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- (71) Applicants: **VIB VZW** [BE/BE]; Rijvisschestraat 120, 9052 Gent (BE). **UNIVERSITEIT ANTWERPEN** [BE/BE]; Prinsstraat 12, 2000 Antwerpen (BE). **KATHOLIEKE UNIVERSITEIT LEUVEN, KULEUVEN R&D** [BE/BE]; Waaistraat 6 - bus 5105, 3000 Leuven (BE).
- (72) Inventors: **VAN BROECKHOVEN, Christine**; Koning Albertlei 15, 2650 Edegem (BE). **THEUNS, Jessie**; Lage

Kaart 296, 2930 Brasschaat (BE). **VERSTRAETEN, Aline**; Hoogbunderlaan 23, 2550 Kontich (BE). **HEEMAN, Bavo**; Kwakkelstraat 30, 9190 Stekene (BE). **VANGHELUWE, Peter**; Rotselaarsesteenweg 63, 3018 Wijgmaal (BE).

- (74) Common Representative: **VIB VZW**; Rijvisschestraat 120, 9052 Gent (BE).

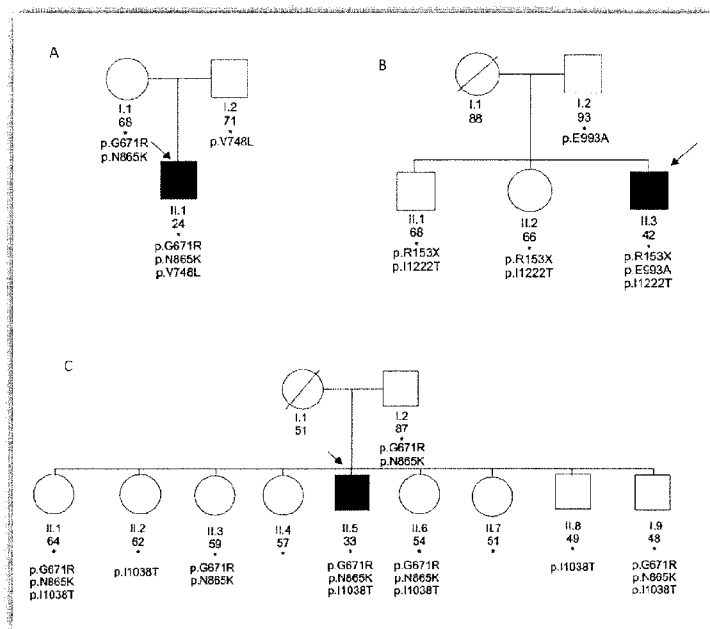
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- (54) Title: A NOVEL GENE IN NEURODEGENERATIVE DISEASE

Figure 1



(57) Abstract: The present application relates to the field of neurodegenerative diseases, particularly Parkinson's disease (PD), most particularly early-onset PD. Using whole genome sequencing in familial early-onset PD, a novel recessive gene could be identified: ATP10B encoding a lysosomal phosphatidylcholine (PC) lipid flippase. Determining expression of this gene, or the presence of mutations in this gene, may be used in diagnosis of neurodegenerative disease. Furthermore, restoring or increasing expression levels or function of ATP10B is envisaged as therapy.



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A novel gene in neurodegenerative disease**Field of the invention**

The present application relates to the field of neurodegenerative diseases, particularly Parkinson's disease (PD), most particularly early-onset PD. Using whole genome sequencing in early-onset PD, a novel recessive gene could be identified: ATP10B. Determining functional expression of this gene, the presence of mutations in this gene or activity of the gene product, may be used in diagnosis of neurodegenerative disease. Furthermore, restoring functional expression or activity of ATP10B is envisaged as therapy.

Background

Parkinson's disease (PD) is a degenerative disorder of the central nervous system. The motor symptoms of Parkinson's disease result from the death of dopamine-generating cells in the substantia nigra, a region of the midbrain; the cause of this cell death is unknown. Early in the course of the disease, the most obvious symptoms are movement-related; these include shaking, rigidity, slowness of movement and difficulty with walking and gait. Later, cognitive and behavioral problems may arise, with dementia commonly occurring in the advanced stages of the disease. Recently, it has been postulated that nonmotor symptoms may predate the onset of neurologic manifestations (Stern et al., 2012). Other symptoms include sensory, sleep and emotional problems. PD is more common in the elderly, with most cases occurring after the age of 50. However, early-onset Parkinson's disease (generally onset between 21-40 years), and juvenile-onset Parkinson's disease (onset before age 21) also exist.

The pathology of the disease is characterized by the accumulation of a protein called alpha-synuclein into inclusions called Lewy bodies in neurons, and from insufficient formation and activity of dopamine produced in certain neurons within parts of the midbrain. Lewy bodies are the pathological hallmark of the idiopathic disorder, and the distribution of the Lewy bodies throughout the Parkinsonian brain varies from one individual to another. The anatomical distribution of the Lewy bodies is often directly related to the expression and degree of the clinical symptoms of each individual. Diagnosis of typical cases is mainly based on symptoms, with tests such as neuroimaging being used for confirmation.

In most individuals, Parkinson's disease is idiopathic, which means that it arises sporadically with no known cause. However, about 15% of individuals have a familial form of PD. Studying these families allowed identification of several genes that are associated with the disorder. The best characterized of these genes are SNCA (encoding α -synuclein), PARK2 (encoding the parkin protein), PARK7 (encoding DJ-1), PINK1 (PTEN-induced putative kinase 1) and LRRK2 (leucine-rich repeat kinase 2).

Many more genes are still being associated with increased risk of PD, or even as a causative gene – for recent overviews of PD-associated risk loci, see the PDgene database (Lill et al., 2012) or Puschmann, 2013. However, many cases remain of unknown origin. Thus, it would be advantageous to identify further genes that contribute to the etiology of PD or possibly even other neurodegenerative disorders.

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Summary

It is an object of the invention to provide methods of diagnosing a neurodegenerative disease in a subject, comprising determining the expression levels or activity of ATP10B and/or determining the presence of one or more mutations in the ATP10B gene in a sample of said subject.

10 Particularly, the neurodegenerative disease is Parkinson's disease. Most particularly, the neurodegenerative disease is early-onset Parkinson's disease.

In case expression levels of ATP10B are monitored, altered expression is indicative of the presence of neurodegenerative disease. 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
15 determining mRNA levels of ATP10B) or using ELISA or Western Blot (e.g. for determining protein levels of ATP10B).

Additionally or alternatively, the presence of one or more mutations in the ATP10B gene may be determined. Most particularly, the mutations will have a deleterious effect on ATP10B gene function, either by altering (e.g. decreasing) expression levels of the gene, or by decreasing or altering its function
20 or activity. According to particular embodiments, at least one of the mutations of which the presence is determined is a mutation in the Had-like domain of ATP10B.

The methods can be performed using direct sequencing methods or others. According to particular embodiments, detection of mutations is done using sequencing, a hybridization assay or PCR-based assay, such as the MastR™ assay.

25 Of note, as shown in Example 4 herein, in several EOPD patients heterozygous mutations are detected in only one of the known autosomal recessive PD genes. This is also the case for other neurodegenerative conditions. Possibly, the combination of heterozygous mutations in known genes with heterozygosity in the newly identified ATP10B gene increases susceptibility to neurodegenerative disease, such as (EO)PD. Thus, in these cases EOPD is the result of mutations in two or more genes, and the methods of diagnosis
30 will focus on identifying digenic mutation carriers. According to these particular embodiments, the methods entail, in addition to determining the expression levels of ATP10B and/or determining the

presence of one or more mutations in the ATP10B gene in a sample of said subject, further determining the expression levels and/or the presence of one or more mutations in another gene. Particularly, genes known to causally contribute to neurodegenerative disease are envisaged. By way of non-limiting example, for EOPD, these genes can e.g. be PARK2, PINK1, DJ-1, GBA, SCA2, EIF4G1.

- 5 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 the ATP10B gene. According to alternative/additional embodiments, kits are provided comprising suitable means for determining the expression levels of ATP10B.

- 10 In yet another aspect, the ATP10B protein, or nucleic acid encoding said protein, is provided for use as a medicament. Most particularly, the ATP10B protein, or nucleic acid encoding said protein, is provided for use as a diagnostic.

According to specific embodiments, the ATP10B protein, or nucleic acid encoding said protein, is provided for use in treating neurodegenerative disease. Most particularly, the neurodegenerative disease is Parkinson's disease.

- 15 This is equivalent as saying that methods of treating neurodegenerative disease (particularly Parkinson's disease) in a subject in need thereof are provided, comprising restoring the levels of ATP10B in said subject (e.g. increasing ATP10B levels in case of a loss of function). It is specifically envisaged that the levels of ATP10B are increased by administering the ATP10B 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 ATP10B can also be restored to normal levels by administering a compound that restores ATP10B expression. For instance, in case of reduced ATP10B levels, the levels can be increased. In case of excessive ATP10B expression (e.g. because of a gain of function), the levels can be decreased.

- 25 Accordingly, in a further aspect, methods to screen for compounds that restore ATP10B expression are provided, comprising:

- Adding a compound to a sample of cells with decreased functional expression of ATP10B;
- Evaluating the expression of ATP10B after addition of the compound.

In a further aspect, methods are also provided to screen for compounds that restore ATP10B activity, comprising:

- 30
- Adding a compound to a sample of cells with decreased activity of ATP10B;
 - Evaluating the activity of ATP10B after addition of the compound.

According to particular embodiments, it is envisaged to screen for compounds that enhance ATP10B transcriptional activity. When the transcriptional activity is enhanced, ATP10B expression will also increase. By screening transcriptional activity specifically, however, (e.g. by asserting effect on the promoter and/or enhancer sequences) it is possible to use a marker or reporter gene as indicator of promoter activity. This is often more straightforward and more easily quantifiable than measuring ATP10B levels.

Thus, according to these embodiments, methods to screen for compounds that increase functional ATP10B expression are provided that comprise the steps of:

- adding a compound to a sample of cells which comprise the ATP10B promoter and/or regulatory regions, optionally functionally linked to a reporter gene said cells;
- evaluating the activity of the ATP10B promoter and/or regulatory regions.

In cases where the promoter/regulatory region is functionally linked to a reporter gene (e.g. luciferase or another enzyme), the activity of the reporter gene can be used as a measure for the ATP10B promoter activity. The skilled person is well aware of suitable reporter genes. Also, the terms promoter and/or regulatory regions of a gene is known by the skilled person.

As shown in Example 6, ATP10B is a phosphatidyl choline flippase and consequently a reduced expression and/or reduced activity of ATP10B leads to reduced uptake of phosphatidyl choline in the cell. Therefore, it is also envisaged that the effect of reduced ATP10B expression or activity can be compensated by compounds that restore phosphatidyl choline uptake.

Accordingly, in a further aspect, methods are provided to screen for compounds to compensate for a reduced phosphatidyl choline uptake due to a reduced expression of ATP10B, comprising:

- Adding a compound to a sample of cells with decreased functional expression of ATP10B;
- Evaluating the uptake of phosphatidyl choline after addition of the compound

to identify a compound that compensates the reduced phosphatidyl choline uptake due to reduced expression of ATP10B.

Accordingly, in a further aspect, methods are provided to screen for compounds to compensate for a reduced phosphatidyl choline uptake due to a reduced activity of ATP10B, comprising:

- Adding a compound to a sample of cells with decreased activity of ATP10B;
- Evaluating the uptake of phosphatidyl choline after addition of the compound

to identify a compound that compensates the reduced phosphatidyl choline uptake due to reduced activity of ATP10B.

Brief description of the Figures

Figure 1. Compound heterozygous variations identified in a) Family DR621, b) family DR754 and c) Family DR741.

5 **Figure 2.** Haplotype sharing of the Flanders-Belgian compound heterozygous ATP10B p.G671R and p.N865K mutation carriers

Figure 3. *In silico* predictions and conservation of the identified ATP10B variations in DR621, DR741 and DR754.

Figure 4. Location of the probable pathogenic identified ATP10B missense variations.

10 **Figure 5.** *In silico* predictions and conservation of the identified ATP10B missense variations in d11527, d13119, d12867.

Figure 6. ATP10B expression levels in different human tissues, relative to total brain ATP10B levels.

Figure 7. ATP10B expression levels in different human brain tissues, relative to total brain ATP10B levels.

15 **Figure 8.** ATP10B expression levels in brain samples of patients and controls. Abbreviations: ns.—not significant. Boxplots represent relative ATP10B expression levels. Median as well as minimum and maximum values are shown. Statistical comparison of expression levels performed using the Mann–Whitney U test. * - $p < 0.01$.

Figure 9. ATP10B promotes phosphatidylcholine (PC) lipid uptake suggesting ATP10B is a PC lipid flippase. **(A)** Phylogenetic analyses of the P4 ATPase protein family using core sequences classified ATP10B into class 5, with known PC and PS lipid flippases. Orthologs with known substrate and the designated subdivision into classes are highlighted on the right hand side. **(B)** The transient overexpression pattern of ATP10B, WT vs. D433N and CDC50A in HeLa cells was determined by western blotting. Non-transfected cells were used as a negative control for antibody specificity **(C)** The PC-based lipid uptake activities (2 μ M NBD-PC) of non-transfected HeLa cells (NT), as well as HeLa cells transiently transfected with ATP10B vs. D433N were determined by flow cytometry using an Attune flow cytometer at 0, 30 and 60 min time points. Fluorescence intensity was measured after extracellular quenching with sodium dithionite for 15 minutes at a final concentration of 10 mM. **(D)** ATP10B significantly increased uptake of PC, in comparison to NT and D433N following 60 min exposure to 2 μ M NBD-PC. Flow cytometry analysis of ATP10Bs capacity to modulate the uptake of either 2 μ M NBD-PE **(E)** or 2 μ M NBD-PS **(F)** demonstrated a reduced uptake potential. All data are the mean of 3 independent experiments.

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The immunoblot depicts a representative image of a minimum of 3 independent experiments. Statistical comparison performed using one-way ANOVA; * - $p < 0.05$, ** - $p < 0.005$, *** - $p < 0.0001$.

Figure 10. Disease related ATP10B mutants inhibit PC uptake. (A) Predicted topology of ATP10B with specific P4 and P5 inserts, conserved P-type ATPase core regions and highlighted position of early-onset PD mutations E993A, G671R/N865K, I1038T, R153X and V748L. **(B)** ATP10B disease mutations, transiently transfected into HeLa cells, were assessed in comparison to non-transfected (NT) cells and cells overexpressing the WT vs. the catalytically inactive variant (D433N) for their capacity to modulate the uptake of NBD-PC over 0, 30 and 60 min time intervals, before extracellular signals were quenched using 10 mM sodium dithionite. Mean fluorescent intensities were acquired using an Attune flow cytometer. **(C)** Bar graph representation of the hampered NBD-PC uptake of disease related variants of ATP10B in comparison to WT, D433N and NT cell controls. Data are the mean of experimental outputs. Statistical comparison performed using one-way ANOVA; * - $p < 0.05$, ** - $p < 0.005$, *** - $p < 0.0001$.

Figure 11. ATP10B localizes to lysosomes. HeLa cells were transiently co-transfected with an expression vector for N-terminally GFP-tagged ATP10B vs. D433N and the expression vector for CDC50A. Following transfection cells were rested prior to fixation and stained with anti-EEA (early endosomal marker), **(A)**, anti-LAMP-1 (lysosomal marker), **(B)** and anti-CD63 (late-endosomal marker), **(C)** primary antibodies and visualized with Alexa 594 conjugated anti-rabbit or anti-mouse antibodies on Olympus IX81 fluorescence microscope. Images provided are a representation of a cellular cohort, visualized over a minimum of 3 independent experiments.

Figure 12. Presence of ATP10B leads to an increase of lysosomal mass. (A) The mean fluorescence per cell was estimated by flow cytometry after staining with LysoTracker Red DND-99 and incubation at 37°C for 30 minutes. In contrast to HeLa cells transiently transfected with I1038T, R153X and V748L mutations, cells that were transfected with WT ATP10B exhibited a significant increase of lysosomal mass, compared to non-transfected control cells. **(B)** HeLa cells transiently overexpressing the WT ATP10B and N-terminally GFP-tagged WT ATP10B exhibit a pattern with morphologically enlarged and tubular vesicles (pointed with the arrow), unlike the cells transfected with D433N, which exhibit smaller punctae-like patterns.

Detailed 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 term "Parkinson's disease" as used herein refers to the second most common neurodegenerative disorder after Alzheimer's disease, with manifestations including resting tremor, muscular rigidity, bradykinesia, and postural instability. Additional features are characteristic postural abnormalities, dysautonomia, dystonic cramps, and dementia. Also, non-motor symptoms in Parkinson's disease (PD) are frequent, increase the patients' disability and have an important negative impact on their quality of life (Stern et al., 2012). The most frequent non-motor symptoms reported include urinary urgency (59.2 %), nocturia (56.9 %), insomnia (45.8 %), attention problems (45.5 %) and orthostatism (41.2 %) (Crosiers et al., 2012). The disease is progressive and usually has an insidious onset in mid to late adulthood. The phrase "early onset Parkinson's disease" as used herein refers to Parkinson's disease with an onset before 50 years of age. According to particular embodiments, EOPD may also refer to PD with an onset before 45 years of age, or before 40 years of age.

"ATP10B" as used herein refers to the ATP10B gene (Gene ID: 23120 in humans, chromosomal location 5q34) or its encoded protein product, an ATPase, class V, type 10B. According to particular embodiments,

the sequence of the (human) ATP10B gene corresponds to SEQ ID NO:1; the protein it encodes is given in SEQ ID NO:2.

The “ATP10B gene product” as used herein typically refers to what is transcribed or translated from the ATP10B gene, such as ATP10B mRNA and ATP10B protein. The different isoforms or variants of ATP10B mRNA and the resulting ATP10B isoforms or variants are envisaged within the term ATP10B gene product. For determining ATP10B gene product levels, typically the ATP10B gene (or gene product) to be detected will be a functionally active gene, in order to accurately assess ATP10B activity from its expression levels. However, it is also envisaged that functionally inactive forms of ATP10B are detected, e.g. mutated versions of ATP10B or inactive variants of ATP10B, or isoforms with decreased activity. The same applies for restoring (e.g. increasing) levels of ATP10B gene product: this may be wild type ATP10B, or a functionally less active form.

With “functional expression” of ATP10B, 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 loss of function mutations. A “loss-of-function” or “LOF” mutation as used herein is a mutation that 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, frameshift 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 ATP10B gene if these interfere with gene function. A null mutation is a LOF mutation that completely abolishes 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 so that it is degraded before translation occurs from the transcript. Third, at the protein level, e.g. because of protein instability, proteins with reduced functionality or activity (e.g. enzymatic activity, such as ATPase or lipid flippase activity), 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).

“Haploinsufficiency” as used herein refers to the situation in which reduced gene function, resulting from a LOF mutation, causes an abnormal phenotype. Haploinsufficiency is the mechanism by which a heterozygous LOF mutation is associated with a dominantly inherited trait.

The phrase “nonsense-mediated mRNA decay” or NMD as used herein refers to a surveillance mechanism that rapidly degrades mRNA species harboring premature termination codons (PTCs) to prevent cells from producing C-truncated and nonsensical proteins. PTCs located >50–55 bp upstream of the most 3′ exon–exon junction of the transcript are recognized.

ATP10B is a P-type ATPase. P-type ATPases comprise 4 functionally important domains: the A-domain is the actuator domain that regulates dephosphorylation of the enzyme; the P-domain is the phosphorylation domain that contains the aspartic acid residue for autophosphorylation; the M-domain is the membrane domain needed for the substrate recognition, binding and transport and the N-domain is the nucleotide binding domain for the binding and delivery of ATP. The folding pattern and the locations of 8 critical amino acids for phosphorylation in P-type ATPases has the haloacid dehalogenase (HAD) fold characteristic of the HAD superfamily. The HAD fold is often referred to as the HAD-like domain. According to particular embodiments, this HAD-like domain corresponds to amino acids 418 to 1109 of the ATP10B protein sequence, most particularly amino acids 418 to 1109 of SEQ ID NO:2.

The present application is the first to show that mutations in ATP10B (which lead to a loss of function) are found in patients with neurodegenerative disease, most particularly early onset Parkinson’s disease, and are causative of the disease.

Accordingly, in a first aspect, methods are provided of diagnosing a neurodegenerative disease in a subject, comprising determining the functional expression levels of ATP10B and/or determining the presence of one or more mutations in the ATP10B gene in a sample of said subject.

Although the mutation is primarily identified in Parkinson’s disease, it is well established that carriers of such mutations are often also at a higher risk of other neurodegenerative diseases, cf. e.g. the progranulin missense mutations found in AD. Further characterization of the ATP10B gene is ongoing in patients with FTLD, Alzheimer, dementia with Lewy bodies and others.

According to particularly envisaged embodiments, the neurodegenerative disease is Parkinson's disease. According to even more particular embodiments, the Parkinson's disease is early-onset Parkinson's disease. Early-onset Parkinson's disease is typically Parkinson's disease with an age at onset of 50 years or younger. According to particular embodiments, EOPD is PD with an AAO of 40 years or younger. It is particularly envisaged that patients with a homozygous mutation in ATP10B, or which are compound heterozygous carriers of ATP10B mutations and a mutation in another recessive PD-associated gene will have an earlier age at onset than heterozygous carriers of ATP10B mutations.

A subject typically is a vertebrate subject, more typically a mammalian subject, most typically a human subject. A sample of the subject will typically contain cells (or at least cellular material) of the subject, to evaluate the expression or presence of mutations in the genetic material. A sample can be obtained from any tissue to establish the presence of germline mutations, and can be a tissue sample, or a fluid sample (e.g. blood, saliva). Particularly when expression of the ATP10B gene is assessed, it is envisaged to use a sample from a tissue which can be linked to the presence of neurodegenerative disease, such as a brain sample or a CSF sample. The samples can be used as such or can be pre-processed (e.g. lysed) using methods routinely used in the art.

When the methods of diagnosing a neurodegenerative disease involve detection of functional expression of ATP10B, typically, these methods will further include a step involving correlating the levels of ATP10B gene product to the risk of presence or development of neurodegenerative disease, particularly correlating decreased levels (or even absence) of ATP10B gene product to increased risk of neurodegenerative disease. However, the reverse can also be true: concluding from an observation that the ATP10B gene product levels are not decreased, or are even increased, in the sample, that there is no increased risk of neurodegenerative disease, or in some instances even a decreased risk of neurodegenerative disease.

Decreased levels of ATP10B gene product are typically decreased versus a control. The skilled person is capable of picking the most relevant control. This will typically also depend on the nature of the disease studied, the sample(s) that is/are available, and so on. Suitable controls include, but are not limited to, similar samples from subjects not having neurodegenerative disease, or a set of clinical data on average ATP10B gene product levels in the tissue from which the sample is taken. As is evident from the foregoing, the control may be from the same subject, or from one or more different subjects or derived from clinical data. Optionally, the control is matched for e.g. sex, age etc.

With 'decreased' levels of ATP10B gene product as mentioned herein, it is meant levels that are lower than are normally present. Typically, this can be assessed by comparing to control. According to

particular embodiments, decreased levels of ATP10B are levels that are 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 100%, 150%, 200% or even more low than those of the control. According to further particular embodiments, it means that ATP10B gene product is absent, whereas it normally (or in control) is expressed or present. In other words, in these embodiments determining the presence of ATP10B gene product is equivalent to detecting the absence of ATP10B gene product. Typically, in such cases, a control will be included to make sure the detection reaction worked properly. The skilled person will appreciate that the exact levels by which ATP10B gene product needs to be lower in order to allow a reliable and reproducible diagnosis may depend on the type of sample tested and of which product (mRNA, protein) the levels are assessed. However, assessing the correlation itself is fairly straightforward.

Instead of looking at decreased levels compared to a healthy control, the skilled person will appreciate that the reverse, comparing to a control with disease, can also be done. Thus, if the ATP10B gene product levels measured in the sample are similar to those of a sample with neurodegenerative disease, (or are e.g. comparable to ATP10B gene product levels found in a clinical data set of neurodegenerative disease), this may be considered equivalent to decreased ATP10B gene product levels compared to a healthy control, and be correlated to an increased risk of neurodegenerative disease. In the other case, if ATP10B gene product levels are higher than those of a control with neurodegenerative disease, this can be said not to correlate with an increased risk of neurodegenerative disease, or even to be correlated with a decreased risk of disease. Of course, ATP10B gene product levels may be compared to both a negative and a positive control in order to increase accuracy of the diagnosis.

The ATP10B gene product whose levels are determined will typically be ATP10B mRNA and/or ATP10B protein. When ATP10B mRNA is chosen as the (or one of the) ATP10B gene product whose levels are determined, this can be the total of all ATP10B mRNA isoforms, or one or more specific mRNAs. Alternatively or additionally, the ATP10B gene product of which the levels are determined may be ATP10B protein. As protein is translated from mRNA and the mRNA exists in multiple isoforms, the same considerations apply: the total ATP10B levels may be determined, or those of specific isoforms only (e.g. using an antibody against the different C-termini). Most particularly, all ATP10B protein isoforms may be detected (e.g. using an antibody against a common epitope). Of note, it is envisaged as well that both ATP10B mRNA and ATP10B protein are determined. In this case, the isoforms to be detected can be all isoforms for both mRNA and protein, identical isoforms (wholly overlapping), or different isoforms (partly or not overlapping), depending on the setup of the experiment. With identical isoforms, it is meant that the mRNA isoform encodes for the corresponding protein isoform.

Additionally or alternatively, the presence of one or more mutations in the ATP10B gene will be determined in a sample of said subject. Typically, the one or more mutations will be deleterious mutations or loss-of-function mutations. The presence of these mutations is indicative of an increased risk of developing, or of the presence of neurodegenerative disease (depending i.a. of the age of the subject when the presence of mutations is determined). According to particular embodiments, at least one of the mutations whose presence is determined is in the HAD-like domain of ATP10B.

The risk of presence or of developing a neurodegenerative disease is even larger if a mutation is present in more than one allele of ATP10B, or if there is also a mutation present in another PD-linked gene, particularly another recessive PD-linked gene (e.g. PARK2). Thus, the methods may further comprise determining the presence of mutations in other genes.

As will be shown in the Example section, there are different mutations in ATP10B that are deleterious. In order to accurately determine the risk of neurodegenerative disease, it is envisaged that the presence of more than one, and up to all, of these mutations are checked. This can be done by sequencing of the entire ATP10B coding sequence, or it can be done using multiple hybridization reactions or PCR reactions (the latter may also be multiplex PCR, wherein more than one mutation can be determined in the same assay). According to particular embodiments, detection of mutations is done using sequencing, a hybridisation assay or PCR-based assay, such as the MastR™ assay.

According to a further aspect, kits are provided suitable for practicing the methods presented herein, i.e. kits for determining the expression levels of ATP10B and/or determining the presence of one or more mutations in the ATP10B gene in a sample of said subject. For determining expression, these kits will typically contain at least one agent specifically binding to ATP10B protein (e.g. an ATP10B antibody), or to ATP10B mRNA (e.g. primers). For determining the presence of one or more mutations, the kits will typically comprise at least one primer or probe suitable to determine the presence of one or more mutations in the ATP10B gene. Obviously, kits may contain further material, such as pharmaceutically acceptable excipients or buffers, as is established in the art.

According to a further aspect, the ATP10B protein, or nucleic acid encoding said protein, is provided for use as a medicament. According to one embodiment, the ATP10B protein, or nucleic acid encoding said protein, is provided as a diagnostic. Indeed, as described in the methods above, the levels and/or presence of mutations in ATP10B may be used as diagnostic tool to establish the presence of neurodegenerative disease. According to specific embodiments, the ATP10B protein, or nucleic acid encoding said protein, is provided as a diagnostic for diagnosing PD, particularly early onset PD.

However, it is also envisaged that the ATP10B protein, or nucleic acid encoding said protein, is provided for use as a medicament to treat neurodegenerative disease. Indeed, restoring correct levels of ATP10B levels will prevent the onset of, or treat, neurodegenerative disease. Most particularly, the ATP10B protein, or nucleic acid encoding said protein, is provided for use in treating Parkinson's disease.

- 5 This is equivalent as stating that methods are provided of treating Parkinson's disease in a subject in need thereof, comprising increasing the levels of ATP10B in said subject. According to particular embodiments, the levels of ATP10B are increased by administering the ATP10B protein, or nucleic acid encoding said protein, to said subject. According to yet further particular embodiments, the levels of ATP10B are increased using gene therapy, particularly gene therapy wherein the nucleic acid encoding
10 said protein is administered to a subject in need thereof.

As shown in Example 6, ATP10B is a phosphatidyl cholin flippase. Therefore, this invention also provides the use of a PC flippase, or nucleic acid encoding said PC flippase, or a small molecule with PC flippase activity as a medicament to treat neurodegenerative disease. More particularly, the PC flippase or the nucleic acid encoding said PC flippase or the small molecule with PC flippase activity is provided for use
15 in treating Parkinson's disease. Examples of proteins with PC flippase activity are ATP10A, ATP10C, ATP10D, ATP8B1 and ATP8B2.

Alternatively, ATP10B levels can also be increased by administering a compound to a subject. Without being bound to a particular mechanism, compounds can increase ATP10B expression e.g. by increasing ATP10B transcriptional activity, by stabilizing the ATP10B mRNA or protein, or by influencing other genes
20 or gene networks (e.g. by blocking ATP10B inhibitors).

Accordingly, methods to screen for compounds that increase ATP10B expression are provided, comprising:

- Providing a sample of cells with decreased functional expression of ATP10B; or providing a sample of cells which express a mutant form of ATP10B;
- 25 - Adding a compound to said cells;
- Evaluating the expression of ATP10B after addition of the compound.

According to particular embodiments, it is envisaged to screen for compounds that enhance ATP10B transcriptional activity. When the transcriptional activity is enhanced, ATP10B expression will also increase. By screening promoter/enhancer activity specifically, however, it is possible to use a marker or
30 reporter gene as indicator of transcriptional activity. This is often more straightforward and more easily quantifiable than measuring ATP10B levels.

Thus, according to these embodiments, methods to screen for compounds that increase ATP10B expression are provided that comprise the steps of:

- providing a sample of cells which contain the ATP10B promoter, optionally functionally linked to a reporter gene;
- 5 - adding a compound to said cells;
- evaluating the activity of the ATP10B promoter.

In cases where the promoter is functionally linked to a reporter gene (e.g. luciferase or another enzyme), the activity of the reporter gene can be used as a measure for the ATP10B promoter activity. The skilled person is well aware of suitable reporter genes. The term "promoter" as used herein comprises
10 regulatory elements, which mediate the expression of a nucleic acid molecule. For expression, the nucleic acid molecule must be linked operably to or comprise a suitable promoter which expresses the gene at the right point in time and with the required spatial expression pattern. The term "operably linked" as used herein refers to a functional linkage between the promoter sequence and the gene of interest, such that the promoter sequence is able to initiate transcription of the gene of interest.

15 Data shown in this application, more precisely in Example 6, surprisingly revealed that ATP10B acts as a PC flippase. "Flippases" are transmembrane lipid transporter proteins located in the membrane responsible for aiding the movement of phospholipid molecules between the two leaflets that compose a cell's membrane. A "PC flippase" is a flippase that aids the movement of phosphatidylcholine. A lipid
20 uptake assay demonstrated that the mutated version of ATP10B reduced the uptake of phosphatidylcholine lipid in the cells tested. As reduced ATP10B levels or expression of non-functional ATP10B is clearly linked with neurodegenerative disease e.g. Parkinson's disease and given that reduced ATP10B levels or expression of non-functional ATP10B leads to reduced uptake of phosphatidylcholine in a lipid uptake assay, increasing the uptake of PC might compensate for reduced expression of ATP10B
25 or reduced activity of ATP10B and might be a new basis of a therapy to halt further progression of neurodegenerative disease or treat neurodegenerative disease as Parkinson's disease. Therefore, in this invention methods are provided to screen for compounds that compensate for a reduced PC uptake due to a reduced expression and/or activity of ATP10B, comprising adding a compound to a sample of cells with decreased functional expression of ATP10B and/or decreased activity of ATP10B and evaluating the
30 uptake of PC 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

1. Whole genome sequencing of an early-onset idiopathic PD patient to identify the underlying disease cause

A nuclear PD family (DR621) composed of an early-onset patient (d12522-age at onset (AAO) 24 years) and both unaffected parents was outsourced to Complete Genomics for whole genome sequencing (Figure 1A). After successive filtering regarding sequence quality, variation frequency and the possible inheritance patterns, the remaining variant combinations were genotyped in 869 Flanders-Belgian control individuals, revealing 8 candidate genes with a high potential to cause disease in this particular family (Table 1).

Candidate gene prioritization using various software programs suggested *ATP10B* and *BCAN* as most promising candidate genes. To be as unbiased as possible however, we sequenced the coding exons and exon-intron boundaries of all candidate genes in 99 Flanders-Belgian early-onset PD (EOPD) patients (AAO≤50y, 15.7% of the entire Flanders-Belgian PD cohort). In *ATP10B* we identified compound heterozygous variations in two additional EOPD patients (d12421/DR741.1-AAO33y (Fig. 1C) and d12621/DR754.1-AAO42y (Figure 1B), Table 2), clinically similar to the index patient of family DR621 (Figure 1a). Mutations in the major causal PD genes (e.g. SNCA, LRRK2, Parkin, PINK1) were excluded in these three patients. In the two additional patients, no variations were identified in the other seven candidate genes, in-line with the proposed inheritance models in this patient cohort.

Table 1. Overview of the 8 candidate genes with their respective variations.

Gene	Gene function	Inheritance pattern	Impact	MAF in controls	Predictions	Protein domain
<i>ATP10B</i>	ATP synthesis	Comp heterozygous	p.N865K	0.021	Inconsistent	Cytoplasmatic domain
			p.V748L	0.002	Inconsistent	Cytoplasmatic domain
			P.G671R	0.021	Inconsistent	Cytoplasmatic domain
<i>ZNF323</i>	Gene regulation	Comp heterozygous	p.R350H	0.001	Inconsistent	-
			p.R61Q	0.003	Inconsistent	SCAN box
<i>PSME1</i>	Immunoproteasome assembly	Comp heterozygous	p.N50S	0.005	Neutral	-
			p.R235K	0.002	Inconsistent	-
<i>ABCC12</i>	ATP transport	Comp heterozygous	Spl. Acc. region	0.004	-	-
			p.V850A	0.001	Neutral	-
<i>PKD1L2</i>	Ion channel component	Comp heterozygous	p.P1065R	0.023	Inconsistent	REJ domain
			p.L474F	0.001	Inconsistent	REJ domain
<i>LOC653354</i>	-	X-linked	?	0.000	-	-
<i>TMSB15B</i>	Cytoskeleton organization	X-linked	p.T44A	0.000	Neutral	-
<i>BCAN</i>	Differentiation nervous system	Homozygous	p.L595I	0.016	Neutral	-
<i>ANKLE1</i>	-	Homozygous	p.E257Q	0.014	Neutral	-

*Only males are taken into account for the exclusion of these variants (N=406)

Table 2. Overview of the identified compound heterozygous mutation carriers and their phenotype

ID	Origin	AAO	FH	Ldopa+	RT	BK	RI	AS	Variations	Exons	Had-like	1000G/hom	MAF _{CON}	MAF _{EOPD}	PPhen2	Conserv
d12522	Belgium	24	no	x		x	x	x	p.G671R	15	x	0.012/0	0.022	0.025	-	-
									p.V748L	15	x	0.002/0	0.001	0.010	+	+
									p.N865K	17	x	0.011/0	0.022	0.025	+	+
d12421	Belgium	33	no	x		x	x	x	p.G671R	15	x	0.012/0	0.022	0.025	-	-
									p.N865K	17	x	0.011/0	0.022	0.025	+	+
									p.I1038T	19	x	-	0.001	0.010	+	-
d12621	Belgium	42	no	NA	x	x	x	x	p.R153X	6		0.002/0	0.004	0.005	NA	NA
									p.E993A	19	x	-	0.000	0.010	-	+
									p.I1222T	24		0.005/0	0.013	0.020	+	+

1000G/hom: The frequency of the variant the 1000 Genomes project followed by the number of homozygous individuals.

EVS/hom: The frequency of the variant in the Exome Variant Server project followed by the number of homozygous carriers.

- 5 The Exome Variant Server samples are mainly from European-American or African-American origin. AAO: Age at onset. FH: Familial History. Ldopa+: Levo-dopa response. RT: Rust tremor. BK: Bradykinesia. RI: Rigidity. AS: Asymmetric onset. NA: Unknown. MAFCON: minor allele frequency in control individuals. MAF_{EOPD}: minor allele frequency in EOPD patients.

The amino acid substitutions p.G671R/p.N865K were observed in two Flanders-Belgian compound heterozygous individuals. Consequently, we performed haplotype sharing (d12522, d12421 and their
 10 parents) using 4 chromosome 5 markers flanking *ATP10B* (genomic location of *ATP10B* is at 5q34) originating from the in-house optimized linkage panel, in addition to 7 highly polymorphic markers (heterozygosity $\geq 70\%$) from the Marshfield marker map and 5 self-designed markers. A common haplotype (estimated size: 13 Mb) was observed between both Flanders-Belgian p.G671R and p.N865K
 15 mutation carriers. Remarkably, the non-shared marker beneath the pink bar (*ATP10B*) resides in *ATP10B* intron 1 (Figure 2).

The particular combinations of variations in d12522, d12421 and d12621 were not observed in a Flanders-Belgian control group consisting of 869 individuals. Furthermore, the majority of the mutated amino acid positions are strongly conserved and prediction programs for non-synonymous variants suggest deleterious effects (Figure 3). Only p. E993A and p.G671R, one of the two variants in linkage
 20 disequilibrium (LD), were predicted to be benign. Yet, to fit the compound heterozygous inheritance model only one of both variations has to be pathogenic. All these data point to a strong likelihood that these variants have a causative deleterious effect.

Interestingly, all *ATP10B* variations observed in d12522, d12421 and d12621 are more frequently observed in heterozygous state in 98 Flanders-Belgian EOPD patients with an average AAO of 43.4 ± 5.9

years versus 869 ethnically matched healthy control individuals with an average AAI of 65.7 ± 14.7 years (Table 2).

In family DR621 we identified three seemingly unaffected compound heterozygous *ATP10B* mutation carriers. Recently, one of them reported to present with a resting tremor in times of stress. However, to date none of these individuals was thoroughly examined by a neurologist, so it is possible that these carriers are affected with a milder form, or develop the disease at a later age.

Since extensive mutation screenings of the known recessive PD genes revealed the existence of gene and exon duplications/ deletions, we designed and optimized three Multiplex Amplicon Quantification (MAQ) (<http://www.multiplicom.com/>) assays covering all *ATP10B* exons, with exception of exon 2 and exon 17. CNV analyses on high-quality gDNA of 78 samples of the Flanders-Belgian EOPD cohort did not reveal homozygous nor heterozygous *ATP10B* exon deletions/duplications.

ATP10B is a P-type ATPase involved in lipid transport that, similarly to all other P-ATPases, contains a HAD-like domain that is thought to be crucial for the catalytic activity of the protein. The compound heterozygous variations that we identified in the index patients of DR621, DR741 and DR754 are located in this domain (Figure 5) or are predicted to disrupt it (p.R153s). Furthermore, the gene causing the very early-onset parkinsonian disorder Kufor-Rakeb (*ATP13A2*) encodes a member of these P-type ATPases as well. Other P-type ATPases linked to neurodegenerative disorders in humans or mice include *ATP1A3* (rapid-onset dystonia parkinsonism), *ATP7A* (Menkes disease), *ATP7B* (Wilson disease), *ATP8A2* (Wabblers-lethal mutant). *ATP10A* was recently described as a candidate modifier for age at onset in familial Alzheimer disease. Based on these clues, we decided to focus on replication of our findings in larger EOPD cohorts.

2. *ATP10B* mutation frequencies: Flanders-Belgian EOPD patients versus control individuals

To identify *ATP10B* compound heterozygous variation carriers in Flanders-Belgian control individuals, we generated sequence data of all *ATP10B* coding exons of 85 healthy individuals with an average AAI of 48.7 ± 12.5 years and 88 controls with an average AAI of 76.5 ± 11.1 years. In these cohorts, we did not identify individuals carrying two or more rare heterozygous variations. An additional control group consisting of 173 individuals with an average AAI of 63.5 ± 13.9 years was screened for all exons encoding the HAD-like domain. Also here, results were negative.

As described in the sections above, the coding regions of *ATP10B* were sequenced in 98 Flanders-Belgian PD patients with an average AAO of 43.4 ± 5.9 years and in 85 Flanders-Belgian healthy individuals with an average AAI of 48.7 ± 12.5 years. In the control group 6.5% of the alleles carried heterozygous non-

synonymous coding variations with a $MAF_{CON} \leq 5\%$. In the EOPD patient group the frequency was slightly higher, namely 10.2%. An allelic association analysis using a Fisher's Exact test did not suggest a statistical significant difference (Table 3) between patients and controls ($p=0.26$).

Table 3. Output of the allelic association analysis of heterozygous ATP10B non-synonymous coding variations with a $MAF_{CON} \leq 5\%$

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.552 ^a	1	.213	.260	.145
Continuity Correction ^b	1.118	1	.290		
Likelihood Ratio	1.580	1	.209		
Fisher's Exact Test					
Linear-by-Linear Association	1.548	1	.213		
N of Valid Cases	364				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 14.31.

b. Computed only for a 2x2 table

Focusing on alleles carrying heterozygous non-synonymous coding variations with a $MAF_{CON} \leq 1\%$, we observed a statistically significant difference (Table 4, $p=0.04$) between EOPD patients (4.1%) and age-matched control individuals (0.6%).

Table 4. Output of the allelic association analysis of heterozygous ATP10B non-synonymous coding variations with a $MAF_{CON} \leq 1\%$

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.560 ^a	1	.033	.042	.032
Continuity Correction ^b	3.229	1	.072		
Likelihood Ratio	5.285	1	.022		
Fisher's Exact Test					
Linear-by-Linear Association	4.547	1	.033		
N of Valid Cases	364				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 4.15.

b. Computed only for a 2x2 table

3. Replication of results in larger EO-PD patient cohorts

3.1 Mixed ethnicity replication samples

In our Department, we have a large collection of PD patient samples (N=348), especially early-onset patients (53.7%), originally sampled for genetic testing and counseling. 134 EO-PD patients (AAO $\leq$ 50 years) were selected from this databank for *ATP10B* sequencing, to identify additional *ATP10B* mutation carriers. Three possible compound heterozygous patients, not from Flanders-Belgian origin, carrying two rare missense variations (p.T161N & p.G648R) were identified (Table 5). Remarkably, we did not observe subjects carrying only one of both variations. In addition to low (p.T161N) and moderate (p.G648R) conservation at the given amino acid positions, prediction programs did not point towards pathogenicity of the identified variations (Figure 5). Segregation analysis is hampered by the lack of DNA of family members and additionally we are not able to genotype population-matched control individuals for these specific variations because of the foreign origin of the index patients. Mutation analysis of the known PD genes revealed the presence of LRRK2 p.G2019S in d11527 and a homozygous deletion of exons 3 and 4 (p.N58_Q178del) in d12867. Together, it seems conceivable that these variations are inherited together and do not cause disease in these individuals.

Table 5. Overview of the mutation carriers, their onset age and family history.

ID	AAO	FH	Mutations'PDgene'	Variations	Exons	Had-like	1000G/hom	PPhen2	Conserv
d11527	32	NA	LRRK2 p.G2019S	p.T161N	7		0.010/1	-	-
				p.G648R	15	x	0.002/0	+	-
d13119	33	no	-	p.T161N	7		0.010/1	-	-
				p.G648R	15	x	0.002/0	+	-
d12867	NA	NA	PARK2 p.N58_Q178del	p.T161N	7		0.010/1	-	-
				p.G648R	15	x	0.002/0	+	-

1000G/hom: The frequency of the variant the 1000 Genomes project followed by the number of homozygous individuals.

AAO: Age at onset. FH: Familial History. NA: Unknown. Mutations'PDgene' shows pathogenic mutations found in the known PD genes.

3.2. European replication samples

We performed mutation analyses of all *ATP10B* exons in a European replication cohort consisting of 744 EOPD samples with an average AAO of 39.3 ± 7.0 years, originating from France, Germany and Austria. Possible compound heterozygous *ATP10B* variations (Table 6) were identified in 4 patient samples (g3775, g3832, g8471 & g8343). Homozygous recessive variations (Table 6) were observed in one affected individual (g8507). Since we have no access to biomaterials of family members of the mutation carriers, variant phase determination is not straightforward. In patient g3832, we confirmed *ATP10B*

compound heterozygosity using the TOPO TA cloning kit for sequencing. In the other patients the inter-mutation distance is too large to reliably amplify and clone the DNA fragment of interest.

Table 6. Overview of the additionally identified compound heterozygous/homozygous *ATP10B* mutation carriers

ID	AAO	FH	RT	BK	RI	Variations	Exons	Had-like	MAF _{CON}	MAF _{EOPD}
g8471	47	no	NA	NA	NA	p.R153s	6	x		0.004
						p.R1449K	26			0.004
g8507	45	no	x	x	x	p.V239M	8			0.002
						p.G671R_Hom	15	x		0.030
						p.N865K_Hom	17	x		0.030
g8343	41	yes	NA	NA	NA	p.G393W	12	x		0.013
						p.G671R	15	x		0.030
						p.N865K	17	x		0.030
g3775	39	no	x	x	x	p.V239M	8		0.000	0.002
						p.G671R	15	x	0.031	0.033
						p.N865K	17	x	0.031	0.033
g3832	39	no	x	x	x	p.G393W	12	x	0.020	0.033
						p.I540T	13	x	0.000	0.002

1000G/hom: The frequency of the variant the 1000 Genomes project followed by the number of homozygous individuals. AAO: Age at onset. FH: Familial History. Ldopa+: Levo-dopa response. RT: Rust tremor. BK: Bradykinesia. RI: Rigidity. AS: Asymmetric onset. NA: Unknown. _Hom: Homozygous variation. MAF_{CON}: minor allele frequency in control individuals. MAF_{EOPD}: minor allele frequency in EOPD patients.

- 10 Interestingly, the variations leading to mutant protein p.G671R/p.N865K are again multiple times observed in both (possible) compound heterozygosity with another rare *ATP10B* variation and in homozygous state. These variations were also observed in two of the Flanders-Belgian *ATP10B* PD families (DR621 & DR741). Unfortunately the lack of gDNA of relatives hampers haplotype sharing analyses of the European replication samples (g8507, g8343 & g3775).
- 15 The specific variation combinations identified in patients g3775 and 3832 were absent in 184 geographically and ethnically matched control individuals with an average AAI of 61.1 ± 12.5 years. Additionally, disease-causing mutations in the known PD genes were excluded in both patients and none of the parents presented with PD-related symptoms supporting a causal role for these (possible) compound heterozygous variations in *ATP10B*.

20 **4. Identification of digenic mutation carriers**

It is well documented that in several EOPD patients heterozygous mutations are detected in only one of the known autosomal recessive PD genes. Possibly, their combination with heterozygosity in another currently unknown autosomal recessive gene increases susceptibility to PD. In a cohort of 233 Flanders-

Belgian EOPD patients, including both the research and DSF sample collection, we identified in one patient (d2687/DR293-AAO39y-Table 7) carrying a heterozygous *PARK2* exon 2 duplication (p.N58CfsX41) a heterozygous variation in *ATP10B* (p.G393W).

Table 7. Overview of an identified digenic Flanders-Belgian mutation carrier and its phenotype

ID	AAO	FH	Gene	Variations	Exons	1000G/hom	PPhen2	Conserv
d2687	39	No	<i>ATP10B</i>	p.G393W	12	0.009/0	+	+
			<i>PARK2</i>	p.N58CfsX41 (ex2dup)	2			

1000G/hom: The frequency of the variant the 1000 Genomes project followed by the number of homozygous individuals. AAO: Age at onset. FH: Familial History. NA: Unknown.

In the European replication cohort we identified multiple possible digenic mutation carriers as well (Table 8).

Table 8. Overview of the identified digenic mutation carriers and their phenotype

ID	AAO	FH	Gene	Variations	Exons	1000G/hom	PPhen2	Conserv
g3766	28	yes	<i>ATP10B</i>	p.G393W	12	0.009/0	+	+
			<i>GBA</i>					
g3770	45	yes	<i>ATP10B</i>	p.G671R	15	0.012/0	-	-
			<i>ATP10B</i>	p.N865K	17	0.011/0	+	+
			<i>SCA2?</i>					
g3815	25	yes	<i>ATP10B</i>	p.G671R	15	0.012/0	-	-
			<i>ATP10B</i>	p.N865K	17	0.011/0	+	+
			<i>EIF4G1</i>					
g3887	27	no	<i>ATP10B</i>	p.G671R	15	0.012/0	-	-
			<i>ATP10B</i>	p.N865K	17	0.011/0	+	+
			<i>PINK1</i>					
g3906	42	no	<i>ATP10B</i>	p.G393W	12	0.009/0	+	+
			<i>GBA</i>					
g8083	38	yes	<i>ATP10B</i>	p.G671R	15	0.012/0	-	-
			<i>ATP10B</i>	p.N865K	17	0.011/0	+	+
			<i>PARK2</i>					
g8119	33	yes	<i>ATP10B</i>	p.R153s	6	0.002/0	NA	NA
			<i>GBA</i>					
g8188	30	no	<i>ATP10B</i>	p.G671R	15	0.012/0	-	-
			<i>ATP10B</i>	p.N865K	17	0.011/0	+	+
			<i>PARK2</i>					
g8235	34	no	<i>ATP10B</i>	p.G393W	12	0.009/0	+	+
			<i>GBA</i>					

1000G/hom: The frequency of the variant the 1000 Genomes project followed by the number of homozygous individuals. AAO: Age at onset. FH: Familial History. NA: Unknown.

5. ATP10B gene expression analyses in different human tissues

In addition to the genetic evidence we aimed at generating initial insights into the biological relevance of ATP10B. To determine the expression pattern of the novel gene we performed reverse transcription qPCR on RNA from a commercially available survey panel comprising multiple human tissues using the ViiA™ 7 Real-Time PCR System (Life Technologies™) and multiple Taqman and SYBR Green assays covering the majority of its reported isoforms. Using Qbase+ software, we normalized against different housekeeping genes (GUSB, ACTB, PPIA, PPK1, RPL13A & HPRT1) and calculated relative expression levels of the gene of interest with the $\Delta\Delta C_t$ method. We detected the highest expression levels in brain, colon, small intestine, trachea and esophagus (Figure 6).

Subsequently we determined ATP10B expression levels in different brain regions including parietal cortex, occipital cortex, temporal cortex, frontal cortex, cerebellum, medulla, substantia nigra and basal ganglia and observed the highest expression in basal ganglia and substantia nigra (Figure 7), fitting the neuropathological hallmarks of PD patient brains.

Substantia nigra and medulla oblongata samples from respectively five and six idiopathic PD patients and four age- and gender-matched control individuals without neurologic pathology were provided by the Antwerp Biobank at the Institute Born-Bunge, University of Antwerp, Belgium. Using qPCR on these samples, we investigated whether *ATP10B* expression levels differed between PD patients and control individuals. These two brain regions were selected as they show the highest *ATP10B* expression levels and represent different stages in PD pathology spreading. Compared with samples of neurologically healthy individuals, a significant decrease ($p = 0.01$) of ATP10B expression was observed in the medulla oblongata of PD patients (Figure 8). ATP10B mRNA levels were also marginally reduced in the substantia nigra ($p = 0.51$).

6. Functional characterization of ATP10B and its mutant forms

6.1. ATP10B WT, but not ATP10B disease mutants, promotes phosphatidylcholine lipid uptake

ATP10B belongs to the family of P-type transport ATPases, which comprises a large number of evolutionary related membrane-integrated pumps that couple ATP hydrolysis to the active transport of a substrate across the membrane. During the catalytic cycle, P-type ATPases form a phosphorylated intermediate on the D of the D-K-T-G-T-[LIVM]-[TIS] signature motif.

The family of P-type ATPases is divided into five subfamilies (P1-P5) according to sequence similarity. P1-P3-type ATPases are ion pumps with well-established functions. For the P5-type ATPases, such as ATP13A2/PARK9, the transported substrate is not yet established. ATP10B belongs to the P4 subfamily containing lipid flippases that establish, maintain or disrupt the vitally important lipid asymmetry

between the two membrane leaflets of various cellular membranes. P4 ATPases interact with members of the evolutionary conserved CDC50 protein family.

ATP10B belongs to the poorly described class 5 (or V) P4-type ATPases (Fig. 9A), which work together with the obligatory regulatory subunit CDC50A. Together with CDC50A, the closely related ATP10A isoform promotes phosphatidylcholine (PC) lipid uptake in the plasma membrane of mammalian cells (Naito et al 2015 J Biol Chem, PMID 25947375), in line with a role as a PC lipid flippase. To determine whether ATP10B might also be a PC lipid flippase, we performed a PC uptake assay as described in Naito et al (2015) with minor modifications. As a negative control, a mutant of ATP10B was generated in which Asp433, the site of autophosphorylation, is replaced by Asn (D433N). This substitution prevents autophosphorylation and therefore transport activity in all studied P-type ATPases.

Lipid flippase activity is typically assessed by measuring the uptake of fluorescently labeled NBD-lipids in mammalian cells overexpressing the flippase of interest. NBD stands for 7-nitrobenz-2-oxa-1,3-diazol-4-yl. Therefore, ATP10B (WT or D433N) was co-expressed with CDC50A in HeLa cells via transient transfection. HeLa cells were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, D6546) culture media containing 1% glutamine and penicillin/streptomycin (Sigma-Aldrich, G7513 and P0781 respectively) as well as 10% fetal bovine serum (FBS, HyClone). Cells were cultured for a maximum of 20 passages and underwent monthly tests for mycoplasma contamination. HeLa cells were grown to 70% confluency before transfection commenced. Cells were transfected (ratio of 3:1 respectively) with gene juice (Millipore), CDC50A (NCBI ID: NM_018247, pcDNA3.0, containing an N-terminal FLAG tag) and ATP10B variants (pcDNA3.0-DEST with ATP10B WT or mutants) for 72 h full culture medium. Cells were then washed and incubated overnight in full media before experimentation. Untransfected cells were taken along as a negative control.

The expression of ATP10B and CDC50A was confirmed after transient transfection (Fig. 9B). To that end, HeLa microsomes were prepared and immunoblotting was performed as previously described (Holemans et al 2015 PNAS, PMID: 26134396). Briefly, western blots of typically 20-25 µg of HeLa microsomes were ran on 4-12% Bis/Tris gel (NuPage, Life technologies) and transferred to a 0.45 µm PVDF membrane (Immobilion-P, Millipore) and probed for ATP10B (Sigma-Aldrich, HPA034574), CDC50A (anti-FLAG antibody from Sigma-Aldrich, F3165) and GAPDH (Sigma-Aldrich, G8795) as loading control. Detection was performed using the applicable HRP based secondary antibodies (BIOKE, 7074S and 7076S). Detections were performed on a Bio-Rad Chemidoc Imager with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, 32106).

The lipid uptake assay was optimized from Naito et al (2015). Following transfection with ATP10B variants in comparison to control, HeLa cells were washed with PBS (Thermo Fisher Scientific, 14190169), detached with TrypLE Express (Thermo Fisher Scientific, 12604021) and harvested by centrifugation. The cells (1×10^6 cells/condition) were washed with PBS, resuspended in 500 μ l Hank's balanced salt solution (HBSS, Thermo Fisher Scientific, 14170112) and incubated for 15 mins at 37°C. An equal volume of 2 μ M (7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)-labeled PC, NBD-phosphatidylethanolamine (PE) or NBD-phosphatidylserine (PS) (Avanti Polar Lipids) were added to the cell suspensions and incubated at 37°C for the required time point. At each subsequent time point, 200 μ l of cell suspension was collected and mixed with 200 μ l of ice-cold HBSS containing 5% BSA (Sigma-Aldrich, A3059). To quench the fluorescence of non-internalized NBD-phospholipids, 10 mM sodium dithionite (Sigma-Aldrich, 157953) in 1 M Tris (Sigma-Aldrich, T1503) pH 10 was added 15 mins prior to acquisition. Next, cell samples were analyzed with an Attune NxT flow cytometer (Life Technologies) measuring the mean fluorescence intensity per cell of 10^4 events.

After 30-60 min of NBD-PC exposure, ATP10B overexpression leads to a 2-3-fold increase in NBD-PC uptake compared to non-transfected (NT) cells or cells overexpressing the D433N mutant (Fig. 9C-D). This shows that NBD-PC uptake is stimulated by ATP10B activity, which is clearly in line with a role as a PC lipid flippase and which resembles the closely related PC lipid flippase ATP10A (Naito et al 2015 J Biol Chem, PMID 25947375). In contrast, ATP10B is unable to promote PS or PE uptake (Fig. 9E-F), two other lipids that are common substrates of P4-type ATPases. Thus, our data suggest that ATP10B is a lipid flippase with PC substrate specificity. The lower PE and PS uptake levels observed in cells overexpressing ATP10B WT or D433N compared to NT is likely attributed to the saturation of the CDC50A protein by ATP10B overexpression, which might hamper the endogenous activity of cellular PE/PS lipid flippases that also depend on CDC50A.

Several of the identified ATP10B disease mutations are found in highly conserved sequence stretches in P4-type ATPases (G671R, N865K, E993A, I1038T), suggesting a loss of ATP10B function (Fig. 10A). To compare the functionality of the ATP10B disease mutants with ATP10B WT and the catalytic dead mutant D433N, we assessed the PC uptake capacity of the ATP10B disease mutants following transient overexpression in HeLa cells (Fig. 10B-C). In contrast to ATP10B WT, but similar to the D433N mutant, none of the disease mutants promote PC lipid uptake, which is in line with a loss-of-function phenotype and which would explain the recessive character of the mutations.

In conclusion, our results demonstrate that the identified genetic mutations in the ATP10B gene, which are associated with early onset PD, are loss of function mutations.

6.2. ATP10B is a lysosomal PC flippase controlling lysosomal mass and morphology

To determine in which subcellular compartment ATP10B is expressed in HeLa cells, we performed immunolocalization and fluorescence microscopy following transient transfection of CDC50A/ATP10B (Fig. 11). HeLa cells were transfected with N- or C-GFP-ATP10B or D433N (pcDNA6.2) using Lipofectamine 2000 (Thermo Fischer Scientific, 11668-027 ratio of 3:1) overnight in serum free media before the assay was terminated by the addition of full culture media. The cells were allowed to recover for a minimum of 6 h before fixation (30 mins, 37°C) with 4% paraformaldehyde. After fixation with 4% paraformaldehyde, cells were washed twice with PBS and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, T9284) and 0.02% SDS (Acros Organics, 230425000) containing PBS for 50 minutes. To minimize aspecific binding, 0.1 M Glycine in PBS was added to the cells after washing with PBS, followed by blocking in PBS containing 1% BSA and 10% FBS. After washing, cells were incubated overnight at 4°C with the primary antibodies: EEA-1 (Becton Dickinson, 610457), CD63 (Bio-Connect Diagnostics, 11-343-C100) or LAMP-1 (Santa Cruz Biotechnology, SC5570) diluted according to manufacturer's recommendation in PBS containing 0.2% Tween-20 (Bio Rad, 170-6531), 0.1% BSA and 1% FBS. Finally, after the samples were washed with 0.2% PBS-T, cells were incubated with Alexa Fluor dyes (Thermo Fisher Scientific). Visualization with the Olympus fluorescent microscope was performed after washing three times with 0.2% PBS-T.

Our results indicate that ATP10B only slightly interacts with the early endosome marker EEA1 (Fig. 11A) or the late endosomal marker CD63 (Fig. 11C). Instead, ATP10B is mainly present in LAMP1-positive organelles (Fig. 11B), a marker of the late endo-/lysosomes, suggesting that ATP10B is targeted to the lysosomal compartment, which is in agreement with Naito et al (2015). In line with the lysosomal distribution of ATP10B, we demonstrate that the overexpression of ATP10B WT alters lysosomal morphology, as indicated by the enlarged lysosomal vesicles and lysosomal tubulation (arrows, Fig. 12B). This phenotype is not observed when the D433N mutant is overexpressed, indicating that the PC lipid flippase activity is responsible for the effect.

The altered lysosomal morphology in ATP10B overexpressing cells might reflect a change in lysosomal mass, which was verified by flow cytometry. Assessment in lysosomal mass was performed with LysoTracker according to the manufacturer's instructions. Briefly, transfected cells were collected and 10^5 cells resuspended in PBS with or without 1 μ M LysoTracker RED DND-99 (Life Technologies, 37°C for 30 mins) to a final volume of 1 ml. Cells were then collected, washed and resuspended in 500 μ l HBSS (Thermo Fisher Scientific) and the fluorescence of 10^4 events acquired using an Attune flow cytometer (Life Technologies).

With this assay we demonstrated that ATP10B increases lysosomal mass (Fig. 12A) in line with an increased uptake of PC lipids in the lysosomal compartment. Instead, the catalytic dead mutant D433N does not affect lysosomal mass (Fig. 12A) or morphology (Fig. 12B). Alterations in lysosomal mass and morphology can therefore be used to assess the functionality of the ATP10B disease mutants since we have already demonstrated that some of our disease mutants do not lead to an increased lysosomal mass (Fig. 12A).

Discussion

Based on the data we produced, it is clear that *ATP10B* is a novel PD gene:

- We identified at least 2 recessive families with patient-specific compound heterozygous variations co-segregating with PD in a selection of 98 Flanders-Belgian EOPD patients (≥ 2.0 %), but no compound heterozygous Flanders-Belgian control individuals (N=173)
- Mutation analyses of 744 European EOPD samples revealed 4 additional possible compound heterozygous variation combinations and 1 homozygous variant carrier.
- Prediction programs and amino acid conservation suggest a pathogenic effect for the majority of the (possible) compound heterozygous or homozygous variations
- By evaluating the PC lipid uptake activity and lysosomal mass, we demonstrate a loss of function of ATP10B EOPD mutations.
- ATP10B seems a rather frequently mutated recessive gene, with rare heterozygous variations seen in both patients and control individuals in addition to patient-specific variations. This phenomenon is also observed for *PARK2* variations and fits the recessive inheritance of *ATP10B* associated disease
- Like *ATP13A2/PARK9*, *ATP10B* encodes a lysosomal P-type transport ATPase and is therefore an interesting candidate gene
- ATP10B is highly expressed in brain, especially in the substantia nigra and basal ganglia fitting the neuropathological hallmarks of PD patient brains

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Claims

1. A method of diagnosing a neurodegenerative disease in a subject, comprising determining the functional expression levels of ATP10B and/or determining the presence of one or more mutations in the ATP10B gene in a sample of said subject.
- 5 2. The method according to claim 1, wherein the neurodegenerative disease is Parkinson's disease.
3. The method according to claim 2, wherein Parkinson's disease is early-onset Parkinson's disease.
4. The method according to any one of claims 1 to 3, wherein at least one of the mutations is in the Had-like domain.
5. The method according to any one of claims 1 to 4, wherein detection of mutations is done using
10 sequencing, a hybridization assay or PCR-based assay, such as the MastR™ assay.
6. A kit comprising at least one primer or probe suitable to determine the presence of one or more mutations in the ATP10B gene.
7. The ATP10B protein, or nucleic acid encoding said protein, for use as a medicament.
8. The ATP10B protein, or nucleic acid encoding said protein, for use in treating Parkinson's disease.
- 15 9. A method of treating Parkinson's disease in a subject in need thereof, comprising increasing the levels of ATP10B in said subject.
10. The method according to claim 9, wherein the levels of ATP10B are increased by administering the ATP10B protein, or nucleic acid encoding said protein, to said subject.
11. The method according to claim 10, which is a gene therapy method.
- 20 12. A method to screen for compounds that increase ATP10B expression or ATP10B activity, comprising:
 - Adding a compound to a sample of cells with decreased functional expression of ATP10B or decreased activity of ATP10B;
 - Evaluating the expression or activity of ATP10B after addition of the compound.
13. A method to screen for compounds that increase ATP10B expression, comprising:
25
 - adding a compound to a sample of cells which comprise the ATP10B promoter, optionally functionally linked to a reporter gene;
 - evaluating the activity of the ATP10B promoter.

- To identify a compound that increases ATP10B expression.

14. A method to screen for compounds to compensate for a reduced phosphatidyl choline uptake due to a reduced expression of ATP10B and/or reduced activity of ATP10B, comprising:

- Adding a compound to a sample of cells with decreased functional expression of ATP10B and/or decreased activity of ATP10B;

5

- Evaluating the uptake of phosphatidyl choline after addition of said compound;
- to identify a compound that compensates for a reduced phosphatidyl choline uptake due to a reduced expression of ATP10B and/or reduced activity of ATP10B.

Figure 1

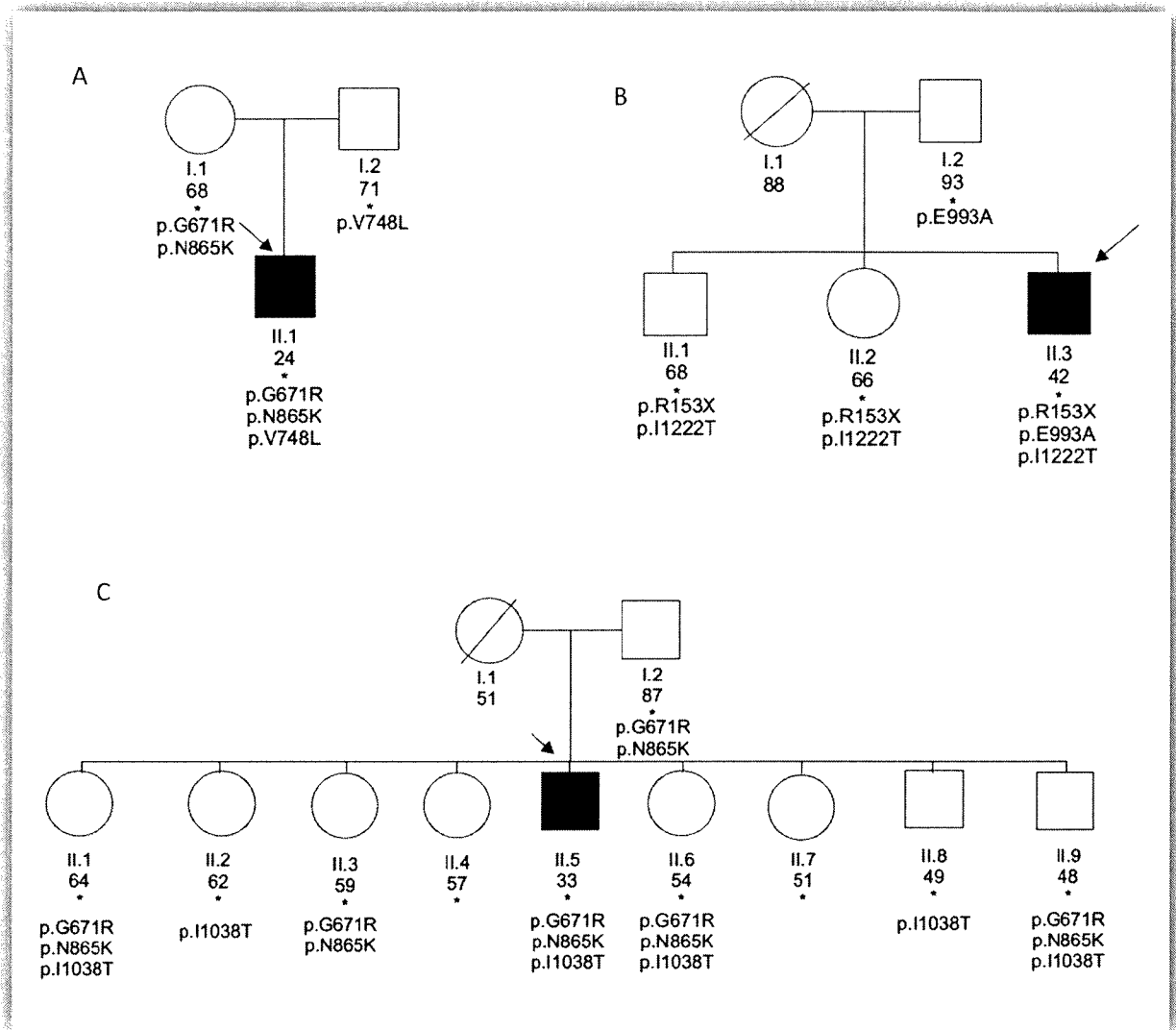


Figure 2

	<u><i>d12522</i></u>			<u><i>d12421</i></u>	
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chr5:143Mb	425/421	421/425		429	421
chr5:144Mb	200/203	203/200		203	203
chr5:146Mb	165	173		173	169
chr5:147Mb	205	207		207	213
chr5:151Mb	331	329		331	329
chr5:152Mb	195	195		195	193
chr5:155Mb	265	257		265	253
chr5:156Mb	326	330		326	334
chr5:158Mb	304	302		304	306
chr5:159Mb	378	368		378	354
chr5:160Mb					
chr5:160Mb	224/230	230/224		232	232
chr5:163Mb	265	279		269	269
chr5:164Mb	233	223		233/225	233/225
chr5:166Mb	308/300	300/308		-	-

Figure 3

- **p.G671R**

- PolyPhen2: Benign
- SIFT: Tolerated

S	G	G	D	S	T	D	D	G	G	Y	Homo sapiens
S	G	G	D	S	T	D	D	G	G	Y	Macaca mulatta
S	G	D	C	S	T	-	D	G	G	Y	Mus musculus
S	G	G	Y	S	T	-	D	G	G	Y	p_Canis lupus familiaris
C	D	N	D	D	G	Y	C	D	S	S	p_Gallus gallus
-	-	-	-	S	G	Q	T	E	G	V	p_Oreochromis niloticus

- **p.N865K**

- PolyPhen2: Prob damaging
- SIFT: Deleterious

D	N	R	D	E	L	L	M	E	T	A	Q	H	L	E	N	Q	Homo sapiens
D	N	R	D	E	L	L	M	E	T	A	Q	H	L	E	N	Q	Macaca mulatta
D	N	R	E	E	L	L	M	E	T	A	Q	H	L	E	N	H	Mus musculus
E	N	R	D	E	L	L	M	E	T	A	Q	H	L	E	N	Q	p_Canis lupus familiaris
D	N	R	D	E	L	L	M	E	T	A	Q	H	L	E	T	K	p_Gallus gallus
E	D	R	E	R	L	V	M	D	T	A	V	Q	L	E	T	N	p_Oreochromis niloticus

- **p.V748L**

- PolyPhen2: Prob damaging
- SIFT: Deleterious

T	V	R	L	P	Q	G	T	C	L	T	F	S	L	Homo sapiens
T	V	R	L	P	Q	G	T	C	L	T	F	S	L	Macaca mulatta
T	V	R	L	P	Q	G	I	C	L	T	F	D	L	Mus musculus
T	V	R	L	P	Q	G	T	C	L	T	F	D	I	p_Canis lupus familiaris
T	V	R	L	P	Q	G	T	L	L	T	F	D	I	p_Gallus gallus
S	V	K	L	P	S	G	E	V	L	D	F	E	V	p_Oreochromis niloticus

- **p.I1038T**

- PolyPhen2: Poss damaging
- SIFT: Deleterious

M	I	V	K	Homo sapiens
M	V	V	K	Macaca mulatta
M	I	V	K	Mus musculus
M	I	V	K	p_Canis lupus familiaris
M	V	V	K	p_Gallus gallus
R	V	V	K	p_Oreochromis niloticus

- **p.E993A**

- PolyPhen2: Benign
- SIFT: Tolerated

P	E	A	G	L	V	I	D	G	Homo sapiens
P	E	A	G	L	V	I	D	G	Macaca mulatta
P	E	I	G	L	V	I	D	G	Mus musculus
P	E	V	G	L	V	I	D	G	p_Canis lupus familiaris
P	E	F	G	L	V	I	D	G	p_Gallus gallus
-	-	-	-	L	V	I	D	G	p_Oreochromis niloticus

- **p.I1222T**

- PolyPhen2: Prob damaging
- SIFT: Deleterious

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D	I	D	V	F	T	F	G	T	P	I	N	T	I	S	L	T	T	I	L	Mus musculus
D	I	D	V	F	T	F	G	T	P	I	N	T	I	S	L	T	T	I	L	p_Canis lupus familiaris
D	I	D	V	F	S	F	G	N	P	I	N	T	V	S	L	L	T	I	L	p_Gallus gallus
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Figure 4

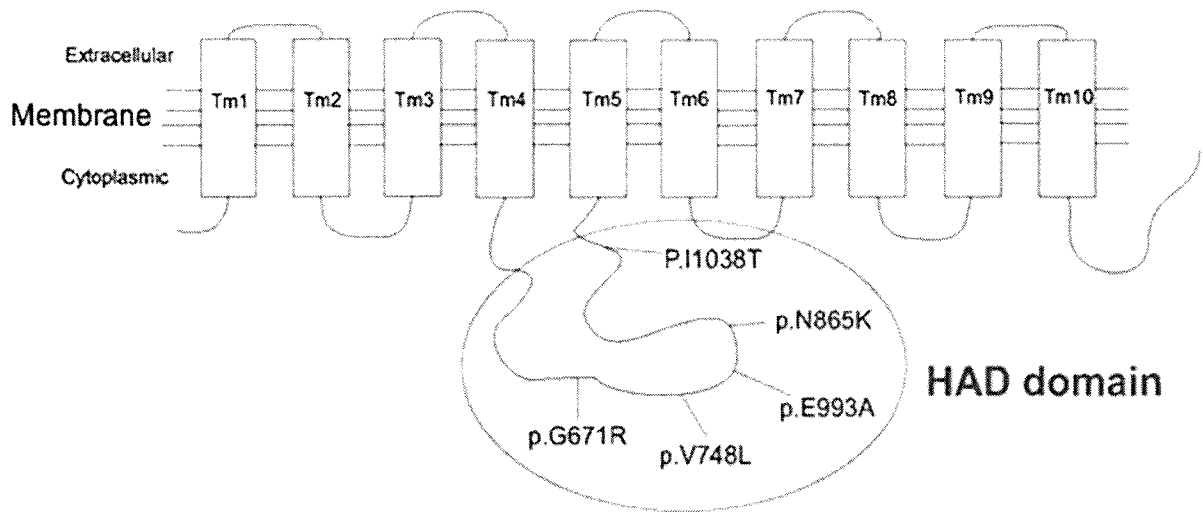


Figure 5

• **p.T161N**

- PolyPhen2: Benign
- SIFT: Tolerated

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T	Y	V	Q	K	C	W	K	D	V	R	V	G	D	F	I	Q	M	K	C	Macaca mulatta
R	Y	M	L	K	R	W	Q	D	V	R	V	G	D	F	V	Q	M	Q	C	Mus musculus
S	Y	V	Q	K	C	W	K	D	V	R	V	G	D	F	I	Q	M	Q	C	p_Canis lupus familiaris
A	Y	V	E	K	C	W	K	D	V	R	V	G	D	F	V	Q	L	Q	C	p_Gallus gallus
Q	E	V	E	R	C	W	K	D	V	R	V	G	D	F	V	K	V	V	C	p_Oreochromis niloticus

• **p.G648R**

- PolyPhen2: Prob damaging
- SIFT: Tolerated

T	D	L	G	E	S	L	G	A	N	V	A	T	T	D	S	Homo sapiens
T	D	L	G	E	S	L	G	A	N	V	A	T	T	D	S	Macaca mulatta
T	D	L	G	E	S	L	G	P	N	L	P	T	I	D	S	Mus musculus
I	D	L	G	E	S	L	G	A	N	M	P	T	T	D	S	p_Canis lupus familiaris
-	-	S	-	-	D	L	G	A	S	F	S	A	K	N	T	p_Gallus gallus
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Figure 6

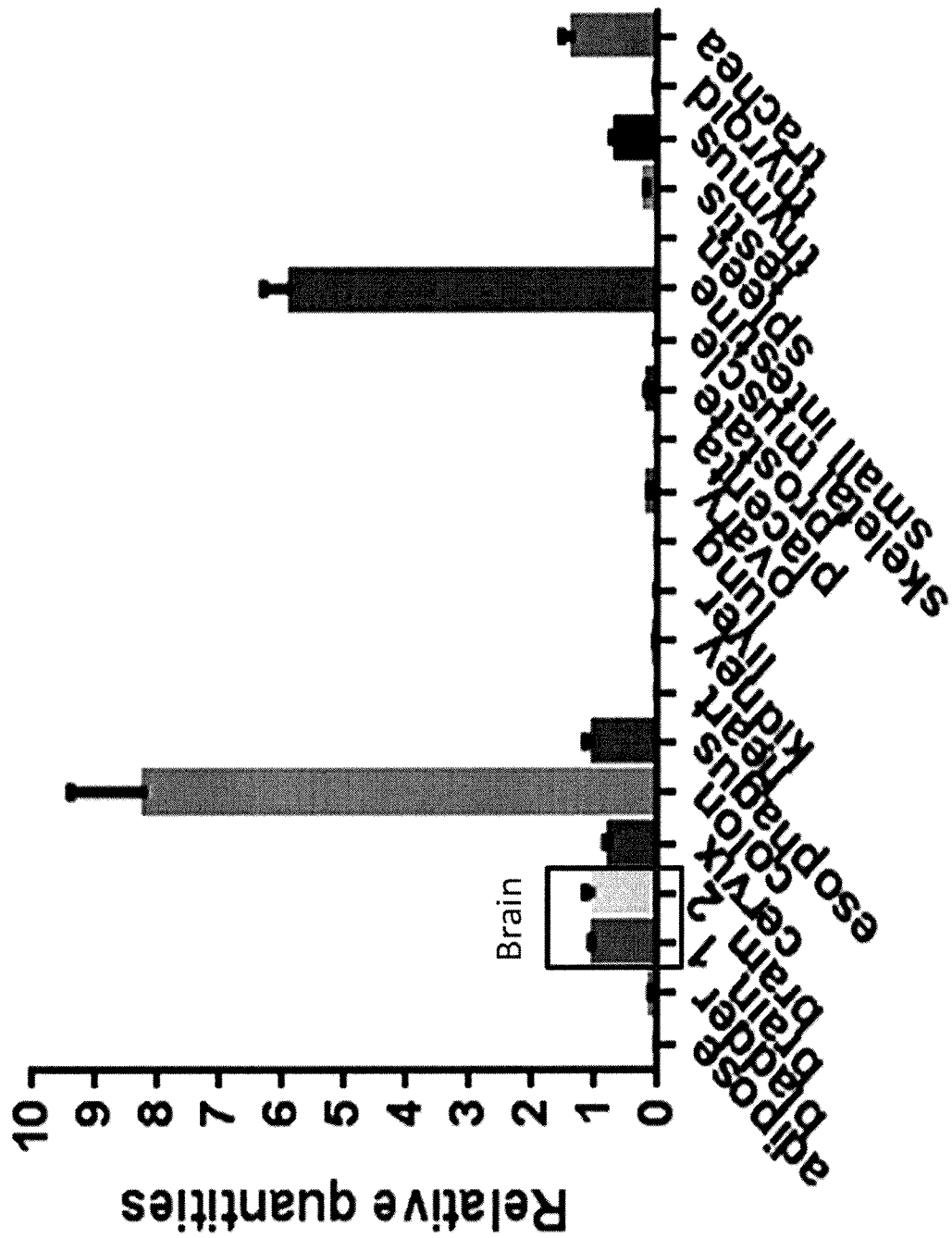


Figure 7

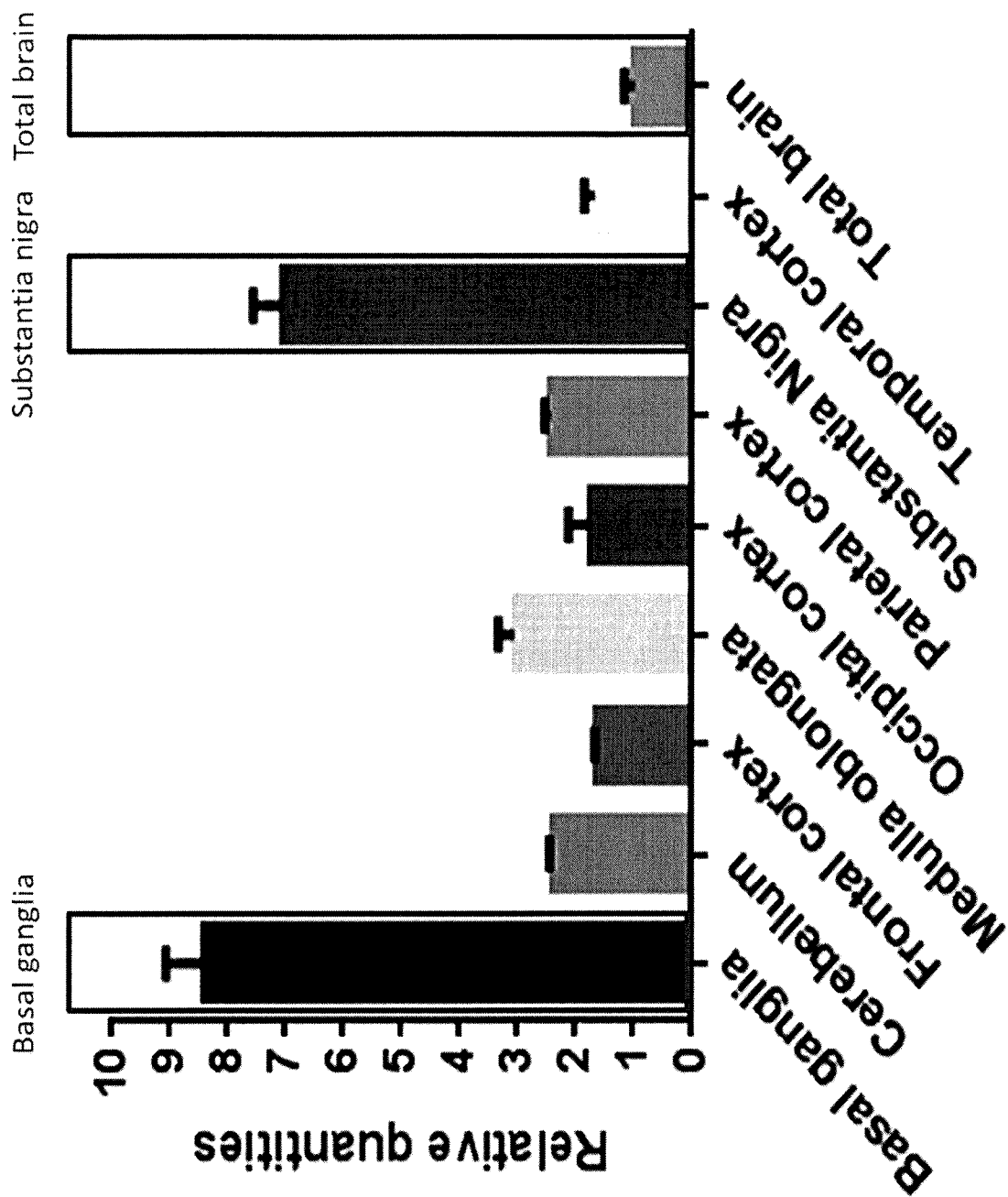


Figure 8

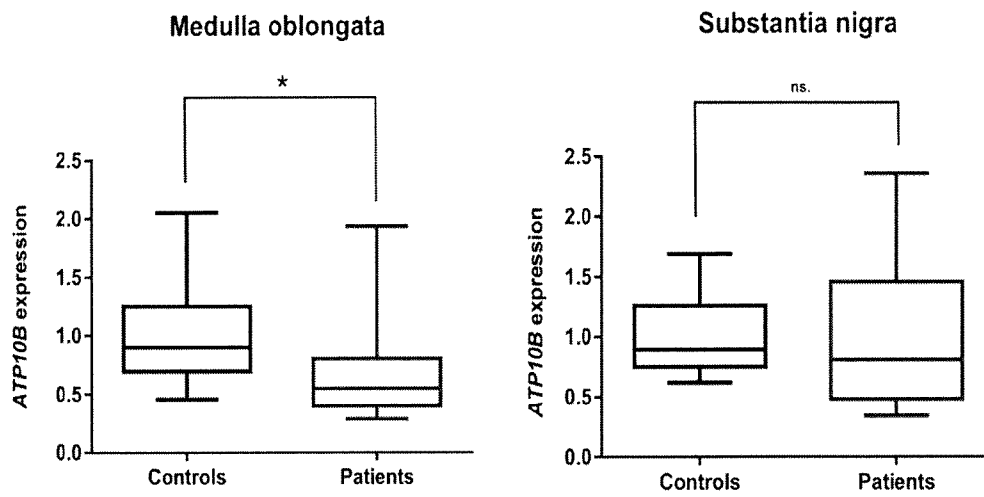


Figure 9

A

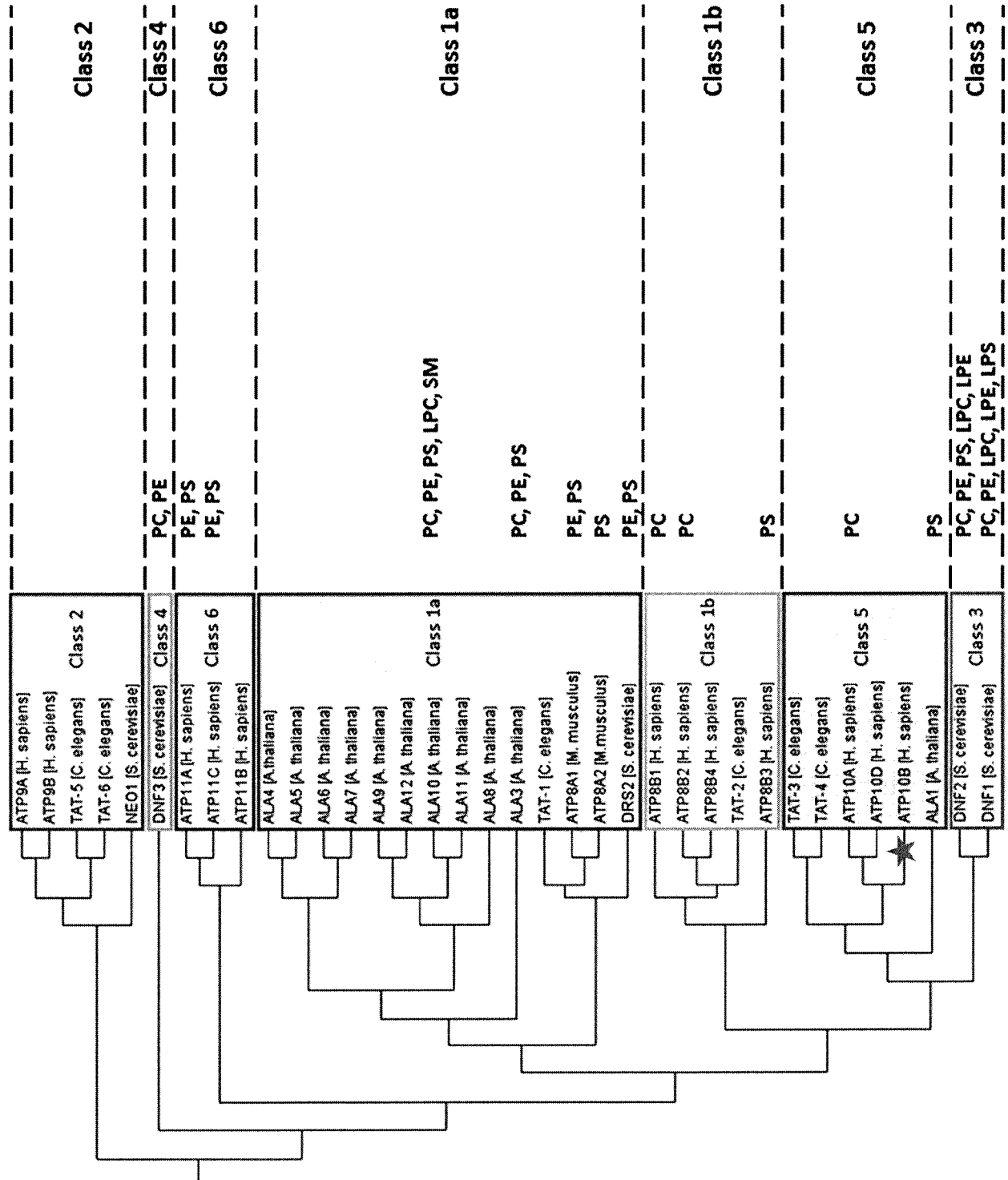


Figure 9 continued

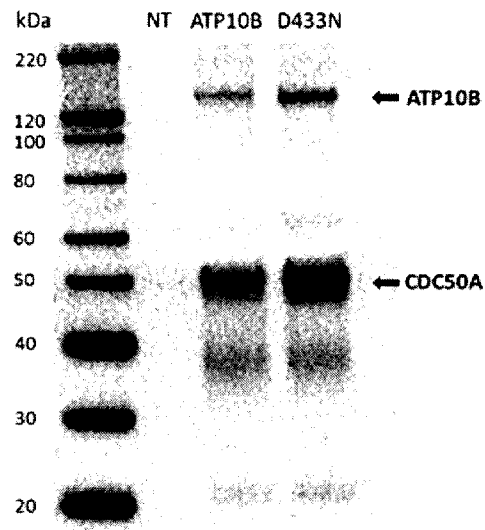
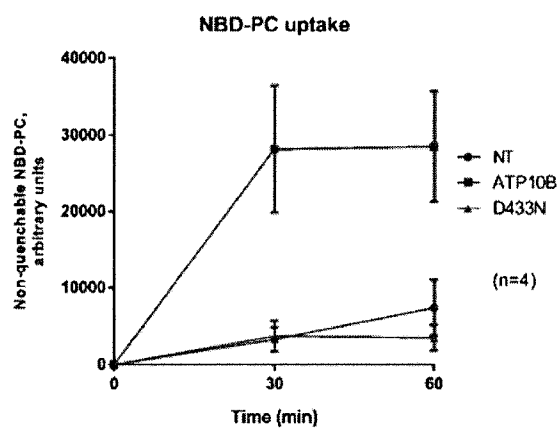
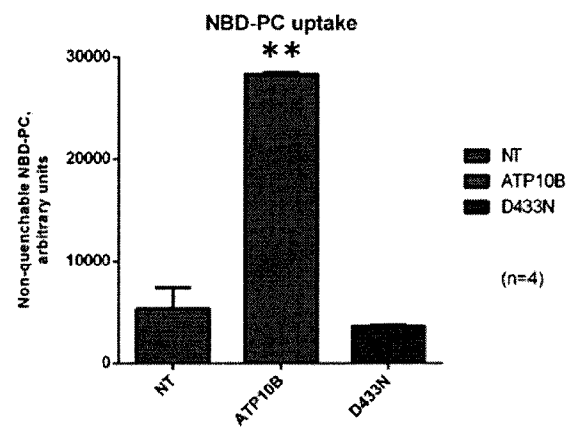
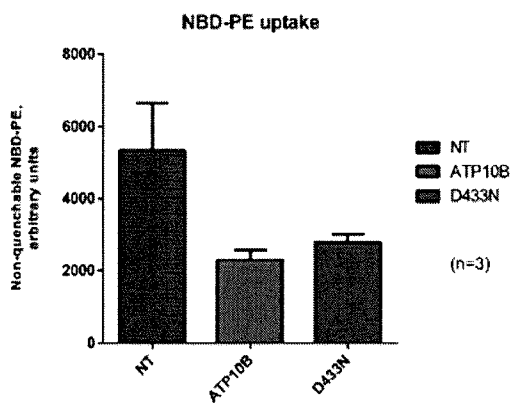
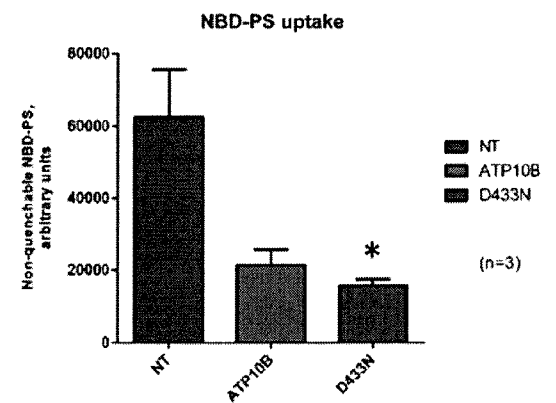
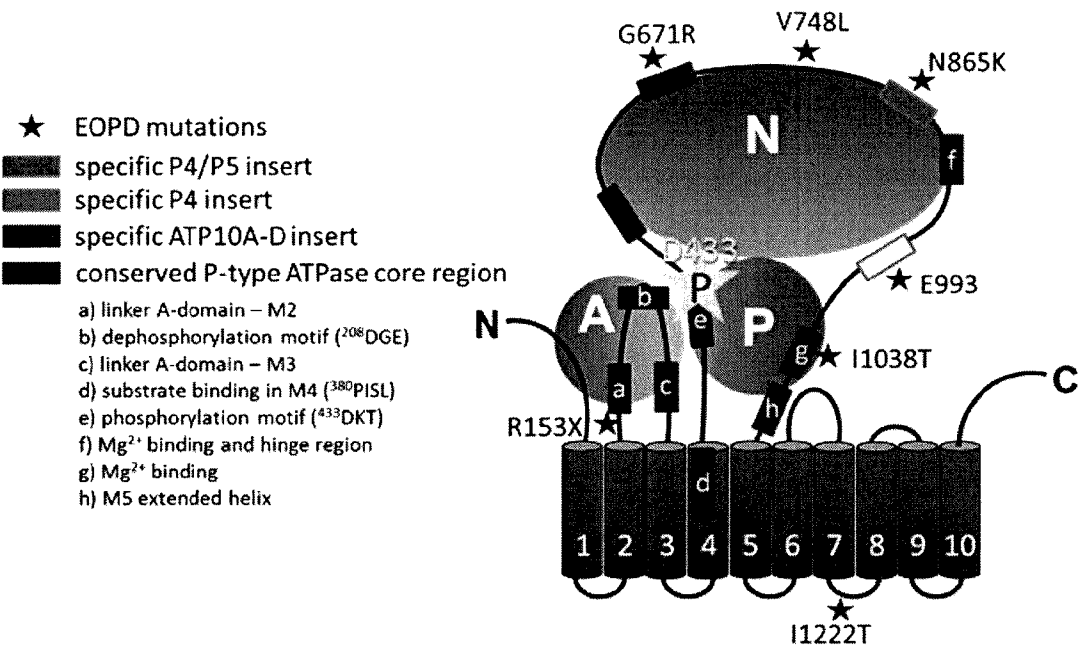
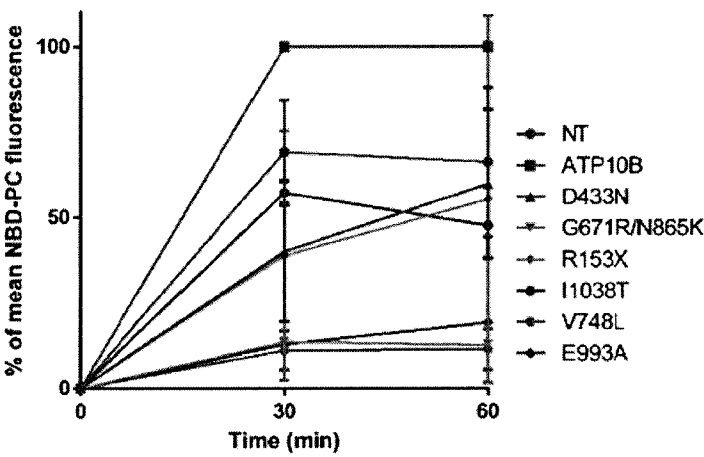
B**C****D****E****F**

Figure 10

A



B



C

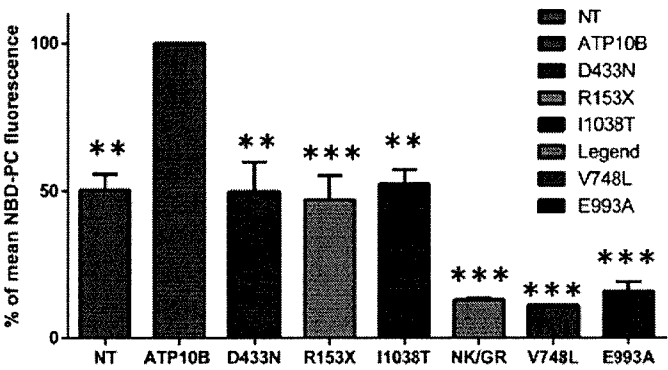


Figure 11

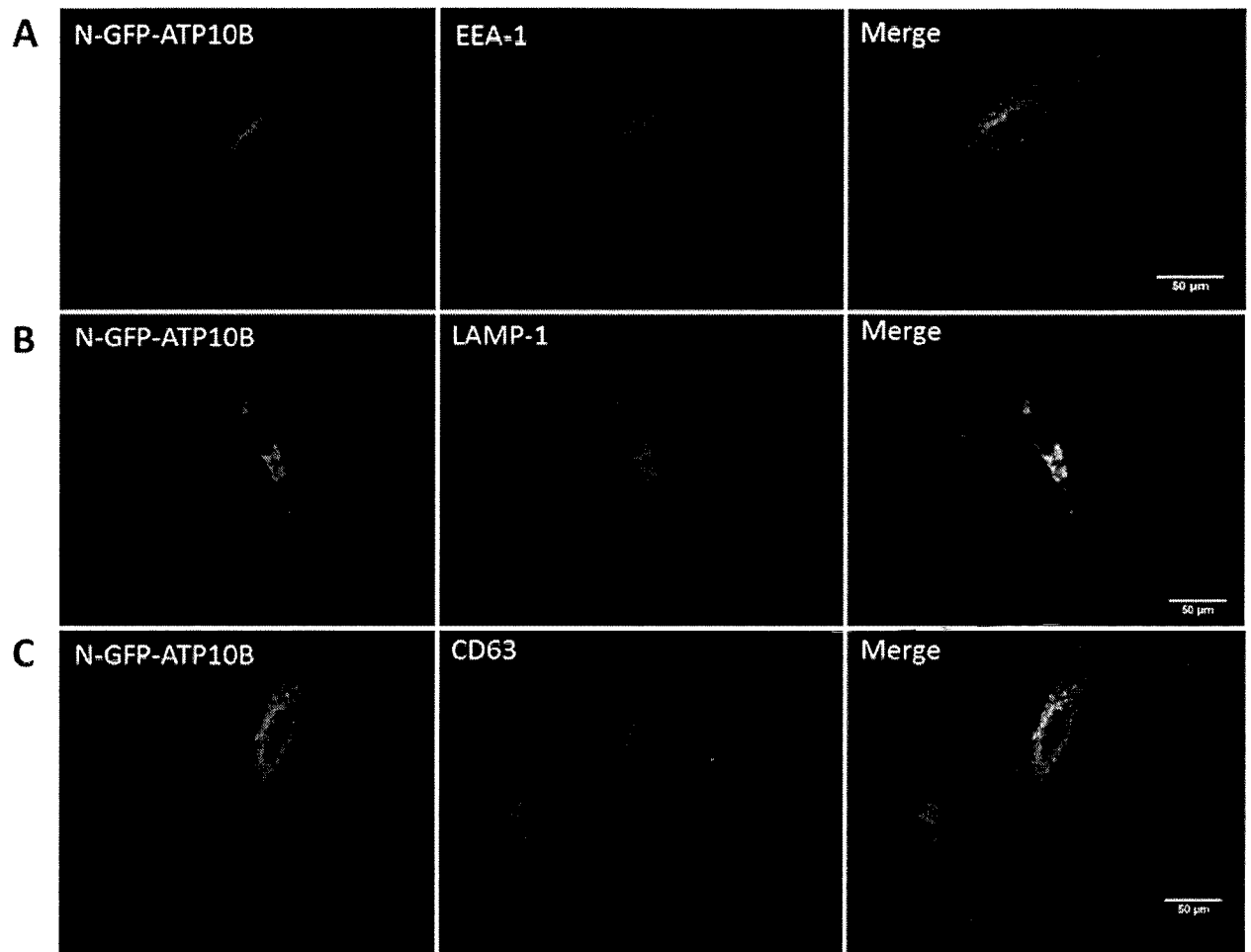
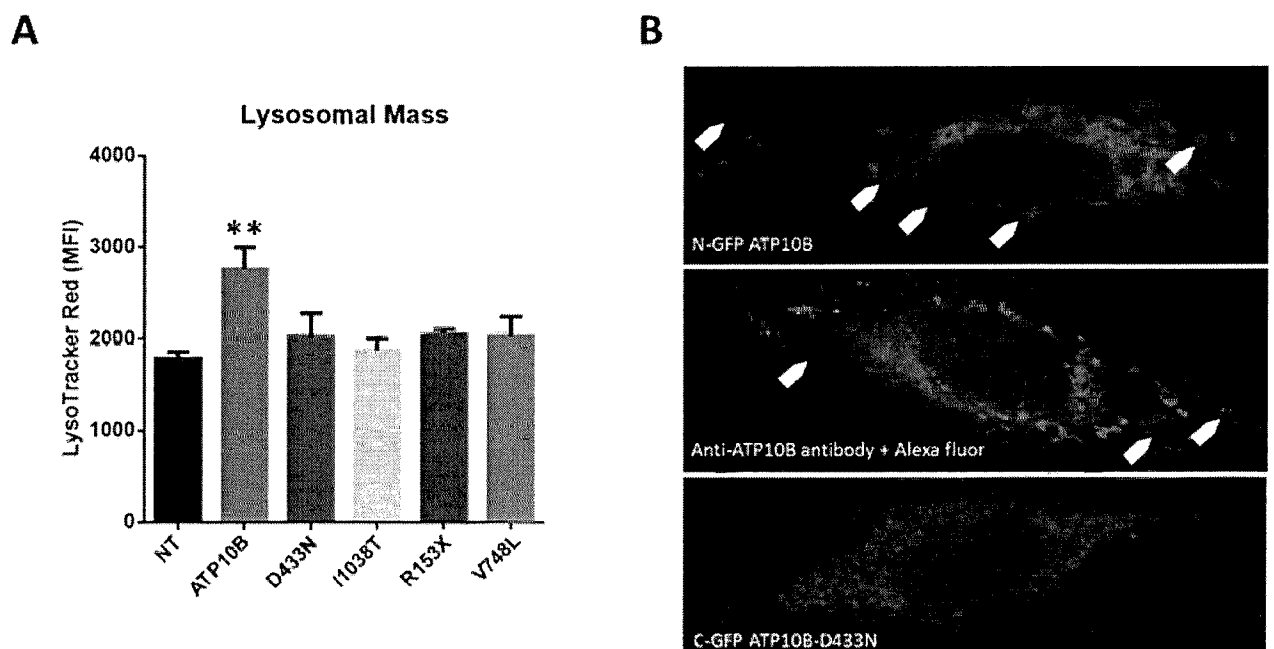


Figure 12



INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2016/058558

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/058558

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12Q1/68 G01N33/68
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 G01N

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, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/091113 A1 (UNIV MCMASTER [CA]) 27 June 2013 (2013-06-27)	6-8
A	abstract paragraphs [0085], [0165]; claims 8,11; tables 4,5	1-5,9-14

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Further documents are listed in the continuation of Box C.



See patent family annex.

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14 July 2016

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Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

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Celler, Jakub

INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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