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Rare heterozygous *de novo* variants in *RAPGEF2* are associated with a neurodevelopmental disorder

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H.J.B., S.B., O.K., K.A.W., X.P., and A.H.B. designed the clinical study and functional experiments. molecular cloning, mutagenesis, generation of the needed reagents, behavioral experiments, immunofluorescence staining, and Western blot analyses performed by A.H.B., X.P., S.B.H., and M.A.C., and supervised by H.J.B., O.K., J.M.S., and S.Y. The clinical and genomic data of probands provided by K.A.W., J.D., E.L., T.M.M., M.S.A., D.A.S., P.Y.B.A., J.P.A., I.E.S., A.K., M.B., M.S.H., A.T.M., E.E., F.H., J.E.P., P.K., E.V., and S.B. (K.A.W. and S.B. lead the clinical team). A.H.B. and K.A.W. contributed to the bioinformatic analyses. Data were analyzed and interpreted by H.J.B., A.H.B., and O.K. The manuscript was written by A.H.B. and K.A.W., and revised by all co-authors, with intensive revisions by O.K., S.B., and H.J.B.

Declaration of interests

The authors declare no competing interests.

Web resources

CADD, <https://cadd.gs.washington.edu/>

ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>

DGV, <http://dgv.tcag.ca/>

DIOPT, <http://www.flymai.org/diopt/>

DOMINO, <https://domino.iob.ch/index.php>

FlyCellAtlas, <https://scope.aertslab.org/#/FlyCellAtlas/>

gnomAD, <https://gnomad.broadinstitute.org/>

MutationTaster, <http://www.mutationtaster.org/>

OMIM, <https://omim.org/>

PolyPhen2, <http://genetics.bwh.harvard.edu/pph2/>

SIFT, <https://sift.bii.a-star.edu.sg/>

MARRVEL, <https://marrvel.org/>

IMPC, www.mousephenotype.org

BDSC, <https://bdsc.indiana.edu/>

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Ethics Declaration

This study was reviewed and approved by the Children's National Hospital institutional review board under the Regeneron protocol. Written informed consents were provided by parents or legal guardians at the enrolling institution.

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Abstract

Purpose: *RAPGEF2* encodes a guanine nucleotide exchange factor (GEF) that activates small GTPases and has not been linked to a Mendelian disorder. *RAPGEF2* is highly intolerant to loss-of-function variants. We report five *de novo* heterozygous variants in *RAPGEF2* in unrelated individuals with developmental delay, attention deficit hyperactivity disorder, epilepsy, dysmorphic features, or other manifestations. We used a *Drosophila* model to assess the functional impact of the identified human variants.

Methods: We generated a *Kozak-GAL4* null allele of the *Drosophila* ortholog of *RAPGEF2*, *PDZ-GEF*, and used the allele to determine the gene expression pattern as well as the LoF phenotypes. We expressed the reference and variant *RAPGEF2* in *PDZ-GEF* mutant background to conduct "humanization" studies.

Results: Our experiments show that *PDZ-GEF* is expressed in the central nervous system. Loss of *PDZ-GEF* leads to severe locomotion defects, aberrant microtubular stability in motor neuron axons, and synaptic overgrowth at neuromuscular junctions in third instar larvae. Mutant animals are lethal at various developmental stages. Importantly, the neurodevelopmental phenotypes can be rescued by expression of the human *RAPGEF2* reference cDNA but not by any of the variants.

Conclusion: Our findings provide functional evidence that the tested *RAPGEF2* variants are LoF alleles and that the *RAPGEF2* variants are associated with a neurodevelopmental disorder.

Keywords

PDZ-GEF; global developmental delay; attention deficit hyperactivity disorder; dysmorphic features; cardiovascular deficit; locomotion defects; microtubule stability; synaptic overgrowth; *Drosophila*

Introduction

The *RAPGEF* (Rap guanine nucleotide exchange factor) gene family encodes proteins that regulate the activity of Rap small GTPase by facilitating the exchange of GDP for GTP. RAPGEFs are often regulated through multiple regulatory domains, and are involved in diverse signaling pathways^{1,2}. *RAPGEF2*, also known as *PDZ-GEF1* is located on chromosome 4q32.1 and is ubiquitously expressed in multiple tissues including the nervous system^{1,3,4}. *RAPGEF2* contains several conserved domains including a CDC25 homology domain (CDC25-HD), a Ras-associated domain, a Ras exchange motif, a PDZ domain, and a cyclic nucleotide binding domain (CNBD) (Figure 1A) essential for its function as a guanine nucleotide exchange factor (GEF) for Rap1 and Rap2. This activity is crucial for cell adhesion, migration, proliferation, and differentiation^{2,5-7}.

PDZ-GEF, the *Drosophila* gene ortholog of human *RAPGEF2* (and also *RAPGEF6*), is highly conserved between *Drosophila* and humans with a DIOPT (DRSC Integrative Ortholog Prediction Tool, version 8)⁸ score of 12/16 and a high degree of similarity (47%) and identity (35%). Similar to *RAPGEF2*, *PDZ-GEF* encodes a GEF for small GTPases and is important for cell adhesion and polarity, cell migration, and synaptic development^{9,10}.

In this study, we identified heterozygous *de novo* variants in *RAPGEF2* in unrelated probands presenting with neurodevelopmental symptoms. We used *Drosophila* to investigate the functional impact of the observed variants. We document that *PDZ-GEF* is expressed in the central nervous system, and loss of *PDZ-GEF* leads to locomotor dysfunction, neurodevelopmental phenotypes, and lethality. These phenotypes can be rescued by expression of human *RAPGEF2* reference cDNA, suggesting that the function of these genes is evolutionarily conserved. In contrast, expression of human *RAPGEF2* variants identified in the affected individuals fail to rescue the observed phenotypes in the mutant background. This finding suggests that the variants in *RAPGEF2* observed in the cohort are loss-of-function (LoF) variants, implicating *RAPGEF2* in a Mendelian disorder.

Materials and Methods

Study cohort

The individuals were recruited through the Children's National Hospital and Genomics Research to Elucidate the Genetics of Rare Diseases (GREGoR) Consortium. This work has been approved by the Children's National Hospital institutional review board under the Regeneron protocol. Written informed consents were provided by parents or legal guardians at the enrolling institution. The clinical data was supplied by the clinicians at each of the clinical centers and collection of individuals' information was facilitated by

the GeneMatcher platform¹¹. Exome sequencing (ES) or Genome sequencing (GS) was performed to detect genetic variants.

Generation of *PDZ-GEF^{Kz-GAL4}*

To investigate the function of *PDZ-GEF* in *Drosophila* we generated a novel allele, *PDZ-GEF^{Kz-GAL4}*, CR71002, using CRISPR-Cas9-directed homologous recombination, replacing the coding regions of *PDZ-GEF* with *Kozak-GAL4-3XP3EGFP* cassette as described¹². This leads to a null allele that expresses a yeast transcription factor, GAL4, under regulatory sequences of *PDZ-GEF*. GAL4 can be used to drive the expression of transgenes under the control of its cognate sequence upstream activating sequence (UAS). Hence, *PDZ-GEF^{Kz-GAL4}* can be used to determine the expression pattern of *PDZ-GEF* by crossing flies with *PDZ-GEF^{Kz-GAL4}* allele with *UAS-fluorescent reporter* flies. In addition, the *PDZ-GEF* coding sequence is fully removed and replaced, thereby generating a complete LoF allele.

Generation of *UAS-RAPGEF2* transgenic stocks

The *UAS-RAPGEF2* transgenic fly lines were generated as previously described¹³. In brief, the *RAPGEF2* reference cDNA sequence (Genebank transcript NM_014247.5) was cloned into the pGW-UAS-HA.attB vector¹⁴ using the Gateway cloning system (Thermo Fisher). The human reference cDNA was used to generate the human cDNA variants by Q5 site-directed mutagenesis (New England Biolabs) and mutagenesis primers (Table S2). All plasmids were validated by Sanger (performed by Azenta lifesciences) or whole plasmid (performed by Plasmidsaurus using Oxford Nanopore Technology with custom analysis and annotation) sequencing. The UAS-human cDNA stocks were generated by inserting vectors into the VK33 docking site by ϕ C31-mediated transgenesis¹⁵.

Drosophila stocks and maintenance

Flies were cultured using standard fly food at 25 °C on a 12-hour light/dark cycle. The *UAS-RAPGEF2* transgenic fly and *Kozak-GAL4* lines were generated in the Bellen, Kanca, and Yamamoto labs. All other fly lines were obtained from the Bloomington *Drosophila* Stock Center (BDSC). Details of the fly strains used in this study are provided in Table S3.

Immunostaining and confocal microscopy

Larval central nervous system (CNS) and adult brain tissues were dissected in 1X PBS and fixed in 4% paraformaldehyde (PFA) solution at room temperature for 20–30 minutes. The 16% PFA stock (Electron Microscopy Sciences, Cat:15710) solution was diluted in 1x PBS to prepare 4% PFA solution. Next, the samples were washed three times with PBS containing 0.2% Triton X-100 (PBST), then blocked in 0.2% PBST with 5% normal goat serum under vacuum for 1.5 hours. Subsequently, samples incubated with primary antibodies including anti-Elav (rat monoclonal 7E8A10, DSHB) at 1:200 dilution and anti-Repo (mouse monoclonal 8D12, DSHB) at 1:50 dilution at 4°C overnight. Next, the samples were washed three times with PBST, and then were incubated with Alexa 647-conjugated secondary antibodies (Jackson ImmunoResearch) at a 1:500 dilution in 0.2% PBST for 2 hours at room temperature. Lastly, the samples were washed three times with PBST and

mounted in RapiClear (SunJin Lab Co.) and imaged using a Zeiss 880 Airyscan confocal microscope¹⁶.

For immunofluorescence at the larval model, third instar larvae were dissected in ice cold Ca²⁺-free hemolymph-like HL-3. In brief, a dorsal superficial midline cut was made on the larvae with dissection scissors (World Precision Instruments), the body wall was slightly stretched, pinned down on a sylgard dish and organs were removed using forceps (Fine Science Tools). Larvae were fixed in 100 μ l 4% PFA (w/v) paraformaldehyde in PBS) at room temperature for 10 minutes. Fixed larvae were washed three times with 1x PBS, before marking them according to genotype, and transferring them into a 1.5 ml Eppendorf tube containing 1x PBS. After dissection, PBS was removed and replaced with the primary antibodies in blocking solution (BS, 0.1% PBST with 5% NGS). Samples were incubated overnight, at 4°C in a rotation wheel. The next day, samples were washed 3 times in PBS for 20 mins at room temperature. Secondary antibodies in BS were added for 2 hours at room temperature, before washing 3 times in PBS for 20 mins each. Samples were mounted in VectaShield Antifade Mounting Medium (Vector Laboratories, Newark, CA, US) mounting medium. Concentration of antibodies: Futsch (mouse, 22C10, DSHB 1 in 100), anti-HRP conjugated with Cy5 (1 in 500), secondary antibodies were used at 1 in 500.

Images were acquired using a Zeiss confocal LSM 710 or 880 microscopes equipped with a AxioCam digital microscope camera, a ConfoCor3 detection module, and PMT detection. Laser lines used were 458/488/514 nm (Argon), or 561/633/440/405 nm (Diode). The following settings were used: pixel size: 100=200 nm, pinhole: 1 AU, line averaging: 1–2 (bidirectional). Z-stacks of whole NMJs or segmental nerves were acquired by manually setting z-values for each NMJ or segmental nerves. NMJs innervating muscles 6/7 of abdominal segments 2 and 3 were imaged. Images were quantified using Fiji.

Quantitative reverse transcription polymerase chain reaction, qRT-PCR

To verify that the *Kozak-GAL4* allele we generated for *PDF-GEF* is a knock-out allele, we utilized qRT-PCR. Primers were designed (Table S2) to cover all isoforms of *PDF-GEF*. In addition, we performed qRT-PCR to assess the expression levels of *RAPGEF2* reference or the modeled variants in *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186; UAS-human cDNA* mutant background (primers listed in Table S2). We used 5 whole larvae for each genotype, then mRNA was isolated using Trizol RNA isolation protocol (TRIzolTM Reagent Invitrogen, 15596018). Subsequently, first-strand cDNA generated using the All-In-One 2X RT MasterMix (abm, G592) and qRT-PCR was performed using iTaq Universal SYBR Green Master Mix (BioRad, 1725120) on a QuantStudioTM 5 Real-Time PCR System, Applied BiosystemsTM.

Larval crawling and larval righting assay

Locomotion is considered as a readout of neuronal system and muscle function in *Drosophila* larvae. For the larval crawling assay, third instar (L3) larvae were placed on an empty plate, and their spontaneous crawling behavior was recorded for one minute using a video camera. The videos were analyzed to determine crawling distance in one minute, providing quantitative measures of locomotor activity. For the righting assay, the L3 larvae

were gently placed on their dorsal side on a plate. The time taken for each larva to right itself to normal crawling position was recorded. This assay evaluates the larvae's ability to coordinate movements necessary to return to a prone position, reflecting neuromuscular function¹⁷.

Statistical analysis

We used GraphPad Prism version 10 (GraphPad Software, USA) to analyse the data. One-Way ANOVA statistical test was applied to compare the means of independent groups. Data are presented as mean \pm standard deviation (SD), with significance levels indicated as follows: n.s. (no significance) for $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Results

Individuals with heterozygous variants in *RAPGEF2* present with global developmental delay, including ADHD, speech and language disorder and intellectual disability

We identified five unrelated individuals, all male, that carry *de novo* heterozygous variants (three missense variants, a frameshift variant, a premature stop codon variant) in *RAPGEF2* (GenBank: NM_014247.5). The individuals in this study, at the time of last assessment were all under 11 years of age and presented with a range of neurological and/or systemic developmental symptoms, including global developmental delay (4/5), motor delay (4/5), speech and language disorder (4/5), intellectual disability (3/5), behavioral abnormalities (3/5), vision deficits (3/5), sleep difficulties with delayed sleep onset and/or decreased duration (4/5), dysmorphic features (3/5), and feeding difficulties (oral aversion requiring G-tube; texture aversion due to reflux/constipation) (4/5). Additional but less frequently observed findings included early onset epilepsy (2/5), recurrent infections (2/5), cardiac abnormalities (1/5), and hypotonia (1/5). Three individuals have missense variants in *RAPGEF2* (RefSeq: NM_014247.5), c.433C>G p.(Arg145Gly), c.454_455delinsAG p.(Val152Arg), and c.758T>C p.(Met253Thr)¹⁸, one individual has a frameshift variant, c.1769dup p.(Leu590Phefs*15), and one individual has a premature stop codon variant, c.3634C>T p.(Arg1212*). The premature stop codon variant is predicted to cause nonsense mediated decay (NMD) or affect the normal folding of the wild-type protein^{19,20}. The p.(Arg145Gly), p.(Val152Arg), p.(Leu590Phefs*15), and p.(Arg1212*) variants were identified by ES and the p.(Met253Thr) variant was identified by GS. Details of the clinical symptoms of individuals are described in Supplemental clinical notes and summarized in Table 1 and Table S1.

In-silico study supports the association of *RAPGEF2* with early onset disorders and pathogenicity of the variants

We used MARRVEL (Model Organism Aggregated Resources for Rare Variant ExpLoration)²¹ to collect information about *RAPGEF2* and its orthologs in other species. *RAPGEF2* is highly constrained for genetic variations. The Z-score for missense variants in *RAPGEF2* is 3.618, suggesting that missense variants in *RAPGEF2* are constrained and could have functional significance. Moreover, the Z-score for loss-of-function (LoF) alterations in this gene is 7.058. This suggests that LoF of *RAPGEF2* may have severe

consequences, and that the gene is essential for normal development. Additionally, DOMINO, a machine-learning based dominant disease gene prediction model, predicts that *RAPGEF2* is “Very Likely” (probability = 0.908) to be associated with an autosomal dominant disorder²².

The CADD scores for missense and LoF variants in our cohort are higher than 20. Additionally, all variants are absent from gnomAD v4.1.0 database²³ and predicted to be deleterious or “pathogenic supporting” by multiple computational prediction tools including SIFT (sorts intolerant from tolerant)^{24,25}, Polyphen-2²⁶, and MutationTaster²⁷. However, the p.(Val152Arg), p.(Arg145Gly), p.(Met253Thr), and p.(Arg1212*) variants are classified as of uncertain significance based on the American College of Medical Genetics (ACMG) guidelines²⁸ due to insufficient evidence (Table 2). All three missense variants map to one of the two cyclic nucleotide binding domains (CNBDs) that are shared by all transcript isoforms (Figure 1A). The affected amino acids are highly conserved between human and *Drosophila* (Figure S1A), suggesting that these residues are important for protein function.

We modeled all identified *RAPGEF2* variants in *Drosophila*, except p.(Leu590Phefs*15), a frameshift variant that results in a premature stop codon. The p.(Leu590Phefs*15) variant is classified as “Likely Pathogenic” based on the ACMG guideline criteria. This variant was recently added to the study and is predicted to undergo nonsense-mediated decay (NMD). We therefore did not model this variant.

***PDZ-GEF^{Kz-GAL4}* is a loss of function allele that facilitates functional characterization of the *RAPGEF2* variants**

We replaced the ORF of *PDZ-GEF* with a *Kozak-GAL4* construct using CRISPR¹². *PDZ-GEF^{Kz-GAL4}* can be used to determine the LoF phenotypes and to conduct “humanization” studies²⁹ by expressing human UAS-*RAPGEF2* cDNA in the mutant genetic background (Figure 1B’). The homozygous *PDZ-GEF^{Kz-GAL4}* or transheterozygous *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* animals die at various developmental stages, but some mutant animals develop to the pupal stage. Hence, we used the *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* third instar (L3) larvae and *yw* L3 larvae as controls to assess expression levels of the *PDZ-GEF* using primers (Table S2). Quantitative reverse transcription PCR (qRT-PCR) detected no expression of *PDZ-GEF* mRNA in the *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* larvae, showing that the generated *PDZ-GEF^{Kz-GAL4}* is indeed a null allele (Figure 1B’’).

***PDZ-GEF* is expressed in neurons and a small subset of glial cells of the central nervous system**

RAPGEF2 is expressed in multiple tissues including the central nervous system (CNS)⁴. Four out of five individuals in our cohort present with neurodevelopmental symptoms. Hence, we sought to determine whether *PDZ-GEF* is expressed in the fly CNS. To examine *PDZ-GEF* expression pattern, we crossed the *PDZ-GEF^{Kz-GAL4}/CyO* animals to a *UAS-mCherry.nls* (nuclear-localized mCherry fluorescent protein). Confocal imaging shows that *PDZ-GEF* is widely expressed in CNS of L3 larvae (Figure 2A’) and adult brains (Figure 2A’’). By co-staining with the neuronal marker (Elav) or the glial marker (Repo), we found that the mCherry signals partially overlap with Elav (Figure 2B’, 2B’’) and a small subset

of Repo-positive cells (Figure 2B''', 2B''''') in larval CNS and adult brains. This expression pattern is consistent with the publicly available single nucleus RNA sequencing data, Fly Cell Atlas³⁰ (Figure S1B). Hence, *PDZ-GEF* is expressed in neurons and a small subset of glia in the fly CNS.

Loss of *PDZ-GEF* causes multiphasic lethality which is rescued by *RAPGEF2* reference cDNA but not by the variants

We observed that the mutants (*PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186*) display multiphasic lethality but a significant fraction of the animals die as pupae (Figure 3A). The lethality is successfully rescued by either a genomic rescue (GR) construct (*Dp(2;3)CH321-57024*) which carries a copy of the *PDZ-GEF* locus inserted in the third chromosome (Table S3), or by expressing a copy of UAS-human *RAPGEF2* reference cDNA in *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* flies. (Figure 3B). These data indicate that *PDZ-GEF* is essential during fly development and that its function is evolutionarily conserved.

To determine if the variants affect protein function, we created four *UAS-RAPGEF2* transgenic fly lines that carry the variants listed in Table 1 (the transgenic fly lines that we generated in our study are listed in Table S3). These variants were tested in *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* flies and their viability was assessed. All the variants in *RAPGEF2* failed to rescue the lethal phenotype (Figure 3C). Hence, the *RAPGEF2* variants affect the function of the gene.

Expression of the Human *RAPGEF2* reference and variants in the *Drosophila* loss of function mutant

To assess the human gene and variants expression levels in flies, we performed qRT-PCR on *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186; UAS-RAPGEF2* larvae. The expression levels of *RAPGEF2* variants *p.(Arg145Gly)*, *p.(Val152Arg)*, and *p.(Met253Thr)* are similar to the *RAPGEF2* reference, suggesting that these variants do not affect the transcript levels. In contrast, the *p.(Arg1212*)* variant results in a severe decrease in mRNA levels. Although the human cDNA does not contain introns, we propose that NMD causes the loss of the transcript, as has been reported for some other transcripts that have premature stop codons^{31,32} (Figure S2A').

To assess the protein levels, we used a commercial antibody (anti-RAPGEF2, Proteintech, 184331-4-RR) and performed Western Blots on *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186; UAS-RAPGEF2* larvae. This antibody is specific for RAPGEF2 as there is no signal in protein extracts from flies that do not express the human transgene. In contrast, flies that express the human reference or variant cDNAs display a clear signal (Figure S2A''). The protein levels of the *p.(Val152Arg)* and *p.(Met253Thr)* variants are comparable to the *RAPGEF2* reference whereas, the *p.(Arg145Gly)* variant showed increased protein levels relative to the reference (Figure S2A'''). Since the mRNA levels were similar between the *p.(Arg145Gly)* variant and reference cDNA expressing samples, this difference in protein level is likely due to post-transcriptional regulation. Note that the protein is expected to be ~167kDa, and we only observe a few minor bands around 167kDa in variant and reference expressing samples. This suggests that *RAPGEF2* undergoes degradation.

Indeed, the degradation of RAPGEF2 has been shown to be essential for its activation and dynamic regulation for proper function in various signaling pathways³³. Finally, we performed immunofluorescence imaging using the same RAPGEF2 antibody and observed that the RAPGEF2 protein is absent when the gene is not expressed or when the *p.(Arg1212*)* variant is expressed. As the RAPGEF2 antibody was designed against an epitope beyond the *p.(Arg1212*)* variant, we did not expect a signal. Expression of the reference cDNA shows that the protein is present at neuromuscular junctions, segmental nerves, and trachea of the L3 larvae. In this assay, the distribution of RAPGEF2 reference, *p.(Arg145Gly)*, *p.(Val152Arg)*, and *p.(Met253Thr)* variant proteins are similar (Figure S2B). In summary, three of the human tested variants and the reference cDNA are expressed in flies. As shown below, the variants affect gene's function.

Loss of *PDZ-GEF* causes locomotor dysfunction that can be rescued by *RAPGEF2* reference cDNA but not by the variants

The locomotor system of *Drosophila* larvae serves as a model to study the interaction between neural and mechanical circuits and was previously shown to be affected by loss of *PDZ-GEF* function³⁴. To investigate locomotor function, we performed larval crawling and larval righting assays¹⁷ in 3rd instar larvae of the following genotypes: *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* (mutant), *yw* (control), and *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186; UAS-RAPGEF2* reference or variant cDNA (rescue). The crawling traces and quantification of the distance that the mutant larvae can crawl within one minute show that the mutant larvae have severely reduced locomotion compared to the *yw* larvae (Figure 3D', 3D''). In addition, we measured the time that the larvae needed to right themselves when turned over. As shown in Figure 3D''', the mutant animals need a significant longer time to recover from their inverted position compared to *yw* control animals.

We then assessed larval crawling and larval righting of animals that express the human reference or variant cDNAs in the *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* mutant background. Expression of the human reference *RAPGEF2* cDNA in the mutant background rescues both phenotypes observed in mutant animals. Interestingly, expressing *RAPGEF2* *p.(Arg145Gly)*, *p.(Val152Arg)*, *p.(Met253Thr)*, or *p.(Arg1212*)* variants all fail to rescue both phenotypes (Figure 3D', 3D'', 3D'''). These data suggest that *PDZ-GEF* is required for proper motor function and that the variants are severe LoF alleles.

Loss of *PDZ-GEF* affects microtubule stability and synaptic development that can be rescued by reference but not by the variant human cDNAs

To further determine the nature of the variants we analyzed other phenotypes associated with *PDZ-GEF* LoF alleles in *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* mutant larvae and mutant larvae rescued by reference or variant *RAPGEF2* human cDNAs. We investigated the intensity of Futsch, a marker for microtubules in motor neuron axons³⁵, and labeled the neuronal membranes with HRP, a neuronal membrane marker³⁶. Our data show that the Futsch intensity is increased in the motor neuron axons of *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* larvae when compared to the *yw* larvae (Figure 4A). This phenotype is not observed in the presence of a copy of human cDNA reference in the mutant background (Figure 4A). In contrast, expressing one copy of *p.(Arg145Gly)*, *p.(Met253Thr)*, or *p.(Arg1212*)* variants

in the mutant genetic background fails to reduce the elevated Futsch intensity observed in mutant motor neuron axons (Figure 4C'). Interestingly, expression of p.(Val152Arg) variant in mutant background decreases Futsch intensity to levels observed in mutant animals that express one copy of the human reference (Figure 4A, Figure 4C', Figure S3A'). These data suggest that p.(Val152Arg) affects the microtubule stability less than the p.(Arg145Gly), p.(Met253Thr), or p.(Arg1212*) variants.

PDZ-GEF functions upstream of Rap1 to restrain synaptic growth. Loss of PDZ-GEF has been shown to lead to overgrowth at larval NMJs⁹. Indeed, the number of satellite boutons, and terminal branches are increased at the NMJs of the *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* mutant 3rd instar larvae when compared to the *yw* larvae (Figure 4B). Expressing one copy of the reference human cDNA in the mutants suppresses the observed increase in boutons and terminal arborization. However, expression of the variants does not (Figure 4B, Figure 4C'', 4C''', Figure S3A''). In line with previous assays, our results suggest the variants severely impair gene function. An increase in the number of satellite boutons and terminal branching at the NMJs of L3 larvae can alter the balance of synaptic inputs and outputs, potentially disrupting neuromuscular transmission and can result in motor dysfunction^{37,38}.

Discussion

In this study, we identified a cohort of five unrelated individuals with *de novo* variants in *RAPGEF2*. Four of the probands in our cohort present with developmental and neurological symptoms. We have a limited clinical description for Proband 2, as the individual lives in Africa with limited access to medical services. Hence, whether the individual shows developmental and neurological symptoms is unknown. Nevertheless, our *Drosophila* data shows that this variant behaves very similarly to the other variants modeled in this study.

Previous studies of *Drosophila PDZ-GEF* mutants showed that loss of *PDZ-GEF* leads to multiphasic lethality associated with systemic developmental defects^{9,39-41}. Surviving L3 larvae, exhibited an overgrowth of the neuromuscular junction (NMJ) with excessive satellite boutons and increased terminal arborizations as well as an increase of microtubule stability in motor neuron axons^{9,42}. These larvae also exhibited decreased locomotor activity. Electro-physiological recordings revealed a significant reduction in excitatory junction potential (EJP) amplitudes at the NMJs of *PDZ-GEF* mutant larvae⁴³.

The observed synaptic overgrowth in *PDZ-GEF* mutant larvae, along with reduced locomotor activity, suggests that *PDZ-GEF* plays a critical role in NMJ maturation and functionality. Indeed, prior studies have demonstrated that PDZ-GEF (Gef26) functions upstream of Rap1 in motor neurons to regulate BMP signaling through Glass bottom boat (Gbb)^{9,44}. Loss of *PDZ-GEF* elevates Gbb signaling, drives synaptic overgrowth, increases microtubule intensity, and impairs endocytic downregulation of Gbb receptors (Tkv, Wit), which contributes to morphological NMJ defects, locomotor disability and synaptic transmission defects^{9,42,45}. These studies also show that supernumerary boutons are often immature and fail to properly integrate into functional circuits. Importantly, Gbb signaling is essential not only for synaptic growth but also for the homeostatic regulation of synapse structure and neurotransmission^{9,44,46}.

Furthermore, the *Caenorhabditis elegans* protein PXF-1 and its mouse ortholog RapGEF2 both play important roles in neuronal development and motor neuron synapse formation^{47–50}. Together, these findings argue that *PDZ-GEF* is essential for regulating microtubule stability, proper synaptic development, and normal locomotion in *Drosophila*^{9,41–43}. The data are also consistent with observations that *Rapgef2* (mouse ortholog of *RAPGEF2*) homozygous mutant mice die at embryonic or preweaning stages. Importantly, *Rapgef2* heterozygous mice show multisystemic developmental defects that include neurological, cardiovascular, behavioral, vision, growth, and digestive issues^{51–53}.

RAPGEF2 has not been associated with Mendelian disorders so far. In-silico studies suggest that this gene might be important for development. There is only 1 individual with a deletion that spans *RAPGEF2* in the DECIPHER⁵⁴ control population and no individual with a microdeletion covering coding exons of *RAPGEF2* in the Database of Genomic Variants (DGV)⁵⁵. The probability of loss of function intolerance (pLI) score for this gene is 1.0. Hence, haploinsufficiency of *RAPGEF2* is likely to result in a significant, early onset phenotype^{23,54,56}. Ishiura et al. (2018) identified an expansion of TTTC pentanucleotide repeats in the intronic regions of *RAPGEF2* and two other genes, which lead to a benign familial adult myoclonic epilepsy 7 (BAFME7, OMIM: 618075?)^{57,58}. This study argued that the pathogenic mechanism of BAFME is due to the repeat expansion which leads to production of a toxic mRNA product, regardless of the genomic location. Hence, BAFME7 is not thought to be caused by the loss of *RAPGEF2* and the BAFME7 associated phenotypes cause a benign form of epilepsy⁵⁷.

Heo et al. (2018), identified a heterozygous *de novo* missense variant in *RAPGEF2*, RefSeq NM_014247.5, *c.4069G>A, p.(Glu1357Lys)*, in an individual with amyotrophic lateral sclerosis (ALS). Motor neuron specific overexpression of the p.(Glu1357Lys) variant protein in *Drosophila* led to reduced microtubule stability and affected mitochondrial distribution in axons and neuromuscular junction (NMJ) synapses suggesting that overexpression of the variant impacts microtubule stability through a toxic gain-of-function mechanism and that the variant may be implicated in ALS⁴². Symptoms of the individuals in our cohort are distinct from ALS patients, and variants in our cohort do not cause an alteration in Futsch intensity when overexpressed in wildtype motor neurons (Figure S3B, S3C). These data suggest different pathogenic mechanism for the variants in our cohort and the variant in Heo et al. (2018) study.

We observed that loss of *PDZ-GEF* leads to locomotor dysfunction and developmental defects in mutant animals, consistent with previous findings^{9,41–43}. In addition, we found that *PDZ-GEF* is widely expressed in *Drosophila* CNS. The expression data and observed phenotypes in the mutant animals are in line with the expression of the gene in humans and clinical symptoms in our study.

We employed a humanization strategy by expressing human *RAPGEF2* reference or variant cDNAs in the mutant background to uncover the impact of the variants on gene function. The expression of the human *RAPGEF2* reference cDNA rescues the lethality, locomotor defects, elevated Futsch intensity in motor neuron axons, and NMJ overgrowth in the mutant background. This data show that the human gene can functionally replace the loss of the

Drosophila gene. In contrast, expression of the variants in the mutant background fails to rescue the observed developmental phenotypes. These data support the pathogenicity of the variants and suggest that the variants are LoF alleles. The p.(Leu590Phefs*15) is a frameshift variant that is predicted to lead to nonsense-mediated mRNA decay (NMD) due to loss of most of the domains. The p.(Arg1212*) variant lies after most of the functional domains and maps to exon 22 of 24 (NM_014247.5); it is therefore likely to cause NMD or a truncated protein. Our data support the pathogenicity of this variant as it behaves similarly to the other variants. Moreover, protein modeling prediction of the p.(Arg1212*) variant suggests that this variant alters the normal folding of the protein (Figure S4A) and hence may interfere with the normal function of the protein. The p.(Arg1212*) variant also affects mRNA stability in our assays, further supporting that the variant causes loss of function. The missense variants, p.(Val152Arg), p.(Arg145Gly), and p.(Met253Thr), cluster within the cyclic nucleotide-binding domain (CNBD), a regulatory domain that modulates GEF activity in response to binding to cAMP but is not itself the site of direct interaction with Rap GTPases. Therefore, while these residues do not appear to lie directly at the GTPase interface, their alteration may indirectly impact GEF activity by affecting conformational regulation of the catalytic domain or disrupting intra and/or inter protein interactions necessary for proper activity⁵⁹. The missense variants are predicted to alter the intramolecular interactions that map to the surface of the protein (Figure S4B) and may alter the function or affinity of the protein with other proteins. Therefore, these variants are likely LoF alleles.

Conclusion

We describe a spectrum of neurodevelopmental symptoms in individuals carrying heterozygous *de novo* variants in *RAPGEF2*. Our study provides functional evidence that LoF variants in *RAPGEF2* contribute to autosomal dominant neurodevelopmental symptoms in the individuals. Several individuals in this case series had other systemic findings which may be part of this condition, but a full phenotypic spectrum of *RAPGEF2* related syndrome remains to be delineated as further individuals are identified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and code availability

This study did not generate datasets. Reagents that we used or generated in the study are available upon request or are deposited in the Bloomington Drosophila Stock Center (BDSC).

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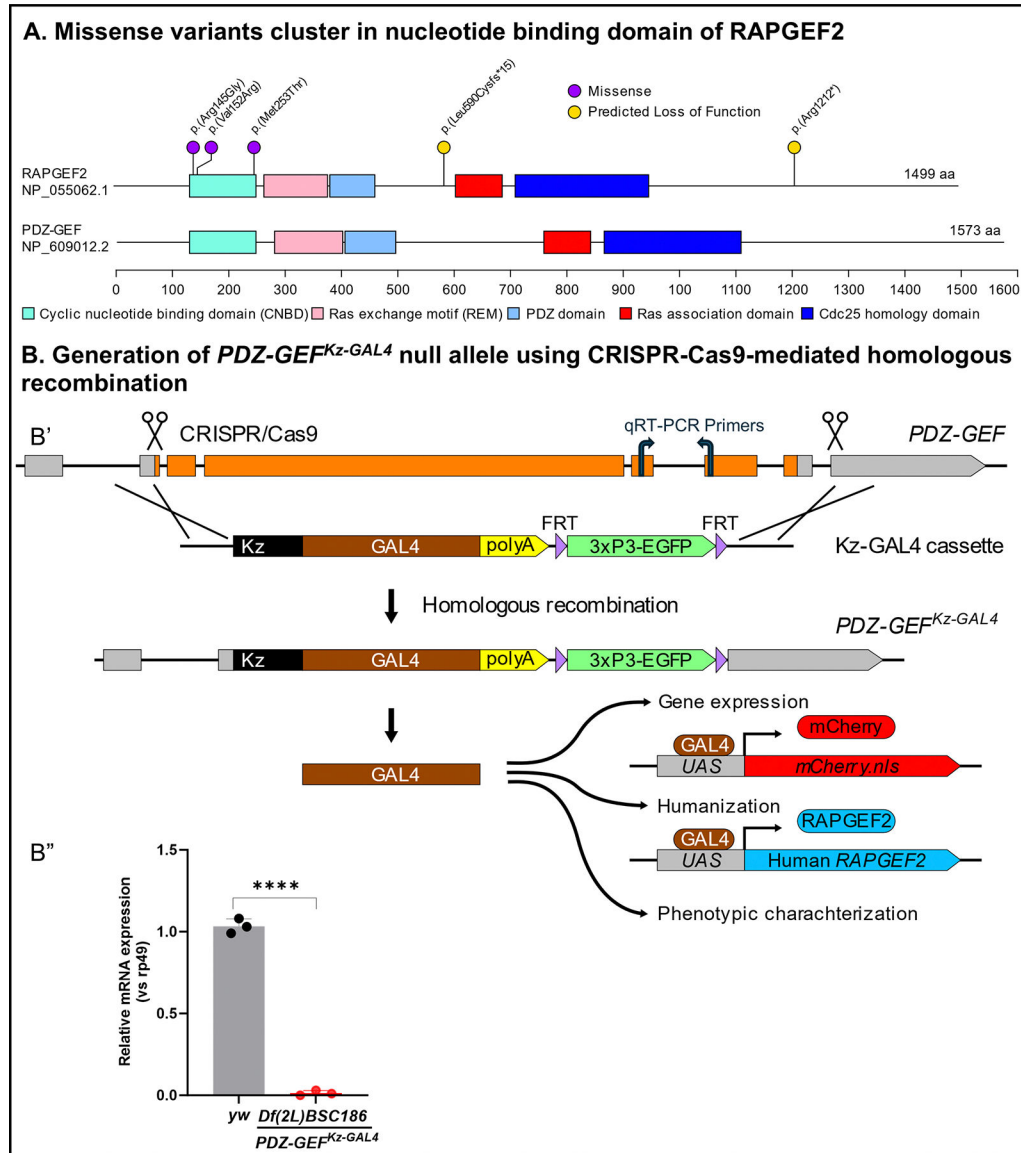


Figure 1. Schematics of RAPGEF2 and PDZ-GEF structural domains, and *PDZ-GEF^{Kz-GAL4}* loss of function allele

A. Schematics of domain organization of RAPGEF2 and PDZ-GEF. Missense variants associated with probands in our cohort are clustered in the Cyclin nucleotide-binding domain (CNBD). Other conserved domains are Ras exchange motif (REM), Cyclin nucleotide-binding domain (CNBD), Ras-association (RA) domain and PDZ domain, and CDC25 homology domain (CDC25-HD) which is responsible for catalyzing the exchange of GDP for GTP.

B. Illustration of the *PDZ-GEF^{Kz-GAL4}* allele generation. The entire coding sequence of *PDZ-GEF* was replaced by a *Kozak-GAL4* cassette using CRISPR-Cas9-directed homologous recombination leading to expression of GAL4 transcription factor under the control of the endogenous regulatory elements of *PDZ-GEF*. The GAL4 can be used for

determining expression pattern of *PDZ-GEF* using *UAS-mCherry.nls*, for expressing *UAS-RAPGEF2* variant or reference cDNA to “humanize” flies, or for characterizing loss of function phenotype (B’). Quantitative reverse transcription PCR from mutant larvae shows that *PDZ-GEF^{Kz-GAL4}* is a null allele (B’’) (3 biological and 2 technical replicates).

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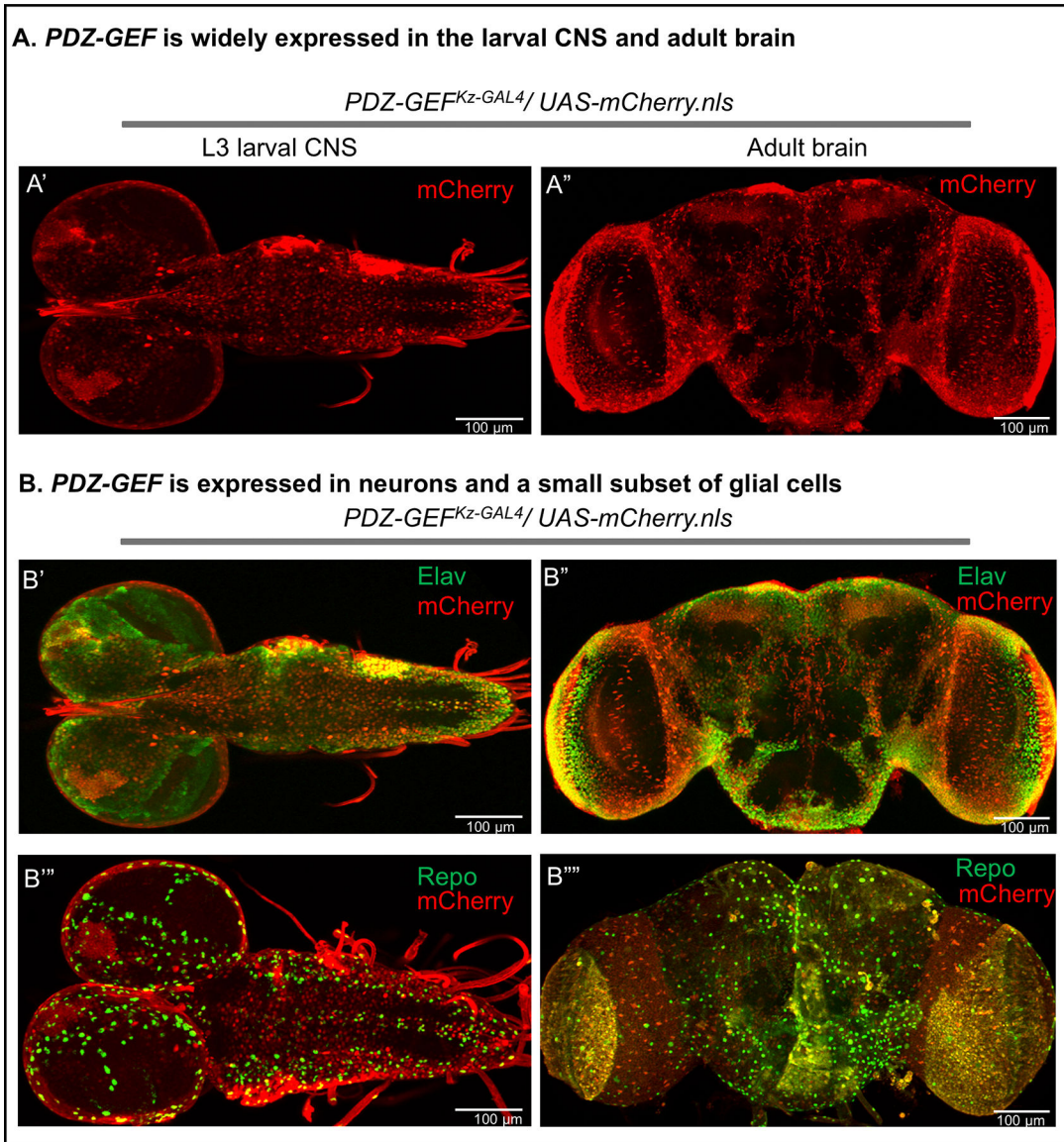


Figure 2.

PDZ-GEF is expressed in subsets of neurons and glia in larval and adult brain

A. *PDZ-GEF* is widely expressed in the larval central nervous system (CNS) (A') and adult brain (A'').

B. Co staining of the neuronal marker (Elav) with *UAS-mCherry.nls* (a fluorescent protein that localizes to the nucleus) driven by *PDZ-GEF^{Kz-GAL4}* shows that *PDZ-GEF* is widely expressed neurons of the L3 larval CNS (B') and adult brain (B''). Co staining of the glial cell marker (Repo) with *UAS-mCherry.nls* (a fluorescent protein that localizes to the nucleus) driven by *PDZ-GEF^{Kz-GAL4}* shows that *PDZ-GEF* is expressed in a small subset of glial cells of the L3 larval CNS (B''') and adult brain (B''')

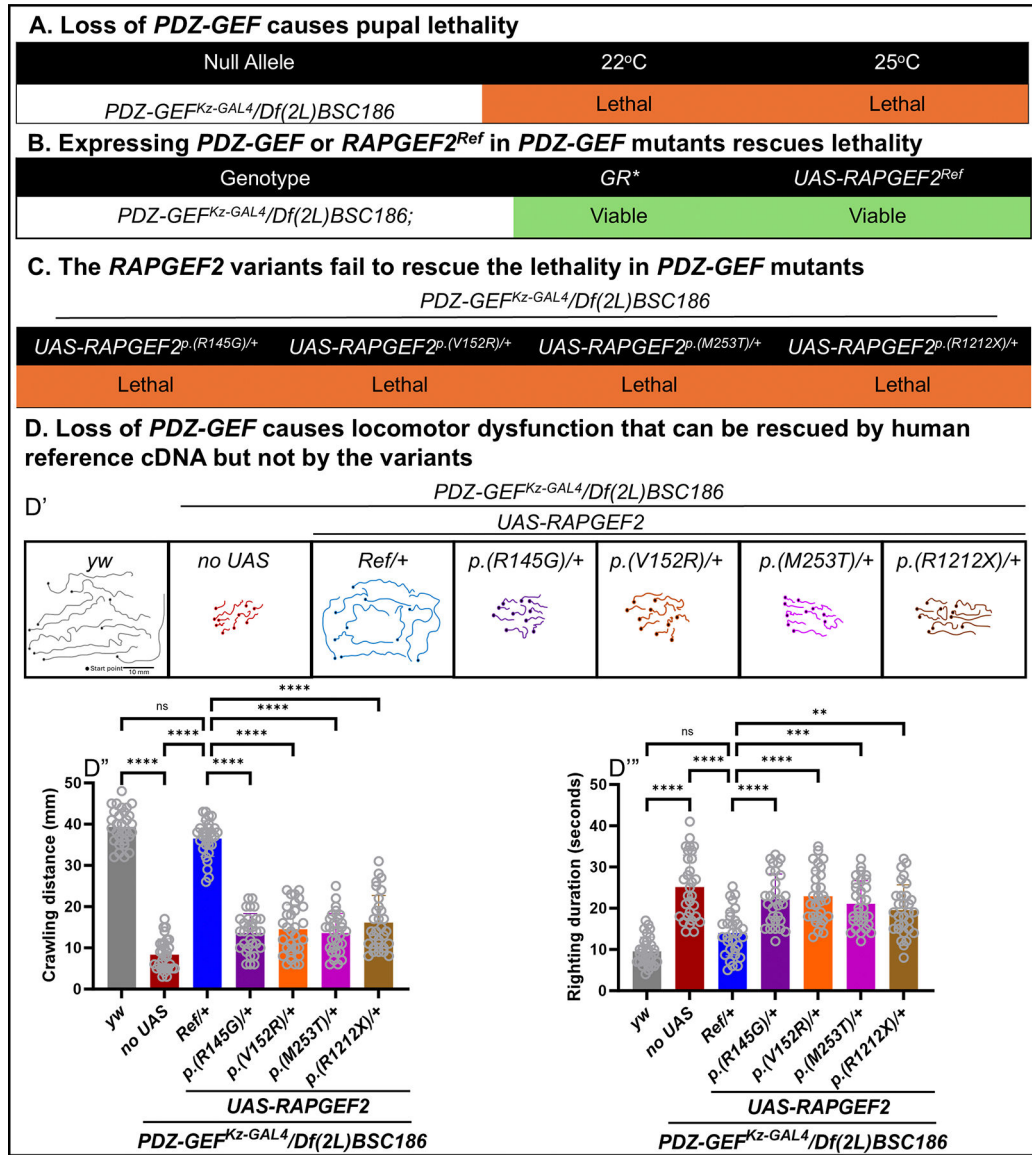


Figure 3:

Loss of *PDZ-GEF* causes lethality and locomotor dysfunction in the L3 larvae that can be rescued by human reference cDNA but not by the variants

A. Loss of *PDZ-GEF* in *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* animals cause lethality.

B. Restoring *PDZ-GEF* expression through a genomic duplication transgene, *Dp(2;3)GV-CH321-57024*, or expressing *UAS-RAPGEF2^{Ref}*, in *PDZ-GEF^{Kz-GAL4}/Df(2L)BSC186* animals rescue lethality.

C. *RAPGEF2* variants associated with probands in our cohort fail to rescue the lethality.

D. Traces and quantification of the locomotion of the L3 larvae show that loss of *PDZ-GEF* leads to decreased larval crawling (D', D'') and delay in recovery from inverted position to the crawling position (D'''). The locomotion defects can be rescued by human reference cDNA but not by the tested variants. D', D'' are traces of larval crawling and quantification

of crawling distance and D'' is quantification of time it takes for larvae to recover from inverted position.

Flies were raised at 25°C. At least 30 larvae were used for each experiment (larval crawling assay and larval righting assay). Data are represented as mean \pm standard deviation (SD). One-Way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; n.s., no significance. GR*: Dp(2;3)GV-CH321-57O24, genomic duplication transgene. p.(Arg145Gly) or p.(R145G), p.(Val152Arg) or p.(V152R), p.(Met253Thr) or p.(M253T) p.(Arg1212*) or p.(R1212X).

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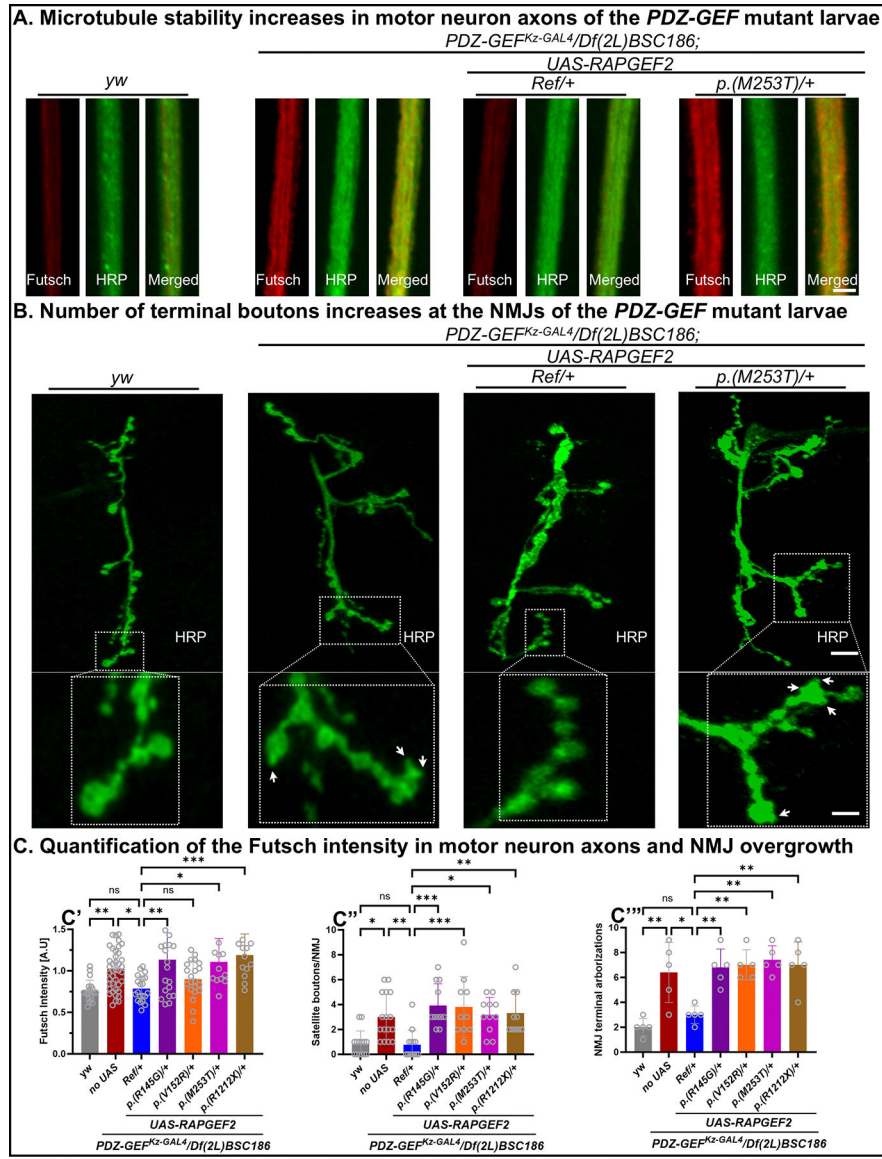


Figure 4. Loss of *PDZ-GEF* leads to increased Futsch intensity in segmental nerves and terminal satellite bouton numbers at the NMJs which can be rescued by human reference cDNA but not by the variant cDNAs

A. Futsch, a microtubule-associated protein, level is increased in motor neuron axons of the *PDZ-GEF* mutant L3 larvae when compared to the control *yw* L3 larvae or mutant animals that express a copy of the reference human cDNA. Mutant flies that express 3/4 variants have increased Futsch intensity in motor neuron axons. The Futsch intensity in motor neuron axons of the p.(M253T) variant is increased, similar to the p.(R145G) and p.(R1212X) variants (see C). Scale bar: 5 μ m.

B. The number of terminal boutons and terminal arborizations are increased in the *PDZ-GEF* mutant L3 larvae as compared to the control *yw* L3 larvae. These phenotype can be rescued by expression of one copy of human reference cDNA but not the variants. Terminal

boutons at the NMJs of the p.(M253T) variant are increased and so the terminal boutons at NMJs of all other variants (see C). Scale bar: 15 μm and 50 μm .

C. Quantification of the Futsch level indicates that human reference cDNA (*RAPGEF2*) rescues the microtubule stability phenotype (increased Futsch level) observed in the *PDZ-GEF* mutant L3 larvae while three of the *RAPGEF2* variants, p.(R145G), p.(M253T), p.(R1212X), fail to rescue the phenotype (C'). The number of satellite boutons and terminal arborizations are increased at the neuromuscular junctions (NMJs) of the *PDZ-GEF* mutant larvae rescued by human cDNA reference, but all variants fail to rescue the over-growth phenotypes (C'', C''').

Flies were raised at 25°C. 5–10 larvae were used for each experiment. Data are represented as mean \pm standard deviation (SD). One-Way ANOVA. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; n.s., no significance. HRP, a neuronal membrane marker; Futsch, a microtubule marker. p.(Arg145Gly) or p.(R145G), p.(Val152Arg) or p.(V152R), p.(Met253Thr) or p.(M253T) p.(Arg1212*) or p.(R1212X).

Table 1:

Clinical features of individuals with heterozygous variants in *RAPGEF2*.

Proband Identification #	1	2	3	4	5
Protein change (NM_014247.5)	p.(Arg1212*)	p.(Arg145Gly)	p.(Val152Arg)	p.(Met253Thr)	p.(Leu590Phefs*15)
Age	9 years	11 years	10 years	6 years	11 years
Developmental Delay	Global developmental delay	ND	+	Developmental coordination disorder	+
Intellectual disability	+	ND	+	-	+
Speech and Language	15–20 words	ND	Approximately 100 words but no phrases	Speech disorder of childhood apraxia of speech and phonological delay. Average receptive language. Mildly impaired expressive language. Spelling deficits.	+
Behavior	ND	ND	Autism, ADHD	Mild anxiety, social difficulties, poor attention and concentration but no official diagnosis of ADHD. Sensory processing disorder.	Mild Autism, ADHD
Seizure semiology	-	-	Refractory drug-resistant epilepsy	-	+
Neurological/Imaging	Hypotonia	Dysgraphia	Toe walking but no overt ataxia or spasticity	-	Borderline myelin maturation
Eye/Vision	+	-	+	+	-
Dysmorphic features	Brachycephaly, epicanthal folds, grimacing smile, prominent and posteriorly rotated ears, overhanging columella, nasal septal deviation, high arched palate, broad first toes, broad thumbs with medial deviation, left preaxial polysyndactyly with duplex/bifid thumb and great toe, flat feet	-	Long eyelashes; widely spaced teeth, mild upturned earlobes, mild right 5th finger clinodactyly	Thin upper lip with smooth philtrum, slight grimacing smile, left ear is slightly anteverted, posteriorly rotated and upper helix is unfolded, slightly downslanting palpebral fissures and ptosis, benign joint hypermobility	-
Cardiac	-	Pulmonary Stenosis	-	-	-

(ND) represents milestone not documented; (-) represents that an abnormal phenotype was reported as absent in the individual; (+) represents an abnormal phenotype was documented in individual but not further described; (ADHD) attention deficit hyperactivity disorder

Table 2.

In-silico study of the variants in *RAPGEF2*

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5
Genomic coordinate (Chromosome 4)	160274664 (hg19) 159353512 (hg38)	160243561 (hg19) 159322409 (hg38)	160243582 (hg19) 159322430 (hg38)	160251101 (hg19) 159329949 (hg38)	160259579 (hg19) 159338427 (hg38)
Variant (NM_001394067.2)	c.4117C>T p.(Arg1373*)	c.916C>G p.(Arg306Gly)	c.937_938delinsAG p.(Val1313Arg)	c.1241T>C p.(Met414Thr)	c.2252T>TT p.(Leu751Phefs*15)
Variant (NM_014247.5)	c.3634C>T p.(Arg1212*)	c.433C>G p.(Arg145Gly)	c.454_455delinsAG p.(Val152Arg)	c.758T>C p.(Met253Thr)	c.1769T>TT p.(Leu590Phefs*15)
Inheritance	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>	<i>De novo</i>
Zygosity	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous
gnomAD v4.1.0	Absent	Absent	Absent	Absent	Absent
CADD	38	25.6	NA	23.9	NA
SIFT	NA	D	NA	D	D
Polyphen-2	NA	D	D	B	NA
MutationTaster	D	D	D	D	D
ACMG Classification	VUS	VUS	VUS	VUS	LP

(D) deleterious; (B) benign; (NA) not available; (VUS) variant of Uncertain Significance; (LP) Likely Pathogenic