A Mutation in *VPS35*, Encoding a Subunit of the Retromer Complex, Causes Late-Onset Parkinson Disease

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To identify rare causal variants in late-onset Parkinson disease (PD), we investigated an Austrian family with 16 affected individuals by exome sequencing. We found a missense mutation, c.1858G>A (p.Asp620Asn), in the *VPS35* gene in all seven affected family members who are alive. By screening additional PD cases, we saw the same variant cosegregating with the disease in an autosomal-dominant mode with high but incomplete penetrance in two further families with five and ten affected members, respectively. The mean age of onset in the affected individuals was 53 years. Genotyping showed that the shared haplotype extends across 65 kilobases around *VPS35*. Screening the entire *VPS35* coding sequence in an additional 860 cases and 1014 controls revealed six further nonsynonymous missense variants. Three were only present in cases, two were only present in controls, and one was present in cases and controls. The familial mutation p.Asp620Asn and a further variant, c.1570C>T (p.Arg524Trp), detected in a sporadic PD case were predicted to be damaging by sequence-based and molecular-dynamics analyses. VPS35 is a component of the retromer complex and mediates retrograde transport between endosomes and the trans-Golgi network, and it has recently been found to be involved in Alzheimer disease.

Parkinson's disease (PD [MIM 168600]) is the second-most common neurodegenerative disorder; it affects 1%–2% of the population above the age of 60.[1] It is characterized by degeneration of dopaminergic neurons in the nigrostriatal pathway and other monoaminergic cell groups in the brainstem. This degeneration leads to bradykinesia, resting tremor, muscular rigidity, and postural instability as well as nonmotor symptoms. Up to 20% of cases with PD are reported to be familial,[2,3] but extended pedigrees with clear Mendelian inheritance are rare. Genetic studies have so far revealed mutations in five genes causing autosomal-recessive (*PARK2* [MIM 602544], *PINK1* [MIM 608309], *PARK7* [MIM 602533]) or autosomal-dominant (*SNCA* [MIM 163890], *LRRK2* [MIM 609007]) forms of PD.[4–9] Whereas the autosomal-recessive forms with early onset and *SNCA* missense mutations or duplications[10] are rare, a single *LRRK2* mutation (RefSeq number NM_198578.3: c.6055G>A [p.Gly2019Ser]) accounts for approximately 1% of sporadic cases of European origin.[11–13] A recent study revealed a strong association of PD with glucocerebrosidase (*GBA*) mutations in carriers for Gaucher [MIM 230800] disease, thus implicating a lysosomal enzyme in the pathogenesis of PD.[14,15] Genome-wide association studies revealed several low-risk susceptibility loci, among them *LAMP3* [MIM 605883] and *HIP1R* [MIM 605613], which have been reported to be implicated in the lysosomal pathway.[16–18]

We identified an Austrian family in which 16 members were affected by PD (family A, Figure 1). PD seemed to be inherited in an autosomal-dominant mode with high penetrance. Seven affected members were available for clinical and DNA investigations. Six of them exhibited at least three of the four cardinal signs of PD (akinesia, resting tremor, rigidity, and postural instability) and showed improvement after dopaminergic treatment. A single affected individual had displayed action tremors since childhood but developed L-Dopa-responsive resting tremors and akinesia only at the age of 62 years. The mean age of onset was 53 years (range 40–68 years) (Table 1). The clinical diagnosis of idiopathic PD was made by movement-disorder specialists who used UK brain bank criteria for PD.[19] All participants gave written informed

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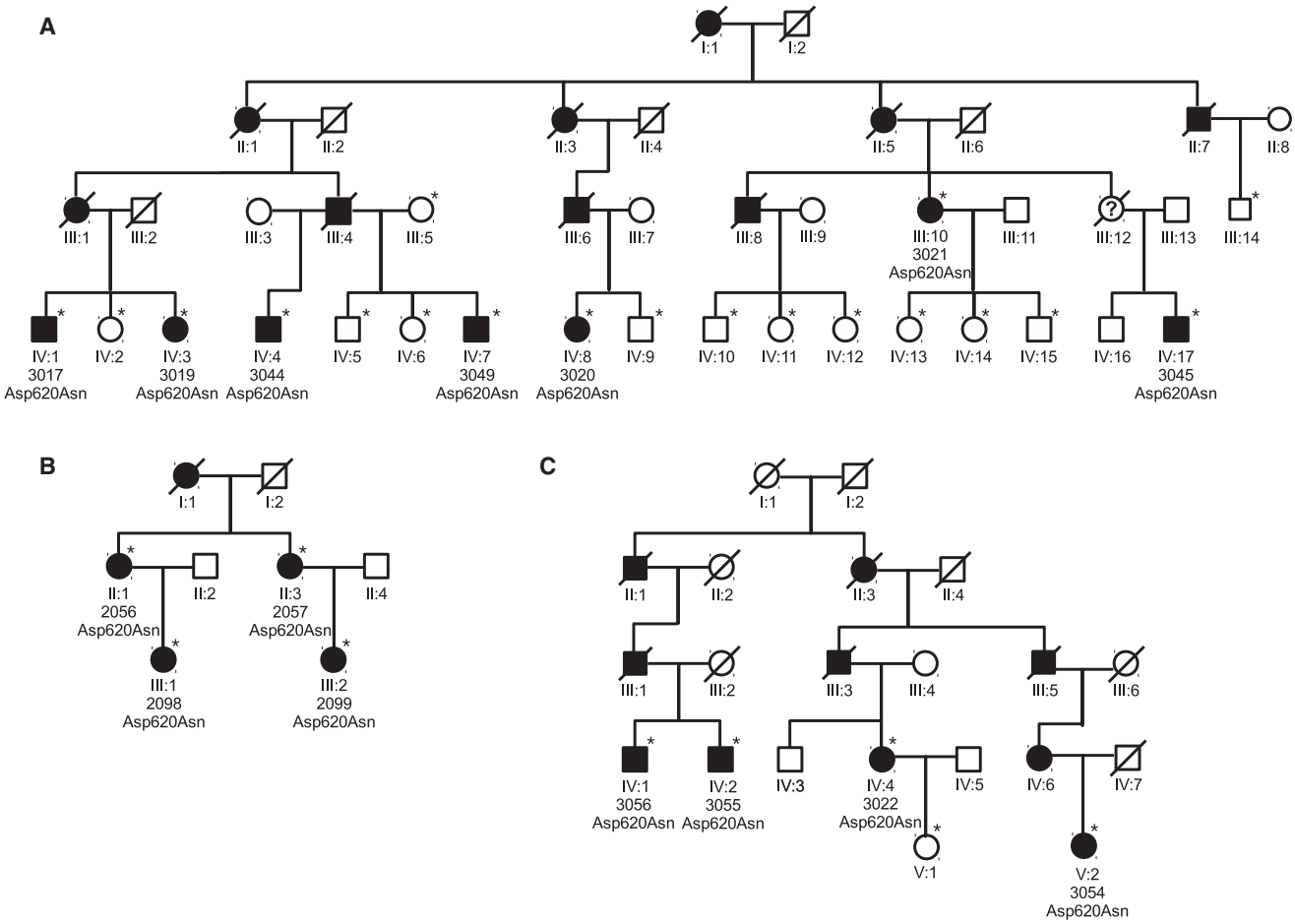

Figure 1. Pedigrees of Families A, B, and C
Unaffected family members are indicated by open symbols, affected members by closed symbols. Asterisks denote individuals genotyped for p.Asp620Asn. To maintain confidentiality, we have not shown genotypes of unaffected individuals. A question mark within a symbol denotes an unknown phenotype. Diagonal bars through symbols denote deceased individuals.

consent. The study was approved by the institutional review board of the Medizinische Universität Wien and the Hessische Landesärztekammer Wiesbaden.

To identify the disease-causing variant, we selected two second cousins (#3017 and #3020) for exome sequencing. We assumed that any rare variants common in both individuals would be disease-causing candidates. Selecting distantly related members of the pedigree should minimize the proportion of alleles shared by descent. Exome sequencing was performed on a Genome Analyzer IIx system (Illumina) after in-solution enrichment of exonic sequences (SureSelect Human All Exon 38 Mb kit, Agilent). We sequenced two lanes of a flowcell for both samples, each as 54 bp paired-end runs. Read alignment was performed with BWA (version 0.5.8) to the human genome assembly hg19 (Table S1, available online). Single-nucleotide variants and small insertions and deletions (indels) were detected with SAMtools (v 0.1.7). We filtered called variants to exclude those present in 72 control exomes from patients with other unrelated diseases. We further excluded all variants that were present in dbSNP 131 and had an average heterozygosity of more than 0.02. Variant annotation was performed with custom scripts. This approach left ten heterozygous nonsynonymous variants shared by both affected individuals (Table 2; see also Table S2).

Only a single heterozygous variant in the *VPS35* gene (RefSeq number NM_018206.4: c.1858G>A [p.Asp620Asn]) fulfilled two further criteria of being possibly causative: (1) it was found in all seven affected members investigated and (2) was absent in approximately 680 KORA S4 general-population samples (Tables 2 and 3).[20] We next screened 486 unrelated PD patients from Austria for the p.Asp620Asn variant by MALDI-TOF mass spectroscopy (Sequenom MassArray system). We detected two additional index patients carrying this mutation (families B and C; Figure 1 and Table 1). The variant was detected in all eight affected individuals investigated in both families. It was not present in a second set of 554 Austrian controls or in an additional 1014 KORA-AGE controls (Table 3). The variant was further detected in three clinically unaffected family members in families A, B, and C. Because the unaffected individuals are all younger than 60 years of age, either they are all presymptomatic or the mutation is nonpenetrant in these subjects.

Table 1. Clinical Findings for PD Patients Carrying Variants in VPS35

Family	Patient	Variation	AaO	DD	IS	B	R	RT	PI	L-Dopa/DA	Other Features
A	3017	p.Asp620Asn	48	7	B	+	+	−	+	+	
A	3019	p.Asp620Asn	40	5	B	+	+	+	+	+	
A	3020	p.Asp620Asn	46	7	PI	+	+	−	+	+	
A	3021	p.Asp620Asn	68	16	PI	+	+	+	+	+	
A	3049	p.Asp620Asn	49	4	RT	+	+	+	−	+	
A	3044	p.Asp620Asn	64	3	PI	+	+	+	+	+	
A	3045	p.Asp620Asn	63	1	RT	+	−	+	−	+	action tremor since childhood
B	2056	p.Asp620Asn	61	15	RT	+	+	+	+	+	fluctuations, dyskinesias
B	2057	p.Asp620Asn	56	8	RT	+	+	+	+	+	fluctuations, dyskinesias
B	2098	p.Asp620Asn	46	0.5	RT	−	−	+	−	untreated	depression, action tremor, pathologic DAT SPECT
B	2099	p.Asp620Asn	51	5	B	+	+	+	−	+	fluctuations, pathologic DAT SPECT
C	3022	p.Asp620Asn	61	5	RT	+	+	+	−	+	dyskinesias
C	3055	p.Asp620Asn	46	12	RT	+	+	+	−	+	
C	3054	p.Asp620Asn	53	9	B	+	+	−	−	+	dyskinesias
C	3056	p.Asp620Asn	43	10	B	+	+	+	+	+	dyskinesias
	211	p.Arg524Trp	37	9	MG	+	+	+	−	+	mild action tremor since youth; 75% motor improvement on levodopa-test; DBS for fluctuations and dyskinesias; pathologic DAT SPECT
	524	p.Leu774Met	51	7	RT	+	+	+	−	+	marked postural tremor
	243	p.Leu774Met	73	9	RT	+	+	+	+	+	dyskinesias, pathologic DAT SPECT
	806	p.Ile241Met	72	2	Postural tremor	+	−	+	+	+	hyposmia (6/12 sniffing sticks), DAT SPECT pathologic, pathologic crying
	90/05	p.Met57Ile	62	13	RT	+	+	+	+	+	dementia (MMSE 23), dysphagia and dysarthria, hyposmia by history, depression

Abbreviations are as follows: AaO, age at onset; DD, disease duration in years; IS, initial symptoms; B, bradykiesia; R, rigidity; RT, resting tremor; PI, postural instability; L-Dopa/DA, response to L-Dopa and/or dopamine agonist; MG, micrographia; DBS, deep brain stimulation.

Cross-species alignment of VPS35 from plants, fungi, invertebrates, and vertebrates showed complete conservation of amino acid Asp620 (Figure S1). The likely consequence of the p.Asp620Asn variant was predicted to be damaging by PolyPhen2,[21] SNAP,[22] and SIFT.[23] We therefore concluded that the variant p.Asp620Asn is indeed very likely to be causative for PD in families A, B, and C.

To determine whether the variant p.Asp620Asn occurred on the same haplotype, we genotyped 20 individuals from families A–C with oligonucleotide SNP arrays (HumanOmni2.5-Quad, Illumina). Haplotyping and linkage analysis were performed with the Merlin software.[24] The haplotypes carrying the variant p.Asp620Asn in families A–C are depicted in Table S3. Family A and B shared a common haplotype across 21 Mb between markers rs1072594 and rs4444336. Family C, however, showed only a common region of 65 kb across *VPS35*. Different alleles were located at markers rs56168099 and rs74459547, 25 kb upstream and 11 kb downstream of *VPS35*, respectively (Table S3). Because the two intragenic markers did not differ, we could not determine whether the three families shared an old common haplotype or whether the mutation has recently arisen on two different haplotypes.

To assess the prevalence of other *VPS35* mutations among PD cases and the general population, we screened all 17 coding exons for variations by dye-binding/high-resolution DNA melting curve analysis (LightScanner HR I 384, Idaho Technology) in 860 cases (484 Austrian and

Table 2. Exome Sequencing: Rare, Heterozygous, Nonsynonymous Variations Shared by Two Individuals of Pedigree A

				Variations		Control Genotypes			
Gene	**Position (hg19)**	**dbSNP**	**Transcript**	**Nucleotide**	**Amino Acid**	**1/1**	**1/2**	**2/2**	**Segregation**
PLK3	chr1:45270359		NM_004073.2	c.1543T>A	p.Ser515Thr	669	0	0	4 of 7
C8A	chr1:57383357	rs41285938	NM_000562.2	c.1723C>T	p.Pro575Ser				5 of 7
ADCY10	chr1:167787479	rs41270737	NM_018417.4	c.4313A>G	p.Asn1438Ser				2 of 7
LAMB2	chr3:49166460		NM_002292.3	c.1724G>A	p.Arg575Gln	647	28	0	5 of 7
NOM1	chr7:156762317		NM_138400.1	c.2503G>A	p.Ala835Thr	670	0	0	3 of 7
KIF22	chr16:29816237		NM_007317.1	c.1780G>A	p.Asp594Asn	665	6	0	6 of 7
SEZ6L2	chr16:29899021		NM_012410.2	c.947G>A	p.Arg316His	660	4	0	7 of 7
VPS35	chr16:46696364		NM_018206.4	c. 1858G>A	p.Asp620Asn	1069[a]	0	0	7 of 7
NLRP1	chr17:5421150		NM_001033053.2	c.3985G>A	p.Val1329Ile	666	4	0	3 of 7
NEURL4	chr17:7221197		NM_001005408.1	c.4109G>A	p.Arg1370Gln				3 of 7

Rare variations revealed by exome sequencing were checked in 670 controls (KORA S4) by MALDI-TOF analysis. The variant allele was denoted as "2," the reference allele as "1."

[a] This number includes additional 554 Austrian control individuals investigated by a TaqMan genotyping assay. Segregation shows the number of affected pedigree A individuals who carry the variant allele.

376 German cases) and 1014 controls. For controls, we used a population-based cohort (KORA AGE) with a mean age of 76 years but excluded eight individuals known to be on medications for PD (Table 3). Exons 2 to 12 are located within a region that is duplicated 12 Mb upstream. Primers were designed to specifically amplify these exons (Table S4). The screening revealed six further rare coding SNVs in addition to p.Asp620Asn (Table 4). Including p.Asp620Asn, we identified four different nonsynonymous missense variants only present in cases, two only present in controls, and one present in cases and controls. Two of the variants unique to PD cases were predicted to be damaging by all three methods (c.1858G>A [p.Asp620Asn]; c.1570C>T [p.Arg524Trp]), and one was predicted by PolyPhen2 to be possibly damaging (c.723T>G, p.Ile241Met). The other variants were predicted to be benign by all methods. Family information was only available for the patient carrying the p.Arg524Trp variant. The only available family member was her mother, aged 74 years. She was found to also carry the variant and showed mild extrapyramidal signs, including intermittent resting tremor of the left fingers and mild postural tremor of both upper limbs, but no bradykinesia. However, a DAT SPECT examination showed normal striatal binding, excluding the possibility of an early stage of PD in this subject. Of note, the screening did not reveal any common nonsynonymous coding SNVs. Furthermore, common nonsynonymous coding SNVs were not found in the 72 control exomes from patients with other unrelated diseases, nor were any recorded in the dbSNP database (version 131).

Table 3. Summary of the Samples Used in This Study

Cohort	**Sample Size**	**Mean Age (SD)**	**Females/Males**
Austrian PD cases[a]	486	58.7 (11.3)	172/314
German PD cases[b]	376	71.1 (9.4)	119/257
KORA S4 controls[c]	680	54.7 (11.9)	280/400
KORA-AGE controls[d]	1014	76.0 (6.6)	508/505
Austrian controls[e]	554	46 (15.2)	254/300

Patients presenting with atypical or secondary (e.g., vascular) parkinsonian disorders as well as patients with known mutations were excluded.

[a] The Austrian cases were recruited at the Department of Neurology, Medizinische Universität Wien, Vienna, as well as in affiliated departments on a consecutive basis. A positive family history for PD was reported from 131 patients. A positive family history was defined by at least one other affected first- or second-degree related family member.

[b] The German PD population originated from the Paracelsus-Elena Klinik, Kassel, a hospital specializing in movement disorders.

[c] This control population was recruited from the KORA S4 survey, comprising individuals who were aged 25–74 years and were examined during 1999–2001.

[d] The KORA-AGE samples were collected in 2009 as a gender- and age-stratified subsample of the KORA S1–S4 studies comprising participants born before 1944. KORA S1–S4 surveys comprise four independent cross-sectional population-based studies in the region of Augsburg, Southern Germany, and were conducted in 5 year intervals. Patients for whom PD was suspected on the basis of questionnaire data were excluded.

[e] These control samples were recruited through the Department of Neurology, Medical University of Vienna, as subjects without known history of a neurological disorder and included, for example, blood donors or unrelated companions or spouses of patients.

VPS35 is a component of the retromer complex and is involved in retrograde transport from the endosomes back to the trans-Golgi network.[25] This multi-protein complex consists of the cargo-recognition VPS26-VPS29-VPS35 heterotrimer and a membrane-targeting heterodimer or homodimer of SNX1 and/or SNX2 (vps5).[25,26] All proteins involved are evolutionarily conserved and have been previously described in *Saccharomyces cerevisiae*. The best characterized cargo proteins of the retromer complex are the cation-independent mannose 6-phosphate receptor

Table 4. Rare *VPS35* Variants in Cases and Controls

ID Cases	KORA AGE Controls	Heterozygous Nucleotide Change	Amino Acid Change	Predicted Impact on Protein			Exon/ Intron	Genomic Position (hg19, chr16)	KORA S4 Controls		
				(i)	(ii)	(iii)			1/1	1/2	2/2
Nonsynonymous											
-	1	c.151G>A	p.Gly51Ser	+	+	+	3	46,716,,039			
90/05	-	c.171G>A	p.Met57Ile	+	+	+	3	46,716,019	670	0	0
-	1	c.245C>G	p.Thr82Arg	+	+	+	4	46,715,367			
806	-	c.723T>G	p.Ile241Met	±	+	+	7	46,711,308	667	0	0
[211]	-	c.1570C>T	p.Arg524Trp	−	−	−	13	46,702,919	671	0	0
[Families A-C]	-	c.1858G>A	p.Asp620Asn	−	−	−	15	46,696,364	669	0	0
243, 524	2	c.2320C>A	p.Leu774Met	+	+	+	17	46,694,455			
Synonymous											
53097	-	c.492A>G	p.Glu164Glu				5	46,714,597	671	0	0
-	1	c.954A>T	p.Gly315Gly				9	46,708,542			
53496	-	c.1881C>T	p.Ala627Ala				15	46,696,341	668	5	0
45, 117, 53626	1	c.2145A>G	p.Leu715Leu				16	46,695,696	666	2	0
53667	-	c.2241C>T	p.Ile747Ile				17	46,694,534	667	2	0
53063	-	c.2346A>G	p.Glu782Glu				17	46,694,429	671	0	0
-	1	c.2361G>A	p.Glu787Glu				17	46,694,414			
Noncoding											
2212	2	c.1-35C>T					5′UTR	46,723,080	667	2	0
-	2	c.1-29C>T					5′UTR	46,723,074			
95, 2206	3	c.3+24A>G					1	46,723,019	662	6	0
159, 528	1	c.102+33G>A					2	46,717,387	668	2	0
[157, 2023]	-	c.103-77T>C					3	46,716,164	668	0	0
-	1	c.199+9T>G					3	46,715,982			
213	-	c.506+6T>C					5	46,714,577	644	0	0
53093	-	c.720+18C>T					6	46,712,773			
-	1	c.914+38T>C					8	46,710,457			
52824	-	c.1161-87A>C					10	46,706,471			
52791	-	c.1161-70G>A					10	46,706,454	668	0	0
-	1	c.1368+16C>T					11	46,706,161			
[2028]	-	c.1369-11G>A					12	46,705,783	669	0	0
-	1	c.1525-17delT					12	46,702,985			
-	1	c.1647+14T>C					13	46,702,828			
320	-	c.2212-45T>C					16	46,694,608	670	0	0
[352]	-	c.2391+7A>G					3′UTR	46,694,377			
-	1	c.2391+8A>G					3′UTR	46,694,376			

Variants for 863 cases and 1014 KORA AGE controls were determined by dye-binding/high-resolution DNA melting curve analysis and confirmed by Sanger sequencing. The table lists the case ID and the number of detected variant alleles of the cases and KORA AGE samples, respectively. Genotypes of identified variants were further investigated by MALDI-TOF analysis in approximately 680 KORA S4 controls. For the KORA S4 samples, the variant allele was denoted as "2," the reference allele as "1." cDNA numbering is based on reference gene NM_018206.4 for *VPS35*, where +1 corresponds to the A of ATG start translation codon. Familial cases are given in square brackets. Three methods were used for predicting the impact of SNPs on the protein. (1) PolyPhen2, (2) SNAP, and (3) SIFT; "+" indicates a benign impact, "±" indicates a possibly damaging impact, and "−" indicates a damaging impact. We detected a further nonsynonymous variant (c.1093C>T [p.Arg365Cys], genomic position 46,708,293) in a patient carrying two *PARKIN* variants (c.exon3_4del and p.Arg275Trp). This variant was not present in 670 KORA S4 and 1014 KORA AGE controls. It is predicted to be possibly damaging by all three methods. This patient's brother is also affected by PD. He carries the 2 PARKIN variants but not the *VPS35* variant.

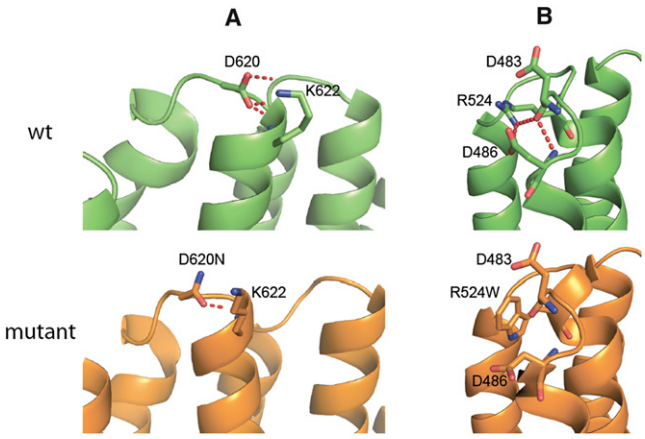

Figure 2. Hydrogen-Bonding Capacities for Wild-Type Asp620 and Arg524 and the Variants p.Asp620Asn and p.Arg524Trp
Hydrogen bonds (HB) are shown as red dashed lines. Asp60 and Arg524 are in green; p.Asp620Asn and p.Arg524Trp are in orange. (A) Asp620 forms a HB to Lys622 and shows an additional salt-bridge interaction. p.Asp620Asn forms fewer HBs, and no electrostatic interaction is possible.
(B) Arg524 forms a HB network with Asp483 and Asp486. This network is broken by the p.Arg524Trp substitution.

(CI-MPR)[27] and Vps10p in mammals and *Saccharomyces cerevisiae*, respectively; these proteins transport hydroxylases to the lysosomes or lysosomal vacuoles. Recently, additional cargo proteins and functions of VPS35 have been described.[28,29] Most interesting in our context is the involvement of the retromer into the retrograde transport of SORL1, a VPS10P-domain receptor protein that has been implicated in Alzheimer disease.[30,31] The crystal structure of the C-terminal part of VPS35 has been resolved.[32] The three variants p.Asp620Asn, p.Arg524Trp, and p.Leu774Met are located in this part of the protein, and we have investigated their impact on protein stability by using molecular dynamics (MD) simulations. We manually introduced the mutations to the crystal structure and modeled the side chains by using scwrl 4.0.[33] All MD simulations were performed via GROMACS 4.5,[34] with the all-atom force field AMBER03[35] and the water model TIP3P[36] as parameters. All three proteins are found on the edge of helices interacting with VPS29. Wild-type residue Asp620 forms frequent hydrogen bonds (HBs) to Lys622, but these bonds are less frequent in the p.Asp620Asn variant (Figure 2A). Similarly, Arg524 is involved in a triple HB network together with residues Asp483 and Asp486, but this network is broken by the introduction of p.Arg524Trp (Figure 2B). Both changes result in the loss of salt bridges and cause the protein to be locally more flexible, as shown by root-mean-square fluctuation (RMSF) profiles (Figure S2). In contrast to the effect predicted for p.Arg524Trp and p.Asp620Asn, the p.Leu774Met variant was not predicted to have a strong impact on protein stability.

In summary, we identified rare *VPS35* missense variants that are potentially pathogenic. One of these variants, p.Asp620Asn, cosegregates with late-onset PD in three unrelated families. The observation that the three families share only a small common haplotype across *VPS35*, the high conservation of VPS35, the predicted structural changes, and the protein's known involvement in lysosomal trafficking together provide strong support for the p.Asp620Asn variant's being causative for late-onset PD, although we identified only a single familial mutation. The penetrance of p.Asp620Asn is high but not complete and might be lower for the other variants. The proportion of PD caused by *VPS35* variants is expected to be low. Although exome sequencing provides perfect access to rare-variant detection, both large families and large collections of cases and controls remain a crucial resource for the identification of disease genes.

Supplemental Data

Supplemental Data include two figures and four tables and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources

The URLs for data presented herein are as follows:

ExonPrimer, http://ihg.helmholtz-muenchen.de/exonprimer.html
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
UCSC Genome Browser, http://genome.ucsc.edu

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