

Bi-allelic Variants in *RALGAP1* Cause Profound Neurodevelopmental Disability, Muscular Hypotonia, Infantile Spasms, and Feeding Abnormalities

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Ral (Ras-like) GTPases play an important role in the control of cell migration and have been implicated in Ras-mediated tumorigenicity. Recently, variants in *RALA* were also described as a cause of intellectual disability and developmental delay, indicating the relevance of this pathway to neurodevelopmental diseases. Here, we report the identification of bi-allelic variants in *RALGAP1* (encoding Ral GTPase activating protein catalytic alpha subunit 1) in four unrelated individuals with profound neurodevelopmental disability, muscular hypotonia, feeding abnormalities, recurrent fever episodes, and infantile spasms. Dysplasia of corpus callosum with focal thinning of the posterior part and characteristic facial features appeared to be unifying findings. *RALGAP1* was absent in the fibroblasts derived from two affected individuals suggesting a loss-of-function effect of the *RALGAP1* variants. Consequently, RalA activity was increased in these cell lines, which is in keeping with the idea that *RALGAP1* deficiency causes a constitutive activation of RalA. Additionally, levels of RalGAPB, a scaffolding subunit of the RalGAP complex, were dramatically reduced, indicating a dysfunctional RalGAP complex. Moreover, *RALGAP1* deficiency clearly increased cell-surface levels of lipid raft components in detached fibroblasts, which might indicate that anchorage-dependence of cell growth signaling is disturbed. Our findings indicate that the dysregulation of the RalA pathway has an important impact on neuronal function and brain development. In light of the partially overlapping phenotype between *RALA*- and *RALGAP1*-associated diseases, it appears likely that dysregulation of the RalA signaling pathway leads to a distinct group of genetic syndromes that we suggest could be named RALopathies.

The Ral guanine nucleotide exchange factor (RalGEF)-Ral GTPase cascade is one of three major downstream pathways that become activated upon Ras signaling. The others include the Raf/mitogen-activated protein kinase (MAPK) cascade and the phosphoinositide 3-kinase (PI3K)-dependent phosphoinositide pathway.¹ *RALGAP1* (previously referred to as *TULIP1* or *GARNL1* [MIM: 608884]) encodes the catalytic subunit of Ral GTPase-activating protein (GAP) complex for two highly related small G proteins, RalA and RalB.^{1,2} Ral proteins draw significant research interest as promising cancer drug targets downstream of Ras.^{3,4} The role of RalA in normal cell physiology centers around the regulation of intracellular-vesicle trafficking. RalA mediates post-Golgi vesicle trafficking toward the

plasma membrane by rearranging the cytoskeleton and via its interaction with the tethering complex exocyst, which positions vesicles for docking and fusion at the plasma membrane.^{5,6} Thus, RalA is implicated in cellular processes, which depend on directional membrane trafficking, such as cell migration, establishment of cell polarity, neuronal polarity, and ciliogenesis. Consequently, RalA is essential for normal brain development. *Rala*-null mouse embryos display exencephaly, which recently has been classified as ciliopathy, between embryonic day 10.5 (E10.5) and E19.5.⁷

RalA activity is regulated in response to extracellular cues. For example, RalA mediates the delivery of vesicles that contain the glucose transporter GLUT4 toward the

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<https://doi.org/10.1016/j.ajhg.2020.01.002>

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plasma membrane in adipocytes treated with insulin and also mediates the formation of lamellipodia in fibroblasts treated with TNF α .^{8,9} As is true for a typical G protein, RalA activity depends on its nucleotide-binding status. Binding to GTP activates RalA, whereas binding to GDP renders RalA inactive.¹⁰ Thus, RalA is regulated by factors that facilitate nucleotide exchange (guanine exchange factors [RalGEFs]) or GTP hydrolysis (RalGAP). In its basal state, RalA is maintained in an inactive GDP-bound form by the RalGAP complex and can be activated either by RalGEF (e.g., downstream of Ras) or by the inactivation of RalGAP (as was described for insulin signaling).¹⁰ The RalGAP complex is a heterodimer of a catalytic subunit, either RalGAP1 or RalGAP2 (the expression of which is cell-type-specific), and a scaffolding subunit, RalGAPB.² It has been demonstrated in several mouse models that the loss of either RalGAP subunit results in constitutive activation of Ral proteins, and physiological outcomes are highly cell-type- and development-stage-specific. It is important to note that the ability to cycle between GTP- and GDP-bound states might be important for RalA function.¹⁰ Thus, although constitutive RalA activation might facilitate certain signaling cascades, it might disrupt the others.

Although the Ral GTPase pathway is well known in cancer biology, its implication in Mendelian disorders has yet to be elucidated. Pathogenic variants in *RALA* (MIM: 179550) have been identified as a cause of an autosomal-dominant inherited syndrome typified by speech and motor delays, intellectual disability, early-onset epilepsy, short stature, facial dysmorphism, and finger and toe abnormalities (e.g., long fingers with extensible joints, finger overlap, and toe clinodactyly).¹¹ *RALGAP1* is ubiquitously expressed; the highest expression levels are in the brain, ovaries, and uterus (GTEx: phs000424.v7.p2 on 02/07/2019). *RALGAP1* has been suggested as a candidate gene for neurodevelopmental disorders because microdeletions, including deletion of *RALGAP1*, were identified in individuals with developmental delay, microcephaly, seizures, muscular hypotonia, and mild brain atrophy. In these cases, *RALGAP1* was the only deleted gene with high expression in the brain.^{12,13} A morpholino oligonucleotide knockdown of the homolog *tulip1* in a zebrafish model resulted in severe developmental impairment of the brain and comparably mild abnormalities of the whole body.¹²

Here, by identifying bi-allelic variants in *RALGAP1* in four unrelated families, we establish that the loss of *RALGAP1* disrupts the RalGAP complex, leads to constitutive RalA activation, and causes a severe neurodevelopmental disease with profound muscular hypotonia, infantile spasms, and feeding difficulties.

Legal guardians of all individuals investigated gave written informed consent for genetic testing and functional studies from tissue material (families A and B). Permission to include clinical photographs was obtained. The study was performed according to the declaration of Helsinki. The local ethical committees at the Heinrich-Heine-University

Düsseldorf (#4957), the Technical University in Munich (#5360/12S), King Faisal Specialist Hospital and Research Center (RAC #2080006), and the Sheba Medical Center (#7786-10-SMC) approved the genetic studies and the studies on tissue samples derived from individuals 1 and 2, where applicable.

The clinical phenotype of the affected individuals is summarized in Table 1. Detailed case reports can be found in the Supplemental Information. Pedigrees and clinical images are depicted in Figure S1 and Figure 1, respectively.

All individuals were born after uneventful pregnancies. Only one child was born preterm. Birth parameters were normal in two individuals, and two children were born small for gestational age. All four children presented with respiratory distress requiring transient non-invasive ventilation directly after birth. One child required a tracheostomy as a result of persistent respiratory problems. Congenital profound muscular hypotonia was a unifying clinical feature. During the first year of life, three individuals had frequent episodes of high fever. A dysregulation of central temperature control was suspected. In two individuals, recurrent viral infections were noted, but there were no abnormalities on immunologic work-up. In one child, hypohidrosis was diagnosed. All individuals had feeding abnormalities requiring tube feeding. Infantile spasms starting between the ages of 8 and 12 months were observed in three out of four children. In this context, it should be noted that the only child without clinical evidence of epilepsy is still very young (8 months at last followup appointment). Seizures responded to adrenocorticotrophic hormone (ACTH) treatment in one child, and two children suffered from drug-resistant epilepsy. Profound developmental delay was observed in all children, none of whom reached any major developmental milestones. Brain MRIs in the affected individuals revealed dysplasia of the corpus callosum; focal thinning of the posterior part (splenium) was a unifying CNS finding (Figure 1 and Figure S2). In addition, a wide spectrum of unspecific abnormalities, including gray-matter heterotopias, ectopic posterior pituitary, signal abnormalities in basal ganglia, and stratum subependymale, was observed (see Figure S3). Only one child showed signs of global brain atrophy (Figure 1). Interestingly, moderate dysmorphic facial features were observed in all of the children. They had a rather consistent phenotype of upslanting palpebral fissures, mild epicanthus, a short nose with a flattened nasal bridge, and slightly anteverted nares (see Figure 1). The oldest child is now 5 years old and is severely disabled. He is unable to sit, crawl, or pull up. Head control is still inadequate, and truncal muscle tone is profoundly reduced. He did not acquire language, and he never interacted with his environment.

Exome sequencing (ES) was performed at four clinical sites in Munich, Tel-Aviv, Chapel Hill, and Riyadh. For family A, trio ES was performed with the Sure Select Human All Exon kit V5 (index individual) and V6 (parents; Agilent) for exome enrichment and a HiSeq2500 (index individual)

Table 1. Clinical Characteristics of Individuals with <i>RALGAP1</i> Variants				
Family	Family A	Family B	Family C	Family D
ID	Individual 1	Individual 2	Individual 3	Individual 4
Age at last examination	4 1/2 years	8 months	18 months	17 months
Gender	male	female	male	male
Genome molecular analysis	exome sequencing	exome sequencing	exome sequencing	exome sequencing
Variant (NM_194301.4)	c.3227A>G;1126C>T, (p.Asn1076Ser;Arg376*)	c.4992del;4992del, (p.Phe1664Leufs*11; Phe1664Leufs*11)	c.610G>T;610G>T, (p.Glu204*;Glu204*)	c.5732C>G;(5732C>G), (p.Ser1911*;Ser1911*)
Pathogenicity	pathogenic	pathogenic	pathogenic	pathogenic
Familial History and Perinatal Period				
Ancestry	German	Palestinian	German and Irish	Saudi
Consanguinity	no	yes	no	yes
Pregnancy	unremarkable	unremarkable	poor maternal weight gain	preterm delivery
Prematurity	no	no	no	born at 34 weeks
Perinatal period	neonatal respiratory distress	neonatal respiratory distress	neonatal respiratory distress	neonatal respiratory distress
Growth Parameters				
Birth length	56 cm (97 th centile)	N/A	45.5 cm (5 th centile)	43 cm (<5 th centile)
Birth weight	3,875 g (79 th centile)	3,000 g (20 th centile)	2,240 g (5 th centile)	2,115 g (<5 th centile)
Head circumference	36.5 cm (93 th centile)	N/A	33.5 cm (13 th centile)	33.5 cm (10 th centile)
Age at examination (y)	4 1/2 years	3 months	18 months	17 months
Current height	111 cm (75 th centile)	51 cm (<1 st centile)	78.1 cm (6 th centile)	75 cm (5 th centile)
Current weight	16.4 kg (25 th centile)	2.9 kg (<1 st centile)	9.52 kg (7 th centile)	12.5 kg (75 th centile)
Current head circumference	51.5 cm (52 th centile)	35 cm (<1 st centile)	46.8 cm (7 th centile)	48 cm (37 th centile)
Motor development	severely delayed	severely delayed	severely delayed	severely delayed
Speech development	none	none	none	none
Neurological Findings				
Muscle tone	profound muscular hypotonia	profound muscular hypotonia	profound muscular hypotonia	profound muscular hypotonia
Head control	poor	poor	poor	poor
Spasticity	no	yes	no	yes
UL/LL tendon reflexes	reduced/reduced	reduced/reduced	reduced/reduced	increased
Seizures	infantile spasms	no	infantile spasms	infantile spasms
Brain MRI	focal thinning of corpus callosum, gray matter heterotopias	focal thinning of corpus callosum, thin pituitary stalk, global brain atrophy	focal thinning of corpus callosum, ectopic pituitary	focal thinning of corpus callosum
Skin findings	dry skin and hypohidrosis	none	none	none
Ophthalmologic findings	bilateral cataract	none	cortical vision impairment (no cataracts)	suspected cortical vision impairment (not confirmed)
General Clinical Findings				
Heart	normal	normal	normal	mild tricuspid valve regurgitation and mild mitral valve regurgitation.
Lungs	normal	tracheostomy	laryngomalacia, oxygen by nasal canula during sleep	recurrent apnea, tracheomalacia with intermittent stridor

(Continued on next page)

Table 1. Continued

Family	Family A	Family B	Family C	Family D
ID	Individual 1	Individual 2	Individual 3	Individual 4
Gastrointestinal tract	feeding difficulties, gastrostomy tube	feeding difficulties, nasogastric tube	feeding difficulties, gastrostomy tube	feeding difficulties, gastrostomy tube, gastro-esophageal reflux
Urinary tract	bilateral vesicoureteric reflux grade II	normal	normal	vesicoureteric reflux, right grade III / IV and left grade III
Recurrent fever / frequent viral infections	yes	yes	no	yes
Limbs	drop foot	normal	soft tissue syndactyly of fingers 3 and 4 bilaterally, partial syndactyly of toes 2, 3, and 4 on the right foot	normal
Craniofacial features	thick eyebrows, deep-set eyes, short nasal bridge with anteverted nares, large mouth with prominent lower lip, protruding tongue	bushy eyebrows and a low anterior hairline	flat occiput and brachycephaly, high anterior hair line with frontal balding, tall forehead, full cheeks, medial eyebrow flare, left epicanthal fold, pointed superior helices of ears, upturned nasal tip	horizontal eyebrows, low-set ears, depressed nasal bridge, and large mouth with prominent lips, and slight retrognathia
Additional clinical findings	gingival hyperplasia	mild hepatomegaly	No	gingival hyperplasia

Abbreviations are as follows: y = year; n/a = not assessed; UL = upper limb; LL = lower limb; and MRI = magnetic resonance imaging.

and HiSeq4000 (parents) system (Illumina) for sequencing. The average read depth was 131×. Reads were aligned to the UCSC human reference assembly (hg19) with the Burrows-Wheeler algorithm (BWA v.0.5.9). More than 98% of the target sequences were covered to a read depth of at least 20×. Single-nucleotide variants (SNVs) as well as small insertions and deletions were detected with SAMtools v.0.1.19. In-house custom Perl scripts were used for variant annotation.

In family B, trio ES was performed with the Sure Select Human All Exon Kit V6 on a HiSeq2500 sequencing machine (Illumina). Reads were aligned to the UCSC human reference assembly (hg19) with the BWA-MEM algorithm (BWA v.0.7.15). Variants were called with Genomic Analysis Toolkit (GATK) variant caller version 3.8; best practices as recommended by the Broad Institute were used.¹⁴ Variant annotation was performed with KGGSeq.¹⁵

For family C, trio ES was done on a clinical basis through GeneDx with XomeDxPlus. A *RALGAP1* homozygous variant was identified but classified as a variant of uncertain significance because no disease was known to be associated with it at that time. 100% of the coding region was covered for this gene at a minimum of 10×, with no indication of a multi-exon deletion or duplication involving this gene.

For family D, index-only ES was performed in parallel with array-based autozygosity mapping as described before.¹⁶

In family A, we filtered for homozygous and compound-heterozygous variants with a minor-allele frequency <0.1% in our in-house database of 15,000 individuals as well as for *de novo* variants that had a minor-allele frequency <0.01%.

A search for bi-allelic variants in family A identified changes in six genes (Table S1), whereas a filter for *de novo* variants did not identify protein altering variants. Among the biallelic variants, variants in *NEB* and *BRCA1* were associated with autosomal recessive inherited conditions and were classified as variants of uncertain significance. Because there was no phenotypic overlap between the condition of our index individual and previously published individuals these variants were considered to not be associated with the child's condition. Of the remaining variants, only the compound-heterozygous variants in *RALGAP1* (GenBank: NM_194301.4) were predicted to result in a loss of function c.3227A>G(p.Asn1076Ser) and c.1126C>T(p.Arg376*). The parents were heterozygous carriers for one of the variants each. Variants were confirmed by Sanger sequencing in the index individual and his parents (Figure S1).

We next sought to identify further families with bi-allelic variants in *RALGAP1* by using the web-based collaboration platform GeneMatcher.¹⁷ We identified additional individuals who came from three unrelated families and had an overlapping phenotype and bi-allelic loss-of-function variants in *RALGAP1* in Israel (family B), the USA (family C), and Saudi Arabia (family D, Figure 1). In family B, a homozygous frameshift variant, c.4992delT(p.Phe1664Leufs*11), was detected; in family C, the homozygous nonsense variant c.610G>T(p.Glu204*) was detected.

In family D, the combination of ES and array-based homozygosity mapping revealed a homozygous nonsense variant, c.5732C>G(p.Ser1911*), within an autozygosity block (see Figure S4) that is unique to the affected

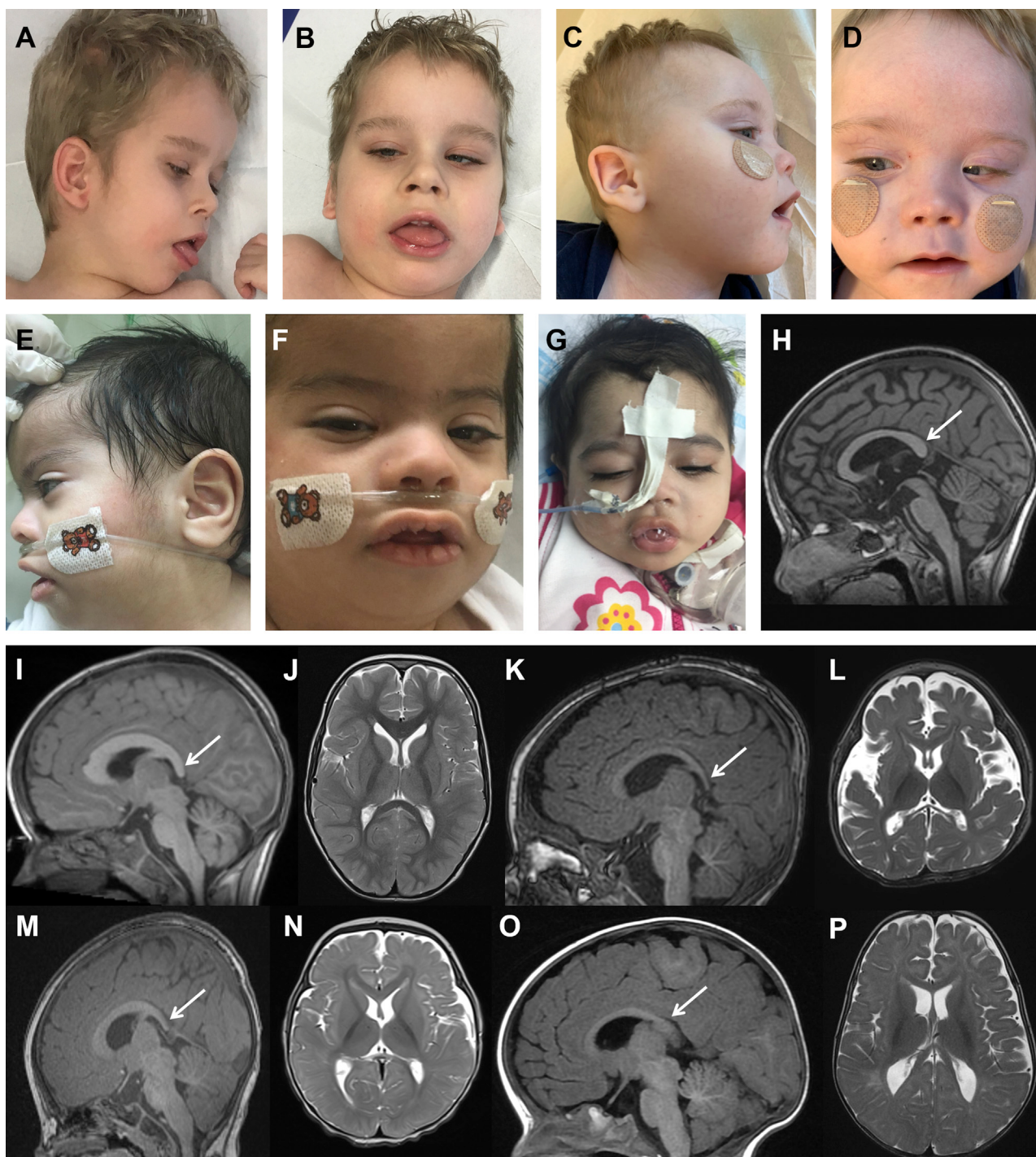


Figure 1. Clinical Details of Affected Individuals

(A–G) Clinical photographs of individuals with pathogenic variants in *RALGAP1*. (A and B) Individual 1 at the age of 4 1/2 years. (C and D) Individual 3 at the age of 18 months. (E and F) Individual 4 at the age of 17 months. (G) Individual 2 at the age of 8 months. All children suffered from severe developmental disability with profound muscular hypotonia and feeding abnormalities. Facial features included upslanting palpebral fissures, mild epicanthus, a short nose with a flattened nasal bridge, and slightly anteverted nares.

(H) Brain MRI image (T1-weighted sagittal view) of a healthy individual at the age of 4 1/2 years. The white arrow indicates the posterior part (splenium) of the corpus callosum.

(I–P) Brain MRI images of individuals with bi-allelic variants in *RALGAP1* (T1-weighted sagittal as well as T2-weighted axial views). (I and J) Individual 1 (age 4 1/2 years). (K and L) Individual 2 (age 8 months). (M and N) Individual 3 (age 10 months). (O and P) Individual 4 (age 8 months). Of note, in all individuals with pathogenic variants in *RALGAP1*, a moderate dysplasia of the corpus callosum with focal thinning of the posterior part was detectable (white arrows; compare with normal findings in panel [H]). Further details (including age-related normal values) regarding this finding are depicted in [Figure S2](#). In addition, individual 2 showed global brain atrophy (L). Other variable or unspecific CNS abnormalities in individuals with *RALGAP1* deficiency are depicted in [Figure S3](#).

individuals. Additional information on the *RALGAPA1* variants can be found in [Table S2](#).

To further support the relevance of *RALGAPA1* variants, we used living skin fibroblasts derived from the affected individuals from families A and B. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; life technologies) supplemented with 10% fetal bovine serum (FBS)(Life Technologies) and 1% penicillin-streptomycin (Life Technologies) at 37°C in a humidified atmosphere of 5% CO₂.

For immunoblotting, whole-cell lysates were prepared with cell-lysis reagent CellLyticM (Sigma-Aldrich). A monoclonal antibody against RalGAPA1 (rabbit; 1:3000; EPR13592-38; Abcam) was used. GAPDH served as a loading marker (mouse; 1:4000; Thermo Fisher Scientific, catalog number AM4300). BM chemiluminescence blotting substrate (Roche) was used for visualization. Methodological details can be also found in the [Supplemental Information](#). As depicted in [Figure 2](#), a RalGAPA1 protein signal was nearly absent in fibroblasts of affected individuals, suggesting a loss-of-function effect in the investigated cell lines. To determine whether the variants result in nonsense-mediated decay (NMD), we additionally performed qRT-PCR for *RALGAPA1* expression for individual 1, and this analysis revealed a reduction to 54% of the transcriptional level ([Figure S5](#)). This indicates that transcripts with the nonsense variant undergo NMD, whereas the missense variant is expressed, and reduced protein amounts are rather a consequence of protein instability.

As a next step, we aimed to gain further insight into the functional consequences of RalGAPA1 deficiency. Of note, the RalGAP complex facilitates GTP hydrolysis on RalA and thus converts RalA into an inactive GDP-bound form. The loss of RalGAP subunits in mice results in constitutively active GTP-bound RalA.¹⁸ This can be exemplified by the deletion of the catalytic subunit RalGAPA2 in the bladder or RalGAPA1 in muscles, as well as the deletion of scaffolding subunit RalGAPB in adipose tissue.^{8,18,19} Thus, we tested RalA activation status in human fibroblasts with RalGAPA1 deficiency. For RalA activity measurements, the fibroblasts were starved in FBS-free media and then in PBS. Fresh cell lysates were used for GTP-bound RalA pulldown with RalBP1 beads (Millipore) according to the manual. Samples 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). Individual proteins were detected with the specific antibodies and visualized on film via horseradish-peroxidase-conjugated secondary antibodies and Western Lightning Enhanced Chemiluminescence (Perkin Elmer Life Sciences). Further details are explained in the [Supplemental Material and Methods](#).

In fibroblasts with RalGAPA1 deficiency, RalA activity was increased in comparison to that in control fibroblasts ([Figure 2](#)). This finding is in line with the idea that RalGAPA1 deficiency causes constitutive activation of RalA. Additionally, amounts of RalGAPB, a scaffolding sub-

unit of RalGAP complex, were dramatically reduced in samples without the RalGAPA1 subunit, indicating a dysfunctional RalGAP complex and the necessity of RalGAPA1 for RalGAPB stability. *RALGAPB* expression analysis via RT-qPCR revealed no downregulation at the transcriptional level, supporting this hypothesis (see [Figure S6](#)).

Constitutive activation of the RalA pathway might have important consequences for cell physiology. Importantly, it has been shown that constitutively active RalA retains lipid-raft microdomains in detached cells at the plasma membrane and promotes anchorage-independent growth signaling.²⁰ It was suggested that this mechanism might play a role in tumor and metastasis formation. To further investigate this aspect in individuals with RalGAPA1 deficiency, we analyzed the localization of lipid-raft components in detached fibroblasts by using the fluorescence marker CTxB-Alexa 594 (for details, see [Supplemental Information](#)). Of note, CTxB (cholera toxin B subunit) constitutes the membrane-binding subunit of cholera toxins and serves as a well-established marker of lipid rafts.²¹ CTxB Alexa 594 cell-surface fluorescence was visualized by ZEISS ApoTome imaging, and quantitative data were obtained by fluorescence-activated cell sorting (FACS) analysis. As depicted in [Figure 2](#), fibroblasts with variants in *RALGAPA1* retained significantly higher levels of surface lipid-raft components than did several control cell lines. Raw data that were used for quantification can be found in [Figure S7](#). FACS analysis of CTxB-stained fibroblasts confirmed these results with a shift toward higher fluorescence intensity, indicating plasma membrane retention of lipid rafts in RalGAPA1-deficient fibroblasts (see also [Figure S8](#)). This finding is in agreement with previous research and indicates that cell-growth signaling might be altered in individuals with RalGAPA1 deficiency.²⁰

Taken together, the experiments described above confirmed a loss-of-function character of the *RALGAPA1* variants and are in line with an autosomal-recessive inheritance pattern. Given the loss-of-function nature of the variants we describe here and the strikingly consistent associated phenotype, we conclude that the loss of RalGAPA1 in the individuals reported here is most likely responsible for the observed clinical abnormalities. This hypothesis is strengthened by the gnomAD gene-constraint metrics showing a depletion of loss-of-function and missense variants (observed-expected ratio of loss-of-function variants in *RALGAPA1* = 0.12; 0.69 for missense variants).²² This finding indicates that *RALGAPA1* is a disease-associated gene. In light of the constraint metrics, the identification of one missense variant in our study group suggests that both loss-of-function and missense variants can cause disease.

Regarding the functional consequences of RalGAPA1 deficiency, it is suggestive that alterations in intracellular trafficking downstream of constitutively active RalA and possibly RalB are involved in disease pathophysiology. However, cell physiological abnormalities caused by

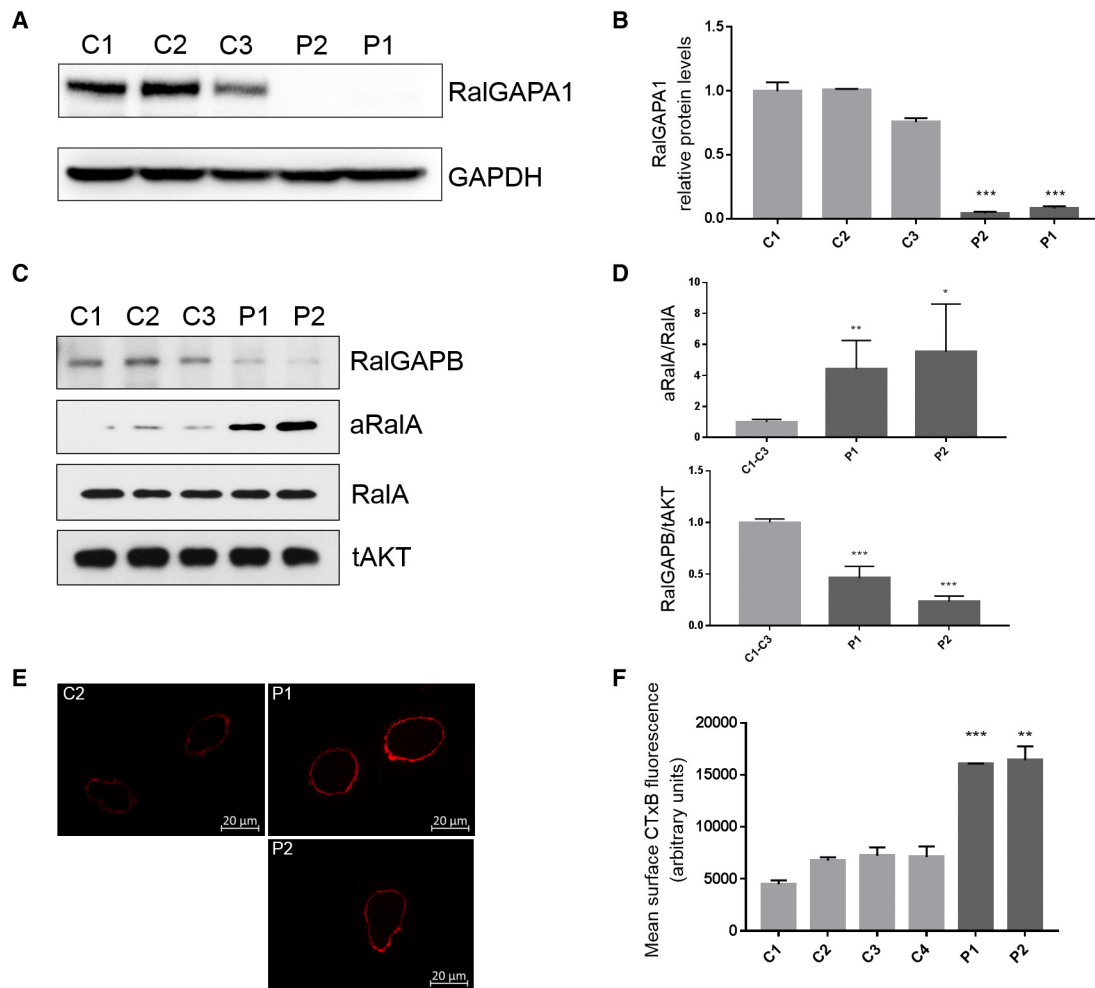


Figure 2. Functional Consequences of RalGAPA1 Deficiency

(A) Representative immunoblot image of fibroblasts derived from two individuals with pathogenic variants in *RALGAP1* (P1 [derived from individual 1] and P2 [derived from individual 2]) and three control fibroblast lines (C1–C3). GAPDH served as a loading marker. Molecular weight: RalGAPA1 = 230 kDa; GAPDH = 36 kDa.

(B) Quantitated density of immunoblot bands from four independent experiments. Average values are presented as the mean $\pm$ SEM; *** p < 0.001 significantly different from controls. In P1 and P2 fibroblasts, RalGAPA1 amounts are drastically reduced.

(C) Representative immunoblot image of the amounts of active GTP-bound RalA (aRala) (these amounts were determined by a pull-down of aRala with RalBP1 beads) in comparison to RalGAPB, RalA, and tAKT. Molecular weight: RalGAPB = 165.2 kDa; RalA = 23.5 kDa; and AKT = 55.7 kDa.

(D) Quantification of immunoblot results from three independent experiments. Average values are presented as means $\pm$ SEM; * p < 0.05, ** p < 0.01, and *** p < 0.001 significantly different from controls (C1–C3, pooled control). Amounts of GTP-bound RalA are increased in P1 and P2 cell lines compared to controls (C1–C3). Moreover, amounts of RalGAPB are reduced in P1 and P2 cell lines. The amounts of AKT (AKT serine-threonine kinase 1) are not altered.

(E) Microscope images (optical sections) of CTxB Alexa 594 cell-surface fluorescence in detached human skin fibroblasts. Images were obtained with an Axio Observer Z1 microscope with ApoTome 2 (63 $\times$ objective lens, Zeiss). Images depict typical examples of control fibroblasts (C2) and two fibroblast lines derived from affected individuals (P1 and P2).

(F) Quantification of CTxB Alexa 594 cell-surface fluorescence by FACS analysis. Results depicted represent data from three independent experiments. Average values are presented as means $\pm$ SEM; ** p < 0.01 and *** p < 0.001 significantly different from controls. P1 and P2 fibroblasts show higher levels of CTxB Alexa 594 fluorescence, indicating a disturbance of lipid-raft clearance from the plasma membrane in detached cells.

constitutively active RalA will be highly dependent on cell type. Which cell types are affected is dependent on whether RalGAPA1 is a major catalytic subunit of the RalGAP complex in the respective tissue because RalGAPA2 might be predominant. In light of the above results, RalGAPA1 deficiency appears to have a crucial impact on neuronal function and development. Directly after birth,

all individuals identified in this study displayed neuromuscular abnormalities (e.g., respiratory problems, feeding difficulties, muscular hypotonia) that later developed into severe psychomotor disability. Three individuals developed early-onset epilepsy. All children had mild-to-moderate dysmorphic facial features, and one had soft tissue syndactyly of fingers (see Figure 1 and Figure S9). Brain

MRIs revealed dysplasia of the corpus callosum as well as additional variable CNS manifestations (e.g., gray-matter heterotopias, ectopic posterior pituitary) that suggested an impact on brain maturation (Figure 1 and Figures S2 and S3). Characterization of the *RALGAP1* homolog in mice (*Ralgap1*, also known as *GRIPE*) indicated that its expression is upregulated in the developing brain during embryogenesis and remains high in the adult brain, especially in neurons of the cortex and hippocampus.²³ In addition, knockdown of the *RALGAP1* homolog in zebrafish resulted in severe brain developmental delay.¹² Finally, *RalGAPB* knockout (which also leads to *RaA* activation) in mice is embryonic lethal (unpublished data). These data highlight the importance of *RALGAP1* for neuronal development and differentiation.

On the basis of the complexity of the *RaA* pathway and its downstream effectors, the mechanisms behind the impact of disturbed *RaA* signaling on the nervous system will be difficult to dissect. Nevertheless, one important experimental result might be the disturbance that we observed in lipid-raft microdomains in *RalGAP1*-deficient fibroblasts. It is well known that lipid remodeling is crucial for the development of the central nervous system via compartmentalization, signal transduction, cell adhesion, and axonal spreading.^{24,25} Accordingly, altered lipid-raft trafficking might be a critical aspect of neuronal development in individuals with *RALGAP1* variants.

Interestingly, the pathophysiology of *RalGAP1* deficiency might be at least in part similar to the mechanism of *RALA* variants published by Hiatt et al., 2018.¹¹ The majority of *RALA* variants cluster in the GDP-GTP binding pocket and are thus predicted to impair GDP-GTP binding. Three out of four *RALA* mutants that were tested in biochemical assays had a dramatic reduction in effector binding, and all mutants had a significantly reduced GTPase activity. The question of whether reduced effector binding together with decreased GTPase activity increases *RaA* signaling was not examined by the authors, but one *RALA* mutant was definitely constitutively activated, a finding similar to the *RaA* activation that we observed in *RalGAP1*-deficient fibroblasts in this study. In general, the phenotype of the individuals with *RALA* variants was milder than in the children reported here. However, the core phenotype observed in individuals with variants in *RALGAP1* resembles the phenotype of individuals with *RALA* variants in previous studies (e.g., this phenotype includes intellectual disability, developmental delay, muscular hypotonia, and early-onset epilepsy). Furthermore, facial abnormalities (e.g., a short nose, a flattened nasal bridge, and gingival hypertrophy) appear to be a common feature. In addition, more than half of the children reported as having *RALA* variants had a dysplastic corpus callosum (shortening and/or thinning), which appears to be also a unifying MRI feature in individuals with *RalGAP1* deficiency. Interestingly, in some of the individuals with *RALA* variants, finger abnormalities, as also

seen in individual 3, were observed.¹¹ These striking similarities also pose an additional argument that *de novo* mutations in *RALA* might have the same downstream effect as the loss of *RALGAP1*.

In light of the overlapping phenotype between *RALA*- and *RALGAP1*-associated diseases, it becomes evident that dysregulation of the *RaA* signaling pathway leads to a distinct group of genetic syndromes that we suggest could be named *RALopathies*. This group of disorders could be expanded by the identification of further *RALA*-related disease genes.

In addition to the neurological disease manifestation, profound muscular hypotonia was a unifying clinical feature of affected individuals. This is of interest because *RaA* and the *RalGAP* complex have been implicated in the regulation of glucose uptake in response to insulin.⁸ In mice, muscle-specific *Ralgap1* deletion caused *RaA* activation, leading to increased glucose and the uptake of free fatty acids by muscles, thus improving whole body glucose handling and lipid homeostasis. However, the study did not address the impact of *RalGAP1* deletion on muscle physiology or contractile ability. Additionally, the genetic approach used in that study did not allow the authors to address the role of *RalGAP1* in muscle development because *Cre* recombinase under myosin light chain 1f promoter is expressed in mature muscle only. No abnormalities in glucose metabolism were identified in the children we report on here.

Another disease feature of *RalGAP1*-deficient individuals was recurrent episodes of fever with or without viral infections, as was reported in three individuals. This might be related to the severe developmental disability (e.g., increased risk for infections as a result of immobility; risk of aspirations). However, fever episodes and viral infections started so early in life and occurred at such a high frequency that they triggered extensive immunological work-up in two of the individuals. In this context, it is important to note that the *RALGAP1* homolog in mice binds E12, a transcriptional regulator of immunoglobulin genes.²³ Therefore, although no specific immune defect could be identified in the affected individuals, a possible role of *RALGAP1* in immune function might be expected.

Finally, on the basis of the overactivation of *RaA* in autosomal-recessive *RalGAP1* deficiency, it is tempting to speculate that inhibition of the *Ral* pathway via the inhibition of *Ral* prenylation²⁶ or the phosphorylation of *RaA* by Aurora A or protein kinase C²⁷ might have a therapeutic effect. However, it will be very difficult to titrate the effect of such inhibitor approaches (especially against the background wherein *RaA* downregulation might also cause a neurological phenotype). Moreover, in light of the neonatal disease onset, it is suggestive that the variant already affects prenatal development in a way that might not be compensated for by a postnatal start of treatment. Further studies in knockout animal models of *RALGAP1* will be helpful in addressing this issue.

Accession Numbers

The accession numbers for the data reported in this paper are as follows: for individual 1, ClinVar: VCV000691798 and VCV000691794; for individual 2, ClinVar: VCV000691795; for individual 3, ClinVar: VCV000691796; and for individual 4, ClinVar: VCV000691797.

Supplemental Data

Supplemental Data can be found online at <https://doi.org/10.1016/j.ajhg.2020.01.002>.

Acknowledgments

First and foremost, all authors thank the families for participating in the study. The study was supported by a grant from the German Research Foundation/Deutsche Forschungsgemeinschaft (DI 1731/2-2 to F.D.) and by a grant from the “Elterninitiative Kinderkrebsklinik e.V.” (Düsseldorf; #701900167). We thank Katharina Mayerhaner for excellent sample handling, Veronika Treffer for Sanger sequencing, and the next-generation core facility of the Helmholtz-Zentrum Munich, especially Elisabeth Graf, Riccardo Berutti, and Sandy Lösecke, for their support. Moreover, we thank Nechama Shalva and Michalle Soudack of the Sheba Medical Center for their support. We also thank Niema Ibrahim for her hard work in helping to recruit family D and collect clinical data. F.D. would like to thank Ernst Otto Fischer, 1973 Nobel Prize laureate for Chemistry, for his support and inspiration.

Declaration of Interests

The authors declare no competing interests.

Received: September 27, 2019

Accepted: January 6, 2020

Published: January 30, 2020

Web Resources

ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>
GeneMatcher, <https://www.genematcher.org>
gnomAD Browser, <https://gnomad.broadinstitute.org/>
OMIM, <https://www.omim.org/>

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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 thermodyregulation 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.

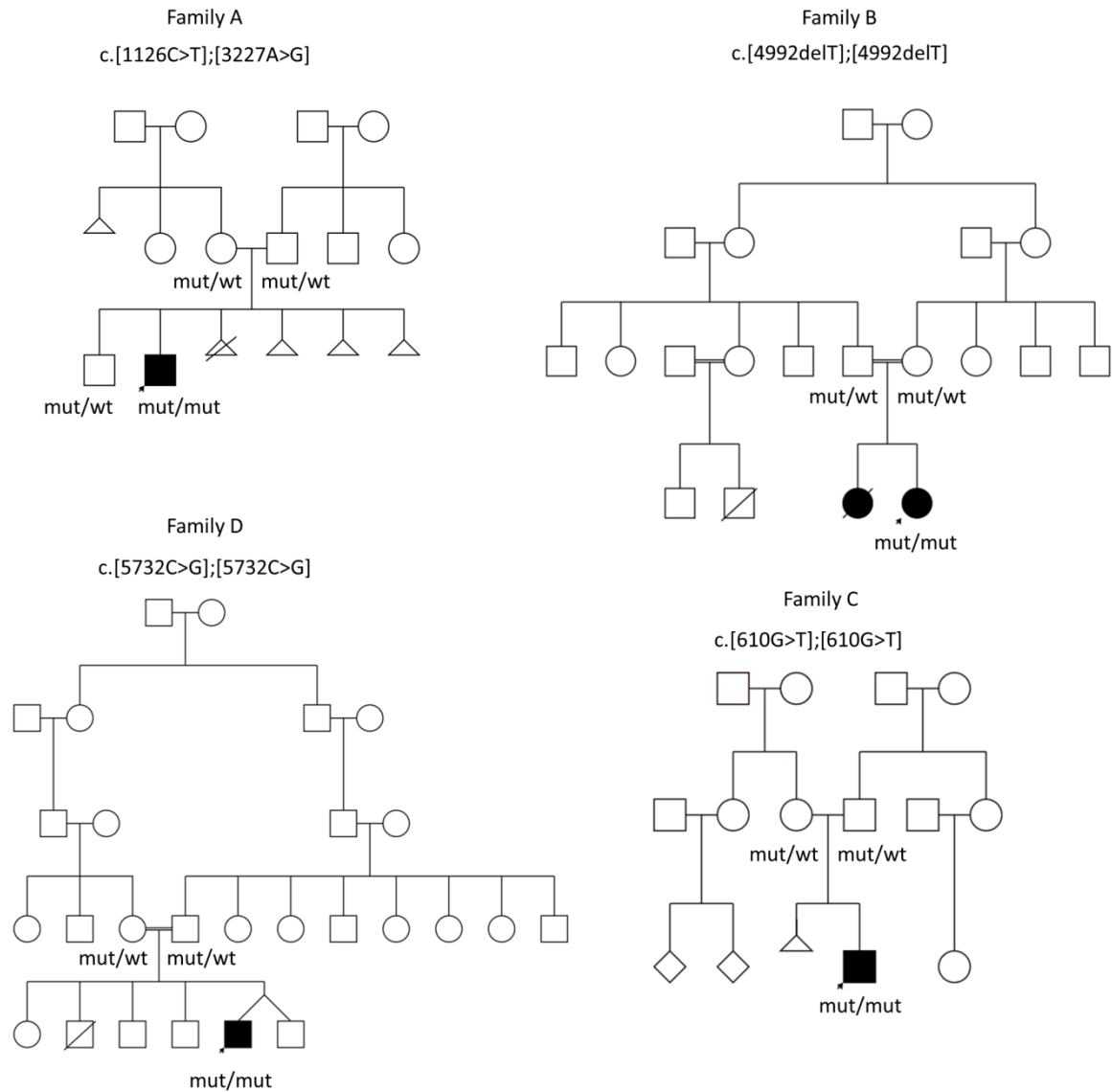
During the neonatal period, the child developed tracheomalacia with on and off stridor as well as recurrent apneas that were associated with poor feeding. These symptoms were progressive and became evident at age of 5 months when he required being on oxygen supplementation. Subsequently, muscular hypotonia 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.

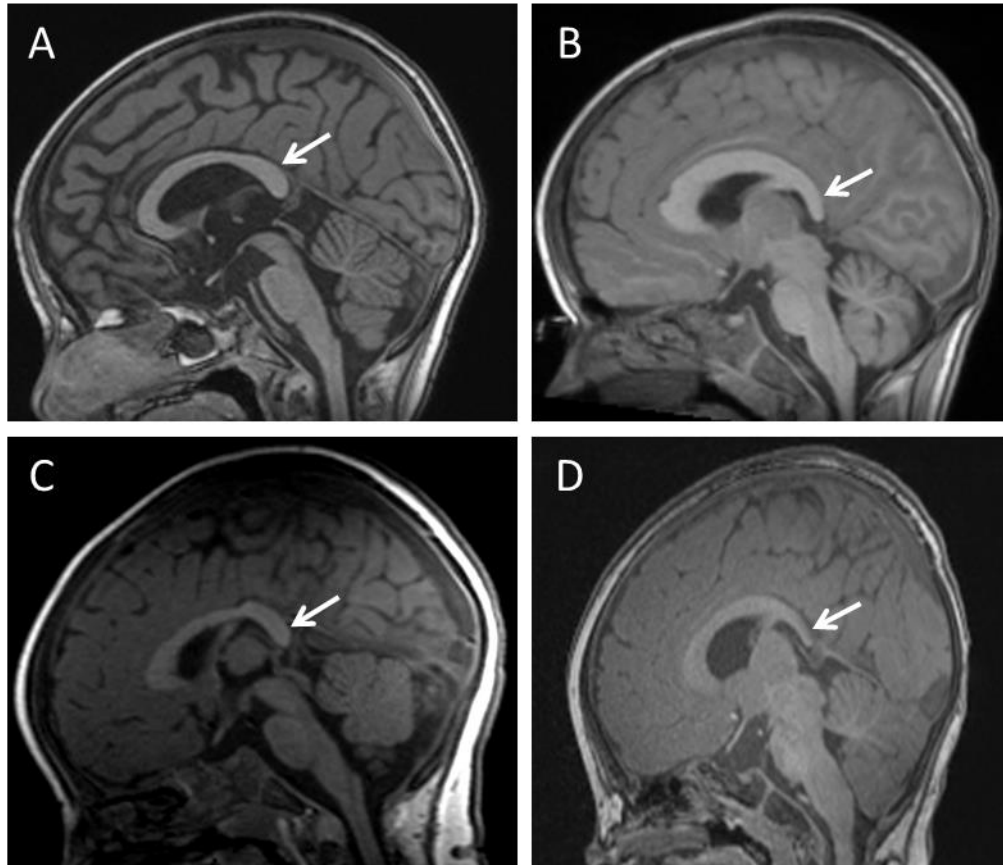
At the current age of 17 months, he is chronically admitted to the hospital (with oxygen dependency, on PEG-tube feeding and status post fundoplication due to failed swallowing assessment and moderate gastro-esophageal reflux). His growth parameters: length 75 cm [5th P], weight 12.5 kg [75th P], head 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:

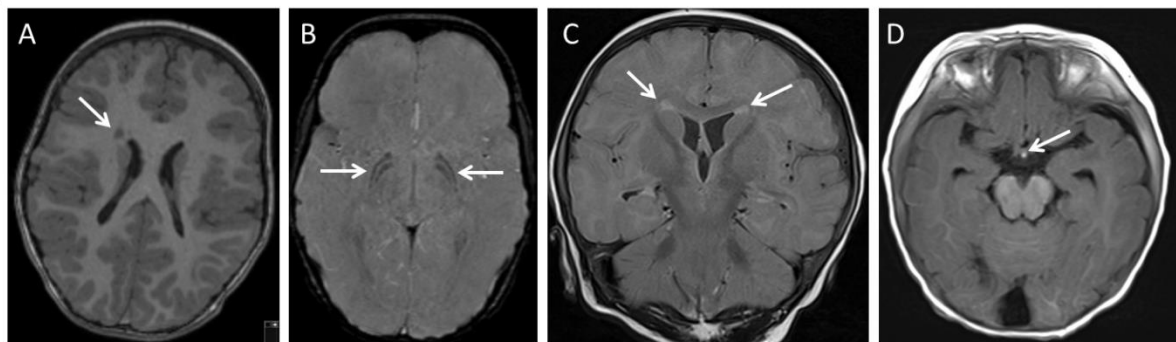


Supplementary Figure 1: Pedigrees of the investigated families. Variants identified are depicted over each pedigree; mut/mut indicates homozygosity or compound-heterozygosity for the pathogenic variant(s) whereas mut/wt indicates heterozygous carriers. Individuals without further indication of carrier 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.

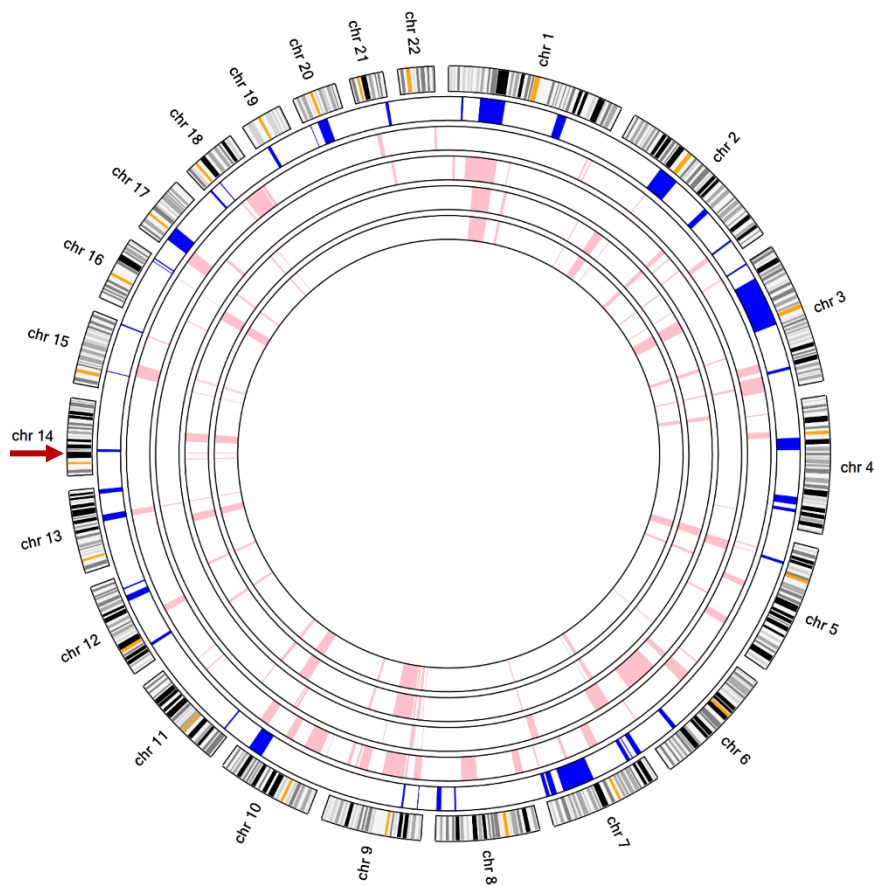
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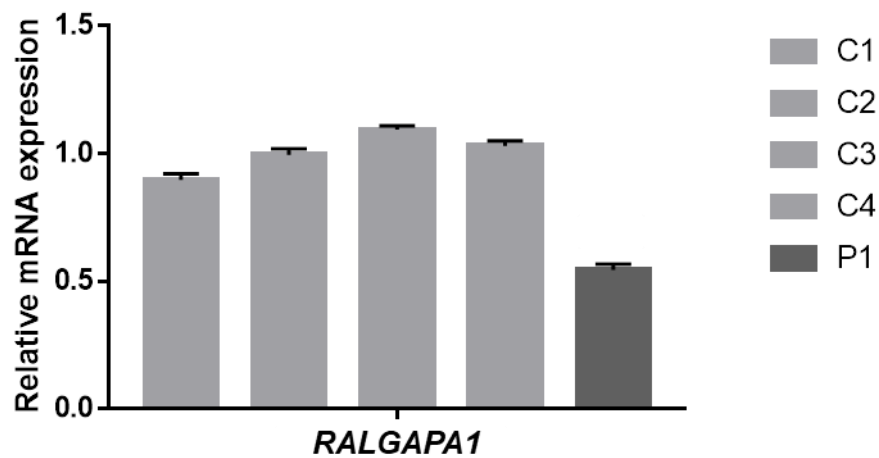
Supplementary Figure 2: Corpus callosum abnormalities in *RalGAPA1*-deficiency. A) Brain MRI image (T1-weighted sagittal view) of a healthy individual at the age of 4 1/2 years. B) Brain MRI image (T1-weighted sagittal view) of individual 1 at the age of 4 1/2 years. Please note the morphological differences 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).



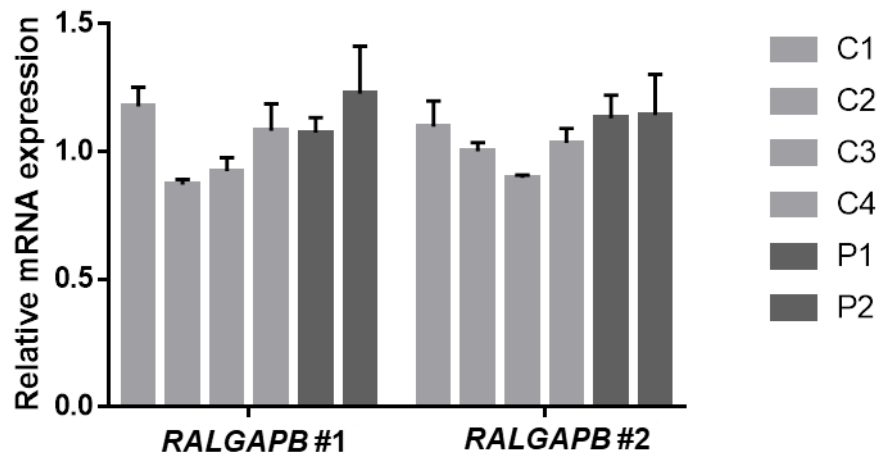
Supplementary Figure 3: Variable CNS findings in individuals with variants in *RALGAP1*. A) Brain MRI image (T1-weighted axial view) of individual 1 at the age of 4 1/2 years shows nodular heterotopia (white arrow). B) Susceptibility-weighted MRI imaging (axial view) of individual 1 at the age of 4 1/2 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).



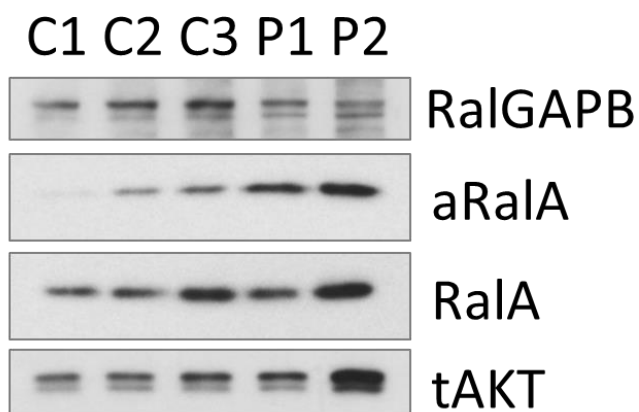
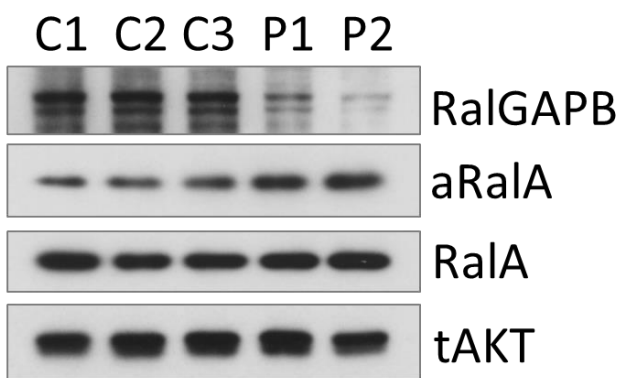
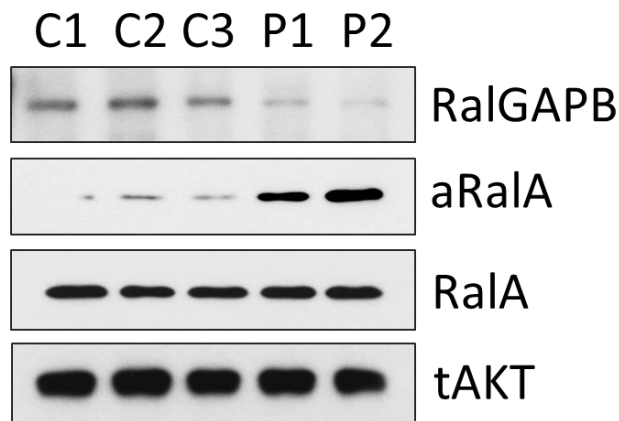
Supplementary Figure 4: Homozygosity mapping of Family D. The figure shows homozygous regions of the index patient of family D (outer ring) and the four unaffected siblings (inner rings). Homozygous regions are highlighted in blue for the index patient and pink for the unaffected siblings. The homozygous 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.

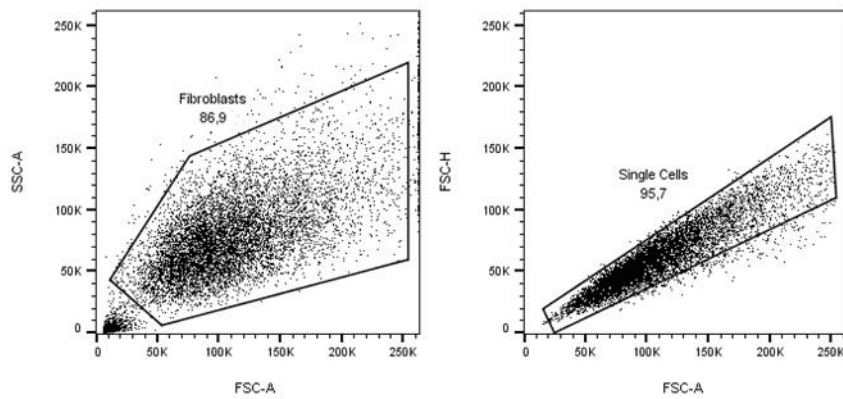


Supplementary Figure 6: Comparison of relative mRNA amount of *RALGAPB* between individual 1 and 2 with control fibroblasts. Relative mRNA amount was detected by two different primer pairs (*RALGAPB* #1 and #2) and normalized against three reference genes (HPRT, GAPDH, β 2M) using the Pfaffl method. 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.

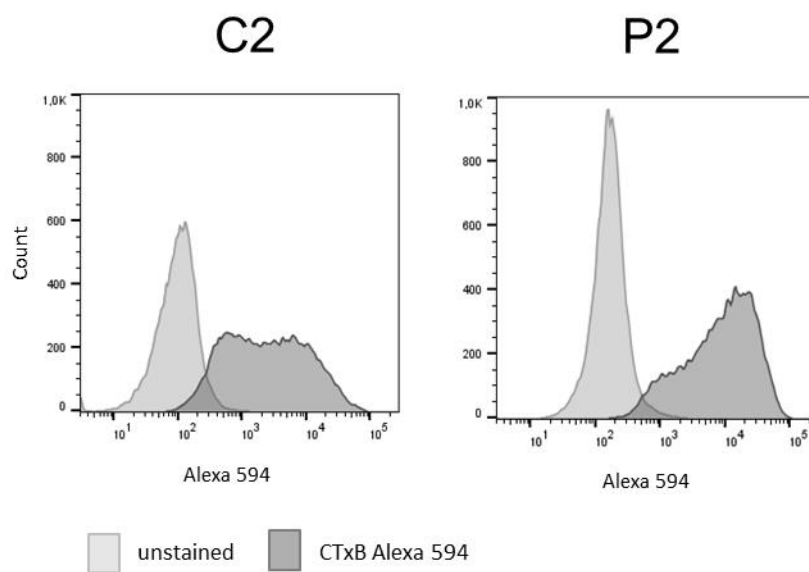


Supplementary Figure 7: RalGAPB, aRala, Rala, and AKT immunoblotting. Panels represent immunoblot images that were used for quantification of aRala/Rala and RalGAPB/tAKT ratios. For description of methodological details see below. For interpretation of findings see main text.

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	CACAGAAGTATGGCTCGCTTC	

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.
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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.