DNA Variant in the *RPGRIP1L* Gene Influences Alternative Splicing

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Abstract

The retinitis pigmentosa GTPase regulator interacting protein 1-like (*RPGRIP1L*) gene encodes a ciliary protein that is critical for processes related to brain development, including development of left-right asymmetry, sonic hedgehog signaling, and neural tube formation. *RPGRIP1L* is a risk factor for retinal degeneration, and rare, deleterious variants in the *RPGRIP1L* gene cause Joubert syndrome and Meckel syndrome, both autosomal recessive disorders. These syndromes are characterized by dysfunctional primary cilia that result in abnormal development – and even lethality in the case of Meckel syndrome. Genetic studies have also implicated *RPGRIP1L* in psychiatric disorders by suggestive findings from genome-wide association studies and findings from rare-variant exome analyses for bipolar disorder and de novo mutations in autism. In this study we identify a common variant in *RPGRIP1L*, rs7203525, that influences alternative splicing, increasing the inclusion of exon 20 of *RPGRIP1L*.

We detected this alternative splicing association in human postmortem brain tissue samples and, using a minigene assay combined with in vitro mutagenesis, confirmed that the alternative splicing is attributable to the alleles of this variant. The predominate *RPGRIP1L* isoform expressed in adult brains does not contain exon 20; thus, a shift to include this exon may impact brain function.

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Introduction

Retinitis pigmentosa GTPase regulator interacting protein 1-like (*RPGRIP1L*) encodes for a protein that localizes to the basal bodies and transition zone of primary cilia [1, 2]. *RPGRIP1L* is critical for proper primary cilium function, as knockout mice have a ciliopathy phenotype and die at birth [1, 3]. Vierkotten et al. [1] showed that *RPGRIP1L* is necessary for multiple processes during embryonic development, including left-right asymmetry development, neural tube formation, and cilium-related sonic hedgehog signaling.

Variants affecting *RPGRIP1L* have been linked to multiple diseases including Joubert syndrome, Meckel syndrome, COACH (cerebellar vermis aplasia, oligophrenia,

congenital ataxia, coloboma, and hepatic fibrosis) syndrome, nephronophthisis, and retinal degeneration [2]. Joubert syndrome and Meckel syndrome, both ciliopathies [3], are both autosomal recessive disorders and are thought to represent a spectrum of the same disorder, with Meckel syndrome being on the extreme end of the spectrum, as it is lethal and is characterized by renal cystic dysplasia, polydactyly, and central nervous system malformation. Joubert syndrome is a severe but nonlethal condition, often diagnosed by the “molar tooth sign” brain malformation phenotype visible on magnetic resonance imaging [4]. Other core hallmarks of Joubert syndrome include ataxia, hypotonia, and intellectual disability [4]. Abnormal eye movement and function, cognitive impairment, development delay, polydactyly, and renal and liver diseases have also been reported as features of the disorder [4]. Individuals with Joubert and Meckel syndromes are at risk for retinal degeneration, and rare [5] and common variants in *RPGRIP1L* have been implicated in retinal degeneration [6].

Primary cilia are essential integrators and conveyors of signal transduction and are critical for multiple aspects of brain development and function including cortical development, neural progenitor development, early neuronal connectivity, and neuronal migration and differentiation [7, 8]. Knockdown experiments in the murine developing cortex implicate the murine homologue of *RPGRIP1L* in neuronal migration and, consequently, correct placement of neurons in the developing cortex [7]. Given these key functions in brain development, primary ciliary function in general [9, 10], and *RPGRIP1L* specifically, have been implicated in neuropsychiatric disorders. Injecting NMDAR antagonists, psychotomimetics often used to model psychiatric phenotypes, into rat and mouse brains causes a decrease in primary cilium length and downregulates *RPGRIP1L* mRNA expression, among other primary cilium-related genes [11]. Further, in multiple genome-wide association studies (GWAS), markers in *RPGRIP1L* were identified as suggestive of schizophrenia and bipolar disorder [12, 13], with the most significant *p* values of $\sim 10^{-7}$ for bipolar disorder [14]. *RPGRIP1L* was also identified by rare-variant exome analyses for bipolar disorder [15] and de novo mutations in autism [16]. Further supporting *RPGRIP1L* as a risk factor for psychiatric disorders are findings of a higher rate of neuropsychological and behavioral symptoms among patients with Joubert syndrome [17]. *RPGRIP1L* has also been found to physically interact in cells with NIMA-related kinase 4 (NEK4), a gene associated with psychiatric disorders [13], at the basal bodies of the cilia and is thought to act as a

scaffolding protein to recruit proteins to the primary cilia [18]. Schizophrenia-associated single nucleotide variants (SNVs) were shown to be associated with differentially spliced *NEK4* transcripts in the CommonMind Consortium data set [19].

Because of the key role of *RPGRIP1L* in neural cell function and brain development, we sought to identify functional variants in *RPGRIP1L* affecting gene and protein expression as possible contributors to disease. Given that the majority of the risks for complex traits are thought to alter gene expression including splicing [20], we mined data sets of predicted splicing variants to identify SNVs predicted by [21], or correlated with [22, 23], alternative splice isoforms and identified rs8050354 as significantly correlated with alternative splicing of exon 20 (position hg19 chr16:53,672,222–53,672,323; exon 20 as annotated in RefSeq, and exon 22 in GTEx). This variant was associated with bipolar disorder and schizophrenia at $p = 4.48 \times 10^{-5}$ in a recent joint analysis [24]. We showed that alternative splicing of exon 20 was correlated with this SNV in adult human brain tissues. Using minigene assays combined with in vitro mutagenesis, we identify rs7203525, an SNV in high linkage disequilibrium (LD) with rs8050354, to be responsible for the alternative splicing in SH-SY5Y cells. The predominate *RPGRIP1L* isoform expressed in adult brains in the GTEx database does not contain exon 20; thus, a shift to include this exon may impact brain function.

Methods

Human Brain Tissue Samples

The aim of this study was to identify alternative splicing changes associated with disease risk. Because the risk SNVs analyzed in this study are common in the population, brain samples from controls carry the risk alleles, and therefore were used to study the relationship between these variants and alternative splicing. Brain tissue samples from 7 schizophrenia patients, 4 bipolar patients, and 79 controls were obtained from the Harvard Brain Tissue Resource Center, the Douglas-Bell Canada Brain Bank, and the Eunice Kennedy Shriver National Institute of Child Health and Human Development (Table 1).

As the purpose of this study was to investigate the effect of common risk variants on alternative splicing, all analyses were performed with the samples separated by genotype regardless of the disease status. The control postmortem brain tissue samples were from healthy individuals <65 years of age with no neurological or psychiatric disorder. The postmortem interval for these tissues was <24 h. The exclusion criteria for the control brain tissue samples included individuals where a neurological disease may have influenced the tissue quality, such as stroke, prolonged hypoxic death, Alzheimer's disease, or other psychiatric or neurological disorders. The gender, age, ethnicity, and cause of death were provided for

each individual. Two brain regions relevant to psychiatric disease were analyzed for this study: (1) dorsolateral prefrontal cortex (DLPFC) Brodmann area 9 and (2) the hippocampus.

Splicing SNV Identification

We mined the splicing prediction and correlation data sets included the SPIDEX database, three data sets from GTEx (Altrans, sQTLseeR, and protein-truncating variants), and a data set of DLPFC sQTLs identified in the CommonMind Consortium data [21, 23, 25–27]. These splicing data sets consist of variants associated with a splicing change based on RNA-Seq and genotype data (GTEx and DLPFC sQTLs) or of variants that are predicted by a computational model to cause a splicing change based on 1,393 features from the two flanking introns and exons of the target exon (SPIDEX) [21]. Identified SNVs were mined for their relationship to associated markers for psychiatric disease [13, 24].

The predicted *RPGRIP1L* alternative splicing variants identified were then validated in a genotyped panel of human brain tissue samples. *RPGRIP1L* transcript isoform expression was tested based on the splicing data set predictions using DLPFC and hippocampal cDNA from the human brain tissue samples. Primers were designed flanking the alternatively spliced exon of *RPGRIP1L* in order to size-separate the PCR-amplified fragments by gel electrophoresis (forward primer: 5'-TCGTGGATATCATGCCA-CATCA-3'; reverse primer: 5'-GCTTTGTTCTGCAAGCT-GAC-3'). Each PCR reaction consisted of 4 ng of cDNA, 2X PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and 0.5 μ M of the primers in exons 19 and 21 of *RPGRIP1L*. An initial denaturation was performed at 95 °C for 3 min, followed by 40 cycles consisting of a 30-s denaturation at 95 °C, a 30-s anneal at 55 °C, and a 30-s extension at 72 °C. A final extension step was performed at 72 °C for 10 min. The PCR amplification was then run on a 2% agarose gel, and the presence of splicing isoforms was identified based on size separation of amplified fragments, with the *RPGRIP1L* transcript containing exon 20 amplifying a 239-bp fragment and the *RPGRIP1L* transcript skipping exon 20 amplifying a 137-bp fragment. All identified alternative transcripts were gel purified and sequenced.

Upon identification of the alternatively spliced isoform, a quantitative digital droplet PCR (ddPCR) assay was designed in order to quantify the proportion of each splicing isoform in each sample. Probe-based assays were designed, with one assay amplifying the *RPGRIP1L* isoform with the exon spliced in using a HEX fluorescent dye for the probe, and a second assay amplifying the exon spliced out using a FAM fluorescent dye for the probe (forward primer: 5'-AGCTGACCTTCAGATAGTAAAGAC-3'; reverse primer: 5'-AGAAGGTATCTTTCGTGGATATCAT-3'; probe for full length: 5'-TGGGAACATGTGGAACAGTCAG-CA-3'; probe for exon skipping: 5'-ACTGCCTTCTTGTAACACTCTGATGT-3'). Each ddPCR reaction consisted of 10 ng of cDNA, 2X ddPCR Supermix for Probes (No dUTP) (Bio-Rad Laboratories, Hercules, CA, USA), 20X FAM probe mix, and 20X HEX probe mix. An initial denaturation was performed at 95 °C for 10 min, followed by 40 cycles consisting of a 30-s denaturation at 95 °C and a 1-min combined anneal and extension step at 58.5 °C. A deactivation step was performed at 98 °C for 10 min, followed by a hold at 4 °C. These assays were run as duplex ddPCRs on each brain sample. Each droplet with either the full length or the exon-skipped transcript, determined by either FAM or HEX fluorescence, was then counted. The fractional abundance “FA” of the

Table 1. Demographics of the individuals from whom the brain sample tissues were collected

	Schizophrenia	Bipolar disorder	Control
Samples, <i>n</i>	7	4	79
Gender	7 male 0 female	3 male 1 female	50 male 29 female
Mean age, years	42.3	44.25	40.2
Ethnicity	7 Caucasian	4 Caucasian	69 Caucasian 9 African American 1 mixed ethnicity

exon-skipped transcript compared to the total number of transcripts amplified was determined by the following equation: $FA = A/(A + B)$. “A” refers to the copies per microliter of the alternatively spliced transcript, in this case the exon-skipped transcript, and “B” refers to the copies per microliter of the full-length transcript, in this case the transcript with the exon spliced in.

The fractional abundance was determined for each individual brain sample and was then run as an ANOVA with sex, RNA integrity number (RIN), age, ethnicity, postmortem interval, and genotype of the candidate SNVs as factors. Only samples with an RIN >6.1 were used in this analysis (mean 7.41 ± 0.67 , range 6.1–9.2). Sex, RIN, age, ethnicity, and postmortem interval were not significantly related to the alternative isoform and were not included in the further analyses. The fractional abundance of each sample compared to their genotype was also plotted as a boxplot using “plotly” in R. For each plot, the upper and lower hinges are the 3rd and 1st quartiles, respectively, and the upper and lower whiskers are the largest and smallest values contained within 1.5 times the interquartile range. Outliers are values that extend above or below 1.5 times the interquartile range.

SNV Genotyping

DNA from the brain tissue samples was genotyped using the Illumina OmniExpress Platform (~713,599 SNVs genotyped). PLINK version 1.9 was used for quality control and analyses (<http://pngu.mgh.harvard.edu/purcell/plink/>) [28]. SNVs were removed if they deviated from Hardy-Weinberg equilibrium ($p < 0.0001$), had a minor allele frequency <0.05, or had a call rate <98%. Ethnicity of the samples was checked using principal component analysis. The genotypes were imputed using the Michigan Imputation Server (<https://imputationserver.sph.umich.edu/index.html>) using the Haplotype Reference Consortium (HRC version r1.1 2016) [29]. SNPs with a low imputation score ($r^2 < 0.3$) were removed and a final round of quality control was performed. Approximately 5.3 million SNPs remained after imputation and quality control. For the SNVs used in this study, rs8050354 was genotyped (T minor allele frequency 0.314) and rs7203525 was imputed (T minor allele frequency 0.289, imputation $r^2 = 0.99876$).

RNA Extraction from Brain Tissue Samples and SH-SY5Y Transfected Cells

RNA was extracted using TRIzol reagent and the PureLink RNA Mini Kit following the manufacturer’s protocol, with the ad-

ditional DNase step added to ensure removal of genomic DNA (Invitrogen, Carlsbad, CA, USA). Eighty-nine DLPFC and 65 hippocampal brain samples had high-quality RNA and were used to make cDNA. cDNA was synthesized using the ImProm-II Reverse Transcriptase protocol (Promega, Madison, WI, USA). For cDNA synthesis of RNA from transfected cells, the RTRHC R primer (5'-GCTTTGCAGCAACAGTAAC-3') specific to the RHCglo plasmid was used instead of oligo(dT) in order to synthesize cDNA specific to the transcribed exons in from the plasmid, and not the endogenous cDNA.

Minigene Assays

A minigene assay was designed to test the candidate SNVs significantly associated with the alternative splicing change. The region surrounding the alternatively spliced exon 20 as well as the candidate SNV was cloned into the *Sall* and *XbaI* restriction sites of the RHCglo splicing vector using forward and reverse primers in introns 19 and 20 of *RPGRIP1L* (forward primer: 5'-ATATATGTCGACACTCAGAGATACTGTTTGGATG-3'; reverse primer: 5'-ATATATTCTAGAGAGACTCAGAAAACAACCACTT-3') [30]. Both clones, either containing the major or the minor allele of the candidate SNV, were confirmed by sequencing.

Clones with either allele of the candidate SNV were transfected into SH-SY5Y cells separately according to the manufacturer's protocol (Lonza Group, Basel, Switzerland). RNA was extracted from the cells 24 h after transfection. RT-PCR was performed using a reverse primer specific to the RHCglo plasmid as the RT primer, and reverse and forward primers specific to the RHCglo plasmid for the PCR (forward primer: 5'-CATTCACCACAT-TGGTGTG-3'; reverse primer: 5'-GCTTTGCAGCAACAGTAAC-3'). Each PCR reaction consisted of 4 ng of cDNA, 2X PCR Master Mix (Thermo Fisher Scientific), and 0.5 μ M of the forward and reverse primers. An initial denaturation was performed at 95 °C for 3 min, followed by 40 cycles consisting of a 30-s denaturation at 95 °C, a 30-s anneal at 55 °C, and a 30-s extension at 72 °C. A final extension step was performed at 72 °C for 10 min. The PCR amplification was then run on a 2% agarose gel, and the presence of splicing isoforms was identified based on size separation of amplified fragments, with the transcript containing exon 20 at 290 bp and the transcript skipping exon 20 at 188 bp. All identified alternative transcripts were gel purified and sequenced.

Site-directed mutagenesis was performed according to the manufacturer's protocol (Q5[®] Site-Directed Mutagenesis Kit; New England Biolabs, Ipswich, MA, USA). Forward primers containing the nucleotide change for the substitution of the risk by the non-risk allele (or vice versa) were used (forward major allele primer: 5'-ATAAAAAATGACATACATGC-3'; forward minor allele primer: 5'-ATAAAAAATAACATACATGC-3'; reverse primer: 5'-TTATTACTAAATCCCAAATCATT-3'). Plasmids with the risk allele were changed to the non-risk allele and vice versa, and the sequence changes were confirmed by sequencing. The plasmids were then transfected separately into SH-SY5Y cells and tested for their effect on alternative splicing using PCR primers designed for the exons of the RHCglo vector as described above.

Quantification of the cDNA from the minigene assays was performed using the Bioanalyzer High Sensitivity DNA Analysis assay. A PCR was performed with forward and reverse primers specific to the RHCglo plasmid (forward primer: 5'-CATTCACCA-CATTGGTGTG-3'; reverse primer: 5'-GCTTTGCAGCAACA-

GTAAC-3'). Each PCR reaction consisted of 4 ng of cDNA, 2X PCR Master Mix (Thermo Fisher Scientific), and 0.5 μ M of the forward and reverse primers. An initial denaturation was performed at 95 °C for 3 min, followed by 35 cycles consisting of a 30-s denaturation at 95 °C, a 30-s anneal at 55 °C, and a 30-s extension at 72 °C. A final extension step was performed at 72 °C for 10 min. The PCR amplification was then run on the DNA Bioanalyzer, and the concentration of the peaks from the fragment with exon 20 spliced in as well as the fragment with exon 20 spliced out was determined. The percent spliced in "PSI" of exon 20 was determined by the following equation: $PSI = B/(A + B) \times 100\%$. "A" refers to the concentration of the peak of the transcript with exon 20 spliced out, and "B" refers to the concentration of the peak of the transcript with exon 20 spliced in.

Results

Association of the Alternatively Spliced *RPGRIP1L* Isoform with Genotypes

The SNV rs8050354 in *RPGRIP1L* was reported by the DLPFC sQTLs data set [23] to be associated with alternative splicing, leading to *RPGRIP1L* exon 20 retention. This SNV was associated with schizophrenia and bipolar disorder in a combined analysis ($p = 4.48E-05$) [24]. Because rs8050354 is in intron 24 of *RPGRIP1L* and the predicted skipped exon is exon 20, we sought to identify SNVs closer to the skipped exon that would be more likely to be the causal variant for the alternative splicing event by identifying SNVs in high LD with rs8050354. rs7203525 is in intron 19 of *RPGRIP1L* and is in high LD with rs8050354 ($r^2 = 0.83$, $D' = 0.96$) [31]; therefore, we studied this SNV in relation to *RPGRIP1L* exon 20 skipping as well as rs8050354.

In 92 genotyped brain tissue samples, PCR cDNA amplification identified two *RPGRIP1L* transcripts: (1) a 239-bp fragment corresponding to the *RPGRIP1L* transcript including exon 20 and (2) a 137-bp fragment corresponding to a smaller *RPGRIP1L* transcript with exon 20 spliced out (Fig. 1a). We found striking variation in the levels of these two *RPGRIP1L* transcripts between individuals, which appeared by inspection to be correlated with the genotypes of rs8050354 and rs7203525. When these two isoforms were then quantified in each of the brain tissue samples using ddPCR, the *RPGRIP1L* transcript with exon 20 spliced out was significantly decreased in the DLPFC of individuals with the schizophrenia and bipolar disorder risk alleles rs8050354(T) and rs7203525(T) with a dose allele effect (rs8050354: $p < 2E-16$, $F(2, 84) = 135.9$; rs7203525: $p < 2E-16$, $F(2, 84) = 490.3$) (Fig. 1b). The *RPGRIP1L* transcript with exon 20 spliced out was also significantly decreased in the hippo-

Fig. 1. Schizophrenia and bipolar disorder risk alleles associated with a decrease in *RPGRIP1L* exon 20-skipped transcript. **a** cDNA PCR amplification using forward and reverse primers in exons 19 and 21 of *RPGRIP1L*, respectively, amplified a 239-bp fragment corresponding to the full-length *RPGRIP1L* transcript and a 137-bp fragment corresponding to a smaller *RPGRIP1L* transcript isoform excluding exon 20. **b** The splicing ratio of the *RPGRIP1L* exon 20-spliced-out transcript compared to all *RPGRIP1L* transcripts amplified shows a significant decrease with the schizophre-

nia and bipolar disorder risk allele rs7203525(T) in an allele dose-dependent manner in the dorsolateral prefrontal cortex (DLPFC). **c** The splicing ratio of the *RPGRIP1L* exon 20-spliced-out transcript compared to all *RPGRIP1L* transcripts amplified shows a significant decrease with the schizophrenia and bipolar disorder risk allele rs7203525(T) in an allele dose-dependent manner in the hippocampus. Hom., homozygous; Het., heterozygous; Maj., major; Min., minor.

campus of individuals with the schizophrenia and bipolar disorder risk alleles rs8050354(T) and rs7203525(T) with a dose allele effect (rs8050354: $p < 2E-16$, $F(2, 60) = 99.61$; rs7203525: $p < 2E-16$, $F(2, 60) = 419.3$) (Fig. 1c).

rs7203525 Influences Alternative Splicing of RPGRIP1L

A minigene assay was performed to identify the causal variant of *RPGRIP1L* exon 20 skipping. The *RPGRIP1L* region including intron 19 (which contains rs7203525), exon 20, and intron 20 was PCR amplified in two different individuals, one homozygous for the major allele haplotype containing rs7203525(C) and the other homozygous for the minor allele haplotype containing rs7203525(T), and then cloned into the RHCglo vector (Fig. 2a). These plasmids were transfected into the human

neuroblastoma cell line SH-SY5Y, and RNA was extracted 24 h later to allow for expression. PCR amplification of the cDNA from each of these transfections identified a greater proportion of exon 20 skipping with the major allele haplotype containing rs7203525(C) (Fig. 2b), consistent with the findings from the brain tissue samples.

One additional SNV (rs77511317) is located in the *RPGRIP1L* region cloned within the minigene construct. To distinguish the causal variant from the rs77511317 variants located on the splicing haplotype, we used site-directed mutagenesis to change the rs7203525 allele (C) on the major allele haplotype to the minor allele and the minor allele (T) on the minor allele haplotype to the major allele (Fig. 2a). These plasmids were then transfected into SH-SY5Y cells and RNA was extracted. PCR amplification of the cDNA from each of these transfected cells

Fig. 2. *RPGRIP1L* variant rs7203525 influences alternative splicing. **a** Schematic of *RPGRIP1L* RHCglo plasmids. The plasmids consist of intron 19, exon 20, and intron 20 of *RPGRIP1L* cloned in between the two endogenous exons of the RHCglo vector. The *RPGRIP1L* major allele haplotype contains rs7203525(C), and the *RPGRIP1L* minor allele haplotype contains rs7203525(T). The *RPGRIP1L* mutant plasmids had rs7203525 altered through site-directed mutagenesis such that the *RPGRIP1L* major allele haplotype mutant contained rs7203525(T) and the *RPGRIP1L* minor allele haplotype mutant contained rs7203525(C). Dashed lines are indicative of the continuation of the RHCglo plasmid. The forward

and reverse primers in the endogenous exons of the RHCglo minigene plasmid used for cDNA amplification are indicated with arrows. **b** *RPGRIP1L* variants influence splicing. cDNA from SH-SY5Y cells transfected with the *RPGRIP1L* RHCglo plasmids amplified a 290-bp fragment corresponding to the RHCglo exons with *RPGRIP1L* exon 20 spliced in, and a 188-bp fragment corresponding to the RHCglo exons with *RPGRIP1L* exon 20 spliced out. A higher proportion of the spliced-out fragment was observed with the *RPGRIP1L* plasmid containing rs7203525(C) regardless of the other single nucleotide variants on the haplotype.

identified a greater proportion of exon 20 skipping with the C allele of rs7203525 regardless of the rs77511317 alleles on the haplotype (Fig. 2b), confirming that the rs7203525 allele is critical for alternative exon 20 splicing of *RPGRIP1L*.

Discussion

In this study we identified a variant in *RPGRIP1L*, rs7203525, critical for alternative exon 20 splicing of the *RPGRIP1L* gene. Specifically the minor allele in Caucasian populations, T, increases the percentage of isoforms with RefSeq-annotated exon 20 retention. The predominant *RPGRIP1L* isoform (ENST00000262135.4) expressed in adult brains in the GTEx database does not

contain exon 20; thus, this variant shifts expression of the common isoform to an alternative form. The full-length *RPGRIP1L* transcript encodes for a protein with 1,315 amino acids, while the *RPGRIP1L* transcript skipping exon 20 causes an in-frame deletion of 34 amino acids and results in a protein with 1,281 amino acids. Two sequence motif-based protein prediction methods (InterPro and Pfam) and one automated function prediction method (PredictProtein) were employed to analyze functional differences between the full-length and alternatively spliced *RPGRIP1L* protein sequences [32–35]. The deletion of 34 amino acids resulting from exon 20 skipping of *RPGRIP1L* does not affect any known domains identified by InterPro or Pfam. PredictProtein identified two protein-binding regions in the full-length *RPGRIP1L* protein sequence and four protein-binding regions in

the exon 20-skipped *RPGRIP1L* protein sequence. As *RPGRIP1L* has been found to interact with NEK4 [18], this difference in protein-binding regions between the two isoforms may have implications for NEK4 binding; however, this is unclear from the prediction data. Therefore, the effect on *RPGRIP1L* protein function from exon 20 skipping of this transcript is currently unknown, and functional studies would be necessary to elucidate the exact biological differences between these two proteins.

The *RPGRIP1L* protein plays an important role in primary ciliary function, and it contributes to sonic hedgehog signaling as well as ciliary proteasomal and autophagic activity [1, 2, 36, 37]. The developmental and brain malformation phenotypes of Joubert syndrome and Meckel syndrome caused by loss-of-function variants in *RPGRIP1L* implicate this gene as critical for these processes. As *RPGRIP1L* is a gene that is necessary for embryonic development and brain formation, the variant identified in this study may affect development as well, and may even influence the Joubert syndrome phenotypes. Further, this variant shows a trend for association with both schizophrenia and bipolar disorder and may contribute to a risk for psychiatric disorders.

The role of the primary cilia has been increasingly indicated in the risk for psychiatric disorders. One of the most thoroughly studied risk genes for psychiatric disorders is disrupted in schizophrenia 1 (*DISC1*), implicated by a translocation segregating in a large, multiply affected pedigree [38]. *DISC1* has been found to localize to, and play a role in, the function of primary cilia [39]. *DISC1* localized to the basal bodies of the primary cilia in rat striatal neurons and a fibroblast cell line, and knockdown of *DISC1* in these cells resulted in decreased primary cilia, an effect which was restored after reexpression of *DISC1* [39]. Interestingly, that study also showed that the dopamine receptors D1R and D2R, both highly implicated in psychiatric disorders, localized to the primary cilia on these cells [39]. A more recent study by Marley and von Zastrow [9] showed that knockdown of 20 genes associated with psychiatric disorders either through interactions with *DISC1*, rare variants associated with psychiatric disorders, or association by GWAS also resulted in decreased ciliated cells. Interestingly, transfecting these cells with one of the top schizophrenia GWAS-associated genes, *MIR137* [13], increased ciliation and the length of the cilia [9]. Furthermore, lithium, the most common and effective treatment of bipolar disorder, has also been found to increase the length of primary cilia in striatal neuronal cell culture and in the mouse brain [40]. Thus, multiple lines of evidence implicate the primary cilia in

psychiatric disorders, and a shift to this *RPGRIP1L* isoform in the brain may also alter primary ciliary function.

Interestingly, *RPGRIP1L* knockout murine embryonic fibroblasts have decreased ciliary proteasome activity, and treatment with sulforaphane leads to a recovery of the ciliary proteasome activity [2, 37]. Sulforaphane, found in cruciform plants, particularly in broccoli sprouts, has garnered interest from clinical trials for cancer, diabetes, schizophrenia, and autism [41–45]. Of particular interest, sulforaphane has been found in mice to prevent cognitive deficits induced by phencyclidine, an NMDAR antagonist that mimics symptoms of schizophrenia and has also been reported to cause impaired primary cilia and altered levels of *RPGRIP1L* mRNA in rat and mouse brains [11]. This provides an interesting avenue of future study with regard to the potential contribution of *RPGRIP1L* and dysfunctional primary cilia to psychiatric disease.

Also relevant to psychiatric disorders and brain function, *RPGRIP1L* has also been found to be important for left-right asymmetry development [1] – implicated in schizophrenia by decades of research including, more recently, structural and functional imaging [46, 47]. Oertel et al. [48] identified reduced left compared to right asymmetry in the temporal lobe of schizophrenia patients when compared to controls based on gray matter volume and functional activation. First-degree unaffected relatives of schizophrenia patients were also studied, and they showed an intermediate reduced-asymmetry phenotype, suggesting a genetic component. Thus, the role of this gene and alternative isoforms are interesting candidates for study of variation in left-right brain asymmetry and laterality.

In summary, we identified an SNV, rs7203525, that influences an alternative splicing event in *RPGRIP1L*, leading to altered ratios of isoforms with exon 20 retention. As *RPGRIP1L* is an important developmental gene, this identification paves the way for future studies to unravel the altered protein function of these two *RPGRIP1L* isoforms and determine how they contribute to embryonic and neural processes, as well as to the *RPGRIP1L* disorders Joubert syndrome and Meckel syndrome.

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Statement of Ethics

The study was approved by the Hospital for Sick Children and University Health Network Research Ethics Boards.

Disclosure Statement

The authors declare no conflicts of interest.

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