THE PNKD GENE IS ASSOCIATED WITH TOURETTE DISORDER AND TIC DISORDER IN A MULTIPLEX FAMILY

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ABSTRACT OF THE DISSERTATION

THE PNKD GENE IS ASSOCIATED WITH TOURETTE DISORDER AND TIC DISORDER IN A MULTIPLEX FAMILY

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Tourette Disorder (TD) is a highly heritable neuropsychiatric and neurodevelopmental disorder characterized by the presence of both motor and vocal tics. Disruptions of particular genes have been identified in subsets of TD patients. However, none of the findings have been replicated, probably due to the complex and heterogeneous genetic architecture of TD that involves both common and rare variants. To understand the etiology of TD, functional analyses are required to characterize the molecular and cellular consequences caused by mutations in candidate genes. Such molecular and cellular alterations may converge into common biological pathways underlying the heterogeneous genetic etiology of TD patients.

In CHAPTER 1, we review specific genes implicated in TD etiology, discuss the functions of these genes in the mammalian central nervous system and the corresponding behavioral anomalies exhibited in animal models, and importantly, review functional analyses that can be performed to evaluate the role(s) that the genetic disruptions might play in TD. Specifically, the functional assays include novel cell culture systems, genome

editing techniques, bioinformatics approaches, transcriptomic analyses, and genetically modified animal models applied or developed to study genes associated with TD or with other neurodevelopmental and neuropsychiatric disorders.

In CHAPTER 2, we reported that a rare heterozygous nonsense mutation at the *PNKD* gene co-segregating with individuals affected by TD or Tic disorders in a multiplex family was identified by whole exome sequencing. 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. We found that transcript and protein levels of the *PNKD* long isoform were reduced in neurons derived from the individuals with TD probably due to nonsense-mediated mRNA decay. Additionally, we demonstrated that the PNKD long protein monomer oligomerizes with itself as well as interacts the synaptic active zone protein RIMS1α. Therefore, we concluded that the reduction of the PNKD long protein in the neurons of TD patients in this multiplex family may contribute to TD.

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My interest in biology didn't stop at the failure of an experiment I did at a very young age. In retrospect, as Charles Darwin once described one of his own experients, it was "a fool's experiment", i.e. extracting chlorophyll by soaking smashed green leaf plants in water. Eventually, I didn't go much further with the plant biology but pursued human genetics in graduate school here at Rutgers University, where my independence as a researcher was nurtured by mentorship and friendship that I am deeply grateful for.

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Overview: Tourette Disorder

Tourette Disorder (TD) is a childhood-onset neurodevelopment disorder characterized by the presence of both motor and vocal tics. Prevalence ranges from 1–3 percent and is found across many ethnic groups around the world (1). However, a recent meta-analysis of previous TD prevalence studies re-estimates the population prevalence of TD to be 0.3-0.9 percent (2). Males are affected 3 to 4 times more often than females (3-5). A high percentage of TD patients have comorbid conditions, most commonly attention-deficit/hyperactivity disorder (ADHD) and obsessive-compulsive disorder (OCD), and to a lesser extent autism spectrum disorders (ASDs).

Consistent evidence from family and twin studies suggests a significant genetic contribution to TD, most likely the result of complex and heterogeneous inheritance involving both common and rare variants, though most of specific findings still require replication. The neurobiological basis of TD is not well understood, but appears to involve alterations in the development, structure, and/or functioning of cortico-striato-thalamo-cortical circuits (6). Specific genes have been found to be associated with TD. It is unclear if mutations in these genes cause TD and, if so, how these alterations affect the function or structural development of the nervous system. Here I review the neurobiology of TD, describe the biological functions of those genes previously associated with TD, and discuss the various functional analyses that are required for evaluating and establishing the pathogenicity of these putative genetic causal variants for TD.

Neurobiology of Tourette Disorder

Alterations of the cortico-striato-thalamo-cortical (CSTC) circuits are considered the neuropathological basis of tic generation (6, 7). These alterations are apparent in functional and structural imaging studies (8), histopathological studies of specific neuronal populations (9), and defective inhibition in various electroneurophysiological experimental paradigms (10, 11). In addition to the male predominance, the developmental features of TD pose an explanatory challenge, with tics usually not appearing until 4-6 years of age and most often, but not always, improving spontaneously by late adolescence.

Neurotransmitter pathways that modulate the activity and the output of the CSTC circuits in the basal ganglia have been the focus of intensive investigation, driven in part by the quest for more effective pharmacological interventions. The most supported neurotransmitter dysregulation hypothesis in TD involves the hyperactivity or imbalance of dopamine signaling in the striatum (12). Within the basal ganglia, dopamine is released to the striatum by dopaminergic neurons originating from the substantia nigra. In the striatum, the effect of dopamine on subsequent neural signal transmission is modulated by the striatal medium spiny neurons expressing either D1 or D2-like dopamine receptors (13). The dopamine hypothesis is supported by the clinical observation that dopamine D2 receptor antagonists effectively reduced tics in some TD patients (14, 15). Also, dopamine pathway dysregulation was reported in postmortem TD brain samples (16, 17) and in the brains of living TD patients (12, 18).

Due to their excitatory and inhibitory effects within CSTC circuits, glutamatergic and GABAergic pathways have also been studied in TD. In postmortem samples, lower levels of glutamate in subcortical brain regions were reported (19). However, it is unclear whether TD is associated with hyper- or hypo- glutamate levels (20). For the GABAergic pathway, an altered number and distribution of striatal GABAergic neurons were described in TD postmortem brain samples (21).

Disrupted serotonin signaling has been implicated in OCD, a common comorbid condition among TD patients. Selective serotonin reuptake inhibitors (SSRIs) have proven effective in reducing OCD symptoms (22) and are also usedto treat TD patients with comorbid OCD (23). Interestingly, sequence variants at the serotonin transporter gene were found in both OCD and TD patients (24), suggesting alterations in the serotonin pathway as one possible mechanism in the etiology of TD.

Until the recent discovery of a dominant-negative nonsense mutation in the *HDC* gene co-segregating with TD in a large family, histaminergic (HA) neurotransmission was not considered a top candidate for TD etiology (25). However, other findings provide additional support for the involvement of HA neurotransmission in TD. For example, single nucleotide polymorphisms (SNPs) within the *HDC* gene region showed association with TD (26). Also, rare copy number variants (CNVs) found in TD patients were enriched in chromosomal regions harboring HA pathway genes (27). Furthermore, mice lacking the *Hdc* gene exhibited tic-like behaviors (28). Even though there was no evidence for direct actions of serotonin and histamine on movements, it is proposed that serotonin and histamine pathways might indirectly regulate movements by modulating the dopamine system in the substantia nigra. In particular, both serotoninergic and

histaminergic innervations are observed in the substantia nigra (29, 30). Also, serotonin and histamine receptors are expressed on nigrostriatal dopaminergic neurons (30, 31).

Aside from the neurotransmitter dysregulation hypothesis in TD, developmental and neuro-immunological findings also provide a context for assessing the relevance of potential gene findings. Altered distribution of parvalbumin interneurons (21) and reduced numbers of parvalbumin and cholinergic interneurons in basal ganglia were observed in the postmortem brain samples of TD patients , suggesting another possible, perhaps developmental, mechanism for alterations of CSTS circuits. Additionally, a dysregulated brain-immune system involving microglia cells was suggested to contribute to TD (32). Gene expression of inflammatory factors was examined using postmortem basal ganglia samples from TD patients and controls (33). An elevated expression of the *CD45* gene was observed in TD patients even though the elevation was not statistically significant. CD45 is a surface marker of microglia and its expression is increased due to the activation of microglia. In another study, transcriptome analysis of postmortem striatum of TD patients and controls revealed upregulation of microglia-related genes (34).

Genes disrupted in patients with Tourette Disorder

In this section, I will review 15 genes that have been associated in TD (Table 1); to suggest how we might move beyond association to establish a role in TD pathogenesis, I will examine what is known about the biological effects of these genes. I group these genes into several categories: 1) neurite outgrowth: *SLITRK1*; 2) histamine pathway: *HDC*; 3) serotonin pathway: *SERT, HTR1A HTR2B*; 4) glutamate pathway: *SLC1A3*; 5)

synaptic signal transduction and cell-adhesion pathway: *NLGN4*, *CDH2*, *CNTNAP2/CASPR2, DPP6*; 6) mitochondrial functions: *IMMP2L, MRPL3*; and 7) genes associated with other diseases : *DNAJC13 (Parkinson's Disease), OFCC1(Orofacial Clefts), HCRTR2 or OX2R (Excessive Daytime Sleepiness)*. The diverse functions of these genes - ranging from neurotransmitter synthesis, neuronal migration, synaptic plasticity, cell adhesion, protein transportation and synthesis - highlight the complexity of unravelling the pathogenesis of TD. However, in addition to the genetic disruptions discussed here, large structural variations, for example copy number variants (CNVs), have also been investigated in TD patients. Genes disrupted by these structural variants have been proposed as potential TD associated genes (35-37).

Neurite outgrowth

SLIT and NTRK-Like Family, Member 1 (*SLITRK1*)

In a TD patient with comorbid ADHD, a *de novo* chromosome 13 inversion was identified (38). The *SLITRK1* gene was mapped close to the breakpoints. Targeted sequencing the *SLITRK1* gene in another 174 unrelated TD patients identified a noncoding variant (var321) and a frameshift mutation (38) not found in a large control sample. The frameshift mutation led to impaired dendrite growth in neurons and the var321 variant may cause reduced SLITRK1 protein expression (38). While the var321 and additional novel variants within *SLITRK1* were found in other TD patients, these associations were not replicated in subsequent studies (39-49).

Members of the SLITRK protein family are transmembrane proteins. They are structurally homologous to the SLIT and the TRK proteins which are involved in axon

guidance pathway and neurodevelopment (50). The *SLITRK1* gene is highly expressed in developing and mature neuronal tissues and promotes neurite outgrowth in culture (51). The SLITRK1 protein is localized to the postsynaptic densities and has been hypothesized to affect synapse formation at excitatory synapses through interactions with the presynaptic cellular adhesion molecule LAR-RPTP (52, 53). In *Slitrk1* knockout mice, while no stereotypic behaviors were observed, anxiety-like and depression-like behaviors which were attenuated by chemicals modulating noradrenergic neurotransmission were observed (54). Neurochemical abnormalities were also detected in *Slitrk1* knockout mice: norepinephrine and its metabolites were significantly increased in the prefrontal cortex and the nucleus accumbens while choline and acetylcholine levels were significantly lower in the striatum (54). Taken together, the evidence suggests that the *SLITRK1* gene might play a role in neurochemical modulation.

Histamine pathway

Histidine decarboxylase (*HDC*)

Histamine neurotransmission was first linked to the etiology of TD when a rare nonsense mutation within the *HDC* gene was discovered in a multiplex family in which the father and all eight children were diagnosed with TD. The mutation resulted from a G to A transition at the 9th exon of the *HDC* gene and led to a premature stop codon (W317X) (25). The heterozygous W317X mutation co-segregated with all affected individuals in this family. The W317X mutation was not found in 3360 unrelated individuals unaffected with TD or another 720 TD patients, suggesting this is a very rare cause for TD. *In vitro* enzymatic assay demonstrated that the truncated protein produced by the mutation lost

histidine decarboxylase activity and had a dominant-negative effect on the activity of wild-type HDC protein. After the initial finding, more TD patients were screened for mutations in the *HDC* gene in different studies. Only an intronic variant and two synonymous variants were identified in a study involving 120 TD patients (55). However, an association of the *HDC* gene and TD phenotypes was reported in a study including 520 TD nuclear families (26). Also, rare genic copy number variants (CNVs) identified in 460 TD patients were enriched for histaminergic pathway genes (27), supporting the potential involvement of the histamine pathway in TD etiology.

In the adult human central nervous system, the *HDC* gene is exclusively expressed in the soma and axon varicosities of histaminergic neurons mostly originating from the tuberomammillary nucleus in the posterior hypothalamic region of the brain (30). The HDC homodimer converts L-histidine into histamine in the soma of histaminergic neurons. Histamine-containing neuronal fibers are seen in many brain areas in rodents and human including cerebral cortex (56, 57). Therefore, loss-of-function mutations at the *HDC* gene will likely cause a lack of histamine in the widespread brain regions receiving histaminergic innervation. In addition to serving as a neurotransmitter, histamine is a neuromodulator, inhibiting dopamine release by striatal dopaminergic neurons through binding to the H3 receptors expressed on these neurons in mice (58). Given the hypothesis that hyperactivity of nigrostriatal dopaminergic neurons is responsible for tic generation (12), it is reasonable that histamine dysregulation may contribute to TD.

Since HDC protein functions as a homodimer (59), individuals harboring the W317X mutation have approximately 25% residual histidine decarboxylase activity remaining

compared to the healthy controls. Therefore, the *Hdc* knockout mice may recapitulate the behavioral outcomes caused by the W317X mutation in humans. As expected, the *Hdc* knockout mice show tic-like stereotypic behaviors after psychostimulant administration and reduced pre-pulse inhibition (28). Interestingly, the striatal dopamine level was increased in the *Hdc* knockout mice during the dark cycle, which could be decreased by administration of histamine in the knockout mice. Also, higher levels of dopamine D2 receptor occupancy were found in the basal ganglia of TD patients carrying the W317X mutations, the *Hdc* knockout mice and the *Hdc* heterozygous mice, indicating that the dopamine release in the basal ganglia of the brain might be disinhibited due to histamine depletion (28). Taken together, parallel studies in TD subjects and mice demonstrated that lack of histamine results in dopamine dysregulation, providing a potential mechanism for the proposed role of dopamine disruption in TD (12).

Serotonin pathway

Serotonin transporter (*SLC6A4* or *SERT*)

Given the effectiveness of the selective serotonin reuptake inhibitors (SSRIs) in reducing OCD symptoms, the *SERT* gene has been studied as candidate gene for OCD (60-64). Serotonin-transporter-linked polymorphic region (5-HTTLPR) polymorphisms and a gain-of-function missense mutation I425V have been associated with OCD (65-69). Sequence variants of the *SERT* gene were first associated with TD in a two-generation pedigree (24). In this family, the heterozygous “long” 5-HTTLPR variant and the linked I425V mutation perfectly segregated with TD individuals in a dominant pattern. The

"long" 5-HTTLPR produces higher *SERT* mRNA level compared to the "short" 5-HTTLPR (70). The I425V mutation results in constitutive activation of the serotonin transporter protein whose activity is regulated by cGMP-dependent protein kinase (71). Therefore, carrying both "long" 5-HTTLPR and the I425V mutation in *cis* is expected to have a synergistic effect that increases the expression of *SERT* mRNA and increases the amount of activated SERT protein.

In the mammalian central nervous system, the *SERT* gene is primarily expressed in the serotonergic neurons that originate from the raphe nucleus in the hindbrain and project widely to other parts of the nervous system, descending to the spinal cord and ascending to the forebrain (72). The human SERT protein is a transmembrane protein (73, 74). Cell surface expression of SERT protein can be regulated by SERT antagonists and substrates (75). The serotonergic axons can innervate and regulate other neurotransmission systems. For example, serotonin can facilitate or inhibit dopamine release in the striatum in a direct or indirect manner (76). Therefore, dysregulation of SERT expression on the plasma membrane may affect dopamine transmission (77). So far, no other sequence variants in the *SERT* gene have been associated with TD and no corresponding transgenic mice are available for *in vivo* studies.

Serotonin receptor 1A (*HTR1A)* and serotonin receptor 2B (*HTR2B*)

In addition to the serotonin transporter gene, other serotonin pathway genes have been examined in TD patients. A missense mutation causing an amino acid change from arginine to leucine was identified in the serotonin receptor 1A gene (*HTR1A*) in one TD patient. However, the mutation was not predicted to change the receptor activity (78).

Two novel non-synonymous missense variants and three known SNPs in the serotonin receptor 2B (*HTR2B*) gene were also found in 132 Caucasian and 128 Chinese Han TD individuals, though the associations were not statistically significant (79).

There are currently 14 known serotonin receptors and these are categorized into seven classes (80, 81). The *HTR1A* and *HTR2B*, both of which are G protein coupled receptors, belong to class I and class II, respectively. The HTR1A receptor is highly expressed in the brain. Lower brain expression of the HTR1A receptor has been associated with mood disorders in humans. *Htr1a* knockout mice exhibit depression and anxiety-like behaviors and have been used for antidepressant drug screening (82, 83). The HTR1A receptors are located at both pre-synaptic and post-synaptic neurons in the CNS. Activation of the presynaptic HTR1A receptors on the serotonergic neurons leads to inhibition of serotonergic neuron firing and reduced serotonin release whereas activation of the post-synaptic HTR1A can modulate the release of other neurotransmitters (84). Compared to the HTR1A receptor, the role that HTR2B plays in the CNS is not well understood. However, there is evidence suggesting that HTR2B may regulate serotonin transporter (SERT) activity by phosphorylating SERT protein (85). In mice, the HTR2B receptor may be involved in modulating serotonin release from the serotonergic neurons. (86). Taken together, dysfunction of the HTR1A or the HTR2B receptor might lead to abnormal serotonin release in the CNS.

Glutamate pathway

Excitatory amino acid transporter 1 (*SLC1A3* or *EAAT1*)

Altered cortico-striatal-thalamo-cortical (CSTC) circuitry is believed to provide a possible neurobiological basis for TD (7, 87). Glutamate is the major excitatory neurotransmitter in CSTS circuitry. A missense mutation (E219D) in the glutamate transporter gene (*SLC1A3*) was associated with TD in a case-control study (88). In the same study, cells transfected with the E219D mutant glutamate transporter gene exhibited increased glutamate uptake activity compared to cells transfected with the wild type gene. The proposed mechanism for the increased glutamate uptake activity was elevated glutamate transporter expression at the plasma membrane due to the E219D mutation. However, whether TD might be associated with hypo- or hyper- glutamate activity is still controversial.

One of the five subtypes of glutamate transporters, EAAT1, is responsible for the reuptake of the excitatory neurotransmitter, L-Glutamate, from the synapses back into cells. Dysfunction of glutamate transporters may lead to imbalanced extracellular glutamate levels, further affecting downstream glutamate neurotransmission or causing glutamate excitotoxicity to neurons (89, 90). EAAT1 is primarily expressed in glial cells. Regionally, EAAT1 proteins are found in neocortex, striatum, cerebellum and spinal cord (91). *Eaat1* knockout mice showed hyperactivity and reduced acoustic startle response compared with the wild type mice (92) but did not exhibit the altered prepulse inhibition behavior which has been found in TD patients (93). No *Eaat1* gain-of-function mutant mice are available to test the "hypo-glutamate activity" hypothesis in TD.

Synaptic signal transduction and cell-adhesion pathways

Neuroligin 4, X linked (*NLGN4* or *NLGN4X*)

Mutations in the neuroligin family members have been identified in patients with neuropsychiatric disorders such as autism spectrum disorder (94-97) and schizophrenia (98). A small deletion in the *NLGN4* gene was detected in a mother and her two sons (99), one of whom was diagnosed with autism while the other was diagnosed with TD and ADHD. Their mother, who also carried the deletion, had a learning disability, depression and anxiety. The deletion spanned exons 4, 5 and 6 of the *NLGN4* gene, resulting in a truncated protein. No other known genes were affected by the deletion.

Neuroligins (NLGNs) are cell adhesion molecules located on the plasma membrane of the presynaptic and postsynaptic neurons. By interacting with neurexins, another family of cell adhesion proteins, NLGNs modulate proper signal transmission between presynaptic and postsynaptic neurons (100). Reduced expression of NLGNs in cultured neurons or mice cause deficits in synaptic maturation and plasticity (101, 102). *Nlgn4* knockout mice have been studied at both the behavioral and cellular levels. Because *Nlgn4* gene disruptions had been associated with ASD, the *Nlgn4* knockout mice were tested for ASD-like behaviors. As expected, *Nlgn4* knockout mice exhibited deficits in social interactions and reduced ultrasonic vocalizations compared to the wild type mice (103). In another study, the *Nlgn4* knockout mice displayed reduced neural network response upon stimulation in both excitatory and inhibitory circuits (104). More interestingly, the *Nlgn4* knockout mice also showed stereotypic repetitive behaviors and increased obsessive compulsive like behaviors (105), supporting the possibility that disruption of the *NLGN4* gene might play a role in TD or related disorders.

Cadherin 2, Type1, N-Cadherin (*CDH2*)

Cadherin 2, also known as N-Cadherin, is another cell adhesion protein that has been associated with TD. CDH2 participates in neuron-neuron communication and in synaptogenesis. In a recent study, exons of the *CDH2* gene were sequenced in 160 OCD probands and 160 controls (106). Four variants in the *CDH2* gene were identified in subjects with OCD or TD. Two mutations were of particular interest: the N706S and the N845S variants. N706S is a rare and novel mutation close to the predicted proteolytic cleavage site of the CDH2 protein while the N845S variant is located at the β-catenin interacting region. Both mutations reduced the CDH2 protein level by more than 50% in transfected HEK293 cells (106), suggesting possible adhesion deficits in cells.

CDH2 is a calcium dependent cell-cell adhesion glycoprotein. CDH2 primarily mediates neurite outgrowth and axon guidance of neurons on myotubes (107) and on the surface of astrocytes (108, 109). The cytosolic domain of the CDH2 protein forms complexes with catenin proteins (110) and these complexes regulate synaptogenesis in both pre- and post-synaptic neurons (111, 112). Additionally, the cleaved C-terminal domain of the CDH2 is a repressor of CBP/CREB-mediated transcription whose target genes are critical in neural development and plasticity (113). The complete knockout the *Cdh2* gene is an embryonic lethal in mice whereas *Cdh2* heterozygous null mice do not exhibit obvious morphological defects during development (114). Conditional knockout of the *Cdh2* gene in the cerebral cortex of mice caused disrupted cortical structure (115). Thus far, behavioral analyses in the *Cdh2*+/- mice or *Cdh2* conditional knockout mice have not been conducted.

Contactin Associated Protein-like 2 (*CNTNAP2/CASPR2*)

Variants in the *CNTNAP2* gene have been associated with ASD in several family-based studies (116-119). Also, putative deleterious mutations were found in the *CNTNAP2* gene in ASD patients (120). In one family, an insertion on chromosome 7p35-p36, disrupting intron 8 of the *CNTNAP2* gene, was shared by a father and his two children, all three of whom were diagnosed with TD (121). However, a translocation disrupting intron 11 of *CNTNAP2* did not cause TD phenotypes in another three generation family (122).

CNTNAP2, a transmembrane protein, belongs to the neurexin superfamily and is highly expressed in neurons and localized to the axonal membrane of the juxtaparanodal region. The CNTNAP2 protein interacts with clustered Shaker-type potassium channels and plays an important role in the axon-glia septate-like junction (123). It is required for normal action potential propagation along myelinated axons of the neurons (124) and it has been hypothesized that malfunctions of the CNTNAP2 protein leads to deficits of electric signal transduction between neurons (121, 125, 126).

Behavioral assessments of *Cntnap2* knockout mice led to stereotypic motor movements (127). Interestingly, a reduced number of GABAergic interneurons was reported in the striatum of the *Cntnap2* knockout mice which is consistent with previous postmortem studies showing a reduction in the number of striatal GABAergic interneurons in TD patients (21). Therefore, understanding the molecular mechanism of neuronal loss caused by disruption of CNTNAP2 may help pinpoint biological pathways altered in TD.

Dipeptidyl-peptidase 6 (*DPP6*)

A heterozygous microdeletion at the first exon of the *DPP6* gene was identified in a boy with TD as well as the boy's father and paternal uncle both of whom were diagnosed with

tic disorder and ADHD (128). The microdeletion led to decreased *DPP6* mRNA level in the boy's blood cells. *DPP6* has also been associated with other neuropsychiatric disorders such as ASD (95, 129) and schizophrenia (130).

DPP6 is a transmembrane protein belonging to a family of serine proteases. However, DPP6 does not have protease activity (131). DPP6's expression is enriched in the brain and different isoforms have different distributions in the brain. (132). DPP6 proteins interact with A-type voltage-gated potassium channels, specifically the Kv4 subunit (133, 134). The A-type potassium channels participate in the modulation of dendritic signal transmission (135, 136). Moreover, the A-type potassium channel was reported to control the tonic dopamine release by substantia nigra dopaminergic neurons (137). Even though DPP6 has no peptidase activity, it may have novel functions and play essential roles in Kv4 intracellular trafficking, membrane expression and proper function of the A-type potassium channels (138). *Dpp6* knockout mice show abnormal synaptogenesis (139) and knocking down the *Dpp6* gene specifically in the mouse brain caused impaired learning and memory abilities (140).

Mitochondria functions

Inner Mitochondrial Membrane Peptidase 2 Like (*IMMP2L*)

A familial translocation segregating with TD or tics (141) and a *de novo* duplication in a TD patient with other developmental and mental phenotypes implicated *IMMP2L* as a possible TD candidate gene (142). This was the first mitochondria-related gene in TD. Subsequently, a cryptic deletion eliminating exons 1 to 3 of the *IMMP2L* gene and 21 other genes was identified in a TD patient with learning and speech difficulties (143).

Also, a case-control study of copy number variations reported intragenic deletions at the *IMMP2L* gene in seven TD patients (144). Among the seven, three deletions at intron 3 led to production of a shorter *IMMP2L* mRNA transcript due to alternative splicing. In the same study, the expression of both the short and the long transcripts was examined by reverse transcription PCR in 19 regions of the human brain. While the long transcript was ubiquitously expressed in all 19 brain regions, the short transcript was selectively expressed at a lower level in only 10 brain regions, suggesting that the short transcript might have a more specific role in the human central nervous system (144).

The human IMMP2L protein is one of the catalytic subunits of the inner mitochondrial membrane peptidase (IMP) complex (145, 146). The IMP complex participates in the cleavage of the inner mitochondrial membrane targeting signal sequence from its protein substrates, allowing maturation of the substrates. Loss of any subunit will cause the decomposition of the whole complex (147). Expression and function of the mammalian IMMP2L protein have been studied in animal models and human tissues. *Immp2l* knockout mice exhibit mitochondrial dysfunction, increased ischemic brain damage and infertility (148, 149).The human *IMMP2L* mRNAs are ubiquitously expressed in various tissues except for adult liver and lungs. Unlike other TD-associated genes, there is no enriched *IMMP2L* mRNA expression in the brain compared to other tissues (142). However, linking mitochondrial dysfunction to TD might lead to further speculation about the varied etiology of TD.

Mitochondrial Ribosomal Protein L3 (*MRPL3*)

Whole exome sequencing of a multiplex TD family showed three rare novel non-synonymous mutations in the *MRPL3, DNAJC13*, and the *OFCC1* genes (150). The three variants were not found in controls or dbSNPs/1000 Genomes databases. However, a targeted-sequencing study of the same three variants in Han Chinese TD patients did not replicate these findings.

MRPL3 is a mitochondrial ribosome protein involved in mitochondrial protein translation (151). Compound heterozygous mutations in the *MRPL3* gene were associated with mitochondrial respiratory chain deficiency in a pedigree of French origin (152) but no psychiatric diseases were reported.

Genes associated with other diseases

DnaJ (Hsp40) Homolog, Subfamily C, Member 13 (*DNAJC13*)

A missense variant (A2057S) in the *DNAJC13* gene was found to segregate with TD or chronic tic disorder (CTD) in a multiplex pedigree (150). Subsequently, a novel missense mutation Asn855Ser in the *DNAJC13* gene was found in a multi-generation family with Parkinson's disease (95) and in an additional four other PD patients (153). Human DNAJC13 is a membrane-associated protein involved in endocytosis, specifically in the process of early endosome-mediated membrane trafficking (154, 155). Knocking down the *DNAJC13* gene in mammalian cells did not cause obvious dysfunction of endocytosis. However, introducing a C-terminus truncated mutant DNAJC13 protein into the cells did affect the normal distribution and formation of early endosomes (155). No *DNAJC13* knockout animal model is available.

Orofacial Cleft 1 Candidate 1 (*OFCC1*)

After an initial study suggesting that the *OFCC1* gene led to orofacial clefts (156), it was later linked to schizophrenia (157). Recently, a genome-wide association study (GWAS) of OCD found a significant association with *OFCC1* (158). Sequence variants in *OFCC1* have been found in patients with neurodevelopmental or neuropsychiatric disorders: a missense mutation (R129G) segregating with TD and CTD in a multiplex family (150); a nonsense and a missense mutations were found in a single autism family (159).

The function of the OFCC1 protein is unclear but one study suggested that the OFCC1 protein was an interacting partner and methylation substrate of protein arginine methyltransferase 1 (160). However, *Ofcc1* knockout mice didn't show any behavioral abnormalities (157).

Hypocretin (Orexin) Receptor 2 (*HCRTR2/OX2R*)

The coding regions of the orexin-1/hypocretin-1 (*OX1R*) receptor gene, the orexin-2/hypocretin-2 (*OX2R*) gene and the prepro-orexin gene were examined in patients with Excessive Daytime Sleepiness, TD or ADHD and healthy controls (161). A C29T nucleotide change in the *OX2R* gene producing a Pro10Ser amino acid substitution was detected in only one TD patient with comorbid ADHD. The Pro10Ser variant reduces responsiveness of the Orexin2 receptor to its ligands, Orexin-A and Orexin-B. The Orexin receptor 2 is a G-protein coupled receptor that participates the regulation of feeding and sleep-wakefulness in mammalian brains (162). *Ox2r* knockout mice do not show any tic-like behaviors (163).

Functional analyses of genes disrupted in Tourette Disorder

As indicated in the previous section, evidence for TD susceptibility genes exists. The mutations discussed were found in only a small number of individuals with TD and replication remains elusive. This lack of replication may be due to the extreme locus heterogeneity, similar to what has been found in ASD (164). What evidence beyond stronger statistical association might help establish their potential role in TD pathogenesis? Some of these genes are found within neurobiological pathways that are presumed to be disrupted in TD (e.g., neural signal transmission/modulation) while others are found in novel pathways. Hence, the evidence that these genes are true susceptibility genes remains insufficient. Consequently, more convincing functional studies are needed to determine how variants in these genes could contribute to TD. In this next section, we review the *in vitro* and *in vivo* functional studies and techniques (beyond the knock out experiments referred to above) that will likely be useful to evaluate the consequences of mutations found in these presumptive TD susceptibility genes and for discovery of cellular and molecular phenotypes in disease.

Transgenic mammalian cell lines

Functional studies using neuronal cells from individuals with TD can provide insights into the molecular basis of TD and potentially, help clarify the biological pathways altered in TD. However, one of the difficulties is the inability to obtain relevant biomaterials (e.g., neurons or neural stem cells) from affected individuals. Since TD is not a lethal disorder, there is very limited access to neural tissue from individuals with TD, particularly from individuals with a known causal variant.

Transgenic human non-neuronal cell lines have been used to characterize the cellular and molecular phenotypes resulting from specific mutations. Typically, plasmids carrying a wild type or a mutant gene of interest are delivered into the cell lines. Once the protein products of the transgene are expressed in the cells, assays are developed to evaluate the functional consequences of the mutant proteins. For example, in the *CDH2* gene, both the wild type and mutant *CDH2* genes were cloned into expression plasmids and subsequently delivered into human embryonic kidney (HEK293) cells and reduced expression of mutant CDH2 proteins was reported (106). While easily done, functional studies in non-neuronal cell lines are suboptimal for a variety of reasons. During transgenesis, the gene of interest is often transiently overexpressed or is controlled by a conditional and/or inducible gene expression system (165). Therefore, the level of transgene expression might not faithfully represent the endogenous gene expression level. Also, the expression levels of many genes are tissue-specific (166). This is particularly relevant for genes with multiple transcript isoforms where the isoform expression pattern in transgenic cell lines may not be comparable to patterns in neurons. Furthermore, because transgenic cells would not be expected to have the same gene expression profile as neurons, they may not provide relevant cellular environment for the transgene to execute its genuine neuronal function(s). Neuronal samples from TD patients are therefore preferred for *in vitro* functional studies but these are very difficult if not impossible to obtain. A recent technological advance, induced pluripotent stem cells, now makes functional studies of neuronal samples with a known causal variant possible.

Induced pluripotent stem cells (iPSCs)

Use of iPSC-derived neuronal cells to model neuropsychiatric and neurodevelopmental disorders:

The relatively new reprogramming technique that converts human somatic cells into induced pluripotent stem cells (iPSCs) allows researchers to model diseases *in vitro* using patient-derived cells. Since the first generation of iPSCs from mouse fibroblasts (167), the ability to produce differentiated cells from iPSCs has been intensively studied and improved. Recently, iPSC neuronal differentiation has become routine (168). Generating patient-specific neurons carrying mutations in disease candidate genes is invaluable for researchers who wish to study the cell-autonomous effects of mutations and to understand the cellular basis of neurological and neuropsychiatric disorders. To date, no study using iPSC-derived neurons to model TD has been published. However, mutations associated with other neuropsychiatric disorders have been studied in iPSC-derived neurons and cellular abnormalities have been demonstrated (169-173). For example, Rett syndrome is a neurodevelopmental disorder occurring mainly in females characterized by mental retardation. Loss-of-function mutations in the methyl-CpG binding protein 2 (*MeCP2*) gene were reported in the majority of Rett Syndrome cases (169). Therefore, neurons with functional null mutation in the *MeCP2* gene were generated from the iPSCs of an individual with Rett Syndrome. In culture, the neurons showed smaller soma size (170). Similarly, iPSC-derived neurons have been used to understand ASD. In an individual with ASD, a balanced translocation spanning the transient receptor potential 6 channel (*TRPC6*) gene was identified. Neurons derived from the iPSCs exhibited decreased *TRPC6* expression, altered morphology and reduced Ca^{2+} influx (171). In schizophrenia,

the *DISC1* gene has been considered an important risk factor (174) and in iPSC-derived *DISC1* mutant neurons, presynaptic vesicle release was impaired (172).

Unfortunately, *in vitro* neural differentiation from iPSCs yields mixed populations of neurons rather than a homogenous population. For diseases with clear neuropathology, pure cultures of the specific neuron types involved in the disorder are preferred in order to recapitulate disease-related cellular phenotypes. For instance, Parkinson's Disease (95) is characterized by loss of substantia nigra dopamine neurons (175). Cultures containing a high percentage of dopaminergic neurons were generated from PD patients who carry monogenic mutations (176-179) and defects in cellular functions such as autophagy, mitophagy, oxidative stress response, dopamine release were found in these neurons. In patients with amyotrophic lateral sclerosis (ALS), motor neurons degenerate. Therefore, motor neurons were derived from iPSCs of ALS patients carrying known casual mutations (173, 180). As hypothesized, the mutant motor neurons exhibited neurite degeneration (180).

Use of single-cell analysis to overcome culture heterogeneity:

One method to overcome cell culture heterogeneity is to analyze single cells, all of the same type. Looking at the transcriptome of single cells using microarray or RNA-seq analysis holds promise of detecting gene dysregulation in particular populations of neurons, which might not be identified by analyzing heterogeneous mixtures of cells. For instance, single-cell gene expression analysis of iPSC-derived dopamine neurons from PD patients with a LRKK2 mutation unveiled dysregulation of oxidative stress genes in mutant dopamine neurons (181). In another study, neurons were generated from the

iPSCs of Timothy Syndrome patients with a mutation in the *CACNA1C* gene. Single cells were taken from the culture containing mixed neuron populations and gene expression was analyzed by microarray. As a result, the distribution of neuron subtypes was altered in the Timothy Syndrome neuronal culture compared to the control cells (182). Compared to microarray, RNA-seq is gaining greater popularity for analyzing the transcriptome of single cells due to its ability to unbiasedly detect any transcript in cells within a broader dynamic range of expression. Generally, there are four important steps to achieve single-cell RNA-seq: 1) single cell isolation, 2) RNA capture, 3) cDNA synthesis and, 4) next generation sequencing. The microfluidic system is becoming popular for single-cell RNA-seq because it can isolate single cells, lyse the cells, purify RNA, synthesize cDNA or even conduct gene expression analysis all in one run (183, 184).

Use of iPSC-derived cerebral organoids to model neuropsychiatric and neurodevelopmental disorders:

In contrast to PD or ALS, the neuropathology of many neurodevelopmental and neuropsychiatric disorders, such as TD or ASD, is unclear or is heterogeneous (18). As described above, mutations associated with TD indicate dysregulations of various neurotransmitter pathways or of neural circuits involving multiple brain regions. Hence, studying specific types of neurons may not help to explain the pathogenesis of TD. The recently developed “cerebral organoid” cellular system enables the differentiation of iPSCs into a three-dimensional miniature organ in a bioreactor, with minimum external interferences (185). Such self-organized spherical structures resemble the human brain at very early stages of development. In comparison to monolayer neuronal cultures, the

cerebral organoids contain more diverse neuronal populations that define distinct brain regions. Also, within the cerebral organoid, neuronal migration and human specific brain structures (e.g., the outer subventricular zone) were observed (186). Therefore, the cerebral organoid has been used to model neurodevelopmental diseases. For microcephaly, premature neural differentiation was recapitulated in organoids derived from iPSCs of microcephaly patients (186). In cases of idiopathic ASD, overproduction of GABAergic inhibitory neurons in patient-derived cerebral organoids was reported (187). At the molecular level, the cellular phenotype was explained by overexpression of the transcription factor FOXG1. However, use of cerebral organoids to model neurodevelopmental diseases has limitations. The various "brain regions" in the organoids are fairly disorganized. Therefore, the cerebral organoid would not be suitable to study neural circuits. Furthermore, neuronal cells within the organoids are mostly neural progenitor cells and their differentiation is restricted by limited growth of the organoids which in turn is probably due to the lack of internal nutrient and oxygen supply. More importantly, each organoid is "unique" because the self-organization process is random and is not controlled by external factors. This "uniqueness" will generate variation among organoids, which may mask phenotypic differences between normal and patient-derived cells.

Use of genome editing to generate isogenic control iPSC lines:

One challenge to identifying the phenotypic effects of a given mutation in iPSC-derived neurons is finding an appropriate control sample. While age, gender, and ethnicity-matched control samples with the wild-type allele are typically available, they are not matched for all of the other common genomic variants. Failure to control for such

variability in genetic background can lead to spurious results. The recent technological advance of highly-specific genome editing now allows the production of more comparable isogenic controls for functional studies. Several genome editing systems, such as zinc finger nuclease (188, 189), TALEN and CRISPR-Cas9 (190, 191), are able to reverse the mutation to wild-type at one genomic locus at a time in iPSCs. Comparing neuronal cells generated from mutant iPSCs and their edited, isogenic control neuronal cells with the mutation removed allows identification of molecular and cellular changes that are due only to the mutation (179). However, "off target" mutations at unrelated loci inadvertently introduced by editing remain a potentially important technological hurdle (192).

Gene expression and gene network analyses

The major goal of genomic sequencing of patients with neurodevelopmental and neuropsychiatric disorders is to identify disease-associated mutations. Once such genes are found, systematic approaches including genome-wide gene expression analysis and gene network analyses can be used to implicate common biological pathways altered in patients with different mutant genes.

Gene expression analysis:

Gene expression analysis, primarily through the RNA-seq approach, aims to quantify transcript level of target genes or of the whole transcriptome in biological samples from patients and healthy controls to identify genes dysregulated in human diseases (193). For neurodevelopmental disorders, postmortem brain samples are often used for the transcriptomic analyses. The first transcriptomic analysis of TD patients' postmortem

striatum samples revealed that interneuron disruption might be involved in the pathophysiology of TD (34). However, to evaluate particular mutations, postmortem samples meeting specific research criteria are usually difficult or virtually impossible to obtain. With the emergence of somatic cell reprogramming techniques, iPSC-derived neurons with and without a putative disease-causing mutation can be produced *in vitro* (194) . The transcriptomes of these iPSC-derived neurons can be compared by microarray or RNA sequencing (RNA-seq) (171, 172). To further dissect the cellular phenotype at single neuron level and to detect abnormalities only shown in particular populations of neurons, single-cell transcriptome analysis can also be performed (181, 182). Multiple bioinformatics tools have been developed for RNA-seq data to detect differentially expressed genes (DEGs) from distinct cell types or under different experimental conditions (195-197). Among these RNA-seq analysis methods, none outperforms the others in all aspects. Selecting an optimal method for a study requires an understanding of the benefits and limitations of each method as well as the parameters of the study (197). Once the DEGs between experimental conditions are determined, gene and pathway annotation tools, gene and protein expression and interaction databases can help to explore the gene pathways underlying the disorder. Gene and pathway annotations tools such as IPA (www.qiagen.com/ingenuity), KEGG (198, 199), DAVID (200, 201), ConsensusPathDB (202) report biological pathways in which DEGs are enriched or reduced and take these into account to predict how these pathways might be affected. However, the data from which these tools were constructed come from non-neuronal samples which could lead to associations not found in neuronal tissues or failure to detect neuronal associations (203). In order to annotate neuronal gene expression in a temporal

and spatial manner, human brain gene expression databases, for example, the Allen Human Brain Atlas (204), BrainSpan (205), GTEx (206) were built using microarray and RNA-seq data from postmortem brain samples. Mapping DEGs identified in neuronal samples of patients with neurodevelopmental disorders to human brain gene expression databases revealed specific brain regions and neural developmental stages that were affected (34, 207). A more detailed human brain gene expression atlas that annotates gene expression at the single cell level has been initiated by a group in Stanford University (208). Single cell RNA-seq was used to analyze neurons from human adult and fetal cerebral cortex and it identified more diverse populations of neurons within the cortical region (208). Constructing a comprehensive human brain gene expression database at single neuron resolution is quite challenging due to limited access to healthy human brain samples and the high cost of single-cell RNA-seq. Therefore, collaborative work with standardized experimental protocols is required.

Gene network analysis:

Differential gene expression from transcriptome analysis is sample-dependent and tissue-specific. In order to explore the etiology of complex neurodevelopmental disorders such as TD, disease-associated genes can be mapped to gene networks to visualize relationships between disease candidate genes and, further, to pinpoint annotated or novel pathways. The gene networks can be gene co-expression networks (204, 205), gene regulatory networks (http://www.***braineac***.org/), protein-protein interaction networks (209) or networks constructed with combined criteria (202, 210). For example, in ASD, disease-associated genes have been evaluated using spatiotemporal gene co-expression networks constructed from BrainSpan (205) and were found to be enriched in sub-

networks that represent specific brain regions and time periods during human brain development (211). Furthermore, ASD-associated mutations identified by previous genetic studies were mapped to a "background network" which scores each pair of human genes based on very comprehensive information about every known human gene (210). Cell types and brain areas affected in ASD were implicated (212). With a growing number of mutations associated with TD, the same approaches could be utilized in TD.

Animal models

Another approach to the study of the functional effects of a specific mutation in a gene is to use animal models. Most often, a putative disease gene is knocked out or modified in the animal model. Then, the mutant and wild type animals from the same genetic background are compared. (Some such animal knock-out studies of putative TD genes have been described above). Conventional animal models are designed to study a small number of genes, usually one or two genes at a time. If, however, TD is caused by the combined effect of multiple variant genes, multi-transgenic animals, whose genomes are modified at multiple loci, would be required. Although more challenging, generation of such multi-transgenic animals can be achieved by genome editing as well. (213, 214).

In order to model TD in animals, the following criteria should be met: 1) the gene to be studied is strongly associated with the disease; 2) the gene and the neurological component phenotypes involved in TD are relatively well conserved between humans and the animal 3) the gene is thought to have similar functions in both humans and the model animals; and 4) the disease phenotype can be experimentally characterized in the model animals by biochemical and/or behavioral approaches (215). One mouse model in

TD that meets these criteria is *HDC*. As indicated previously, a rare dominant nonsense mutation W317X in *HDC* cosegregated with all TD individuals in a two generation pedigree. (25). Subsequently, a study using *HDC* knockout mice demonstrated behavioral and molecular abnormalities caused by the loss of the HDC activity in the brain (28). The knockout and heterozygous mice showed tic-like stereotypic movements after psychostimulant administration. Also, the striatal dopaminergic pathway was dysregulated due to the HDC deficiency. Specifically, the dysregulation of dopamine receptors in the basal ganglia region of the *HDC* knockout and heterozygous mice recapitulated the dysregulation of the same types of dopamine receptors in TD patients carrying the W317X mutation.

Summary and Future Directions

TD is likely caused by a complex multigenic inheritance pattern that includes locus and allelic heterogeneity of both common and rare variants that interact with environmental factors (14, 216, 217). Despite long-standing interest in the genetic contribution to TD, the overall genetic architecture of TD remains elusive. Some of the genes identified as causal of TD are involved in neurotransmitter pathways presumed to be altered in TD, while others are novel. In this respect, the genetics of TD may resemble that of other complex neuropsychiatric disorders. Indeed, there is evidence of some overlap with subsets of similar genes involved in multiple disorders Furthermore, there also appears to be an increased rate of comorbidity between some such disorders, such as TD and ASD (27, 218). It may also be that current DSM-based psychiatric nosology does not sufficiently "carve nature at its joints," and that other classification schemes, such as

Research Domain Criteria (RDoC) (219-223), might reveal etiologically clearer groupings of disorders or patients.

International consortiums including the Tourette International Collaborative Genetics (TIC Genetics), the Tourette Syndrome Association International Consortium for Genetics (TSAICG), the European Multicenter Tics in Children Studies (EMTICS), the European Society for the Study of Tourette Syndrome (ESSTS), the Tourette Syndrome Genetics The Southern and Eastern Europe initiative (49) and sharing repositories (New Jersey Center for Tourette Syndrome Repository) (224, 225) have initiated large collaborations to collect many patient and family samples in an effort to understand the genetics of TD. Continued efforts in gene discovery from large open-access repositories are needed to find additional risk variants.

Table1. Genes disrupted in TD

Biological Pathways	Gene Name	Disruption(s) found in TD patients and References
Neurite outgrowth	*SLITRK1*	Inversion, frameshift variant, Var321 (38) Synonymous variant: 708C>T (39) 3'-UTR variant: 3383G>A (43) 3225 T>C (41) D397G (44)
Histamine	*HDC*	W317X (25) Intronic transition and synonymous variants: 426C>A, 1743G>A (55)
Serotonin	*SERT* *HTR1A* *HTR2B*	I425V and 5-HTTLPR (24) R219L (78) M63R, R449Q (79)
Glutamate	*SLC1A3/EAAT1*	E219D (88)
Synaptic signal transduction and cell-adhesion	*NLGN4/NLGN4X* *CDH2* *CNTNAP2* *DPP6*	Deletion across exon 4, 5 and 6(99) N706S, N845S (106) Intronic insertion (121) Microdeletion at exon 1 (128)
Mitochondrial functions	*IMMP2L* *MRPL3*	Translocation (141) De novo duplication (142) Translocation and cryptic deletion eliminated the exon 1 to 3 (143) Intragenic deletions (144) S75N (150)
Genes associated with other diseases	*DNAJC13(Parkinson's disease)* *OFCC1(Orofacial clefts)* *HCRTR2/OX2R(Excessive Daytime Sleepiness)*	A2057S (150) R129G and a novel variant at 5'UTR (150) P10S (161)

CHAPTER 2:

The *PNKD* gene is associated with Tourette Disorder and Tic Disorder in a multiplex family

Introduction

We are interested in pinpointing TD-associated rare sequence variant(s) with large effect size and further pinpointing potential candidate genes for TD. Fifteen of such genes have been discussed in CHAPTER 1. Particularly, we focused on identifying rare sequence variants co-segregating with individuals affected by TD and Tic Disorder in multiplex families. To achieve this goal, we performed whole exome sequencing (WES) on selected members (including both affected and unaffected individuals) of multiplex families. By using a bioinformatics tool, we were able to obtain rare segregating sequence variants and to obtain candidate gene lists under both dominant and recessive models of inheritance. In one family heavily affected by TD and Tic disorders, a novel nonsense mutation within the Paroxysmal Nonkinesigenic Dyskinesia (*PNKD*) gene was identified, which appears to be a strong candidate contributing to TD in this family.

To evaluate the impact(s) that the *PNKD* nonsense mutation might have on gene expression and cellular functions of neurons, we generated induced pluripotent stem cells (iPSCs) from selected family members and differentiated these iPSCs into neuronal cells *in vitro*. With the iPSC-derived neurons from unaffected and affected family members, we were able to measure the endogenous transcript and protein expression of the PNKD long (L) isoform and we examined the subcellular localization of the PNKD (L) protein. In addition, we demonstrated the self-oligomerization of the PNKD (L) protein monomer.

Since it has been shown that PNKD (L) protein interacts with and stabilizes RIMS1/2 proteins in mice (226), we showed that the human PNKD (L) protein interacts with human synaptic protein RIMS1α in transfected human cells. Also, human PNKD (L) and RIMS1α proteins co-localize in the transfected human iPSC-derived neurons.

Methods and Materials

Human Subjects

A three-generation multiplex family was recruited through the Tourette International Collaborative Genetics (TIC Genetics) (225) study and New Jersey Center for Tourette Syndrome (NJCTS) (224) and informed consent was provided by all research participants. This study was approved by institutional review board of Rutgers University.

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) was used to capture the targeted region for sequencing, which was performed on Illumina HiSeq2000 at RUCDR Infinite Biologics®. The variants in each individual were called by Geospiza's GeneSifter (PerkinElmer). For Sanger sequencing, the genomic region harboring the nonsense mutation was amplified by PCR. Sequencing primers (PNKD-Seq-F and PNKD-Seq-R) are shown in Table S1.

pVAAST Analysis

The pedigree Variant Annotation, Analysis and Search Tool (pVAAST) program was used to prioritize candidate genes (227). The variants in the VCF files were converted to Genome Variation Format (GVF) with the vaast_converter. Genotypes with quality scores (GQ) of less than 30 were converted to no calls. The variants were annotated using the Variant Annotation Tool (VAT). Then, annotated variants for the entire pedigree were combined into one condensed file using Variant Selection Tool (VST). Lastly, the pVAAST analysis was conducted under both dominant and recessive modes of inheritance. Single nucleotide variants and short insertion/deletions overlapping coding and splice sites were included in the analysis, which was performed allowing up to 1% prevalence of a variant in the background sample. The background sample consists of 427 genomes from the 1000 Genomes project that were sequenced using the Complete Genomics platform (228). It includes a wide variety of ethnicities, including 80 Africans, 96 Europeans, 93 Chinese and several other populations. Candidate genes were ranked based on their disease-causing probability and evidence of their segregation with the phenotype in the pedigree. The candidate gene list and detailed commands for pVAAST runs can be found in Table S3.

Generation and maintenance of induced pluripotent stem cells (iPSCs)

CD4+ T cells from peripheral blood draws were isolated using the CD4+ T cells isolation kit (Miltenyl Biotec) and subsequently stimulated with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific) for 4 days in T cell growth medium (RPMI 1640 medium + 15%FBS + 1% glutamine + 1% Pen/Strep). To reprogram T cells into iPSCs, stimulated CD4+ T cells were transduced with Sendai virus vectors carrying transgenes: Oct4, Sox2, Klf4 and c-Myc (CytoTune-iPS reprogramming kit, Thermo Fisher

Scientific). Two days after transduction, CD4+ T cells were plated on X-ray-inactivated mouse embryonic fibroblast feeder cells in T cell growth medium. Two days later, the medium was replaced with the KOSR medium (20% Knockout serum replacement, 20ng/mL bFGF, 1% GlutaMax and 1% NEAA in Knockout DMEM/F12) and iPSC colonies appeared around 9 days post-transduction. iPSC colonies were then manually picked and seeded onto plates coated with Matrigel (BD Biosciences) and cultured in mTeSR medium (Stemcell Technologies) for expansion. iPSCs were passaged weekly using Dispase (Stemcell Technologies).

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

At day 0, iPSCs were split 5:1 (passage 1) onto Matrigel coated plates using Dispase and were cultured in 1:1 mTeSR/NBM medium (NBM: Neurobasal Medium supplemented with 1% N2, 2% B27 without vitamin A, 1% Insulin Transferrin Selenium and 2mM L-Glutamine) with 500ng/ml Noggin (PeproTech) for 5 days during which medium was refreshed every 2 days. At day 5, culture medium was changed to NBM with 500ng/ml Noggin and was refreshed every two days until the cells become 90% confluent. At approximately day 10 to 12, cells were scraped off the plates manually with cell scrapers and were plated (passage 2) onto 20ug/ml laminin (Sigma)-coated 10cm dishes and were subsequently cultured in NBM medium without Noggin. NBM medium was changed every 2 days and was replaced by NPM medium (50% DMEM/F12 with GlutaMAX, 50% Neurobasal Medium, 0.5% N2, 0.5% B27 without vitamin A and 20ng/ml bFGF) when the cells became 60%-70% confluent. At around day 24, the culture dishes became 90% confluent and cells from one 10cm dish were passaged (passage 3) onto 3 T75 flasks coated with Matrigel using Accutase (Stemcell Technologies). After 3-4 days, when the

T75 flasks became confluent, the monolayer cells were plated onto 10cm dishes for RNA and protein extractions, or plated onto glass coverslips for immunocytochemistry, or cryopreserved in mFreSR (Stemcell Technologies) for storage.

To generate neurons, the neural progenitor cells were plated on 10cm culture dishes as a monolayer in NPM. NPM was then replaced with neural differentiation medium (NDM) (NDM: Neurobasal Medium with 1x B27 Supplement (Thermo Fisher Scientific) and 10ng/ml human recombinant BDNF (Peprotech). NDM was changed every 2 days until cells were collected for functional assays.

Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using the RNeasy Mini kit (QIAGEN) and concentration was measured with a NanoDrop ND-1000 Spectrophotometer. cDNA was made from 1ug total RNA using TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific). The reverse transcription reaction was conducted under the following conditions: 25°C for 10 min, 48°C for 30min, 95°C for 5 min. Real-time quantitative PCR was performed on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Primers information is shown in Table S1.

Immunocytochemistry

To perform immunocytochemistry, iPSCs were plated on plastic chamber slides (Nunc Lab-Tek) coated with Matrigel, NPCs were plated on glass coverslips coated with Matrigel and neurons were plated on glass coverslips coated with poly-L-ornithine (15ug/ml)/laminin (1ug/ml)/fibronectin (2ug/ml) mix-coated glass coverslips (Sigma). Cells were fixed with 4% paraformaldehyde for 10 min and were permeabilized with 1x

PBS solution containing 0.5% Triton X-100 and 1% BSA for 5 min. Cells were blocked in 1x PBS solution containing 1% BSA overnight at 4°C. Information of primary and secondary antibodies was shown in Table S2. Primary and secondary antibodies were both diluted in 1 x PBS solution containing 0.2% Triton X-100 and 1% BSA. To mount iPSCs, SlowFade Gold Antifade Mountant (ThermoFisher Scientific) was added onto chamber slides and glass coverslips applied. For NPCs and neurons, coverslips were mounted onto glass slides and sealed with nail polish. Images were taken using Zeiss LSM 510 META confocal microscope (Carl Zeiss).

Plasmids construction and Transfection

pcDNA4/TO expression vector was used to overexpress the PNKD (L) isoform. The human PNKD (L) isoform cDNA was cloned from PNKD Human cDNA ORF Clone (RC206179) (OriGene). By PCR, restriction enzyme sites with a FLAG tag or a Myc tag sequence were added to the PNKD (L) cDNA sequence as shown: 5'-EcoRV-FLAG-PNKD-XhoI-3' and 5'-EcoRV-PNKD-Myc-Xhol-3'. After the PCR products and pcDNA4/TO were double-digested by EcoRV and XhoI, ligation was facilitated by T4 DNA ligase (New England Biolabs). To express human RIMS1α in cells, RIMS1 transcript variant 1, Human cDNA ORF Clone (RC213013) (OriGene), was used. We also cloned GFP into the pcDNA4/TO vector and used GFP expression to monitor transfection efficiency.

To transfect human 293FT cells (Thermo Fisher Scientific), ~$6x10^6$ cells were plated on a 10cm dish the day before transfection. On the day of transfection, 10ug DNA plasmid was mixed with 30ul 1mg/ml PEI solution (Sigma) and 300ul Opti-MEM reduced serum

medium. The mixture was incubated at room temperature for 30 min before being added into each dish. Two days after transfection, cells were collected for RNA extraction or lysed in protein lysis buffer.

To transfect neurons, cells were plated onto poly-L-ornithine/laminin/fibronectin-coated 12mm coverslips in 24 well plates. When neurons attached well to the coverslips, plasmid DNA, Lipofectamine 2000 transfection reagent and Opti-MEM reduced serum medium were mixed and incubated at room temperature for 30 min. The mix was then added to the neuron cultures and after 2 d, neurons were fixed for immunocytochemistry.

Western blot and co-immunoprecipitation

Cells were lysed in RIPA lysis buffer (229) for western blot analysis or in RIPA IP lysis buffer for co-immunoprecipitation (Co-IP). Anti-FLAG M2 Magnetic beads (Sigma) were used to pull down FLAG-tagged proteins. The whole cell lysates or eluates of the anti-FLAG M2 beads were resolved on NuPAGE 10% Bis-Tris or 3-8% Tris-acetate mini gels (Thermo Fisher Scientific).

Calcium imaging

Fluorescent calcium indicator dye Fluo4 AM and cell viability dye calcein red-orange AM (Thermo Fisher Scientific) were used for calcium imaging experiments. Cells were cultured on 12 mm coverslips and the dyes were dissolved in DMSO and added to the cell culture medium to achieve a final concentration of 2 µM. Cells were incubated for 20 min with both dyes. Free dye was washed out with pre-warmed HBSS (Thermo Fisher Scientific). Cells then were incubated for 30 min in DMEM medium (Thermo Fisher Scientific) for de-esterification of the Fluo4 AM and calcein red-orange dyes. During the

imaging session, the coverslip was placed in the Quick Release Imaging Chamber (Warner Instruments) and the medium was replaced with pre-warmed HBSS. 100mM KCl Tyrode's solution was perfused to depolarize the neuronal cultures. Images were acquired using a Zyla sCMOS camera (Andor) mounted on an Olympus IX71 using a 20X 0.75 NA objective. Images were taken at 0.25 sec/0.5sec intervals for 1 min. Images for calcium imaging were processed and intensity was quantified using the NIH ImageJ software.

Results

Clinical evaluations of the TD multiplex family

In this white 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. Of the five siblings, four (including the proband) met DSM-IV-TR (230) criteria for TD and one sibling met criteria for a chronic tic disorder not specified in the DSM, namely chronic tic disorder-combined subtype (225), defined as a history of a single motor (rather than multiple motor) and at least one vocal tic, with onset prior to age 18 and persistence for at least a year. The sibship consists of an older sister (5001), a set of MZ twin females (5002 and 5003) which includes the proband, and a younger set of DZ twins- one male (5005) and one female (5004). The proband's MZ twin was not sequenced. The father (4001) was also diagnosed with chronic tic disorder- combined subtype. The paternal grandmother (3002) was diagnosed with a chronic motor tic disorder and paternal grandfather (3001) was diagnosed with a tic disorder, not otherwise specified (onset age 66), consisting of a recent-onset recurrent motor tic of less than a

year's duration. Both paternal grandparents and the three oldest siblings were diagnosed with OCD. The father was diagnosed with subclinical OCD (consisting of obsessions and compulsions that were non-impairing and only mildly distressing (231) while the mother (4002) was diagnosed with OC symptoms (defined as non-interfering, non-distressing obsessions and compulsions) (232). The youngest sibling was also diagnosed with subclinical OCD and probable autism spectrum disorder.

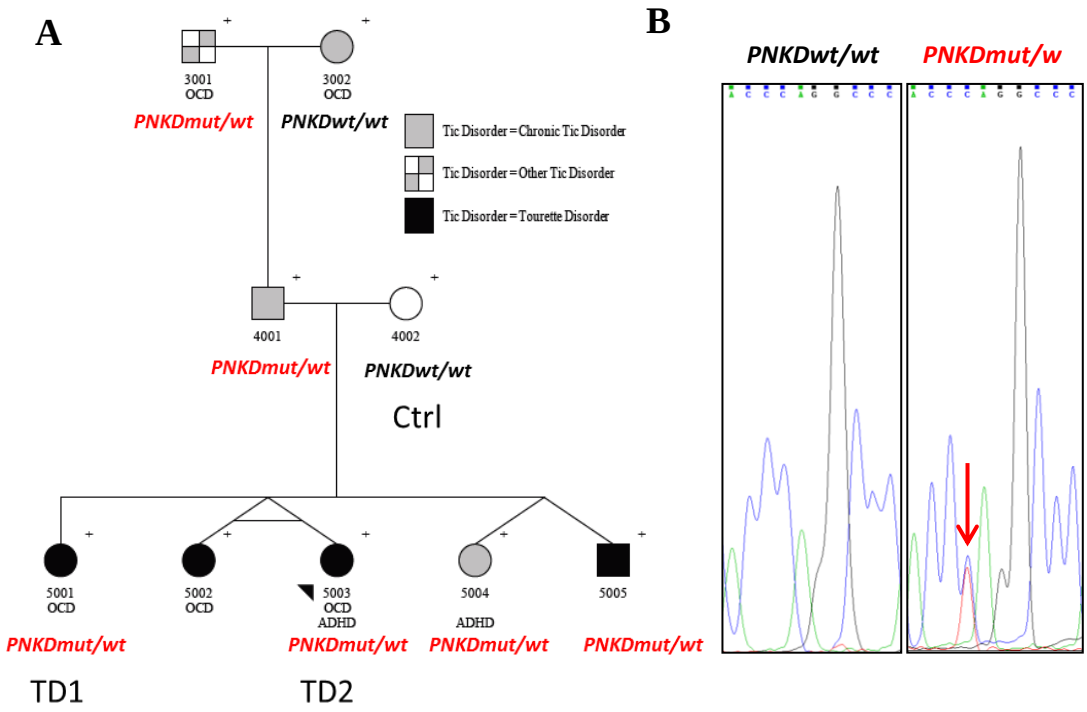


Figure 1. A rare heterozygous nonsense mutation (C to T transition) was identified in a TD multiplex family by whole exome sequencing.

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 validated by Sanger sequencing in subjects 3001, 4001, 5001, 5003, 5004 and 5005.

A rare nonsense mutation in PNKD is associated with TD and Tic disorder in a multiplex family

We performed WES on individual 4001, 4002, 5001, 5003 (proband), 5004 and 5005. We generated more than 60 million 100bp paired-end reads for each sample with an average coverage of 53x (Table S4). About 200,000 variants were identified in each individual. Using 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. In addition to the population information, pVAAST also directly incorporates the variant inheritance pattern within the family into the likelihood ratio test for gene prioritization (227). We performed pVAAST analysis under both dominant and recessive modes of inheritance. A total of 145 and 169 genes were scored under each mode, respectively. Under the dominant mode of inheritance, *PNKD* has the highest score. All 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 (Sanger seuquencing results of 4002 and 5003 are shown in Figure 1B), thus validating the complete segregation of this variant with individuals affected by TD or Tic disorders in this family. As reported by the Exome Aggregation Consortium (ExAC) database (229), this variant was only found in 4 alleles from 120802 total alleles, giving an allele frequency of $3.3x10^{-5}$.

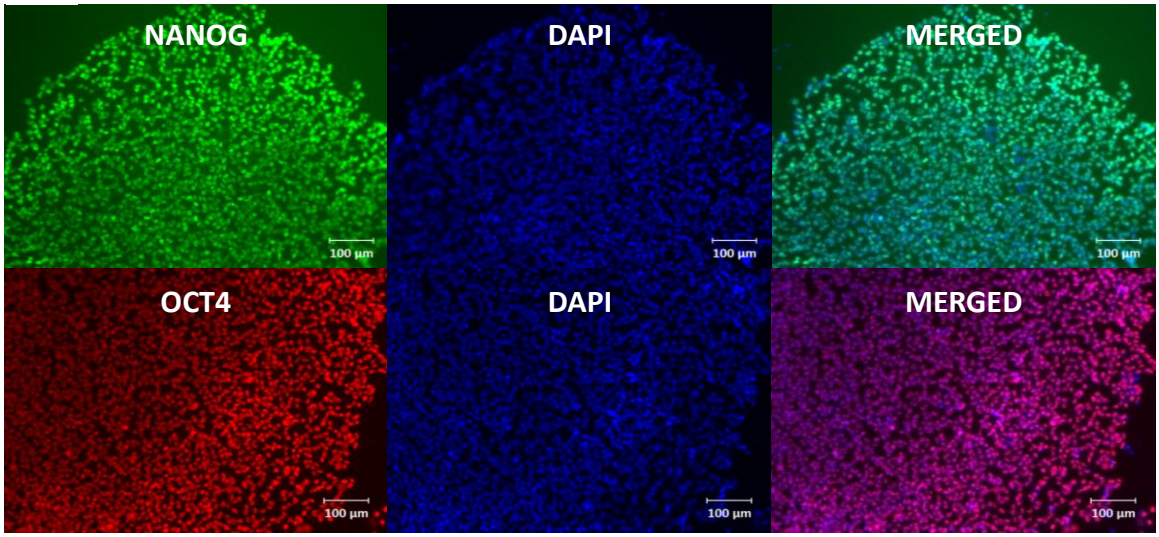
Mutations in *PNKD* have been associated with familial paroxysmal nonkinesigenic dyskinesia (PNKD), a neurological movement disorder causing episodic involuntary movement attacks (233-235). Because of the deleterious nature of the nonsense mutation, the segregation pattern of the variant within the pedigree, and the neurological movement disorder associated with *PNKD*, we selected *PNKD* as our top candidate gene for further investigation.

Neuronal cells were generated from the human subjects through iPSC intermediates

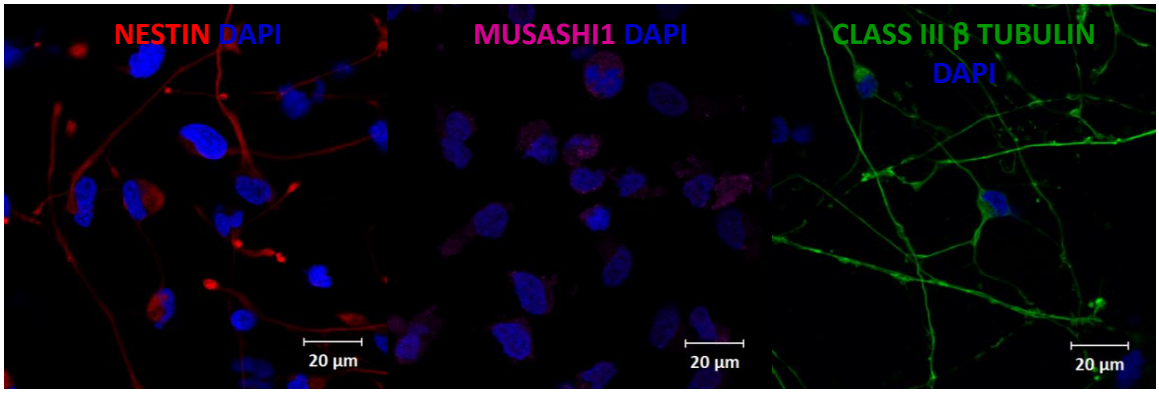
To study the functional impact of the identified variants, we generated neuronal cells from three members in the pedigree (4002-Ctrl; 5001-TD1; 5003 (proband)-TD2) through iPSC intermediates. The iPSCs were further differentiated into NPCs. For generation of D30 neurons, the NPCs were cultured in NDM for 30 days. The iPSC colonies expressed pluripotency markers: Oct4 and Nanog (Figure 2A). As shown in Figure 2B, the iPSC-derived NPCs were stained positive for neural progenitor markers Nestin and Musashi1 as well as neuronal marker Class III β-tubulin by immunocytochemistry. The D30 neurons expressed neuronal markers MAP2 and Class III β-tubulin (Figure 2C). By western blot analysis, synaptic vesicle protein synapsin I (SYN1), which regulates neurotransmitter release, was also detected in D30 neurons (Figure 2C). To characterize the type of neurons in the D30 cultures, we stained for glutamatergic neuron marker VGLUT1, GABAergic neuron marker VGAT and glia cell marker GFAP (Figure 2D). Immunocytochemistry showed that there were subpopulations of neurons stained positive for VGLUT1, VGAT or GFAP in the D30 neuron culture, indicating heterogeneity. We also examined the calcium influx of the D30 neurons from the Ctrl, TD1 and TD2 subjects. All three lines of D30 neurons exhibited spontaneous

calcium influx and potassium-stimulated calcium influx when depolarized with the 100mM KCl Tyrode's solution (Figure 2E), further indicating that the iPSC-derived D30 neurons have appropriate neuronal activities.

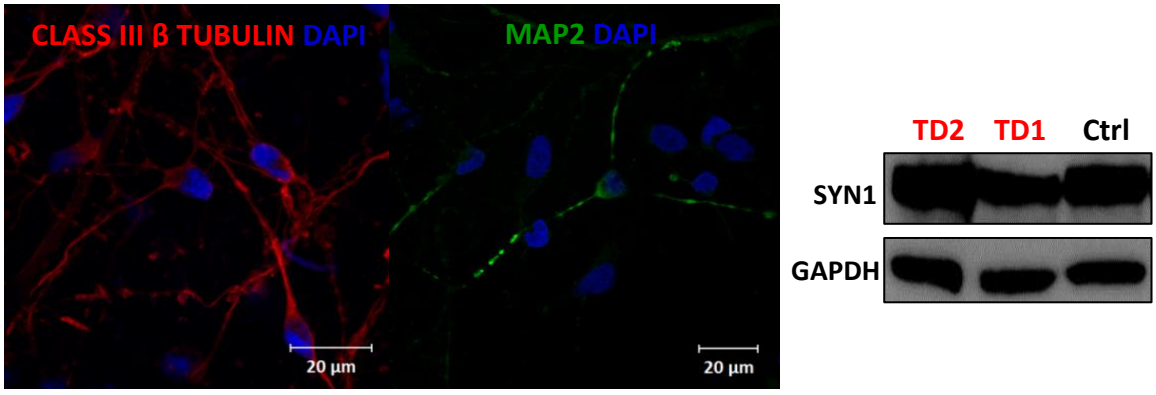
A



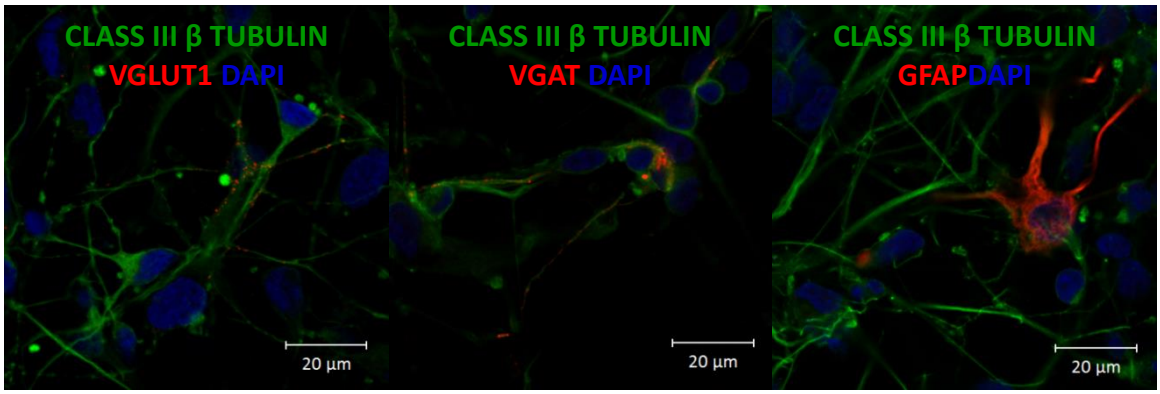
B



C



D


CLASS III β TUBULIN
VGLUT1 DAPI
20 µm
CLASS III β TUBULIN
VGAT DAPI
20 µm
CLASS III β TUBULIN
GFAPDAPI
20 µm

E

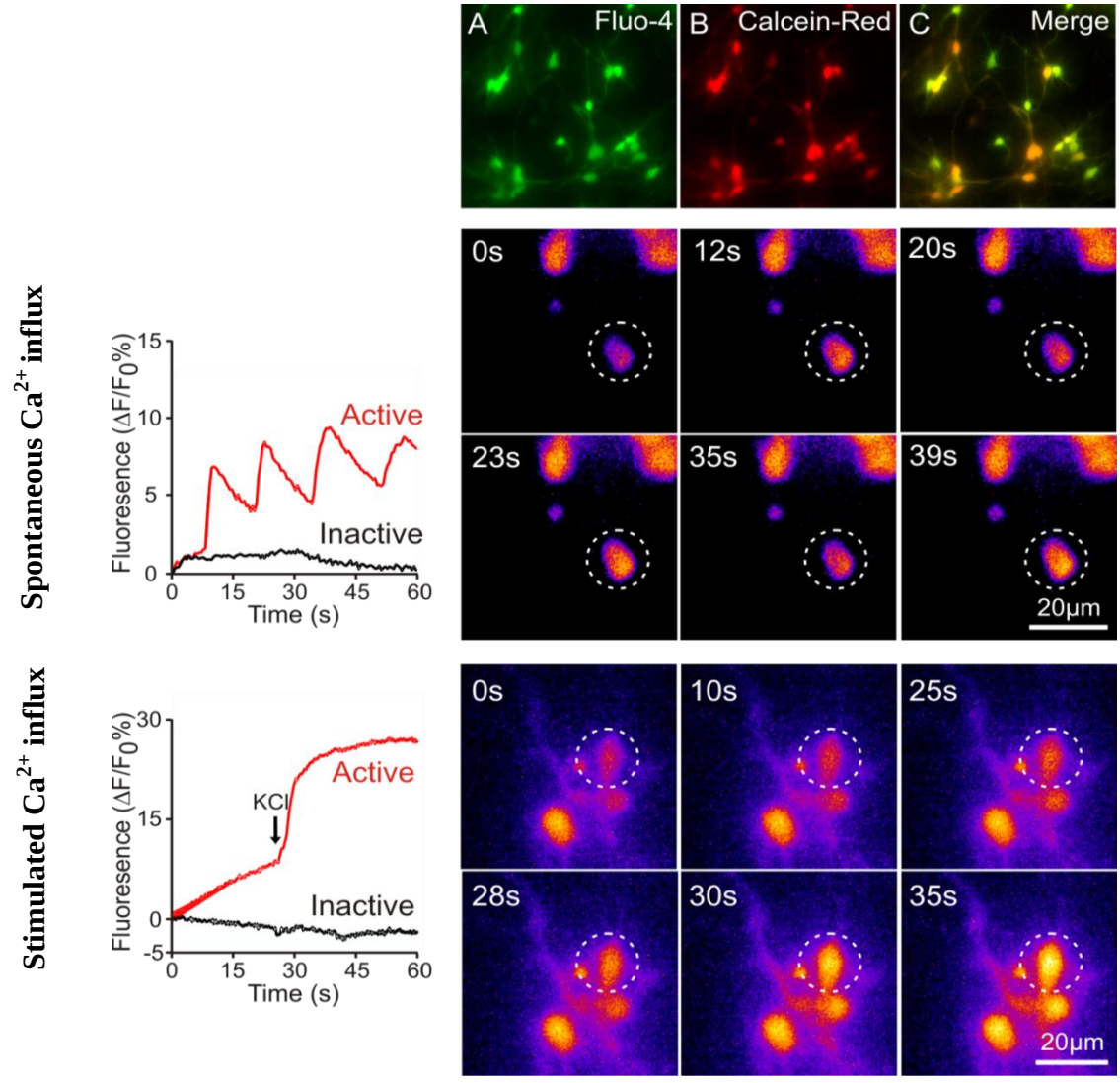

A Fluo-4
B Calcein-Red
C Merge
Spontaneous Ca2+ influx
Fluoresence (ΔF/F0%)
Active
Inactive
Time (s)
0s
12s
20s
23s
35s
39s
20µm
Stimulated Ca2+ influx
KCl
0s
10s
25s
28s
30s
35s
20µm

Figure 2 Functional neurons were generated from one control and two TD-affected subjects.

A. The iPSC colonies expressed pluripotency markers: Nanog and Oct4.

B. Neural progenitor cells (NPCs) derived from iPSCs were stained positive for NPCs markers: Nestin and Musashi1. Also, NPCs expressed neuronal marker: Class III β-tubulin.

C. Day 30 neurons expressed neuronal markers MAP2 and Class III β-tubulin as well as synaptic vesicle protein synpasin I (SYN1).

D. Glutamatergic neuron marker VGLUT1, GABAergic neuron marker VGAT or glial cell marker GFAP was detected in subpopulations of the iPSC-derived neurons.

E. Calcium imaging showed that the iPSC-derived D30 neurons exhibit spontaneous and potassium-stimulated calcium influx.

The human PNKD (L) isoform is enriched in iPSC-derived neurons and is reduced in TD patients' neurons, suggesting nonsense-mediated decay

In human, the *PNKD* gene encodes multiple protein isoforms. There are 12 exons within the human *PNKD* locus. 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 locates at the exon 6, therefore affecting both the *PNKD* (L) and *PNKD* (M) transcripts. Genotyping-Tissue Expression (GTEx) project (http://www.gtexportal.org/home/) indicates that the *PNKD* (L) transcript is enriched in the human brain where the *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. In order to characterize the *PNKD* gene expression, all *PNKD* transcripts were measured in lymphoblastoid cell lines (224) (LCL), iPSC, NPC and D30 neurons from the same subject by RT-qPCR. We designed primers that distinguished different transcripts and the

positions of each primer are shown in Table S1. In Figure 3, we showed the *PNKD* transcript expression in LCL, iPSC, NPC and D30 neurons from the three subjects. The *PNKD* (L) transcript was not detected in LCL and iPSC. The expression of the *PNKD* (L) transcript appeared at relatively low-levels in NPC whereas the expression increased greatly in the D30 neurons (Figure 3A). However, expression of the *PNKD* (L) transcript significantly increased from iPSC to NPC and from NPC to D30 neurons in the Ctrl subject (Figure 3B), indicating *PNKD* (L) is enriched in human neurons. The *PNKD* (S) transcript was detected in all the cell types with relatively higher expression and the *PNKD* (S) transcript expression increased significantly with neuronal differentiation of iPSC (Figure 3B). The p-value was 0.0018 from iPSC to NPC and was 0.012 from NPC to D30 neurons respectively. The *PNKD* (M) transcript is expressed in all the cell types examined but its expression was relatively low in neuronal cells (Figure 3C). By western blot, the PNKD (L) protein expression was also examined 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 but not the in LCL or iPSC (Figure 3E).

As we have shown in Figure 3A and 3C, of the two transcripts affected by the nonsense mutation, only the *PNKD* (L) isoform is enriched in human neuronal cells. Nonsense mutations could produce loss-of-function, gain-of-function or dominant negative phenotypes. To investigate what effects the nonsense mutation might have on the PNKD (L) isoform, we measured the transcript levels and protein expression in the D30 neurons. To account for the technical variabilities of differentiation, 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 *PNKD* (L) transcript level expressed in the Ctrl subject's neurons, indicating the possibility of nonsense-mediated decay (NMD) (236) (Figure 3D). Consistent with the transcript expression, the PNKD (L) protein was also reduced in the TD patients' neurons compared to the Ctrl (Figure 3E).

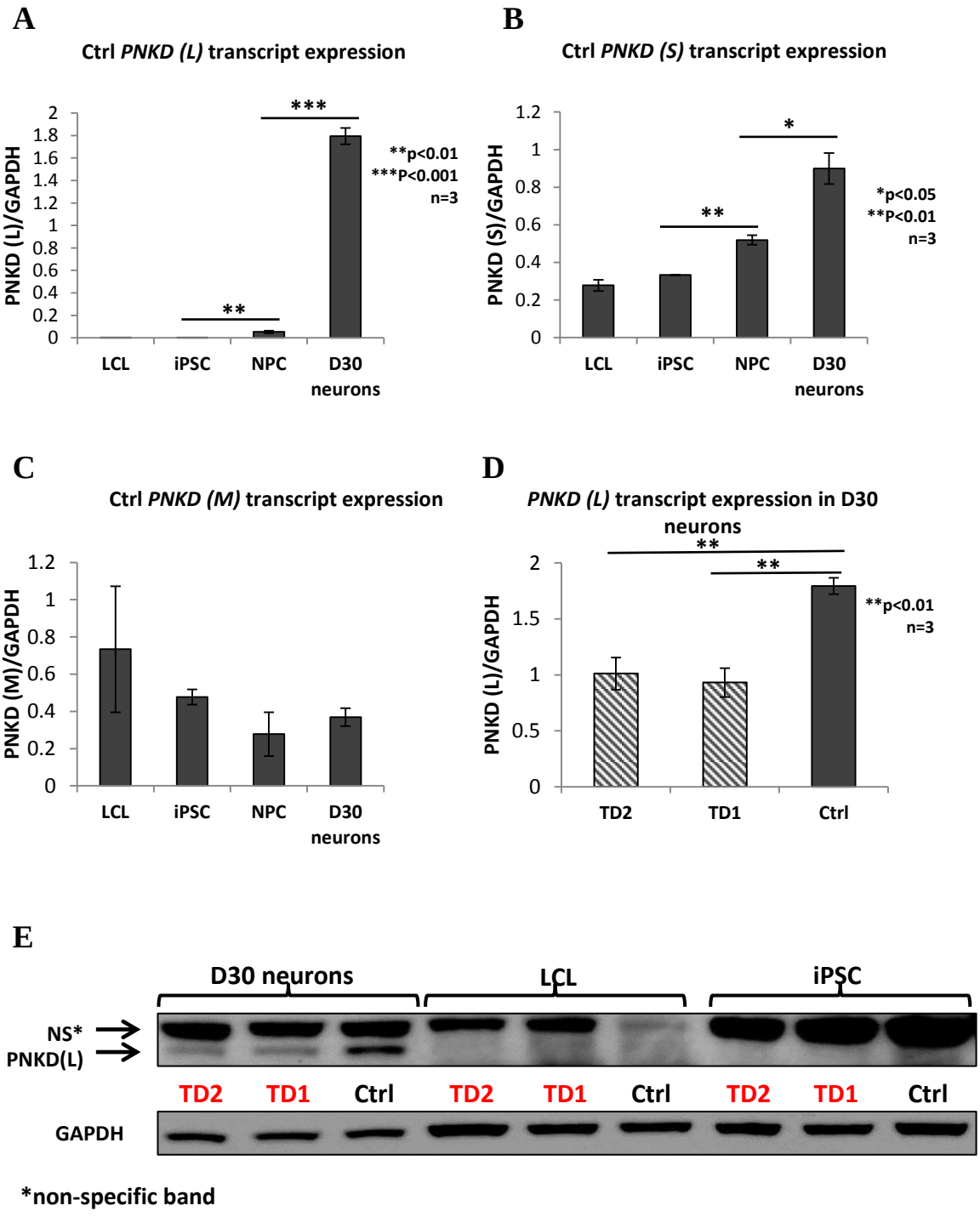


Figure 3 Examine *PNKD* expression in LCL, NPC, iPSC and iPSC-derived D30 neurons by RT-qPCR.

A. *PNKD* (L) transcript expression increased from iPSC to NPC and from NPC to D30 neurons in the Ctrl subject, indicating that *PNKD* (L) was enriched in neuronal cells;

B. *PNKD* (S) transcript is expressed in all the cell types of all three subjects. *PNKD* (S) transcript level increased during neural differentiation

C. *PNKD* (M) transcript is expressed low in iPSCs, NPCs and iPSC-derived neurons.

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

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 Ctrl subject.

The human PNKD (L) protein self-oligomerizes and interacts with RIMS1α protein

To speculate how *PNKD* nonsense mutation might affect the protein function of PNKD (L) protein, we would like to test whether PNKD (L) proteins function as a monomer or oligomers. A previous study showed that the human PNKD (L) protein interacts with itself (237). We transfected the 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 pulled down by anti-FLAG M2 magnetic beads. The PNKD (L)-Myc was also pulled down only in the presence of FLAG-PNKD (L), indicating the self-oligomerization of the PNKD (L) protein (Figure 4A). Therefore, reduction of PNKD (L) protein expression to half in cells might lead to less than half of the functional PNKD (L) oligomers existed in cells.

It has also been reported that the PNKD (L) protein interacts with the presynaptic proteins RIMS1/2 in mouse cortex (226). Based on the mouse and human protein sequence conservation, the PNKD (L) and RIMS1α proteins likely interact in human. We transfected the 293FT cells with plasmids expressing: GFP, RIMS1α-FLAG, PNKD (L)-Myc or RIMS1α-FLAG+PNKD (L)-Myc. Co-IP was performed using the whole cell lysates and, as shown in Figure 4B, the PNKD (L)-Myc was pulled down only in the presence of RIMS1α-FLAG, suggesting their interaction.

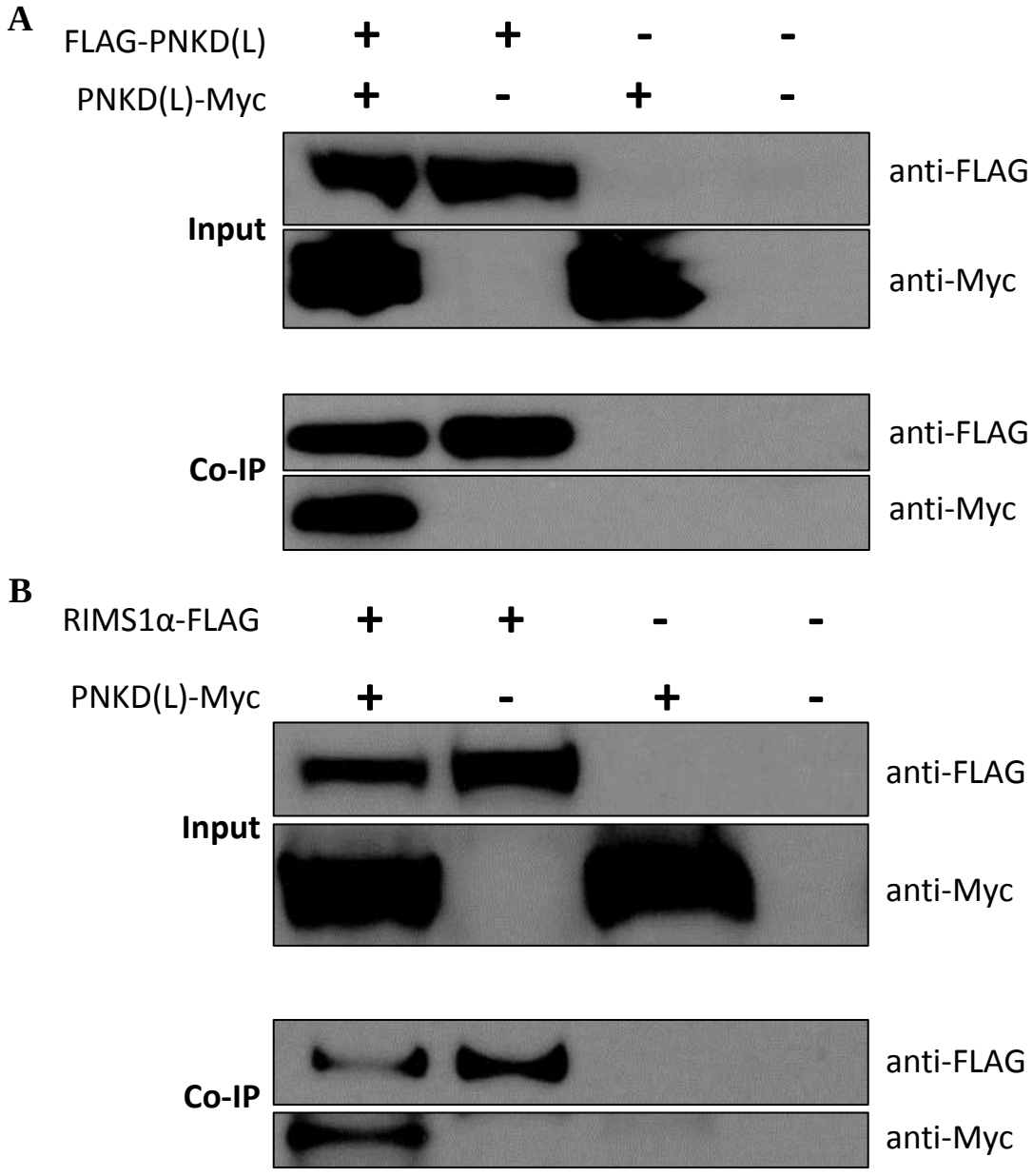


Figure 4 The human PNKD (L) protein oligomerizes with itself and interacts with the RIMS1α protein.

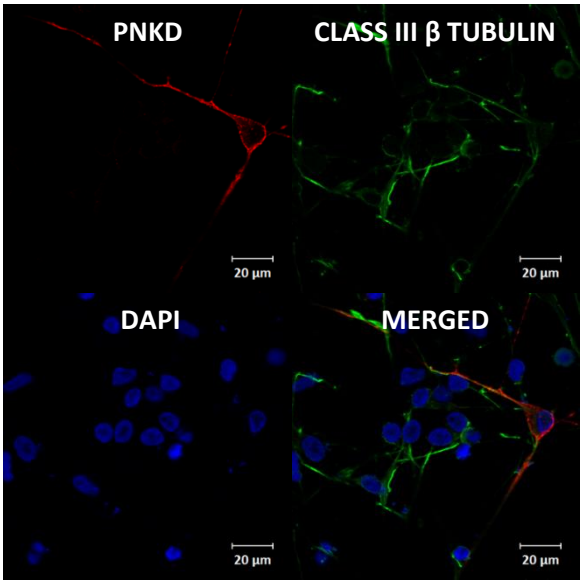
A. 239FT cells overexpressing GFP, FLAG-PNKD(L), PNKD(L)-Myc or FLAG-PNKD(L) + PNKD(L)-Myc were lysed for Co-IP. The PNKD(L)-Myc was pulled down by the anti-FLAG magnetic beads only in the presence of the FLAG-PNKD(L).

B. 239FT cells overexpressing GFP, RIMS1α-FLAG, PNKD(L)-Myc or RIMS1α-FLAG+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 RIMS1α-FLAG.

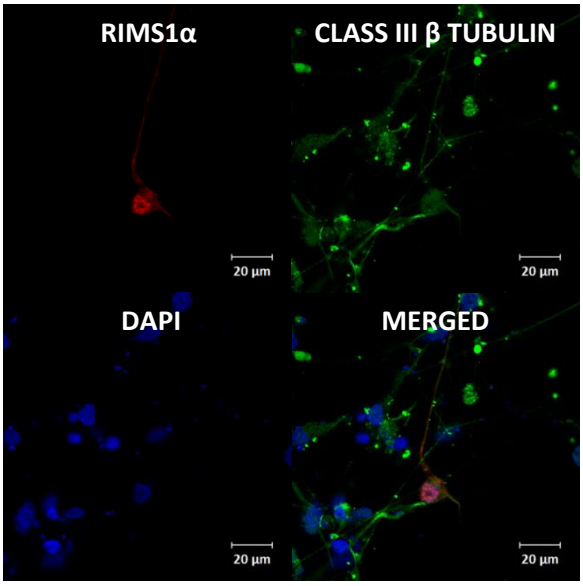
PNKD (L) isoform protein is plasma membrane-associated and co-localizes with RIMS1α and in iPSC-derived neurons

Endogenous PNKD protein has been detected in presynaptic terminals, postsynaptic regions and dendrites of cortical neurons from adult mice, supporting that PNKD and RIMS protein might function in the same pathway at the synapse(226). The subcellular localization of the human PNKD (L) protein has been examined by overexpression in transformed human cell lines (234, 237, 238). However, the subcellular localization of PNKD (L) in differentiating human neurons has not been shown. Due to the low endogenous expression of PNKD (L), we overexpressed the PNKD (L) with the FLAG tag at its N-terminus in iPSC-derived neurons by transfection. We also transfected the iPSC-derived neurons with plasmid expressing the human RIMS1α protein with the Myc tag at its C-terminus. Immunocytochemistry showed that PNKD (L) protein was enriched in the plasma membrane and neurites whereas and the expression was weaker in the cell body (Figure 5A). The RIMS1α protein could be detected in both cell body and neurites in the iPSC-derived neurons (Figure 5B), which was consistent with the endogenous RIMS1 expression pattern in iPSC-derived neurons (Figure S1). Co-transfection of the PNKD (L) and the RIMS1α showed that these two proteins co-localized in the cell body and neurites (Figure 5C).

A


PNKD
CLASS III β TUBULIN
20 µm
20 µm
DAPI
MERGED
20 µm
20 µm

B


RIMS1α
CLASS III β TUBULIN
20 µm
20 µm
DAPI
MERGED
20 µm
20 µm

C

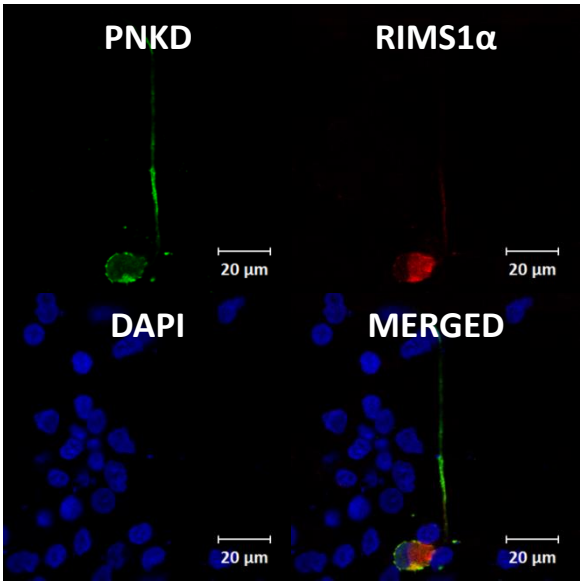


Figure 5 PNKD (L) protein is plasma membrane-associated and co-localizes with the RIMS1α protein in the iPSC-derived neurons.

A. iPSC-derived neurons were transfected with plasmid expressing the FLAG-PNKD(L) protein. anti-FLAG antibody was used to detect the PNKD (L) protein whose expression was enriched in the plasma membrane and the neurites.

B. RIMS1α-Myc was overexpressed in the iPSC-derived neurons by transfection. The anti-Myc antibody was used to detect the RIMS1α protein which is localized in the cell body and neurites of the iPSC-derived neurons.

C. Plasmids expressing the FLAG-PNKD(L) and RIMS1α-Myc were co-transfected into the iPSC-derived neurons. anti-PNKD(L)(C-terminus) and anti-Myc antibodies were used to detect the PNKD (L) and the RIMS1α proteins which were co-localized in the cell body and the neurites of the iPSC-derived neurons.

RIMS1α transcript is enriched in iPSC-derived neurons and is not affected by the reduction of the PNKD (L) isoform.

GTEx indicates that the *RIMS1α* transcript is exclusively expressed in the brain. We measured the level of the *RIMS1α* transcript in the LCL, iPSC, NPC and the iPSC-derived D30 neurons of subjects Ctrl, TD1 and TD2 by RT-qPCR. As shown in Figure 6A, the *RIMS1α* transcript is barely detected in LCLs and has extremely low expression in iPSC and NPC. As expected, the *RIMS1α* transcript is highly enriched in iPSC-derived

D30 neurons. We have shown that PNKD (L) and the RIMS1α protein interacted in 293FT cells and co-localized in iPSC-derived neurons. Additionally, it has been shown that PNKD (L) stabilizes RIMS1/2 proteins in mouse (226). Therefore, we measured the endogenous RIMS1 protein in the iPSC-derived D30 neurons from three independent differentiation processes. As shown in Figure 6C, the RIMS1 protein was 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 RIMS1α transcript in human neurons, we specifically measured the *RIMS1α* transcript expression in iPSC-derived D30 neurons from TD1 and TD2 (Figure 6B). There is no significant change of the RIMS1α transcript expression in control and TD neurons, further justifying the previous finding that PNKD (L) protein affects the stability of the RIMS1 proteins.

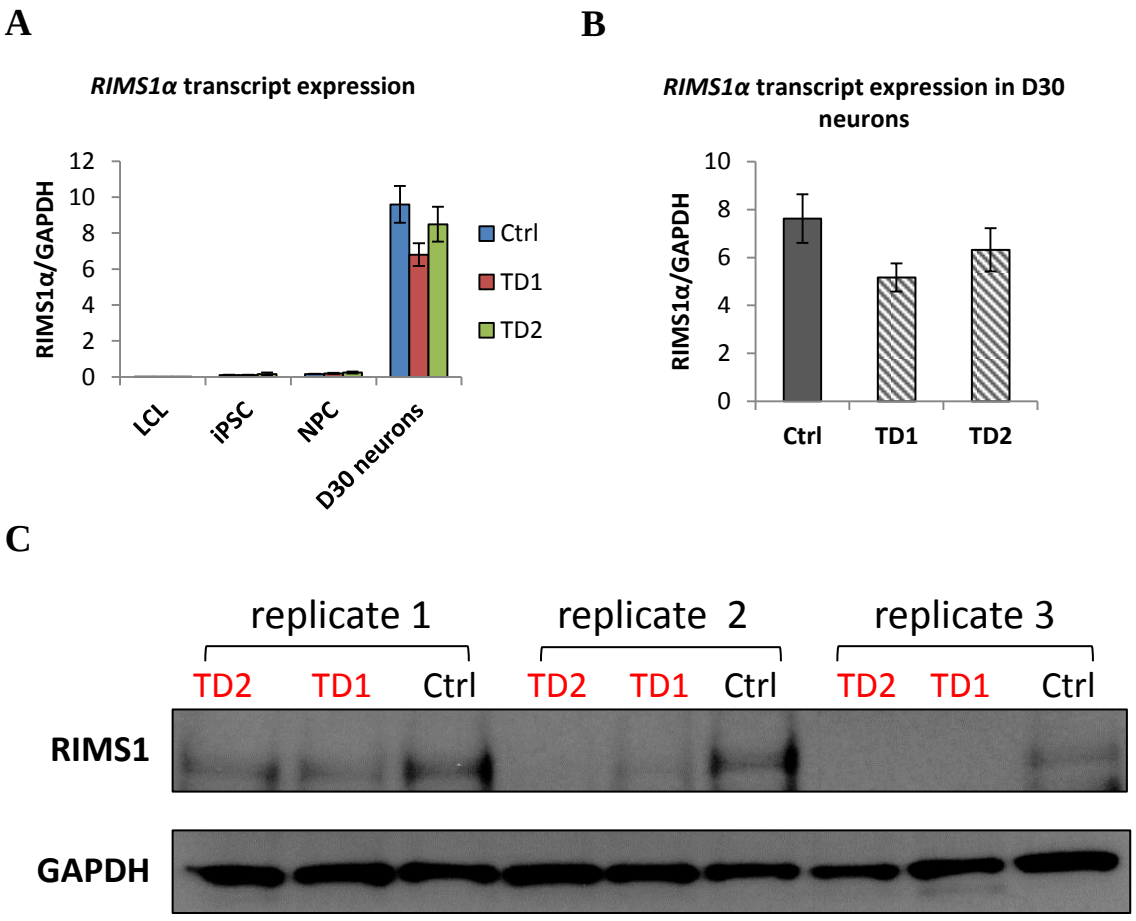


Figure 6 Examine the endogenous *RIMS1α* expression in Ctrl subject's and TD patients' cells.

A. *RIMS1α* transcript was not expressed in LCL, was expressed low in iPSC and NPC and was enriched in iPSC-derived D30 neurons.

B. RT-qPCR was conducted to measure the *RIMS1α* transcript in iPSC-derived D30 neurons of subjects Ctrl, TD1 and TD2. D30 neurons from three independent differentiations were collected. When comparing the expressions of the RIMS1α transcript of Ctrl and TD neurons, there was no significant difference.

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

Discussion

In this study, we identified a segregating nonsense mutation at the *PNKD* gene in a multiplex pedigree and proposed that *PNKD* is a candidate gene for TD and Tic disorders in this family. Furthermore, we characterized the nonsense mutation in iPSC-derived neurons and demonstrated the self-oligomerization of the PNKD (L) protein and its interaction with the synaptic active zone protein RIMS1α. In addition to what we have shown above, we also examined the spatial and temporal expression of the *PNKD* gene by analyzing the public available human brain transcriptomic data. Also, we discussed the possibility of a dominant negative effect of the *PNKD* nonsense mutation in cells. Lastly, we speculated the role that the *PNKD* nonsense mutation might play in TD etiology by discussing *PNKD* mutations identified in other neurological disorders.

Expression of the PNKD and RIMS1 in the human brain

The BrainSpan (http://www.brainspan.org/) developmental transcriptome data shows that *PNKD* gene expression increases with human brain development (Figure S2A). The gene

expression level was represented by Reads Per Kilobase of transcript per Million mapped reads (RPKM) as shown at the y-axis in Figure S2. We also examined *PNKD* expression in developing brains by isoform. Figure S2B 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 S2C 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 is much lower and not coordinated with the *PNKD* (S) transcript at all ages. Unfortunately, there is no exon that is specific to the *PNKD* (L) transcript. Figure S2D 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 S3, not every human subject could provide all 26 samples. Still, for this particular exon shared by the *PNKD* (L) and the *PNKD* (M) transcripts, we listed the brain structure which has the highest expression in each human subject (Table S5). Interestingly, brain structures implicated in the neuropathogenesis of TD including striatum (STR) (7, 8, 21, 87, 239-246), primary motor cortex (area M1, area 4) (M1C) (247-255), primary somatosensory cortex (area S1, areas 3,1,2) (S1C) (8, 248, 253, 256, 257) and mediodorsal nucleus of thalamus (MD) (258) have the highest exon expression at certain stages during brain development, suggesting that disruption of the PNKD (L) protein function might affect neural activities in these brain structures. For the *RIMS1* gene, we also plotted the *RIMS1* gene expression using gene-level reads (Figure S3A) and *RIMS1α* expression using exon-level reads (Figure S3B) using the RNA-Seq data from the BrainSpan. Both the *RIMS1*

gene total expression and the *RIMS1α* expression are enriched in the cerebellar cortex (CBC) region in some of the subjects. We also looked at the *PNKD* and *RIMS1* expressions at the Human Brain Transcriptome database (http://hbatlas.org/) where gene expression was measured by microarray at exon level using over 1340 samples from 57 human brains. The *PNKD* expression was shown in Figure S4A where an increasing trend was seen along human brain maturation. Also, *PNKD* might have higher expression in the striatum than other brain regions. For *RIMS1*, an increase of expression was seen from period 1 (4 ≤ Age < 8 PCW) to period 6 (19 ≤ Age <38 PCW) (Figure S4B). Then the RIMS1 expression reached a plateau for brain regions of mediodorsal nucleus of the thalamus (MD), striatum (STR), amygdala (259), hippocampus (260) and 11 areas of neocortex (NCX) except for the cerebellar cortex (CBC). The CBC has higher expression of *RIMS1* from period 8 (birth ≤ Age <12 postnatal months) to period 15 (60 years +) than other brain regions. We were able to model the expression features of different *PNKD* transcript isoforms during early brain development by measuring the expression of *PNKD* (L), the *PNKD* (M) and the *PNKD* (S) transcripts in human iPSC, NPCs and D30 neurons (Figure 3). In our study, 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 most pronounced.

Effect of the PNKD nonsense mutation

We observed reduced PNKD (L) transcript and protein levels in the mutant iPSC-derived D30 neurons, indicative of nonsense-mediated decay. To eliminate the possibility of a dominant-negative mechanism for reduction of PNKD oligomer activity due to a putative N-terminal PNKD protein fragment binding full length fragment(s), we investigated

whether the nonsense-mediated decay was complete by examining the existence of the *PNKD* (L) transcript harboring the nonsense variant in iPSC-derived neurons. We reverse-transcribed total RNA extracted from the control and the mutant 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 not genomic DNA could be amplified. The PCR product was purified from the agarose gel and Sanger sequenced. As shown in Figure S5A, the *PNKD* (L) transcript carrying the nonsense variant was detected, indicating that the nonsense-mediated decay does not eliminate all the mutant *PNKD* (L) transcripts in the TD1 and the TD2 neurons. However, it was difficult to determine whether the mutant *PNKD* (L) transcript was translated into truncated protein due to the extremely low endogenous levels of the mutant *PNKD* (L) transcript, which probably resulted from nonsense-mediated decay in iPSC-derived neurons. 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 S5B the truncated PNKD protein lost the ability to localize to the plasma membrane and did not co-localize with the wild type PNKD (L) protein, mitigating against the possibility of a dominant negative effect of the truncated protein. Therefore, we hypothesize that the nonsense mutation identified in *PNKD* is simply a loss-of-function mutation and that the phenotype is likely due to haploinsufficiency at critical times in development. We have shown that the *PNKD* (L) transcript expression increases along neuronal maturation and, thus, expression of the mutant *PNKD* (L) transcript might increase as well. Therefore, in

neurons that express the *PNKD* (L) transcript at a much higher level, testing for a truncated PNKD (L) protein and its possible neuron-specific interactions will be useful. The putative truncated PNKD (L) protein shares the first 79 amino acids of their protein sequence with the PNKD (S) protein. The first 75 amino acids of the PNKD (S) protein have been reported to modulate the MEK/ERK signaling (261, 262) or be involved in MLC2/FAK/Akt signaling (263) in the cytoplasm of human cancer cells. Investigating whether the PNKD (S) protein is involved in the MAPK signaling in human neurons will help us to predict the role that the truncated PNKD (L) protein might have due to their sequence similarity. If the truncated PNKD (L) got translated in neurons where the *PNKD* (L) transcript were expressed at much higher level, and affected the MEK/ERK and MLC2/FAK/Akt signaling, the nonsense mutation might be gain-of-function. In addition, the RIMS1α interacting sequence of PNKD (L) has not been identified. In neurons where the truncated PNKD (L) protein got expressed, whether the mis-localized truncated protein would interfere with normal RIMS1α protein trafficking to the synapses will also redefine the effect of the *PNKD* nonsense mutation.

The PNKD gene and disease

The *PNKD* gene is known for its association with the movement disorder paroxysmal nonkinesigenic dyskinesia (PNKD) which is a neurological disorder causing episodic involuntary movement attacks. As shown in Table 2, recurrent missense mutations A7V (234, 264-271) and A9V (234, 265-267, 272-274) were associated with the PNKD disorder in unrelated familial cases from different ethnic groups. Another heterozygous mutation A33P (275) was detected in one PNKD patient who also suffered cerebellar ataxia. In a study investigating mitochondrial disease related genes, a missense mutation

G89R in the *PNKD* gene was identified in a boy affected by intermittent ataxia, diarrhea, exercise intolerance and speech articulation problems (276). 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 (277). Among all the disease-associated *PNKD* mutations, mutations affecting the PNKD (L) and PNKD (S) isoforms including A7V, A9V, A33P were associated with the PNKD disorder in an autosomal dominant manner 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* (278). Our TD/Tic disorder-associated *PNKD* nonsense mutation affects the PNKD (L) and the PNKD (M) isoforms and leads to reduction of the PNKD (L) in the TD patients' neurons. Similar to our nonsense mutation, the P341Rfs*2 mutation generated a premature stop codon at the C-terminus of the PNKD (L). However, the P341Rfs*2 mutation was originally identified in the *PNKD* (L) transcript in the fibroblasts of the subjects (277), suggesting the nonsense-mediated decay did not occur or was incomplete and a truncated protein might exist in patients' neurons. Longer than the truncated protein caused by our nonsense mutation, the truncated protein due to the P341Rfs*2 mutation presumably preserves the region that was required for PNKD (L) self-oligomerization (237) and therefore it might interfere with the wild type PNKD (L) protein in neurons.

Interestingly, previous studies showed that the frequency of migraine headache was higher in TD patients than the general population (279, 280), suggesting overlaps of the etiology of these two disorders. Also, association between paroxysmal kinesigenic dyskinesia (PKD: an involuntary movement different from but similar to PNKD disorder) and TD was reported. A 34-year-old TD patient who was diagnosed with TD at age of 6 started to suffer PKD-like attacks (281). Similarly, a suspected TD patient developed PKD after he started to have motor tics and OCD symptoms (282). Also, in a study conducting clinical evaluation of 121 idiopathic PKD patients, TD/Tic disorders were reported to be present in some sporadic PKD patients (283). Taken together, disorders like PNKD, PKD, familial hemiplegic migraine, TD and Tic disorders, whose diagnostic criteria and clinical phenotypes are different, may be caused by distinct mutations disrupting the same gene or be comorbid disorders in the same patient. In other words, the same gene or a functionally associated gene could be involved in multiple disorders. Reasons for this pleiotropic effect of a gene are manifold: 1) clinical phenotypes of different disorders may not be well defined (284). In DSM-5, paroxysmal dyskinesia is under differential diagnosis of Tic disorders (285). It is possible that these two disorders are biologically correlated to some extent. 2) a pleiotropic gene contributes to the development of multiple disorders with other genetic factors. The genetic etiology of TD has been proposed to be polygenic. In this particular TD family, the *PNKD* mutation might require other mutations to cause TD. 3) a pleiotropic gene might encode multiple protein isoforms. Different mutations at the same gene locus might impair different or different combination of the protein isoforms and thus lead to different clinical phenotypes. One study shows that protein isoforms from the same gene locus can interact

with diverse protein partners and thus having divergent cellular functions (286). Each of the PNKD isoforms has its own spatial and temporal expression pattern in the human brain. Also, the subcellular localizations of the PNKD isoforms were different in human cells but not well defined in human neurons. We have shown that PNKD (L) protein localized to the membrane of cell body and neurites in human neurons where ion channels are known to be expressed in mammalian central nervous system (287). The PNKD (S) was shown to be localized in cytoplasm (262) and even was predicted to be associated with mitochondria in mammalian cells (237). Therefore, to unveil the role that the *PNKD* gene might play in the pathogenesis of those neurological or neurodevelopmental disorders, one must better understand the functions of the different PNKD isoforms in neurons.

Significance and Future directions

This is the first study reporting a rare nonsense mutation co-segregating with TD and Tic disorders in a multiplex family and affecting the neuronal isoform of *PNKD* gene that was previously associated with neurological and movement disorders. We examined the expression of the *PNK*D gene and its transcript isoforms in the human brain using publicly available data, but knowledge of temporal and spatial expression, post-translational modification(s), function of PNKD (L) isoform in the human brain is still lacking. Also, the function of the PNKD (S) isoform which is highly expression in human brain is unknown. In addition, whether PNKD isoforms interact with or compensate the expression of one another in neuronal cells is unclear. Answering above questions will help to understand the biological basis of *PNKD* gene related diseases.

To recapitulate the phenotypes caused by the *PNKD* nonsense mutation in animals, mice bearing the same mutation have been made by the CRISPR/Cas9 gene editing technique in Dr. Jay Tischfield's lab. These mice are being tested to look for differences in obsessive–compulsive, anxiety-related or prepulse inhibition behaviors. To elucidate the biological pathways the *PNKD* nonsense mutation could affect in human neurons of early development, we have collected multiple batches of iPSC-derived D30 neurons from both unaffected and affected individuals in this family and performed transcriptomic analyses through RNA-Sequencing (RNA-Seq). Global gene expression profiles of control and mutant neurons will be compared and differentially expressed genes (DEGs) will be identified. Pathway and co-expression network analyses using the DEGs might provide evidence on molecular alterations in TD neurons and facilitate discovery of cellular phenotypes for TD. Also, studying the *PNKD* nonsense mutation in more mature human neurons, for example, iPSC/ESC-derived 3D neuronal spheres or cerebral organoids will be a very useful system to discover mutation-associated cellular phenotype(s) since the neurons could survive for longer period of time *in vitro* and were electrophysiologically mature (288).

Limitations

In this study, we argued that *PNKD* nonsense mutation is probably a contributor for TD and Tic disorder in this family based on the following evidence: 1) *PNKD* nonsense mutation perfectly segregates with TD and Tic disorder in this family, 2) *PNKD* nonsense mutation is a very rare loss-of-function mutation, 3) other mutations in *PNKD* gene have been associated with movement disorder paroxysmal nonkinesigenic dyskinesia and other neurological disorders and 4) PNKD (L) protein interacts with synaptic protein RIMS1α

and therefore may function with RIMS1α in common pathway(s) at synapses of neurons. However, to consider *PNKD* gene as a candidate gene for TD based on what we have shown so far, limitations in this study need to be recognized. First, that a mutation perfectly segregated with TD and Tic disorder in this family was the primary criterion we used to prioritize mutations identified by whole exome sequencing. However, due to the fairly small sample size (7 members were sequenced) and lack of multiple unaffected individuals in this family, the great majority of the mutations that fit the segregation pattern was probably not related to TD. We prioritized the *PNKD* nonsense mutation based on its extreme rarity in general population; however, it is not possible to infer TD causality based only on its low allele frequency due to the limited sample size. Secondly, since TD is proposed to be polygenic, it is possible that the *PNKD* nonsense mutation is necessary but not sufficient to cause TD. Especially in family studies, there could be unknown genetic predispositions to disease. To answer this question, one needs to identify a TD-associated cellular phenotype(s) in *PNKD* mutant neurons. The phenotype(s) must not be present in the unaffected neurons. If the phenotype(s) could be rescued by correcting the *PNKD* mutant allele to wild type allele in mutant neurons, or be introduced into unaffected neurons by mutating the *PNKD* wild type allele using genome editing techniques, for example, CRISPR/Cas9, the mutation is considered to be responsible for the phenotype(s). If not, other factors need to be taken into account to explain the phenotype(s). Thirdly, assuming that *PNKD* nonsense mutation caused a phenotype in the neurons we study, this particular phenotype might be one of many TD-associated phenotypes because current *in vitro* systems are unable to generate neurons comparable to the diverse and mature neuronal populations in human brain. Nevertheless,

to add evidence showing that *PNKD* is a candidate gene for TD, we are performing whole exome sequencing on more TD multiplex families and on the trios of simplex families, and hoping to identify recurrent mutations in *PNKD*.

Table 2. Mutations at the *PNKD* gene and Diseases

Mutation	Exon location	Isoforms impacted	Phenotype	Reference
A7V (het[a])	1	long and short	Paroxysmal nonkinesigenic dyskinesia	(234, 264-271)
A9V (het)	1	long and short	Paroxysmal nonkinesigenic dyskinesia	(234, 265-267, 272-274)
A33P (het)	2	long and short	Paroxysmal nonkinesigenic dyskinesia and cerebellar ataxia	(275)
G89R	5	long and medium	Mostly gastro-intestinal dysmotility but a couple of patients exhibiting movement disorders (ataxia, dyskinesia)	(276)
P341Rfs*2 (het)	12	long and medium	Familial hemiplegic migraine	(277)

[a] heterozygous mutation

APPENDICES

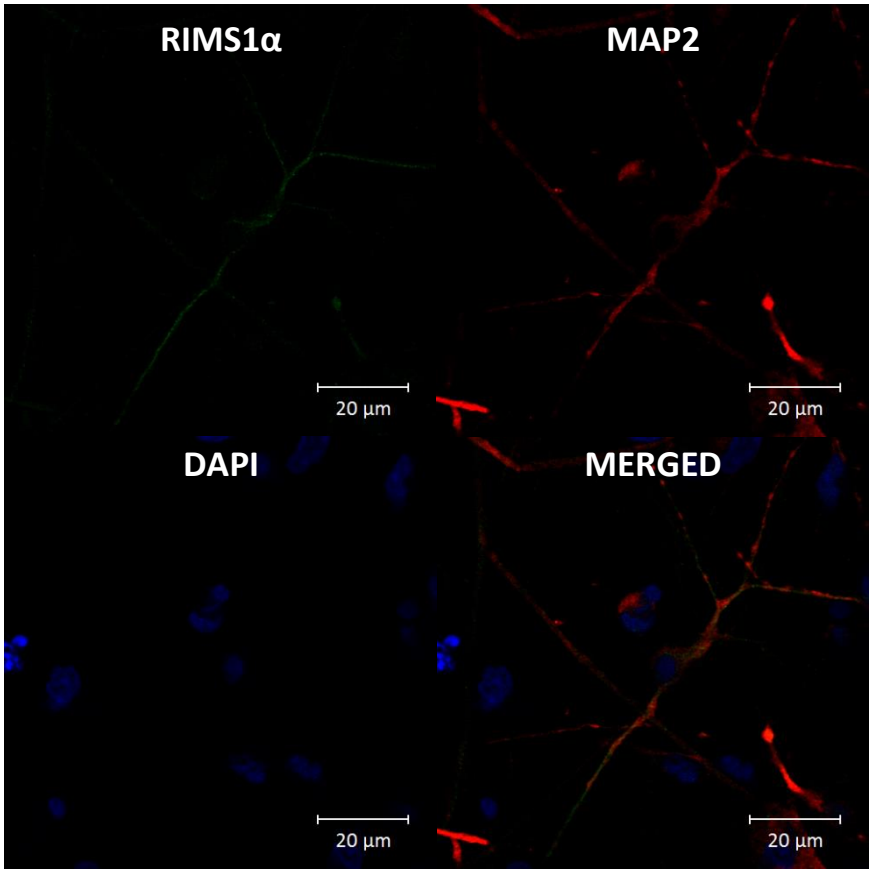
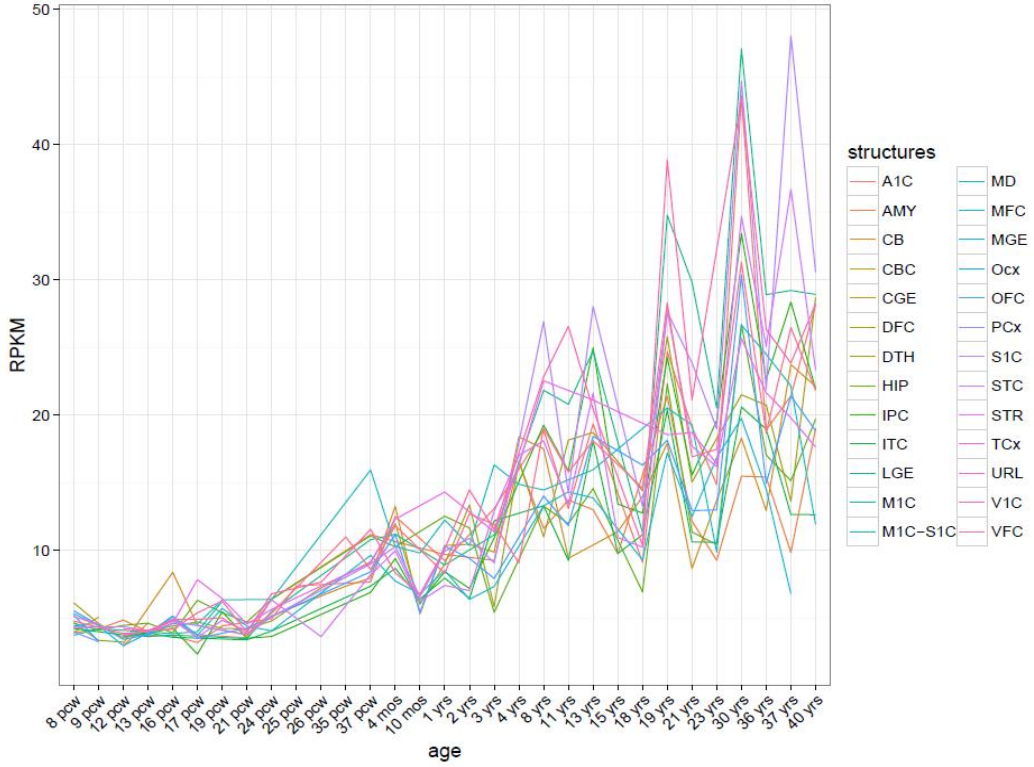


Figure S1 Endogenous RIMS1 protein expression in iPSC-derived neurons

Endogenous RIMS1 protein was detected in the cell body and the neurites of the iPSC-derived neurons however the expression was very weak.

A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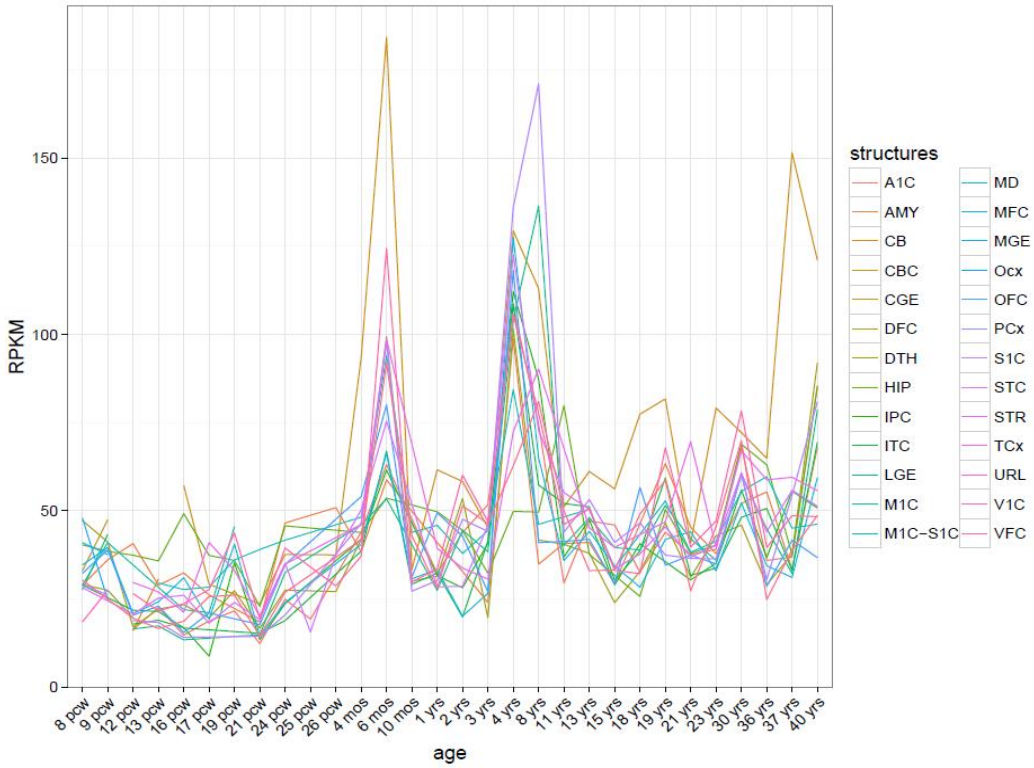
40
30
20
10
8 pcw
9 pcw
12 pcw
13 pcw
16 pcw
17 pcw
19 pcw
21 pcw
24 pcw
25 pcw
26 pcw
35 pcw
37 pcw
4 mos
10 mos
1 yrs
2 yrs
3 yrs
4 yrs
8 yrs
11 yrs
13 yrs
15 yrs
18 yrs
19 yrs
21 yrs
23 yrs
30 yrs
36 yrs
37 yrs
40 yrs
age
structures
A1C
AMY
CB
CBC
CGE
DFC
DTH
HIP
IPC
ITC
LGE
M1C
M1C−S1C
MD
MFC
MGE
Ocx
OFC
PCx
S1C
STC
STR
TCx
URL
V1C
VFC

B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100
50
0
8 pcw
9 pcw
12 pcw
13 pcw
16 pcw
17 pcw
19 pcw
21 pcw
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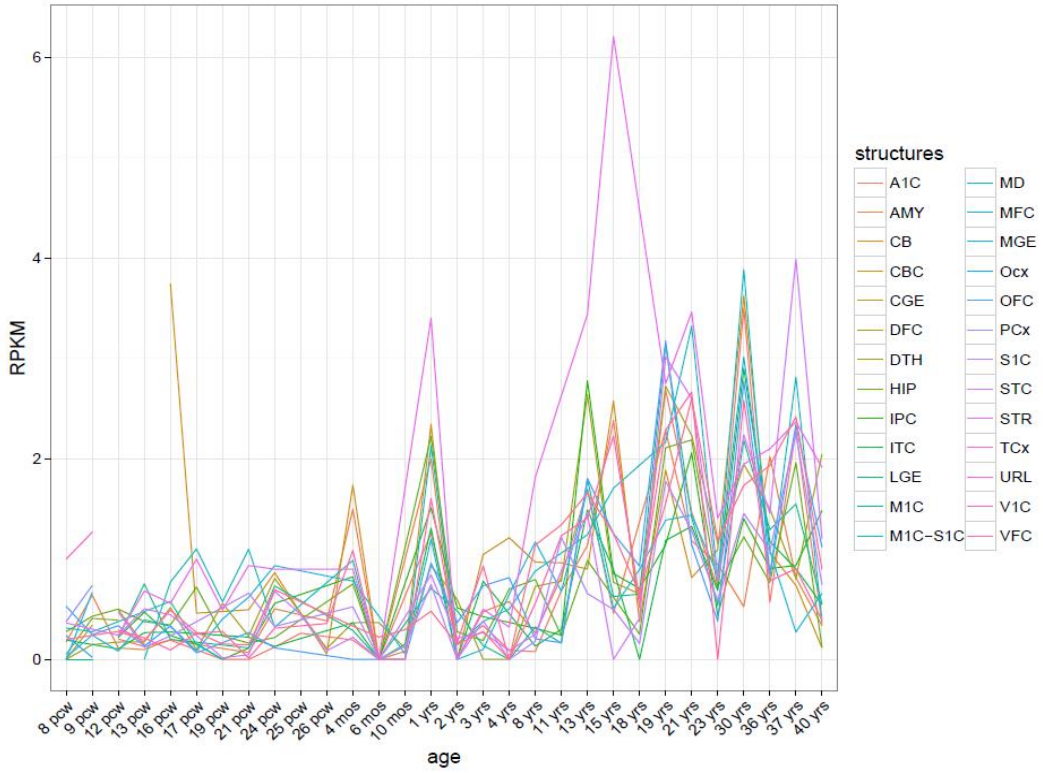
25 pcw
26 pcw
4 mos
6 mos
10 mos
1 yrs
2 yrs
3 yrs
4 yrs
8 yrs
11 yrs
13 yrs
15 yrs
18 yrs
19 yrs
21 yrs
23 yrs
30 yrs
36 yrs
37 yrs
40 yrs
age
structures
A1C
AMY
CB
CBC
CGE
DFC
DTH
HIP
IPC
ITC
LGE
M1C
M1C−S1C
MD
MFC
MGE
Ocx
OFC
PCx
S1C
STC
STR
TCx
URL
V1C
VFC

C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0
2
4
6
age
8 pcw
9 pcw
12 pcw
13 pcw
16 pcw
17 pcw
19 pcw
21 pcw
24 pcw
25 pcw
26 pcw
4 mos
6 mos
10 mos
1 yrs
2 yrs
3 yrs
4 yrs
8 yrs
11 yrs
13 yrs
15 yrs
18 yrs
19 yrs
21 yrs
23 yrs
30 yrs
36 yrs
37 yrs
40 yrs
structures
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AMY
CB
CBC
CGE
DFC
DTH
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LGE
M1C
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PCx
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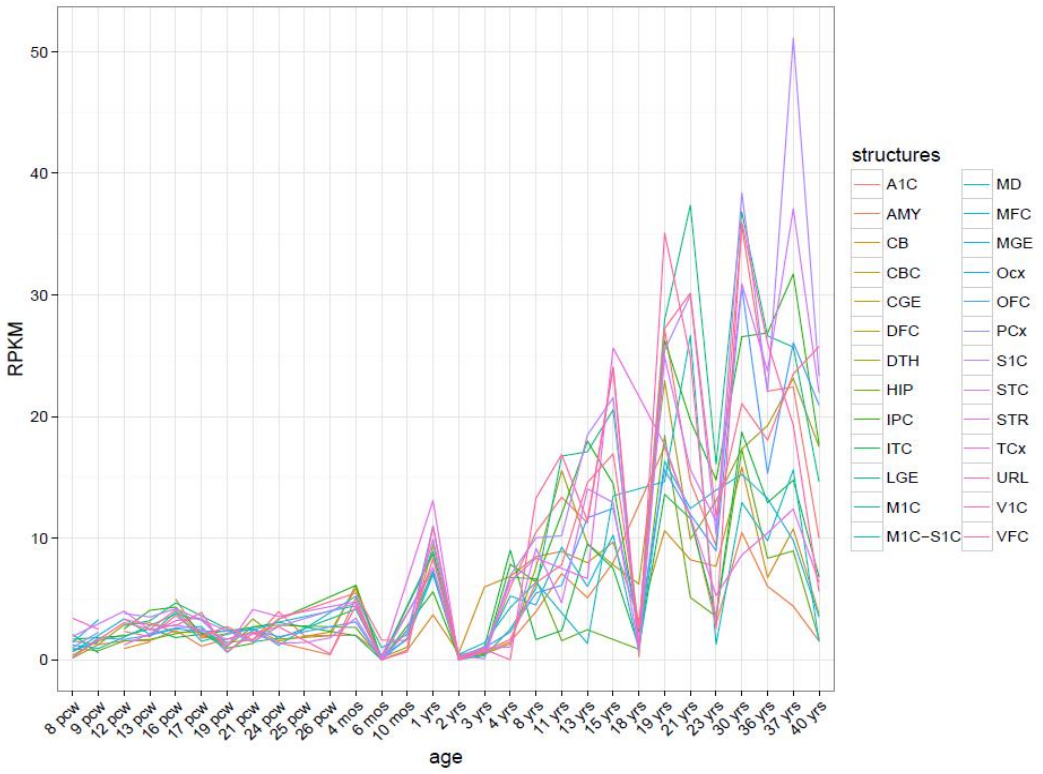
STR
TCx
URL
V1C
VFC

D


RPKM
0
10
20
30
40
50
age
8 pcw
9 pcw
12 pcw
13 pcw
16 pcw
17 pcw
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24 pcw
25 pcw
26 pcw
4 mos
6 mos
10 mos
1 yrs
2 yrs
3 yrs
4 yrs
8 yrs
11 yrs
13 yrs
15 yrs
18 yrs
19 yrs
21 yrs
23 yrs
30 yrs
36 yrs
37 yrs
40 yrs
structures
A1C
AMY
CB
CBC
CGE
DFC
DTH
HIP
IPC
ITC
LGE
M1C
M1C−S1C
MD
MFC
MGE
Ocx
OFC
PCx
S1C
STC
STR
TCx
URL
V1C
VFC

Figure S2 *PNKD* expression in human brain development

A. The "RNA-Seq Genecode v10 summarized to genes" dataset was downloaded from the BrainSpan website. RPKM values of *PNKD* gene were extracted and plotted using the "age" and "structures". There are 31 ages shown at the x-axis and 26 brain structures represented by color coded lines. In general, the *PNKD* gene expression increases during human brain development.

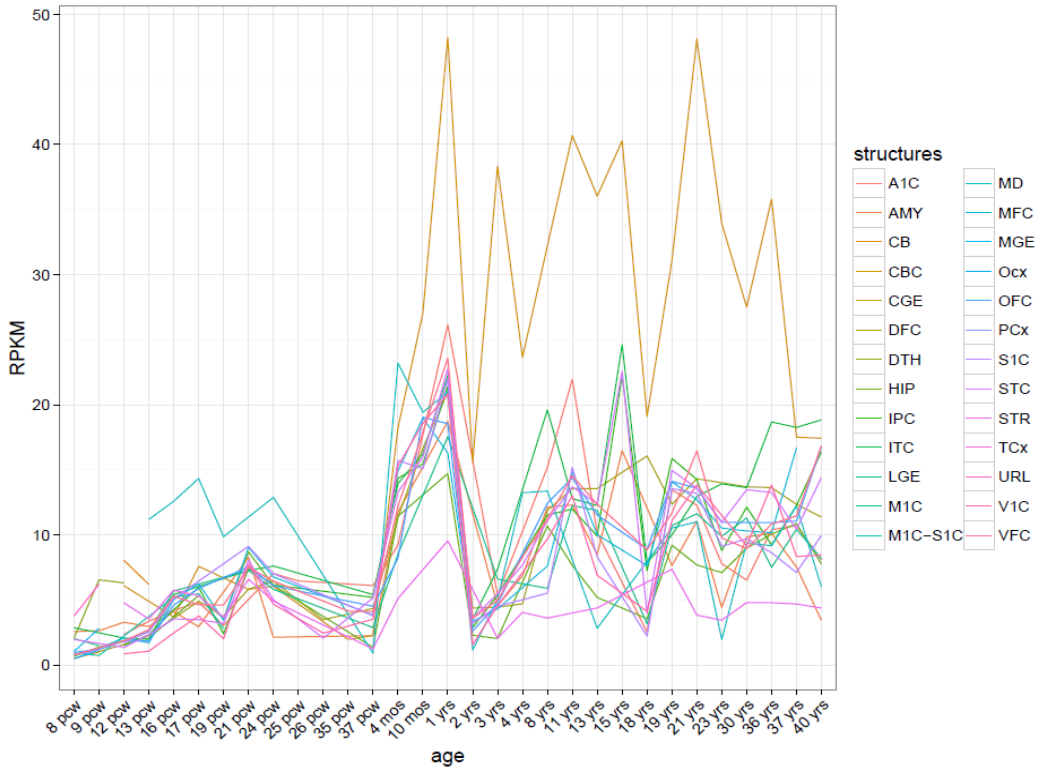
B. The "RNA-Seq Genecode v10 summarized to exons" dataset was downloaded from the BrainSpan website. RPKM values of the 13 exons of the *PNKD* gene were extracted and plotted using the "age" and "structures". There are 30 ages shown at the x-axis and 26 brain structures represented by color coded lines. The exon 3 is only included in the *PNKD* (S) transcript. The panel B shows the RPKM values of the exon 3 expression, which has higher expression in the 6-month and 4-year old brains.

C. Panel C shows the RPKM values of the exon 4 which is only included in the *PNKD* (M) transcript. The expression of the exon 4 is low in the human brain and doesn't exhibit a significant increase along with the brain maturation.

D. Panel D shows the PRKM values of the exon 6 which is shared by the *PNKD* (L) and the *PNKD* (M) transcripts. The expression of the exon 6 increases dramatically during human brain development. Since the *PNKD* (M) transcript is expressed low in the human brain, the expression increase of the exon 6 is probably due to the expression increase of the *PNKD* (L) transcript.

RPKM: Reads Per Kilobase of transcript per Million mapped reads

A


RPKM
50
40
30
20
10
0
8 pcw
9 pcw
12 pcw
13 pcw
16 pcw
17 pcw
19 pcw
21 pcw
24 pcw
25 pcw
26 pcw
35 pcw
37 pcw
4 mos
10 mos
1 yrs
2 yrs
3 yrs
4 yrs
8 yrs
11 yrs
13 yrs
15 yrs
18 yrs
19 yrs
21 yrs
23 yrs
30 yrs
36 yrs
37 yrs
40 yrs
age
structures
A1C
AMY
CB
CBC
CGE
DFC
DTH
HIP
IPC
ITC
LGE
M1C
M1C−S1C
MD
MFC
MGE
Ocx
OFC
PCx
S1C
STC
STR
TCx
URL
V1C
VFC

B

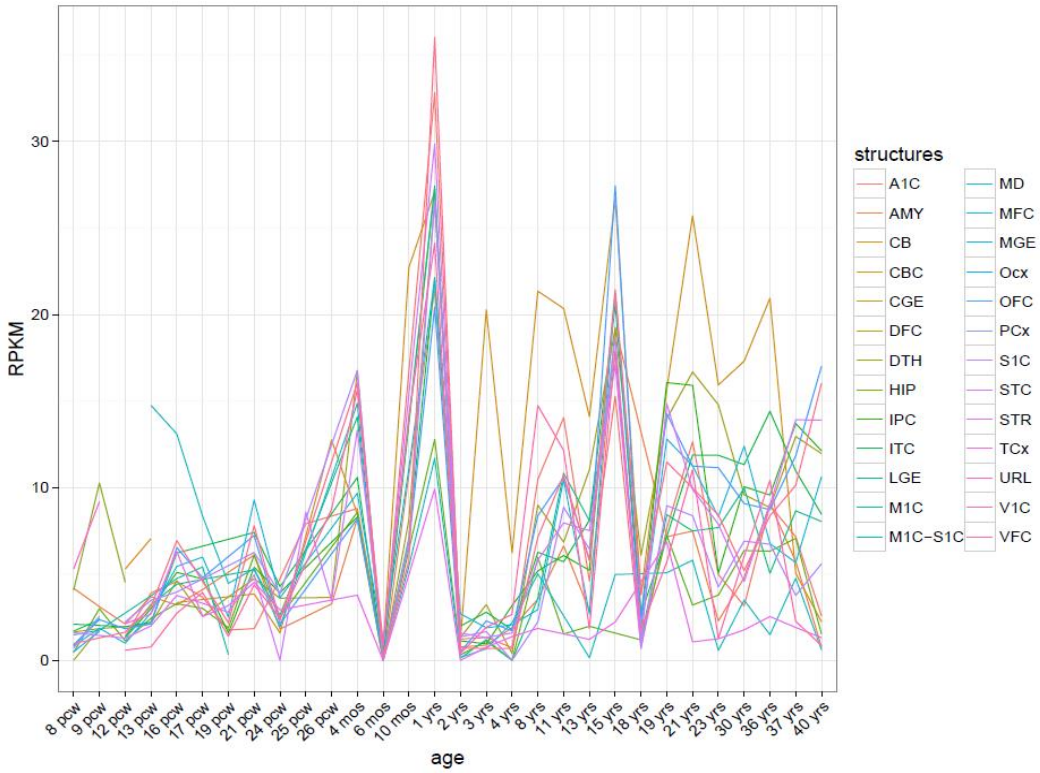
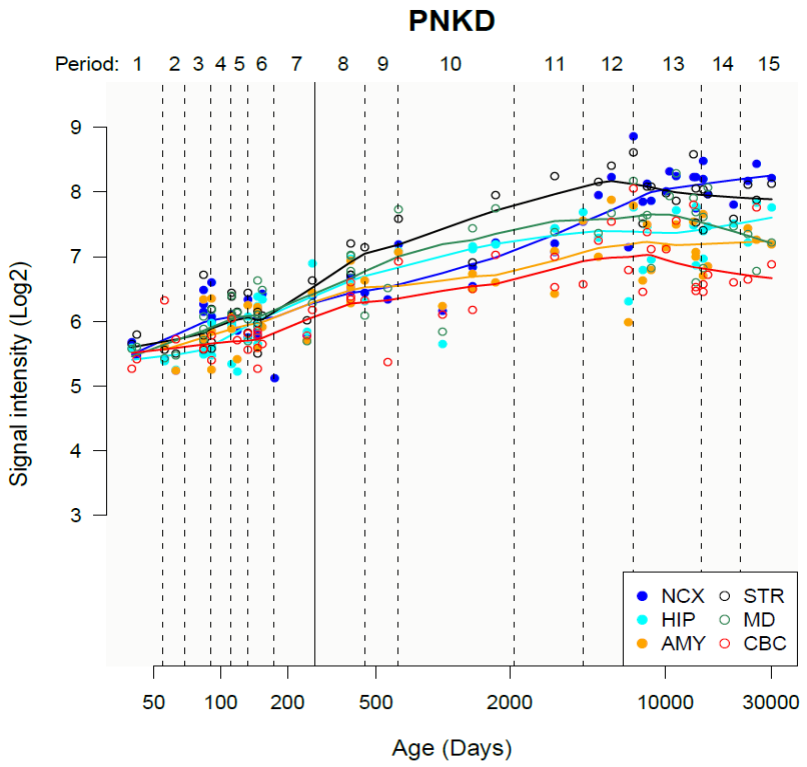

RPKM
30
20
10
0
8 pcw
9 pcw
12 pcw
13 pcw
16 pcw
17 pcw
19 pcw
21 pcw
24 pcw
25 pcw
26 pcw
4 mos
6 mos
10 mos
1 yrs
2 yrs
3 yrs
4 yrs
8 yrs
11 yrs
13 yrs
15 yrs
18 yrs
19 yrs
21 yrs
23 yrs
30 yrs
36 yrs
37 yrs
40 yrs
age
structures
A1C
AMY
CB
CBC
CGE
DFC
DTH
HIP
IPC
ITC
LGE
M1C
M1C−S1C
MD
MFC
MGE
Ocx
OFC
PCx
S1C
STC
STR
TCx
URL
V1C
VFC

Figure S3 *RIMS*1 expression in human brain development

A. The "RNA-Seq Genecode v10 summarized to genes" dataset was downloaded from the BrainSpan website. RPKM values of the *RIMS1* gene were extracted and plotted using the "age" and "structures". There are 31 ages shown at the x-axis and 26 brain structures represented by color coded lines. In general, the *PNKD* gene expression increases during human brain development.

B. The "RNA-Seq Genecode v10 summarized to exons" dataset was downloaded from the BrainSpan website. RPKM values of exon 3 of the *RIMS1* gene were extracted and plotted using the "age" and "structures". The exon 3 is only included in the *RIMS1α.* There are 30 ages shown at the x-axis and 26 brain structures represented by color coded lines.

A


PNKD
Period: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Signal intensity (Log2)
9
8
7
6
5
4
3
NCX
HIP
AMY
STR
MD
CBC
50 100 200 500 2000 10000 30000
Age (Days)

B

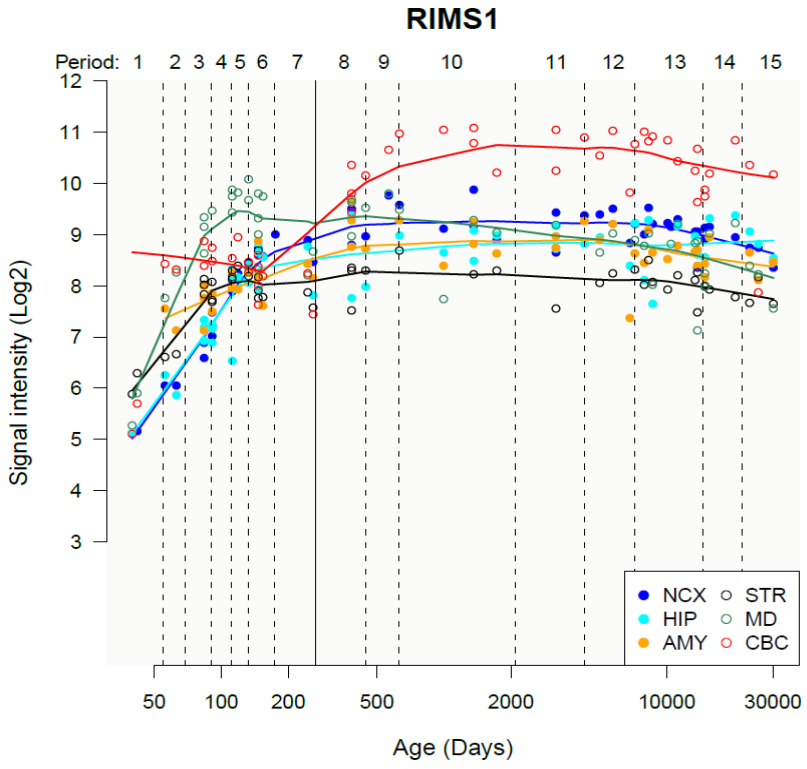

RIMS1
Period: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Signal intensity (Log2)
12
11
10
9
8
7
6
5
4
3
NCX
HIP
AMY
STR
MD
CBC
50 100 200 500 2000 10000 30000
Age (Days)

Figure S4 *PNKD* and *RIMS1* expression from the Human Brain Transcriptome (HBT) database.
Gene expression was measured in 6 brain regions at 15 developmental periods. For each developmental period, multiple samples were analyzed (experimental details can been found in http://hbatlas.org/files/nature10523-s1.pdf)
NCX: neocortex; STR: striatum; HIP: hippocampus; MD: mediodorsal nucleus of the thalamus; AMY: amygdala; CBC: cerebellar cortex.

A. *PNKD* expression increases from period 1 to period 12 during human brain development. STR might have higher *PNKD* expression from period 8 to 12 than other brain regions.

B. *RIMS1* expression increases dramatically from period 1 to period 6 and reaches to a plateau in all brain regions except for CBC. The CBC has higher *RIMS1* expression from period 8 to period 15 than other brain regions.

Period 1 (Embryonic development, 4< Age <8 PCW)

Period 2 (Early fetal development, 8< Age <10 PCW)

Period 3 (Early fetal development, 10< Age <13 PCW)

Period 4 (Early mid-fetal development, 13< Age <16 PCW)

Period 5 (Early mid-fetal development, 16< Age <19 PCW)

Period 6 (Late mid-fetal development, 19< Age <24 PCW)

Period 7 (Late fetal development, 24< Age <38 PCW)

Period 8 (Neonatal and early infancy, birth ≤ Age <6 postnatal months)

Period 9 (Late infancy, 6< Age <12 postnatal months)

Period 10 (Early childhood, 1< Age <6 years)

Period 11 (Middle and late childhood, 6< Age <12 years)

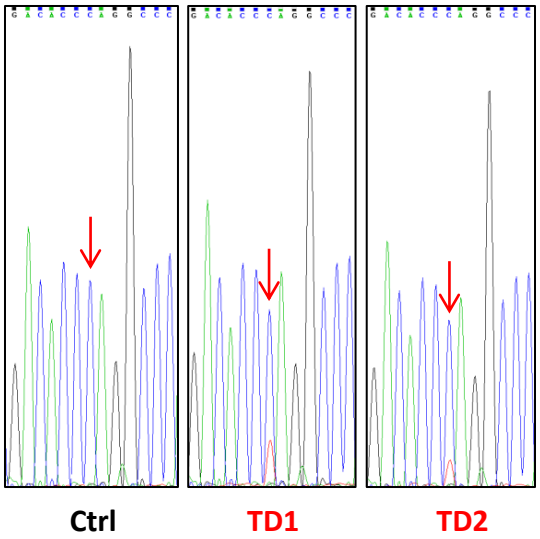
Period 12 (Adolescence, 12< Age <20 years)

Period 13 (Young adulthood, 20< Age <40 years)

Period 14 (Middle adulthood, 40< Age <60 years)

Period 15 (Late adulthood, 60 years +)

A



B

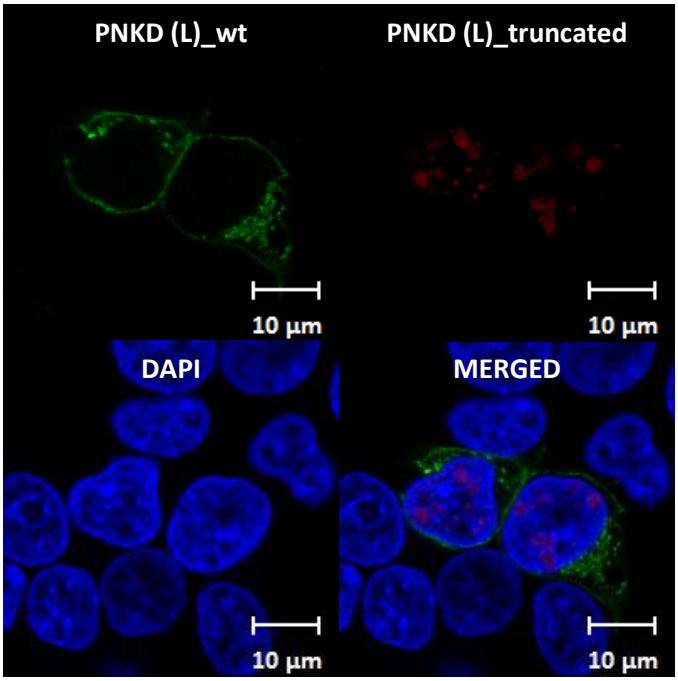


Figure S5 *PNKD* mutant transcript was detected in TD neurons and was translated into truncated protein in 293FT cells.

A. *PNKD* (L) transcript harboring the nonsense variant was detected in the TD1 and TD2 iPSC-derived neurons by Sanger sequencing, suggesting the nonsense-mediated decay was not complete.

B. By overexpressing the *PNKD* (L) wildtype and mutant transcripts together in 293FT cells, localization of the PNKD (L)_wt and PNKD (L)_truncated proteins can be visualized by immunocytochemistry.

Table S1 Primers information

Sanger sequencing		
PNKD-Seq-F	5'-GGTCACCAAAGGAATGGAGA-3'	
PNKD-Seq-R	5'-AAGGGTCAGCCCTCTCTAGC-3'	
PNKD transcript-F	5'-TGGCTCGGGTACCTCTTCTA-3'	
PNKD transcript-R	5'-TGACCCCTTCCTTTTCAATG-3'	
RT-qPCR		
PNKD(L)-F	5'-GCCACAGCTAACAAGGCTTC-3'	Exon 2
PNKD(L)-R	5'-GGCCTTTAGGGTAGCGATTC-3'	Exon 5
PNKD(M)-F	5'-GCTCCTCCTCGTCCTTGTC-3'	Exon 4
PNKD(M)-R	5'-AGGTACCCGAGCCAGGTG-3'	Exon 5
PNKD(S)-F	5'-CCTGGGCCATCGGCTT-3'	Exon2 and Exon 3
PNKD(S)-R	5'-GCTCTCATACTCGCCCGTGT-3'	Exon 3
RIMS1α-F	5'-GCCATGCTCAAGTGTGTTGT-3'	
RIMS1α-R	5'-ATTCCACAAGTCGGAGCATC-3'	
GAPDH-F	5'-ACCACCAACTGCTTAGCACC-3'	
GAPDH-R	5'-ATGATGTTCTGGAGAGCCCC-3'	
Molecular Cloning		
FLAG-PNKD-F	5'TCCCGATATCCCGATGGACTACAAAGACGATGACGACAAG ATGGCGGCGGTGGTAGCTGC-3'	
FLAG-PNKD-R	5'-TCTCCCCTCGAGTCACTTGCTCTTGTGCATATCCTTC-3'	
PNKD-Myc-F	5'-TCTCCCGATATCCCGATGGCGGCGGTGGTAGCTGC-3'	
PNKD-Myc-R	5'-CCTCGAGTTACAGATCCTCTTCTGAGATGAGTTTCTGCTCCTTGCTCTTGTGCATATCCT-3'	
LNCX_F	5'-AGCTCGTTTAGTGAACCGTCAGATC-3'	
BGH_R	5'-TAGAAGGCACAGTCGAGG-3'	

Table S2 Antibodies information

Primary Antibodies	**Application**	**Source**
Nanog	ICC	EMD Millipore (MABD24)
Oct4	ICC	EMD Millipore (MAB4401)
Nestin	ICC	EMD Millipore (MAB5326)
Mushashi1	ICC	EMD Millipore (MABE268)
Class III β Tubulin (Mouse)	ICC	EMD Millipore (MMS-435P)
Class III β Tubulin (Rabbit)	ICC	Sigma (T2200)
MAP2	ICC	EMD Millipore (AB5622)
RIMS1	WB	Synaptic systems (140003)
Anti-DDDK tag antibody [F-tag-01] – (Equivalent to FLAG antibody from Sigma)	WB for co-IP (PNKD (L) self-oligomerization)	Abcam (ab18230)
Anti-FLAG M2 antibody	WB for co-IP (RIMS1α and PNKD (L) Interaction)	Sigma (F3165)
Myc-Tag (9B11) Mouse mAb	WB ICC	Cell Signaling Technology
Synapsin I	WB	Sigma (S193)
VGLUT1	ICC	Synaptic systems (135511)
VGAT	ICC	Synaptic systems (131011)
GFAP	ICC	Synaptic systems (173011)
GAPDH	WB	Abcam (ab9482)
Secondary Antibodies	**Application**	**Source**
Cy3-conjugated AffiniPure F(ab')$_2$ Fragment Goat Anti-Mouse IgG (H+L)	ICC	Jackson ImmunoResearch (115-166-003)
Goat anti-Rabbit IgG (H+L) Superclonal Secondary Antibody, Alexa Fluor 488 conjugate	ICC	Thermo Fisher Scientific (A27034)

Table S3 pVAAST commands

Command - dominant	
VAAST -d 1e4 -m pvaast --indel --codon_bias --splice_site --fast_gp -pv_control TS0011_d.ctl -l phastcons-hg19-vertebrate.txt -o TS0011_d refGene_hg19.gff3 1kg-cgi-427_gene_only.cdr	
Control File - dominant (TS0011_d.ctl)	
#---------- Basic options----------	
input_ped_cdr_files:	TS0011.ped TS0011.cdr
pedigree_representatives:	3
unknown_representatives:	yes
additional_cases:	
inheritance_model:	dominant
#---------- Performance Tuning ----------	
informative_site_selection:	3
simulate_genotyping_error:	yes
genotyping_error_rate:	1.00E-04
penetrance_lower_bound:	0.6
penetrance_upper_bound:	1
#---------- Gene and Variant Filtering ----------	
max_prevalence_filter:	0.01
lod_score_filter:	yes
clrt_score_filter:	no
nocall_filter:	yes
nocall_filter_cutoff:	2
inheritance_error_filter:	yes
Command - recessive	
VAAST -d 1e4 -m pvaast --indel --codon_bias --splice_site --fast_gp -pv_control TS0011_r.ctl -l phastcons-hg19-vertebrate.txt -o TS0011_r refGene_hg19.gff3 1kg-cgi-427_gene_only.cdr	
Control File - recessive (TS0011_r.ctl)	
#---------- Basic options----------	
input_ped_cdr_files:	TS0011.ped TS0011.cdr
pedigree_representatives:	3
unknown_representatives:	yes
additional_cases:	
inheritance_model:	recessive
#---------- Performance Tuning ----------	
informative_site_selection:	3
simulate_genotyping_error:	yes
genotyping_error_rate:	1.00E-04
penetrance_lower_bound:	0.6
penetrance_upper_bound:	1
#---------- Gene and Variant Filtering ----------	
max_prevalence_filter:	0.01
lod_score_filter:	yes
clrt_score_filter:	no
nocall_filter:	yes
nocall_filter_cutoff:	2
inheritance_error_filter:	yes

Table S4 Statistics of the whole exome sequencing

SeqID	PID	ID	Sample ID	Total reads	Mean target coverage	Variants passed QC	Affected
28036	TS0011	NJT00036	4001	62135534	50.2	200298	2
27886	TS0011	NJT00021	4002	66022486	49.7	215897	1
28242	TS0011	NJT00022	5001	87865300	64.3	264505	2
27887	TS0011	NJT00023	5003	70384372	58.6	232068	2
28037	TS0011	NJT00232	5004	61150770	43.4	205672	2
27895	TS0011	NJT00588	5005	65183680	51.5	223182	2

Table S5 Brain structures with the highest *PNKD* exon 6 expression for each human subject

Expression (RPKM)	donor_id	age	gender	structure_ acronym	structure_name
6.462002	12890	4 mos	M	MD	mediodorsal nucleus of thalamus
1.610548	12297	6 mos	F	VFC	ventrolateral prefrontal cortex
2.451382	12977	10 mos	M	ITC	inferolateral temporal cortex (area TEv, area 20)
13.10173	12830	1 yrs	F	STR	striatum
0.550969	12979	2 yrs	F	CBC	cerebellar cortex
6.174245	12836	3 yrs	F	CBC	cerebellar cortex
5.789877	12980	3 yrs	M	CBC	cerebellar cortex
9.016101	12298	4 yrs	M	ITC	inferolateral temporal cortex (area TEv, area 20)
10.020554	12841	8 yrs	M	S1C	primary somatosensory cortex (area S1, areas 3,1,2)
19.472764	12981	8 yrs	M	V1C	primary visual cortex (striate cortex, area V1/17)
16.872481	12289	11 yrs	F	V1C	primary visual cortex (striate cortex, area V1/17)
18.477811	12831	13 yrs	F	S1C	primary somatosensory cortex (area S1, areas 3,1,2)
25.635981	12299	15 yrs	M	STR	striatum
6.22946	12984	18 yrs	M	DFC	dorsolateral prefrontal cortex
35.09306	12832	19 yrs	F	V1C	primary visual cortex (striate cortex, area V1/17)
37.374642	13057	21 yrs	F	M1C	primary motor cortex (area M1, area 4)
16.095879	12300	23 yrs	M	M1C	primary motor cortex (area M1, area 4)
38.378502	12290	30 yrs	F	S1C	primary somatosensory cortex (area S1, areas 3,1,2)
26.877621	12302	36 yrs	M	IPC	posteroventral (inferior) parietal cortex
51.100412	12303	37 yrs	M	S1C	primary somatosensory cortex (area S1, areas 3,1,2)
25.051741	12291	40 yrs	M	S1C	primary somatosensory cortex (area S1, areas 3,1,2)
28.768026	12304	40 yrs	F	STC	posterior (caudal) superior temporal cortex (area TAc)

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