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(54) **Title:** METHODS FOR DIAGNOSIS OF NEURODEGENERATIVE BRAIN DISEASES

(57) **Abstract:** The present application relates to the field of human genetics, particularly in relation to the field of neurodegenerative brain diseases. Specifically, the present invention relates to methods and materials to detect human neurodegenerative brain diseases, more particularly Frontotemporal lobar degeneration (FTLD). Provided herein are diagnostic assays for the detection of neurodegenerative brain diseases, kits for performing these assays, and methods to treat these diseases.



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Methods for diagnosis of neurodegenerative brain diseases

Field of the invention

The present application relates to the field of human genetics, particularly in relation to the field of neurodegenerative brain diseases. Specifically, the present invention relates to methods and materials to detect human neurodegenerative brain diseases, more particularly Frontotemporal lobar degeneration (FTLD). Provided herein are diagnostic assays for the detection of neurodegenerative brain diseases, kits for performing these assays, and methods to treat these diseases.

Background

Frontotemporal lobar degeneration (FTLD) is a heterogeneous group of neurodegenerative dementias and mainly affects people younger than 65 years of age. Although several highly penetrant genetic variations (= causative mutations) and more common genetic variations that act as genetic risk factors are associated with genes known to be involved in the pathogenesis of FTLD, together these genes only explain a fraction of the occurrence of the disease. Indeed, in about 60% of the families with FTLD, the genetic cause is still unknown. Thus, there is a need to find the remaining genes that harbor causative or risk mutations/variations for FTLD. Not only may these help in diagnosis, but such genes may identify new molecular pathways offering therapeutic opportunities for FTLD.

Summary

In order to identify novel risk genes for FTLD, we performed whole genome sequencing (WGS) on an affected sib pair suffering from early onset FTD (age at onset 40 and 42 years). Analysis of the filtered WGS variants in an extended Belgian FTD patient (n=590) and control cohort (n=1,314) revealed the presence of the p.A444P missense mutation in the vacuolar protein sorting 13 homolog C gene (VPS13C) in five unrelated FTD patients while it was absent in control individuals. Screening of the 86 coding exons of VPS13C in the Belgian FTD population resulted in the identification of 17 additional missense mutations, two small deletions and one frameshift mutation. All these variants were absent in age- and geographically matched control individuals. Immunofluorescence staining and immunoblotting analysis on lymphoblast cell lines of patients carrying patient-only VPS13C missense mutations demonstrated a decreased expression of endogenous VPS13C protein in patients compared to mutation-negative controls. In conclusion, we identified 21 novel mutations in the VPS13C gene in 25 of 590 FTD patients (4.2%), that were absent in a large control series (n=1,314). Tested mutations were associated with decreased expression of endogenous protein in patient-derived cells. This is both

suggestive for a loss of function mechanism, and allows screening for diagnosis by assaying VPS13C protein levels.

Brief description of Figures

Figure 1: VPS13C transcripts and protein. (A) Schematic presentation of the four transcripts of VPS13C. Coding regions are shown as thick blocks while untranslated regions are shown as thinner blocks. Thin lines represent intronic regions. **(B)** Schematic presentation of the VPS13C protein with the known protein domains (Chorein, DUF1162, Apt1 and ATG_C) annotated with their amino acid coordinates. Mutations observed in FTD patients-only are indicated by red vertical lines above the protein diagram.

Figure 2: Decreased endogenous protein expression of VPS13C in mutation carriers compared to healthy controls. (A) Epstein Barr Virus transformed lymphoblast cells of a mutation-free control and patient DR481.1 carrying the *VPS13C* A444P missense mutation (table 2), immunostained for endogenous VPS13C. **(B)** Double-blind quantification of the corrected total cell fluorescence per cell averaged over two independent experiments per individual, both including 10 randomly selected cells per slide. Vertical lines indicate standard deviation (SD). Control: mutation-free control (n = 4); 'P + C': group patients carrying a mutation that was also observed in at least 1 control. 'Patient-only': group of mutations observed in patients only. **(C)** Western blot analysis of mutation-free controls (n = 3), patients carrying a VPS13C mutation that was present in both patients and controls (p.N2563S and p.S2904L) and patients-specific mutations (p.W395C/p.A444P, p.A444P, p.N2517Y and p.T1218A). **(D)** Quantification of VPS13C expression, normalized for the expression of α -tubulin. *** 0.0001 <P < 0.001; ****P < 0.0001

Figure 3: Cellular localization of endogenous VPS13C in HeLa cells. HeLa cells were double-stained with anti-VPS13C antibody in green and in red an antibody staining either **(A)** Giantin (Golgi); **(B)** PDI (Endoplasmic reticulum); **(C)** CD63 (late endocytic multivesicular bodies); or **(D)** LAMP1 (lysosome). In the overlay images of the right panel nuclei are stained in blue with DAPI. Endogenous VPS13C showed a diffuse staining pattern in the nucleus and in the cytoplasm.

Figure 4: VPS13C immunoreactivity in frontal cortex of a VPS13C mutation carrier and a healthy control individual. (A) Frontal cortex sections of a healthy control individual and patient DR481.1 (p.A444P mutation) were stained against VPS13C. Endogenous VPS13C was mainly observed in the astrocytes that were part of the BBB (Subpial and around blood vessels). In addition, astrogliosis in the deeper cortical layers was observed in patient DR481.1 while it was absent in the frontal cortex of a healthy control individual. **(B)** Immunoreactivity against astrocytes was confirmed performing an immunofluorescent double staining of VPS13C and GFAP, an astrocyte-specific marker in the frontal

cortex of DR481.1. **(C)** Schematic presentation of the protein localization of the two different epitopes recognized by the different VPS13C antibodies (orange). The four known protein domains of VPS13C are also shown. **(D)** Frontal cortex sections of a healthy control individual stained with two different VPS13C antibodies, Novus NBP1-94043 and NBP1-94044 (Novus Biological). NBP1-94043 showed VPS13C immunoreactivity only in the astrocytes while NBP1-94044 showed VPS13C immunoreactivity in the neurons.

Figure 5: VPS13C turn-over assay in HEK293T cells transfected with either wild type VPS13C, p.W395C or p.A444P.

Figure 6: EGFR assay performed on HEK293T cells expressing either wild type VPS13C, p.W395C mutant VPS13C or p.A444P VPS13C.

Description

Definitions

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. The drawings described are only schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn on scale for illustrative purposes. Where the term "comprising" is used in the present description and claims, it does not exclude other elements or steps. Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated.

Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

The following terms or definitions are provided solely to aid in the understanding of the invention. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Plainsview, New York (1989); and Ausubel et al., Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999), for definitions and terms of the art. The definitions provided herein should not be construed to have a scope less than understood by a person of ordinary skill in the art.

The terms “frontotemporal lobar degeneration (FTLD)” or “frontotemporal dementia (FTD)” are used as synonyms herein, and refers to the disease phenotype classified under G31 in ICD-10.

The term “VPS13C” as used herein refers to the vacuolar protein sorting 13 homolog C gene (Gene ID: 54832 in humans), and its encoded protein, as well as the mRNA that is transcribed from the gene. The VPS13C gene encodes a single-pass type II membrane protein that is a member of the S9B family in clan SC of the serine proteases. This protein has no detectable protease activity, most likely due to the absence of the conserved serine residue normally present in the catalytic domain of serine proteases. However, it does bind specific voltage-gated potassium channels and alters their expression and biophysical properties. Alternate transcriptional splice variants, encoding (at least) three different isoforms, have been characterized. Unless specifically mentioned otherwise, the term VPS13C encompasses the different isoforms. The phrase “mutation in the VPS13C gene” or “mutation in VPS13C” as used herein refers to mutations in the coding sequence of the gene as well as mutations in the non-coding regions (e.g. introns, promoter region, UTR). Examples of mutations include, but are not limited to, substitutions, insertions, deletions, indels, amplifications, inversions, copy-number-variations (CNV). Mutations can have different effects, e.g. loss-of-function (up to complete loss-of-function, i.e. amorphic mutations), gain-of-function, dominant negative mutation, and so on. Importantly, mutations resulting in decreased transcription or translation are also regarded as loss-of-function mutations. Note that mutations as used herein covers both mutations that are causative of the disease (high penetrant, pathogenic mutations) as those that only confer an increased risk of developing the disease (i.e. low to median penetrant mutations). According to particularly envisaged embodiments, the mutations are null mutations.

Although not limited thereto, like for other genes, a mutation in the VPS13C gene will typically be a small scale mutation, i.e. only comprise one or a few nucleotides (note that, in case of frameshift or nonsense mutations, a change of even one nucleotide in the genomic sequence may lead to a much larger difference in the resulting gene product). Larger mutations are envisaged as well within the definition.

With “functional expression” of VPS13C, it is meant the transcription and/or translation of functional gene product. “Functional expression” can be deregulated on at least three levels. First, at the DNA level, e.g. by absence or disruption of the gene, or lack of transcription taking place (in both instances preventing synthesis of the relevant gene product). The lack of transcription can e.g. be caused by epigenetic changes (e.g. DNA methylation) or by loss of function mutations. A “loss-of-function” or “LOF” mutation as used herein is a mutation that prevents, reduces or abolishes the function of a gene product as opposed to a gain-of-function mutation that confers enhanced or new activity on a protein.

LOF can be caused by a wide range of mutation types, including, but not limited to, a deletion of the entire gene or part of the gene, splice site mutations, frame-shift mutations caused by small insertions and deletions, nonsense mutations, missense mutations replacing an essential amino acid and mutations preventing correct cellular localization of the product. Also included within this definition are mutations in promoters or regulatory regions of the VPS13C gene if these interfere with gene function. A null mutation is an LOF mutation that completely abolishes the function of the gene product. A null mutation in one allele will typically reduce expression levels by 50%, but may have severe effects on the function of the gene product. Note that functional expression can also be deregulated because of a gain of function mutation: by conferring a new activity on the protein, the normal function of the protein is deregulated, and less functionally active protein is expressed.

Second, at the RNA level, e.g. by lack of efficient translation taking place – e.g. because of destabilization of the mRNA (e.g. by UTR variants) so that it is degraded before translation occurs from the transcript. Or by lack of efficient transcription, e.g. because a mutation introduces a new splicing variant.

Third, at the protein level, e.g. because of protein instability, proteins with reduced functionality or activity (e.g. enzymatic activity, or binding activity, such as the binding to voltage-gated potassium channels), truncated proteins, and/or proteins with altered function (e.g. as a result of a gain of function mutation). For instance, while a truncated protein may be equally expressed as the wild type counterpart, this will typically result in a decrease in functional expression (or functional expression levels), since the truncated protein will be less active (or less functional).

Notably, another way in which functional VPS13C may be decreased, is by affecting post-translational modifications, such as glycosylation. Indeed, it is known that correct glycosylation is important for protein function, and we have identified several mutations in NBD patients that affect glycosylation. Thus, mutations affecting glycosylation of VPS13C and thereby its function are also envisaged as mutations affecting functional levels of VPS13C. Again, the essence is that a decrease in VPS13C functionality and/or its levels will increase risk of, or even cause, a neurodegenerative brain disease.

It is an object of the invention to provide methods of diagnosing a neurodegenerative brain disease (NBD) in a subject, particularly of diagnosing FTLD, comprising determining the presence of one or more mutations in the VPS13C gene and/or determining the functional expression levels of VPS13C in a sample of said subject. Indeed, a decrease in functional levels of VPS13C (through any mechanism: e.g. by sole presence of mutated VPS13C (and no wild type VPS13C), or by decreased levels of wild type VPS13C) will affect the risk of developing a neurodegenerative brain disease (particularly in case of a

relatively small decrease) or even be causative of FTLD (particularly in cases where functional VPS13C levels are significantly affected).

In case expression levels of VPS13C are monitored, altered expression is indicative of the presence of a NBD. Expression levels can be assessed using methods well known in the art. Without being limited to a particular technology, this can be using quantitative RT-PCR (e.g. for determining mRNA levels of VPS13C) or using ELISA or Western Blot (e.g. for determining protein levels of VPS13C). As shown in the Examples section, several antibodies already exist for detection of VPS13C, and it is well within the capabilities of the skilled person to create further antibodies (or other affinity reagents to detect VPS13C protein, such as, but not limited to, nanobodies, peptides or peptidomimetics, alphabodies, aptamers, and DARPins).

Additionally or alternatively, the presence of one or more mutations in the VPS13C gene may be determined. Most particularly, the mutations will have a deleterious effect on VPS13C gene function, either by altering (e.g. decreasing) expression levels of the gene, or by decreasing or altering its function.

It is particularly envisaged that the one or more mutations are selected from mutations affecting the coding regions of VPS13C, particularly exonic mutations. Mutations affecting the coding regions of VPS13C typically are selected from missense mutations, nonsense mutations, frame-shift mutations and indels. According to particular embodiments, mutations affecting translation into VPS13C protein or transcript stability that are not in the coding regions of VPS13C are also envisaged. Typically, these are mutations in the translation initiation codon or in regulatory sequences such as those regulating splicing, transcript stability or turn-over. According to these embodiments, the VPS13C mutations are selected from mutations affecting transcript stability, translation or protein function and/or conformation.

According to particular embodiments, one or more of the mutations affect residues that are conserved between VPS13A and VPS13C. According to alternative particular embodiments, at least one of the mutations is selected from those listed in Table 2.

The methods can be performed using direct sequencing methods or others. According to particular embodiments, detection of mutations is done using (e.g. DNA and/or cDNA) sequencing, a hybridization assay or PCR-based assay, a cytogenetic method, or pulsed-field gel electrophoresis. According to particular embodiments the sample in which mutations in VPS13C are determined is selected from blood, fibroblasts or tissue. According to further embodiments the sample in which

expression of VPS13C is determined is selected from tissue, fibroblasts, iPS cells, neuronal cells or cell lines, and urine.

According to a further aspect, kits are provided comprising at least one primer or probe suitable to determine the presence of one or more mutations in VPS13C. According to alternative/additional
5 embodiments, kits are provided comprising suitable means for determining the expression levels of VPS13C, e.g. RT-qPCR primers and Taqman probes. When detection is at the protein level, kits typically comprise at least one reagent chosen from e.g. an antibody, a nanobody (single domain antibody), an aptamer, a peptide or peptidomimetic, an alphabody, and a DARPin directed against VPS13C.

In yet another aspect, the VPS13C protein, or nucleic acid encoding said protein, is provided for use as
10 a medicament. Most particularly, the VPS13C protein, or nucleic acid encoding said protein, is provided for use as a diagnostic.

According to specific embodiments, the VPS13C protein, or nucleic acid encoding said protein, is provided for use in treating FTLD as described herein. Treating can also mean delay the onset of, or prevent the onset of, FTLD.

15 This is equivalent as saying that methods of treating FTLD in a subject in need thereof are provided, comprising restoring the levels of VPS13C in said subject (e.g. increasing VPS13C levels in case of a loss-of-function). It is specifically envisaged that the levels of VPS13C are increased by administering the VPS13C protein, or nucleic acid encoding said protein, to said subject. This can for instance be achieved using a gene therapy method.

20 However, it is also envisaged that levels of VPS13C can also be restored to normal levels by administering a compound that restores VPS13C expression. For instance, in case of reduced VPS13C levels, the levels can be increased. In case of excessive VPS13C expression (e.g. because of a gain-of-function), the levels can be decreased.

Accordingly, in a further aspect, methods to screen for compounds that restore VPS13C expression are
25 provided, comprising:

- Providing a sample of cells with decreased functional expression of VPS13C; or providing a sample of cells expressing a mutant form of VPS13C;
- Adding a compound to said cells;
- Evaluating the expression of VPS13C after addition of the compound.

It is to be understood that although particular embodiments, specific configurations as well as materials and/or molecules, have been discussed herein for cells and methods according to the present invention, various changes or modifications in form and detail may be made without departing from the scope and spirit of this invention. The following examples are provided to better illustrate particular embodiments, and they should not be considered limiting the application. The application is limited only by the claims.

Examples

Materials and methods

Belgian FTD patient and control cohort

The Belgian FTD cohort consisted of 590 genealogically unrelated index patients (mean onset age 62 ± 10 years; 45.7% female) recruited in the framework of the Belgian Neurology (BELNEU) consortium, a multicenter collaboration of dementia expertise centers in Belgium (Gijssels et al., 2012, Van Langenhove et al., 2013). Index patients were evaluated according to a standard protocol including a detailed clinical history of patients and family, neurological examination, and neuroimaging. Clinical diagnosis of FTD was reached in consensus by two neurologists according to the international Lund and Manchester group criteria for FTD (Neary et al., 2005). For 3.0% of patients, neuropathology examination was available confirming the clinical diagnosis. A positive family history of dementia, defined by the presence of at least one first-degree relative with dementia, was recorded in 29.5% of patients. Screening of the known FTD genes revealed the presence of 38 GRN mutations (6.4%), 8 MAPT mutations (1.3%), 40 C9orf72 repeat expansions (6.8%), 8 TBK1 mutations (1.3%), 2 VCP mutations (0.3%) and 1 CHMP2B mutation (0.2%) (Cruts et al., 2006, van der Zee et al., 2007, van der Zee et al., 2008, van der Zee et al., 2009, Gijssels et al., 2012, Van Langenhove et al., 2013) (Gijssels et al, unpublished data).

In FTD family DR479, genomic DNA for WGS was available of the index patient DR479.1 with onset age of 40 years, and a sibling diagnosed with FTD with onset age 42 years. Both FTD patients were diagnosed according to the international Lund and Manchester group criteria for FTD (Neary et al., 2005) and were negative for mutations in the known FTD genes GRN, C9orf72, MAPT, TBK1, VCP and CHMP2B, as well as for mutations in the ALS genes TDP-43 and FUS. In the index patient, mutations in all major genes associated with neurodegenerative brain diseases were excluded by massive parallel sequencing of a panel of 16 genes (list of genes is available upon request).

The control cohort consisted of 1,314 unrelated age- and geographically matched individuals (mean age at inclusion 66 ± 13 years, 57.9% female). These control individuals were either recruited from partners of patients visiting the Memory Clinic of ZNA Middelheim and Hoge Beuken, Antwerp and screened for neurological or psychiatric antecedents or neurological complaints, or were

community-recruited control individuals with a Mini Mental State Examination (MMSE) score >26 (Folstein et al., 1975), and lacking familial history of dementia.

All participants and/or their legal guardian provided written informed consent for participation in clinical and genetic studies. The clinical study protocol and the informed consent forms of patient
5 ascertainment were approved by the Ethics Committee of the respective hospitals involved in cohort sampling. The genetic study protocols and informed consent forms for genetic studies were approved by the Ethics Committees of the University of Antwerp and the University Hospital of Antwerp, Belgium.

10 *Whole genome sequencing*

WGS was performed by combinatorial probe anchor ligation (cPAL) chemistry (Complete Genomics Inc., Mountain View CA, USA) to independently assay each base from patterned nanoarrays of self-assembling DNA nanoballs, with an average read coverage of 45 to 87x (Drmanac et al., 2010). Read mapping and variant identification was performed using Complete Genomics' proprietary software.
15 GenomeComb (Reumers et al., 2012) was used to select variants of high quality based on a sequence coverage of at least 20x, a variant call score (Complete Genomics Inc.) of ≥ 60 dB, i.e. a probability of $\geq 106:1$ describing the likelihood of the variant call compared to the second most likely call, and genomic location outside repeat regions marked as simple or satellite repeats by RepeatMasker v3.0 (Smit et al., 1996-2010). Next, we selected intragenic variants with predicted impact on the encoded
20 protein sequence, excluding intronic, noncoding and synonymous variants. Subsequently, variants in public variant databases (1000 Genomes Project Phase 1 Release and dbSNP v138) with a minor allele frequency (MAF) of >1% were excluded. The selected variants were prioritized for further analyses based on predictions of damaging effects on protein function using prediction programs SNAP (Screening for Non-Acceptable Polymorphisms, <https://www.rostlab.org/services/snap/>), SNPs&GO
25 (<http://snps-and-go.biocomp.unibo.it/snps-and-go/>) and PMUT (<http://mmb2.pcb.ub.es:8080/PMut/>) (Ferrer-Costa et al., 2005, Bromberg and Rost, 2007, Calabrese et al., 2009). All variants that were predicted to be damaging by at least one prediction program were selected.

Variant Genotyping

30 Filtered and prioritized WGS variants were genotyped by MassArray® iPLEX assays, followed by Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight (MALDI-TOF) mass spectrometry (Sequenom Inc., Hamburg, Germany). FASTA input files for primer design were generated using GenomeComb (Reumers et al., 2012). Polymerase chain reaction (PCR) and extension primers were designed using

Assay Design 3.0 Software (Sequenom). Genotypes were scored both automated (MassArray Typer version 4.0) and by a researcher.

Mutation screening

5 PCR-based amplification of 86 exons comprising the complete VPS13C coding DNA sequence (CDS) was performed by the Multiplex Amplification of Specific Targets for Resequencing (MASTR) technique (Multiplicom N.V., Niel, Belgium). Targets were amplified in four multiplexed PCR reactions and amplicons were uniquely labeled by a pair of tags based on the Nextera XT shotgun library preparation protocol (Illumina, San Diego, CA, USA) and containing sample-specific indices (Lange et al., 2014). 384
10 uniquely barcoded sequencing libraries were pooled and sequenced in one run on the MiSeq platform using the MiSeq V2 chemistry generating paired-end sequence reads of 250 nucleotides (Illumina, San Diego, CA, USA). After sample demultiplexing, sequence reads were mapped using the Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009, 2010) to a minigenome consisting of the combined target sequences extracted from the human genome reference sequence hg19. Sequence variants were
15 called using the Genome Analysis Toolkit (GATK) (McKenna et al., 2010, DePristo et al., 2011) and variants were annotated using GenomeComb (Reumers et al., 2012).

All rare variants with MAF <1% were validated by PCR-based amplification of genomic DNA followed by Sanger sequencing using the BigDye® Terminator Cycle Sequencing kit v3.1 (Applied Biosystems) on an ABI3730 automated sequencer (Applied Biosystems). Primers were designed using Primer3 (Rozen
20 and Skaletsky, 2000). gDNA numbering of VPS13C variants was relative to nucleotide 1 in GenBank Accession Number NG_027782.1. Coding variants were numbered relative to the translation initiation codon in the largest VPS13C transcript (GenBank Accession Number NM_020821.2). Amino acid changes were numbered according to the largest VPS13C isoform (GenPept Accession Number NP_065872.1).

25

In silico predictions

To predict the impact of non-synonymous variants in the VPS13C gene, the Sorting Intolerant From Tolerant (SIFT) Related sequences prediction program was used (Ng and Henikoff, 2003). Therefore, a list of VPS13C fasta sequences from different vertebrates was used to predict the pathogenicity of the
30 variants. In addition, we calculated the Combined Annotation Dependent Depletion (CADD_Phred) scores for all novel, non-synonymous VPS13C missense variants (Kircher et al., 2014).

Cell culture

Human cervical carcinoma (HeLa) cells were cultured in Modified Eagles medium (MEM, Life Technologies), supplemented with 15% fetal calf serum (Sigma Aldrich), 2 mM L-glutamine (Life Technologies) and 500 U/500 µg penicillin/streptomycin (Life Technologies). Lymphoblasts, immortalized by Epstein Barr virus transformation of lymphocytes collected from whole blood on lithium heparin according to standard procedures (Brouwers et al., 2007, Gijssels et al., 2008), were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Life Technologies), supplemented with 15% fetal calf serum (Sigma Aldrich), 2 mM L-glutamine (Life Technologies) and 500 U/500 µg penicillin/streptomycin (Life Technologies).

Immunocytochemistry

Lymphoblast cells (1x10⁵/ml) were attached to coated microscope glass slides (Superfrost Plus®, Menzel-Gläser) using a Cytospin™ 4 cytocentrifuge (Thermo Scientific). Next, the cells were fixed in 4% paraformaldehyde in PBS, and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 2 min. Fixed cells were blocked with 1% bovine serum albumin (Merck) combined with 10% normal mouse or normal swine serum (Dako) in PBS for 1 hour at room temperature. Cells were incubated overnight at 4°C with goat anti-VPS13C (1:250, Santa Cruz). To visualize the primary antibody, the donkey-anti goat Alexa Fluor 488 secondary antibody was used (1:500, Invitrogen), and 4',6-diamidino-2-phenylindol (DAPI, Bio-Rad, Hercules, CA, USA) was used as a nuclear counterstain.

HeLa cells (1x10⁵/ml) were grown on coverslips, fixed for 20 min with 4% paraformaldehyde in PBS and blocked for 1 h in normal donkey serum (1:500, Merck). Cells were then double stained overnight at 4°C with goat anti-VPS13C (1:250, Santa Cruz) and antibodies against Golgi apparatus (rabbit anti-Giantin, 1:1000, Covance), endoplasmic reticulum (mouse anti-PDI, 1:100, Abcam) or endosomes (mouse anti-CD63 and mouse anti-LAMP1, 1:250, Abcam). To visualize the primary antibodies, the cells were incubated with secondary antibodies conjugated to Alexa Fluor 488 (1:500, Invitrogen) or Alexa Fluor 594 (1:500, Invitrogen) for 1 h at room temperature. DAPI was used as a nuclear counterstain.

qRT-PCR

Total RNA was isolated from lymphoblast cells or crushed frozen human brain using the Ribopure kit (Ambion) and treated with DNase (Turbo DNase Kit; Ambion). First-strand cDNA was synthesized with SuperScript III First-Strand Synthesis System (Invitrogen) utilizing random hexamer and oligo dT primers. PCR was carried out for 48 cycles with VPS13C-specific forward and reverse primers using a Power SYBR Green PCR Master Mix (Life Technologies) on an ABI Viia™7 Real-Time PCR System (Applied Biosystems).

Western blot

Cultured lymphoblast cells were washed with ice-cold PBS and lysed in modified radioimmune precipitation buffer (RIPA: 1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 0.5% Na-Doc, 1% NP-40, 50 mM Tris-HCL; pH, 8.0) supplemented with protease and phosphatase inhibitor (2x Complete Protease inhibitor cocktail and 1x Phosphostop phosphatase inhibitor cocktail; both from Roche). Lysates were sonicated on ice, cleared at 20,000 g for 15 min at 4°C and supernatants was used for immunoblotting. Protein concentrations were measured using a BCA Protein Assay Kit (Perbio Science). Equal amounts of protein were separated on a 3-8% Tris-Acetate gel (Life Technologies) and electroblotted onto a polyvinylidene difluoride membrane (Hybond P; Amersham Biosciences, Aylesbury, UK). Membranes were blocked in 5% skimmed milk in PBS and probed with a primary antibody against VPS13C. Immunodetection was performed with specific secondary antibodies conjugated to horse-radish peroxidase and ECL-plus chemiluminescent detection system (Amersham Biosciences). The bands were quantified on an ImageQuant LAS4000 imaging system (GE Healthcare Life Sciences). Quantitative data were normalized to the signal obtained for α -tubulin.

Immunohistochemistry

For immunohistochemistry (IHC), 4- μ m-thick sagittal sections were cut using an automated HM355 rotary microtome (Microm International). Sections were deparaffinized, rehydrated, and pretreated with citrate buffer to enhance immunoreactivity. Brain tissue was immunostained overnight at 4°C with a primary antibody to vacuolar protein sorting 13 homolog C (VPS13C; Novus Biologicals) and developed using 3,3'-diaminobenzidine (DAB). Counterstaining for cellular structures was performed with hematoxylin according to standard procedures.

Image acquisition and quantification

IHC images were taken on an Axioskop 50 light microscope (Zeiss) equipped with a CCD UC30 camera (Olympus Inc.). Immunofluorescence images were taken with fixed settings on a LSM700 confocal microscope using the Zen 2009 imaging software (Zeiss, Jena, Germany). Expression of VPS13C was quantified by determine the corrected total cell fluorescence per cell. All images were captured with the same exposure time. Staining and quantification were done blind for disease status, in twofold and each experiment contained all samples twice. An average of 20 randomly selected cells per sample and per experiment were analyzed.

Statistics

All results are reported as means and standard deviation (SD) and P values for the description of the statistical significance of differences were calculated by the Multiple Comparisons of a one-way ANOVA using the GraphPad Prism V6.03 software. Values were considered significant if * $0.01 < P < 0.05$;

** $0.001 < P < 0.01$; *** $0.0001 < P < 0.001$; **** $P < 0.0001$.

Example 1. Whole genome sequencing identifies the p.A444P missense mutation in *VPS13C*

We performed WGS in the index patient and affected sibling of family DR479. Both genome sequences covered at least 96.8% of the human reference genome sequence, resulting in a total of 4.60×10^6 variants of which 4.28×10^6 passed quality filters and were absent in repeat regions (**Table 1**). In first instance, protein coding and splice site variants that were present in a heterozygous state in both affected sibs and absent in dbSNP and 1000 Genomes Project were prioritized on and resulted in a total of 377 variants (**Table 1**). Further prioritization of these variants based on impact on protein function (e.g. no synonymous variants) and mutation prediction programs SNAP, SNPs&GO, and PMUT resulted in a total of 81 variants (**Table 1**).

Screening of the 81 prioritized variants in 966 control individuals demonstrated that 29 variants were observed with MAF $>1\%$, leaving 21 variants absent in controls and 31 variants observed in one control individual (0.1%) for further analysis. The 52 variants with a MAF $<1\%$ in controls were screened in the complete cohort of 590 Belgian FTD patients. All variants except one were absent in additional FTD patients. *VPS13C* p.A444P was observed in four additional FTD patients, resulting in a mutation frequency for this variant of 0.8% (5/590).

Example 2. *VPS13C* mutation frequency of 4.2% in Belgian FTD patients

VPS13C has four major transcript variants of which the CDSs are encoded by 86 exons (**Figure 1A**). Sequencing of all 86 coding exons in 590 unrelated FTD patients and 1,314 control individuals revealed 17 additional missense mutations, two small deletions and one frameshift mutation which were not detected in 1,314 control individuals (**Table 2 & Figure 1B**). Together these 21 patient-specific mutations were identified in 25 of 590 index patients (mean age at onset 58 years $\pm$ 15 years; 60% female; 4.2%, **Table 2**). Two missense mutations, p.L1729V and p.I1830T, were present in the 1000 Genomes data with a frequency of 0.0% suggesting its heterozygous presence in only one sample. Additionally, two different missense mutations, p.V348A and p.A444P, were present in dbSNP in absence of frequency data. The affected sib pairs of DR479 were compound heterozygous for p.W395C/p.A444P. In addition to patient-specific missense mutations, we also identified five missense variants that were present in both patients and controls with a MAF $<1\%$ (**Table 3**).

All 21 novel coding patient-specific mutations were scattered all over the complete length of the protein (**Figure 1B**), affecting amino acids that were highly conserved amongst species including chimpanzee, rhesus monkey, mouse, cow and chicken (**Figure 2**). When considering amino acid conservation between VPS13 paralogs, the mutations were enriched for amino acid positions that were conserved in VPS13A (10/21 (48%)) compared to the rare variants that were present in both patients and controls (0/5 (0%)) (**Table 3 & 4**), but not in VPS13B or VPS13D (data not shown). Further, *in silico* prediction of pathogenicity using SIFT predicted no pathogenic effect (tolerated) for only one patient-specific missense mutation, p.I1830T (**Table 4**), compared to three out of five of the variants present in both patients and controls (p.K171E, p.N2563S and p.R2936S, **Table 3**). All other novel patient-specific missense mutations were predicted to be pathogenic (17/18 (94%); **Table 4**). In addition, a pathogenicity score based on the CADD_Phred score (Kircher et al., 2014) scored only five patient-specific missense mutations (p.L1729V, p.I1830T, p.I1854V, p.R2542G and p.D2731Y) below 20. All other missense mutations and the frameshift mutation scored higher than 20, meaning that those substitutions are predicted to be among the 1% most deleterious substitutions in the human genome. Furthermore, two patient-specific missense mutations (p.W395C and p.R1329Q) scored higher than 30, 33 and 34 respectively, resulting in the assumption that these missense mutations were among the 0.1% most deleterious substitutions in the human genome (**Table 4**). The variants that were identified in both patients and controls scored relatively low compared to the patient-specific missense mutations with only two scores above 20 (p.N1205Y and p.N2563S), one score above ten (p.R2936S) and two scores below ten (p.K171E and p.C1187S) (**Table 3**). These data suggest that the patient-specific mutations are more likely pathogenic compared to the variants identified in both patients and controls.

Together, five patients carried the p.A444P mutation (**Table 2**). Haplotype sharing analysis of chromosome 15q21 STR markers flanking *VPS13C* in these five patients showed two different haplotypes: STR genotypes of patients DR903.1 and DR1062.1 were compatible with a shared region of 1.2 Mb with the phase-determined haplotype in family DR479. STR genotypes of DR290.1 and DR481.1 were compatible with a shared haplotype of maximally 5.2 Mb that differed from the DR479 haplotype. Sharing of a smaller haplotype by all p.A444P carriers could not be excluded because all five patients were homozygous for all common SNPs in the *VPS13C* gene identified during our gene sequencing effort. Based on SNP genotype frequencies in the International HapMap Project (2003, 2004, Deloukas and Bentley, 2004), the frequency of this haplotype was estimated at 0.027% (data not shown).

Example 3. A significant reduced expression of endogenous VPS13C in FTD patients compared to healthy controls

Expression of endogenous VPS13C protein was studied by immunocytochemistry in lymphoblast cell lines of five patients carrying patient-specific VPS13C mutations, two patients carrying a mutation that was seen in both patients and controls, and four mutation-negative control individuals. All mutation-negative controls (n = 4) showed a similar level of endogenous VPS13C. VPS13C expression levels in patients carrying a mutation that was seen in both patients and controls showed no significant difference compared to control levels (p.N2563S (MAF <1%) and p.S2904L (MAF >1%), **Figure 2A & 2B**). However, all cells derived from patient-specific mutation carriers resulted in significantly decreased endogenous VPS13C levels of 44–80% compared to mutation-negative controls (**Figure 2A & 2B**). Cells derived from patient DR479.1, compound heterozygous for p.W395C/p.A444P showed a decrease in VPS13C levels of 44%, compared to 55% in the patients carrying only the p.A444P mutation, suggesting that the p.W395C missense mutation has a detrimental impact on the protein. This significantly reduced expression of endogenous VPS13C in lymphoblast cell lines of patient DR479.1 was confirmed using immunoblotting, where the VPS13C protein is almost absent (**Figure 2C & 2D**). The protein levels of the other patient-specific mutations showed variable expression on a Western blot, but patient-specific mutations all resulted in decreased VPS13C protein levels, whereas this is not the case for mutations present in both patients and controls. A difference in gene expression was not observed at transcript level (data not shown).

Example 4. Subcellular expression of endogenous VPS13C in Golgi and endoplasmic reticulum

Because the cellular localization of endogenous VPS13C is still unknown, we investigated its subcellular localization in HeLa cells. Immunofluorescence was used to visualize endogenous VPS13C and a variety of marker proteins for subcellular compartments, including giantin (Golgi apparatus), PDI (endoplasmic reticulum), LAMP1 (late endosomes and lysosomes) and CD63 (endosomal marker). Staining of endogenous VPS13C showed a diffuse cytoplasmic and nuclear staining pattern (**Figure 3**). In addition, colocalization of endogenous VPS13C was mainly observed with the Golgi marker Giantin (**Figure 3A**) and the endoplasmic reticulum marker PDI (**Figure 3B**). No co-staining was observed with the endosomal markers LAMP1 and CD63 (**Figure 3C and 3D**).

Example 5. Both glial and neuronal expression of VPS13C observed in human frontal cortex

Since the localization of VPS13C in human brain is still not elucidated, we performed immunohistochemistry on frontal cortex of both mutation-negative controls and patients carrying a patient-specific mutation in *VPS13C* (**Figure 4**). In first instance, the analysis showed VPS13C immunoreactivity in the astrocytes located at the blood brain barrier (Subpial) in both patient and

control brains. In addition, activated astrocytes located in the deeper cortical layers of the frontal cortex of FTLD patients were also immunoreactive against VPS13C (**Figure 4A**). This immunoreactivity against astrocytes that are located at the blood brain barriers confirmed by performing an immunofluorescent double staining against VPS13C and glial fibrillary acidic protein (GFAP), an antibody specific for astrocytes (**Figure 4B**).

When a different antibody against human VPS13C was used, a different staining pattern was observed. The antibody that showed a glial staining pattern recognized an epitope located at amino acids 2574 – 2662 (Novus NBP1-94043; Novus Biologicals; **Figure 4C**). However, when we used an antibody that recognized a different epitope, located at amino acids 1270 – 1357 (Novus NBP1-94044; Novus Biologicals; **Figure 4C**), the staining pattern changed to a complete neuronal pattern instead of a glial pattern (**Figure 4D**). Both antibodies were tested on a western blot to determine its specificity and both recognized the 420 kDa band that corresponds to VPS13C (data not shown).

Example 6. Effect of mutations on protein stability and function

To investigate whether the identified patient-only missense mutations have an effect on protein stability, we performed a VPS13C turn-over assay in transfected HEK293T cells. Protein production was blocked by adding cycloheximide at time point 0 and protein lysates were generated at time points 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours. Both mutants tested (p.W395C and p.A444P) show a faster degradation of the transfected VPS13C compared to wild type transfected VPS13C, suggesting an effect of these two missense mutations on protein stability (see **Figure 5**).

VPS13C mutations have no effect on EGFR degradation

CHMP2B missense mutations are shown to accumulate in endosomes and have an effect on the trafficking and degradation of cargo proteins such as the epidermal growth factor receptor (EGFR). We tested whether the VPS13C mutants p.W395C and p.A444P have a similar effect on EGFR degradation. However, we were not able to show a faster or slower breakdown of EGFR in HEK293T cells expressing VPS13C mutations compared to the wild type VPS13C (see **Figure 6**).

Furthermore, modeling of VPS13C patient-only missense mutations in Vps13 in *Saccharomyces cerevisiae* (yeast) demonstrated a deleterious effect on sporulation, CPY protein sorting, and/or mitochondrial homeostasis (data not shown)

Discussion

WGS was performed on a sib pair suffering from early-onset FTD (age at onset 40 and 42 years of age). We selected WGS because, at that time, sequence coverage of the applied technology was higher (95%) than that of the exome sequencing technologies (65–72%) (Drmanac et al., 2010, Hoischen et al.,

2010). Applying a carefully designed genetic and functional variant filtering strategy, we brought down the number of prioritized variants compatible with autosomal dominant inheritance to 81, of which 52 occurred in less than 1% of geographically matched control individuals. Assuming the presence of founder mutations in the Belgian FTD patients, variant sequencing of an extended series of 590 FTD patients supported only one mutation, i.e. p.A444P in the vacuolar protein sorting 13 homolog C gene (*VPS13C*). The founder effect was supported by two extended STR-defined background haplotypes and possibly one smaller SNP-based haplotype extending across the complete gene. Subsequent sequencing of all 86 coding exons and the exon-intron boundaries of *VPS13C* in the Belgian FTD patient population resulted in the identification of in total 18 novel missense mutations, two small in-frame deletions and one frameshift mutation predicted to result in a premature termination codon, that were not detected in 1,314 age- and geographically matched control individuals. Of note, 17 of the 18 missense mutations were predicted pathological by SIFT, 18 were at amino acid positions that were fully conserved in *VPS13C* orthologs of five vertebrates ranging from chimpanzee to zebra fish on the evolutionary scale, and ten were conserved in human *VPS13A*. In addition, 14 missense mutations received a score of more than 20 using the CADD_Phred score, suggesting that these variants are among the 1% most deleterious substitutions in the human genome (Kircher et al., 2014). These data suggest that at least some of these novel coding mutations are at functional or structural clinically relevant amino acid positions and are unlikely to represent benign polymorphisms. 25 of 590 Belgian FTD patients were carrying a novel coding mutation in *VPS13C* resulting in a mutation frequency of 4.2%. This report is the first to describe clinical mutations in the *VPS13C* gene and describe a link to FTLD. A role for *VPS13C* in other neurodegenerative diseases, namely Parkinson's disease (PD) and Alzheimer's disease (AD) has been tentatively suggested. In PD, a large-scale meta-analysis of genome-wide association studies showed a significant association with a variant (rs2414739) located 155 kb downstream of *VPS13C* (Nalls et al., 2014). We genotyped this variant in our Belgian FTD patient and control cohorts but were not able to find an association ($p = 0.167$, OR = 1.367, 95% CI = 0.878 – 2.130, data not shown). In AD, data of magnetic resonance imaging (MRI) phenotypes were linked to a sample of genome-wide common single nucleotide polymorphisms (Meda et al., 2012). They concluded that *VPS13C* is part of a gene network that was negatively correlated with decreased brain volume in late-onset AD (Meda et al., 2012). Furthermore, loss-of-function mutations in another vacuolar sorting protein, i.e. *CHMP2B*, alias vacuolar protein sorting 2B (*VPS2B*), a component of the endosomal sorting complex required for transport III (ESCRT-III), are known to cause FTD (Skibinski et al., 2005, van der Zee et al., 2008). In our *in vitro* immunohistochemistry studies of lymphoblast cells, *VPS13C* did not co-localize with *CHMP2B* or endosomal protein markers (data not shown), suggesting it is not likely to be

part of the ESCRT-III complex. However, it cannot be excluded that VPS13C and CHMP2B are involved in the same pathway.

VPS13C belongs to a human family of four genes A to D with strong homology to yeast Vps13p, involved in trafficking of membrane proteins between the trans-Golgi network (TGN) and the pre-
5 vacuolar compartment, corresponding to the multivesicular body in animal cells (Redding et al., 1996, Brickner and Fuller, 1997, Velayos-Baeza et al., 2004). The *VPS13C* gene comprises 86 coding exons and alternative splicing results in four different transcripts. All transcripts are ubiquitously expressed, while the longest transcript containing exons 6 and 7 (NM_020821.2) is the predominant splice variant in brain, suggesting brain-specific gene functions (Velayos-Baeza et al., 2004). Further, VPS13C is a
10 vertebrate-specific member of the VPS13 gene family and is likely to be arisen from a gene duplication event of *VPS13A* with which it shows 41% protein sequence identity. Loss-of-function mutations in *VPS13A* are known to cause Chorea-Acanthocytosis (ChAc) (Danek et al., 2005), a multisystemic disorder associated with neurodegeneration of the central nervous system. ChAc is primarily associated with subcortical dementias and patients carrying mutations in the *VPS13A* gene show
15 neurological symptoms similar to FTD, such as changes in behavior and personality, apathy and hyperactivity, and impulsivity and depression (Danek et al., 2005). In addition, both *VPS13A* and *VPS13C* are shown to be a component of the phagosome (Jacobs et al., 2006). Strikingly, among the paralogs of the human *VPS13* gene family, the patient-only *VPS13C* mutations in our study were enriched for amino acid positions that were conserved in *VPS13A* compared to the rare variants
20 present in patients and controls. Also similar to *VPS13C*, knowledge of functional protein domains in *VPS13A* is limited to the N-terminal chorein domain and the C-terminal DUF1162 and ATG_C domains, also observed in *VPS13C*. Therefore, functions of these members of the VPS13 gene family may be related. ChAc mutations are scattered all over the protein and comprise mainly frameshift mutations and small insertion and deletions, but also missense mutations associated with a recessive mode of
25 disease inheritance. Interestingly, in lymphoblasts of FTD patients carrying a *VPS13C* mutation, endogenous *VPS13C* was significantly reduced by 40 to 80% compared to mutation-free cells and cells harboring *VPS13C* mutations observed in both patients and controls. This also suggests that the disease mechanism in *VPS13C*-associated FTD is associated with reduced protein function, similar to the mechanism in *VPS13A*-associated ChAc. Possibly, in contrast to *VPS13A*, at least one function of
30 *VPS13C*, e.g. a brain-specific function, is dosage sensitive whereby a hypomorphic allele leads to the clinical phenotype. Alternatively, autosomal dominant mutations in *VPS13A* result in exon skipping and a premature termination of translation, while mutations in *VPS13C* may result in a decreased expression of *VPS13C* (Saiki et al., 2003).

In addition, we performed a study to determine the subcellular localization of endogenous VPS13C because its cellular localization was not yet reported. We found co-staining of endogenous VPS13C with markers for the Golgi apparatus and the endoplasmic reticulum. This cellular localization is similar to that of VPS13A and VPS13B, which are both reported to co-localize with GM130, a marker for the Golgi apparatus (Seifert et al., 2011, Hayashi et al., 2012). The observation is also consistent with the presence of an apt-1 Golgi localization signal ranging from amino acids 3206 to 3404 at the C-terminal end. We were not able to detect co-staining of endogenous VPS13C with late endosomal and lysosomal markers such as LAMP1 and CD63. This could indicate that VPS13C is mainly expressed in the early-endosomes responsible for the cycling of protein between the TGN and lysosomes, as was already shown in yeast (Brickner and Fuller, 1997). Furthermore, we demonstrated that VPS13C is abundantly expressed in the frontal cortex of the human brain. Dependent on which antibody was used, VPS13C immunoreactivity was observed in neurons or astrocytes. Immunoreactive astrocytes were primarily located at the blood brain barrier in a control and FTLN brain, while activated astrocytes located in the deeper cortical layers were also immunoreactive in an FTLN brain using the Novus NBP1-94043 antibody (Novus Biological). The Novus NBP1-94044 antibody (Novus Biological) showed VPS13C immunoreactivity in the neurons of the cortex. No glial staining was observed. One can argue that at least one of these antibodies shows an aspecific staining pattern of VPS13C, however, we performed a Western blot analysis to test this and both antibodies recognized a 420 kDa band that corresponds to VPS13C. All commercial available antibodies are raised against one of these two epitopes and show similar staining patterns. Therefore, antibodies should be raised against a different epitope of VPS13C to be able to elucidate whether VPS13C is localized in the neurons or in the astrocytes in human brain. Further, an immunohistochemical staining of VPS13A in human brain is not yet reported. However, a previous reports on the localization of VPS13A in brain using subcellular protein extractions showed that VPS13A was predominantly expressed in the microsomal and synaptosomal fractions (Kurano et al., 2007). Furthermore, it seems that the patient-only mutations have an effect on the endogenous expression of VPS13C. The patient-only missense mutations that we identified decrease the expression level of VPS13C in the patient-specific lymphoblasts while the mutations that are present in both patients and controls were predicted neutral in SIFT analysis and did not have an effect on the expression of VPS13C. This implies that the patient-only mutations have a possible effect on protein stability. This reduction in protein expression is also seen in patients with a *VPS13A* mutation, suggesting a similarity between at least *VPS13A* and *VPS13C*. However, further research is needed to conclude if VPS13C has a similar role as VPS13A in the human phagosome and to elucidate the effect of the VPS13C missense mutations in FTD.

Tables

Table 1: Whole-genome sequencing variant filtering results in DR479

Filter	Number of variants ¹
Total	4.60x10 ⁶
Quality	4.34x10 ⁶
STR & microsatellite	4.28x10 ⁶
Coding & splice site	24,805
Zygosity	7,875
dbSNP & 1000 Genomes Browser	377
Variant effect prediction	81

¹Numbers indicate the total number of variants shared by the two patients of DR479 after each filtering step.

Table 2: Patient-specific *VPS13C* missense mutations and clinical characteristics of mutation carriers

<i>VPS13C</i> mutation	Genomic position	rs number	Patient	Sex ¹	Family History ²	Age at onset (years)	Age (years) ³	Disease duration (years)
p.L27P	g.5171T>C	-	DR1066.1	F	U	78	86	≥8
p.I295Sfs37X	g.54865A>G	-	DR1067.1	F	S	41	46	≥5
p.P318S	g.54935C>T	-	DR1105.1	F	S	73	77	≥4
p.V348A	g.56736T>C	rs138433394	DR902.1	M	U	54	66	≥12
p.W395C / p.A444P	g.58053G>C	-	DR479.1	M	F	42	52	≥10
		-	DR479.2	M	F	40	47*	7
p.A444P	g.64879G>C	rs371007037	DR290.1	M	F	75	82*	7
			DR903.1	F	S	29	42	≥13
			DR481.1	F	S	53	68*	15

VPS13C mutation	Genomic position	rs number	Patient	Sex ¹	Family History ²	Age at onset (years)	Age (years) ³	Disease duration (years)
			DR1062.1	M	S	79	89*	10
p.A761T	g.86831G >A	-	DR746.1	F	S	62	70*	8
p.K1071_ A1072deli nsT	g.100765 _100767d elGAA	-	DR1068.1	F	S	58	64	≥6
p.T1218A	g.103621 A>G	-	DR149.1	F	F	54	64*	10
p.R1329Q	g.105948 G>A	-	DR1063	M	S	80	84	≥4
p.D1693E	g.119682 C>A	-	DR905.1	F	U	70	85	≥15
p.L1729V	g.123435 T>G	rs370438 226	DR906.1	F	S	44	66	≥22
p.I1830T	g.124707 T>C	rs143258 929	DR907.1	M	F	68	74	≥6
p.I1854V	g.124778 A>G	-	DR908.1	F	F	48	52	≥4
p.P1914d el	g.128856 delCCT	-	DR700.1	F	F	62	66	≥4
p.L2057K	g.135849 T>C	-	DR1064.1	F	S	31	32	≥1
p.A2118V	g.138212 C>T	-	DR346.1	F	S	73	78*	5
p.N2517Y	g.146088 A>T	-	DR32.1	F	F	57	69	12
p.R2542G	g.146163 C>G	-	DR909.1	M	U	58	69	≥11
p.D2731Y	g.149579 G>T	-	DR1065.1	M	S	63	67	≥4
p.T2941S	g.155266 A>T	-	DR615.1	M	F	54	72	≥18

¹F: Female, M: Male;²F: Familial, S: Sporadic, U: Unknown;

³Age at last examination is shown. In case of decease, age at death is shown with an asterisk.

Table 3: Amino acid comparison with the VPS13 paralog VPS13A and pathogenicity predictions of missense variant present in both patients and controls

<i>VPS13C</i> mutation	Genomic position	rs number	# Patients (%)	# Controls (%)	<i>VPS13A</i>	CADD_Phred Score ¹	SIFT Prediction	RI ²
p.K171E	g.41683A >G	rs150832 196	1 (0.17)	3 (0.25)	-	9.1	Neutral	0.14
p.C1187S	g.103052 G>C	-	1 (0.17)	3 (0.25)	-	5.8	Pathologica 	0.00
p.N1205Y	g.103582 A>T	rs199794 907	1 (0.17)	1 (0.08)	-	26.3	Pathologica 	0.00
p.N2563S	g.147258 A>G	rs201120 398	3 (0.51)	3 (0.25)	G	24.2	Neutral	0.16
p.R2936S	g.155253 A>T	-	1 (0.17)	1 (0.08)	K	11.8	Neutral	0.08

5 ¹CADD_Phred score >20 means that the variant is predicted to be among the 1% most deleterious substitutions in the human genome;

²Reliability index: variants with a RI ≤ 0.05 are predicted to be pathogenic, >0.05 are predicted to be tolerated

10 **Table 4: Amino acid comparison with the VPS13 paralog VPS13A and pathogenicity prediction of patient-specific mutations**

<i>VPS13C</i> mutation	Genomic position	rs number	<i>VPS13A</i>	CADD_Phred score ¹	SIFT Prediction	RI ²
p.L27P	g.5171T>C	-	L	22.7	Pathological	0.01
p.I295Sfs37X	g.54865A>G	-	V	25.3	-	-
p.P318S	g.54935C>T	-	P	27.1	Pathological	0.00
p.V348A	g.56736T>C	rs138433394	V	23.3	Pathological	0.00
p.W395C	g.58053G>C	-	W	33.0	Pathological	0.00

VPS13C mutation	Genomic position	rs number	VPS13A	CADD_Phred score ¹	SIFT	
p.A444P	g.64879G>C	rs371007037	A	27.5	Pathological	0.00
p.A761T	g.86831G>A	-	S	24.5	Pathological	0.01
p.K1071_A10 72delinsT	g.100765_100 767delGAA	-	-	-	-	-
p.T1218A	g.103621A>G	-	-	25.7	Pathological	0.00
p.R1329Q	g.105948G>A	-	-	34.0	Pathological	0.00
p.D1693E	g.119682C>A	-	D	22.9	Pathological	0.00
p.L1729V	g.123435T>G	rs370438226	L	11.9	Pathological	0.02
p.I1830T	g.124707T>C	rs143258929	L	14.8	Neutral	0.26
p.I1854V	g.124778A>G	-	V	10.3	Pathological	0.05
p.P1914del	g.128856delCC T	-	T	-	-	-
p.L2057K	g.135849T>C	-	L	29.3	Pathological	0.00
p.A2118V	g.138212C>T	-	A	29.7	Pathological	0.00
p.N2517Y	g.146088A>T	-	H	20.4	Pathological	0.00
p.R2542G	g.146163C>G	-	R	19.6	Pathological	0.00
p.D2731Y	g.149579G>T	-	P	15.9	Pathological	0.00
p.T2941S	g.155266A>T	-	I	22.6	Pathological	0.01

¹CADD_Phred score >20 means that the variant is predicted to be among the 1% most deleterious substitutions in the human genome;

²Reliability index: variants with a RI ≤ 0.05 are predicted to be pathogenic, >0.05 are predicted to be tolerated

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Claims

1. A method of diagnosing the presence of and/or risk of developing a neurodegenerative brain disease in a subject, comprising determining the presence of one or more mutations in the VPS13C gene and/or determining the functional expression levels of VPS13C in a sample of said subject.
- 5 2. The method according to claim 1, wherein the neurodegenerative brain disease is frontotemporal lobar degeneration (FTLD).
3. The method according to claim 1 or 2, wherein the one or more mutations are selected from mutations affecting the coding region of VPS13C, particularly exonic mutations.
4. The method according to claim 3, wherein the mutations affecting the coding region of VPS13C are
10 selected from missense mutations, nonsense mutations, frameshift mutations, insertions and deletions.
5. The method according to any one of claims 1 to 4, wherein the one or more mutations affect residues that are conserved between VPS13A and VPS13C.
6. The method according to any one of claims 1 to 5, wherein at least one of the mutations is selected
15 from those listed in Table 2.
7. The method according to any one of claims 1 to 6, wherein detection of mutations is done using sequencing, a hybridization assay or PCR-based assay, a cytogenetic method, and pulsed-field gel electrophoresis.
8. The method according to any one of claims 1 to 6, wherein detection of functional expression
20 levels is done using an ELISA.
9. The method according to any one of claims 1 to 8, wherein the sample is selected from blood, fibroblasts, tissue, neuronal cells or cell lines, iPS cells, and urine.
10. A kit comprising at least one primer or probe or at least one affinity reagent suitable to determine the presence of one or more mutations in the VPS13C gene and/or the functional expression
25 levels.
11. The VPS13C protein, or nucleic acid encoding said protein, for use as a medicament.
12. The VPS13C protein, or nucleic acid encoding said protein, for use in treating FTLD.

13. A method of treating FTLD in a subject in need thereof, comprising increasing the functional expression levels of VPS13C in said subject.
14. The method according to claim 13, wherein the levels of VPS13C are increased by administering the VPS13C protein, or nucleic acid encoding said protein, to said subject.
- 5 15. The method according to claim 14, which is a gene therapy method.
16. A method to screen for compounds that increase VPS13C expression, comprising:
 - Providing a sample of cells with decreased functional expression of VPS13C; or providing a sample of cells expressing mutated forms of VPS13C;
 - Adding a compound to said cells;
 - 10 – Evaluating the expression of VPS13C after addition of the compound.

Figure 1

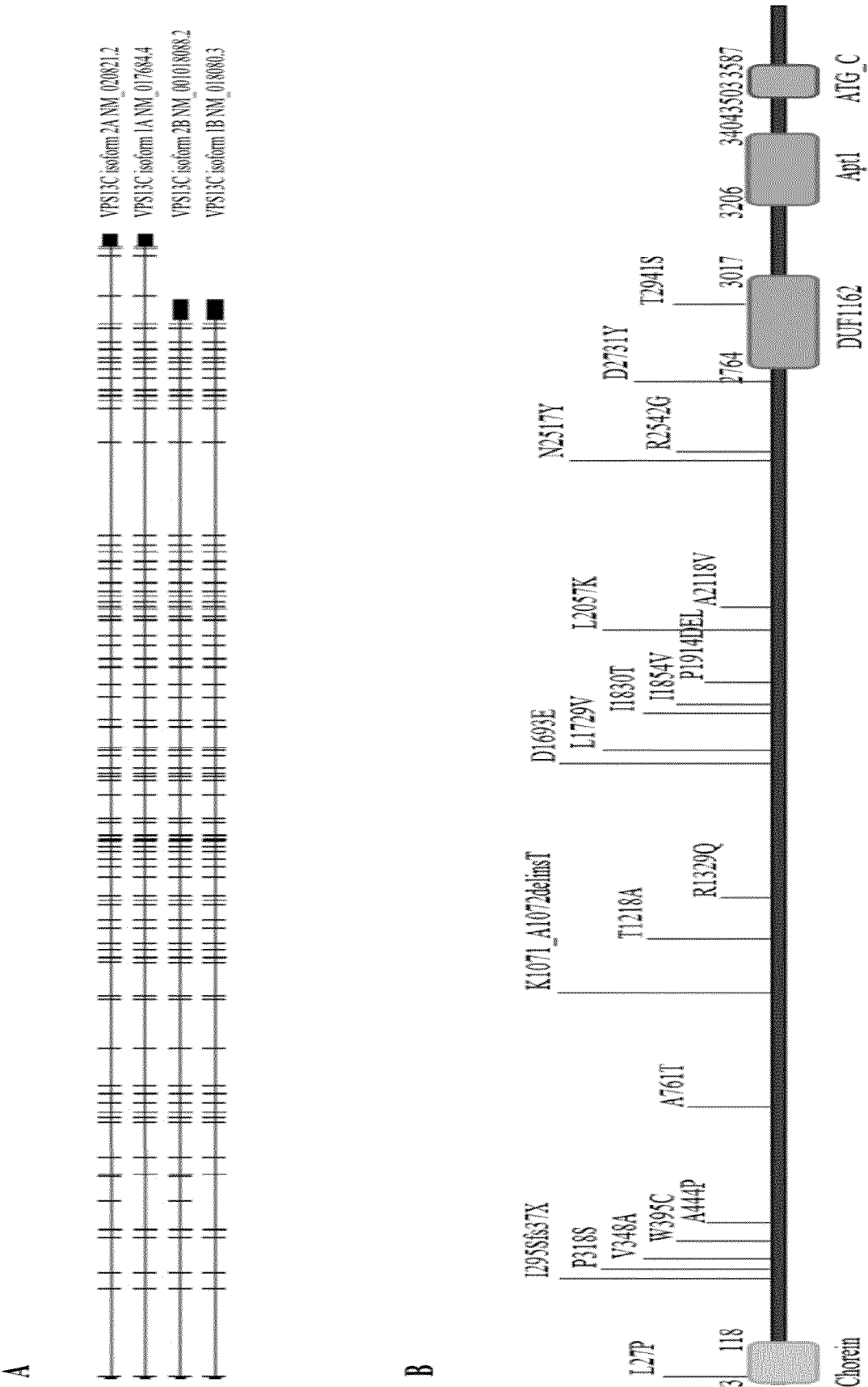


Figure 2

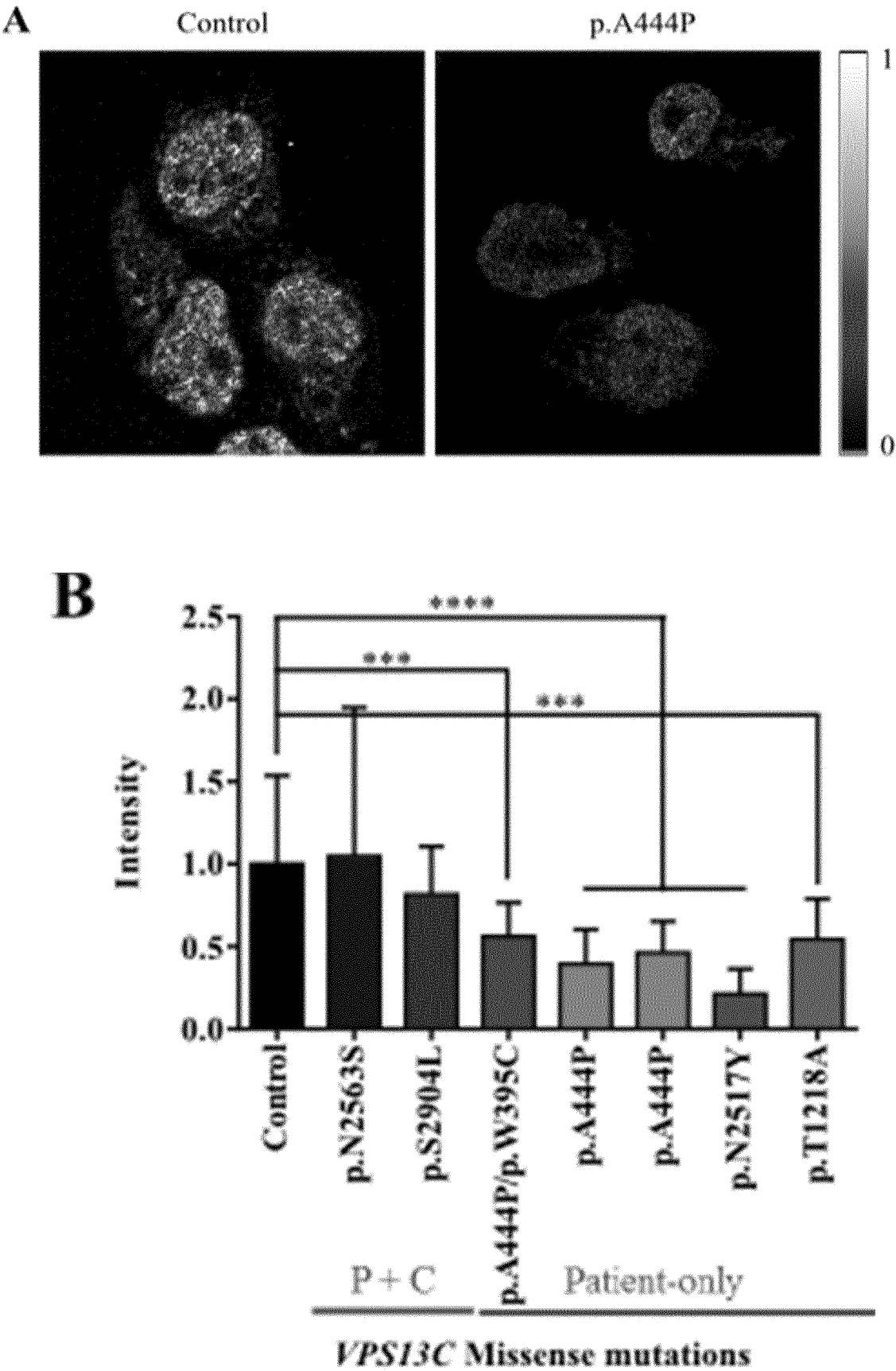


Figure 2 continued

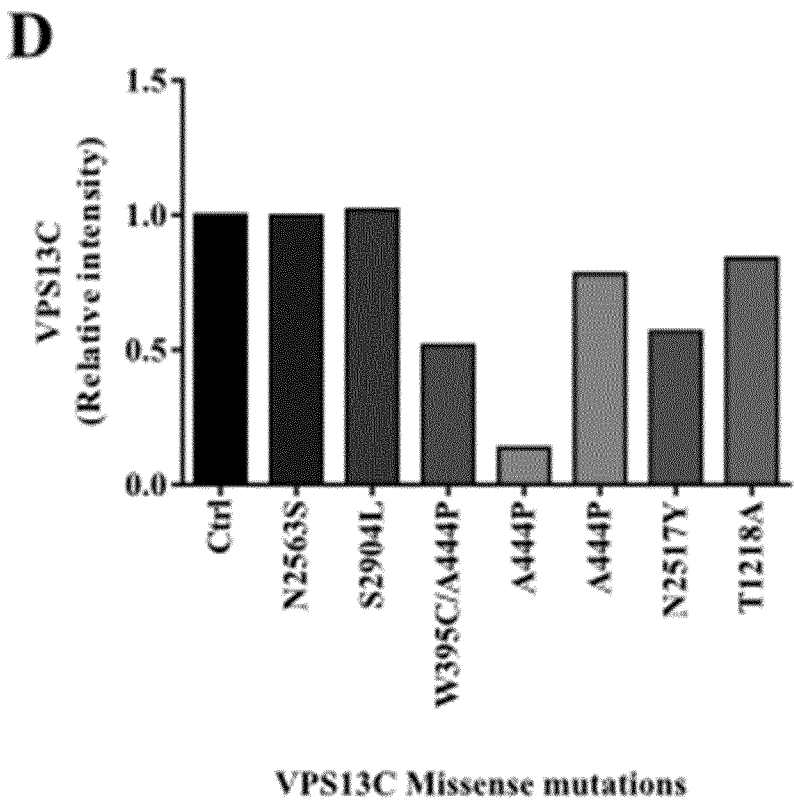
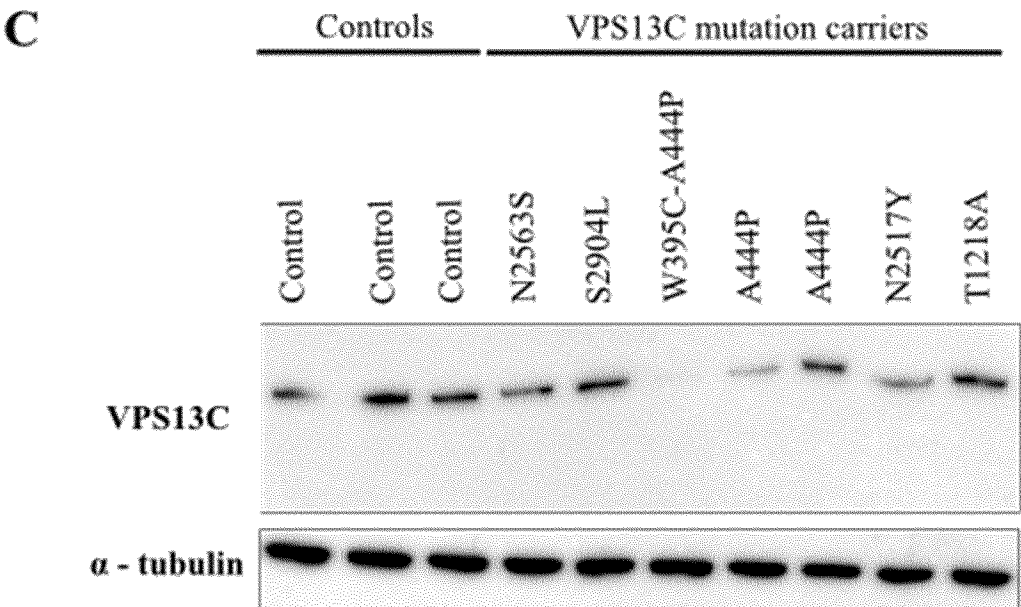


Figure 3

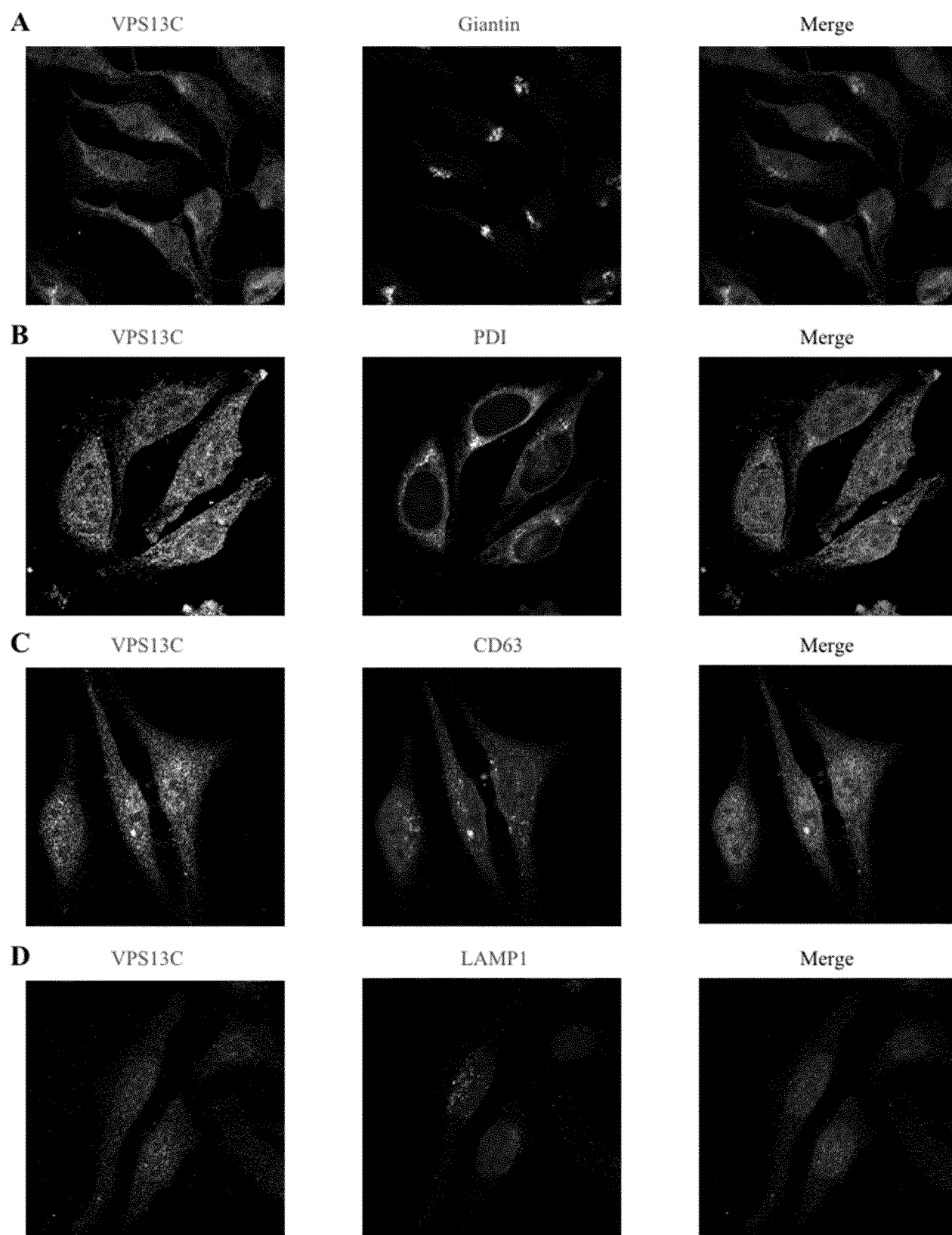


Figure 4

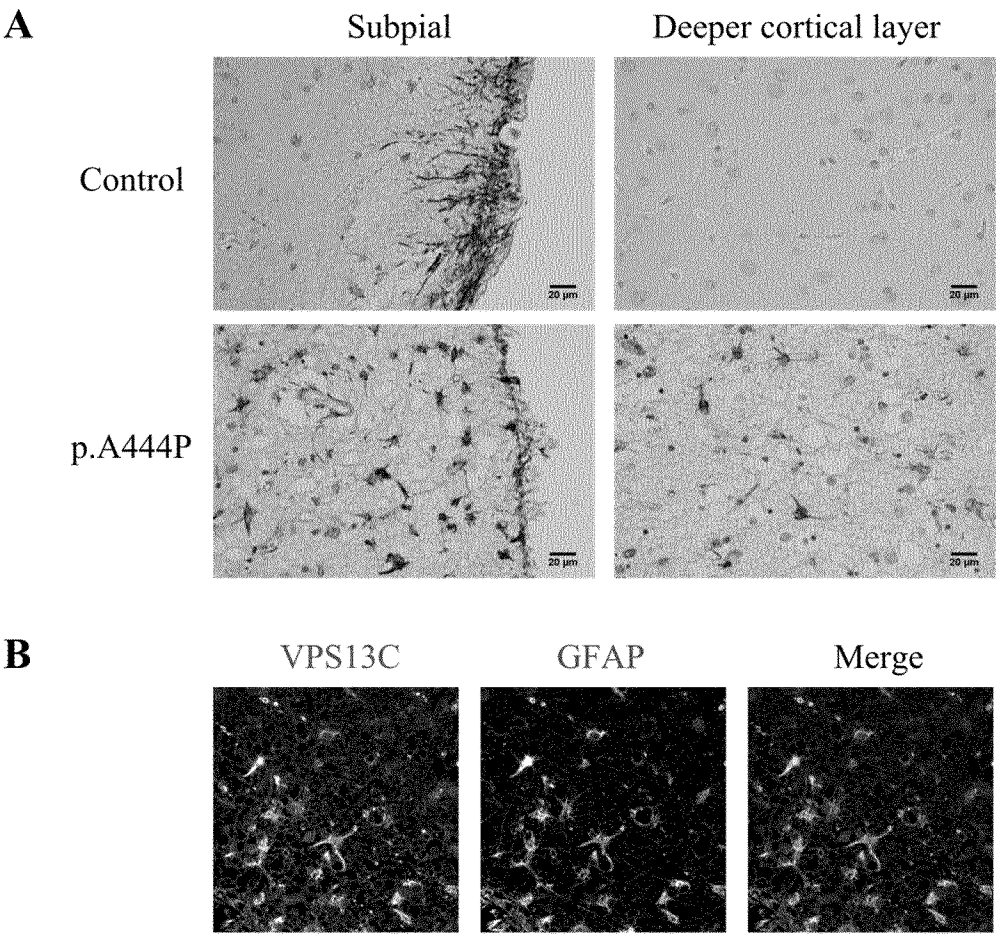


Figure 4 continued

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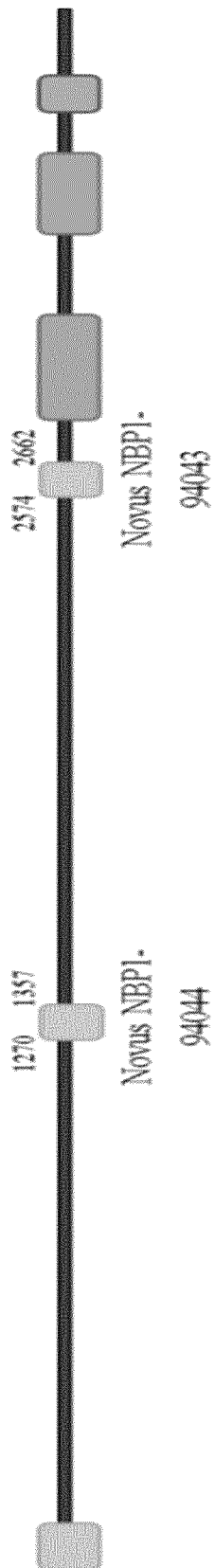


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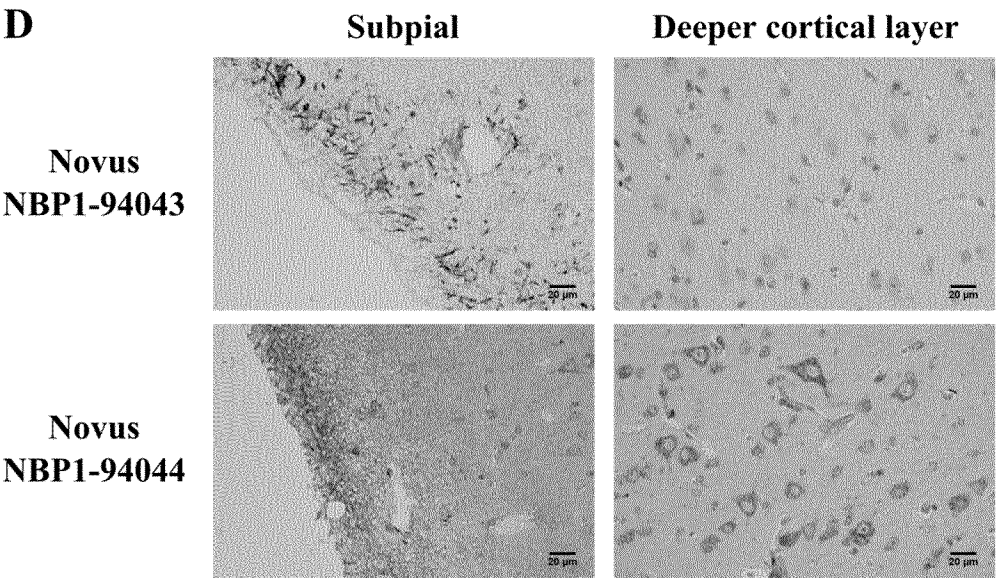


Figure 5

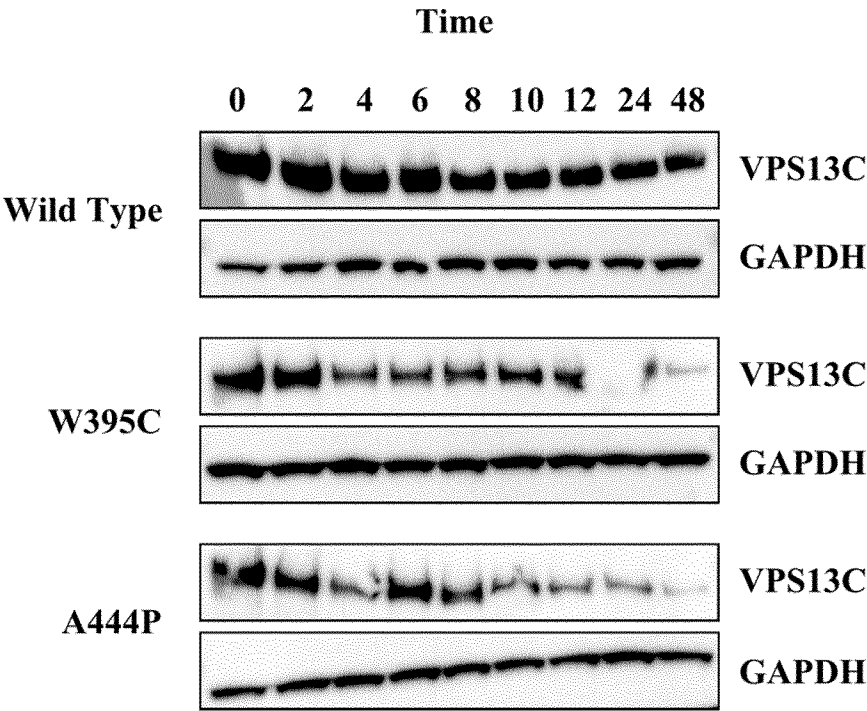
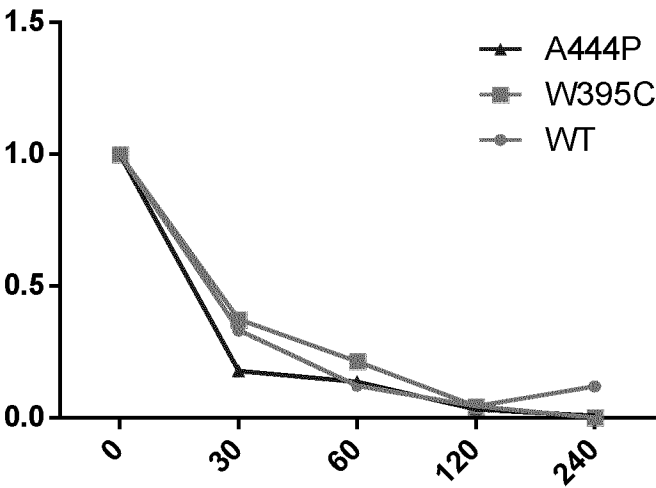
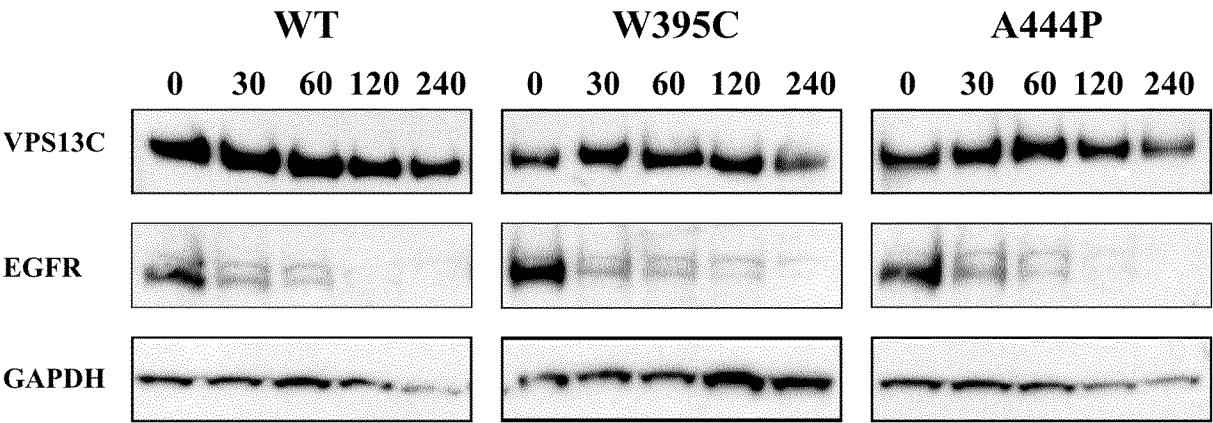


Figure 6



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/074626A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHRISTINE VAN BROECKHOVEN: "0.8 Integrated approach to identify FTLD genes and modifiers", AM J NEURODEGENER DIS, vol. 3, no. 1, 5 September 2014 (2014-09-05), page 10, XP055239133, the whole document ----- -/--	1-9, 11-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

7 January 2016

Date of mailing of the international search report

14/01/2016

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Authorized officer

Helliot, Bertrand

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/074626

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STÉPHANIE PHILTJENS ET AL: "0.11 Next generation sequencing identifies mutations in VPS13C associated with decreased expression of endogenous protein in Frontotemporal lobar degeneration", AM J NEURODEGENER DIS, vol. 3, no. 1, 5 September 2014 (2014-09-05), page 13, XP055239135, the whole document	1-4,6,7,9,11-15
X	----- WO 2013/142286 A1 (PHILADELPHIA CHILDREN HOSPITAL [US]) 26 September 2013 (2013-09-26) page 6, lines 25-30 page 16, lines 21-27 page 16, line 33 - page 17, line 3 abstract the whole document -----	10,16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2015/074626

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013142286	A1	26-09-2013	NONE
