Genotype-phenotype correlations in *RHOBTB2*-associated neurodevelopmental disorders

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ABSTRACT

Background

Missense variants clustering in the BTB domain region of *RHOBTB2* cause a developmental and epileptic encephalopathy (DEE) with early-onset seizures and severe intellectual disability.

Methods

By international collaboration we assembled individuals with *RHOBTB2* variants and a variable spectrum of neurodevelopmental disorders (NDDs). By western blotting we investigated the consequences of missense variants *in vitro*.

Results

In accordance with previous observations, *de novo* heterozygous missense variants in the BTB domain region lead to a severe DEE in 16 individuals. We now identified also *de novo* missense variants in the GTPase domain in six individuals with apparently more variable neurodevelopmental phenotypes with or without epilepsy. In contrast to variants in the BTB domain region, variants in the GTPase domain do not impair proteasomal degradation of RHOBTB2 *in vitro*, indicating different functional consequences.

Furthermore, we observed bi-allelic splice-site and truncating variants in nine families with variable neurodevelopmental phenotypes, indicating that complete loss of *RHOBTB2* is pathogenic as well.

Conclusion

By identifying phenotype-genotype correlations regarding location and consequences of *de novo* missense variants in *RHOBTB2* and by identifying bi-allelic truncating variants, we further delineate and expand the molecular and clinical spectrum of *RHOBTB2* related disorders including both autosomal dominant and recessive NDDs.

Keywords: developmental and epileptic encephalopathy, neurodevelopmental disorder, RHOBTB2, intellectual disability, movement disorder, seizures

INTRODUCTION

In 2018, *de novo*, heterozygous missense variants clustering in the BTB domain region of *RHOBTB2* were found to cause a developmental and epileptic encephalopathy (DEE64, MIM# 618004).[1] This severe neurodevelopmental disorder (NDD) is characterized by early-onset seizures, severe to profound intellectual disability (ID), movement disorders, and postnatal microcephaly.[1] *RHOBTB2* is an atypical Rho GTPase, containing a GTPase and two BTB domains. It interacts via the BTB domains with a Cullin3 dependent ubiquitin ligase complex, mediates its own ubiquitination, and recruits other substrates to the complex.[2] So far, only one substrate has been identified, the RNA-binding protein Musashi-2, encoded by *MSI2*. Overexpression of *RHOBTB2* resulted in enhanced ubiquitination and thus decreased protein levels of Musashi-2, while knockdown of *RHOBTB2* resulted in increased levels of Musashi-2.[3]

RHOBTB2 missense variants initially identified in individuals with DEE were shown to result in abundant levels of mutant RHOBTB2 *in vitro*, probably due to impaired proteasomal degradation.[1] Consistent with these findings, flies with increased levels of the *Drosophila* orthologue *RhoBTB* showed seizure susceptibility *in vivo*.[1] Based on the observation of recurrent missense variants clustering in the BTB domain region and heterozygous, large deletions of *RHOBTB2* apparently not being associated with a disease phenotype, a rather specific effect of the missense variants was suggested.[1]

Additional to the initial report of 10 cases in 2018,[1] 23 independent individuals with (likely) *de novo* missense variants in *RHOBTB2* and a neurodevelopmental/neurological phenotype were reported.[4-13] Most of the identified variants (28/33) are located in the BTB domain region, either in the first BTB domain or at the dimer interface of the second BTB domain,[1] and are associated with a relatively homogeneous DEE phenotype including epilepsy, severe to profound ID and further neurological abnormalities.[4-8,10-13] Two missense variants were identified in the GTPase domain (p.(Glu35Lys), p.(Arg116Cys)), one of them in an individual with developmental and epileptic encephalopathy and the other in an individual with a dystonic movement disorder without ID and with only a single febrile seizure.[9] Three reported missense variants were not located in any of the known domains. Two of them involved neighboring amino acid positions 239 and 241 (p.(Trp239Cys), p.(Ser241Tyr)) and were associated with a prominent dystonia phenotype with variable ID with or without epilepsy.[4] The third variant (p.(Thr659Ala)) was identified downstream of the BTB domain region and was associated with developmental delay and infantile spasms.[10]

By assembling data on 23 cases with *de novo* missense variants in either the BTB domain region (16x) or the GTPase domain (6x) or in between (1x), we now further delineate the mutational and clinical spectrum of *RHOBTB2*-related autosomal-dominant NDDs. Variants in the GTPase domain are associated with a more variable phenotype compared to variants in

the BTB domain region. They also behave differently *in vitro*, thus suggesting a genotype-phenotype correlation. Additionally, identification of nine families with bi-allelic splice-site or truncating variants in *RHOBTB2* and variable intellectual disability and neurological abnormalities indicate that complete loss or truncation of *RHOBTB2* is causative also of an autosomal-recessive NDD.

MATERIALS AND METHODS

Data collection

After the initial report in 2018,[1] we assembled mutational and clinical data of 36 additional individuals with *RHOBTB2* variants by personal communication with clinicians or parents, and by GeneMatcher.[14] Individuals from family 2 were included in a previously published study,[15] the other cases have not been reported before. Variants in *RHOBTB2* were identified by panel or (trio) exome sequencing in either diagnostic or research settings (Supplemental Table 1). Consent for publication of molecular and clinical data was obtained from the individuals, their parents or legal guardians. Ethics approval for this study was obtained from the ethical review board of the University of Bern or respective institutional review boards of the testing centers (Supplemental Table 1). The described variants are annotated based on the longest isoform of *RHOBTB2* (GenBank: NM_001160036.2, NP_001153508.1, NC_000008.10). As also isoform NM_015178.3 has been used in variant databases and some publications, we indicate variants also for this isoform in Supplemental Tables 1 and 2.

In-silico analyses and structural modelling

The model of RHOBTB2 was retrieved from the AlphaFold protein structure database (https://alphafold.ebi.ac.uk/entry/Q9BYZ6).[16,17] Residues at the sites of mutation are modeled with high or very high confidence in the structure. A putative GEF binding site was mapped based on the crystal structure of the RhoA- PDZ-RhoGEF complex (PDB: 3KZ1).[18] RasMol was used for structure analysis and visualization.[19]

Constructs

To investigate the variants p.(Ala474Gly), p.(Arg483His), and p.(Arg511Gln), we used the His-cMyc-tagged expression plasmids from the initial report.[1] Novel variants p.(Arg116Cys), p.(Arg154Gln), p.(Arg183Met), p.(Ala471Cys), and p.(Arg507Cys) from this study and p.(Trp239Cys), p.(Ser241Tyr), and p.(Tyr306Asp) from the literature were introduced using a modified version of the Quick-Change site directed mutagenesis kit (Stratagene, Agilent, Santa Clara, CA, USA) into the same plasmid as described previously.[1] Additionally, an HA-tagged Cullin3 vector[1] was used. An expression vector for Musashi-2, containing

FLAG-tagged human MSI2 was obtained from Sino Biologicals, Beijing, China (pCMV3_FLAG-MSI2:HG13069-NF).

Protein expression analyses

Protein levels of wildtype and mutant RHOBTB2 and MSI2 were determined as described previously[1] and in more detail in Supplemental Methods.

For statistical analysis, all values >5 were set to 5. Statistical analysis was performed using the one sample t-test with the hypothetical mean set to 1 followed by Bonferroni correction.

Co-immunoprecipitation

For co-immunoprecipitation, HEK293 cells were transiently co-transfected with 1 µg His-cMyc-tagged wildtype or mutant *RHOBTB2* and 0.5 µg HA-tagged *Cullin3* or FLAG-tagged *MSI2* per 6-well. After 48 hours, cells were treated with 25 µM MG-132 proteasome inhibitor (Sigma-Aldrich) for 4 hours and harvested with lysis buffer (100 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA and 1 % Triton X-100) with protease inhibitor (Sigma-Aldrich). For immunoprecipitation, lysates were diluted with 1x TBS and incubated with 15 µl Protein A Mag Sepharose bead suspension (GE Healthcare, Chicago, IL, USA) and 1.6 µg anti-Myc antibody (M4439, Sigma-Aldrich) at 4°C overnight. Subsequently, the beads were washed once with lysis buffer and three times with 1x TBS, followed by elution with 1x Lämmli buffer. Protein separation and western blotting was performed as described above. The amount of co-precipitated Cullin3 was quantified using the Image Lab software (Bio-Rad), and normalized to the amount of precipitated RHOBTB2 and compared to the wildtype. Statistical analysis was performed using the one sample t-test with the hypothetical mean set to 1 followed by Bonferroni correction.

RESULTS

Mutational and clinical spectrum of *de novo* heterozygous missense variants in *RHOBTB2*

We were able to assemble data on 23 individuals with *de novo* heterozygous missense variants in *RHOBTB2* that can be categorized into different groups based on the location of the variants and the associated phenotypes. Detailed clinical information and genomic and cDNA position description of the variants are provided in Supplemental Table 1 and summarized in Table 1 and Figure 1A.

De novo, heterozygous missense variants in the BTB domain region

Sixteen individuals harbored seven different, heterozygous missense variants in the BTB domain encoding region of *RHOBTB2* (Figure 1, Supplemental Table 1), located in the first

BTB domain (3x) or at the interface of the second BTB domain (4x). In 14 individuals, the variant was shown to be *de novo*, for three individuals this information was not available. Six variants were recurrent and were either identified in another individual within this study and/or were described previously,[1,5] the (p.(Ala471Cys) variant is novel. All missense variants in the BTB domain region were classified as pathogenic or likely pathogenic according to ACMG guidelines (Supplemental Table 1).[20] Of note, several individuals additionally carried variants of unknown significance in other known disease genes (Supplemental Table 1), and a contributory effect of these variants to the phenotype cannot be excluded.

Individuals with missense variants in the BTB domain region presented with a severe developmental and epileptic encephalopathy. In 13 individuals, onset of epilepsy was within the first six months of life, and in one individual seizures started at the age of 6 years. Seizure types included generalized tonic-clonic or focal seizures, with status epilepticus reported in two individuals. Eight individuals were treated with levetiracetam. Seizures were reported to be treatment-responsive in most individuals, but were refractory in two.

Developmental delay and intellectual disability were noted in 15 individuals, for one individual this information is missing. Developmental regression occurred in four individuals, correlating with onset of seizures. All but one affected individuals presented with severe language delay and lacking or severely impaired speech capacities. Motor development was also severely impaired with limited or lack of ambulation in most individuals. Microcephaly or a rather small head circumference were observed in all individuals for whom data was available.

Twelve individuals had movement disorders including ataxia, dyskinesia and choreoathetosis. Response to treatment with acetazolamide was reported for one individual in this study. In seven individuals (post-ictal) hemiparesis occurred, in one subject after head injury. Brain MRI anomalies included hypoplastic corpus callosum, atrophic changes in temporal lobes or delayed occipital myelination.

Other common features included muscular hypo- or hypertonia (12/14 individuals) and behavioral abnormalities, autism-spectrum-disorder or stereotypic movements (11/14 individuals). Minor facial dysmorphisms were noted in the majority of individuals but were rather non-specific.

<u>*De novo*, heterozygous missense variants in the GTPase domain</u>

Additionally, we assembled data on variants residing outside the BTB domain region. Five different *de novo* missense variants in six individuals were identified within the GTPase domain (Figure 1, Supplemental Table 1). One of them recurrently occurred in two individuals (p.(Arg116Cys)) in this study and was published previously in another individual.[9] Another variant was located in close proximity (p.(Asp114His)). Two missense variants affected the same amino acid residue (p.(Arg154Gln) and p.(Arg154Leu)). The missense variant

p.(Arg183Met) is located close to a splice site and is predicted to lead to loss of the splice donor (score 0.95).[21] Aberrant splicing by loss of exon 6 was confirmed in an *in vitro* splice assay (Supplemental Methods and Supplemental Figure 1A). However, as patient-derived material (blood or cells) was not available, the *in vivo* effects of this splicing variant situation remain unclear.

None of the missense variants in the GTPase domain was present in gnomAD (gnomAD v2.1.1).[22] They all affected highly conserved amino acid residues (Figure 1B) and were predicted to be deleterious by several *in silico* prediction tools (Supplemental Table 2). The recurrent p.(Arg116Cys) variant, the variants affecting the same amino acid position (p.(Arg154Gln), p.(Arg154Leu)) and the variant at position 114 in the GTPase domain were classified as likely pathogenic according to ACMG guidelines.[20] (Supplemental Methods and Supplemental Table 1) The variant p.(Arg183Met) remained of unknown significance due to the possible effect on splicing that might result in nonsense-mediated mRNA decay of the mutated allele.

All six individuals with variants in the GTPase domain presented with a variable neurodevelopmental phenotype (Table 1, Supplemental Table 1). Seizures only occurred in two individuals with good response to anti-seizure treatment. When data were available, cognitive impairment ranged from learning difficulties (1x) to mild (1x) and moderate (2x) ID. While speech delay occurred in most, two individuals could speak in complete but rather short sentences. Developmental regression was reported in two cases, co-occurring with spasms in one of them. Dystonic movements or hemiparesis were reported in a single individual each, and brain MRI anomalies in two individuals. Behavioral abnormalities and minor, non-specific facial dysmorphism were common. Head circumferences was normal in three individuals, and macrocephaly was noted in one.

<u>*De novo* missense variants not located in any of the known domain regions</u>

Another *de novo* missense variant (p.(Pro262Leu)) was located between the GTPase and the BTB domains. Pathogenicity remains unclear. The individual's phenotype included neonatal onset epilepsy with 30-40 seizures per day and myoclonic jerks which were not fully controlled. Profound intellectual disability and neurological impairment were present, with absent ambulation and speech at age of 4 years. Other features included microcephaly, severe spastic paraparesis with little voluntary movement and brain MRI anomalies such as diffuse cerebral atrophy. This individual additionally harbored a hemizygous missense variant in *UBE2A*, a gene associated with severe neurodevelopmental phenotypes (intellectual disability disorder, X-linked, syndromic, Nascimento type, MIM# 300860). However, the variant is located in a non-canonical transcript and its clinical significance is uncertain.

Mutational and clinical spectrum of bi-allelic splice-site and truncating variants in *RHOBTB2*

We also collected data of 13 individuals from nine independent families with homozygous (eight families) or compound heterozygous (one family), potential loss-of-function variants in *RHOBTB2* (Figure 1A, Supplemental Figure 2, Supplemental Table 1). Ten variants were nonsense, frame-shifting or located in or close to splice sites. Aberrant splicing was confirmed by an *in vitro* splice assay (Supplemental methods) for three splice-site variants (c.258+4A>C, c.1568-1G>A, c.2032+1G>C) (Supplemental Figure 1B and C). However, as patient-derived material (blood or cells) was not available, the *in vivo* effects of this splicing variant situation remain unclear.

Of note, four of the truncating variants are located in the pen-ultimate or ultimate exon, therefore possibly escaping nonsense mediated mRNA decay,[23] as predicted for the other, more N-terminal truncating variants. For these C-terminal variants, truncation and thus a gain-of-function mechanism cannot be excluded. Five of the recessive variants are listed in gnomAD in a heterozygous state with a very low frequency (p.(Arg179*) and p.(Arg670*) reported in two alleles, p.(Tyr700*) and p.(Trp105*) reported in one allele each, and c.258+4A>C reported in eight alleles).[22] According to ACMG criteria, seven of these variants were classified as likely pathogenic and three as variants of unknown significance (Supplemental Table 1).

All individuals with bi-allelic variants in *RHOBTB2* presented with variable neurodevelopmental phenotypes. Cognitive impairment ranged from learning difficulties to moderate ID. Speech delay was common. Seizures or febrile seizures occurred in all but two individuals. Response to treatment ranged from poor/partial (3/6) to good (3/6). Most of the individuals started to walk within the first two years of life, often with unsteady gait or a movement disorder. Microcephaly was reported in five of nine individuals for whom information was available.

Predicted structural consequences of heterozygous missense variants in *RHOBTB2*

In 2018, structural modeling of variants in the BTB domain region predicted impairment of intramolecular stability and formation, destabilization of the first BTB domain and interference with dimer formation of the second BTB domain, the latter associated with variants at position 510 and 511, located at the dimer interface of the second BTB domain.[1]

We now performed structural analysis of the GTPase domain, based on a model generated with AlphaFold (Figure 1C). Most affected residues (Asp114, Arg116, Arg154) are located on the surface of the GTPase domain, making them candidates for protein-protein interactions with interaction partners and/or substrates. As homologous, typical GTPases such as RhoA, interact with guanine nucleotide exchange factors (GEFs) to catalyze release of GDP, we

exemplarily mapped the PDZ-RhoGEF binding site on the RHOBTB2 GTPase domain, revealing that Asp114 and Arg116 would be located in the RHOBTB2-GEF interface and that Arg154 is close to the interface (Figure 1C). We refrained from a more detailed analysis of the interactions, because the exact physiological interaction partner recognizing this surface patch is not yet known and as a GTPase activity for RHOBTB2 has not been shown.[2,24-26] Regardless of the protein recognized by this surface region, we expect that variants have a significant impact as they all lead to a loss of positive charges ((p.Arg116Cys), p.(Arg154Gln), p.(Arg154Leu)) or negative charges (p.Asp114His), thereby disrupting the electrostatic complementarity of protein-protein interactions. In the model, Arg183 forms a salt-bridge with Glu189. Since this interaction cannot be formed by the uncharged Met183, the p.(Arg183Met) exchange rather destabilizes the GTPase domain itself.

Interaction with Cullin3 or Mushashi-2 is not impaired by *RHOBTB2* missense variants

To further analyze the functional consequence of missense variants on the RHOBTB2 protein level, we selected variants from the different domains and from regions between the known domains (GTPase: p.(Arg116Cys), p.(Arg154Gln), p.(Arg183Met), between: p.(Trp239Cys), p.(Ser241Tyr), BTB cancer: p.(Tyr306Asp), BTB DEE: p.(Ala471Cys), p.(Ala474Gly), p.(Arg483His), p.(Arg507Cys), p.(Arg511Gln)) identified in this and previous studies.[1,2,4,5]

As RHOBTB2 interacts with the scaffold protein Cullin3 to assemble into an ubiquitin ligase complex, we first tested if the variants resulted in impaired binding towards Cullin3. We confirmed reduced binding for the cancer variant p.(Tyr306Asp) as demonstrated before.[2] Consistent with previous findings[1] we did not observe impaired binding to Cullin3 resulting from variants in the BTB domain region (p.(Ala471Cys), p.(Ala474Gly), p.(Arg483His), p.(Arg507Cys), p.(Arg511Gln)). We now also tested binding to Cullin3 for variants located within the GTPase domain or between the domains (p.(Arg116Cys), p.(Arg154Gln), p.(Arg183Met), p.(Trp239Cys)), and did not observe an alteration, either (Figure 2A). Moreover, we also did not observe altered binding to the only known substrate of RHOBTB2, Musashi-2 for any of the mutant constructs (Supplemental Figure 3).

Proteasomal degradation is only impaired by missense variants in the BTB domain region

We next determined protein levels of wildtype and mutant RHOBTB2 and observed increased levels for mutant RHOBTB2 carrying any of BTB domain region variants, as described previously.[1] The differences in protein quantity decreased upon addition of proteasomal inhibitor, supporting the hypothesis of impaired proteasomal degradation as cause of protein abundancy. Protein levels of mutant RHOBTB2 carrying a variant outside the BTB domain, however, were unaltered compared to wildtype. Our findings therefore indicate different

consequences on proteasomal degradation of RHOBTB2 depending on localization of missense variants (Figure 2B).

DISCUSSION

By assembling data on individuals with either *de novo* missense or inherited, bi-allelic splice-site and truncating variants in *RHOBTB2*, we further delineate and expand the *RHOBTB2*-associated NDD spectrum. We are able to define several *RHOBTB2*-related genotype-phenotype correlations, based on a) clinical manifestations b) location, nature and functional consequences of variants, and c) inheritance pattern.

Consistent with initial reports,[1,5] individuals with *de novo* missense variants clustering in the BTB domain encoding region of *RHOBTB2* present with a rather homogeneous, severe developmental and epileptic encephalopathy, including early-onset epilepsy, intellectual disability, microcephaly, paroxysmal movement disorders and MRI anomalies. Furthermore, transient neurological deficits such as hemiparesis, stroke-like episodes, and brain MRI anomalies were reported in several individuals, often occurring post-ictally.[1,4,5,11,12] Head trauma also appears to trigger encephalopathic episodes in individuals with *RHOBTB2* associated DEE.[27,28] In our study, hemiparesis was observed in a post-ictal setting in six individuals and once after head injury (individual 22).

Apart from the "typical" DEE phenotype associated with *de novo* missense variants in the BTB domain region, we found growing evidence for a broader spectrum of neurodevelopmental phenotypes associated with *de novo* missense variants located in other domains or regions of *RHOBTB2*. In addition to two previously reported missense variants,[9] we now identified five further *de novo* missense variants in the GTPase domain. The phenotype associated with variants in the GTPase domain is more variable and/or milder compared to that of individuals harboring variants in the BTB domains. Affected individuals presented with mild to moderate ID, and seizures or movement disorders occurred less frequently. Microcephaly, which is commonly seen in individuals with BTB domain region variants, was not observed in any of the individuals with variants in the GTPase domain. Interestingly, even macrocephaly was reported in one subject in the latter group. Thus, our data show that the GTPase domain manifests as a second variant hotspot for a *RHOBTB2*-associated NDD. However, the small number of missense variants outside the BTB domain region still limits definitive conclusions. Furthermore, we identified several families with bi-allelic truncating variants in *RHOBTB2.* Affected individuals presented with variable neurodevelopmental phenotypes including intellectual disability and seizures. Therefore, *RHOBTB2* is not only implicated in autosomal-dominant NDDs due to *de novo* missense variants but also in an autosomal-recessive NDD caused by bi-allelic truncating/loss-of-function variants. *RHOBTB2* therefore adds to the growing list of genes associated with both autosomal-dominant and autosomal-recessive

NDDs, such as PLXNA[29] (Dworschak-Punetha neurodevelopmental syndrome, MIM# 619955) or ACTL6B[30] (Developmental and epileptic encephalopathy 76, MIM# 618468, Intellectual developmental disorder with severe speech and ambulation defects, MIM# 618470). The phenotype of individuals with bi-allelic truncating variants in *RHOBTB2* overlaps with those carrying *de novo* missense variants regarding intellectual disability and epilepsy. However, it is more variable and less specific than the DEE resulting from *de novo* missense variants in the BTB domain region.

Though a gain-of-function effect due to truncation and lack of nonsense-mediated mRNA decay for bi-allelic variants in the last or pen-ultimate exon might be possible, a general loss-of-function effect is most likely at least for bi-allelic splice-site and truncating variants in the more N-terminal regions of RHOBTB2. In contrast, such a loss-of-function effect for the *de novo* missense variants is rather unlikely as heterozygotes of the familial truncating variants do not show a phenotype, as *RHOBTB2* is predicted to be tolerant towards loss-of-function variants (pLI=0.01[22]) and as heterozygous deletions of *RHOBTB2* were observed in unaffected individuals.[1,31] In accordance, not reduced but instead abundant RHOBTB2 protein levels were shown to result from pathogenic missense variants in the BTB domain region, previously.[1] This might be due to impaired proteasomal degradation of RHOBTB2. Missense variants in the BTB domain region might therefore have a very specific, initial loss-of-function effect on the ubiquitin-proteasome pathway but result in a gain-of-function effect by increased RHOBTB2 levels.

As we now observed phenotypic differences in individuals carrying *de novo* missense variants also in other domains or regions of *RHOBTB2*, we investigated if these missense variants might have different functional consequences from those in the BTB domain region. While the BTB domains are known to interact with Cullin3 and with other RHOBTB2 molecules to form homo and heterodimers with RHOBTB2 or RHOBTB3 molecules,[32] the function of the GTPase domain is still elusive. It is distinguished from typical Rho GTPases by various sequence alterations and lack of crucial amino acids for GTP binding and catalytic transformation.[26,33] Therefore, it is controversially discussed if this domain has GTP binding capacities and/or GTPase activity.[2,24-26] Similar to variants residing in the BTB domain region we did not observe impaired binding to ubiquitin ligase complex scaffold protein Cullin3 for variants located in the GTPase domain. This would be in line with none of the DEE or NDD related variants being located in the specific Cullin3 binding region in the first BTB domain.[1,2] While variants in the BTB domain region result in impaired proteasomal degradation of RHOBTB2 *in vitro* as shown before[1] and confirmed in this study, we did not observe such an effect when testing variants from the GTPase domain or outside the domains. This suggests a different functional consequence of missense variants in the GTPase domain compared to missense variants in the BTB domain region.

Missense variants in the BTB domain region were predicted to impair the intramolecular stability of RHOBTB2 and dimer formation of the second BTB domain.[1] Structural modeling for the GTPase domain variants now suggests that p.(Arg183Met) decreases stability of the GTPase domain itself, whereas the remaining variants at positions 114, 116 and 154 more likely affect protein-protein interactions (Figure 1C). Alternatively, these variants may also affect intramolecular domain interactions, which have been proposed to regulate the active and inactive state of RHOBTB2.[32] Here, the GTPase domain interacts with the first BTB domain, keeping the protein in an inactive state. Conformational changes could be induced by interaction with specific ligands or substrates, allowing RHOBTB2 to assemble into a Cullin3 ubiquitin ligase complex.[25,26,32]

Since the physiological binding partner of the interface is yet unknown, we investigated the impact of the missense variants on the only known substrate of RHOBTB2 to date, Musashi-2, *in vitro*. Musashi-2 has been shown to interact with the C-terminal region of RHOBTB2.[3] In accordance, our results did not indicate impaired interaction for any of the tested missense variants. However, it is still possible that variants in the GTPase domain lead to a destabilization of this protein-protein interaction or affect an alternative type of protein-protein interaction (e.g. intramolecular interactions or interactions with GEF proteins).

Although the exact mechanism remains elusive, our observations and investigations indicate a different functional consequence resulting from variants in the GTPase domain compared to variants in the BTB domain region and may possibly contribute to the different severity and manifestation of associated phenotypes. While the functional consequence of clustering missense variants in either the BTB domain region or the GTPase domain might be rather specific, no clear categorization into loss-of-function, gain-of-function or dominant-negative effects is possible, so far.

Conclusion

By identifying a phenotype-genotype correlation regarding location and consequences of *de novo* missense variants in *RHOBTB2* and the resulting neurodevelopmental phenotype and by newly identifying bi-allelic truncating variants, we further delineate and expand the molecular and clinical spectrum of *RHOBTB2*-related NDDs.

DATA AVAILABILITY

Novel variants in this paper will be submitted to LOVD.

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AUTOR CONTRIBUTIONS

Mutational and clinical data acquisition and analysis: F.L., R.M., R.Ba., A.G., M.R., J.B.R., M.K., T.H., M.H., F.K., D.H., C.M., B.K., S.B., C.B., B.B.Z., E.A., E.H.S., V.K.G., V.M.S., S.B., M.C.K., M.A.S., A.Ku., E.A.M., K.B., O.K., K.L.P., A.Ko., K.B., S.R., R.D.C., M.E., G.M., N.T., Z.S., G.M.M., J.O., U.G., A.B., L.v.W., R.A.J., Y.H., P.G., C.A.A., M.C., R.Bo., M.B., R.A., F.A.M., A.M.T., F.Ab., F.S.A., E.M.K., J.R.A., T.S., N.H., E.G.K., F.As., S.I., S.E., H.H., H.S., C.Z.; Experimental work: F.L, R.B.; Structural modeling: H.S.; Writing: F.L., C.Z. The manuscript was read and revised by all co-authors.

ETHICS DECLARATION

Ethics approval for this study was obtained from the ethical review board of the University of Bern and respective institutional review boards of the testing centers (Supplemental Table 1). Research in this report was conducted in a manner consistent with the principles of research ethics, such as those described in the Declaration of Helsinki and/or the Belmont Report. Consent for publication of molecular and clinical data was obtained from the individuals, their parents or legal guardians.

CONFLICT OF INTEREST

J.B.R. Serves on the editorial board for the journal Neurology and has received consulting fees from Supernus Pharmaceuticals.

All other authors declare no conflicts of interest.

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Figure legends

Figure 1

Overview of published and novel variants in *RHOBTB2*. A. Schematic drawing of RHOBTB2 with domains and identified missense variants clustering in the GTPase or BTB domain region (GenBank: NM_001160036.2) above the scheme and bi-allelic splice-site and truncating variants below the scheme. Domains were identified and re-colored based on SMART prediction.[34,35] Variants in grey were described previously,[1,4-13,15] variants in black have been identified in this cohort. Recurrent variants are underlined. p.(Arg154*) has been published before,[15] compound heterozygous variants are marked by a +. Possible genotype-phenotype correlations based on phenotype severity and variant location are indicated by blue, grey and red boxes. #, variants included in experiments. The cancer variant p.(Tyr306Asn),[2] marked with a C, results in impaired binding to Cullin3.

B. Conserved positions of the affected amino acids in the GTPase domain according to the UCSC genome browser.[36,37] **C.** Structural model of the GTPase domain. The domain is shown in ribbon presentations with α-helices in red and β-sheets in green. The variant sites (Asp114, Arg116, Arg154, Arg183) and one interacting glutamate are shown in space-filled presentation (atom-type coloring) and are labelled. The potential GEF binding site is exemplarily illustrated for PDZ-RhoGEF (white space-filled presentation).

Figure 2

Consequences of missense variants regarding binding to Cullin3 and proteasomal degradation of RHOBTB2. A. Co-immunoprecipitation of His-cMyc-tagged RHOBTB2 and HA-tagged Cullin3 shows equal co-precipitation of Cullin3 for both wildtype and mutant RHOBTB2, except for the cancer variant p.(Tyr306Asn). Cells were treated with the proteasomal inhibitor MG132, and co-immunoprecipitation was performed with an antibody against Myc. A representative image from five independent experiments is shown. For quantification, Cullin3 bands after co-immunoprecipitation were normalized to the corresponding RHOBTB2 bands and compared to RHOBTB2 wildtype. Error bars represent the standard error. Statistical analysis was performed using the one sample t-test with the hypothetical mean set to 1 followed by Bonferroni correction ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Uncropped blots are provided in Supplemental Figure 4. **B**. Representative western blot from three independent experiments after transfection of wildtype and mutant His-cMyc-tagged RHOBTB2 shows impaired proteasomal degradation for both cancer and BTB domain region variants but not for the others. Experiments were performed with (top) and

without (bottom) proteasomal inhibitor MG132. For quantification, RHOBTB2 bands were normalized to the loading control GAPDH and compared with RHOBTB2 wildtype. Error bars represent the standard error. For statistical analysis all values >5 were set to 5. Statistical analysis was performed using the one sample t-test with the hypothetical mean set to 1 followed by Bonferroni correction ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Uncropped blots are provided in Supplemental Figure 5.