Regulatory rare variants of the dopaminergic gene **ANKK1** as potential risk factors for Parkinson’s disease

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Parkinson’s disease (PD) is characterized by cerebral dopamine depletion that causes motor and cognitive deficits. The dopamine-related gene **ANKK1** has been associated with neuropsychiatric disorders with a dopaminergic deficiency in the striatum. This study aims to define the contribution of **ANKK1** rare variants in PD. We found in 10 out of 535 PD patients 6 **ANKK1** heterozygous rare alleles located at the 5′ UTR, the first exon, intron 1, and the nearby enhancer located 2.6 kb upstream. All 6 **ANKK1** single nucleotide variants were located in conserved regulatory regions and showed significant allele-dependent effects on gene regulation in vitro. **ANKK1** variant carriers did not show other PD-causing Mendelian mutations. Nevertheless, four patients were heterozygous carriers of rare variants of **ATP7B** gene, which is related to catecholamines. We also found an association between the polymorphic rs7107223 of the **ANKK1** enhancer and PD in two independent clinical series (\( P = 0.007 \) and 0.021). rs7107223 functional analysis showed significant allele-dependent effects on both gene regulation and dopaminergic response. In conclusion, we have identified in PD patients functional variants at the **ANKK1** locus highlighting the possible relevance of rare variants and non-coding regulatory regions in both the genetics of PD and the dopaminergic vulnerability of this disease.

Parkinson’s disease (PD) is a neurodegenerative disorder characterized by progressive movement disability and a variety of non-motor symptoms. PD patients show tremor, rigidity, bradykinesia, and postural instability caused by the selective death of dopaminergic neurons in the **substantia nigra**1. The dopamine deficiency in PD also impairs a wide network of brain areas that cause cognitive deficits. In addition, other neurotransmitters are also implicated including the cholinergic system, which partially explains cognitive deficits in advanced stages2. Genetic studies of familial and sporadic PD patients have identified more than 27 PD-associated genes3,4. How-ever, there is considerable missing heritability and therefore the need to identify novel genes.

Single nucleotide variations (SNVs) of the Ankyrin repeat and kinase domain containing I gene (**ANKK1**; MIM*608774) have been reported to be associated with neuro-psychiatry disorders characterized by a dopaminergic deficiency in the striatum5. In particular, the **TaqIA** SNV (rs18000497) has been previously linked to alcoholism, antisocial traits6, and other psychiatric disorders5. **TaqIA** has also been associated with both PD...
vulnerability\(^7,8\) and clinical features of PD patients such as dopamine agonist response\(^9\) or impulse control disorders as a side effect of pharmacological treatment\(^10,11\).

The ANKK1 gene and protein have been related to the dopaminergic system\(^12\)-\(^14\). In silico analysis of the ANKK1 locus identified share haplotypic blocks with the neighbor gene DRD2 (Dopamine Receptor D2 gene)\(^15\) and it has been proposed that ANKK1/DRD2 locus is a genomic substrate for cognitive traits\(^16\). TagI, ANKK1 SNV is in linkage disequilibrium with DRD2 variants that regulate the expression of the long and the short isoforms of the DRD2\(^17\) and its distribution in the ventral striatum\(^18\). The C957T DRD2 SNV (rs6277) is a marker of a regulatory sequence at the 5' UTR of the ANKK1 gene\(^16\). Additionally, an association between TagI and striatal activity of the aromatic L-amino acid decarboxylase has been described\(^19\). Therefore, there is evidence that variations of the ANKK1 gene have a significant impact on the functioning of the dopaminergic system in the brain.

Given that cerebral dopamine depletion is a hallmark of PD here we evaluate in PD patients the eight exons and 5' regulatory regions of ANKK1 as a new PD risk candidate gene. We identified PD-related regulatory SNVs in the ANKK1 locus.

Results

PD-related ANKK1 rare variants affect gene expression. Firstly, we studied variation at the eight exons of ANKK1 from 71 PD patients of the FJD series using DHPLC and Sanger sequencing. We found three heterozygous SNVs in two patients. PD-03 carried the coding variant c.10G>T located at exon 1 (rs35657708, p.Asp4Tyr) while PD-01 had the variants c.185 + 43A>C (rs769430211) and c.185 + 45G>C (rs780054479) at intron 1 (Fig. 1a). The cloning and sequencing of PD-01 intron 1 showed that the two variants were in cis (Fig. S1g). The screening of c.10G>T and c.[185 + 43A>C;185 + 45G>C] by DHPLC in samples from the DNBN-CIIII series revealed they were absent in 288 ethnic-matched healthy controls and 200 PD patients. We found in one PD patient from DNBN-CIIII the c.185 + 30G>A SNV (rs545702309) (Fig. 1a). After, we screened these variants in the independent HC PD series of 264 patients. Two samples (PD-07 and PD-08) were heterozygous for c.10G>T and PD-06 carried the allele c.[185 + 43A>C;185 + 45G>C]. In addition, we found the novel rare variant c.6G>A in PD-04 and the rare variant c.185 + 50 G>A in PD-05 (Fig. 1a). All ANKK1 variants were absent in 288 ethnic-matched healthy controls.

The in silico study of ANKK1 SNVs using CADD showed that c.10G>T, c.185 + 43A>C and c.185 + 45G>C, found in the two clinical series, have the highest C-scores thus suggesting a higher likelihood of deleterious effect on ANKK1 gene regulation (Table S1). We also found these variants occur in well-conserved nucleotides among primates (Fig. S2). The transcription factor binding sites (TFBSs) prediction tool JASPAR, and the Encyclopedia of DNA Elements (ENCODE) data, showed that all variants disrupt TFBSs leading to loss or gain of multiple TFBSs (Fig. 1, Table S2). Further analysis of this region using the ENCODE data showed epigenetic marks associated with promoter activity including accessible chromatin and TFBSs (Fig. 1b).

Next, we performed a luciferase reporter assay (LRA) to validate the functional consequences of the PD-related ANKK1 rare variants. For each variant and WT, luciferase reporter constructs were generated and transfected into HEK293T cells to determine the transcriptional activity. All variants showed significant allele-dependent effects on gene regulation when compared to WT. c.-6G>A, c.10G>T, c.185 + 30G>A and c.[185 + 43A>C;185 + 45G>C] drove lower expression upon reporter activity while the opposite effect was observed for c.185 + 50 G>A (Fig. 2a). Given that c.[185 + 43A>C;185 + 45G>C] showed the lowest expression levels and is immediately adjacent to the binding site of the transcription factor MZF-1, we studied the impact of this variant upon MZF-1 binding. We found a significant increase of luciferase activity in cells co-transfected with ANKK1 alleles constructs and plasmid MZF-1-GFP. We found that WT and c.-6G>A alleles showed similar expression levels while c.[185 + 43A>C;185 + 45G>C] was still significantly lower (Fig. 2b) thus confirming the negative effect of this variant upon ANKK1 transcription activity.

We next measured ANKK1 RNA levels in a sample of peripheral blood cells from PD patients that carry c.-6G>A, c.10G>T, or c.[185 + 43A>C;185 + 45G>C]. In contrast with LRA results, only the patient carrying the c.[185 + 43A>C;185 + 45G>C] allele was different from the WT which suggests that the effect of ANKK1 could be tissue-specific (Fig. 2c).

ANKK1 variants were differentially affected upon dopaminergic treatment. Since the expression of ANKK1 is closely related to the functioning of the dopaminergic system\(^11\), we also studied the effect of apomorphine (dopaminergic agonist) treatment upon ANKK1 variants. In LRA using apomorphine no effect was found upon expression of WT, c.-6G>A, c.10G>T, and c. [185 + 43A>C; 185 + 45G>C] alleles. In contrast, c.185 + 30G>A showed a significant upregulation while we observed in c.185 + 50 G>A the opposite effect decreasing the expression up to WT levels (Fig. S3). To further study apomorphine impact upon ANKK1 variants, an electrophoretic mobility shift assay (EMSA) was performed. In untreated cells, two DNA–protein complexes appeared as a band shift pattern in WT and c.185 + 30G>A while c.[185 + 43A>C; 185 + 45G>C] showed a single band shift (Fig. 2d, Fig. S4a). Apomorphine treatment caused a strikingly decreased binding. EMSA using Primary Normal Human Epidermal Keratinocytes (NHEK) nuclear proteins confirmed quantitative and qualitative differences between the WT and the variants c.185 + 30G>A and c.[185 + 43A>C; 185 + 45G>C] (Fig. 2d, Figure S4b).

rs7107223 SNV located at ANKK1 enhancer is associated with PD. Since the ANKK1 study in PD patients identified rare SNVs that affect transcriptional activity, we hypothesized that other nearby regulatory genomic elements could also be involved in PD vulnerability. ENCODE data showed a regulatory element at 2.6 Kb from the ANKK1 ATG start codon (Fig. 3a, Fig. S5a). This region shows chromatin modification marks that indicate enhancers and conserved elements in primates (6 elements) and M. musculus (248-bp region)
Figure 1. ANKK1 SNVs related to PD are located in the conserved 5’ regulatory regions of the gene. (a) Sequence of ANKK1 Exon 1 (capital letters) and intron 1 (lowercase) annotated with rare PD-related SNVs (bold), Kozak sequence (square), and transcription factor binding sites (TFBSs) identified by JASPAR (grey arrows). (b) ENCODE chromatin modifications marks of ANKK1 exon 1/intron 1 region in Human Skeletal Muscle Myoblasts (HSMM, green), Human Umbilical Vein Endothelial Cells (HUVEC, blue), Normal human epidermal keratinocytes (NHEK, violet) cell lines. Histone acetylation and methylation marks for H3K27Ac, H3K4me3 and H3K4me1 indicate promoters and enhancers. DNaseI hypersensitivity indicates regulatory regions and TFBSs.
variant in GBA, a known risk factor for PD. We also found patients with rare variants of
Nuclear Receptor Subfamily 4 Group A Member 2), in NR4A2 (Leucine-rich repeat kinase 2) genes (Supplementary Methods). None of the patients car-
gorates deletions or duplications in 8 PD genes and the presence of both A30P in the
lyzed with an NGS custom panel and MLPA. The panel includes 24 PD-related genes and MLPA analysis inter-
ANKK1 better know the genetics underlying PD in patients that carry
SNVs were located in conserved regulatory regions, showed significant
within regulatory regions. All 6 ANKK1 in PD showed that 10 out of 535 patients (1.9%) carried rare variants
ANKK1 locus
Patients carrying ANKK1 variants do not have mutations in PD-causing Mendelian genes. To
better know the genetics underlying PD in patients that carry ANKK1 rare SNVs, these samples were also ana-
yzed with an NGS custom panel and MLPA. The panel includes 24 PD-related genes and MLPA analysis interrog-
ates deletions or duplications in 8 PD genes and the presence of both A30P in the SNCA (α-Synuclein) and
G2019S in the α-Synuclein) and LRRK2 (Glucosidase, beta, acid) and
Table S3). Notably, four patients had rare heterozygous variants
PD-08 that shows the earliest age of onset of the disease and cognitive decline, carries a pathogenic
variant in GBA, a known risk factor for PD. We also found patients with rare variants of LRRK2 gene.
PD patients who carry ANKK1 rare SNVs showed significant variability in main clinical symptoms, motor
complications, and cognitive decline, with limited phenotype/genotype correlation, and the age of onset was from
early to classical onset (Table S7). Altogether and the lack of family history in most of these patients suggest that
ANKK1 rare variants are risk factors of PD vulnerability and not Mendelian cause of disease.

Discussion
In this work, the study of the ANKK1 locus in PD showed that 10 out of 535 patients (1.9%) carried rare variants
within regulatory regions. All 6 ANKK1 SNVs were located in conserved regulatory regions, showed significant
allele-dependent effects on gene regulation, and had extremely low frequency or were absent in international or
Spanish databases of control populations.

ANKK1 variants’ contribution to the vulnerability of PD should be investigated in independent patients’
series. Despite this limitation, we believe that the finding of PD-related rare variants in regulatory regions
deserves greater attention in the future search for genetic risk factors of PD. On the one hand, there is a need
to identify and analyze rare genetic variants because they could be moderate risk factors contributing to PD
vulnerability. On the other, genome-wide association studies (GWAS) in human diseases have shown that the
majority of disease-associated variants lie outside protein-coding regions. In PD, a GWAS meta-analysis reported
11 out of 17 novel loci in linkage disequilibrium with variants predicted to affect TFBs and 3 loci of genes that
encode TFs. Focusing on the dopaminergic system, the study of the epigenome landscape of PD has shown

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**Figure 3.** ANKK1 enhancer activity is affected by rs7107223 alleles. (a) ANKK1 locus showing both an enhancer located 2.68 Kb upstream (wave pattern-block) and coding exons (white blocks). (b) ENCODE chromatin modifications marks of ANKK1 enhancer in HUVEC (blue), NHEK (violet), and HSMM (green) cell lines. Histone acetylation and methylation marks for H3K27Ac, H3K4m1, and H3K4m3 indicate an enhancer. DNaseI hypersensitivity indicates regulatory regions and TFBSs. (c) Human binding motif of RELA and relative scores and sequence for RELA BS in the rs7107223 region. Data obtained from JASPAR database. (d) RLA of rs7107223 alleles constructs or after co-transfection with T7-RelA plasmid. (e) EMSA of rs7107223 alleles using radiolabeled probes (P), molar fold excess of non-labeled competitors (C) as a control and RELA as a competitor. (f) RLA of g.113384813G>A alleles constructs. HEK293T cells untreated and treated with apomorphine were used in D, E and F. Data are shown as mean ± SEM (N = 4). Asterisks indicated statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001), one-way ANOVA followed by Tukey correction was conducted.
hypermethylation of regulatory regions associated with downregulation of a network of TF including FOXA1 that is related to dopaminergic neurons21. It has been reported that PD is associated with deregulations of a dopamine-modulated gene network in the striatum that is relevant for non-motor and motor symptoms22. Our finding of PD-related rare ANKK1 variants also highlights the possible contribution of primary dopaminergic dysfunction as a condition of PD vulnerability. Moreover, PD patients carrying regulatory ANKK1 variants do not have disease-causing mutations in PD Mendelian genes. Five patients carry rare variants in NR4A2 and ATP7B genes, which could affect catecholaminergic homeostasis in the brain. The nuclear receptor NURR1 encode by NR4A2 is expressed in midbrain dopamine neurons during neurodevelopment and in adulthood and, its down-regulation contributes to dopamine neuron dysfunction23. The ATPase ATP7B is also related to the dopaminergic pathway in the brain since this protein contributes to catecholamine metabolism24. Four patients had rare heterozygous variants in ATP7B that could contribute as risk factors in catecholaminergic neuron dysfunction in the early stages of PD. Indeed, it has been reported that single ATP7B regulatory SNV may confer susceptibility for late-onset major depression and parkinsonism25 as well as ATP7B coding mutations in early-onset PD26. Our findings in PD patients in ANKK1 and other catecholaminergic-related genes support the role of genetic variability in the dopaminergic pathway as a risk factor for the disease. On the other hand, 2 patients carry rare variants in the GBA or LRRK2 genes. GBA is one of the most recognized PD modifying genes in sporadic cases4, and the ambiguous classification of the LRRK2 variant does not allow reaching the genetic diagnosis. These results altogether suggest these rare variants, including regulatory ANKK1 variants, contribute to the PD pathophysiology by increasing PD susceptibility.

In this work, we also found an association between PD and the functional rs7107223 SNV located at the enhancer nearby the ANKK1 promoter. Our functional studies showed that while RELA binding to this enhancer was genotype-independent, the transcriptional effect of TF binding was observed only for the rs7107223 T allele and it was dependent on dopaminergic stimulation. These findings suggest the link between dopaminergic function and the regulation of this enhancer. Moreover, rs7107223 is in strong linkage disequilibrium with rs11214594, rs17600713, and rs12360992 located at intron 116. These ANKK1 polymorphisms are predicted to have a potential functional impact on the gene expression in the human brain (http://icatnp.org/). Therefore rs7107223 not only is a functional SNV associated with PD but also is a marker for regulatory ANKK1 SNVs at intron 1. rs7107223 is also linked to the rs6277 SNV of the DRD2 gene27. This SNV has a marked impact on the variability in DRD2 binding features27. Since PD shows dopamine depletion in neural pathways innervating the striatum, thalamus, and cortex, it is possible that rs7107223 is also a functional marker of DRD2 variability that would facilitate or hinder compensatory changes in the brain and they may be relevant for clinical phenotype variability in PD patients.

In summary, we have identified functional PD-related rare and polymorphic SNVs in the ANKK1 locus that act in cis regulatory DNA elements of ANKK1. The knowledge of ANKK1 as a new susceptibility gene for PD shows the relevance of both rare variants and non-coding gene regulatory regions in PD genetics and will help to understand pathophysiological aspects of dopaminergic vulnerability in PD.

**Methods**

**Study participants.** The study included two independent clinical series of Spanish PD patients. 71 patients were recruited from the Neurology Department of the Fundación Jiménez Díaz (FJD), Madrid, Spain (45 males and 26 females with disease onset between the ages of 35 and 77 years, X = 57.31; σ = 10.54), and 264 patients were from the Hospital Clinic (HC), Barcelona, Spain (157 males and 107 females with disease onset between the ages of 16 and 86 years, X = 53.40; σ = 11.91). The inclusion criteria were PD diagnosis according to the London Brain Bank28. The study also included 260 PD patients (aged between 41 and 98 years, X = 69.48; σ = 9.51) from the DNA National Bank Carlos III (DNBCCI) at the University of Salamanca, Spain and, 288 healthy controls (aged between 55 and 73 years, X = 60.03; σ = 3.33). The control sample is a collection of DNAs from healthy donors (not diagnosed with any relevant disease) and representative of the population residing in Spain.

The study has been approved by the Clinical Research Ethics Committee of “Institut de Recerca Sant Joan de Déu”, Barcelona (PIC-93-16) and “Fundación Jiménez Díaz”, Madrid (PIC76/2015-FJD). All methods were performed in accordance with the relevant guidelines and regulations set forth by the Declaration of Helsinki. Informed written consent was obtained from all participants.

**ANKK1 mutation screening.** Genomic DNA from peripheral blood was extracted following conventional protocols with proteinase K. ANKK1 genetic variants at 5’-UTR region and exons 1, 3, 4, 6, and 7 were screened by denaturing high-performance liquid chromatography (DHPLC), while exons 2, 5, and 8 (larger and more polymorphic) by Sanger sequencing. The software Gene Runner (version 3.05, Hastings Software) was employed.
for primer’s design using ANKK1 gene reference sequence NM_178510.1. ANKK1’s exons and their flanking intronic regions were amplified by PCR at the specific annealing temperature of each pair of primers (Table S5). The 5’-UTR region of ANKK1 was amplified by touchdown PCR (TD-PCR).

For DHPLC we used the WAVE System (Transgenicom, Nebraska, USA) according to the manufacturer’s instructions. The conditions for DHPLC were established using Transgenicom Navigator software and adjusted by adding positive and negative controls in each sample set. Sanger sequencing was performed on positive amplicons.

**Genotyping of rs7107223.** Touch-down (TD)-PCR was performed using the primers rs7107223-F and rs7107223-R (Table S5) and PCR products digested with TfiI (New England Biolabs, Massachusetts, USA). After, genotypes were visualized in 1.8% agarose gel in Tris–acetate-EDTA (TAE) 1X containing GelRed (1:10,000, Biotium). The web tool SNPStats (https://www.snpstats.net/start.htm?q=snpstats/start.htm) was used to analyze the association under five genetic models (codominant, dominant, over-dominant, recessive, and log-additive) with an adjustment of age29.

**Genetic panel and multiplex ligation-dependent probe amplification (MLPA) analysis.** Some PD samples were genotyped in a custom AmpliSeq primer panel for 24 genes (Supplementary methods) designed online (Ion AmpliSeq Designer v.1.2.8, https://ampliseq.com; Thermo Fisher Scientific). Library construction was performed according to the Ion AmpliSeq Library Kit 2.0 protocol and the Ion OneTouch System protocol (PNMAN0006957, rev. 6.0; Thermo Fisher Scientific) following manufacturer instructions. Sequencing was performed using the Ion Personal Genome Machine_ (PGM) 200 sequencing kit protocol (PN4474246, rev. D; Thermo Fisher Scientific). The sequencing runs were performed using 520 flows for 130 cycles. For data analysis and variant prioritization pipeline, we used the Torrent Suite (TS) software version 4.0. Five main steps were performed: quality control, alignment, variant calling, variant annotation, and filtering.

MLPA was used for some PD samples to identify large deletions or duplications. MLPA kit P051-D1 (MRCL- Holland, Amsterdam, The Netherlands) was used according to manufacturer instructions. The P051-D1 set includes ATP13A2 (exon 2 and 9), PARK2 (exons 1–12), PARK7 (exons 1–7), PINK1 (exons 1–8), SNCA (exons 1–6; point mutation A30P), LRRK2 (point mutation G2019S).

**Constructions of expression plasmids and luciferase reporter assays (LRA).** Oligonucleotide primers containing engineered KpnI sites were designed for PCR-based amplification of genomic DNA from patients to generate the pGL3 plasmids for each variant (Supplementary methods and Table S6). MZF-1-GFP plasmid containing the Myeloid Zinc Finger 1 gene was kindly provided by Dr. Jiawei Zhou and the pEGFP-N1 (Clontech, Mountain view, California, USA) as a mock. T7-RelA plasmid containing RELA (part of the NF-kappa-B complex) was a gift from Warner Greene (Addgene plasmid # 21984).

HEK293T cells were grown in DMEM containing 10% (v/v) fetal bovine serum, 1% penicillin–streptomycin, and 2 mM L-glutamine (Lonza, Basel, Switzerland). NHEK cells were kindly provided by Dr. Pilar Sepúlveda Sanchís. Cells were incubated at 37°C in 5% CO₂. For LRA, HEK293T cells were transfected with 500 ng of luciferase expression pGL3 plasmids and 40 ng of renilla expression construct (pRL-TK vector, Promega) using FuGene HD reagent (Promega, Madison, Wisconsin, USA). For co-transfection experiments, 500 ng of MZF-1-GFP or T7-RelA plasmid were transfected with 500 ng of pGL3 plasmids and 40 ng of pRL-TK. In some cases, 24 or 48 h post-transfection, cells were treated with the dopamine receptor agonist (R)-(-) apomorphine (APO) (Sigma, Saint Louis, Missouri, USA) at 10 μM in complete medium with 0.5% serum for 8 h. After 48 h, cells were lysed and luciferase and renilla activity were measured using the Dual Luciferase Reporter Assay System (Promega) and the Wallac Victor2 spectrophotometer (Perkin Elmer, Waltham, Massachusetts, U.S.). Each experiment was performed in triplicate at least 6 times. The ratio of luciferase to renilla activity and the fold increase relative to a mock (control luciferase expression vector) were calculated. Data are expressed as the mean ± standard errors of the mean (SEM) and values of P<0.05 were considered significant. Differences were evaluated by one-way ANOVA followed by Tukey’s multiple comparisons post-hoc test using GraphPad v6.0 (CA, USA).

**Electrophoretic mobility shift assay (EMSA).** HEK293T (untreated and treated with apomorphine) and NHEK nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce). Double-stranded oligonucleotides (Table S7) were labeled with [γ-32P] ATP using a T4 polynucleotide kinase (Promega, Madison, Wisconsin, USA) and purified on a Microspin G-25 column (Roche, Basilea, Switzerland). For EMSA, 5 μg of nuclear extract were incubated with 1 μg of poly deoxyinosine-deoxycytosine and 15 fmol of the 32P-labeled probes in a 20 μl binding reaction containing 1X buffer (50 mM glycerol; 10 mM Tris–HCl, pH 7.6; 500 mM KCl; 10 mM EDTA; and 1 mM dithiothreitol) for 30 min at room temperature. For the specificity control, a molar excess of unlabeled probe over the radiolabeled oligonucleotide was added to the binding reaction.

After, the reaction mixtures were mixed with loading dye and separated on a 6% nondenaturing polyacrylamide gel (29:1 acrylamide-bisacrylamide) and electrophoresed in a 0.25X Tris-boric buffer at 150 V for 1.5 h. The gel was dried and exposed to X-ray film for autoradiography.

**RNA extraction and RT-PCR form peripheral blood.** Whole venous blood was collected into PaxGene Blood RNA Tube (PAX tubes; BD Vacutainer, Plymouth, UK) and the RNA was extracted using the PaxGene Blood RNA System Kit (PreAnalytiX, QIAGEN) following the manufacturer’s guidelines. PolyT primers were used for the RT reaction using the Maxima First Strand cDNA Synthesis Kit (Fermentas). All quantitative real-time PCR reactions were carried out in triplicate on a 7500 Fast Real Time PCR System (Applied Biosystems, CA, USA).
Foster City, California, USA). Gene expression was analyzed using FastStart Universal SYBR Green Master Rox (Roche). Amplification was performed using 200 ng of cDNA per reaction, at 50°C for 2 min, 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, 60°C for 1 min and 72°C for 30 s (extension Samples were quantified by the standard-curve method and normalized to the GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and PPIA (Peptidylprolyl Isomerase A (Cyclophilin A)) housekeeping genes. Results were analyzed with SDS 2.1 software (Applied Biosystems). Baseline values of amplification plots were set automatically and threshold values were kept constant to obtain normalized cycle times and linear regression data. The primers used are shown in Table S6. Data are expressed as the mean ± standard errors of the mean (SEM) and values of P < 0.05 were considered significant. Differences were evaluated by one-way ANOVA followed by Tukey’s multiple comparisons post-hoc test using GraphPad v6.0 (CA, USA).

In silico studies. Combined Annotation Dependent Depletion (CADD) was used for scoring the deleteriousness of single nucleotide variants (https://cadd.gs.washington.edu/). For the identification of regulatory regions of the ANKK1 gene, we used data from The Encyclopedia of DNA Elements as a part of the ENCODE consortium (genome build GRCh37/hg19). To search for potential transcription factor binding sites (TFBSs) affected by SNVs we use the JASPAR (http://jaspar.genereg.net/) and TRANSFAC (http://www.gene-regulation.com/cgi-bin/pub/databases/transfac.cgi; version 7.0) public databases. We accept TFBS with a minimum relative score threshold of 0.8.

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Author contributions
E.P.S., P.G.R., D.M.R., M.E., I.P.N., J.P., M.J.M. and J.H. have contributed to the data acquisition and analysis. E.P.S., F.P. and J.H. have contributed to the experimental design. F.P. and J.H. have contributed to the conception, funding and writing the original draft of the manuscript. All authors have done a critical review of the manuscript.

Competing interests
The authors declare no competing interests.

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