting for ~50% and 4% of familial cases in Europe, respectively.[2,3] A significant proportion of cases remain genetically unexplained.

EO AR PD is linked to mitochondrial dysfunction. The mitochondrial kinase PINK1 and the E3 ubiquitin-protein ligase Parkin cooperate in mitochondrial quality control.[4] They promote the removal of dysfunctional mitochondria in a process termed mitophagy that may also involve FBXO7.[4,5] In addition, they play a role in a vesicular trafficking pathway targeting damaged mitochondrial components to the lysosome.[6] To identify additional PD-associated genes involved in AR EO parkinsonism, we performed homozygosity mapping and exome sequencing in consanguineous PD families and isolated individuals and used data mining in

the exomes of 1,348 unrelated PD individuals. Five truncating mutations in the *vacuolar protein sorting 13C* gene (*VPS13C* [MIM 608879]) were identified in three unrelated PD isolated individuals. We provide evidence that depletion of the VPS13C protein exacerbates mitochondrial vulnerability to stress.

Subjects and Methods

Participants

Gene discovery cohort

We selected nine PD families (with ≥ two affected siblings) and 43 unrelated isolated individuals according to the following criteria: 1) individuals diagnosed by neurologists according to the UK Parkinson's Disease Society Brain Bank (PDSBB) clinical diagnostic criteria[7] and onset ≤55 years in at least one affected family member; 2) with no mutations in known PD-associated genes; and 3) with confirmed consanguinity (inbreeding coefficient $F \neq 0$ computed with the FEstim program[8]). Sixteen families or isolated subjects were European, 16 North African, 19 Turkish and one Lebanese. A total of 66 PD affected individuals (23 family members and 43 isolated subjects) and 39 unaffected relatives were included for the genome-wide screen study. Sixty-two affected and 10 unaffected individuals were subsequently selected for whole exome sequencing.

Validation cohort

Exome data were obtained from a series of 1,348 additional PD individuals (99% unrelated, 99% of European ancestry, 60% males, mean age at onset 41.7 ± 11.0 years), including 249 French PD probands (57% males, age at onset ≤40 years, 50 individuals with atypical forms of parkinsonism) recruited by the French network for the study of PD genetics (PDG), and 530 matched control subjects (95% of European ancestry, 65% males, mean age at

examination: 45.1 ± 10.7 years), from the International Parkinson Disease Genomics Consortium (IPDGC).

Hundred Turkish control subjects without family history of PD (43% males, mean age at examination: 60.4±13.9 years) served to check for the absence of the identified variant in family A originated from Turkey.

A flow diagram detailing the selection criteria of PD individuals and controls, and the different experimental steps of the study is provided (Figure S1).

Study Approval

Informed consent was obtained from all participants, and the genetic studies were approved by local ethics committees (INSERM, CCPPRB du Groupe Hospitalier Pitié-Salpêtrière, Paris, France).

Neuropathological assessment

The autopsy of the affected individual II-1 in family B (Figure 1) was performed approximately 36 h *post mortem*. Brain tissues were fixed for six weeks in 10% buffered formalin, extensively sampled and processed as previously described[9]. Immunohistochemistry was performed by a Ventana Benchmark automate. We used hematoxylin-eosin staining for histopathology. For immunochemistry, the antibodies used were: anti-Tau (in-house AD2,[10] 1 ng/mL), anti-β-amyloid (4G8, 1:1,000, Sigma, France), anti-α-synuclein (LB509, 1:500, Abcam, France), anti-ubiquitin (1:1,000, Dako, France), anti-TDP-43 (1:500, Protein Tech, France), and anti-glial fibrillary acid protein (GFAP, 1:20,000, Dako, France). The degree of neuronal loss and the frequency of α-synuclein-immunoreactive and other inclusions were determined semi-quantitatively by visual inspection, in comparison to brains of three aged-matched controls (2 males and 1 female) from the Lille Neurobank collection.

Molecular Studies

Whole Genome Homozygosity Linkage Mapping

Genome-wide screens were performed on all available affected (n = 66) and unaffected (n = 39) individuals from the gene discovery cohort using the Illumina HumanCytoSNP-12 v2.1 DNA Analysis BeadChip microarrays that contain ~300,000 single nucleotide polymorphisms (SNPs) and ~1,300 markers of common copy number variations (CNVs). Homozygosity tracks (> 2 Mb) were visualized with the Homozygosity Detector module, and CNV with the Illumina cnvPartition module. The inbreeding coefficients F were computed with the FEstim program.[8] Samples from individuals with confirmed consanguinity ($F \neq 0$) were subjected to exome sequencing.

Whole Exome Sequencing

Exons from 62 affected (19 relatives and 43 isolated individuals) and 10 unaffected family members from the gene discovery cohort, and all the 1,348 affected and 530 control individuals from the validation cohort were captured using different exome enrichment kits from fragmented genomic DNA and sequenced as indicated in Table S1. Ten-fold mean sequencing depth was achieved in 96.4% and 88.8% of baited regions in PD individuals and controls, respectively, thirty fold mean sequencing depth was achieved across ~75% of targeted regions.

Human reference sequence UCSC hg19 was used for sequence alignment and variant calling with the Burrows-Wheeler Aligner[11] and the Genome Analysis Toolkit.[12] PCR duplicates were removed prior to variant calling using Picard software. Variants were annotated with ANNOVAR software[13] (exomes from validation cohort) or SnpEff and SnpSift programs (exomes from gene discovery cohort). Data were analyzed with Ingenuity® Variant Analysis

(IVA) TM software from Ingenuity System. Effects on mRNA splicing by putative splice variants (+/-5 base pairs around splice junctions according to IVA threshold) were analyzed with Splice Site Finder, MaxEntScan, NNsplice, Genesplicer and Human Splicing Finder[14–16] all included in Alamut v3 software. The human VPS13C protein and its closest homologs were aligned with Alamut v3 software, computed by Ensembl and aligned with MUSCLE.

An in-house pipeline crossed the output data from IVA and Homozygosity Mapping to filter and identify the variants of interest (Figure S2). These variants were visualized using the Broad Institute Integrative Genomics Viewer (IGV) and verified by bidirectional Sanger sequencing using primers designed with Primer3 (Table S2) on an ABI 3730 automated sequencer (Life Technologies, Carlsbad, CA, USA). Sanger sequencing confirmed the absence of mutations in origin-matched controls and determined the genetic status of unaffected relatives. Mutation nomenclature follows Human Genome Variation Society (HGVS) recommendations: the longest *VPS13C* transcript 2A cDNA nucleotides ("c.") are numbered from the adenine of the first ATG translation initiation codon as nucleotide +1 (GenBank reference sequence NM_020821.2).

Splicing defect analysis by RT-PCR

Keratinocytes from affected individual V-2 in family A or peripheral blood lymphocytes from affected individual II-1 in family C (Figure 1) were used for splicing defects analyses. Hair follicles were plucked under sterile conditions and cultured in flasks coated with MG (1:10, Corning, Tewksbury, USA). Keratinocytes were grown on 20 mg/mL collagen IV (Sigma-Aldrich, St. Louis, USA) -coated dishes containing EpiLife medium with the HKGS supplement (Life Technologies, Carlsbad, USA). Total RNA extraction was carried out with the RNeasy Kit (Qiagen, Hilden, Germany), according to the manufacture's manual. RNA (250 ng) was reverse-transcribed into cDNA using iScript reverse transcription supermix

(Bio-Rad). Primers are listed in Table S2. RT-PCR products were Sanger-sequenced directly or after sub-cloning into the pJET1.2/blunt vector (Thermo Scientific).

Studies in mammalian cells

Mammalian expression vectors, siRNAs, cell culture, and transfection

COS-7 and HEK293T cells were grown in Dulbecco's Modified Eagle Medium + Glutamax (Life Technologies) supplemented with 10% goat serum (Life Technologies) and 1% Penicillin-Streptomycin (Life Technologies). Cells were plated, at 80% confluence, on glass coverslips (Thermo Scientific) in 24-well cell plates for immunofluorescence, 6-well plates for qPCR, 10 cm-Petri dishes for subcellular fractionation by differential centrifugation and for the mitochondrion isolation kit, or 75 cm2-flasks for continuous sucrose gradient and Percoll gradient purification. Cells were co-transfected with siRNAs (15 to 30 nM) and expression vectors using Lipofectamine 2000 (Life Technologies), in an antibiotic free medium, according to the manufacturer's instructions. The siRNA used were: Hs_VPS13C_5 and Hs_VPS13C_6 (si*VPS13C*, Qiagen); PINK1 stealth siRNAs (si*PINK1*, Invitrogen); AllStars negative control siRNA (siControl, Qiagen). Their efficacy was controlled by quantitative real-time RT-PCR (Figure 5F). The expression vectors were: pcDNA3-HA-PARK2, pcDNA3-HA-PINK1, pcDNA3-HA[17] and pEGFP-C1 (Life Technologies). Where indicated, the cells were incubated with 10 μM CCCP (Sigma).

Subcellular fractionation, trypsin digestion assay and western blot analyses

For sucrose density gradient confluent HEK293T cells from five 75 cm^2-flasks were harvested and disrupted with a Dounce homogenizer (80 manual strokes) in 10mM Tris-HCl buffer, pH 7.6 containing 10% w/v sucrose, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), supplemented with protease and phosphatase inhibitors (0.2

mM sodium orthovanadate, 4 mg/ml sodium fluoride, 5.4 mg/ml β-glycerophosphate, and Complete cocktail 1X – 11836145001, Roche). After three centrifugations at 600 x g for 5 min to remove cell debris, the cell lysate was layered on a 20-60% linear sucrose gradient in Tris-HCl, pH 7.6, containing 10 mM EDTA, as previously described[18]. After 18 h of centrifugation at 100,000 x g, successive 0.8 ml fractions were collected. Proteins were precipitated on ice with 10 % trichloroacetic acid, pelleted by centrifugation at 13,000 x g for 45 min and resuspended in 100 µl of loading buffer (Tris pH 6.8 60mM, SDS 4%, β-mercaptoethanol 5%, glycerol and bromophenol blue).

Total protein fractions were obtained from cells lysed in 210 mM mannitol, 70 mM sucrose, 5 mM Tris pH 7.4, 0.2 mM EGTA, 0.1 mM EDTA, 0.5 mM DTT, 0.1% BSA and protease and phosphatase inhibitors after centrifugation at 600 x g for 5 min at room temperature. Mitochondrion-enriched fractions were obtained by differential centrifugation (HEK293T) or magnetic isolation (COS-7) and digested with trypsin (Sigma), as previously described.[17,19]

For isolation of pure mitochondria, cells were lysed in 250 mM mannitol, 5 mM HEPES pH 7.4, 5 mM EGTA with protease and phosphatase inhibitors and the crude mitochondrial fraction was layered on top of a 30% Percoll gradient, as previously described.[20] Protein concentrations were determined with Bio-Rad protein assays (Bio-Rad, 500-0006), based on the Bradford method. Samples were boiled in protein sample buffer, resolved by SDS-PAGE, transferred onto a nitrocellulose membrane (Protran, Whatman) and analyzed by western blotting with selected primary and secondary antibodies (Table S3). Membranes were incubated with enhanced chemiluminescence substrate (Pierce); chemiluminescent and fluorescent signals were revealed on film (ECL, Amersham Hyperfilm) or captured with Odyssey Imaging (Li-COR) systems and quantified with ImageJ software (NIH). Total or cytoplasmic fractions were normalized to α-tubulin, mitochondrial fractions to PMPCB. Three to six independent fractionation experiments were quantified.

Analysis of mitochondrial respiration

Cellular oxygen consumption was measured using high resolution respirometry (OROBOROS Oxygraph-2k, Austria) in a temperature-regulated chamber at 37°C. Oxygen consumption was measured in intact COS-7 cells at a density of $2.5x10^6$ cells in 2 mL of respiration assay medium (1x DMEM, GlutaMAX™ , GIBCO) containing 4.5 g/L D-Glucose and 4 mM L-glutamine, by sequential additions of 1 µg/mL oligomycin, 2.5 µM CCCP and 5 µM rotenone/10 µM antimycin A. We determined the following mitochondrial parameters: *basal oxygen consumption* (= basal cellular respiration - non-mitochondrial respiration), *proton leak* (= oligomycin-inhibited respiration - non-mitochondrial respiration), *maximal respiratory capacity* (= maximal uncoupled respiration - non-mitochondrial respiration), *reserve respiratory capacity* (= maximal uncoupled respiration - basal respiration), and *non-mitochondrial respiration* (rotenone/antimycin A-inhibited respiration). Cells were then lysed to quantify the protein content using the Bradford reagent, which was used to normalize the oxygen consumption data. The results were expressed in pmol of O_2/s/mg of total protein.

Immunostaining, ΔΨmt, respiration, mitochondrial morphology, and Parkin-dependent mitophagy

Immunocytochemical stainings were performed as described previously using the antibodies and dilution conditions indicated in Table S3. Changes in ΔΨmt were evaluated with the potentiometric dye tetramethylrhodamine methyl ester (TMRM) as described in[19]. Mitochondrial morphology was analyzed on COS-7 cells immunostained for the B subunit of the mitochondrial processing peptidase (PMPCB) using an image-processing algorithm and two descriptive parameters to assess mitochondrial length and branching: aspect ratio, calculated as the ratio between major and minor axes of each mitochondrial object,

representing its length; and form factor, calculated as perimeter^2/(4π × area), representing a combined evaluation of the length and degree of branching of the mitochondrial network.[21,22] For Parkin-dependent mitophagy, cells were immunostained for PMPCB or the outer mitochondrial membrane protein TOMM20 and quantified as described.[17] Images were acquired with an Olympus FV-1000 confocal microscope (× 60 oil immersion objective, NA 1.35) and analyzed using ImageJ analysis software (NIH).

Quantitative real-time RT-PCR

To demonstrate the efficiency of the siRNA-mediated silencing of endogenous *VPS13C* in HEK-293T cells, total RNA was isolated from cells transfected with control or *VPS13C* or *PINK1* siRNA using the RNeasy plus Mini Kit (Qiagen) and QIAshredder (Qiagen). RNA from each sample (500 ng) was reverse-transcribed into cDNA using iScript reverse transcription supermix (Bio-Rad). Real-time PCR was performed with the LightCycler ® 480 System (Roche Applied Science) and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Results were analyzed using LightCycler 480 sw 1.5 quantification Software (Roche Applied Science). *Beta-actin* (ACTB) was used as the reference gene for normalization. Primers are listed in Table S2.

Statistical analysis

Statistical significance was established at $p < 0.05$ and determined with an unpaired t-test in Figures 4A (Aspect Ratio) and S6A, matched t-test in Figures 4C, 5B and 5D, Mann–Whitney–Wilcoxon test in Figure 4A (Form Factor), one-way ANOVA in Figures 6 and S5, or two-way ANOVA in Figures 4B, 5E and 5F.

Results

Truncating mutations in *VPS13C* cause AR parkinsonism

Genome-wide screens in an initial series of 66 affected and 39 unaffected subjects, using DNA microarrays identified a mean of 16.3 regions of homozygosity ≥2 Mb on the 22 autosomes of each consanguineous individual (gene discovery cohort). No rare deleterious large genomic rearrangements were detected. Exomes were subsequently sequenced in the 62 affected individuals with confirmed consanguinity (inbreeding coefficient $F \neq 0$) and 10 unaffected family members to identify homozygous variants which: 1) in priority, would disrupt the protein function (frameshifts, stop codons or splicing variants); 2) were rare [minor allele frequency (MAF) < 1%] in dbSNP137, the National Heart Lung and Blood Institute (NHBLI), Exome Sequencing Project (ESP) database, and the 1000 Genomes Project; 3) were shared by affected siblings when available; 4) were heterozygous in parents and/or heterozygous or wild-type in unaffected siblings when available; 5) fell within homozygous intervals; and 6) were absent in the homozygous state from DNA of 530 controls. We identified rare or undescribed homozygous truncating variants within 32 genes, each found in a single affected individual with consanguinity that fulfilled all these prioritization criteria (Table S4). We screened these candidate genes for additional homozygous or compound heterozygous mutations in a validation cohort. *VPS13C* (also known as KIAA1421, NM_020821.2) on chr15q22 was mutated in a consanguineous Turkish PD individual (V-2 in family A) from the gene discovery cohort, and in two additional French PD isolated individuals (II.1 in family B, and II.1 in family C) from the validation cohort (Figure 1A). In addition, we identified a total of 80 rare (MAF < 1% in public databases) single heterozygous mostly nonsynonymous variants in *VPS13C* from the validation cohort, including 14 present in at least one of the 530 European controls (Table S5). No additional homozygous or compound heterozygous variants were found in the 31 other candidate genes.

The affected individual V-2 in family A harbored a homozygous splice-site mutation c.8445+2T>G, intron 61 in *VPS13C* confirmed by Sanger sequencing; eight unaffected relatives, including the mother, had heterozygous c.8445+2T>G mutations or wild-type sequences (Figure 1A, Figure S3A). Affected individuals in families B and C were compound heterozygotes (Figure 1A, Figures S3B and S3C): the affected individual II-1 in family B with c.806_807insCAGA, exon 11 (p.Arg269Serfs*14) / c.9568G>T, exon 69 (p.Glu3190*) variants; the affected individual II-1 in family C with c.4165G>C, exon 37 (p.Gly1389Arg) / c.4777delC, exon 43 (p.Gln1593Lysfs*7) variants. Direct sequencing of *VPS13C* in the three unaffected siblings (II-2, II-3, and II-4) in family B and the unaffected mother (I-1) in family C showed that they all carried heterozygous mutations or wild-type alleles (Figure 1A, Figure S3), indicating that all variations were located on different alleles. The five *VPS13C* variants were absent in dbSNP137, 1000 Genomes Project, EVS (Table S6A) and Exome Aggregation Consortium (ExAC) databases (Table S6B), and our European control exomes, except for the missense p.Gly1389Arg variant found on one control chromosome. In addition, the c.8445+2T>G mutation was absent from 200 Turkish control chromosomes. No disruptive bi-allelic variants were found in our 530 control subjects; one disruptive homozygous variant (rs199602573) was found in the EVS database and ExAC populations (1/6,246 and 2/61,547, respectively), indicating that *VPS13C* homozygous disruptive variants are extremely rare in non-PD populations (Tables S6 and S7).

The c.4165G>C and c.8445+2T>G mutations were predicted *in silico* to modify donor splice sites, one base upstream and two bases downstream, respectively, of splice junctions (Figure S4A). Reverse-transcription PCR analysis of potential splicing defects confirmed the predictions (Figure S4B). RNA from the homozygous individual of family A showed at least three shorter transcripts, lacking up to 231 nucleotides at the end of the exon 61. In the subject with the heterozygous variant, shorter transcripts were barely visible, probably due to a high

instability of these aberrant RNAs. In family C, the longer transcript was found in the subject with the heterozygous variant, containing 14 additional nucleotides from intron 37.

VPS13C contains 86 exons spanning a 208-kb genomic region and has two main transcript variants, 1A (NM_017684.4) and 2A (NM_020821.2) (Figure 1B). While the transcript 1A, lacking exons 6 and 7 and encoding a 3,710-amino acid protein, is expressed in most tissues, including brain and peripheral blood cells, the longest transcript 2A encodes a brain-specific 3,753-amino acid protein.[23] Two additional isoforms with uncharacterized expression pattern are reported in Ensembl (NM_018080.3 and NM_001018088.2) and lack the 4 last exons. All the isoforms contain the splice site variants found in families A and C. VPS13C contains a chorein domain at its N-terminus, a DUF1162 domain of unknown function and a putative autophagy-related domain (Figure 1B). Except for the c.8445+2T>G variant, which is located in the DUF1162 domain, none of the variants were found in the predicted domains.

Clinical and pathological characteristics of affected individuals harboring *VPS13C* mutations

The three affected individuals harboring *VPS13C* mutations had early disease onset (25 to < 46 years) and typical parkinsonism (akineto-rigid syndrome, rest tremor, good levodopa response). Disease progression, however, was particularly severe, with early cognitive decline, loss of response to treatment, axial symptoms, and dysautonomia. Affected subjects were bedridden within 15 years of clinical onset. Pyramidal signs and motor deficits were observed in two affected individuals. Brain MRI was normal early in the disease, then bilateral atrophy was observed in the frontal, parietal and temporal lobes (Table 1). Post-mortem examination of the brain of the affected individual II-1 in family B, who died at age 49 of a bronchopneumopathy by gulp, showed mild frontal atrophy, including the primary motor area (Figures 2A-2C). The pathology resembled diffuse Lewy body disease. Alpha-

synuclein and ubiquitin positive-Lewy bodies were observed in the brainstem, limbic system, hippocampus and all cortical associative areas, including the parieto-occipital region (Figures 2D-2F, Table S8). Tau-immunoreactive neurofibrillary tangles and neurites were seen in the brainstem, hippocampus and primary motor cortex (Figure 2G, Table S8). There were no glial-, α-synuclein-, Aβ– or TDP-43-immunoreactive inclusions.

Loss of *VPS13C* function affects mitochondrial morphology, transmembrane potential and respiration

To investigate the function of VPS13C, we explored its subcellular distribution in human HEK293T by sucrose gradient fractionation (Figure 3A). VPS13C was enriched in the low-density fractions 1 and 2 containing the early endosomal marker EEA1 and most of the cytosolic protein Parkin. VPS13C was also found in higher density fractions containing membrane and soluble markers of the Golgi apparatus (GOLGA2), the ER (Calnexin, BiP) and mitochondria (TOMM70, PMPCB, PINK1). Here, it was most abundant in fractions 8-10, containing the greatest proportion of TOMM70 and PMPCB. The mitochondrial localization of VPS13C was confirmed in mitochondrion-enriched fractions and pure mitochondria from HEK293T and COS-7 cells (Figures 3B and 3C). Limited trypsin digestion of mitochondrion-enriched fractions caused concomitant loss of VPS13C and the outer mitochondrial membrane receptor TOMM70 under conditions preserving the outer mitochondrial membrane channel TOMM40 and mitochondrial matrix enzyme PMPCB, indicating that VPS13C is located on the mitochondrial surface (Figure 3C).

We then investigated the impact of loss of *VPS13C* function on mitochondrial morphology, transmembrane potential and respiration, reported to be affected in models of *PINK1* or *PARK2* deficiency.[24,25] The siRNA-mediated silencing of *VPS13C* in COS-7 cells reduced *VPS13C* mRNA levels to no more than 25% of the control condition (Figure S6) and was

associated with perinuclear redistribution of mitochondria and mitochondrial fragmentation, as confirmed by quantitative image analysis (Figure 4A). Evaluation of the mitochondrial transmembrane potential (ΔΨmt) with the potentiometric dye tetramethylrhodamine methyl ester (TMRM), revealed a significant decrease in the mean fluorescence intensity of mitochondria in cells depleted of VPS13C (Figure 4B). The ΔΨmt decrease was accompanied by an increase in maximal respiration rates and respiratory reserve, as assessed by high resolution respirometry in intact cells (Figure 4C). Similar results were obtained in HEK293T cells (data not shown).

Loss of *VPS13C* function exacerbates PINK1/Parkin-dependent responses to mitochondrial depolarization

We further investigated the relationship between *VPS13C* and *PINK1* and *PARK2*, both at the transcript and protein levels, with respect to their well-characterized response to mitochondrial damage. PINK1 accumulates on mitochondria and recruits Parkin to initiate mitophagy in response to mitochondrial dysfunction.[4] Mitochondrial depolarization, triggered by the protonophore CCCP, partially redistributed VPS13C from mitochondria to the cytoplasm without significantly changing *VPS13C* transcript levels (Figures 5A, 5B and 5F - left panel); under these conditions PINK1 accumulated on mitochondria, as expected.[4] *VPS13C* silencing did not affect PINK1 levels under basal conditions, but it exacerbated CCCP-induced mitochondrial accumulation of PINK1 without impacting *PINK1* mRNA abundance (Figures 5C, 5D and 5F - middle panel). Moreover, *VPS13C* silencing enhanced mitochondrial translocation of Parkin triggered by CCCP (Figures 5C and 5E). It also upregulated Parkin protein abundance in the cytosol without affecting *PARK2* transcript levels at 3 h of CCCP treatment (Figure 5E and F - right panel). *PARK2* expression increases in response to mitochondrial damage caused by mitochondrial toxins, including CCCP.[26,27]

Here, *PARK2* transcript levels tended to be higher at 48 h of CCCP treatment (Figure 5F - right panel). This response was significantly enhanced following silencing of *VPS13C* or *PINK1*, suggesting greater mitochondrial damage. *PINK1* silencing was also associated with down regulation of *VPS13C* transcript levels under basal conditions (Figure 5F - left panel), an effect that was reversed by PINK1 overproduction (Figure S5), indicating the existence of multiple regulatory loops between VPS13C, PARK2, and PINK1. Consistent with the above described effects on PINK1 and Parkin, *VPS13C* silencing exacerbated PINK1/Parkin-mediated mitophagy triggered by CCCP in COS-7 cells, a model that we previously validated for the study of this process (Figures 6 and S6).[17,19]

Discussion

This study establishes *VPS13C* mutations as a monogenic cause of EO AR parkinsonism. Homozygous or compound heterozygous truncating mutations in three PD individuals, absent from or present in the heterozygous state in available unaffected family members and in a very large number of controls, strongly support the pathogenicity of *VPS13C* in EO parkinsonism. We identified three affected individuals harboring *VPS13C* mutations and could not perform co-segregation analyses, due to the lack of additional affected relatives in the corresponding families. However, the affected individuals shared a specific, rare, and extremely distinctive phenotype consisting of EO parkinsonism with very rapid progression and dementia, which argues strongly for the pathogenicity of *VPS13C* mutations. The initial phenotype, EO parkinsonism and a good response to levodopa treatment, is similar to that of PD individuals with *PARK2, PINK1* or *DJ-1* mutations. However, the affected individuals rapidly became bedridden because of the worsening of motor dysfunction and loss of response to treatment. Dysautonomia and pyramidal signs were observed in two affected individuals, also distinguishing the phenotype from the classical, slowly progressive EO PD.

The presence of numerous α-synuclein and ubiquitin-positive-Lewy bodies in the brainstem, limbic system and many cortical areas was reminiscent of diffuse Lewy body disease, consistent with a motor phenotype associated with dementia. α-synuclein Lewy bodies are absent in most PD individuals with *PARK2* mutations,[28] but were observed in the single autopsy case with *PINK1* mutations reported.[29] Tau-immunoreactive neurofibrillary tangles and neurites were also observed in cases with *PARK2* mutations.[28] These features define *VPS13C*-associated EO parkinsonism as a clinical, pathological and genetic entity belonging to the group of synucleinopathies. In addition, our study also provides 31 potential candidate genes harboring disruptive homozygous mutations in a single PD affected individual. However, genetic replication and functional validation are still needed to confirm their relevance to PD.

Alterations in other members of the *VPS13* family cause AR neurodegenerative disorders: *VPS13A* (MIM 605978) (CHAC [MIM 200150]) is mutated in chorea-acanthocytosis characterized by progressive neurodegeneration and red cell acanthocytosis;[30] *VPS13B* (MIM 607817) (COH1 [MIM 216550]) is mutated in Cohen syndrome characterized by psychomotor retardation, microencephaly and eye abnormalities.[31] *VPS13A*, *VPS13B,* and *VPS13C* are also mutated in gastric and colorectal cancers with unstable microsatellites.[32]

VPS13C belongs to a family of large VPS13 proteins (VPS13A-D) similar to yeast Vps13p.[23] Like yeast Vps, mammalian vacuolar sorting proteins are crucial for vesicular transport.[33] Initial studies linked yeast VPS13 orthologs to the delivery of proteins to the vacuole, the mammalian lysosome equivalent.[34] Mutations in *VPS35*, encoding a core component of the retromer complex regulating endosomal protein sorting, are implicated in AD late-onset PD.[35,36] VPS35 is also involved in protein trafficking from mitochondria to peroxisomes through mitochondria-derived vesicles.[37] PINK1 and Parkin play a role in this transport route, which delivers damaged mitochondrial cargo directly to lysosomes in response to

mitochondrial stress.[6] However, the machinery regulating cargo selection and sorting into vesicles remains to be identified. VPS13C may be involved in this process. Such a mechanism would be consistent with its mitochondrial localization and the observed relocation to the cytosol in response to mitochondrial damage.

Several other observations in mammalian cells suggest that, like PINK1 and Parkin, VPS13C plays a role in mitochondrial maintenance. VPS13C depletion led to reduction of $\Delta\Psi$mt and mitochondrial fragmentation in cell lines. Moreover, it enhanced maximal respiration rates suggesting compensatory adaptation aimed at preserving $\Delta\Psi$mt levels. In neuronal cells, which produce ATP mainly through mitochondrial oxidative phosphorylation and are unable to switch to glycolysis under acute mitochondrial stress, such changes may in the long term exacerbate generation of reactive oxygen species and trigger irreversible mitochondrial damage.[38,39] VPS13C depletion also upregulated PINK1/Parkin-dependent mitophagy and, similarly to PINK1 depletion, it enhanced the previously reported transcriptional upregulation of Parkin in response to toxin-induced mitochondrial dysfunction. Overall, these data suggest that loss of *VPS13C* function increases mitochondrial vulnerability to stress and thereby activates PINK1/Parkin-dependent mitochondrial quality control pathways. Based on the inverse relationship between VPS13C and PINK1 protein levels on the mitochondrial surface, we cannot exclude that VPS13C also acts as a negative regulator of PINK1.

Mitochondrial function is ensured by a series of interconnected finely orchestrated pathways, activated in response to different degrees of mitochondrial dysfunction.[40] Excessive mitophagy has been associated with α-synuclein-dependent neurodegeneration.[41] Further work is required to clarify the role of VPS13C in mitochondrial maintenance and dissect its possible relation to PINK1/Parkin-dependent pathways.

Enrichment of VPS13C in cell fractions containing the early endosomal marker EEA1 suggests broader roles for VPS13C in vesicular trafficking. A more general involvement in

endosomal-lysosomal trafficking, possibly counteracting α-synuclein pathology as recently reported for VPS35,[42] might explain the diffuse α-synuclein pathology and rapid progression to dementia in individuals with *VPS13C* mutations. Such a mechanism would potentially represent a unifying link with cellular pathways involved in AD PD.

In summary, we describe truncating mutations in *VPS13C* associated with EO parkinsonism with rapid progression and widely distributed Lewy bodies. A meta-analysis of PD genome-wide association studies (GWAS) recently identified a susceptibility allele ~250 kb from the *VPS13C* gene but not associated with either CpG methylation or mRNA expression,[43] suggesting that *VPS13C* can either cause a monogenic form of EO parkinsonism or confer genetic susceptibility to PD. While we are confident that our work strongly implicates *VPS13C* mutations in PD, further genetic studies in other populations are needed to confirm their pathogenicity. The development of animal models in which *VPS13C* is stably inactivated will help dissect the mechanisms by which loss of *VPS13C* function affects the survival of dopaminergic neurons.

Supplemental Data

Supplemental Data include six figures and seven tables and can be found with this article online at http:/.

Consortia

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We declare that we have no conflicts of interest for this study.

Web Resources

The URLs for data presented herein are as follows:

1000 Genomes Database, http://www.1000genomes.org/

ANNOVAR, http://annovar.openbioinformatics.org/

Broad Institute IGV**,** https://www.broadinstitute.org/igv/

BWA, http://bio-bwa.sourceforge.net/

dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/

Ensembl, http://www.ensembl.org/

ExAC Browser, http://exac.broadinstitute.org

GATK, http://www.acronymfinder.com/Genome-Analysis-Toolkit-(software)-(GATK).html

Human Splicing Finder (HSF), http://www.umd.be/HSF3/HSF.html

Ingenuity® Variant Analysis (IVA) TM software from Ingenuity System, http://www.ingenuity.com/variants

MUSCLE, http://www.ebi.ac.uk/Tools/msa/muscle/

NCBI Gene, http://www.ncbi.nlm.nih.gov/gene/

NHBLI Exome Sequencing Project (ESP), Exome Variant Server, http://evs.gs.washington.edu/EVS/

OMIM, http://www.omim.org/

Picard, http://picard.sourceforge.net/

Primer3**,** http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi

SnpEff, http://snpeff.sourceforge.net/

SnpSift, http://snpeff.sourceforge.net/SnpSift.html

UCSC Genome Browser, https://www.genome.ucsc.edu/

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Figure legends

Figure 1. Identification of *VPS13C* mutations

(A) Pedigrees of families with *VPS13C* mutations. Black symbols represent individuals with PD, open symbols, those unaffected. Arrows point to probands who underwent whole exome sequencing. AE: age at examination; AD: age at death; AO: age at onset.

(B) Schematic representation of *VPS13C* and its variations. *VPS13C* spans 208kb and contains 86 exons encoding a 3753-amino acid protein with a chorein domain at its N-terminus, a DUF1162 domain of unknown function and a putative autophagy-related domain. The five variations found in the 3 probands are indicated. Numbers above the gene identify the exons containing *VPS13C* variations. Alternative splicing a and b represent skipping of exons 6+7, and exon 82 respectively. Transcripts 1A, NM_017684.4: splicing a + b; 2A, NM_020821.2: splicing b; 1B, NM_018080.3: ends at exon 82; 2B, NM_001018088.2: splicing a and ends at exon 82.

Figure 2. Neuropathology in the proband of family B with c.806_807insCAGA / c.9568G>T *VPS13C* mutations shows abundant α-synucleinopathy

(A-C) Macroscopic appearance of the left hemisphere (fixed): lateral view (A); medial view (B); coronal section at the level of the cerebral peduncle (C).

(D, E) Lewy bodies in pigmented neurons in the *substantia nigra* (D, arrow, hematoxylin-eosin (HE) staining) and the parietal neocortex (E, arrowhead, HE staining).

(F) Representative image of α-synuclein-immunoreactivity in the frontal cortex showing abundant Lewy bodies and neurites.

(G) Tau-immunoreactive neurofibrillary tangles in the primary motor cortex. Scale bars for microscopic images: 50 µm.

Figure 3. A pool of VPS13C is located on the outer mitochondrial membrane

(A) Sucrose gradient fractionation illustrating the subcellular distribution of the endogenous VPS13C protein in HEK293T. Note the enrichment of the protein in fractions 1 to 3 and 8 to 10. Soluble endoplasmic reticulum (ER, BiP) and mitochondrial (PMPCB) markers in fractions 1 and 2 reflect organelle damage during fractionation.

(B) Western blot showing VPS13C immunoreactivity in mitochondria purified by Percoll gradient centrifugation from HEK293T cells (pM fraction). Note the enrichment in VPS13C and the mitochondrial markers, TOMM70 and PMPCB, in the pM fraction compared to the mitochondrion-enriched fraction (M). T: total lysate.

(C) Limited trypsin treatment of mitochondrion-enriched fractions (M) from HEK293T or COS-7 cells caused loss of VPS13C and the mitochondrial surface marker TOMM70; the outer mitochondrial membrane channel TOMM40 and the matrix marker PMPCB are preserved.

Figure 4. *VPS13C* silencing impacts mitochondrial morphology, transmembrane potential and respiration

(A) Representative immunofluorescence staining illustrating mitochondrial perinuclear redistribution and fragmentation in COS-7 cells silenced for *VPS13C* (si*VPS13C*, 30 nM) compared to cells treated with control siRNA (siControl, 30 nM): green, mitochondrial matrix marker PMPCB; red, α-Tubulin. *VPS13C* silencing reduced *VPS13C* mRNA levels to no more than 25% of the control condition (see Figure S6). Scale bar 10 µm. Quantification of Aspect Ratio and Form Factor (see Methods and [21,22]) shows reduced mitochondrial network complexity in si*VPS13C* treated cells (means ± SEM; **p < 0.01; ***p < 0.001, of n = 88 or 86 cells scored per condition).

(B) Analysis of the relative TMRM fluorescence of mitochondria in COS-7 cells transfected as in (A), illustrating the decrease in ΔΨmt in cells depleted for VPS13C. n = 40 cells per condition from one experiment representative of three carried out. ***p < 0.001.

(C) Oxygen consumption rates in intact COS-7 cells transfected with siControl or si*VPS13C*. The left panel shows the oxygen flux corrected for instrumental background from one representative experiment. The graph in the right panel displays the respiration rates. Absence of *VPS13C* is associated with increased maximal respiration (= maximal uncoupled respiration under CCCP - non-mitochondrial respiration in the presence of the mitochondrial complex I and III inhibitors, rotenone and antimycin A) and reserve capacity (= maximal uncoupled respiration - basal respiration before the addition of the complex V inhibitor oligomycin). Means ± SEM; *p < 0.05, of 6 independent experiments.

Figure 5. Loss of VPS13C function enhances mitochondrial accumulation of PINK1, recruitment of Parkin and *PARK2* upregulation in response to CCCP

(A-B) Western blot (A) and (B) corresponding VPS13C protein levels (normalized to α-Tubulin or PMPCB) in cytosolic (C), mitochondrion-enriched (M) and total (T) cell fractions from HEK293T cells treated or not with CCCP (10 µM, 3 h). VPS13C levels decreased significantly in mitochondria following CCCP treatment, but tended to increase in cytosol (means ± SEM; *p < 0.05, of 6 independent fractionation experiments).

(C-E) Western blot (C) and (D, E) corresponding normalized protein levels in cytosolic and mitochondrion-enriched fractions from HEK293T transfected with 30 nM of control siRNA (siControl) or siRNA targeting *VPS13C* (si*VPS13C*). (D) CCCP treatment resulted in accumulation of PINK1 (endogenous) in mitochondrion-enriched fractions (M) following treatment with siControl (-) and, more significantly, with si*VPS13C* (+). (E) Accumulation of Parkin (endogenous) on depolarized mitochondria was also strongly enhanced in cells treated

with si*VPS13C*. In addition, Parkin levels were upregulated in the cytosolic (C) fractions, particularly in untreated cells (means ± SEM; *p < 0.05 of 4 independent fractionation experiments).

(F) Quantitative real-time RT-PCR showing relative mRNA levels, normalized to α-actin (*ACTB*), in HEK293T cells treated with control siRNA (siControl), or siRNA targeting *VPS13C* (si*VPS13C*) or *PINK1* (si*PINK1*), under basal conditions or following CCCP treatment. Note the more than 30% decrease in *VPS13C* mRNA levels after *PINK1* silencing under basal conditions, but not after CCCP treatment (left panel). Note also that *VPS13C* and *PINK1* silencing enhance the upregulation of *PARK2* mRNA at 48 h of CCCP treatment (right panel); means ± SEM of 3-9 replicates per condition from 2 independent experiments (**p < 0.01; ***p < 0.001 compared to siControl within each condition of CCCP treatment; ###p < 0.001 between the indicated conditions of CCCP treatments).

Figure 6. Loss of *VPS13C* function exacerbates PINK1/Parkin-dependent mitophagy

(A) Immunofluorescence staining of a representative experiment illustrating PINK1/Parkin-dependent mitophagy in COS-7 cells overproducing Parkin and silenced for *VPS13C* or *PINK1* (20 nM siRNA) after CCCP treatment (10 µM for 48 h): red, Parkin; green, mitochondrial matrix marker PMPCB. Open arrows indicate loss of mitochondrial networks; white arrows show preserved networks. Scale bar 10 µm.

(B) Quantification of mitophagy in the conditions described in (A), expressed as the proportion of COS-7 cells without PMPCB (black bars) or TOMM20 (grey bars) staining; the si*VPS13C* treatment increased and si*PINK1* decreased the proportion. In the absence of exogenous Parkin (- Parkin; cells overproducing the control protein EGFP) or CCCP (not shown) all the cells harbored normal mitochondrial PMPCB staining, whether or not *VPS13C*

was silenced (means ± SEM; *p < 0.05, **p < 0.01 of 3 independent experiments; 100 cells scored per condition).

(C) Proportion of COS-7 cells without PMPCB staining after transfection with half-doses (10 nM) of each siRNA and 48 h of CCCP treatment. The mitophagy-promoting effect of *VPS13C* depletion was abolished by concomitant silencing of *PINK1* (means ± SEM; *p < 0.05 of 3 independent experiments; 100 cells scored per condition).

Table 1. Clinical Characteristics of Affected Individuals Harboring *VPS13C* Mutations

	V-2/family A	**II-1/family B**	**II-1/family C**
Origin	Turkish	French	French
Consanguinity	Yes	No	No
Gender	Female	Male	Female
Age at onset (years)	< 46	33	25
Symptoms at onset	Depression; Asymetric akineto rigid syndrome; No dystonia	Asymetric akineto rigid syndrome and rest tremor; Limb dystonia	Asymetric akineto rigid syndrome; Limb dystonia
Response to levodopa	Yes, partial	Yes, at early stage	Yes, at early stage (evaluated at 75% initially)
Complications with treatment	No motor fluctuation nor dyskinesia	Fluctuation and dyskinesia, ICD, somnolence	No motor fluctuation nor dyskinesia
Evolution	Severe with early cognitive decline with spatial disorientation (MMSE 21), slurred speech and hallucinations at the age of 51; Axial symptoms	Severe with early cognitive decline (MMSE 18 at the age of 40); Axial symptoms (FOG at the age of 35, falls at the age of 39); Dysautonomia (at the	Severe with early cognitive decline, slurred speech before the age of 39; Severe axial symptoms at early stage; Bedridden at the age of 31;

	(postural instability, FOG and falls) and dysautonomia with urinary incontinence at the age of 54; Bedridden, unable to speak, apathetic, confused, cachexic with dysphagia at the age of 58	age of 35); Bedridden at the age of 43, death at the age of 49 of bronchopneumopathy by gulp	Gastrostomia at the age of 37 Her father died at 52 of a pancreatic cancer
Atypical symptoms associated	Brisk tendon reflexes on the lower limbs but no pyramidal syndrome	Motor neuron signs with pyramidal syndrome and limb atrophy at late stage	Motor neuron signs with spastic tetraplegia
Cerebral MRI	Asymmetric atrophy in frontal, parietal and temporal areas at the expense of the left side	Normal (performed at early stage)	Normal (performed at early stage)

1 ICD, impulse control disorder; MMSE, mini-mental state examination; FOG, freezing of gait;
2 MRI, magnetic resonance imaging.

Figure 1

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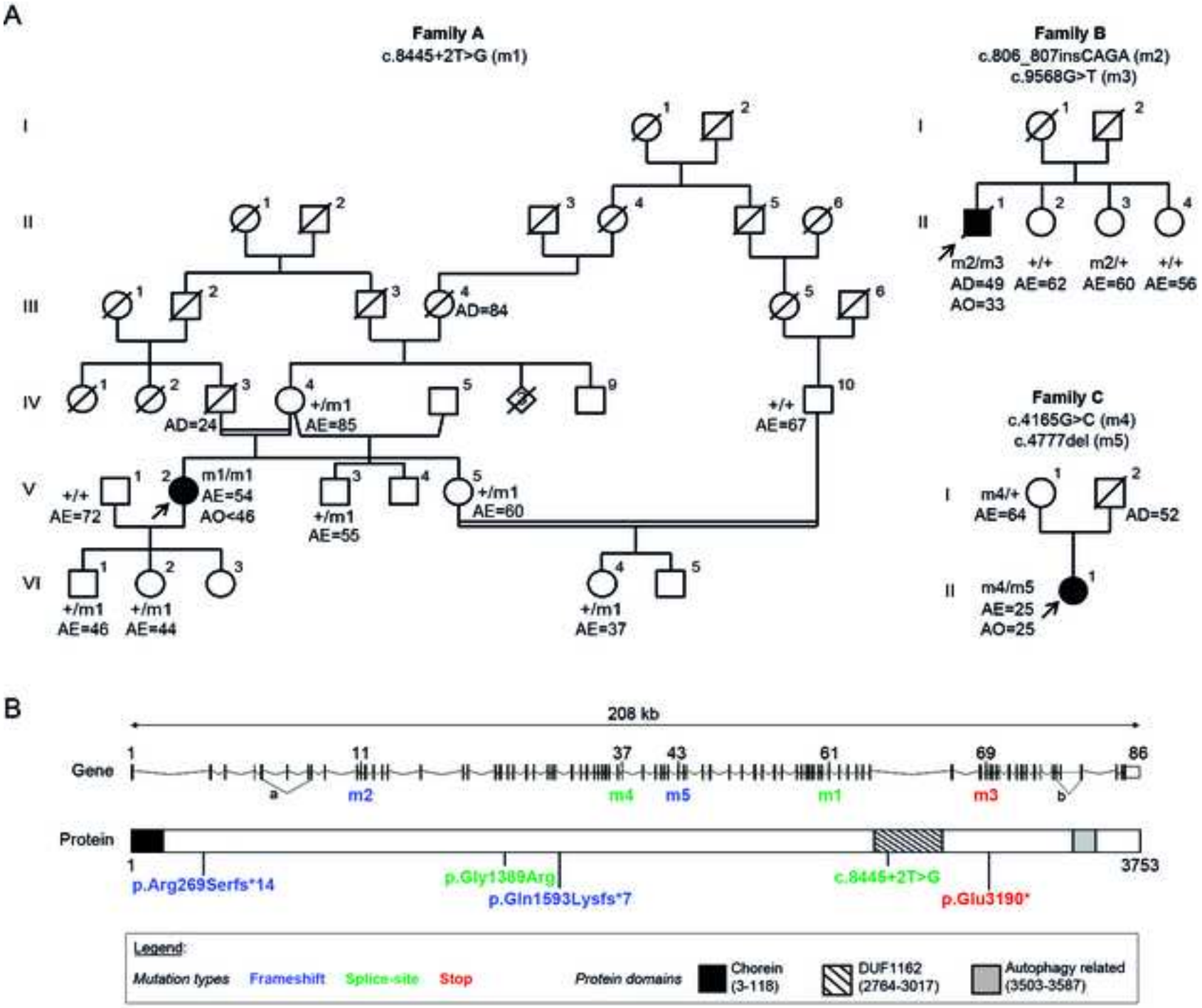

Figure 2

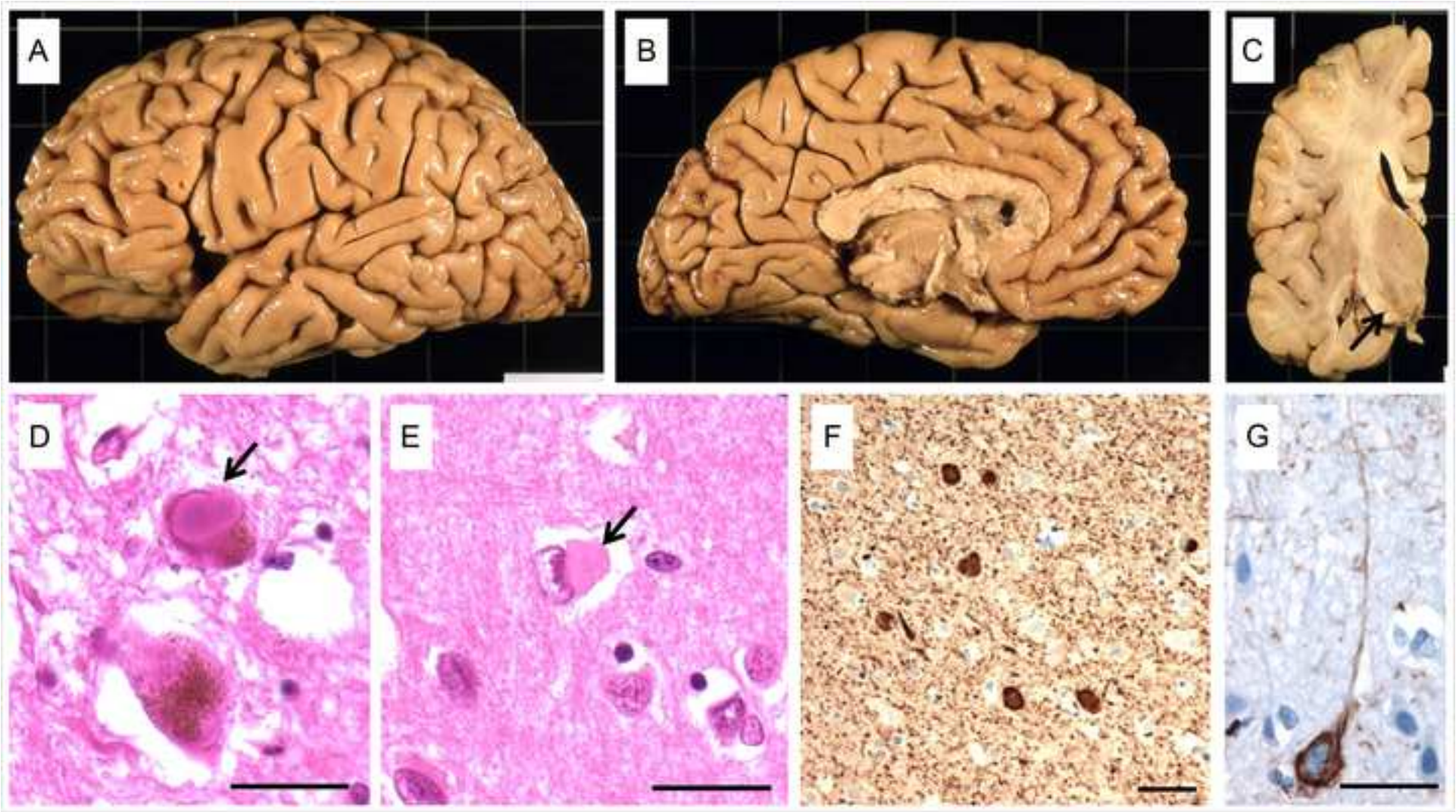

Figure 3

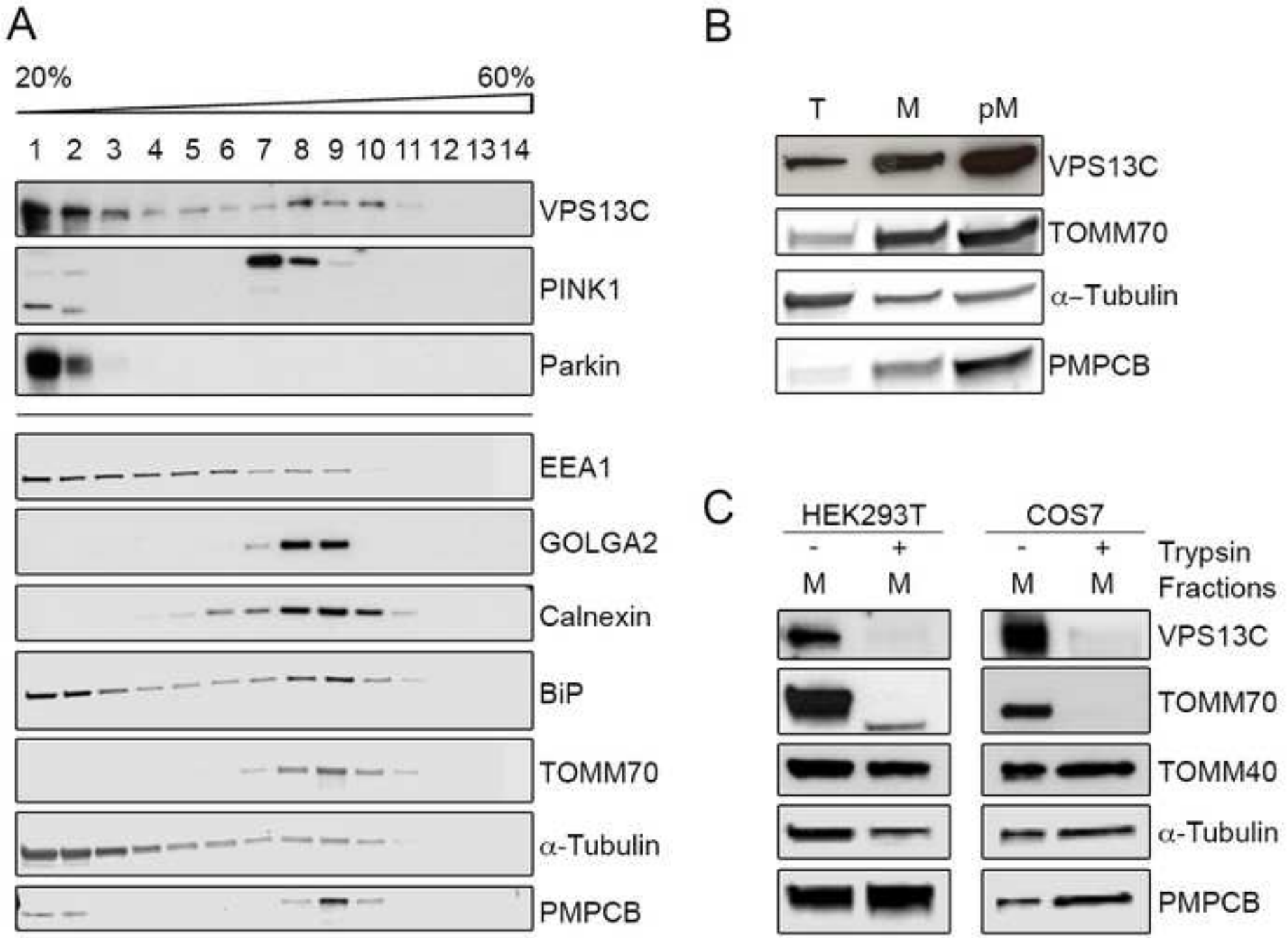

Figure 4

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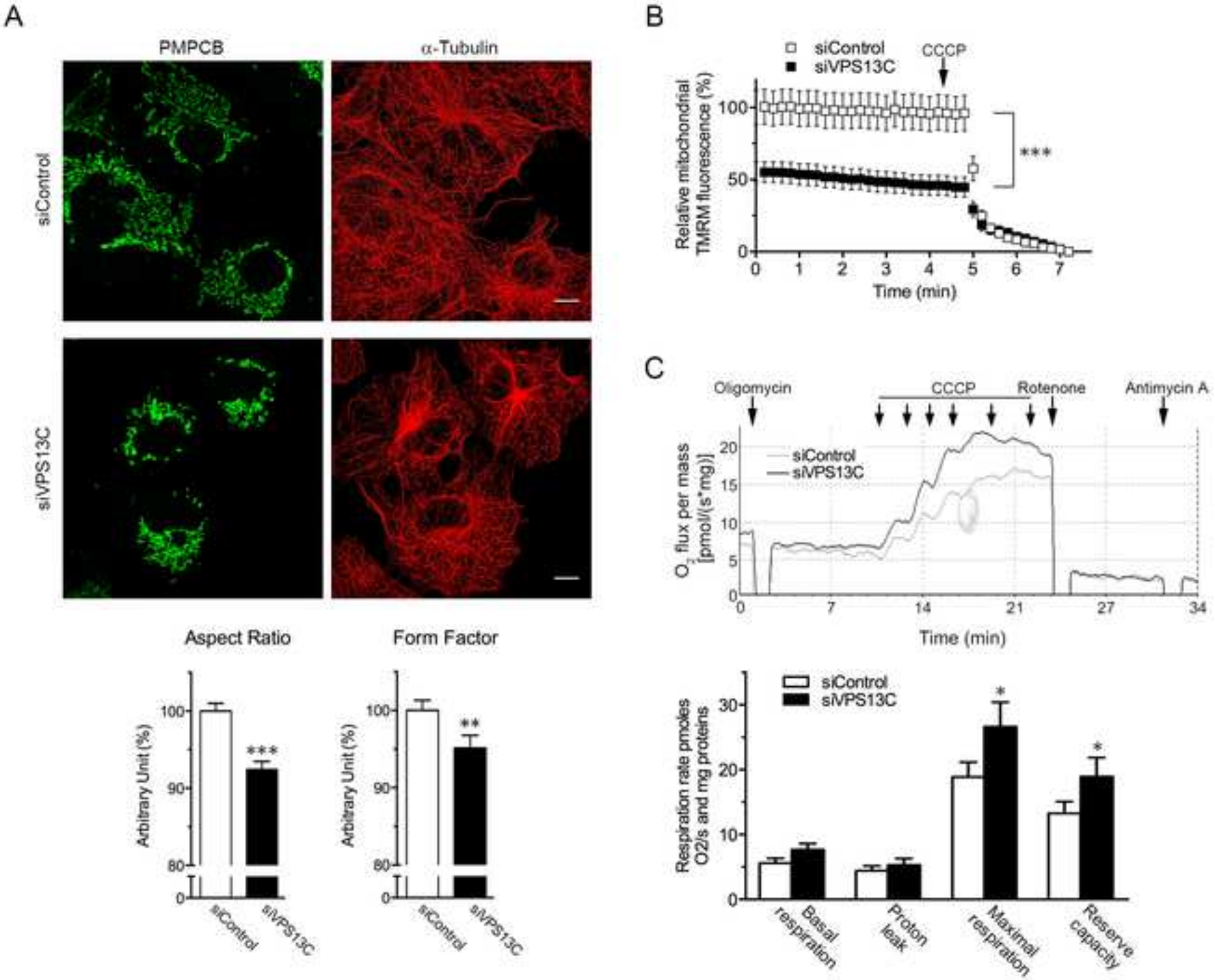

Figure 5

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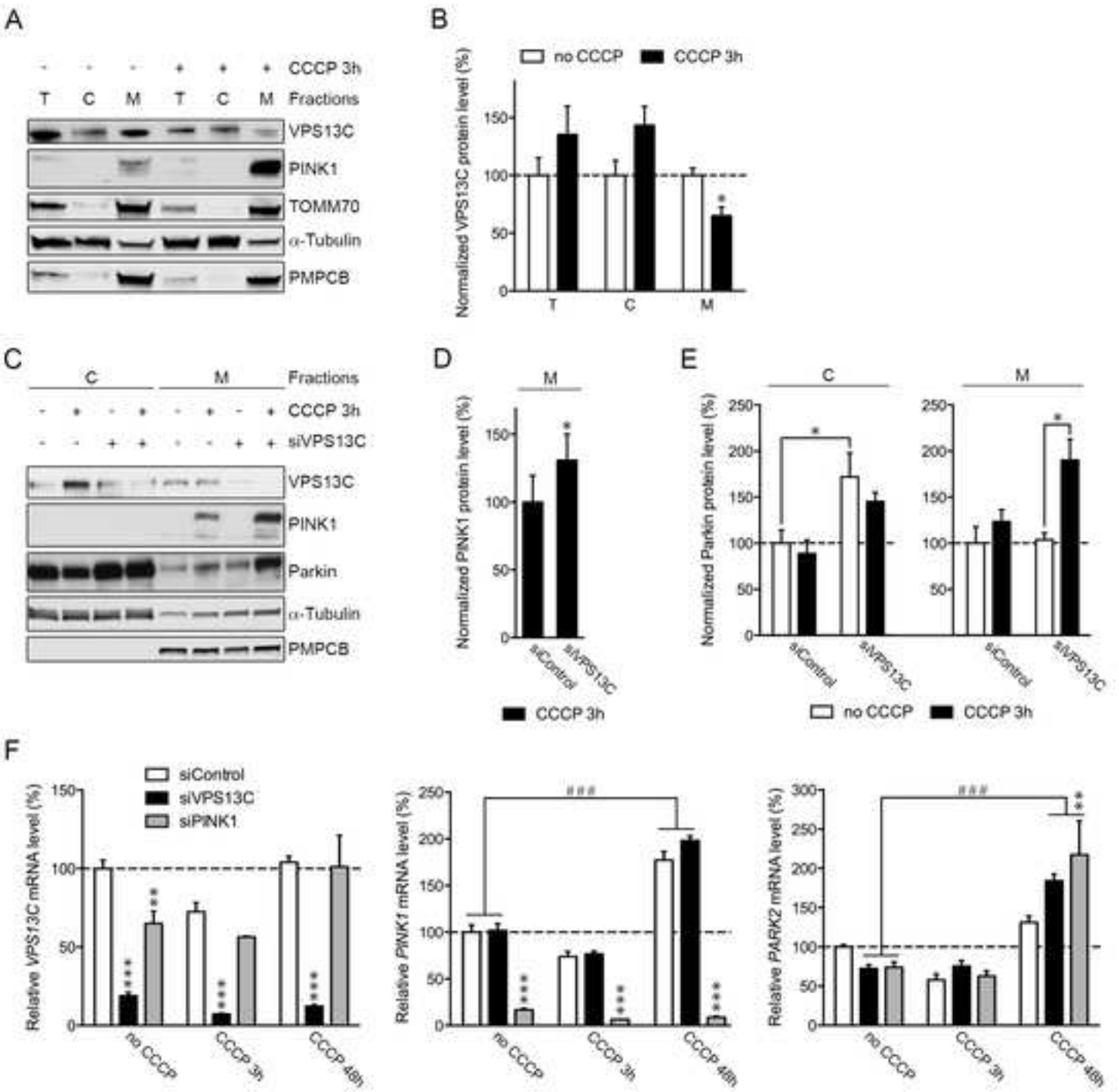

Figure 6

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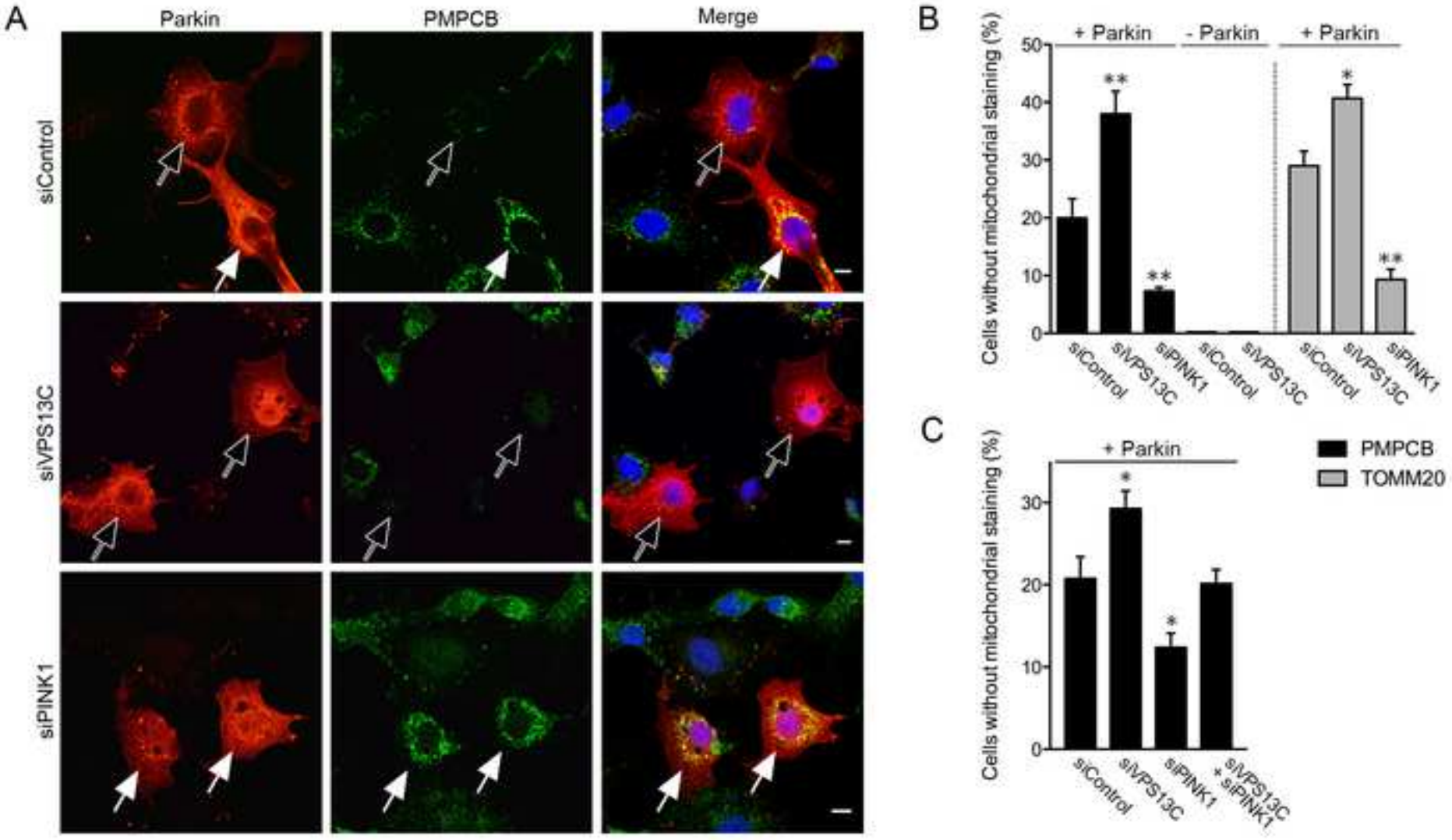

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Spreadsheets_Tables S1-S8.xlsx