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## The *PNKD* gene is associated with Tourette Disorder or Tic disorder in a multiplex family

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### Abstract

Tourette Disorder (TD) is a childhood-onset neuropsychiatric and neurodevelopmental disorder characterized by the presence of both motor and vocal tics. The genetic architecture of TD is believed to be complex and heterogeneous. Nevertheless, DNA sequence variants co-segregating with TD phenotypes within multiplex families have been identified. This report examines whole exomes of affected and unaffected individuals in a multiplex TD family to discover genes involved in the TD etiology. We performed whole exome sequencing on six out of nine members in a three-generation TD multiplex family. Putative deleterious sequence variants co-segregating with TD patients were identified by our in-house bioinformatics pipeline. Induced pluripotent stem cells (iPSCs) were generated from one unaffected and two TD affected individuals. Neurons were derived from the iPSCs and biochemical assays were conducted to evaluate possible molecular differences between affected and unaffected. A rare heterozygous nonsense mutation in *PNKD* was co-segregated with TD in this multiplex family. Transcript and protein levels of the *PNKD* long isoform were reduced in neurons derived from the individuals with TD due to the nonsense mutation, indicating nonsense-mediated mRNA decay. We demonstrated that the *PNKD* long isoform monomer oligomerizes with itself as well as interacts with the synaptic active zone protein RIMS1 $\alpha$ . We concluded that reduced *PNKD* long isoform levels are detected in all affected individuals and we provide evidence for a mechanism where by this might contribute to the TD phenotype.

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Supplemental information is available at *Molecular Psychiatry*'s website

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

## INTRODUCTION

Tourette Disorder (TD) is a heritable early-onset neurodevelopmental disorder characterized by the presence of both motor and vocal tics with unclear neuropathogenesis as well as complex genetic architecture<sup>1</sup>. The etiology of TD appears to be polygenic and heterogeneous, probably involving dozens of common variants with small effect size or rare variants with larger effect size<sup>2</sup>. An initial genome-wide association study (GWAS) did not identify common variants meeting significance thresholds<sup>3</sup>. However, a linkage study on a multiplex family identified a rare dominant-negative nonsense mutation (W317X) at the L-histidine decarboxylase (*HDC*) gene<sup>4</sup>. In addition, targeted sequencing of the region where chromosomal abnormalities were found in a TD patient identified the *SLITRK1* gene, in which rare recurrent variants were further identified<sup>5</sup>. To identify potential TD-associated rare sequence variant(s) with large effect size we focused on whole exome sequencing of multiplex families. In one particular family heavily affected by TD and Tic disorders, a novel nonsense mutation within the Paroxysmal Nonkinesigenic Dyskinesia (*PNKD*) gene was identified, which appeared to be a strong TD candidate.

To evaluate the impact(s) that the *PNKD* nonsense mutation might have on gene expression and cellular functions, we generated induced pluripotent stem cells (iPSCs) and differentiated them into neuronal cells *in vitro*. With the iPSC-derived neurons from unaffected and affected family members, we were able to measure the endogenous transcript level and protein expression of the neuronal-specific PNKD long (L) isoform and we examined its subcellular localization. In addition, we demonstrated the self-oligomerization of the PNKD (L) protein monomer and its interaction with synaptic protein RIMS1 $\alpha$  in transfected human cells and their co-localization in the human iPSC-derived neurons.

## MATERIALS AND METHODS

### Human Subjects

A three-generation multiplex family was recruited through the Tourette International Collaborative Genetics (TIC Genetics)<sup>6</sup> study and New Jersey Center for Tourette Syndrome (NJCTS)<sup>7</sup> with informed consent by all participants. The study protocol has been approved by Rutgers University Institutional Review Board.

### The whole exome sequencing (WES) and Sanger Sequencing

Genomic DNA was extracted from the bloods of subjects 3001, 3002, 4001, 4002, 5001, 5003 and 5005 and the saliva of subject 5004. WES was performed on subjects 4001, 4002, 5001, 5003 (proband), 5004 and 5005 (Figure 1A). For the WES library preparation, the SureSelect Human All Exon V5+UTR kit (Agilent Technologies, CA) was used to capture the targeted region for sequencing on the Illumina HiSeq2000. Variants were called by Geospiza's GeneSifter (PerkinElmer, MA). For Sanger sequencing, the genomic region harboring the nonsense mutation was PCR-amplified with primers shown in Table S1.

### **pVAAST Analysis**

The pedigree Variant Annotation, Analysis and Search Tool (pVAAST) program prioritized candidate genes<sup>8</sup> under both dominant and recessive modes of inheritance (see supplement).

### **Generation and maintenance of iPSCs**

iPSCs were generated from the CD4<sup>+</sup> T lymphocytes using CytoTune-iPS reprogramming kit<sup>9</sup> iPSCs were cultured on Matrigel-coated plates in mTeSR (see supplement).

### **Generation and maintenance of neural progenitor cells (NPCs) and neurons**

NPCs were generated from iPSCs using a neural rosette-based protocol. Neurons were derived from NPCs by culturing the NPCs in neural differentiation medium (see supplement).

### **Real-time quantitative PCR (RT-qPCR)**

Total RNA was extracted using the RNeasy Mini kit (QIAGEN). cDNA was made from 1ug total RNA using TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific, MA). RT-qPCR was performed on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, CA). Primer information is shown in Table S1.

### **Plasmids construction and Transfection**

Human PNKD (L) cDNA was cloned from cDNA ORF Clone (RC206179) (Origene, MD) and was inserted into the pcDNA4/TO vector. By PCR, a FLAG tag or a Myc tag was added to the PNKD (L) cDNA sequence as shown: 5'-FLAG-PNKD-3' or 5'-PNKD-Myc-3'. To express human RIMS1 $\alpha$ , RIMS1 transcript variant 1, cDNA ORF Clone (RC213013) (Origene, MD) was used. The expression of *GFP* cloned into the pcDNA4/TO vector monitored transfection efficiency. To transfect neurons plated on poly-L-ornithine/laminin/fibronectin-coated 12mm coverslips, we used Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, MA). To transfect 293FT (Thermo Fisher Scientific, MA) cells plated in 10cm dishes, we used 1mg/ml PEI solution (Sigma-Aldrich, MO). A room temperature mixture of plasmids, transfection reagents and Opti-MEM reduced-serum medium were added to the cultures. Two days later, cells were collected for RNA extraction, or lysed in protein lysis buffer or fixed for immunocytochemistry.

### **Western blot and co-immunoprecipitation**

Cells were lysed in RIPA buffer for western blot analysis or in RIPA IP buffer for co-immunoprecipitation (Co-IP). Anti-FLAG M2 Magnetic beads (Sigma-Aldrich, MO) eluted FLAG-tagged proteins. Total protein lysates or eluates of the anti-FLAG M2 beads were resolved on NuPAGE 10% Bis-Tris or 3–8% Tris-acetate gels (Thermo Fisher Scientific, MA).

### **Electrophysiology**

Whole cell patch clamp experiments were performed as described elsewhere<sup>10, 11</sup>. Whole cell patch clamp recordings were performed in human neurons derived from iPSCs in oxygenated artificial cerebrospinal fluid (ACSF) at 30 °C. The ACSF contains (in mM): 125

NaCl, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 2.5 Glucose, 22.5 Sucrose, 2.5 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>. The whole-cell pipette solution contained (in mM) 126 K-Gluconate, 4 KCl, 10 HEPES, 0.3 Na-GTP, 4 Mg-ATP, 0.05 EGTA and 10 Phosphocreatine (pH 7.2, adjusted with KOH). When whole cell currents were recorded, voltage clamp mode was used, and cells were held at -70 mV and a depolarization protocol from -100 to +90 mV in 10 mV intervals were applied. When spontaneous potentials were recorded current clamp I=0 was used. Whole cell patch clamp recordings were performed using an Axon 700B amplifier. Data were filtered at 2 kHz, digitized at 10 kHz and collected using Clampex 10.2 (Molecular Devices, CA).

## RESULTS

### Clinical evaluations of the TD multiplex family

In this nonconsanguineous three-generation pedigree (Figure 1A), the proband (5003) and all four of her full siblings participated, as well as both parents and paternal grandparents (see supplement for clinical data).

### A rare nonsense mutation in *PNKD* is associated with TD or Tic disorder

We performed WES on individual 4001, 4002, 5001, 5003 (proband), 5004 and 5005, generating more than 60 million 100bp paired-end reads for each, with an average coverage of 53x (Table S3). We identified about 200,000 variants per individual. From these variants, we prioritized candidate genes using the pVAAST program, which identifies genes that contain highly differentiated functional variants between the cases and a reference control population of normal individuals. pVAAST also directly incorporates the variant inheritance pattern within the family into the likelihood ratio test for gene prioritization<sup>8</sup>. A total of 145 and 169 genes were scored under dominant and recessive modes of inheritance, respectively (Table S4). Under the dominant mode, *PNKD* has the highest score. Tourette or Tic Disorder affected family members shared a heterozygous nonsense mutation (chr2: 219204814 C/T), which is not present in the unaffected individuals and absent in the background sample. By Sanger sequencing, we confirmed the heterozygous C to T transition in *PNKD* is also present in subject 3001, and validated the genotypes in 4001, 5001, 5003, 5004 and 5005 (an example is shown in Figure 1B). The Exome Aggregation Consortium (ExAC) database<sup>12</sup>, indicates that this variant was found in 4 of 120802 alleles, giving an allele frequency of  $3.3 \times 10^{-5}$ . In our recent study where WES of 511 TD trios were completed<sup>13</sup>, this *PNKD* nonsense mutation was not identified, suggesting its rarity.

Missense mutations in *PNKD* are associated with familial paroxysmal nonkinesigenic dyskinesia (PNKD), a neurological movement disorder causing episodic involuntary movement attacks<sup>14-16</sup>. Because of the deleterious nature of the nonsense mutation, its segregation pattern within the pedigree, and the neurological movement disorder associated with *PNKD*, we selected *PNKD* for further investigation.

### Neuronal cells were generated from the human subjects through iPSC intermediates

We generated NPCs and subsequently neurons from iPSCs of three members (4002-Uaf; 5001-TD1; 5003 (proband)-TD2) and one unrelated unaffected subject (Urel\_Uaf) without

the mutation (Figure S6A). Karyotypes of all iPSC lines were normal (Figure S1). Sendai virus was not detected in all iPSC lines (Table S6). Pluripotency of iPSCs were examined by gene expression analysis (Table S6), flow cytometry (Figure S2A–D) and immunocytochemistry (Figure S4A). The iPSC-derived NPCs were stained positive for neural progenitor markers Nestin and Musashi1 as well as neuronal marker Class III  $\beta$ -tubulin by immunocytochemistry (Figure S4B). Flow cytometry also showed that the majority of NPCs expressed markers Nestin and Sox2 (Figure S3). For generation of D30 neurons, the NPCs were cultured in NDM for 30 days. The D30 neurons expressed neuronal markers MAP2 and Class III  $\beta$ -tubulin (Figure S5F). Western blot detected synaptic vesicle protein synapsin I (SYN1), which regulates neurotransmitter release, in D30 neurons (Figure S5G). To characterize the type(s) of neurons in D30 cultures, we stained for glutamatergic neuron marker VGLUT1, GABAergic neuron marker VGAT and glia cell marker GFAP (Figure S4C). Immunocytochemistry showed subpopulations of neurons stained positive for VGLUT1, VGAT or GFAP, indicating heterogeneity. We also examined the calcium influx of the D30 neurons from the Uaf, TD1 and TD2 subjects. All three exhibited spontaneous calcium influx and potassium-stimulated calcium influx when depolarized with the 100mM KCl Tyrode's solution (Figure S5A–E), further indicating that the iPSC-derived D30 neurons have appropriate neuronal phenotypes.

To explore whether neurons derived from iPSCs of controls as well as TD patients have properties resemble mature neurons, we performed patch-clamp recordings of neurons at 30–37 d after replating the 15 d of neurons on glial cells. These neurons expressed neuronal markers MAP2, Class III  $\beta$  tubulin (Figure 2A). Under voltage-clamp mode we observed both fast, inactivating inward and outward currents, which probably correspond to opening of voltage dependent  $\text{Na}^+$  and  $\text{K}^+$  -channels, respectively (Fig 2B). More importantly, a large proportion of these neurons can fire spontaneous action potentials (Figure 2C). We also conducted IHCs and revealed that synapsin punctas were found in these cultures (Figure 2D). The basic membrane properties of these human neurons were comparable among different cell lines (Fig. 2E–G).

### **The human *PNKD* (L) isoform is enriched in iPSC-derived neurons and is reduced in TD patients' neurons**

The 12 exons within the human *PNKD* locus encode multiple protein isoforms<sup>17</sup>. The *PNKD* (L) transcript contains exons 1, 2 and 5–12. The *PNKD* medium (M) transcript contains exons 4–12 and the *PNKD* short (S) transcript contains exons 1–3. The nonsense mutation is in exon 6, therefore affecting both the *PNKD* (L) and *PNKD* (M) transcripts. The Genotyping-Tissue Expression (GTEx) project (<http://www.gtexportal.org/home/>) indicates that *PNKD* (L) transcript is enriched in the human brain where *PNKD* (M) transcript is weakly expressed. The *PNKD* short (S) transcript has higher expression than the long transcript in the human brain but the *PNKD* (S) transcript is also highly expressed in other human tissues. To characterize *PNKD* gene expression, all *PNKD* transcripts were quantitated by RT-qPCR in lymphoblastoid cell lines<sup>7</sup> (LCL), iPSC, NPC and D30 neurons from subjects Uaf, TD1 and TD2. Primers distinguished different transcripts and their positions are shown in Table S1. In Figure 3, we show *PNKD* transcript expression in the Uaf subject. The *PNKD* (L) transcript was not detected in LCL and iPSC but appeared at

low-levels in NPC, whereas its expression increased greatly in the D30 neurons (Figure 3A). *PNKD*(L) transcript expression significantly increased from iPSC to NPC and from NPC to D30 neurons (Figure 3A). The *PNKD*(S) transcript was ubiquitously detected with relatively higher expression and it increased significantly with neuronal differentiation of iPSC (Figure 3B). The *PNKD*(M) transcript is expressed by all cell types examined, but its expression was relatively low in neuronal cells (Figure 3C). PNKD (L) protein expression was examined by western blot in LCL, iPSC and D30 neurons of all three subjects. Consistent with the transcript expression data, the PNKD (L) protein was only detected in D30 neurons (Figure 3E). Thus, of the two *PNKD* isoform transcripts affected by the nonsense mutation, the (L) but not the M isoform is enriched in human neuronal cells. Nonsense mutations could produce loss-of-function, gain-of-function or dominant-negative phenotypes. To investigate effects of the nonsense mutation on the PNKD (L) isoform, we measured the transcript levels and protein expression in D30 neurons. For each subject, D30 neurons from three independent differentiations were included in the RT-qPCR analysis. The *PNKD*(L) transcript levels in the two TD patients' neurons were reduced to about half of the level of the Uaf subject's neurons, suggesting nonsense-mediated decay (NMD)<sup>18</sup> (Figure 3D). Consistent with the transcript expression, the PNKD (L) protein was also reduced in the TD patients' neurons compared to the Uaf (Figure 3E).

Furthermore, we have observed that treating TD neurons with 100ug/ml NMD inhibitor cycloheximide (CHX) eliminated the expression difference of *PNKD*(L) transcript between unaffected and TD neurons (Figure S6B), supporting our NMD hypothesis.

### **The human PNKD (L) protein self-oligomerizes and interacts with RIMS1 $\alpha$ protein**

A previous study showed that the human PNKD (L) protein interacts with itself<sup>19</sup>. We transfected 293FT cells with plasmids expressing: GFP, FLAG-PNKD (L), PNKD (L)-Myc or FLAG-PNKD (L)+PNKD (L)-Myc. Forty eight hours after transfection, cells were lysed for co-immunoprecipitation (Co-IP). The FLAG-PNKD (L) was eluted by anti-FLAG M2 magnetic beads. The PNKD (L)-Myc co-eluted with FLAG-PNKD (L), indicating the self-oligomerization of the PNKD (L) protein (Figure 4A). It has been reported that the PNKD (L) protein interacts with the presynaptic proteins RIMS1/2 in mouse cortex<sup>20</sup>. Based on the mouse and human protein sequence conservation, the PNKD (L) and RIMS1 $\alpha$  proteins also likely interact in human. We transfected the 293FT cells with plasmids expressing: GFP, RIMS1 $\alpha$ -FLAG, PNKD (L)-Myc or RIMS1 $\alpha$ -FLAG+PNKD (L)-Myc. Co-IP was performed using the whole cell lysates and, as shown in Figure 4B, the PNKD (L)-Myc was eluted only in the presence of RIMS1 $\alpha$ -FLAG, suggesting their interaction.

We also examined the subcellular localization of PNKD (L) and RIMS1 $\alpha$  in neurons. Due to the low endogenous expression of PNKD (L), by transfection we overexpressed the PNKD (L) with the FLAG tag at its N-terminus in iPSC-derived neurons. We also transfected the iPSC-derived neurons with plasmid expressing the human RIMS1 $\alpha$  protein with the Myc tag at its C-terminus. Immunocytochemistry showed that PNKD (L) protein was enriched in the plasma membrane and neurites whereas expression was weaker in the cell body (Figure S7A). The RIMS1 $\alpha$  protein could be detected in both cell body and neurites (Figure S7B).

Co-transfection of the PNKD (L) and the RIMS1 $\alpha$  showed that these two proteins co-localized in the cell body and neurites (Figure S7C).

### **RIMS1 $\alpha$ transcript is enriched in iPSC-derived neurons and is not affected by the reduction of the PNKD (L) isoform**

GTEX indicates that the *RIMS1 $\alpha$*  transcript is exclusively expressed in the brain. Using RT-qPCR we measured the level of the *RIMS1 $\alpha$*  transcript in LCL, iPSC, NPC and D30 neurons of Uaf, TD1 and TD2. As shown in Figure 5A, the *RIMS1 $\alpha$*  transcript is barely detected in LCL and has extremely low expression in iPSC and NPC. As expected, the *RIMS1 $\alpha$*  transcript is highly enriched in D30 neurons. We have shown that PNKD (L) and the RIMS1 $\alpha$  protein interact in 293FT cells and co-localized in iPSC-derived neurons. Additionally, it has been shown that PNKD (L) stabilizes RIMS1/2 proteins in mouse<sup>20</sup> Therefore, we measured the endogenous RIMS1 protein in D30 neurons from three independent differentiation processes. As shown in Figure 5C, the RIMS1 protein was only reduced in TD1 and TD2 neurons, correlating with the reduction of the PNKD (L) protein. To investigate whether the reduction of PNKD (L) affects the expression of the RIMS1 $\alpha$  transcript in human neurons, we measured the *RIMS1 $\alpha$*  transcript in D30 neurons from Uaf, TD1 and TD2 (Figure 5B). There is no significant change of the RIMS1 $\alpha$  transcript expression in Uaf or TD neurons, ruling out a transcriptional regulatory mechanism.

## **Discussion**

### **Expression of the PNKD and RIMS1 in the human brain**

BrainSpan (<http://www.brainspan.org/>) developmental transcriptome data shows that *PNKD* gene expression (RPKM) increases with human brain development (Figure S8A). We also searched *PNKD* expression in developing brains by isoform. Figure S8B shows the expression of an exon only included in the *PNKD* (S) transcript, which has peaks at 6 months and at 4 years of age. Figure S8C shows the expression of an exon only included in the *PNKD* (M) transcript. By comparing the RPKM values, the expression of the *PNKD* (M) transcript at all ages is much lower and not coordinated with the *PNKD* (S) transcript. Unfortunately, there is no exon that is specific to the *PNKD* (L) transcript. Figure S8D shows the expression of an exon shared by the *PNKD* (L) and the *PNKD* (M) transcripts, which increases greatly during human brain development. The increase is probably due to the increased expression of the *PNKD* (L) transcript considering the uniformly low expression of the *PNKD* (M) transcript. Even though 26 brain structures are listed in Figure S8, not every human subject could provide all 26 samples. Still, for the exon shared by *PNKD* (L) and *PNKD* (M) transcripts, we listed the brain structure which shows the highest expression in each subject (Table S5). Interestingly, brain structures implicated in the neuropathogenesis of TD, including striatum (STR)<sup>21–32</sup>, primary motor cortex (area M1, area 4) (M1C)<sup>33–41</sup>, primary somatosensory cortex (area S1, areas 3,1,2) (S1C)<sup>32, 34, 39, 42, 43</sup> and mediodorsal nucleus of thalamus (MD)<sup>44</sup> have the highest expression at certain stages during brain development, suggesting that disruption of PNKD (L) protein function might affect neuronal development or activity in these brain structures. We also plotted *RIMS1* gene expression using gene-level reads (Figure S9A) and *RIMS1 $\alpha$*  expression using exon-level reads (Figure S9B) using the RNA-Seq data from the

BrainSpan. Both the *RIMS1* gene total expression and the *RIMS1a* expression are enriched in the cerebellar cortex (CBC) region in some of the subjects. Additionally, we examined the *PNKD* and *RIMS1* expression in the Human Brain Transcriptome database (<http://hbatlas.org/>) where gene expression was measured by microarray at exon level using over 1340 samples from 57 brains. *PNKD* expression is shown in Figure S10A, where an increasing trend is seen with human brain maturation. Also, *PNKD* might have higher expression in the striatum than in other brain regions. For *RIMS1*, an increase of expression was seen from period 1 to period 6 (Figure S10B). *RIMS1* expression reached a plateau for mediodorsal nucleus of the thalamus (MD), striatum (STR), amygdala<sup>45</sup>, hippocampus<sup>46</sup> and 11 areas of neocortex (NCX), except for the cerebellar cortex (CBC). The CBC has higher expression of *RIMS1* from period 8 to period 15 compared to other brain regions. We modeled the expression of *PNKD* transcript isoforms during very early brain development by measuring *PNKD* (L), the *PNKD* (M) and the *PNKD* (S) transcripts in human iPSC, NPCs and D30 neurons (Figure 3). Consistent with brain observations, both the *PNKD* (L) and the *PNKD* (S) transcripts increased during neural differentiation, suggesting these two isoforms have neuronal functions, especially the former as its increase is tissue-specific and most pronounced.

### Effect of the PNKD nonsense mutation

We observed reduced *PNKD* (L) transcript and protein levels in the mutant D30 neurons, suggestive of nonsense-mediated decay. We investigated a possible dominant-negative mechanism for reduction of *PNKD* multimer activity due to a putative N-terminal *PNKD* protein fragment binding to full length fragment(s). Therefore, we determined whether nonsense-mediated decay was complete by measuring *PNKD* (L) transcript with the mutation in iPSC-derived neurons. We reverse-transcribed total RNA extracted from Uaf and TD iPSC-derived neurons into cDNA and used the cDNA as template to PCR-amplify the region containing the nonsense variant. The forward and reverse PCR primers located at different exons. In addition, the reverse primer spanned an exon-exon junction. Therefore, only cDNA and not genomic DNA could be amplified. The PCR product was purified from the agarose gel and Sanger sequenced. As shown in Figure S11A, the *PNKD* (L) transcript carrying the nonsense variant was detected, indicating that the nonsense-mediated decay doesn't eliminate all the mutant *PNKD* (L) transcripts. However, we were unable to determine whether the mutant *PNKD* (L) transcript is translated into truncated protein. To get an idea of the possible subcellular localization of putative *PNKD* truncated protein, we overexpressed the mutant *PNKD* (L) transcript in 293FT cells where it was translated into a truncated protein. As shown in Figure S11B the truncated *PNKD* protein lost the ability to localize to the plasma membrane and didn't co-localize with the wild type *PNKD* (L) protein, arguing against a dominant-negative effect. Therefore, we hypothesize that the nonsense mutation exerts its phenotype due to haploinsufficiency at critical times in development. However, in certain brain neurons that express the *PNKD* (L) transcript at a much higher level, testing for a truncated *PNKD* (L) protein might be useful.

### The PNKD gene and disease

The *PNKD* gene is known for its association with the neurological movement disorder paroxysmal nonkinesigenic dyskinesia (PNKD) which causes episodic involuntary



movement attacks<sup>14–16</sup>. As shown in Table 1, recurrent missense mutations A7V<sup>15, 47–54</sup> and A9V<sup>15, 48–50, 55–57</sup> were associated with the PNKD disorder in familial cases from different ethnic groups. Transgenic mice expressing *Pnkd* carrying the human disease-causing mutations A7V and A9V exhibited alterations of striatal dopamine signaling<sup>58</sup>. Another heterozygous mutation A33P<sup>17</sup> was detected in one patient who suffered PNKD and cerebellar ataxia. Also a missense mutation G89R was identified in a boy affected by intermittent ataxia, diarrhea, exercise intolerance and speech articulation problems<sup>59</sup>. In addition to missense mutations, a single nucleotide deletion (P341Rfs\*2) in the *PNKD* (L) transcript leading to an amino acid change at position 341 (Proline to Arginine) and the generation of a premature stop codon at position 343 was reported to co-segregate with familial hemiplegic migraine<sup>60</sup>. Mutations affecting the PNKD (L) and PNKD (S) isoforms including A7V, A9V, A33P were associated with the classic, autosomal dominant PNKD whereas mutations affecting the PNKD (L) and PNKD (M) isoforms including the G89R and P341Rfs\*2 were associated with ataxia or familial hemiplegic migraine. It has been reported that episodic ataxia type-2 and familial hemiplegic migraine could be caused by mutations in the same gene: *CACNL1A4*<sup>61</sup>. Our TD and Tic disorder-associated *PNKD* nonsense mutation affects the PNKD (L) and the PNKD (M) isoforms leading to reduction of PNKD (L) in the TD neurons. Longer than the truncated protein caused by our nonsense mutation, the truncated protein due to the P341Rfs\*2 mutation (60) presumably preserves the region that was required for PNKD (L) self-oligomerization<sup>19</sup> and therefore it might interfere with the function of PNKD (L) protein. Interestingly, previous studies suggested clinical overlap between migraine and TD<sup>62, 63</sup> or between paroxysmal kinesigenic dyskinesia (PKD, an involuntary movement distinct from PNKD disorder) and TD in multiple cases<sup>64–66</sup>. Taken together, disorders like PNKD, PKD, familial hemiplegic migraine, TD and Tic disorders, whose diagnostic criteria and clinical phenotypes differ, may be caused by distinct mutations disrupting the same gene.

To summarize, we identified a nonsense mutation in the *PNKD* gene segregating with TD and Tic disorders in a multiplex pedigree. We characterized the functional consequences of the mutation in iPSC-derived neurons and demonstrated the self-oligomerization of the PNKD (L) protein and its interaction with the synaptic active zone protein RIMS1 $\alpha$ . In addition, we analyzed the spatial and temporal expression of *PNKD* in publicly available human brain transcriptomic data. Also, we discuss the data-supported alternatives of haploinsufficiency versus a dominant-negative phenotypic mechanism. This report increases the evidence that *PNKD* plays a critical role in neuronal development and function but further studies will be required to fully elucidate the role of the *PNKD* gene in the pathogenesis of neurodevelopmental disorders, including TD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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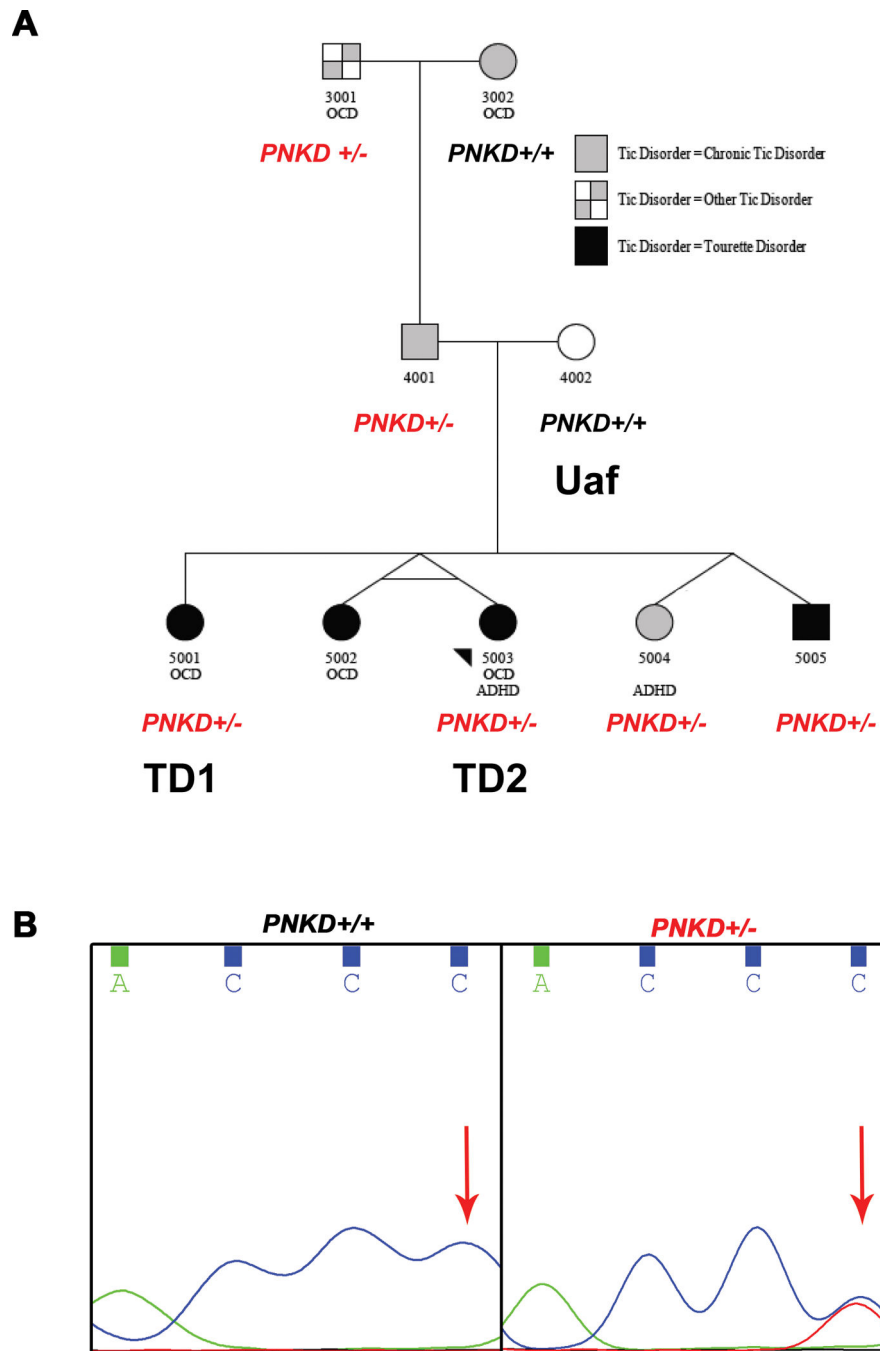
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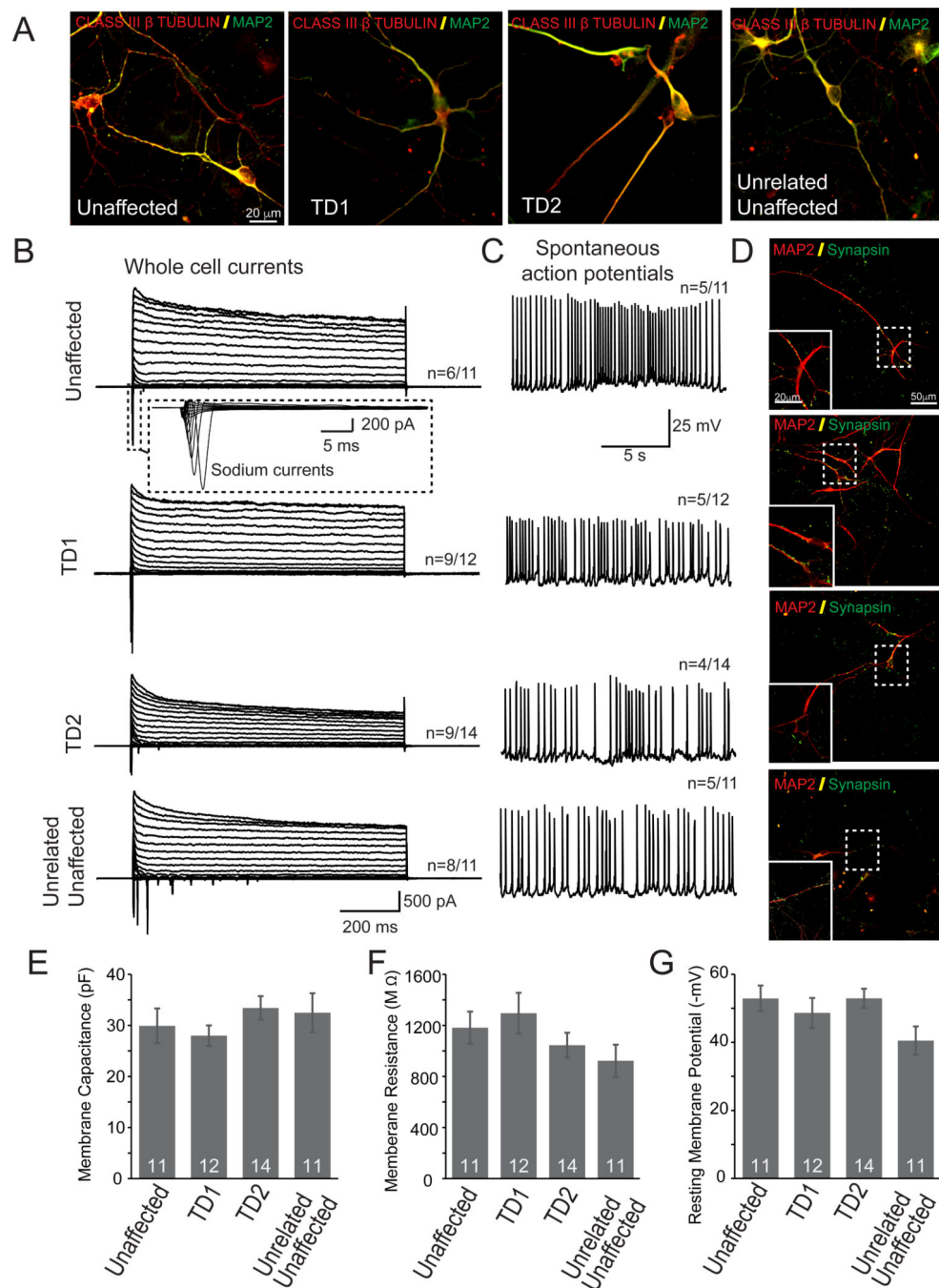
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**Figure 1. A rare heterozygous nonsense mutation (C to T transition) was identified in a TD multiplex family by whole exome sequencing**

A. We performed whole exome sequencing on 4001, 4002, 5001, 5003 (proband), 5004 and 5005.

B. The heterozygous nonsense mutation at the *PNKD* gene was present in subjects 3001, 4001, 5001, 5003, 5004 and 5005.



**Figure 2. Functional characterizations of neurons derived from iPSCs**

A. Neurons derived from unaffected as well as TD subjects express mature neuronal marker MAP2 and Class III  $\beta$ -tubulin.

B. Whole cell currents were recorded from human neurons derived from subjects with and without TD;

C. Spontaneous action potentials were recorded from human neurons derived from subjects with and without TD;

D. Synapsin, a synaptic vesicle marker, was detected in human neurons with and without TD.

E. Membrane capacitance, F, Membrane resistance and G, resting membrane potentials of human neurons derived from subjects with and without TD were quantified.

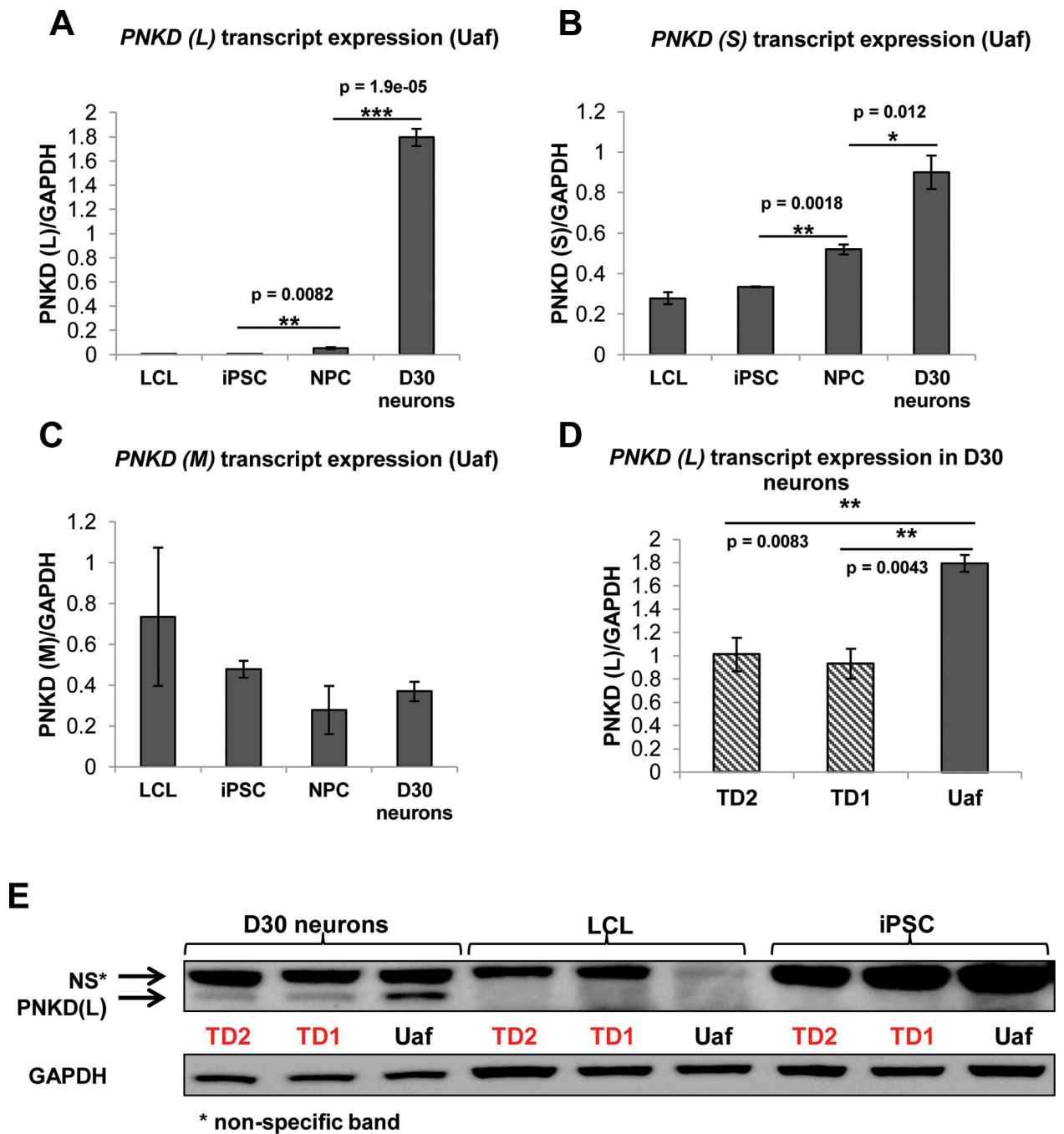
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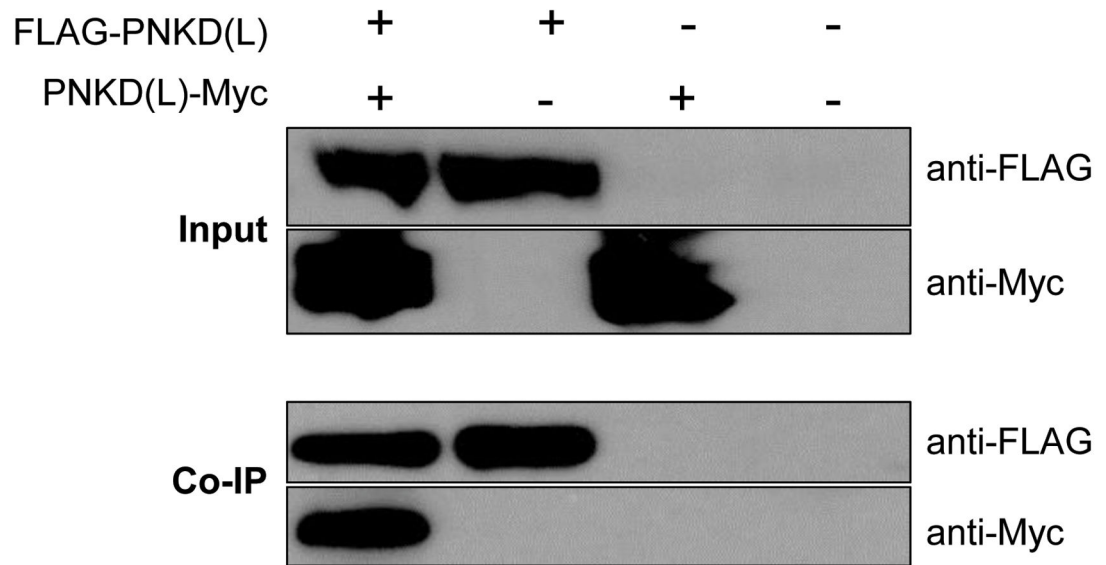
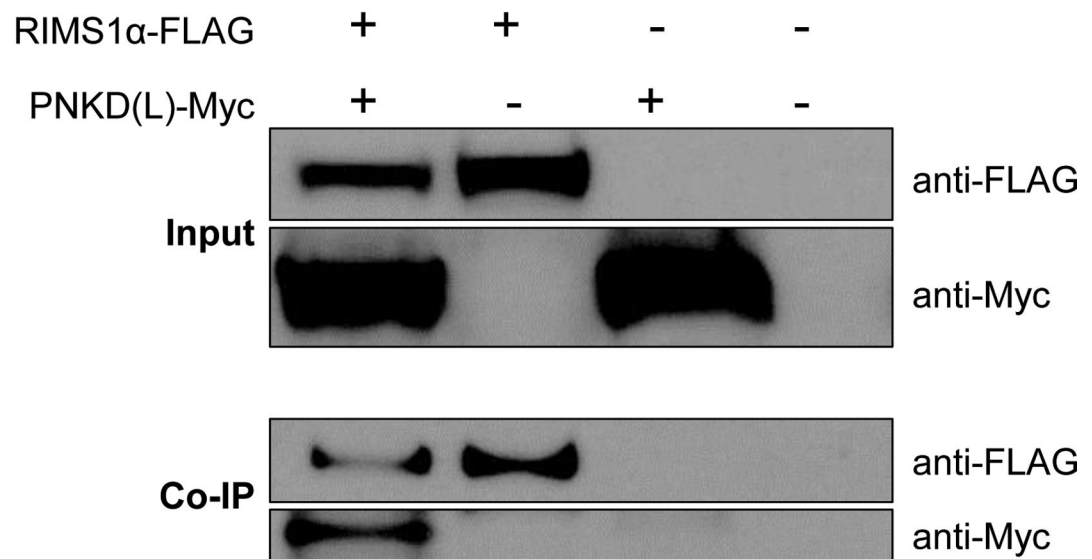




**Figure 3.** *PNKD* expression in LCL, NPC, iPSC and iPSC-derived D30 neurons by RT-qPCR  
 A. *PNKD (L)* transcript expression increased from iPSC to NPC and from NPC to D30 neurons in the Uaf subject, indicating that *PNKD (L)* was enriched in neuronal cells (n = 3).  
 B. *PNKD (S)* transcript is expressed in all the cell types of all three subjects. *PNKD (S)* transcript level increased during neural differentiation (n = 3).  
 C. *PNKD (M)* transcript is expressed at low levels in iPSCs, NPCs and iPSC-derived neurons (n = 3).

D. *PNKD* (L) transcript was significantly reduced in iPSC-derived D30 neurons of TD1 and TD2 subjects compared to the Uaf subject (n = 3).

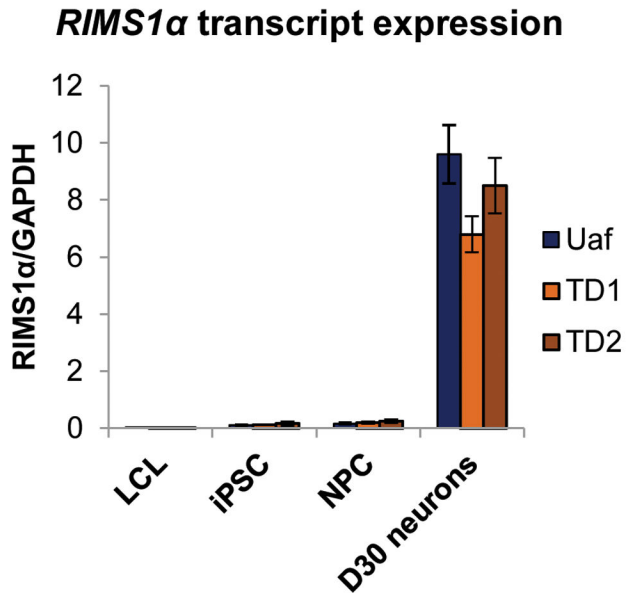
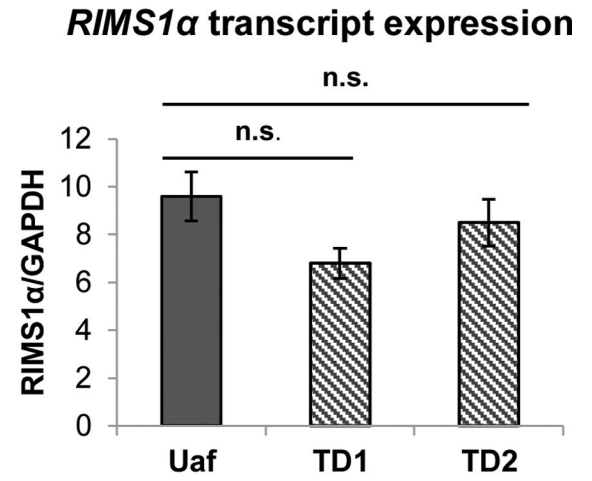
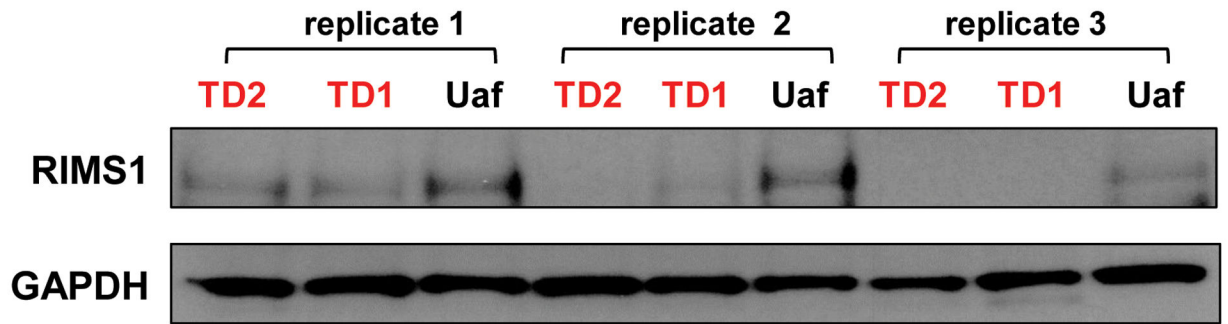
E. *PNKD* (L) protein was detected in D30 neurons but not in LCL or iPSC. *PNKD* (L) protein was reduced in iPSC-derived D30 neurons of subjects TD1 and TD2 compared to the Uaf subject (n = 3).

**A****B**

**Figure 4. The human PNKD (L) protein oligomerizes with itself and interacts with the RIMS1  $\alpha$  protein**

A. 239FT cells overexpressing GFP, FLAG-PNKD(L), PNKD(L)-Myc or FLAG-PNKD(L) + PNKD(L)-Myc were lysed for Co-IP. The PNKD(L)-Myc was pulled down by the anti-FLAG magnetic beads only in the presence of the FLAG-PNKD(L).

B. 239FT cells overexpressing GFP, RIMS1 $\alpha$ -FLAG, PNKD(L)-Myc or RIMS1 $\alpha$ -FLAG +PNKD(L)-Myc were lysed for Co-IP. The PNKD(L)-Myc was eluted by the anti-FLAG magnetic beads only in the presence of the RIMS1 $\alpha$ -FLAG.

**A****B****C**

**Figure 5. Endogenous *RIMS1α* expression in Uaf and TD cells**

A. *RIMS1α* transcript was not expressed in LCL, was expressed at low levels in iPSC and NPC and was enriched in iPSC-derived D30 neurons (n = 3).

B. RT-qPCR measured the *RIMS1α* transcript in iPSC-derived D30 neurons of Uaf, TD1 and TD2 subjects. D30 neurons from three independent differentiations were collected. There was no significant difference between the expression of the *RIMS1α* transcript in Uaf and TD neurons (n = 3).

C. *RIMS1* protein was examined by western blot in D30 neurons from three independent differentiations. *RIMS1* protein was reduced in TD neurons.

**Table 1**Mutations at *PNKD* and diseases

Mutation	Exon location	Isoforms impacted	Phenotype	Reference
A7V (het <sup>a</sup> )	1	long and short	Paroxysmal nonkinesigenic dyskinesia	15, 47–54
A9V (het)	1	long and short	Paroxysmal nonkinesigenic dyskinesia	15, 48–50, 55–57
A33P (het)	2	long and short	Paroxysmal nonkinesigenic dyskinesia and cerebellar ataxia	17
G89R	5	long and medium	Mostly gastro-intestinal dysmotility but a couple of patients exhibiting movement disorders (ataxia, dyskinesia)	59
P341Rfs*2 (het)	12	long and medium	Familial hemiplegic migraine	60

<sup>a</sup>heterozygous mutation