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Exploring the Impact of *RNU4-2* Defects on Neurodevelopmental Disorders in a Korean Population

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ABSTRACT

Neurodevelopmental disorders (NDDs) often remain unexplained due to limited assessment of non-coding genomic elements. Motivated by recent reports implicating *RNU4-2*, which encodes a spliceosomal small nuclear RNA (snRNA), we analyzed whole-genome sequencing data from 15450 Korean individuals, including 2797 unrelated NDD probands. Rare pathogenic *RNU4-2* variants were identified in 20 probands (0.72%), including 17 (85%) with a recurrent n.64_65insT variant. RNA secondary structure modeling and molecular dynamics simulations demonstrated that n.64_65insT disrupts the U4/U6 snRNA duplex and impairs exposure of the U6 ACAGAGA motif required for 5′ splice-site recognition. Whole-blood RNA-seq from carriers revealed increased alternative 5′ splice-site usage and dysregulation of immune, chromosomal, and DNA metabolic gene programs. Clinically, affected individuals presented with global developmental delay, microcephaly, seizures, failure to thrive, and dysmorphic features. These findings establish *RNU4-2*, particularly n.64_65insT, as a cause of early-onset NDD. We advocate for routine assessment of spliceosomal RNA genes in genomic diagnostics and reanalysis of unsolved cases to improve yield and guide counseling in rare neurodevelopmental syndromes.

1 | Introduction

High-throughput sequencing has improved diagnostic yield in neurodevelopmental disorders (NDDs), particularly by enabling the detection of rare single-nucleotide and structural variants missed by chromosomal microarray [1, 2]. Whole-genome sequencing (WGS) offers broader coverage, including non-coding regions, and has revealed new disease-associated loci [3].

Recent studies identified *RNU4-2*, a non-coding gene encoding U4 snRNA of the major spliceosome, as a recurrent cause of syndromic NDD [4, 5]. Pathogenic variants cluster within

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an 18-base pair critical region and account for ~0.4%–0.5% of unsolved NDD cases in European cohorts, placing *RNU4-2* among the most frequently implicated NDD genes, comparable to *MECP2* [6, 7]. Affected individuals typically exhibit microcephaly, growth impairment, dysmorphic features, and global developmental delay. The recurrent variant n.64_65insT has been reported in multiple unrelated patients.

To assess generalizability beyond European populations and investigate potential mechanisms, we screened a large Korean rare disease cohort for *RNU4-2* variants, characterized associated clinical features, and applied structural modeling and transcriptomic profiling to evaluate the impact of spliceosomal disruption.

2 | Method

We analyzed WGS data from 15450 individuals in two Korean rare disease cohorts: the Korea Biobank Rare Disease Cohort (~15000 participants; 2856 with NDD) and a multicenter pediatric cohort (544 individuals; 215 with NDD). After quality control (QC) for ancestry, sex, and relatedness, we retained 2797 unrelated NDD probands. Clinical features were annotated using Human Phenotype Ontology terms.

WGS data (150 bp, paired-end; Illumina NovaSeq) were aligned to GRCh38 and jointly genotyped using Illumina DRAGEN. Rare *RNU4-2* variants were identified and compared against public genome databases.

For transcriptomic profiling, we performed whole-blood RNA-seq on six carriers and ten unaffected parents. Differential expression and alternative splicing analyses were conducted using DESeq2 and rMATS, respectively. Structural modeling, molecular dynamics (MD) simulations, and docking predictions were used to assess the functional impact of recurrent *RNU4-2* variants.

Detailed protocols for study population, QC, sequencing, structural modeling, splicing analysis, and statistical methods are provided in Data S1.

3 | Results

3.1 | Frequency of RNU4-2–Related Neurodevelopmental Disorders

We screened *RNU4-2* in 2797 unrelated NDD probands, focusing on the previously reported 18-bp "critical region" (GRCh38: chr12:120291825–120291842; Figure 1A) [4, 5]. Rare variants in this region were identified in 20 unrelated Korean individuals (0.72%), with the recurrent n.64_65insT variant accounting for 17 (85%) of these (Figures 1B and S1–S2; Tables 1 and S1). Parental sequencing in 17 families confirmed that all proband

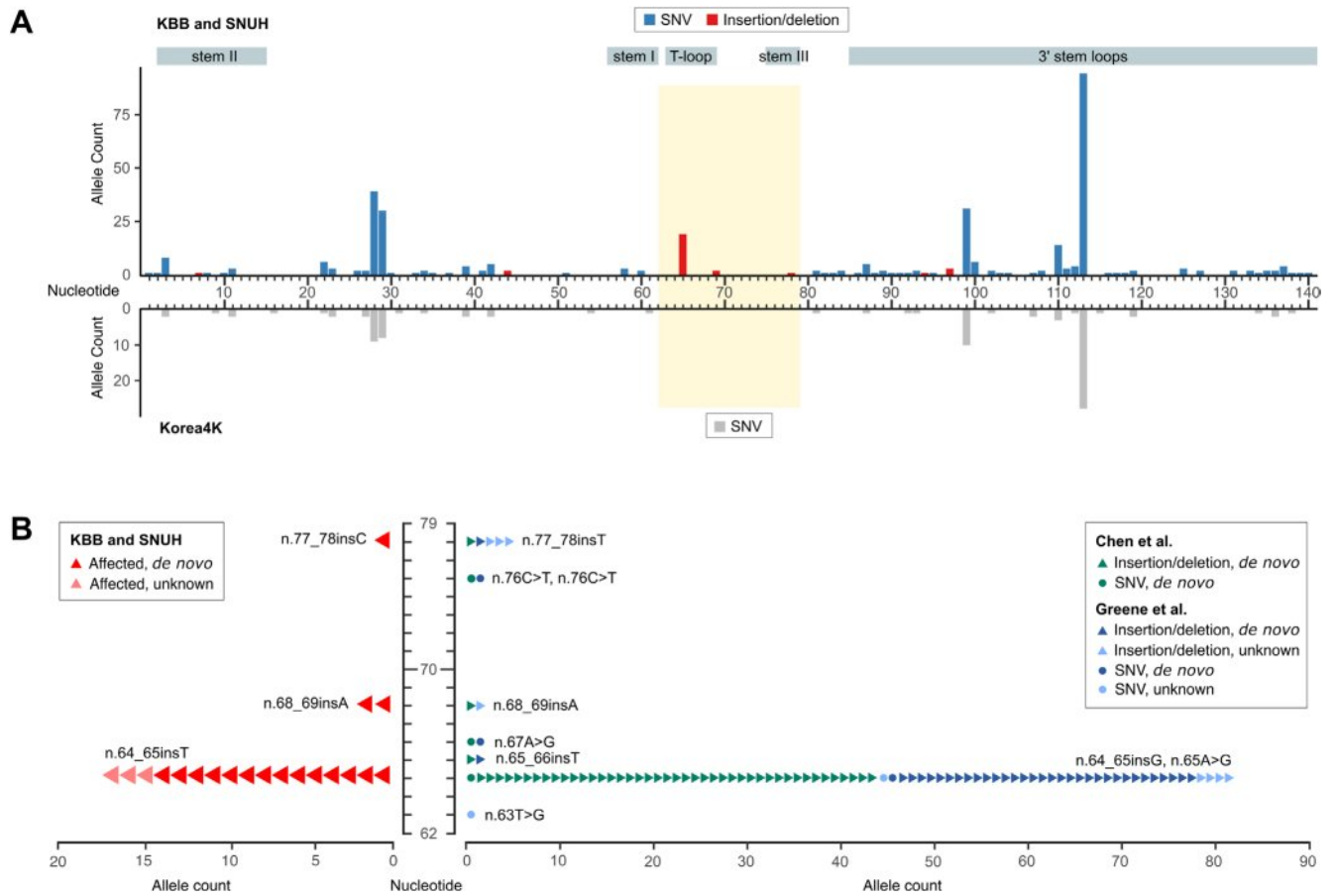

FIGURE 1 | *RNU4-2* variant characterization in a Korean NDD cohort. (A) Allele frequencies of variants in our cohort (top) and a 3617 Korean reference population (Korea4K, bottom). (B) Allele counts of *de novo* variants in the *RNU4-2* hotspot region (18 bp) across NDD probands in our cohort ($n = 2797$; left), Chen et al. ($n = 8841$), and Greene et al. ($n = 5529$; right). [Colour figure can be viewed at wileyonlinelibrary.com]

variants arose *de novo*. In seven out of these 17 families, phasing was possible and demonstrated that the variant originated from the maternal allele (Figure S3–S4). These findings align with prior reports and establish *RNU4-2* as a recurrent non-coding genetic cause of NDD in Korean children [4, 5, 7].

3.2 | Clinical Characteristics of Patients With *RNU4-2* Variants

Among 20 individuals with pathogenic *RNU4-2* variants, detailed clinical data were available for 18 cases across six regional hospitals (Table S2–S3). All were enrolled based on global developmental delay, with or without seizures. Neonatal complications were common: 10 patients (56%) required transfer to neonatal care for reasons including microcephaly and/or brain anomalies ($n = 4$), and multiple congenital anomalies ($n = 6$).

Motor and language delays were universal. Only six individuals (6/18, 33.3%) achieved independent walking at a mean age of 27.0 months, while others, including six patients older than 5 years, remained non-ambulatory. Sixteen of 18 patients were nonverbal or had severe cognitive impairment. Neurological features included seizures (13/17, 77%), dyskinesia (3/16, 19%), and stereotyped movements (6/16, 38%). Two girls with severe symptoms and hand stereotypies were initially managed as Rett syndrome. Among nine patients with treatment data, seizures were generally controlled with 1–3 antiseizure medications in eight.

Brain magnetic resonance imaging (MRI) findings ranged from focal heterotopia to diffuse white matter atrophy (Figure 2A–G). Ophthalmologic abnormalities were present in 10/14 patients (71%), including nystagmus, strabismus, retinal thinning, and optic disc hypoplasia; one child with progressive vision loss attended a school for the blind. Dysmorphic features were seen in 14/18 patients (78%), most commonly tented upper lip, cupped ears, and low nasal bridge (Figure 2H–K).

3.3 | Structural and Functional Impact

To assess the structural consequences of recurrent n.64_65insT, we modeled the U4/U6 snRNA duplex for wild-type (WT) and mutant. In WT, U4-U6 interactions across stem I-III regions correctly position the U6 ACAGAGA sequence for 5′ splice site. In the mutant, secondary structure predictions showed aberrant pairing between the ACAGAGA sequence and U4, which was recapitulated in the 3D model (Figure S5). These alterations suggest that n.64_65insT disrupts proper 5′ splice-site loading by mispositioning the U6 ACAGAGA motif.

3.3.1 | Molecular Dynamics Simulations

We performed 100 ns MD simulations for WT and mutant forms (n.64_65insT, and n.64_65insC). In WT, the U6 ACAGAGA motif remained unpaired and base-stacked, consistent with its role in 5′ splice-site recognition. Both insertions introduced non-native hydrogen bonds: n.64_65insT formed a solvent-exposed bulge at U4, while n.64_65insC more frequently paired with U6 (Figure S6A–C). Across 1000 WT frames, the mutants exhibited three aberrant base pairs absent in the WT (Figure S7).

MMGBSA analysis showed marked duplex destabilization in mutant forms: −177.06 kcal/mol (WT), −141.99 kcal/mol (insT), and −140.41 kcal/mol (insC), with reduced stability across the ACAGAGA region and the insertion site (Figure S8). Solvent-accessible surface area (SASA) analyses indicated increased sequestration of the ACAGAGA motif in mutants, particularly in insT (Figure S6D).

These results indicate that *RNU4-2* insertions disrupt U4/U6 pairing and misposition the ACAGAGA motif, supporting a pathogenic mechanism linked to increased A5SS usage in carriers.

3.4 | Identification of Differentially Expressed Genes and Abnormal Splicing Events

Whole-blood RNA-seq from six carriers (five with n.64_65insT and one with n.68_69insT) and ten unaffected parent controls identified 353 differentially expressed genes (DEGs; adjusted $p < 0.05$, $|\log_2 FC| > 1$; Figures S9 and S10A; Table S4). Upregulated DEGs were enriched for immune-related transcripts and multiple immunoglobulin genes, suggesting systemic immune activation.

Gene set enrichment analysis revealed 78 significantly enriched Gene Ontology Biological Process terms, grouped into five clusters based on semantic similarity: (1) immune

TABLE 1 | Variants in the critical 18–bp region.

Variant	Nucleotide description	NDD probands ($n = 2797$)		Population cohort				
				Korea4K ($n = 3617$)	All of Us ($n = 245\,400$)	gnomAD v4.1 ($n = 76\,215$)	gnomAD v4.1 EAS ($n = 2598$)	TOPMed freeze 8 ($n = 132\,345$)
12:120291826:T:TG	n.77_78insC	1	0.04%	.	.	.	.	.
12:120291835:G:GT	n.68_69insA	2	0.07%	.	.	.	.	.
12:120291839:T:TA	n.64_65insT	17 (3)	0.61%	.	.	.	.	.
Total		**20**	**0.72%**					

Note: Numbers in brackets in NDD probands columns correspond to individuals whose inheritance with unconfirmed inheritance status.
Abbreviations: EAS, East Asian; NDD, neurodevelopmental disorders.

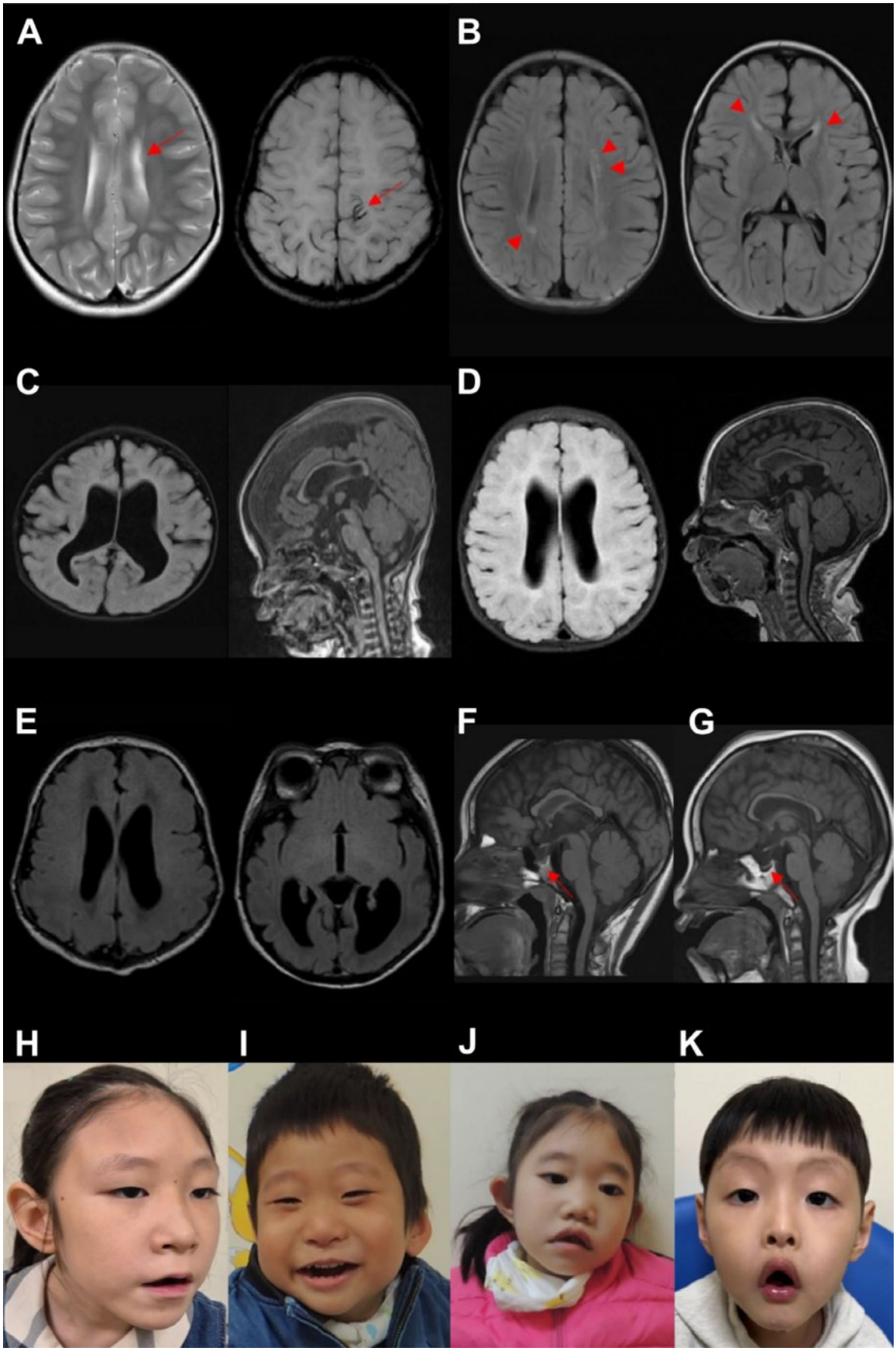

FIGURE 2 | Legend on next page.

FIGURE 2 | Neuroimaging and facial features in patients with *RNU4-2* variants. (A) The red arrows indicate the heterotopia (left) and developmental venous anomaly (right) in Patient 14. (B) The red triangles indicate the periventricular leukomalacia-like lesions identified in Patient 10. (C–E) Diffuse brain atrophy with ventriculomegaly observed in Patients 5 (C), 16 (D), and 6 (E). A dysplastic corpus callosum was also noted in Patients 5 (C) and 16 (D). (F, G) The red arrows indicate the hypoplasia of neurohypophysis identified in Patients 1 (F) and 3 (G). (H–K) Facial features of the patients. A low nasal bridge and tented upper lip were observed in Patients 1 (H), 10 (I), 3 (J), and 11 (K). [Colour figure can be viewed at wileyonlinelibrary.com]

response, (2) rRNA processing, (3) chromosome segregation, (4) organelle organization/fission, and (5) DNA metabolic processes (Figure S10B; Table S5), suggesting a broad cellular stress response.

Splicing analysis revealed a significant increase in alternative 5′ splice-site (A5SS) usage in carriers compared to controls (mean 263 vs. 253 events; Wilcoxon $p = 1.28E-02$; Figure S11). Over-representation analysis of 273 genes with significant A5SS events revealed no pathway-level enrichment, suggesting a broad transcriptome-wide impact. Intersection with 664 high-confidence NDD genes pinpointed *MAP4K4*, which showed the most significant A5SS event (Figure S12; Table S6) [8], reproducing prior findings [5]. This introduced an 8-amino acid insertion into the citron homology domain, which interacts with Rap2A (Figure S13). Protein–protein docking showed reduced binding affinity in the long isoform compared to wild-type (Table S7), suggesting destabilization of MAP4K4-Rap2A and potential impairment of autophosphorylation and neuronal morphology. To validate broader relevance, we re-examined 12 genes previously reported to undergo aberrant splicing in *RNU4-2*–associated disorders [5, 9]. *STX16*, *MAP4K4*, and *JMJD1C* (Table S8) showed significant A5SS, while others did not, likely due to reduced statistical power ($n = 16$ vs. 383 in prior studies).

3.5 | Expression Patterns and Evolutionary Conservation

BrainSpan RNA-seq data showed a distinct expression trajectory of *RNU4-2* compared to its paralog *RNU4-1* and other U4 homologs [10], with consistently higher expression from the prenatal period through early childhood, adolescence, and into adulthood (Figure S14).

ATAC-seq analysis of adult brain tissue showed *RNU4-2* had a non-significant trend toward higher chromatin accessibility than *RNU4-1* (mean 41.8 vs. 39.7, Wilcoxon $p = 0.81$; Figure S15) [11]. This directionally aligns with prior findings of significantly higher *RNU4-2* accessibility in the fetal brain [5]. Combined with expression data, these results support *RNU4-2* as the dominant functional U4 snRNA in the brain.

Finally, comparative genomic analysis further reinforced the importance of *RNU4-2*, showing fewer base differences across species than *RNU4-1* (e.g., mouse: 0 vs. 2; dog: 0 vs. 3; Figure S16). These suggest an evolutionarily conserved and indispensable role for *RNU4-2* in brain development.

4 | Discussion

This study identifies *RNU4-2* as a recurrent non-coding cause of Korean NDD, with a carrier frequency of 0.72%. Although not powered to assess ancestry effects, this recurrence highlights the need for cross-population surveillance of *RNU4-2* variants.

This study integrates structural modeling, MD, and transcriptomics. The n.64_65insT variant was shown to remodel the U4/U6 duplex and reduce accessibility of the U6 ACAGAGA motif, consistent with impaired 5′ splice-site recognition. Blood RNA-seq from carriers revealed increased A5SS usage and enrichment of gene programs related to immune activation, chromosome segregation, and DNA metabolism, supporting spliceosomal disruption as the core pathogenic mechanism for *RNU4-2*-associated NDDs.

It is important to note that the observed immune-related signatures in blood RNA-seq likely reflect systemic activation or cellular shifts, not central nervous system-specific inflammation. Similar peripheral patterns are seen in other NDDs (e.g., *MECP2*-related Rett syndrome) [12], suggesting they may be a parallel biomarker rather than a direct cause of neurodevelopmental dysfunction.

Splicing and expression analysis revealed that nine genes (e.g., *JMJD1C*, *MBOAT2*, *KAT2B*) exhibited significant splicing changes but were downregulated, suggesting loss of function. This likely drives compensatory secondary effects, such as upregulated DNA metabolism and immune pathways, consistent with a hierarchical model where primary spliceosomal disruption cascades into broader transcriptional stress.

All variants were *de novo*; in seven informative trios, the maternal allele was affected. Although no maternal mosaicism was detected, low-level gonadal mosaicism remains possible [13].

Study limitations include the use of blood RNA, modest sample size, and reliance on computational modeling. Future work in iPSC-derived neurons, brain organoid or splicing reporters will be critical to validate these findings and explore therapeutic avenues [14].

In summary, *RNU4-2* variants represent a recurrent and clinically relevant cause of early-onset NDD, supporting routine inclusion of snRNA genes in diagnostic pipelines to improve identification and enable targeted care.

Author Contributions

J.C. and J-.H.C.: Conceptualization and design of the study. S.Y.K., B.C.L., J.S.C., A.C., H.K., H.W., J.S.K., M.K., C.K.C., J.E.L., and J-.H.C.: Data acquisition. J.H., S.Y.K., S.L., T.K., S.J., W-.H.S., J.H.C., J-.H.P., D.J., and G.P.: Data analysis. J.H., S.Y.K., and S.L.: Original draft and figures. J.C. and J-.H.C.: Review and editing.

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Ethics Statement

All participants or their legal guardians provided written informed consent. Individual-level data were fully de-identified, and explicit consent was obtained for publication of clinical images. Bioresources were obtained from the Korea Biobank, which adheres to strict ethical guidelines (CODA_D22004, CODA_D23012, CODA_D23016). The study was approved by the Institutional Review Board of Seoul National University Hospital (2407–195-1559).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Peer Review

For transparency, the peer review documents associated with this article are available at https://doi.org/10.1111/cge.70154.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** cge70154-sup-0001-DataS1.docx. **Figure S1:** Demographic characteristics of the Seoul National University Hospital (SNUH) and Korea Biobank cohort (KBB). **Figure S2:** Kinship analysis of 13 probands with whole-genome sequencing data. **Figure S3:** IGV data of the five individuals with *de novo* variants and their parents, revealing the parent-of-origin. **Figure S4:** Sanger sequencing electropherogram of the two individuals with *de novo* variants and their parents, revealing the parent-of-origin. **Figure S5:** Impact of the n.64_65insT variant on the U4/U6 snRNA interaction. **Figure S6:** Structural dynamics of wild-type and mutant U4/U6 complexes. **Figure S7:** The frequencies of hydrogen bond atom pairs observed during 100 ns simulation. **Figure S8:** Residue-wise decomposition of the binding energy. **Figure S9:** PCA of RNA-seq transcriptomic profiles demonstrating the mitigation of batch effects. **Figure S10:** Analysis of differential gene expression in patients with *RNU4-2* variants. **Figure S11:** The number of splicing events across five types analyzed by rMATS. **Figure S12:** Sashimi plot of *MAP4K4*. **Figure S13:** A5SS event in *MAP4K4*. **Figure S14:** Expression patterns of *RNU4-2* during brain development, based on BrainSpan data. **Figure S15:** Chromatin accessibility levels of *RNU4-1* and *RNU4-2* in human adult brain prefrontal cortex. **Figure S16:** Evolutionary conservation and genomic alignment of *RNU4-2* and *RNU4-1*. **Table S1:** Summary statistics of sequencing data of the SNUH and KBB cohort. **Table S2:** Clinical presentations of patients. **Table S3:** Detailed phenotypic characteristics of *RNU4-2*–related NDD patients. **Table S4:** Differentially expressed genes identified through the comparison of blood transcriptomes between patients and their unaffected parents. **Table S5:** Enriched GO BP terms in mutation carriers. **Table S6:** All A5SS events called by rMATS. **Table S7:** Predicted binding affinities and interfacial contacts between MAP4K4 isoforms and RAP2A using PRODIGY. **Table S8:** Replicated A5SS events previously reported in *RNU4-2*–related studies.