Original research

Clinical, neuroimaging and molecular characteristics of *PPP2R5D*-related neurodevelopmental disorders: an expanded series with functional characterisation and genotype–phenotype analysis

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ABSTRACT

Background Variants in *PPP2R5D*, affecting the regulatory B56δ subunit of protein phosphatase 2A (PP2A), have been identified in individuals with neurodevelopmental abnormalities. However, the molecular and clinical spectra remain incompletely understood.

Methods Individuals with *PPP2R5D* variants were enrolled through Simons Variation in Individuals Project/Simons Searchlight. Data were collected from medical history interviews, medical record review, online validated instruments and neuroimaging review. Genetic variants were biochemically characterised.

Results We studied 76 individuals with *PPP2R5D* variants, including 68 with pathogenic de novo variants, four with a variant of uncertain significance (VUS) and four siblings with a novel dominantly inherited pathogenic variant. Among 13 pathogenic variants, eight were novel and two (p.Glu198Lys and p.Glu200Lys) were highly recurrent. Functional analysis revealed impaired PP2A A/C-subunit binding, decreased short linear interaction motif-dependent substrate binding or both—with the most severe phenotypes associated with variants that completely retained one of these binding characteristics and lost the other—further supporting a dominant-negative disease mechanism. p.Glu198Lys showed the highest C-binding defect and a more severe clinical phenotype. The inherited p.Glu197Gly variant had a mild substrate binding defect, and three of four VUS had no biochemical impact. Common clinical phenotypes were language, intellectual or learning disabilities (80.6%), hypotonia (75.0%), macrocephaly (66.7%), seizures (45.8%) and autism spectrum disorder (26.4%). The mean composite Vineland score was 59.8, and most participants were in the 'moderate to low' and 'low' adaptive levels in all domains.

Conclusion Our study delineates the most common features of *PPP2R5D*-related neurodevelopmental disorders, expands the clinical and molecular spectrum and identifies genotype–phenotype correlations.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Previous studies have identified *PPP2R5D* genetic variants as a cause of developmental disorders in children including intellectual disability and macrocephaly.

WHAT THIS STUDY ADDS

⇒ Our significantly expanded series of individuals with *PPP2R5D* variants enabled us to better characterise the association of this gene with neurodevelopmental disorders, neurobehavioural issues and other notable clinical features, as well as further our understanding of the molecular function and biochemical properties of causal *PPP2R5D* variants.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The clinical, molecular and functional data from this study could have important consequences for clinical evaluations and aid in the future treatment for affected individuals.

INTRODUCTION

De novo genetic variants in major Ser/Thr-specific protein phosphatases, also known as the protein phosphatase 2A (PP2A) family of genes, were first identified in 2015 as causal of several neurodevelopmental disorders (NDDs) and intellectual disability phenotypes.[1–4] PP2A phosphatases are multisubunit enzymes, encoded by 19 human genes, three of which have so far convincingly been implicated in NDDs, namely, *PPP2CA* (MIM# 618354),[5] *PPP2R1A* (MIM# 616362)[1 2 6] and *PPP2R5D* (MIM# 616355).[1 2 7–9] *PPP2CA* encodes the catalytical PP2A Cα subunit, harbouring the dephosphorylating ability of the complex.[10] De novo missense and nonsense *PPP2CA* variants have been reported in 16 individuals, are dispersed throughout the protein and mainly result in loss-of-function either by affecting PP2A complex formation or by inhibiting intrinsic PP2A activity.[5] *PPP2R1A* encodes the PP2A scaffolding Aα subunit, an all-helical structural protein that has no other function than keeping the phosphatase complex together.[10] Most of the 37 reported *PPP2R1A* missense variants affect the intrahelical repeat loops of the Aα protein, resulting in altered binding to a varying

number of specific PP2A regulatory B-type subunits and/or the catalytical C subunit, suggestive of a loss-of-function pathogenic mechanism.[2 6 11 12] Finally, 31 individuals have been reported with genetic variants in *PPP2R5D*, encoding the regulatory PP2A B56δ subunit.[2 8 9 13–20] In general, PP2A regulatory B-type subunits confer tissue-specific and cell-specific expressions, subcellular localisation and substrate specificity to the PP2A complexes and are thus indispensable for PP2A regulation.[21 22] Specifically, the ubiquitous B56δ subunit is highly expressed in the human brain, where PP2A–B56δ holoenzymes play an important role in neuronal signalling processes.[3 23] Although just a few neuronal PP2A–B56δ substrates have been identified,[24–27] B56 subunits define PP2A substrate specificity in part through their high binding affinity for proteins harbouring a short linear interaction motif (SLiM), denoted as LxxIxE motif.[28 29] These SLiM harbouring proteins are postulated to act as PP2A–B56 substrates or substrate scaffolds.[30 31] Biochemical characterisation of six reported *PPP2R5D* variants has revealed mild to severe A/C subunit binding defects in five variants (p.Glu198Lys, p.Glu200Lys, p.Pro201Arg, p.Trp207Arg and p.Glu420Lys),[2 32] while binding to SLiM harbouring substrates was not tested. Moreover, unbiased phosphoproteomic analysis of HEK293 cells with heterozygous knock-in of the p.Glu420Lys variant revealed an increase in AKT–mTOR signalling as a major functional consequence, with AKT kinase as the presumed affected substrate.[32]

In this study, we sought to characterise the clinical and molecular spectrum of *PPP2R5D*-related NDDs by systematically collecting medical, neurodevelopmental and neurobehavioural data using standardised tools on a cohort of 76 individuals with *PPP2R5D* variants registered as part of the Simons Variation in Individuals Project (SVIP)/Simons Searchlight. Variants were confirmed de novo in 68 individuals, with one familial case (four siblings with a maternally inherited variant) and four individuals with variants of uncertain significance (VUS) due to unknown inheritance. Detailed biochemical analysis, in terms of both A/C subunit and SLiM-containing substrate binding, revealed a functional deficit in 13 of 16 identified variants, reclassifying three of four VUSs as likely benign. The data presented in this study substantially expand the known clinical and molecular spectrum of *PPP2R5D*-related NDDs, as well as provide a functional framework for genotype–phenotype analysis aiding in interpretation of novel variants.

METHODS

Phenotypical data collection

Data on participant phenotypes are available from the *PPP2R5D* Simons Searchlight Single Gene Dataset V.7. Standardised medical history interviews were performed by telephone with certified genetic counsellors using previously published methods.[33] *PPP2R5D* genetic variants were identified through clinical exome sequencing or multigene panel sequencing for intellectual disability (ID), developmental delay (DD), autism spectrum disorder (ASD), and clinical genetic test reports were reviewed and verified through Simons Searchlight. Images were analysed for any structural abnormalities in the cerebral cortex, white matter, basal ganglia, thalami, cerebellum, corpus callosum, ventricular size and overall cerebral symmetry.

Evaluated phenotype data

Validated online instruments in the Simons Searchlight include the Rare Epilepsy Network survey (https://www.epilepsy.com/clinical_trials/rare-epilepsy-network), the Third Edition of the Vineland Adaptive Behavioural Scale (VABS), Second Edition (Vineland II), and the Child Behavioural Checklist (CBCL) (2–5 years and 6–18 years). The VABS was either administered by a telephone interview with a trained genetic counsellor or obtained by online questionnaires by primary caregivers. The VABS was analysed by domain and subdomain scores and an adaptive behavioural composite score. The CBCL captures behavioural characteristics of children at two ages: 2–5 years of age and 6–18 years of age. Both age groups report similar information, and the average T-scores of behavioural domains and the numbers of individuals in the normal, borderline-clinical or clinical range are reported. For individuals who took the survey multiple times at different ages, only one response per individual was included and the most recent response was analysed.

To assess clinical severity and derive relevant genotype–phenotype correlations of individuals with *PPP2R5D*-related disorders, we used VABS-3 Adaptive Behaviour Composite (Vineland) scores and a heuristic clinical severity score consisting of five clinical phenotypes based on the most common characteristics of this series. Participants were given one point with each of the following three phenotypes: macrocephaly, seizures, eye or visual conditions, and half points with each of the following two phenotypes: gastroesophageal reflux disease (GERD) or constipation. In parallel, we classified *PPP2R5D* genetic variants into three functional subgroups based on their binding capacity, particularly C-binding and SLiM-dependent substrate (liprin α1) binding. We investigated whether there is a correlation between the clinical severity scores and functional subgroups, as well as among individuals with the most common genotypes (p.Glu198Lys, p.Glu200Lys and p.Trp207Arg, and all individuals with variants at amino acid position 251).

Biochemical assays

All 16 identified *PPP2R5D* variants were generated by PCR-based site-directed mutagenesis (primer sequences provided in online supplemental table 1), cloned into a common backbone vector (pWPXL) and expressed as GFP (green fluorescent protein)-tagged fusion proteins in HEK293T cells. GFP pull-down experiments were completed and analysed by immunoblotting, as previously described.[2] Blots were developed on an ImageQuant LAS4000 scanner (GE Healthcare) using Western Bright ECL (Advansta) and the following antibodies: mouse anti-C and anti-A (kind gift from Professor S Dilworth, Middlesex University, London, UK); mouse anti-GFP (Abcam, clone 9F9.F9), rabbit anti-liprin-α1 (ProteinTech); secondary HRP-coupled anti-mouse (Dako) and anti-rabbit (Cell Signalling). All densitometric quantifications were performed with Image Studio Lite software V.5.2. Data from the variants were always compared with wild-type (WT) values that were set at 100% in each experimental replicate.

Statistical analysis

Statistical analysis and relative graph generation were performed in GraphPad Prism V.9.3.1. The appropriate statistical test was chosen based on the type of comparison of interest. Relevant comparisons of clinical data were performed using the Wilcoxon test. For biochemical assays, all data are from $n \geq 3$. Statistical comparative analysis was assessed with one-sample student t-tests using Graphpad Prism 8.4.2 software; p values below 0.05 were considered significant ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

Table 1 Pathogenic variants in *PPP2R5D* (N=72)

cDNA change	Amino acid change	ACMG classification	Individuals (n)	Mean % A binding	Mean % C binding	Mean % Liprin α1 binding
c.590A>G	p.Glu197Gly	Pathogenic (PM1, PM2, PM5, PP2, PP3, PP5)	5°	86.80	94.05	54.52*
c.589G>A	p.Glu197Lys	Pathogenic (PM1, PM2, PP5, PM5, PP2, PP3)	2	74.42	59.63	39.39***
c.592G>A	p.Glu198Lys	Pathogenic (PP5, PM1, PM2, PP2, PP3)	33	29.75***	9.54***	78.44
c.598G>A	p.Glu200Lys	Pathogenic (PP5, PM1, PM2, PS3, PP2, PP3)	11	59.64**	53.6***	4.01***
c.599_602delAGCCinsGGCA	p.Glu200_Pro201delinsArgHis	Likely pathogenic (PP5, PM2, PP2, PP3)	1	34.44***	5.29***	25.03***
c.619T>C	p.Trp207Arg	Pathogenic (PP5, PM1, PS1, PM5, PM2, PP2, PP3)	4	13.75***	3.38***	63.97
c.632A>C	p.Gln211Pro	Pathogenic (PVS1, PM2, PP5, PM1, PP2, PP3)	2	54.73*	29.88***	8.56***
c.752A>C	p.Asp251Ala	Pathogenic (PM1, PM2, PM5, PP5, PP2, PP3)	3	33.63***	12.75***	59.05*
c.751G>C	p.Asp251His	Pathogenic (PM1, PM2, PM5, PP2, PP3, PP5)	1	9.42***	1.39***	48.30***
c.752A>T	p.Asp251Val	Pathogenic (PM1, PM2, PM5, PP5, PP2, PP3)	4	39.79**	2.599***	69.32**
c.751G>T	p.Asp251Tyr	Pathogenic (PM1, PM2, PM5, PP5, PP2, PP3)	2°°	7.888***	0.6081***	20.97*
c.758G>C	p.Arg253Pro	Pathogenic (PM1, PM2, PM5, PP2, PP3, PP5)	1	2.487***	3.511***	24.31**
c.1258G>A	p.Glu420Lys	Pathogenic (PP5, PM2, PP2, PP3)	4	78.85	88.72	23.04***

Transcript ID: NM_006245. *P≤0.05, **P≤0.01, ***P≤0.001, °Inherited variant seen in one family, °°Inheritance for one individual is not known.

RESULTS

Molecular findings

From the Simons Searchlight data, a total of 16 pathogenic or likely pathogenic *PPP2R5D* variants were identified, only five of which have been previously reported (p.Glu197Lys, p.Glu198Lys, p.Glu200Lys, p.Trp207Arg and p.Glu420Lys)[2 8 9] (table 1). Of those 16 variants, p.Glu198Lys was the most recurrent variant, identified in 33 independent probands, followed by p.Glu200Lys (n=11 probands). Collectively, four variants affecting amino acid Asp251 (p.Asp251Ala, p.Asp251Tyr, p.Asp251His and p.Asp251Val), which are not previously reported and resided immediately adjacently to the reported p.Glu250Lys variant,[18] were present in 10 probands. All pathogenic and likely pathogenic variants were missense with only one non-frameshift indel (p.Glu200_Pro201delinsArgHis) identified. In total, 68 probands had de novo variants, while four individuals from a single family had a novel dominantly inherited pathogenic variant (p.Glu197Gly). All variants are reported in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) as pathogenic or likely pathogenic, were absent from gnomAD V.21.1.1 (https://gnomad.broadinstitute.org/) and were predicted as deleterious using REVEL (with scores >0.8) and CADD (scores >29).[34 35] Four individuals had VUS in *PPP2R5D* (online supplemental table 2), one of which was identical to one of the newly identified de novo variants (p.Asp251Tyr) and shown to affect protein function. We therefore reclassified this variant as pathogenic. All identified genetic variants and their distribution within the crystal structure of PP2A–B56γ and the primary amino acid structure of B56δ are shown in figure 1. On review of all genetic findings, 14 individuals in this series have at least one additional genetic variant besides *PPP2R5D* (online supplemental table 3), most of which are VUS and many of which are inherited from unaffected parents and are therefore not likely causal of disease.

Functional assays

In order to study changes in PP2A–B56δ holoenzyme formation, WT *PPP2R5D* and 16 *PPP2R5D* variants were expressed as GFP-tagged proteins in HEK293T cells and assessed for their ability to interact with endogenous PP2A A and C subunits. Except for p.Ile230Thr (A-binding but not C-binding defect) and p.Leu313Val (C but not A subunit-binding defect), all observed binding effects were fully concordant for PP2A A and C subunits (figure 2A,B). Moreover, in accordance with previous data,[2] mild to severe A/C binding defects were observed for 10 out of 16 *PPP2R5D* variants tested (figure 2A,B). Only variants p.Glu197Lys, p.Glu197Gly, p.Glu420Lys and p.Phe473Leu showed unaffected A/C binding compared with *PPP2R5D* WT (figure 2A,B). We next assessed potential binding deficiencies of the variants to a random, canonical B56 interactor/substrate containing a typical B56 subunit LxxIxE binding motif.[28] Our unpublished, mass spectrometry-based *PPP2R5D* interactomics analyses revealed liprin-α1 (encoded by *PPFIA1*) as a strong PP2A–B56δ interactor containing at least one canonical B56-binding SLiM motif, in accordance with data of others.[28 31 36] Interestingly, although liprin-α1 strongly bound toWT *PPP2R5D*, mild to severe Liprin-α1 binding defects were observed to several (11/16) *PPP2R5D* variants (figure 2C). For p.Trp207Arg, the binding defect was borderline significant (trend: p=0.07). Liprin-α1 binding was unaffected for p.Glu198Lys, p.Leu313Val, p.Phe473Leu and p.Ile230Thr, of which the latter three were VUS. Based on these data, we concluded that out of 16 *PPP2R5D* variants tested, eight showed both an A/C and liprin-α1 binding defect (p.Glu200Lys, p.Glu200_Pro201delinsArgHis, p.Gln211Pro, p.Asp251Tyr, p.Asp251Ala, p.Asp251Val, p.251AspHis and p.Arg253Pro); two showed only an A/C binding defect (p.Glu198Lys and p.Trp207Arg); and three showed only a liprin-α1 binding defect (p.Glu197Lys, p.Glu197Gly and p.Glu420Lys), categorising them all as pathogenic. Based on these data, we were able to stratify variants into three functional subgroups. The first group includes variants that demonstrate exclusively reduced C-binding activity (p.Glu198Lys and p.Trp207Arg); the second group includes variants that demonstrate exclusively reduced liprin-α1 binding activity (p.Glu420Lys, p.Glu197Lys and p.Glu197Gly); and the third group includes variants that demonstrate a reduction of both C-binding and liprin-α1 binding activity (p.Glu200Lys, p.Gln211Pro, p.Asp251Tyr/Val/His/Ala, p.Glu200_Pro201delinsArgHis and p.Arg253Pro). We further functionally interrogated the VUS identified in this study (p.Ile230Thr, p.Leu313Val and p.Phe473Leu), and they either did not show any impairments at all (p.Phe473Leu) or showed a discordant and rather small A-binding or C-binding defect, suggesting they are all likely non-pathogenic and can thus be reclassified as likely benign.

Clinical findings

Data were collected from all 76 participants, with detailed analysis of the clinical data from the 73 individuals with pathogenic or likely pathogenic variants. One individual did not provide

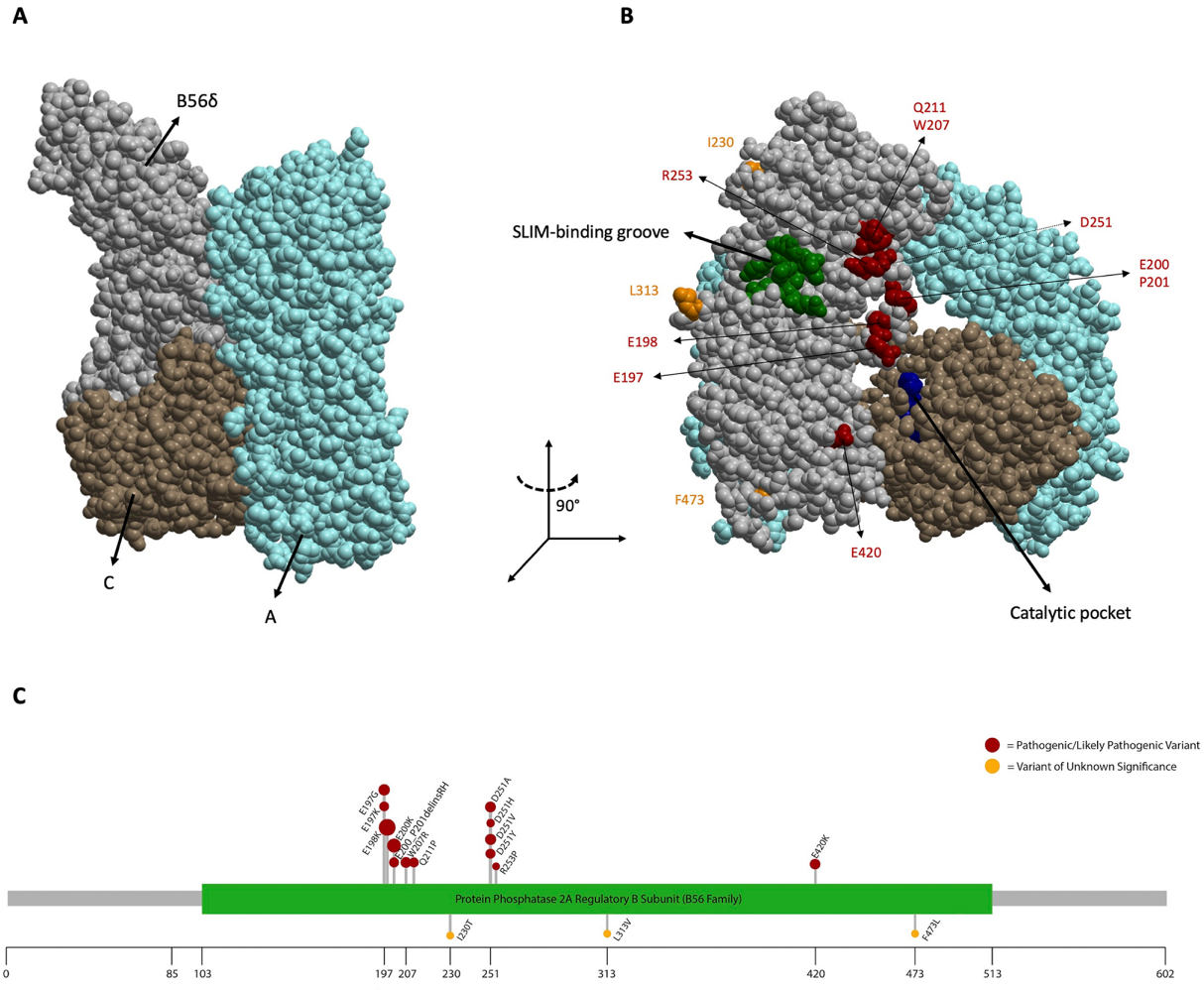

Figure 1 Structural representation of the *PPP2R5D* variants reported in this study within the PP2A holoenzyme and gene diagram of *PPP2R5D*. (A) Side view of the PP2A holoenzyme; brown denotes the catalytical C subunit; blue denotes the scaffolding A subunit; and grey denotes the B56γ1 subunit (most related to B56δ, of which no crystal structure exists). (B) 90° rotation of (A) with red denoting pathogenic variants, orange denoting variants of unknown significance, green denoting the SLiM-binding domain of B56 and blue denoting the catalytical pocket of the C subunit. The structure was generated based on PP2A–B56γ1 crystallographic data (PDB 2IAE)[45] and visualised in Molsoft MolBrowser V.3.9-2d software (ICM-Broser-Pro). All affected residues in B56δ are 100% conserved in B56γ1. (C) Lollipop diagram of the *PPP2R5D* protein showing location of pathogenic and likely pathogenic variants in *PPP2R5D* (in red). The location of variants of uncertain significance within the gene is also shown (in yellow). PP2A, protein phosphatase 2A; SLiM, short linear interaction motif.

sufficient clinical data for inclusion. Of the 72 remaining probands, 39 (54.2%) identified as female and 34 (45.8%) identified as male. Ages at the time of enrolment ranged from 1.3 years to 44.9 years of age. Thirty-eight (52.8%) identified as white; 27 (37.5%) did not indicate their race or ethnicity; six (8.3%) indicated more than one race; and one (1.4%) identified as Asian. From the medical histories of the 72 individuals, hypotonia (75.0%) and macrocephaly (66.7%) were the most commonly reported features. A significant number of individuals had seizures (45.8%), GERD (27.8%), strabismus (27.8%), diarrhoea (23.6%), clumsiness (20.8%), failure to thrive (19.4%) and astigmatism (16.7%). The frequency of all major clinical issues is shown in figure 3A–C, with more detailed data regarding epilepsy and macrocephaly shown in online supplemental tables 4 and 5.

Of the 33 individuals who had seizures (online supplemental table 4), the most common genetic variant was p.Glu198Lys (20/33, 60.6%) and p.Glu197Gly (3/33, 9.1%) . The most common seizure type was tonic clonic (or grand mal) seizures (12/33, 36.4%), followed by myoclonic seizures (10/33, 30.3%). The mean age of onset for seizures was 2.3 years (range birth-17.8 years). Seizure frequency ranged greatly among individuals from >100 episodes per day in the most severe instances to one episode per year. For the three individuals who reported having 100 or more seizures per day, all had different *PPP2R5D* variants as well as types of seizures. One child with the variant p.Glu198Lys had myoclonic seizures starting during infancy with more than 100 episodes occurring daily. The other participant, with p.Asp251Val, had spasms beginning shortly after birth with a frequency of 120 episodes per day. The third individual, with p.Gln211Pro, had simple partial seizures, starting when the individual was a toddler, with a frequency of 200 episodes per day. Six individuals reported current use of antiepileptic drugs (AEDs), and four participants reported a reduction in seizures and one reported improved development with AED therapy. Those who saw a reduction in seizure frequency were on clobazam, lamotrigine or valproic acid, and the individual with improved development was on levetiracetam. Of those individuals who had macrocephaly or megalencephaly (n=48), 28 (58.3%) had the recurrent p.Glu198Lys variant. However,

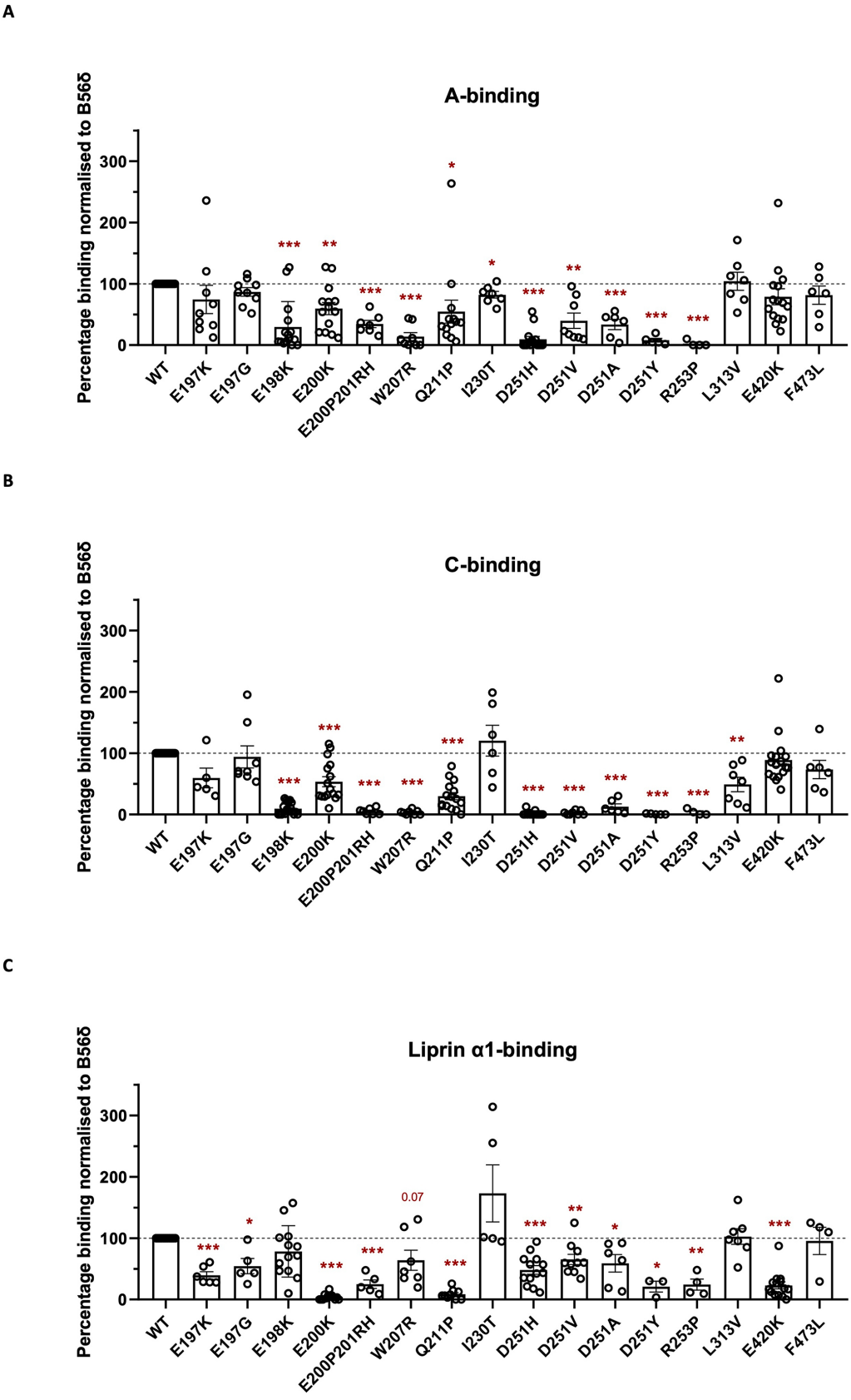

Figure 2 Functional characterisation of *PPP2R5D* variants. GFP-tagged WT and mutant B56δ subunits were purified from transfected HEK293T cells by GFP pulldown. Interaction of endogenous (A) PP2A-Aα subunit, (B) PP2A-Cα subunit and (C) the SLiM-containing substrate liprin-α1 to B56δ variants was determined by immunoblotting. Results were quantified and depicted as the average±SEM of the ratios of the quantified endogenous protein signal to the quantified GFP signal in ratio to the WT B56δ interaction (set to 100% in each experiment), as determined in at least three independent experiments (n≥3). A one-sample t-test (compare to 100%) was used to determine statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001). PP2A, protein phosphatase 2A; SLiM, short linear interaction motif; WT, wild type.

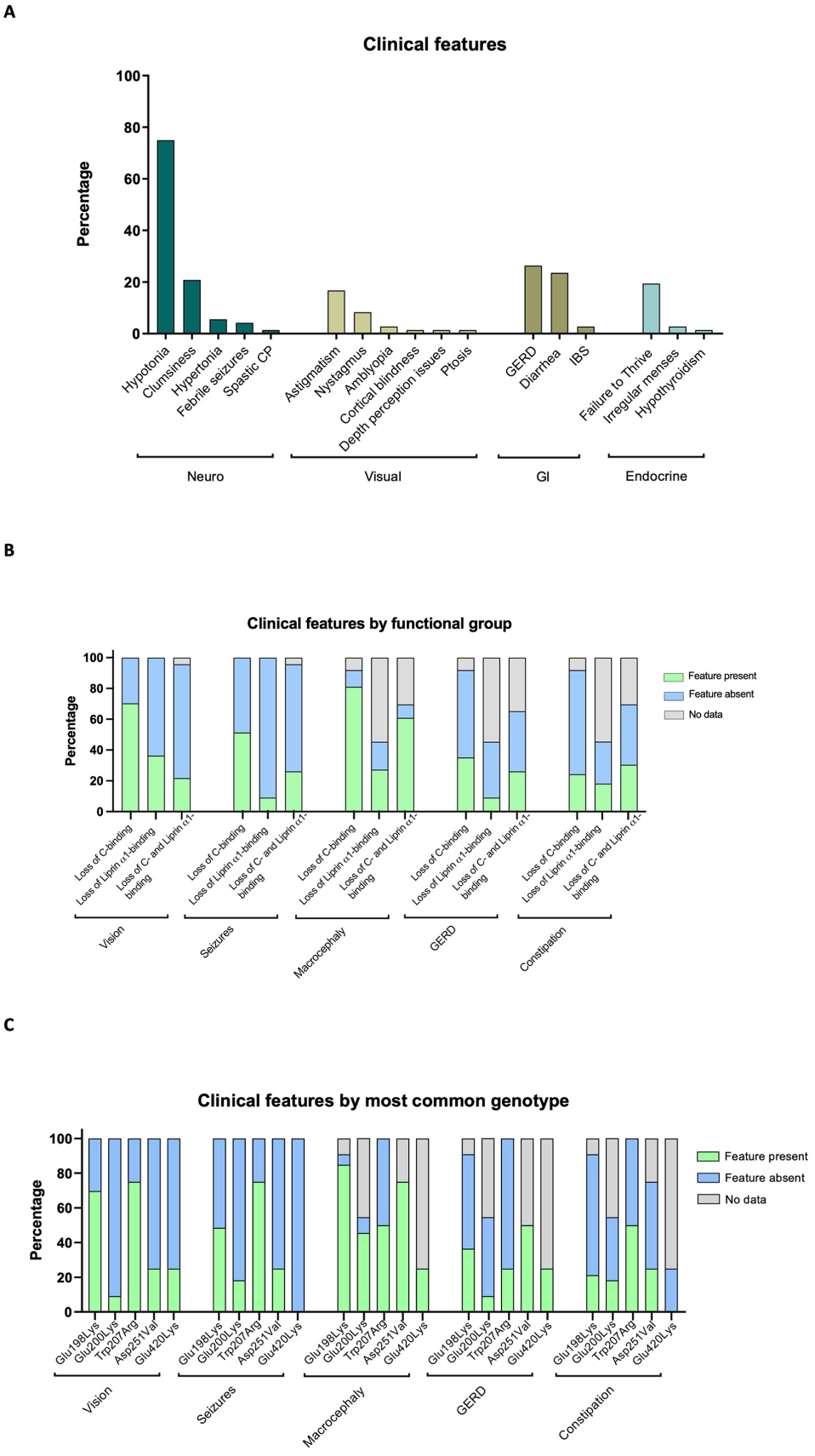

Figure 3 Summary of the most common phenotypical features of individuals with pathogenic and likely pathogenic variants in *PPP2R5D*. (A) Bar graph showing the key phenotypical features reported in individuals with *PPP2R5D* variants across the entire cohort including neurological issues, vision problems, GI issues and endocrine issues. (B) Comparison of the frequency of the five canonical phenotypes used for heuristic genotype–phenotype analysis between the three functional subgroups. (C) Comparison of the frequency of the five clinical phenotypes between the most common genotypes present in this cohort. GERD, gastroesophageal reflux disease; GI, gastrointestinal.

notably two individuals without macrocephaly, as well as three non-respondents had this variant as well. Eight participants with macrocephaly (16.3%) had a genetic variant at amino acid 251 (p.Asp251Ala, p.Asp251His, p.Asp251Tyr and p.Asp251Val). All other variants seen in this cohort affected five or less participants and were also seen in similar numbers among those who did not have macrocephaly or who did not respond (online supplemental table 5).

Brain MRI studies were obtained on 15 individuals as part of their standard clinical care. The most common findings included diffuse macrocephaly or megalencephaly without major structural anomalies. Minor features included focal cortical abnormalities (n=2), cavum septum pellucidum et vergae (n=2), mesial temporal sclerosis (n=1), plagiocephaly (n=1), white matter abnormalities (n=2) and mild ventriculomegaly (n=1) (online supplemental figure 1).

On analysis of neurobehavioural assessments, a total of 48 individuals with pathogenic and likely pathogenic *PPP2R5D* variants completed the Vineland assessment. The average Vineland score was 58.2±13.7. All individuals had low levels below 70, with the exception of one participant who had an adequate (86–114) adaptive level and six with moderate low (71–85) adaptive level. The average score for the 11 subdomains across the cohort was 8.3 (>2 SD below the population average score of 15). Of these subdomains, expressive communication and personal daily living skills had the lowest average scores (7.2 and 6.6, respectively). However, receptive communication, domestic daily living skills and fine motor skills subdomains had the highest average scores (9.6, 9.1 and 9.0, respectively). Detailed Vineland domain scores are graphically shown in figure 4A and online supplemental table 6).

On analysis of the CBCL survey data, 24 out of 72 participants completed the survey for children aged 2–5 years and 14 for children aged 6–18 years. Among the younger cohort, the areas in which several respondents had T-scores in the borderline clinical or clinical range were withdrawn behaviour (16.7% clinical and 25.0% borderline clinical), wants attention (8.3% clinical and 20.8% borderline clinical) and austism (as defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM-5)) (16.7% clinical and 16.7% borderline clinical). Domains in which participant T-scores were in the normal range were anxiety/depression, aggressive behaviour, stress, DSM5 anxiety problems (0% clinical and 4.2% borderline clinical), emotional reactivity and DSM5 oppositional defiant (0% clinical and 12.5% borderline clinical) (online supplemental table 7 and supplemental figure 2). For those aged 6–18 years, domains with an elevated number of respondents with T-scores in the borderline clinical or clinical range were withdrawn behaviour (16% clinical and 24% borderline clinical), wants attention (8% clinical and 20% borderline clinical) and DSM5 autism (16% clinical and 16% borderline clinical). Other areas in which more participants had T-scores in the clinical and borderline clinical range were DSM5 depression (21% clinical and 43% borderline clinical), social problems, DSM5 ADHD (29% clinical and 21% borderline clinical), total problems (36% clinical and 21% borderline clinical), attention problems (36% clinical and 50% borderline clinical) and thought problems (57% clinical and 29% borderline clinical). Areas in which many individuals had a T-Score in the normal range were anxiety/depression, rule-breaking behaviour (0% clinical and 14% borderline clinical) and DSM5 anxiety problems (0% clinical and 7% borderline clinical) (online supplemental table 8 and supplemental figure 3).

Next, we sought to examine genotype–phenotype relationships. Using the heuristic clinical severity score mentioned in the Methods section, among the 55 participants who provided phenotypical data, 85.5% had macrocephaly; 63.6% had eye condition; 49.1% had seizures; 36.4% had GERD; and 32.7% had constipation. The average heuristic score based on the above five phenotypes was 2.33±0.85 (online supplemental table 9). Overall, clinical features were more consistently present in individuals with variants associated with loss of C binding (p.Glu198Lys and p.Trp207Arg) (figure 3B,C). We further clustered all Vineland and CBCL survey data for individuals with the most common genotypes, namely, p.Glu198Lys and p.Glu200Lys variants and variants at amino acid residue 251 (p.Asp251Ala, p.Asp251Tyr, p.Asp251His and p.Asp251Val).

On the Vineland Adaptive Behaviour Scales, the number of responses for these three mutation groups were 25, five and seven for p.Glu198Lys, p.Glu200Lys and variants at amino acid residue 251, respectively. Notably, individuals with p.Asp251Ala, p.Asp251Tyr, p.Asp251His and p.Asp251Val, and p.Glu200Lys variants had better expressive language skills, personal care and social skills compared with individuals with p.Glu198Lys and p.Trp207Arg (figure 4B,C). Similarly, when analysing the CBCL measures for these three mutational groups, the numbers of responses were 18, six and eight for p.Glu198Lys, p.Glu200Lys, p.Asp251Ala, p.Asp251Tyr, p.Asp251His and p.Asp251Val, respectively. Increased aggression was reported more in individuals with p.Glu198Lys and p.Glu200Lys compared with individuals with variants of amino acid residue 251. Individuals with p.Glu200Lys demonstrated increased oppositional behaviour with age. All groups, except those with amino acid changes involving residue 251, had increased attention difficulties and hyperactivity with age (online supplemental figure 2 and 3). When examining responses for the largest mutational group (p.Glu198Lys), all individuals had a mix of stable, increased or decreased aggression, though differences were small. Most children had increasing attention deficit and hyperactivity with age, though caregivers also reported that attention/hyperactivity is not a concern. Most children across all mutational groups had increasing behavioural concerns as they got older (online supplemental figure 2 and 3).

DISCUSSION

The genetic basis of NDDs in children is extremely heterogenous with a rapidly growing number of causal variants being identified underlying these phenotypes. There is a particularly well-established association between specific neurodevelopmental issues and physical features including a notable association between neurodevelopmental issues and brain size abnormalities with many affected individuals harbouring genetic variants in key cell signalling pathways including the Ras/mitogen activated protein kinase (Ras–MAPK), the phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) pathway and others.[20] Variants in genes encoding subunits of the PP2A complex have recently been identified in children with variable degrees of ID, neurobehavioural issues including ASD and brain size abnormalities among other features.[2 5 6 8 9] Genetic variants in the *PPP2R5D* gene specifically have been identified in children with DD, ID, ASD, macrocephaly and epilepsy.[37] Review of previously published data combined with our comprehensive analysis of phenotypical, molecular and functional data, including detailed neurobehavioural assessments, helps further delineate the spectrum of *PPP2R5D*-related NDDs.

PP2A is one of two phosphatases in the body that accounts for up to 90% of Ser/Thr phosphatase activity. The phosphatase is composed of a catalytical C, substrate-binding regulatory subunit

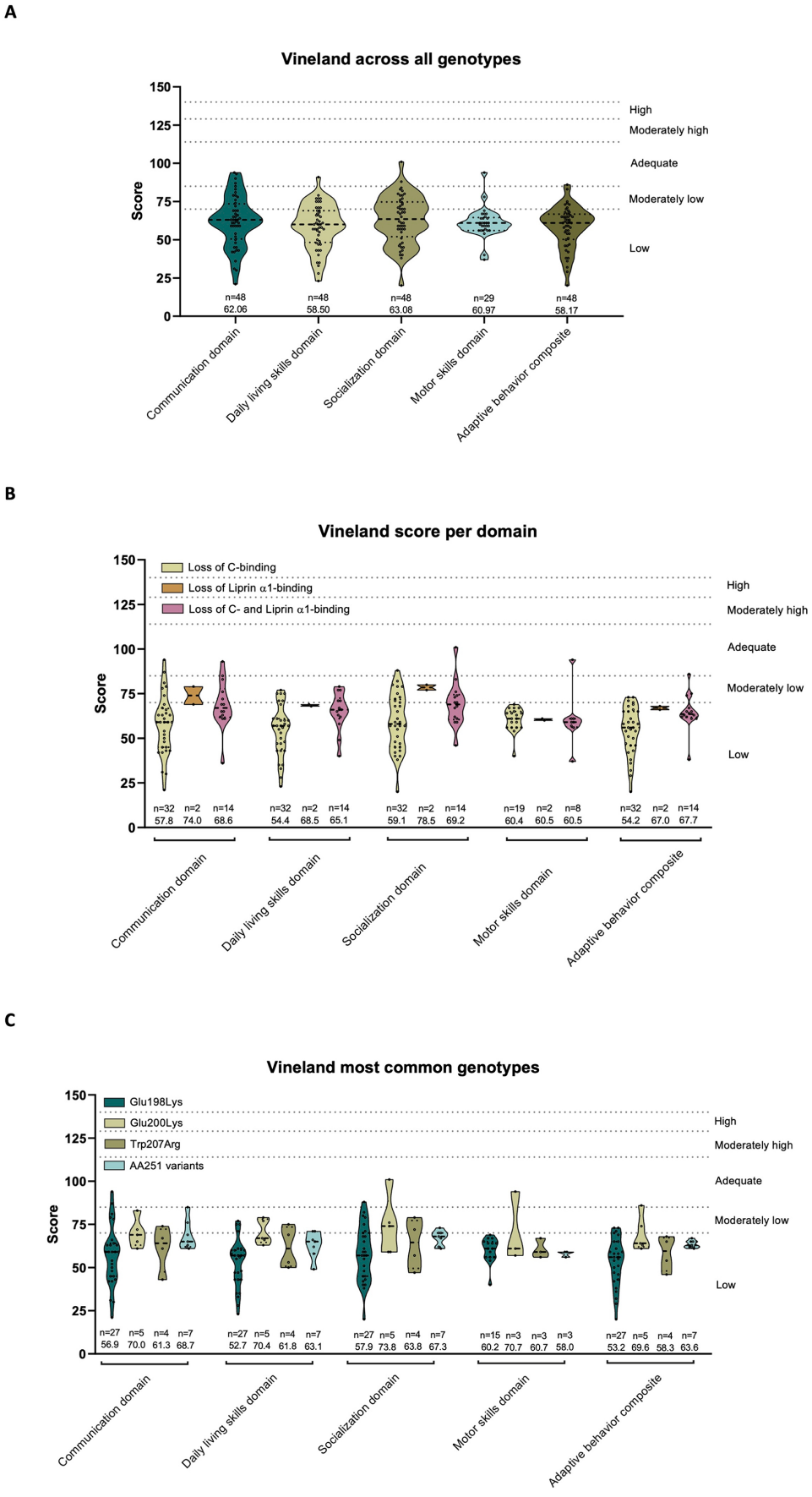

Figure 4 Vineland domain scores of individuals with pathogenic and likely pathogenic *PPP2R5D* variants. Violin plot showing (A) Vineland scores across all Vineland domains in all individuals in this cohort. (B) Comparison of Vineland scores across all domains between individuals within the three functional subgroups. (C) Comparison of Vineland scores across all domains between individuals with the most common genotypes.

Table 2 Summary of previously published *PPP2R5D* series

	Loveday *et al*[8]	Houge *et al*[2]	Shang *et al*[9]	Yeung *et al*[20]	Hetzelt *et al*[13]	Kim *et al*[14]	Walker *et al*[18]	Yan *et al*[19]	Madaan *et al*[15]	Moirangthem *et al*[17]	Maines *et al*[16]	This study
Total N of affected individuals	3	11	7	2	1	3	1	1	1	1	1	72
Hypotonia	N/A	10 (90.1%)	7 (100%)	N/A	1 (100%)	1 (33.3%)	N/A	1 (100%)	1 (100%)	1 (100%)	1 (100%)	54 (75.0%)
Macrocephaly	N/A	7 (63.6%)	6 (85.7%)	N/A	1 (100%)	1 (33.3%)	1 (100%)	1 (100%)	0	1 (100%)	1 (100%)	48 (66.7%)
Language disorder	N/A	11 (100%)	N/A	N/A	1 (100%)	2 (66.7%)	N/A	1 (100%)	N/A	N/A	N/A	43 (59.7%)
Epilepsy	N/A	3 (27.3%)	1 (14.3%)	2 (100%)	1 (100%)	0	N/A	N/A	1 (100%)	N/A	1 (100%)	33 (45.2%)
ASD	N/A	N/A	5 (71.4%)	1 (50%)	N/A	0	N/A	N/A	N/A	N/A	N/A	19 (26.0%)
ID/DD	3 (100%)	11 (100%)	7 (100%)	2 (100%)	1 (100%)	3 (100%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	1	12 (16.7%)
Early-onset Parkinsonism	N/A	N/A	N/A	N/A	1 (100%)	3 (100%)	1 (100%)	N/A	N/A	N/A	N/A	N/A

ASD, autism spectrum disorder; DD, developmental delay; ID, intellectual disability; N/A, not available.

B, and scaffolding subunit A that links B and C. PP2A catalytical C subunit, ubiquitously expressed in almost every tissue, is most abundant in heart and brain,[4 38 39] underscoring its major functions in these two tissues. Many PP2A trimeric complexes are considered tumour suppressive and are functionally inactivated in cancer.[40] Genetic variants, reduced protein levels or functional inhibition caused by increased expression of cellular inhibitors are found in a variety of tumour types.[40 41] The combinatorial assembly of one C (out of two isoforms), one A (out of two isoforms) and one B subunit (out of at least 19 isoforms) gives rise to >70 different combinations of holoenzyme complexes. PP2A complexes encompassing the B56δ regulatory subunit (encoded by *PPP2R5D*) show high expression in the brain and have been shown to, directly or indirectly, regulate dephosphorylation of several neuronal and non-neuronal substrates, including tau,[26] GSK-3β kinase,[25 42] AKT kinase,[32] tyrosine hydroxylase[43] and DARPP-32.[24] As is the case for all B56-type subunits, B56δ in part defines PP2A substrate specificity through high-affinity binding to cellular proteins harbouring a LxxIxE motif, which was identified as a B56-specific SLiM.[28 29] Nevertheless, not all B56δ substrates contain such a SLiM, suggesting that yet other means of substrate specificity regulation should exist.

The majority of *PPP2R5D* variants in the literature and our study were missense variants, affecting three different regions within the B56δ protein (figure 1B): (1) a conserved acidic loop facing the C subunit (193-EFDP**EED**E**P**TLEAA**W**PHLQ-211), (2) another conserved region, residing downstream of the SLiM-binding domain and also facing the C subunit (246-LFDS**ED**P**R**ERD-256) and (3) a conserved helix more downstream in the protein (415-HFQVA**E**RALYYWN-427). The majority of pathogenic variants described here and in the literature are within the acidic loop, changing one of six amino acids (Glu197, Glu198, Glu200, Pro201, Trp207 and Gln211) often into a positively charged lysine or arginine residue.[2 8 9] One variant also showed a two-amino acid substitution in this loop (Glu200-Pro201 was changed into Arg200-His201). Enriched with negatively charged glutamate and aspartate residues, studies show that this loop is thought to aid in binding the subunit to the PP2A A and C subunits.[2] Previous studies and our current data showing impaired A/C subunit binding in p.Glu198Lys, p.Gly200Lys, p.Glu200_Pro201delinsArgHis, p.Trp207Arg, and p.Gln 211Pro are consistent with this hypothesis. However, others have identified this acidic loop as a substrate specifying domain as well,[44] which also seems to be supported by our current data, since liprin-α1 binding was impaired in p.Glu197Lys and p.Glu197Gly (with a milder impairment of the Gly substitution than the Lys substitution) to p.Glu200Lys, p.Glu200_Pro201delinsArgHis, and p.Gln211Pro variants, despite the fact that the SLiM-binding pocket was intrinsically unaffected in these variants. This is suggestive of additional regulation of SLiM-dependent substrate binding to B56δ by the acidic loop. These novel substrate binding data additionally suggest that variants p.Glu198Lys and p.Trp207Arg, which show a significant A/C subunit binding defect but unaltered substrate binding, are predicted to be more efficient in 'substrate-trapping' and exerting a dominant-negative effect (by competing with the WT protein through their retained substrate binding) than any other of the acidic loop variants, which, besides a C subunit binding defect, all also show a moderate (p.Glu197Lys and p.Glu197Gly) to severe (p.Glu200Lys, p.Glu200_Pro201delinsArgHis and p.Gln211Pro) impairment in substrate binding. Accordingly, p.Glu198Lys and p.Trp207Arg variants seem to be associated with the most severe clinical phenotypes.

The second most frequently altered region within B56δ (11/72, 15.3%) and the literature[18] contains three affected amino acids (Glu250, Asp251 and Arg253), which on missense mutation result in a very profound A/C subunit binding defect, and a moderate to severe substrate binding defect, at least for the Asp251 and Arg253 variants, tested here. Phenotypically, individuals with these variants seem to belong to the less severely affected end of the *PPP2R5D* spectrum, probably because their impairment in both substrate and A/C binding makes them less successful in competing with the WT B56δ protein than variants that fully retain one of these characteristics (eg, p.Glu198Lys). Finally, a minority of the cases (4/72, 5.6%) presented with the p.Glu420Lys variant,[9] affecting a more C-terminally located area in the B56δ protein of unknown function. Here too, a dominant-negative disease mechanism is suggested, as our functional analyses demonstrated a severe liprin-α1 binding defect to this variant, while A/C binding was hardly affected, thus allowing efficient competition with the WT B56δ protein for A/C binding. This appears in contrast with data from Papke *et al*,[32] who observed a 50% A/C subunit binding reduction and unaltered AKT binding to this variant.[32] However, as far as we know, AKT does not engage the B56δ SLiM binding pocket to interact with B56δ, suggesting a specific effect of this missense variant on SLiM-dependent substrate binding only. As this is expected to affect multiple B56δ substrates/interactors in the cell, this would suggest a severe impact on the functionality of this variant. Notably and logically, this impact is expected to decrease in p.Glu197Lys and p.Glu197Gly, both other variants showing retained A/C binding, but far less impaired liprin-α1 binding—fully consistent with their decreased clinical severity. The latter is especially apparent for p.Glu197Gly, identified in this study as an inherited variant from a mildly affected mother and transmitted to four children.

Our study has some limitations. Due to ascertainment bias, we cannot exclude the possibility that some individuals included in this study may have been published previously. However, we expect this overlap to be minimal as most families were identified and ascertained recently after these initial publications (summarised in table 2). The Simon VIP datasets do not include detailed growth measurements or clinical photos to assess for facial dysmorphisms. Due to the limited number of brain MRI scans, it is challenging to draw conclusions regarding the prevalence of brain abnormalities in this cohort, though megalencephaly and mild–moderate ventriculomegaly and hydrocephalus were previously noted.[2] Another limitation of this study is that most reported individuals are children and therefore data regarding phenotypical features that may emerge or evolve over time are limited. Establishing a molecular diagnosis in older individuals with ID may prove challenging due to barriers related to access to genetic care and lack of family members available for comparison studies such as trio exome or genome sequencing, and we believe this further underlies the enrichment of a paediatric population in our datasets. Some information was obtained from interviews and surveys administered to caregivers is subject to recall bias. There are also limitations of the biochemical studies. The assays do not directly assess dephosphorylation of the, so far unknown, biologically relevant substrates in these disorders. Additional functional studies might reveal more subtle molecular defects, such as, for example, putative changes in substrate specificity.

In summary, de novo variants in the *PPP2R5D* gene are associated with a broad NDD characterised by neurodevelopmental issues, macrocephaly and hypotonia. Other notable features include seizures, feeding difficulties, vision problems and a wide range of neurobehavioural issues. Genetic variants in other members of the PP2A phosphatase family of genes—*PPP2R1A*, *PPP2CA*, *PPP2R5B* and *PPP2R5C*—also cause neurodevelopmental issues, although it is challenging to determine if these genes cause a similar phenotype to *PPP2R5D* based on the limited number of affected individuals reported in the literature, to date. Additional studies are needed to fully understand the phenotypical and molecular heterogeneity of the PP2A family of genes. Our current clinical, molecular and functional data, based on a significantly expanded series, suggest that not all *PPP2R5D* variants might affect B56δ biochemical properties in the same way, where in some cases variants cause a more severe clinical phenotype (p.Glu198Lys, p.Trp207Arg and p.Glu420Lys), while in others giving rise to less severe clinical presentations (p.Asp251Ala, p.Asp251Tyr, p.Asp251His, p.Asp251Val and p.Glu200Lys). This conclusion is based on a twofold hypothesis. The first is that variants that are only affected in A/C binding or in substrate binding (and thus fully retain either substrate binding or A/C binding) are likely more efficient competitors of the WT protein compared with variants that show binding deficiencies to both A/C subunits or SLiM-engaging substrates. Second, the severity of the former variants relies on the severity of the observed binding defect (eg, p.Glu420Lys>p.Glu197LysK>p.Glu197Gly). However, how these different molecular consequences in specific *PPP2R5D* variants eventually affect B56δ function in brain or other tissues needs to be further established in cell and animal models of the disease. Nevertheless, the identification of potentially different *PPP2R5D* functional subgroups may have important consequences for clinical follow-up and impact future treatment avenues in affected individuals.

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Ethics approval This study involves human participants and was approved by the institutional review board (IRB) at Columbia University. A subset of participants was also enrolled in the Developmental Brain Disorder Research Program at Seattle

Children's Research Institute under an IRB-approved protocol (IRB #13291) for review of brain MRI data. Participants were enrolled through Simons Variation in Individuals Project (SVIP), later renamed Simons Searchlight (https://www.simonssearchlight.org). The participants gave informed consent to participate in the study before taking part.

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