Missense Variants in RHOBTB2 Cause a Developmental and Epileptic Encephalopathy in Humans, and Altered Levels Cause Neurological Defects in Drosophila

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Although the role of typical Rho GTPases and other Rho-linked proteins in synaptic plasticity and cognitive function and dysfunction is widely acknowledged, the role of atypical Rho GTPases (such as RHOBTB2) in neurodevelopment has barely been characterized. We have now identified de novo missense variants clustering in the BTB-domain-encoding region of RHOBTB2 in ten individuals with a similar phenotype, including early-onset epilepsy, severe intellectual disability, postnatal microcephaly, and movement disorders. Three of the variants were recurrent. Upon transfection of HEK293 cells, we found that mutant RHOBTB2 was more abundant than the wild-type, most likely because of impaired degradation in the proteasome. Similarly, elevated amounts of the Drosophila ortholog RhoBTB in vivo were associated with seizure susceptibility and severe locomotor defects. Knockdown of RhoBTB in the Drosophila dendritic arborization neurons resulted in a decreased number of dendrites, thus suggesting a role of RhoBTB in dendritic development. We have established missense variants in the BTB-domain-encoding region of RHOBTB2 as causative for a developmental and epileptic encephalopathy and have elucidated the role of atypical Rho GTPase RHOBTB2 in Drosophila neurological function and possibly dendrite development.

Introduction

Neurodevelopmental disorders (NDDs), comprising intellectual disability (ID), autism spectrum disorders (ASDs), and epileptic encephalopathies, are genetically extremely heterogeneous—more than 1,000 genes have been implicated to date (SysID database).1 Next-generation sequencing technologies have greatly facilitated the identification and confirmation of disease-related genes. Using trio exome sequencing, several landmark studies have confirmed de novo variants as a major cause of NDDs in non-consanguineous populations.2–4 Most NDD-associated genes, whose haploinsufficiency or loss of function (LoF) causes developmental disorders, have been identified by now.6 By contrast, many disease-associated genes in which variants alter protein function still remain to be discovered, given that interpretation of missense variants is often challenging. Other than methods with limited power, such as segregation analysis or computational prediction, the currently most valuable criteria are functional studies and the identification of the same variant or a sufficient number of similar variants in individuals with overlapping phenotypes.

After the initial finding of a de novo missense variant in RHOBTB2 (MIM: 607352) in an individual with early-onset epilepsy, severe ID, movement disorder, and postnatal microcephaly, we used matchmaking platforms and collaborative efforts to assemble a total of ten individuals with a similar phenotype and de novo missense variants in the same gene. All identified variants cluster in the BTB-domain-encoding region of RHOBTB2, and three of them are recurrent. We provide further evidence for their pathogenicity by showing that degradation of mutant RHOBTB2 by the proteasome is impaired in vitro and that...
altered amounts of RhoBTB in the nervous system of *Drosophila melanogaster* result in seizure susceptibility, neurological defects, and disturbed dendrite development.

**Material and Methods**

**Affected Individuals**

Tri- exome sequencing on an Illumina HiSeq 2500 platform in individual 1 was performed to reveal genetic causes of phenotypes similar to Pitt-Hopkins syndrome. After identification of a *de novo* variant in *RHOB2*, we contacted colleagues and searched DECIPHER \(^7\) and GeneMatcher \(^8\) for further individuals with variants in this gene. The resulting variants had been revealed in diagnostic or research settings by trio exome sequencing in various centers worldwide. *De novo* occurrence was confirmed in all individuals. Informed consent was obtained from parents or guardians of all affected individuals. If done in a research setting, the studies were approved by the ethic committees of the respective universities or centers. In *silico* prediction was performed with online programs SIFT, \(^9\) PolyPhen-2, \(^10\) MutationTaster, \(^11\) and M-CAP. \(^12\) Mutational screening of all coding exons of *RHOB2* (conditions and primer sequences are available on request) was performed in approximately 275 individuals with Pitt-Hopkins-like ID but without a pathogenic variant in *TFC4* (MIM: 602272) and in 96 further individuals with severe ID.

**Structural Modeling**

Modeling of the BTB domains was performed with the LOMETS server. \(^13\) For the first and second BTB domain, the template structures 1R28 \(^14\) and 1I3N, \(^15\) respectively, were used. Variants were modeled with Swiss-PIdbViewer. \(^16\) and RasMol \(^17\) was used for structure analysis and visualization.

**Protein Analysis**

Human *CUL3* (MIM: 603136) and *RHOB2* were amplified from cDNA derived from RNA from whole blood and adult lung, respectively (Human Multiple Tissue cDNA Panel I, Clonetech) (primer sequences were 5'-CCCCGCCTTAAATGTGACACC-3' [forward] and 5'-TGATGTTGGAAACTCTCAAAGGG-3' [reverse] for *CUL3* and 5'-CAGTAAACAAGAATATGCACGCG-3' [forward] and 5'-CTGTAGGTTGATGGGTAGTGC-3' [reverse] for *RHOB2*), and cloned into a pCR 2.1-TOPO vector (Thermo Fisher Scientific). After site-directed mutagenesis using a modified version of the QuickChange Site-Directed Mutagenesis protocol (StrataGene), wild-type and mutant *RHOB2* cDNAs were transfected into a pcDNA3.1 expression vector (Thermo Fisher Scientific) and a His-cMyc-tagged version of the pcDNA3.1 vector, respectively, and *CUL3* was cloned into a HA-tagged CMV expression vector. For co-immunoprecipitation, HEK293 cells were grown in 6-well plates and co-transfected with 1 μg of His-cMyc-tagged wild-type or mutant *RHOB2* and with 1 μg of HA-tagged *CUL3* with Lipofectamine 2000 and PLUS reagent (Thermo Fisher Scientific). The empty pcDNA3.1-His-cMyc plasmid was transfected as a negative control. 24 hr after transfection, new medium containing 25 μM of MG-132 proteasome inhibitor (Sigma-Aldrich) was added. After 4 hr, cells were trypsinized, washed three times with 1× PBS, and lysed (buffer: 100 mM TRIS-HCl [pH 8], 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100). Protein concentrations were measured with a Qubit 1.0 fluorometer (Thermo Fisher Scientific), and 1.5 mg of protein per sample was adjusted to 300 μL with 1× TBS. Immunoprecipitation was performed with 20 μL Protein A Mag Sepharose bead suspension (GE Healthcare), which was incubated with the sample and 1.6 μg anti-cMyc antibody (Sigma-Aldrich) at 4°C overnight. As controls, lysates from cells transfected with wild-type *RHOB2* were incubated either with beads only or with beads and mouse IgG. Subsequently, the supernatant was removed, and beads were washed once with lysis buffer and four times with 1× TBS. Samples were eluted with 2× NuPAGE sample buffer and 10% DTT and stored at −80°C.

For overexpression analysis, HEK293 cells were transfected with 1 μg of wild-type or mutant *RHOB2* with or without 1 μg of the *CUL3* construct as described above. 24 hr after transfection, cells were trypsinized, washed three times with cold 1× PBS, and lysed (buffer: 100 mM TRIS, 150 mM NaCl, and 1% Triton X-100 [pH 7.5]). Lysates were frozen at −80°C. MG-132 was added to a final concentration of 5 μM for 16 hr. To exclude major differences in transfection efficiency and to exclude higher protein amounts based on mRNA overexpression, we performed quantitative PCR on RNA from cells taken from the same wells as the protein lysate (Figure S1).

For western blotting, the NuPAGE 4%–12% Bis-Tris Gel System (Thermo Fisher Scientific) was used. After blocking, incubation with primary antibodies was performed overnight at 4°C. Antibodies against *RHOB2* (SABI1407189) and *CUL3* (C0871) were obtained from Sigma-Aldrich and used in 1:400 and 1:2,000 dilution, respectively. Tagged proteins were detected with antibodies against cMyc (M4439) and HA (H6908) (Sigma-Aldrich). Alpha-tubulin or beta-actin (Abcam) was used as a loading control. To reduce background, we cut and incubated blot membranes separately with either an antibody against RHOBTB2 or the loading control. Secondary, horseradish-peroxidase-conjugated antibodies (anti-mouse [Abnova] and anti-rabbit [Bio-Rad]) were applied for 2 hr at room temperature. Blots were scanned on a C-DiGit blot scanner (LI-COR), and images were exported as TIFs for analysis in ImageJ. \(^18\) For quantification, rectangles were drawn around the peak corresponding to RHOBTB2 and used and normalized to those of the corresponding loading control.

**Fly Lines and Conditions**

Flies were raised on standard food containing cornmeal, sugar, and yeast. Knockdown or overexpression was achieved with the UAS/GAL4 system. \(^19\) Given that high breeding temperatures result in stronger knockdown effects, ^20^ crosses were carried out at 28°C to induce knockdown and at 25°C to induce overexpression. Driver lines were obtained from the Bloomington Stock Center or assembled in house and by colleagues (*Act5C-GAL4/Cyo* [ubiquitous expression]; *D42-GAL4* [expressed in motoneurons], BL#8816; *OK6-GAL4* [expressed in motoneurons, type 1 terminals], BL#64199; *Df(3R)Gal80-GAL4* [expressed in muscle], BL#1884; *repo-GAL4* [expressed in glia], BL#7415; *UAS-Drx-2:GFP-GAL4/UAS-mCD8::GFP/ppk-GAL4/Tm3b* [expressed in class IV dendritic arborization (da) neurons], ^21^ *elav-GAL4*; *UAS-Drx-2* [pan-neuronal expression]; *elav-GAL4* [pan-neuronal expression]; *UAS-Drx-2*; *elav-GAL4* [pan-neuronal expression]; and *UAS-Drx-2:G428-GAL4* [expressed in mushroom body]). RNAi lines were obtained from the Transgenic RNAi Project ^22^ (TRIP) (BL#32416).
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