

Supplemental Data

Bi-allelic Variants in *RALGAPA1* Cause Profound Neurodevelopmental Disability, Muscular Hypotonia, Infantile Spasms, and Feeding Abnormalities

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Supplemental information

Supplementary Case reports:

Individual 1, a boy, was born as the second child to healthy non-consanguineous parents of German ancestry. The first child of the family (a boy) was unaffected. The mother had three miscarriages. Birth parameters were normal (weight 3875 g [79th P], lengths 56 cm [97th P], head circumference 36.5 cm [93rd P]). After birth he required transient CPAP respiratory support. During the following weeks, the child appeared very lethargic and stimulation was required for feeding. Subsequently, muscular hypotonia and lack of visual contact were noted. From the age of 4 months, progressive feeding difficulties and failure to thrive became evident. A nasogastric tube was placed at the age of 6 months and later changed into a PEG-tube. Motor and cognitive milestones were not reached (e.g. no rolling over, no crawling, no social interactions or affective expression, etc.).

Laboratory work-up as well as testing for metabolic diseases (including analysis of amino acids in plasma, acyl carnitines, organic acids / purine and pyrimidines in urine, screening for congenital disorders of glycosylation, and analysis of CSF neurotransmitter metabolites) was normal. Chromosomal analysis, array-CGH and methylation testing for Prader-Willi syndrome were without pathological findings.

Because of recurrent viral infections as well as unexplained episodes with high fever an immunologic work-up (Flow cytometry, determination of immunoglobulins, etc.) was done but revealed no abnormalities. A central dysregulation of temperature control was suspected. In addition, unusually dry skin with scaling and hypohidrosis became evident, which might have contributed to the fever episodes.

Whole-body MRI including brain MRI at the age of 9 months revealed small gray matter heterotopias but was otherwise normal. Nerve conduction studies as well as visual evoked potentials were without pathological findings. Eye examination was normal but cortical visual impairment was suspected.

At the age of 12 months the boy developed infantile spasms (West syndrome) and was treated with prednisolone and vigabatrin. Later, also valproate and levetiracetam were administered. During treatment seizure frequency slowly decreased, however, the boy was never seizure-free.

At the age of 1 ½ years a muscle biopsy was performed to exclude a mitochondrial disease. Biochemical OXPHOS analysis of fresh muscle tissue revealed a mildly reduced ATP production rate but was otherwise normal. Histological examination was without specific findings. At the age of 3 years development of bilateral cataracts was noted.

At the current age of 4 1/2 years, the boy is severely disabled (growth parameters: length 111 cm [75th P], weight 16.4 kg [25th P], head circumference 51.5 cm [52th P]). He is unable to sit, crawl, or pull up. Head control is still inadequate and truncal muscle tone is profoundly reduced. He didn't acquire language and he never interacted with his environment. He presented with mild craniofacial dysmorphism consisting of fully eyebrows, deep-set eyes, short nasal bridge with anteverted nares, large mouth with prominent lower lip, protruding tongue and gingival hyperplasia. His skin, especially at extremities, was dry and scaling.

Follow-up MRI at the age of 4 1/2 years confirmed the finding of small nodular gray matter heterotopias (Figure 1). In addition, Signal alterations of substantia nigra and globus pallidus (NBIA-like pattern) became evident. Moreover, focal thinning of corpus callosum was noted.

Individual 2, a girl, was born as the second child of consanguineous parents of Palestinian ancestry. Her older female sibling had died at the age of 4 months (see below). The proband was born following an uneventful pregnancy, via Cesarean section, and her birth parameters were within normal limits. Following birth she remained hospitalized due to failure to gain weight, respiratory distress and hypotonia. She required non-invasive respiratory support for the first 3 weeks of life, and had feeding difficulties.

The patient was first transferred to the Pediatric Intensive Care Unit (PICU) at our center at the age of three months, under the suspicion of a neurometabolic disorder or immune deficiency. Initial evaluation had included echocardiogram which was reportedly considered normal, and an evaluation

for infections agents which demonstrated RSV in nasal swab for respiratory viruses, and *Candida Albicans* in urine cultures. Head ultrasound was notable for lenticulostriate vasculopathy (LSV) and septations in the anterior horns of the lateral ventricles, and ophthalmological evaluation was considered normal. PCR for CMV in urine was negative. An extensive evaluation ensued, which included elaborate metabolic screening tests (including serum lactate levels, ammonia levels, plasma amino acid profile, biotinidase screening, transferring electrophoresis for congenital disorders of glycosylation, very long chain fatty acids, total serum carnitine, urine organic acid profile and urine reducing substances) however these did not yield a diagnosis. In addition, due to persistent high fever and several episodes of documented infections (RSV, *Candida albicans* in urine, *Klebsiella* sepsis), investigations for a possible immune deficiency were also undertaken (including serum immunoglobulin levels, TREC, CGD, T cell receptor repertoire, among other tests) and was within normal limits. Finally, whole exome sequencing was sent for the proband and her mother.

At 8 months of age she was readmitted for further phenotyping. Upon physical examination she did not show evident dysmorphic features, apart from bushy eyebrows and a low anterior hairline. Neurological evaluation showed global developmental delay, as she did not seem to achieve any major developmental milestones, did not smile, roll over or followed gaze. She had decreased axial muscle tone with evident head lag and increased muscle tone in her limbs. Babinsky reflex showed fanning of the toes. She required naso-gastric tube feeding, and received environmental oxygen to her tracheostomy.

Brain MRI revealed a thin pituitary stalk as well as dilatation of cerebral ventricles, cisterns and gyri, consistent with brain atrophy, most prominently affected the temporal lobes. Moreover, focal thinning of corpus callosum was noted. MRS did not show abnormal metabolite values. Echocardiogram, ophthalmological evaluation and computed tomography of the head, thorax and abdomen were considered normal, apart from brain atrophy and mild hepatomegaly.

Family history was notable for an older sister, who was born via Cesarean Section at term and was discharged home from the nursery at 5 days of life. Reportedly, she did not show marked dysmorphic features. Due to recurrent vomiting and failure to thrive, she underwent an upper GI series and

abdominal ultrasound, which did not show evidence of pyloric stenosis. Ophthalmological evaluations noted bilateral glaucoma at 1 month of age and bilateral pallor of the optic disc at 3 months. At 4 months of age, she was found dead in her crib. As no DNA samples were kept, molecular analysis was unfortunately unavailable.

Additionally, a male paternal cousin, also born to consanguineous parents of the same family, was affected by an undiagnosed condition, was never discharged home after birth and had died at 37 days of life, reportedly due to an intracranial hemorrhage.

Individual 3, a boy, was born as the first child to non-consanguineous parents of German-Irish ancestry. He weighed 2240 grams (5th P) after a 37 week uncomplicated gestation. He had neonatal respiratory distress and thermodysregulation requiring hospitalization for the first 13 days of life. He had early problems with oral feedings and central hypotonia and a gastrostomy tube was placed at 3 months-of-age. He was diagnosed with bilateral hypermetropia. He had a genetics evaluation at 8 months of age that noted laryngomalacia, oxygen requirement, severe developmental delay, hypotonia, and failure to thrive. On cytogenomic microarray a 28 kb duplication at 6q23.2 containing the *EYA4* gene was classified as a variant of uncertain significance (VUS) but thought to be likely benign. Whole exome sequencing revealed a VUS of the *RALGAP1* gene (c.610G>T, p.Glu204*). He developed infantile spasms at 10 months of age that responded to treatment with adrenocorticotrophic hormone (ACTH). Brain MRI at that time noted a possible ectopic pituitary and thinning of the corpus callosum. He was seen at 18 months of age for clinical genetics evaluation and noted to have severe hypotonia with intermittent arching of the head and trunk, minimal head control, inability to sit without support, and no pincer grasp. He demonstrated slight ability to sign in an attempt to signal for “more”. Weight was 9.52 kg (7th P), length 78.1 cm (6th P), head circumference 46.8 cm (7th P). Dysmorphic features included a flat occiput and brachycephaly, high anterior hair line with frontal balding, tall forehead, full cheeks, medial eyebrow flare, left epicanthal fold, slightly pointed superior helices of ears, slightly upturned nasal tip, partial soft tissue syndactyly of fingers 3 and 4 bilaterally, to the proximal interphalangeal joint of the left hand and to the mid-

proximal phalangeal area on the right hand (see supplemental Figure 5), and partial syndactyly of toes 2,3 and 4 on the right foot. He had severe neck and truncal hypotonia but some antigravity strength in the legs.

Individual 4, a 17 months-old boy, was product of a twin pregnancy with preterm delivery at 34 weeks gestation to healthy consanguineous parents of Saudi ancestry. The family had a previous child with profound muscular hypotonia that expired at 1 1/2 years of age due to respiratory failure. The other three siblings were healthy and there was no history of miscarriages for the mother. Birth parameters were below normal percentiles 2115 [<5th P], lengths 43 cm [<5th P], head circumference 33.5 cm [10th P]). After birth he required transient CPAP respiratory support and NICU admission because of prematurity.

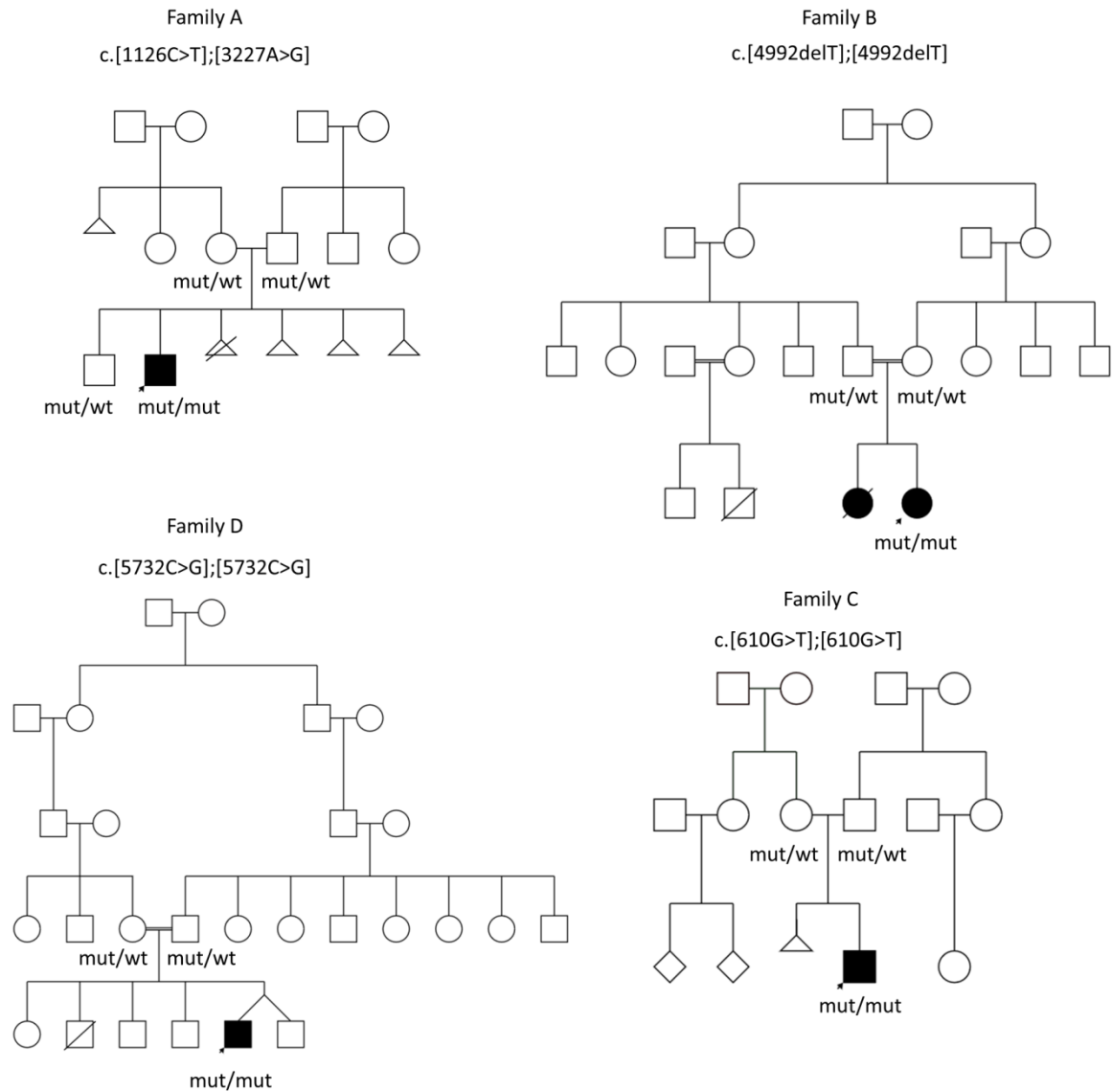
During the neonatal period, the child developed tracheomalacia with on and off stridor as well as recurrent apneas that were associated with poor feeding. These symptoms were progressive and became evident at age of 5 months when he required being on oxygen supplementation. Subsequently, muscular hypotonia and significant global developmental delay were noted. Motor and cognitive milestones were not reached (e.g. no rolling over, no crawling, no social interactions or affective expression, etc.).

Laboratory work-up as well as testing for metabolic diseases (including lactate, ammonia, CPK, amino acids in plasma, acylcarnitine profile, organic acids, transferrin isoelectric focusing) were without pathological findings. Chromosomal analysis, array-CGH and FISH for SNRPN gene for Prader-Willi syndrome were normal. Eye examination was unremarkable but cortical visual impairment was suspected. Echocardiogram showed mild tricuspid valve regurgitation and mild mitral valve regurgitation. Brain MRI indicated thinning of corpus callosum but was otherwise unremarkable.

At the age of 8 months the boy developed infantile spasms (West syndrome) and was treated with different anticonvulsant medications with unsatisfactory control that was aggravated by several episodes of hyperthermia with no associated infections were attributed to central thermal dysregulation.

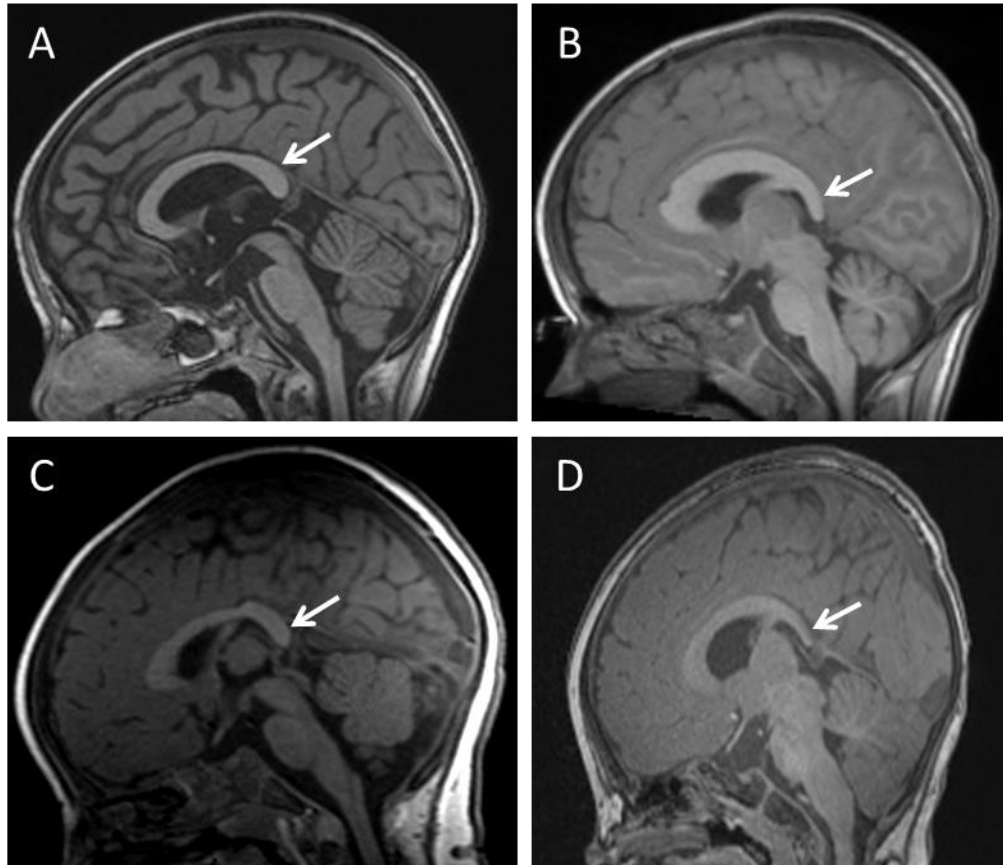
At the current age of 17 months, he is chronically admitted to the hospital (with oxygen dependency, on PEG-tube feeding and status post fundoplication due to failed swallowing assessment and moderate gastro-esophageal reflux). His growth parameters: length 75 cm [5th P], weight 12.5 kg [75th P], head circumference 48 cm [37th P]. He is unable to sit, crawl, or pull up. Head control is still inadequate and truncal muscle tone is profoundly reduced. He didn't acquire language and he never interacted with his environment. He presented with mild craniofacial dysmorphism consisting of horizontal eyebrows, low seated ears, depressed nasal bridge, and large mouth with prominent lips, gingival hyperplasia and slight retrognathia. He had single palmar creases.

Supplementary Figures:

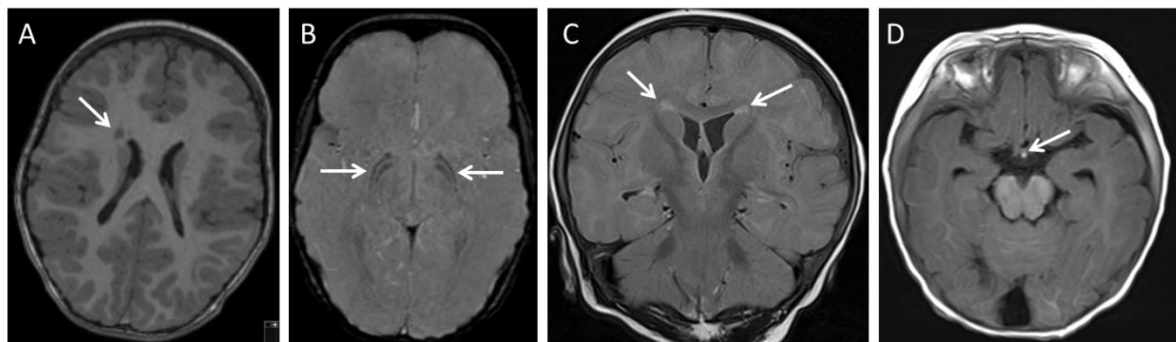


Supplementary Figure 1: Pedigrees of the investigated families. Variants identified are depicted over each pedigree; mut/mut indicates homozygosity or compound-heterozygosity for the pathogenic variant(s) whereas mut/wt indicates heterozygous carriers. Individuals without further indication of carrier status were not tested. Arrow = index patient; solid symbol = affected individual; circles = females; squares = males; triangle = spontaneous abortion, slashed triangle = termination of pregnancy; slashed symbols = deceased.

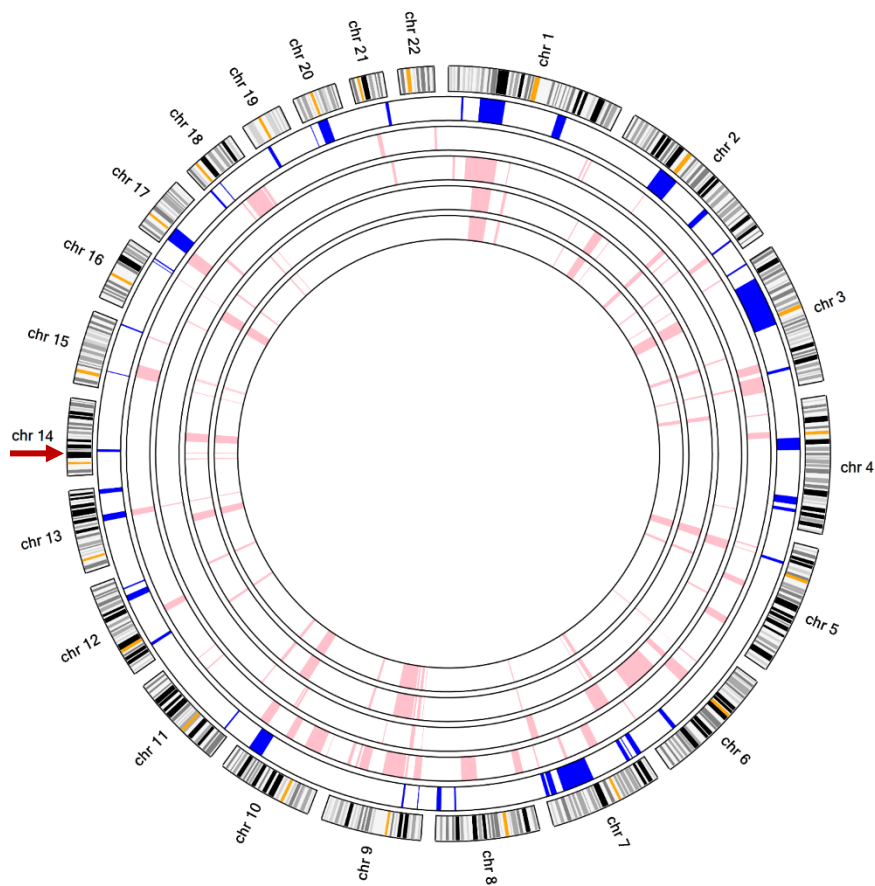
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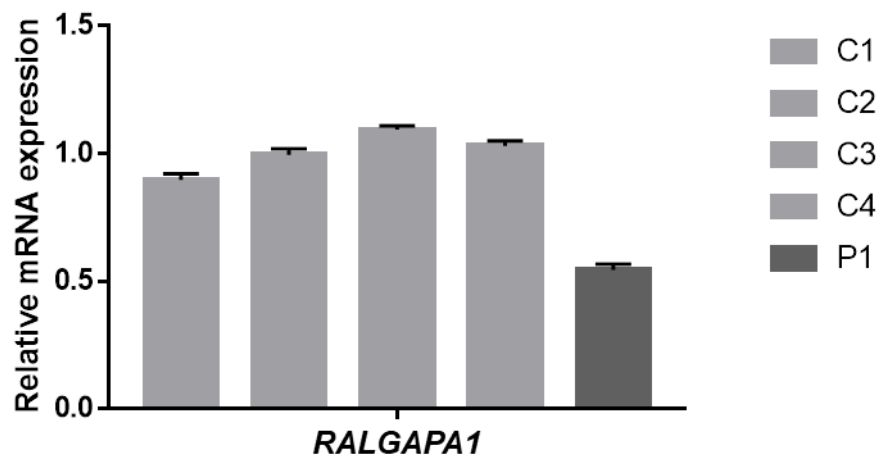
Supplementary Figure 2: Corpus callosum abnormalities in RalGAPA1-deficiency. A) Brain MRI image (T1-weighted sagittal view) of a healthy individual at the age of 4 1/2 years. B) Brain MRI image (T1-weighted sagittal view) of individual 1 at the age of 4 1/2 years. Please note the morphological differences of the posterior part (splenium) of the corpus callosum (white arrows). Measurements of the splenium in individual 1 revealed 4.9 mm (control range 6.9 – 12.2; for details regarding normal values see Garel et al., 2011).¹ C) Brain MRI image (T1-weighted sagittal view) of a healthy individual at the age of 10 months. D) Brain MRI image (T1-weighted sagittal view) of individual 3 at the age of 10 months. A similar pattern with thinning of the splenium was observed (white arrows). Measurements of the splenium in individual 4 revealed 3.0 mm (control range 3.4 - 9.2).



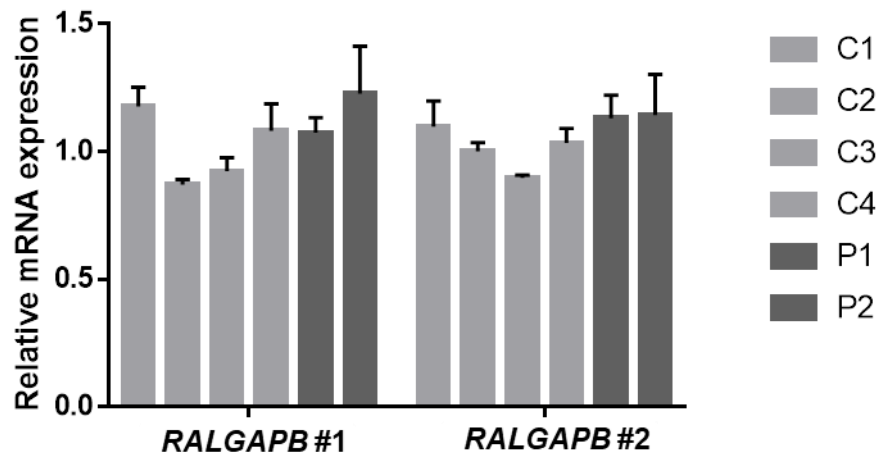
Supplementary Figure 3: Variable CNS findings in individuals with variants in *RALGAP1*. A) Brain MRI image (T1-weighted axial view) of individual 1 at the age of 4 1/2 years shows nodular heterotopia (white arrow). B) Susceptibility-weighted MRI imaging (axial view) of individual 1 at the age of 4 1/2 years indicates low signal intensities in the globus pallidus and substantia nigra respectively (white arrows) suggesting deposition of paramagnetic material. C) T2-weighted MRI imaging (coronal view) of individual 3 at the age of 10 months demonstrating increased signal intensities of the stratum subependymale (white arrows). D) T1-weighted MRI imaging (axial view) of individual 3 at the age of 10 months showing ectopic posterior pituitary (white arrow).



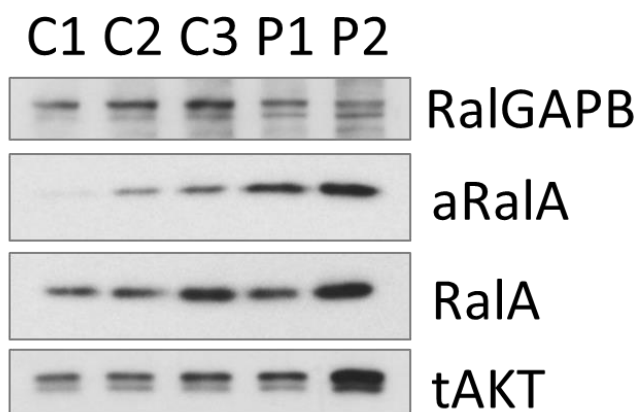
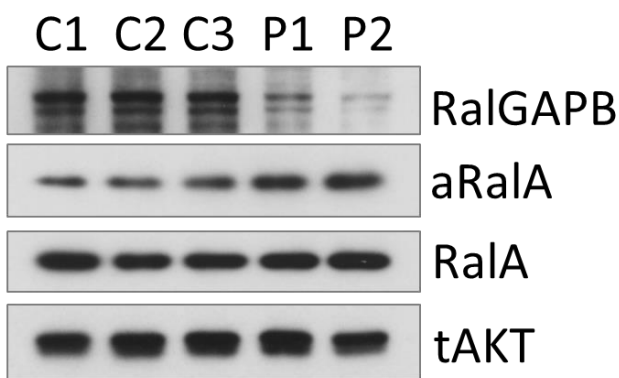
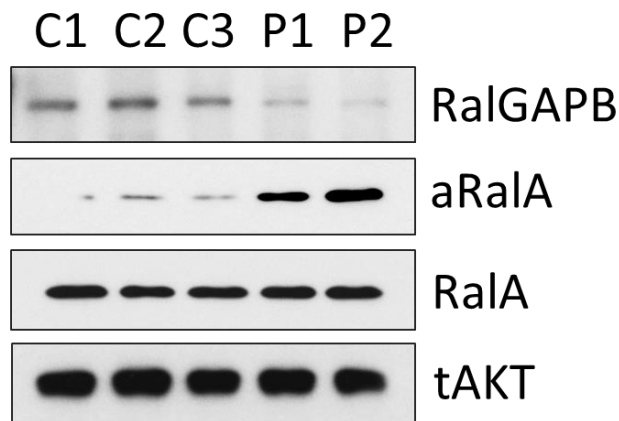
Supplementary Figure 4: Homozygosity mapping of Family D. The figure shows homozygous regions of the index patient of family D (outer ring) and the four unaffected siblings (inner rings). Homozygous regions are highlighted in blue for the index patient and pink for the unaffected siblings. The homozygous region including *RALGAP1* is indicated with a red arrow. Homozygosity mapping revealed that the unaffected siblings are not homozygous for the variant c.5732C>G, p.(Ser1911*) in *RALGAP1* proving segregation in the family.



Supplementary Figure 5: Comparison of relative mRNA amount of *RALGAPA1* between individual 1 and control fibroblasts. Relative mRNA amount was detected by a TaqMan gene expression assay and normalized against TBP using the Pfaffl method. *RALGAPA1* expression was reduced to 54% in patient 1 when compared to four controls implying that transcripts with the nonsense variant undergo nonsense-mediated mRNA decay but the missense variant is expressed. Data is presented as the mean $\pm$ SEM derived from three technical replicates. C1-C4 = controls; P1 = fibroblasts from individual 1.

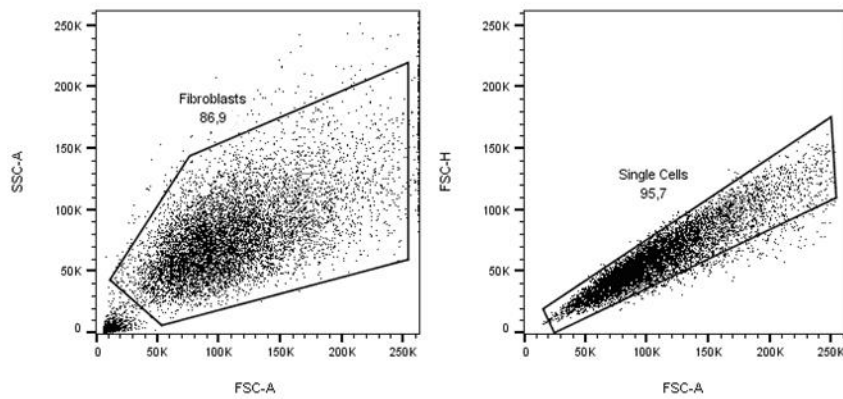


Supplementary Figure 6: Comparison of relative mRNA amount of *RALGAPB* between individual 1 and 2 with control fibroblasts. Relative mRNA amount was detected by two different primer pairs (*RALGAPB* #1 and #2) and normalized against three reference genes (HPRT, GAPDH, β 2M) using the Pfaffl method. No significant expression differences between control and patients could be seen. Student's t-test was used to compare the groups. Data is presented as the mean $\pm$ SEM derived from three biological replicates. C1-C4 = controls; P1 / P2 = fibroblasts from individual 1/2.

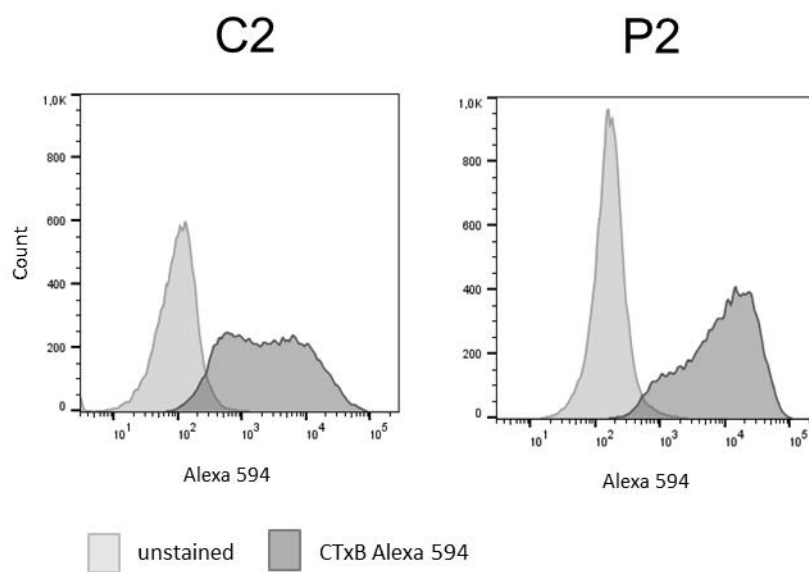


Supplementary Figure 7: RalGAPB, aRala, Rala, and AKT immunoblotting. Panels represent immunoblot images that were used for quantification of aRala/Rala and RalGAPB/tAKT ratios. For description of methodological details see below. For interpretation of findings see main text.

A



B



Supplementary Figure 8: Fluorescence-activated cell sorting (FACS) analysis of CTxB Alexa 594. A) FACS gating strategy showing selection of fibroblasts (left plot) and single cells (right plot). B) FACS histogram plots of control (C2) and patient-derived (P2) fibroblasts with RalGAPA1-deficiency. Light grey curves indicate unstained samples; dark-grey curves indicate CTxB Alexa 594 labelled samples. Please note a shift of patient-derived cells towards higher fluorescence intensity values. For each individual sample 30000 events were recorded.



Supplementary Figure 9: Syndactyly of fingers in individual 3: Soft tissue syndactyly of fingers 3 and 4 bilaterally, to the proximal interphalangeal joint of the left hand (left image; black arrow) and to the mid-proximal phalangeal area on the right hand (right image, black arrow).

Supplementary Tables:

Supplementary Table 1: Biallelic variants identified in individual 1.

	Gene	Transcript	Variant	OMIM	Function	pph2	Sift	heterozygous /homozygous	Inheritance	Coverage	ClinVar	1000 genomes AF
1	RALGAPA1	NM_194301.4	c.3227A>G, p.(Asn1076Ser)		ms	probably damaging	0	het	maternal	53		0
			c.1126C>T, p.(Arg376*)		LoF		1	het	paternal	21		0
2	PKD1	NM_000296.3	c.11335G>A, p.(Asp3779Asn)	601313	ms	benign	0.42	het	maternal	83		0
			c.3772G>A, p.(Ala1258Thr)		ms	possibly damaging	0.13	het	paternal	77		0
3	BRCA1	NM_007294.3	c.3748G>A, p.(Glu1250Lys)	113705	ms	benign	0.06	het	maternal	365	benign	0
			c.2477C>A, p.(Thr826Lys)		ms	possibly damaging	0.02	het	paternal	406	benign	0.000199681
4	NEB	NM_001164507.1	c.20162T>C, p.(Leu6721Pro)	161650	ms	probably damaging	0.19	het	maternal	82	Uncertain significance	0.000199681
			c.9473C>G, p.(Ser3158Cys)		ms	probably damaging	0.01	het	paternal	229	Uncertain significance	0
5	TET2	NM_017628.4	c.323A>T, p.(Gln108Leu)	612839	ms	benign	0.15	het	maternal	389		0
			c.1415C>T, p.(Pro472Leu)		ms	benign	0.21	het	paternal	181		0
6	PCDHB5	NM_015669.2	c.523A>G, p.(Asn175Asp)		ms	probably damaging	0.01	het	maternal	214		0
			c.746T>C, p.(Val249Ala)		ms	benign	0.11	het	paternal	146		0

Abbreviations: ms = missense; LoF = loss of function; het = heterozygous; AF = allele frequency. Pph2 (PolyPhen-2) predicts the effect on the protein with three classes (probably damaging, possibly damaging or benign). SIFT scores less than 0.05 are referred to as “deleterious” and those greater than 0.05 as “tolerated”.

Supplementary Table 2: Variants in *RALGAP1* identified in the present study

family	genomic variant (NC_000002.11)	cDNA (NM_194301.4)	protein effect (NP_919277.2)	mutation type	CADD score	PhyloP score	gnomAD alleles
A	g.36143795T>C	c.3227A>G	p.(Asn1076Ser)	missense	25.5	5.073	absent
A	g.36217916G>A	c.1126C>T	p.(Arg376*)	nonsense	36	6.083	absent
B	g.36096643delA	c.4992delT	p.(Phe1664Leufs*11)	frameshift	NA	2.31	absent
C	g.36226052C>A	c.610G>T	p.(Glu204*)	nonsense	NA	6.085	absent
D	g.36041884G>C	c.5732C>G	p.(Ser1911*)	nonsense	NA	5.93	absent

Supplementary Table 3: Sequences of primer pairs for reference and target genes for *RALGAPB* expression analysis via RT-qPCR

Oligonucleotides	Sequence (5'-3')	Description
HPRT-Forward	TTCCTTGGTCAGGCAGTATAATC	Primer Pair that binds within human HPRT
HPRT-Reverse	GGGCATATCCTACAACAAACTTG	
β2M-Forward	GACTTGCTTTTCAGCAAGGA	Primer Pair that binds within human β2M
β2M-Reverse	ACAAAGTCACATGGTTCACA	
GAPDH-Forward	AGGGCTGCTTTAACTCTGGT	Primer Pair that binds within human GAPDH
GAPDH-Reverse	CCCCACTTGATTTTGGAGGGA	
RALGAPB #1-Forward	TGGATCTCTTGCCATGTCGT	Primer Pair that binds within human RALGAPB (pubmed)
RALGAPB #1-Reverse	TGAGATCCTTCACCATCATCACA	
RALGAPB #2-Forward	TCGCAGCATTCTCATCTCGTCACC	Primer Pair that binds within human RALGAPB (OriGene, CAT# HP213605)
RALGAPB #2-Reverse	CACAGAACTGATGGCTCGCTTC	

Supplementary Material and Methods

Fibroblast cell culture:

Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; life technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (life technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (life technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO₂.

RalGAPA1 immunoblotting:

For RalGAPA1 western blotting, whole cell lysates for SDS-PAGE were obtained by solubilization of cell pellets using CellLytic M Cell Lysis Reagent (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were determined using the BCA protein kit (Thermo Scientific, Waltham, MA, USA). 30µg of whole cell protein were loaded and separated in NuPAGE 3-8% TA gels using NuPAGE tris-acetate SDS running buffer (all from Invitrogen, Carlsbad, CA, USA). After electrophoresis, the gels were processed for western blotting and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 3% skimmed milk powder in TBS-T (1 h) and incubated with primary antibodies against RalGAPA1 (rabbit; 1:3000; EPR13592-38; Abcam, Cambridge, UK) or GAPDH (mouse; 1:4000; AM 4300, Thermo Fisher Scientific, USA) overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase conjugated anti-rabbit IgG or anti-mouse IgG (both from GE Healthcare Life Sciences, Marlborough, MA, United States) as secondary antibodies for 1 h at room temperature. BM Chemiluminescence blotting substrate (Roche, Basel, Switzerland) was used for visualization. Bands were quantified using Image Lab software V 5.2.1 (BioRad). Statistical significance was assessed using Student's t-test.

RalGAPB, RalA, and AKT immunoblotting:

Primary human fibroblasts were grown in high glucose DMEM supplemented with glutamine and 10% serum. For experiments, cells were fasted in FBS-free medium for 3 h and then in PBS for 1 additional h, then the cells were rinsed with PBS and snap frozen. The frozen cells were lysed in a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 1mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 1% triton X-100, phosphatase inhibitor cocktail 2 and 3 (Roche), and protease inhibitor tablet (Sigma). The lysates were passed through an insulin syringe, then incubated with rotation at 4°C for 30 min, and then centrifuged at 15,000 g for 15 min. The protein concentration in supernatants was determined using Bio-Rad Protein Assay Reagent. 5 µg of supernatants were diluted in SDS sample buffer with 50 mM DTT, boiled for 5 min, resolved on SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad).

Antibodies against AKT (#9272, from Cell Signaling) and against RalA (#610222, from BD Transduction Laboratories) were used at 1:1,000 and 1:10,000 dilution respectively. RalGAPB antibody were generated as previously described and were used at 1:1,000 dilution.² Goat anti-rabbit IgG, HRP (ThermoFisher, #31460) and goat anti-mouse IgG, HRP (ThermoFisher, #31430) were used as secondary antibodies at 1:5,000 dilution. Individual proteins were detected with the specific antibodies and visualized on film using horseradish peroxidase–conjugated secondary antibodies and western Lightning Enhanced Chemiluminescence (Perkin Elmer Life Sciences). Film was developed with ECOMAX X-ray Film Processor. Bands were quantified using ImageJ software. Statistical significance was assessed using Student's t-test.

RalA activity assay:

0.5 mg of fresh lysates prepared as described above were used for GTP-bound RalA pulldown with RalBP1 beads (Millipore) according to the manual. At the end of the assay the beads were resuspended in total 50 µl of the lysis buffer (see above) with 50 µM DTT and SDS sample buffer and boiled. 15 µl of generated sample were used for western blotting with anti-RalA antibody as

described above. Bands were quantified using ImageJ software. Statistical significance was assessed using Student's t-test.

Measurement of CTxB Alexa 594 surface fluorescence:

Measurement of CTxB Alexa 594 surface fluorescence was performed as described previously with several modification.³ For CTxB labeling experiments fibroblasts were serum fasted in high glucose DMEM medium (containing Glutamax and pyruvate) supplemented with 0.2 % FBS (life technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (life technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO₂. Next, cells were detached using TrypLE (Thermo Fisher Scientific). Cell suspension was separated into two aliquots (one for unstained FACS control, one for CTxB Alexa 594 labeling). Cells were washed 2x with cold PBS (Thermo Fisher Scientific) and incubated for 15 min on ice. Next, samples were centrifuged at 300xg for 5 min at 4°C, the supernatant was removed. Cell pellets were re-suspended either in PBS containing 10 µg/ml CTxB Alexa 594 (Thermo Fisher Scientific) or PBS without fluorescence marker (unstained control). Samples were incubated for 15 min at 4°C in the dark. Next, cells were washed 2x with cold PBS and fixed with 4% formaldehyde. FACS analysis was performed by using a BD LSR Fortessa cell analyzer (Becton Dickinson, Heidelberg) equipped with BD FACS Diva software (Becton Dickinson, Heidelberg). For each individual sample 30000 events were recorded. Data was analyzed using FlowJo software (Becton Dickinson, Heidelberg). Statistical significance was assessed using Student's t-test. For acquisition of representative microscopy images, a sample of the CTxB Alexa 594 stained cells was added on a glass coverslip and images were obtained by fluorescence microscopy (63 x objective, Zeiss; Axio Observer Z1 microscope with ApoTome 2 and a Perkin-Elmer Ultra View spinning disc confocal microscope equipped with filterset 31; excitation: BP 565/30; emission: BP 620/60 HC; Zeiss).

RNA extraction, cDNA studies and qRT-PCR for *RALGAPA1*

Cryo-asseverd (in DMEM Media with 10% DMSO) fibroblasts were thawed and centrifuged. RNA was isolated from cell pellets using the RNeasy Mini Kit (Quiagen) according to the manufacturer's

instructions. RNA concentration and purity was determined using the NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts) and the 2100 Bioanalyzer System (Agilent). Total RNA (300 ng) was reverse transcribed using the GoScript Reverse Transcriptase kit (Promega) according to manufacturer's instructions.

Relative mRNA amount was quantified by real-time PCR with a QuantStudio7 (Thermo Fisher) with TaqMan Probes (Thermo Fisher) for *RALGAPA1* (Hs04180554_g1), *RALGAPB* (Hs00384265_m1) and *TBP* (Hs00427620_m1) as a reference gene. All PCR experiments were performed with three technical replicates. For quantification, the Thermo Fisher DataAssist software was used.

Analysis of relative mRNA amount of *RALGAPB* via RT-qPCR

According to the RalA activity assay, for experiments, cells were cultured in FBS-deprived medium for 3 h and additionally for 1 h in PBS containing CaCl_2 and Mg^{2+} . Cells were lysed and RNA isolation was performed according to the instructions in the RNeasy Mini Kit (Qiagen). A total amount of 1000 ng RNA was reverse transcribed to cDNA using QuantiTect® Reverse Transcription Kit (Qiagen). Next, qPCR using gene-specific primers was performed with QuantiTect® SYBR® Green Kit (Qiagen) and a 7500 RT-PCR System (Applied Biosystems, Foster City, USA). Two different primer pairs were used for *RALGAPB* (*RALGAPB* #1: Forward: TGG ATC TCT TGC CAT GTC GT; Reverse: TGA GAT CCT TCA CCA TCA TCA CA; sequence generated by pubmed; *RALGAPB* #2: Forward: TCG CAG CAT TCA TCT CGT CAC C; Reverse: CAC AGA ACT GAT GGC TCG CTT C; sequence by Origene, CAT# HP213605). Relative gene expression data was obtained using Paffl method. Primer reaction efficiencies were calculated by means of LinRegPCR software. Values were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine guanine phosphoribosyl transferase (HPRT) and β_2 microglobulin ($\beta_2\text{M}$) levels. Primer sequences are listed in supplementary Table 3.

Supplemental references:

1. Garel C, Cont, Alberti C, Josserand E, Moutard ML, Ducou le Pointe H. Biometry of the corpus callosum in children: MR imaging reference data. *AJNR Am J Neuroradiol.* 2011; 32:1436-43.
2. Chen, X.W., Leto, D., Xiong, T., Yu, G., Cheng, A., Decker, S., Saltiel, A.R. (2011). A Ral GAP complex links PI 3-kinase/Akt signaling to RalA activation in insulin action. *Mol Biol Cell* 22; 141-52.
3. Balasubramanian N, Meier JA, Scott DW, Norambuena A, White MA, Schwartz MA. RalA-exocyst complex regulates integrin-dependent membrane raft exocytosis and growth signaling. *Curr Biol.* 2010; 20:75-9.