

Supplemental Note: Case reports

Clinical description of the affected individuals with the *de novo* p.(Glu41Lys) variant

Individual 1 (Tours)

Second child from unrelated parents, he was born after an uneventful pregnancy with normal birth parameters. He presented severe hypotonia, poor contact and swallowing difficulties. Developmental milestones were significantly delayed as he started to walk at 7 years and was not toilet trained at adulthood. Curiously, even though he was still able to walk, he progressively moved only on his knees. He had severe intellectual disability (ID) with no language and developed behavioral problems with temper tantrums, aggressive behavior and self-harm. On physical examination he had mild microcephaly (-2.5 SD), severe kyphoscoliosis, small genitalia and poorly developed secondary sexual characteristics. In time, he acquired facial dysmorphic features that were not seen in childhood. In particular he had brachycephaly, deep set eyes, short philtrum, prognathism, full lips and macrostomia. Standard diagnostic work-up, including array-CGH, was normal. Brain MRI performed at 37 years 11 months (reviewed by W. Dobyns) showed low forehead, normal extra-axial spaces, normal gyral pattern and cortex except for mildly prominent sulci, mildly small and up-rotated hippocampi, normal basal ganglia and thalami, diffuse and prominent perivascular spaces more numerous posteriorly, mildly enlarged 3rd ventricle, widely separated and mildly enlarged lateral ventricles, very thin anterior commissure, complete agenesis of the corpus callosum (ACC) with thick Probst bundles, normal brainstem, diffuse mild cerebellar hypoplasia with superimposed mild cerebellar atrophy, and normal size posterior fossa. The key features thus were ACC, hippocampal dysplasia, and mild cerebellar hypoplasia.

Individual 2 (Amsterdam)

This patient was the first child of healthy non-consanguineous parents after they had three miscarriages. He was born after an uncomplicated pregnancy of 42 weeks with a birthweight of 3042 grams. His development was very delayed with hypotonia and poor contact. He started sitting up at the age of 1.5 years and was not able to walk at the age of six. He had a severe retardation with no speech and was very uncomfortable. He also had major sleep problems. On physical examination he was very restless and was screaming. Head circumference was at -1.5 SD and he had a high nasal bridge and a short philtrum.

On brain MRI on the age of 4 years and 5 months there were an abnormally thin anterior commissure, complete agenesis of the corpus callosum with secondary radial gyral pattern over both mesial hemispheric surfaces, bilateral large Probst bundles, and mildly small and uprotated hippocampi. The cerebellum and posterior fossa were borderline small.

(Bill Dobyns : REVIEW: MRI at 4 years 5 months showed normal forehead, extra-axial spaces, normal gyral pattern and cortex for except radial pattern over mesial hemispheric surfaces, small and up-rotated hippocampi (but low resolution), normal basal ganglia, thalami and white matter, mildly enlarged 3rd ventricle, widely separated and mildly enlarged lateral ventricles, very thin anterior commissure with small bundle extending up to location of corpus callosum, complete ACC with thick Probst bundles, normal brainstem, and normal to borderline small cerebellum and posterior fossa size. The key features thus were ACC, and hippocampal dysplasia).

Individual 3 (Rotterdam)

The history of this 13 yr old boy has been previously reported, as exome sequencing had revealed an X-linked variant of unknown significance in *SYP*. The variant p.(Thr198Ile) appeared later to be frequently encountered in hetero- and hemizyosity in the general population (GnomAD), and therefore considered not to be responsible for the phenotype. Reanalysis of the full exome data of the Patient and his parents, finally showed the *de novo* *DPYSL5* mutation. At the last observation, at the age of 11 years, he presented a severe neurodevelopmental disorder, cognitive development being consistent with the age of 7 months, severe hypotonia and progressive severe convex kyphoscoliosis, generalized tonic-clonic seizures controlled by sodium valproate, no speech development, strabismus, limited interaction with environment. The brain MRI performed at the age of 14 months showed agenesis of the corpus callosum, hippocampal dysplasia, mild/moderate hypoplasia of cerebellar vermis and hemispheres. MRI of the spine at the age of 11 years revealed progression of the cerebellar atrophy.

Individual 4 (Birmingham)

This patient was the second child of healthy Caucasian parents. Pregnancy and delivery were normal. Hypotonia lead to delay in learning to sit. Left congenital hip dislocation (managed by

brace and later surgery) and global developmental delay were diagnosed at 9 months of age. Growth including occipital-frontal circumference (OFC) has been normal.

As an adult she uses a wheelchair for mobility and has ataxia. She can speak in short phrases, inconsistently indicates her toileting needs. She has periods of agitated behavior if excited and in response to noise. Apart from delayed puberty her general health is good.

Agenesis of the corpus callosum and hypoplastic cerebellum were reported on brain MRI. She was following recruited to the DDD study (Patient inclusion number 258864) where the *de novo* c.121G>A mutation in *DPYSL5* was found.

Individual 5 (Birmingham)

This is a 22 year-old female with agenesis of corpus callosum, dysmorphic features, severe intellectual disability and motor disability. She is the first child from unrelated parents. She was born at 39 weeks of pregnancy following spontaneous labor and ventouse extraction with normal birth parameters. Developmental milestones were significantly delayed with independent sitting achieved at 20 months and only walking aided with a walking frame to date. She has severe ID with no language and developed behavioral problems with aggressive behavior, screaming and a disturbed sleeping pattern. A high pain threshold has been noted by her parents. Autistic features are present but no formal autism spectrum disorder (ASD) diagnosis has been made. She exhibits athetoid movements, truncal ataxia, repetitive stereotypic hand movements and head nodding. She had delayed puberty, with secondary sexual characteristics developing at the age of 14 years but menstruation has not commenced. She has a history of left strabismus, severe gastro-oesophageal reflux and severe scoliosis requiring surgical interventions. She was previously investigated with array-CGH which showed no copy number imbalance and *FOXG1* and *MECP2* gene testing with no pathogenic variants found. She was then recruited for the DDD study (inclusion number DDD-BWH:258661:15-09-16) in which the *de novo* c.121G>A mutation in *DPYSL5* was found.

Individual 6 (Akron)

See Table 1

Individual 7 (Prague)

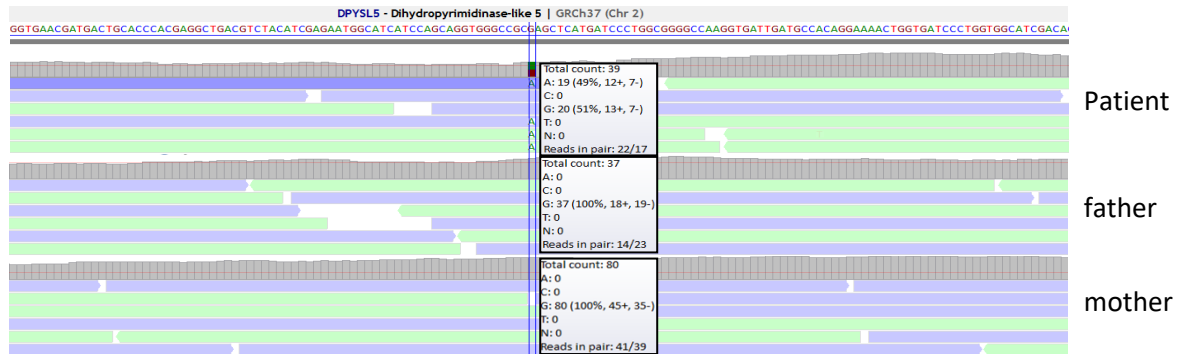
The girl was born in term from the 5th pregnancy of healthy unrelated Caucasian parents. Weak fetal movements and placental abruption were noted during the pregnancy. The weight of the newborn was 2600 g (6th centile) and length was 49 cm (42nd centile). The girl showed unilateral hypotonia. Her developmental milestones were delayed (sitting at 12 months, walking at 5.5 years of age). She developed focal seizures which resolved after prolonged carbamazepine treatment. The girl has never developed speech, she must use diapers and her intellectual disability was evaluated at the last examination at 17.5 years of age as severe. She shows attacks of aggression. Brain examination revealed agenesis of corpus callosum, periventricular leukomalacia, Dandy-Walker malformation, moderate-sized encephalocele and intracranial hypertension. She has microcephaly and strabism but no remarkable facial dysmorphism. Her stature is short with severe kyphoscoliosis and gibbus rotation, and her body shows severe declination to the left side.

Individual 8 (San Francisco)

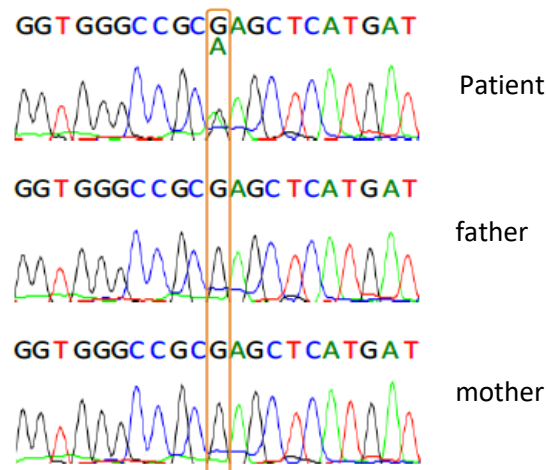
See Table 1

Figure S1. Integrative Genomics Viewer (IGV) screenshots and Sanger sequencing analysis of the *de novo* DPYSL5 c.121G>A substitution (encoding p.(Glu41Lys)) in Individual 1.

A



B



A- Integrative Genomics Viewer (IGV) screenshots of the *de novo* DPYSL5 c.121G>A substitution (encoding p.(Glu41Lys)) in Individual 1 and his parents.

B- Representative Sanger sequencing electropherograms of the region included in exon 2 of DPYSL5 in the Subject and parents' respective genomic DNAs.

Figure S2. Integrative Genomics Viewer (IGV) screenshots of the *de novo* *DPYSL5* c.121G>A substitution (encoding p.(Glu41Lys)) in Individual 2.

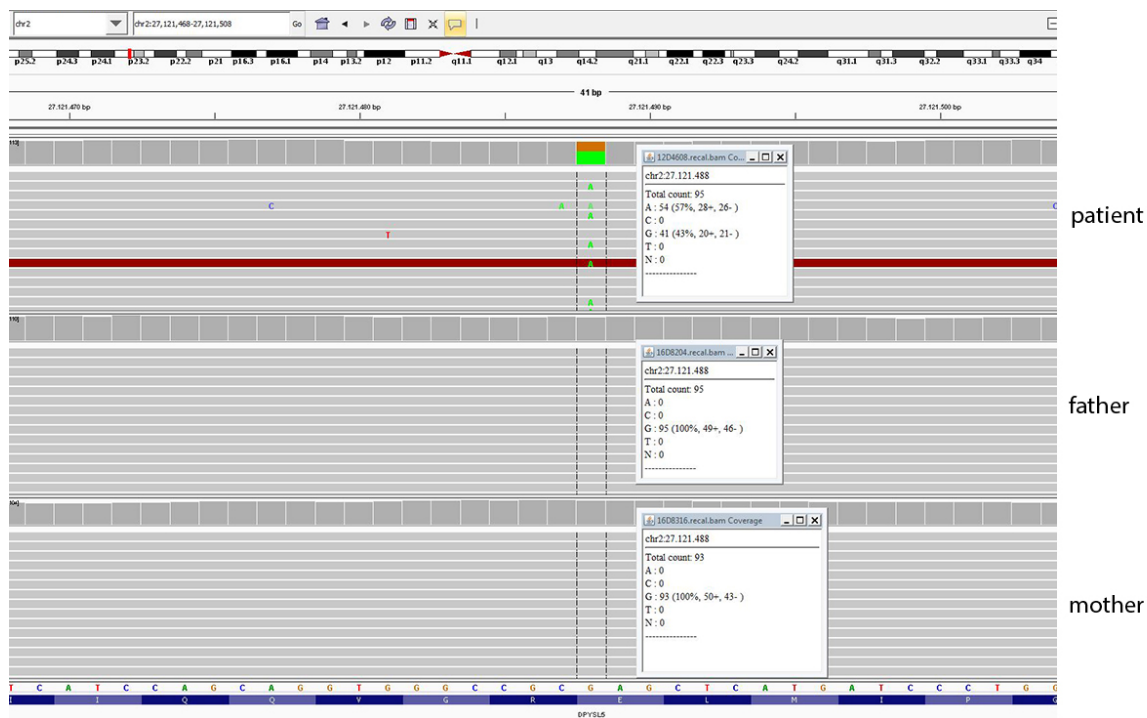
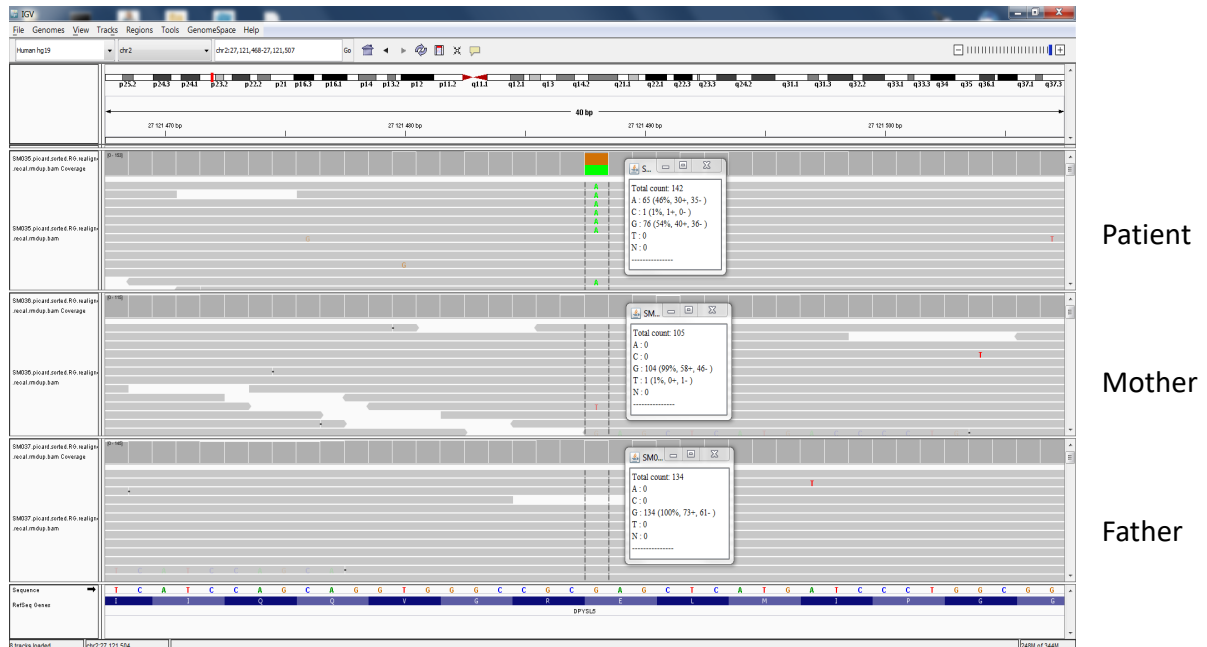
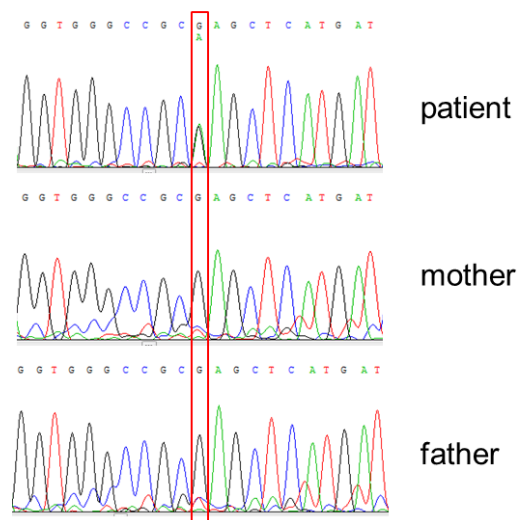


Figure S3. Integrative Genomics Viewer (IGV) screenshots and Sanger sequencing analysis of the *de novo* *DPYSL5* c.121G>A substitution (encoding p.(Glu41Lys)) in Individual 7.

A

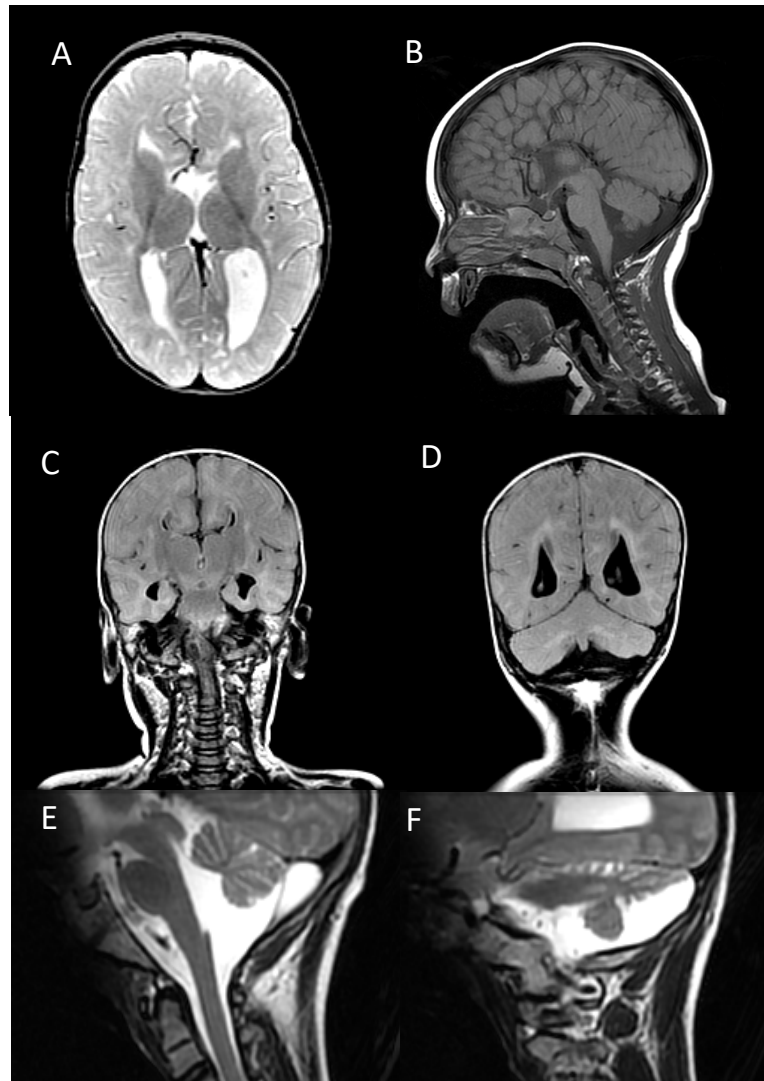


B



A- Integrative Genomics Viewer (IGV) screenshots of the *de novo* *DPYSL5* c.121G>A substitution (encoding p.(Glu41Lys)) in Individual 7 and her parents.
 B- Representative Sanger sequencing electropherograms of the region included in exon 2 of *DPYSL5* in the Subject and her parents' respective genomic DNAs.

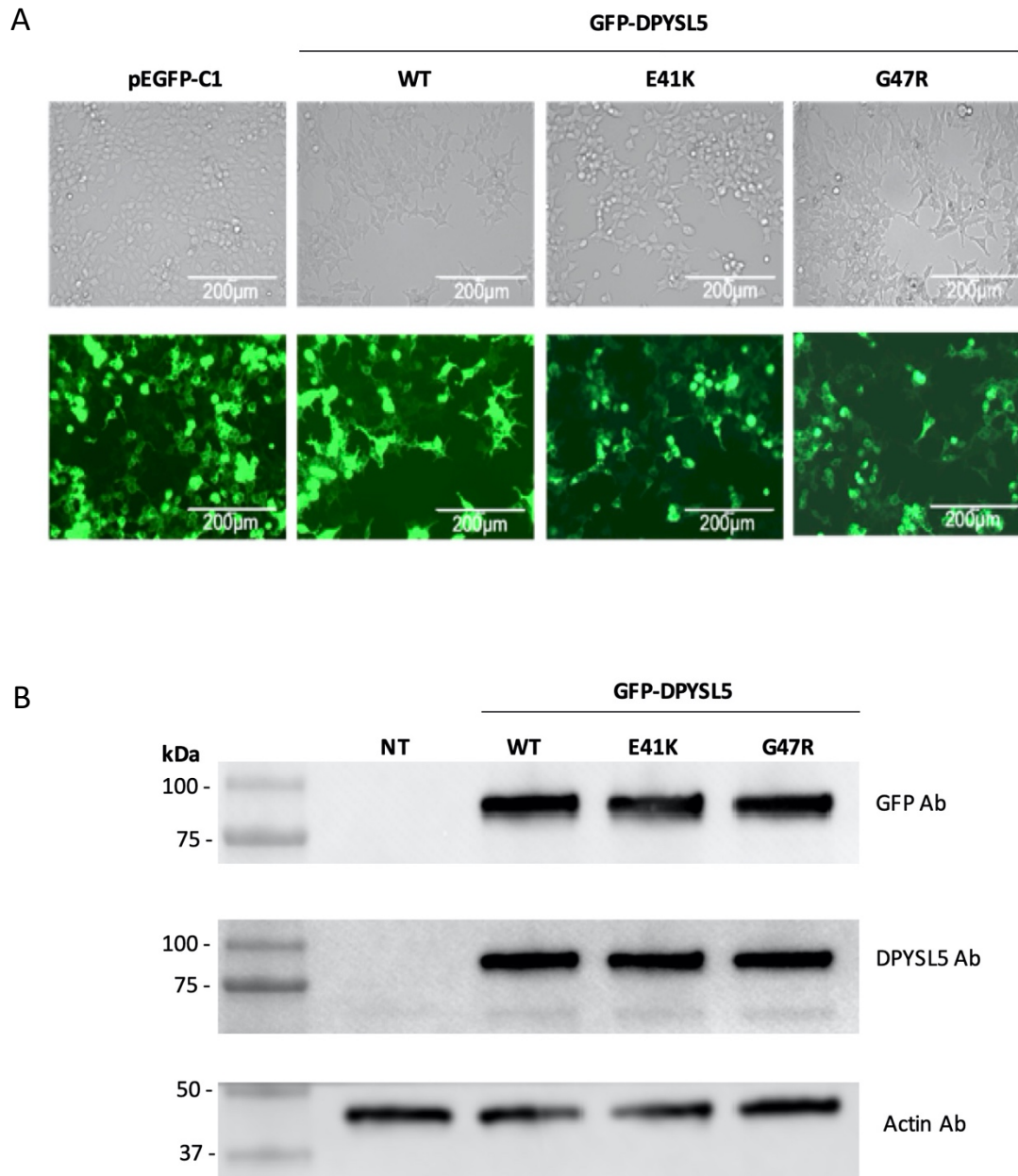
Figure S4. Brain Magnetic Resonance Imaging data from Individual 3 (Rotterdam)



A-D) Brain MRI at the age of 14 months, clockwise: A- T2 weighted image showing ACC and enlarged lateral ventricles. B- Sagittal T1weightedd midline image showing ACC and mild hypoplasia of the inferior cerebellar vermis. C- Coronal FLAIR image showing ACC and bilateral hippocampal dysplasia. D- Coronal FLAIR image showing hypoplasia of the vermis and cerebellar hemispheres.

E,F- MRI at the age of 12 yr, sagittal T2 weighted images showing progressive cerebellar atrophy.

Figure S5. Protein levels and localization of WT and variant forms of GFP-DPYSL5 in transfected HEK293T cells.



A- Images (Phase contrast and Fluorescent GFP) of HEK293T cellular cultures transfected with pEGFP-C1 (control), GFP-DPYSL5 WT, GFP-DPYSL5 p.Glu41Lys (E41K) or GFP-DPYSL5 p.Gly47Arg (G47R) plasmids and observed 48 hours post transfection. Scale bar, 200 µm.

B- Representative Immunoblotting of total protein lysates (30 µg) of untransfected (NT) or transfected HEK293 cells with DPYSL5 plasmids and tested with GFP, DPYSL5 and Actin Antibodies (n=3 independent transfections).

Raw images and data

1. Raw quantified numerical data on neuronal morphology and for statistical analyses used to generate the graphs included in Figure 2b

Axon length

| Axon length (µm) | | | | | Normality and Lognormality Tests | | | | |
|------------------|------|-----|------|------|-------------------------------------|--------|--------|--------|--------|
| | EGFP | WT | E41K | G47R | | EGFP | WT | E41K | G47R |
| | 194 | 104 | 507 | 378 | Test for normal distribution | | | | |
| | 326 | 299 | 683 | 295 | | | | | |
| | 330 | 362 | 492 | 308 | D'Agostino & Pearson test | | | | |
| | 280 | 398 | 510 | 282 | K2 | 2,529 | 0,7233 | 1,612 | 3,227 |
| | 169 | 284 | 200 | 511 | P value | 0,2823 | 0,6965 | 0,4466 | 0,1992 |
| | 691 | 253 | 527 | 316 | Passed normality test (alpha=0.05)? | Yes | Yes | Yes | Yes |
| | 300 | 238 | 990 | 320 | P value summary | ns | ns | ns | ns |
| | 406 | 479 | 400 | 500 | | | | | |
| | 559 | 347 | 202 | 480 | | | | | |
| | 808 | 227 | 335 | 227 | | | | | |
| | 341 | 498 | 405 | 531 | | | | | |
| | 283 | 460 | 321 | 200 | | | | | |
| | 268 | 445 | 792 | 406 | | | | | |
| | 686 | 691 | 642 | 665 | | | | | |
| | 461 | 490 | 336 | 779 | | | | | |
| | | | 679 | 270 | | | | | |
| Number of values | 15 | 15 | 16 | 16 | | | | | |

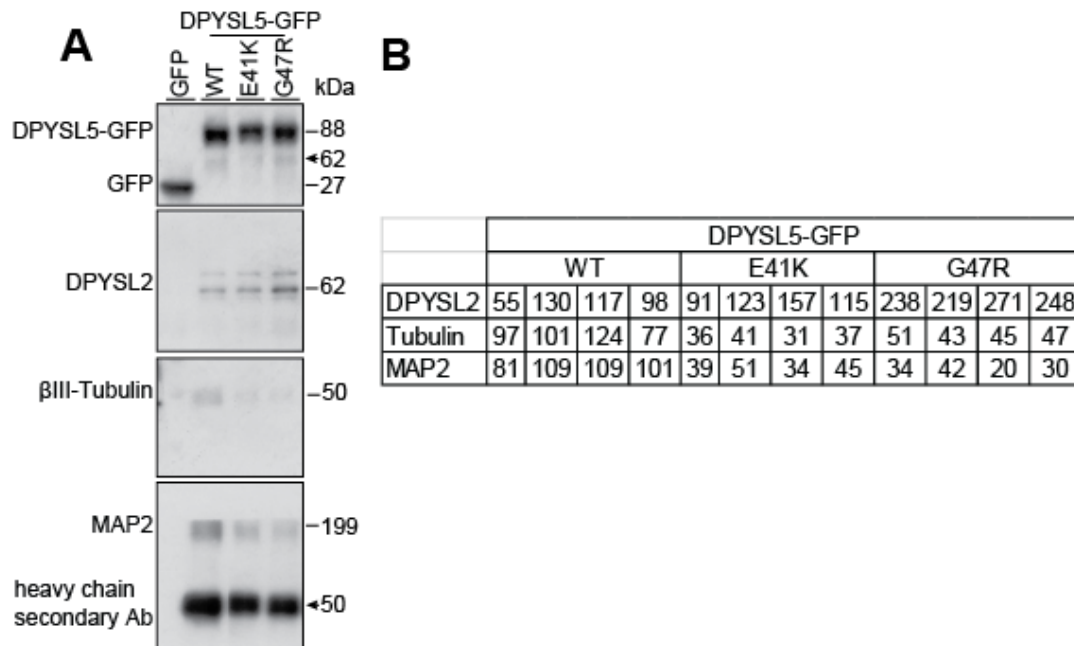
| Ordinary One-Way ANOVA | | | | | ANOVA summary | | | | |
|-----------------------------------|------------|-----------------|--------------|---------|------------------|--------|--|--|--|
| Number of families | 1 | | | | F | 1,488 | | | |
| Number of comparisons per family | 6 | | | | P value | 0,2272 | | | |
| Alpha | 0,05 | | | | P value summary | ns | | | |
| Tukey's multiple comparisons test | Mean Diff, | 95% CI of diff, | Significant? | Summary | Adjusted P Value | | | | |
| EGFP vs. WT | 35,13 | -140,2 to 210,5 | No | ns | 0,9514 | | | | |
| EGFP vs. E41K | -94,51 | -267,1 to 78,07 | No | ns | 0,4749 | | | | |
| EGFP vs. G47R | 2,55 | -170,0 to 175,1 | No | ns | >0,9999 | | | | |
| WT vs. E41K | -129,6 | -302,2 to 42,94 | No | ns | 0,2047 | | | | |
| WT vs. G47R | -32,58 | -205,2 to 140,0 | No | ns | 0,9589 | | | | |
| E41K vs. G47R | 97,06 | -72,72 to 266,8 | No | ns | 0,4369 | | | | |

Total dendrite length

| Total dendritic length (µm) | | | | | Normality and Lognormality Tests | | | | |
|-----------------------------|------|------|------|------|-------------------------------------|--------|--------|--------|--------|
| | EGFP | WT | E41K | G47R | | EGFP | WT | E41K | G47R |
| | 1720 | 683 | 1632 | 1572 | Test for normal distribution | | | | |
| | 1273 | 832 | 1601 | 2437 | | | | | |
| | 2242 | 1257 | 1109 | 1568 | D'Agostino & Pearson test | | | | |
| | 1610 | 692 | 2425 | 2019 | K2 | 0,1005 | 0,832 | 0,3527 | 0,1973 |
| | 1275 | 1169 | 1668 | 467 | P value | 0,951 | 0,6597 | 0,8383 | 0,9061 |
| | 1321 | 945 | 2099 | 1447 | Passed normality test (alpha=0.05)? | Yes | Yes | Yes | Yes |
| | 2395 | 280 | 1765 | 1499 | P value summary | ns | ns | ns | ns |
| | 1782 | 1034 | 1171 | 1135 | | | | | |
| | 1659 | 1253 | 884 | 960 | | | | | |
| | 2276 | 741 | 798 | 1634 | | | | | |
| | 1581 | 822 | 1389 | 1049 | | | | | |
| | 864 | 741 | 956 | 1453 | | | | | |
| | 1617 | 1185 | 1873 | 877 | | | | | |
| | 2413 | 1753 | 1833 | 1839 | | | | | |
| | 1793 | 1320 | 1143 | 1373 | | | | | |
| | | | 1473 | 2016 | | | | | |
| Number of values | 15 | 15 | 16 | 16 | | | | | |

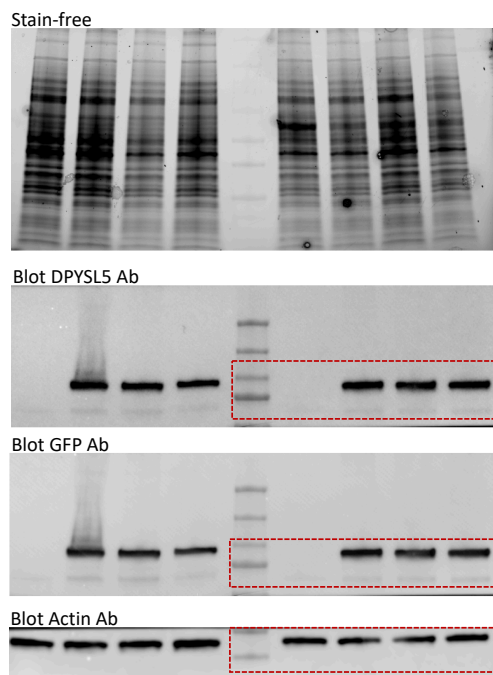
| Ordinary One-Way ANOVA | | | | | ANOVA summary | | | | |
|-----------------------------------|------------|--------------------|--------------|---------|------------------|--------|--|--|--|
| Number of families | 1 | | | | F | 7,377 | | | |
| Number of comparisons per family | 6 | | | | P value | 0,0003 | | | |
| Alpha | 0,05 | | | | P value summary | *** | | | |
| Tukey's multiple comparisons test | Mean Diff, | 95,00% CI of diff, | Significant? | Summary | Adjusted P Value | | | | |
| GFP vs. WT | 740,9 | 312,3 to 1170 | Yes | *** | 0,0001 | | | | |
| GFP vs. E41K | 232,7 | -189,2 to 654,6 | No | ns | 0,4685 | | | | |
| GFP vs. G47R | 262,3 | -159,5 to 684,2 | No | ns | 0,3621 | | | | |
| WT vs. E41K | -508,2 | -930,1 to -86,33 | Yes | * | 0,0121 | | | | |
| WT vs. G47R | -478,6 | -900,5 to -56,71 | Yes | * | 0,0201 | | | | |
| E41K vs. G47R | 29,63 | -385,4 to 444,7 | No | ns | 0,9976 | | | | |

2. Raw DPYSL5 immunoprecipitation data.



A- Raw immunoblot of DPYSL5, MAP2, β III-tubulin and DPYSL2 in immunoprecipitates from CAD cells transfected with the indicated DPYSL5-GFP expressing plasmids. B- Raw quantified numerical data for MAP2, β III-tubulin and DPYSL2 co-immunoprecipitated with DPYSL5-GFP.

3. Full-size gels and western-blots for Figure S5



Supplemental Materials and methods

Inclusion of the Individuals

The ten affected individuals included in the present study were enrolled together with their healthy biological parents in different programs or centers investigating the molecular basis of a neurodevelopmental disorder (NDD) in a research or clinical setting: Individual 1 and biological parents were followed at the Department of Genetics of the University Hospital of Tours (France) and were included in the Western France HUGODIMS research program (ClinicalTrials.gov registration no. NCT02136849; « Inter-regional Project of the Great Western Exploration Approach for Exome Molecular Causes Severe Intellectual Disability Isolated or Syndromic »). Individuals 2 to 8 and their biological parents were enrolled at the following centers: Departments of Pediatric and Clinical Genetics, Amsterdam UMC, Amsterdam, The Netherlands (Patient 2); Department of Clinical Genetics, Erasmus MC University Medical Center, Rotterdam, The Netherlands (Patient 3); West Midlands Regional Clinical Genetics Service, Birmingham Women's and Children's Hospital, National Health Service Foundation Trust, Birmingham, UK (Patients 4 and 5) ; Department of Clinical Genetics, Akron Children's Hospital, Akron, USA (Patient 6); Department of Medical Genetics, Yerevan State Medical University after Mkhitar Heratsi and Center of Medical Genetics and Primary Health Care, Yerevan, Armenia, and Department of Biology and Medical Genetics, Charles University 2nd Faculty of Medicine and University Hospital Motol, Prague, Czech Republic (Patient 7); Departments of Neurology and Pediatrics, Weill Institute of Neuroscience and Institute of Human Genetics, University of California, San Francisco, USA (Patient 8). Individuals 9 and 10 were from the family which allowed the original description of Ritscher-Schinzel syndrome. Connecting the different centers was allowed by GeneMatcher.¹

Whole-Exome sequencing

Genomic DNA was extracted from blood cells through standard procedures. A trio-based exome sequencing strategy was applied to the affected individuals and their parents.

The DNAs of Individual 1 and respective parents were tested at the genomics platform of the Genomics and Bioinformatics Core Facility of Nantes (GenoBiRD, Biogenouest). Library generation was performed with an optimized SureSelect XT Target Enrichment Kit (Agilent Technologies, Santa Clara, CA, USA). The exome was captured by hybridization using the

SureSelect Clinical Research Exome (Agilent Technologies) and sequenced on a HiSeq 2500 (Illumina, San Diego, CA) following the manufacturer's instructions. DNA sequences were mapped to the reference human genome sequence (GRCh37) with BWA. HaplotypeCaller from the Genome Analysis Toolkit was used to perform variant calling. Filtering was performed using Jvarkit and annotations from SnpEff, VEP and dbNSFP in a custom bioinformatic pipeline with an autosomal dominant hypothesis.

Exome sequencing was performed in a trio diagnostic approach for Individual 2 (Amsterdam) and both parents. Libraries were prepared using the Kapa HTP kit (Illumina) and capture was performed using the SeqCap EZ MedExome (Roche NimbleGen). Sequencing was done on an Illumina HiSeq2500. The reads were aligned to the reference sequence (GRCh37/hg19 Feb 2009) and variants identified using pipeline based on BWA-MEM0.7 and GATK 3.3.0. The median coverage of the captured target region was at least 109x and 94.8% of the probe base pairs were covered at >20X. Variant annotation and prioritizing were done using Cartagenia Bench Lab NGS (Agilent Technologies). Variants in all coding exons and 6 nucleotide flanking intronic regions are analyzed for de novo variants (dominant inheritance) and homozygous or compound heterozygous variants (recessive inheritance). Variants with a minor allele frequency of >1% in gnomAD database that are not present as (likely) pathogenic in HGMD database (<https://portal.biobase-international.com/hgmd/pro/start.php>) or ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) database, have not been analyzed.

The whole-exome sequencing study performed in a trio diagnostic approach for Individual 3 (Rotterdam) and both parents has been previously described.²

Genome-wide microarray and whole exome sequencing in trios (subject and parents) was performed for Individuals 4 and 5 as per the Deciphering Developmental Disorders (DDD) study methods.³ The whole-exome sequencing study performed for Individuals 6, 8 and 9 and both parents has been previously described.⁴

For Individual 7, trio exome sequencing was performed at Charles University in Prague. SeqCap EZ MedExome Probes (Roche NimbleGen, Madison, WI) were used for exon capture. The enriched library was paired-end sequenced using the HiSeq2500 system (Illumina, San Diego, CA). The reads were mapped to the reference genome (hg19) using Novoalign. SAM to BAM conversion and PCR duplicate removal were performed using Picard Tools. Genome Analysis Toolkit (GATK) was used for local realignment, base recalibration, variant recalibration and genotyping. Variants were annotated using the GEMINI framework, and

were filtered according to population frequencies in several public databases (gnomAD, ExAC, 1000GP) and in an in-house database of variants identified in previous experiments. The variants were prioritized according to the predicted severity, various inheritance pattern models, and presence in in-house candidate gene lists.

DPYSL5 Protein modeling analyses

The p.(Glu41Lys) and p.(Gly47Arg) variants have been modelled using the *ddg_monomer* module of the Rosetta software.⁵ The input structure was the crystal structure of human DPYSL5 (pdb code 4b91).⁶ Figures were prepared using CCP4MG.⁷

DPYSL5 plasmids and mutagenesis

Full-length human *DPYSL5* cDNA (AF264015) was amplified by PCR and inserted directionally into the pEGFP-C1 vector (Clontech) using *EcoR1* (5') and *Pst1* (3') cloning sites, which generated a fusion protein with a GFP tag at its N terminus (EGFP-DPYSL5). The missense variants p.Glu41Lys (E41K) and p.Gly47Arg (G47R) were generated by site-directed mutagenesis using the Q5[®] Site-Directed Mutagenesis kit (New England Biolabs). The oligonucleotides used to generate each mutation in pEGFP-DPYSL5 were designed using the NEBase Changer (<https://nebasechanger.neb.com>) and include the following sequences: E41K Forward 5'-ggtgggccgcaagctcatgat-3' and E41K Reverse 5'-tgctggatgatgccattctc-3'; G47R Forward 5'-gatccctggcaggcccaaggtgat-3' and G47R Reverse 5'-atgagctcgcggcccacc-3'. The sequences of all constructs were confirmed by automated DNA Sanger sequencing.

Primary neuronal culture, transfection and immunocytochemistry

All mouse experiments were performed according to protocols approved by the University Tours and by the INSERM (Project authorization number 01456.03, Ministry of Research). Hippocampal neuronal cultures from embryonic day 17 (E17) mouse embryos were prepared and plated at a density of 450 cells/mm² as previously described.⁸ The developing neurons were transfected 4 days after plating with EGFP, EGFP-DPYSL5, EGFP-DPYSL5 p.Glu41Lys (E41K) or EGFP-DPYSL5 p.Gly47Arg (G47R) plasmid constructs using Lipofectamine 2000 (Cat# 11668500, Invitrogen) and fixed 48 hours later at 6 days of *in vitro* culture (D.I.V. 6) with a solution containing 4% paraformaldehyde/4% sucrose in PBS. Cultures were then incubated with a blocking buffer (10% donkey serum/0.2% Triton X-100 in PBS) for 1 hour, washed (PBS)

and incubated for 1 hour with the following primary antibodies diluted in blocking buffer: Rabbit polyclonal anti-Tau Ab (1/1000, Cat# 314003, Synaptic Systems), Mouse monoclonal anti-MAP2 antibody (1/200, Cat# M9942, Sigma-Aldrich). After several washes in PBS, neurons were incubated for 45 minutes with the following secondary antibodies diluted in blocking buffer: FluoProbes[®] 594 donkey anti-rabbit Ab (1/300, Cat# FPSD5110, Interchim), Alexa Fluor 405 Goat anti-mouse Ab (1/300, Cat# A-31553, Invitrogen). After several washes in PBS, the fixed and stained neurons were mounted in ProLong[™] Diamond Antifade reagent (Cat# P36391, Invitrogen). The cells were observed using a laser-scanning confocal microscope SP-8 (Leica) and images were generated by the in-built Leica Application Suite X (Leica). The axonal and dendritic measurements were done with Imaris Software as previously described.⁸

Culture and transfection of HEK293T cells

Human embryonic kidney 293T (ATCC CRL-3216) cells were grown in standard cell culture conditions (DMEM/Glutamax with 10% heat-inactivated fetal bovine serum (EuroBio)), 37°C in 5% CO₂. Approximately 350,000 cells were transferred into 6-wells plates and transfected 24 hours after plating with a solution containing 2 µg of plasmid DNA and 4 µL of Lipofectamine 2000 (Cat# 11668027, ThermoFisher). Total proteins lysates were prepared 48 hours post-transfection and analyzed by western blotting using primary mouse monoclonal GFP antibody (Cat# 1181446000, Roche), Affinity-purified rabbit polyclonal DPYSL5/CRMP5 antibody (from J. Honnorat's lab),^{9,10} and horseradish peroxidase (HRP)-conjugated actin antibody (Cat# A3854, Sigma). Membranes were analyzed by chemiluminescence using Clarity ECL substrate (Cat# 1705060, Bio-Rad) using a ChemiDoc Imaging System (Bio-Rad), onboard acquired with Image Lab Touch 2.4 software (Bio-Rad) and quantified using Image Lab 6.1 software (Bio-Rad).

Culture and transfection of catecholamine A differentiated (CAD) cells

Mouse neuron derived CAD cells (ECACC Cat# 08100805) were grown in standard cell culture conditions (DMEM/F12 media supplemented with 10% fetal bovine serum (Hyclone), 37°C in 5% CO₂), as previously described.¹¹⁻¹⁴ CAD cells were transfected with a 1 mg/ml solution of polyethyleneimine (PEI, MW 25000, Sigma) at a ratio of 1 µg of DNA for 3 µg of PEI. Under these conditions, efficiencies above 90% were routinely observed.

Generation of CAD cells lysates and immunoprecipitation

Transfected CAD cells lysates were generated by homogenization and sonication in RIPA buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM MgCl₂, 1% (vol/vol) NP40, 0.5% (mass/vol) sodium deoxycholate, 0.1% (mass/vol) SDS), protease inhibitors (Cat# B14002; Bimake, Houston, TX), phosphatase inhibitors (Cat# B15002, Bimake), and benzonase (Cat#71206, Millipore, Billerica, MA). Protein concentrations were determined using the BCA protein assay (Cat# PI23225, Thermo Fisher Scientific, Waltham, MA). Immunoprecipitation was done as previously described,¹¹⁻¹⁴ with 2 µg of anti-GFP antibody (Cat# AB3080, Millipore, Billerica, MA) incubated overnight before adding protein G magnetic beads (Cat# 10009D, Thermo Fisher Scientific, Waltham, MA), pre-equilibrated with the immunoprecipitation buffer. Incubation was done for 1 hour at 4°C to capture the immuno-complexes. Beads were washed 3 times with RIPA buffer before resuspension in Laemmli buffer with DTT and denaturation (5 minutes at 95°C) prior to immunoblotting.

Indicated samples were loaded on 4-20% Novex® gels (Cat# EC60285BOX, Thermo Fisher Scientific, Waltham, MA). Proteins were transferred for 1h at 120 V using TGS (25 mM Tris, pH=8.5, 192mM glycine, 0.1% (mass/vol) SDS), 20 % (vol/vol) methanol as transfer buffer to polyvinylidene difluoride (PVDF) membranes 0.45 µm (Cat# IPVH00010, Millipore, Billerica, MA), pre-activated in methanol. After transfer, the membranes were blocked at room temperature for 1 hour with TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween 20), 5% (mass/vol) non-fat dried milk, then incubated separately with indicated primary antibodies against CRMP5, CRMP2 (Cat# C2993, Sigma, St Louis, MO), βIII-tubulin (Cat# G7121, Promega, Madison, WI) or MAP2 (Cat# M4403, Sigma, St Louis, MO) in TBST, 5% (mass/vol) BSA, overnight at 4°C. Following incubation with HRP-conjugated secondary antibodies from Jackson ImmunoResearch, blots were revealed by enhanced luminescence (WBKLS0500, Millipore, Billerica, MA) before exposure to photographic film. Films were scanned, digitized, and quantified using Un-Scan-It gel version 6.1 scanning software by Silk Scientific Inc.

Statistical Analyses

Statistical analyses were performed using the GraphPad Prism 6.0 software (La Jolla, CA, USA). For the neuronal morphological study, the data were analyzed using the D'Agostino-Pearson normality test. When the result was positive, the data were then analyzed using an Ordinary one-way ANOVA followed by Tukey's multiple comparisons. For the protein interaction

analysis, a Kruskal-Wallis test with Dunn's post-hoc was performed. $P < 0.05$ was assumed significant.

Supplemental references

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