Bi-allelic loss-of-function variants in BCAS3 cause a syndromic neurodevelopmental disorder

Holger Hengel,1,2 Shabab B. Hannan,1,2 Sarah Dyack,3 Sara B. MacKay,4 Ulrich Schatz,5,6 Martin Flegler,7 Andreas Kurringer,7 Ghassan Balousha,8 Zaid Ghanim,9 Fowzan S. Alkuraya,10,11 Hamad Alzaidan,12 Hessa S. Alsaf,13 Tahadaro Mitani,13 Sevcan Bozdogan,16 Davut Pehlivan,13,14 James R. Lupski,13,14,15 Joseph J. Gleseson,17 Mohammadreza Dehghani,18 Mohammad Y. Mehrjardi,19 Elliott H. Sherr,20 Kendall C. Parks,20 Emanuelu Argill,20 Amber Begtrup,21 Hamid Golehdari,22 Osama Balousha,23 Gholamreza Shariati,24 Neda Mazaheri,25 Reza A. Malamiri,26 Alistair T. Pagnamenta,27 Helen Kingston,28 Siddharth Banka,28,29 Adam Jackson,29 Mathew Osmond,30 Care4Rare Canada Consortium,30 Genomics England Consortium Research,31 Angelika Rieß,32 Tobias B. Haack,32 Thomas Nägele,34 Stefanie Schuster,1,2 Stefan Hauser,1,2 Jakob Admard,32,33 Nicolas Casadei,32,33 Ana Velic,35 Boris Macek,35 Stephan Ossowski,32,33 Henry Houlden,36 Reza Maroofian,36,37,* and Ludger Schöls1,2,37,*

Summary

BCAS3 microtubule-associated cell migration factor (BCAS3) is a large, highly conserved cytoskeletal protein previously proposed to be critical in angiogenesis and implicated in human embryogenesis and tumorigenesis. Here, we established BCAS3 loss-of-function variants as causative for a neurodevelopmental disorder. We report 15 individuals from eight unrelated families with germline bi-allelic loss-of-function variants in BCAS3. All probands share a global developmental delay accompanied by pyramidal tract involvement, microcephaly, short stature, strabismus, dysmorphic facial features, and seizures. The human phenotype is less severe compared with the Bcas3 knockout mouse model and cannot be explained by angiogenic defects alone. Consistent with being loss-of-function alleles, we observed absence of BCAS3 in probands’ primary fibroblasts. By comparing the transcriptomic and proteomic data based on probands’ fibroblasts with those of the knockout mouse model, we identified similar dysregulated pathways resulting from over-representation analysis, while the dysregulation of some proposed key interactors could not be confirmed. Together with the results from a tissue-specific Drosophila loss-of-function model, we demonstrate a vital role for BCAS3 in neural tissue development.

Introduction

BCAS3 microtubule-associated cell migration factor (BCAS3) is a large 928 amino acid, 101 kDa protein encoded by a 25-exon gene, BCAS3 (MIM: 607470), that spans a genomic interval of 714 kb on chromosome 17q23.2. This highly conserved cytoskeletal protein is involved in human embryogenesis as well as in

1Department of Neurology and Hertie-Institute for Clinical Brain Research, University of Tübingen, 72076 Tübingen, Germany; 2Division of Medical Genetics, Department of Pediatrics, Dalhousie University, Halifax, NS B3K 6R8, Canada; 3Maritime Medical Genetics Service IWK Health Centre, Halifax, NS B3R 688 Canada; 4Institute of Human Genetics, Medical University of Innsbruck, Peter-Mayr-Str. 1, 6020 Innsbruck, Austria; 5Institute of Human Genetics, Technical University of Munich, Trogerstr. 32, 81675 Munich, Germany; 6Department of Pediatrics, Landeskrankenhaus Bregenz, Carl-Pedenz-Str. 2, 6900 Bregenz, Austria; 7Department of Pathology and Histology, Al-Quds University, East Jerusalem 19356, Palestine; 8Palestine Medical Complex, Ramallah, Palestine; 9Department of Translational Genomics, Center for Genomic Medicine, King Faisal Specialist Hospital and Research Center, Riyadh 11211, Saudi Arabia; 10Department of Anatomy and Cell Biology, College of Medicine, Alfaisal University, Riyadh 11533, Saudi Arabia; 11Department of Medical Genetics, King Faisal Specialist Hospital and Research Center, Riyadh 11564, Saudi Arabia; 12Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA; 13Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA; 14Department of Medical Genetics, Cukurova University Faculty of Medicine, 01330 Adana, Turkey; 15Laboratory for Pediatric Brain Disease, Howard Hughes Medical Institute, Department of Neurosciences, University of California, San Diego, La Jolla, CA 92039, USA; 16Genetics Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran; 17Abortion Research Centre, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran; 18Department of Neurology and Institute of Human Genetics and Well Institute for Neurosciences, University of California, San Francisco, San Francisco, CA 94158, USA; 19GeneDX, 207 Perry Parkway, Gaithersburg, MD 20877, USA; 20Department of Genetics, Faculty of Science, Shahid Chamran University of Ahvaz, Ahvaz, Iran; 21Faculty of Medicine, Al-Quds University, East Jerusalem 19356, Palestine; 22Department of Medical Genetics, Faculty of Medicine, Al-Quds University, East Jerusalem 19356, Palestine; 23Department of Genetics, Faculty of Science, Shahid Chamran University of Ahvaz, Ahvaz, Iran; 24Department of Paediatric Neurology, Golestan Medical, Educational, and Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; 25Department of Paediatric Neurology, Golestan Medical, Educational, and Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; 26National Institute for Health Research Oxford Biomedical Research Centre, Wellcome Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, UK; 27Manchester Centre for Genomic Medicine, St Mary’s Hospital, Manchester University NHS Foundation Trust, Health Innovation Manchester, Manchester M13 9WL, UK; 28Division of Evolution and Genomic Sciences, School of Biological Sciences, Faculty of Biology, Medicine, and Health, University of Manchester, Manchester M13 9WL, UK; 29Children’s Hospital of Eastern Ontario Research Institute, University of Ottawa, Ottawa, ON K1H 8L1, Canada; 30Genomics England, London EC1M 6BQ, UK; 31Institute of Medical Genetics and Applied Genomics, University of Tübingen, 72076 Tübingen, Germany; 32NGS Competence Center Tübingen, University of Tübingen, 72076 Tübingen, Germany; 33Division of Neuroradiology, University of Tübingen, 72076 Tübingen, Germany; 34Department of Neuromuscular Disorders, Queen Square Institute of Neurology, University College London, London WC1N 3BG, UK

*These authors contributed equally

Correspondence: marroohan@gmail.com (R.M.), ludger.schoels@uni-tuebingen.de (L.S.)

https://doi.org/10.1016/j.ajhg.2021.04.024.
© 2021 The Authors. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
tumorigenesis. The mouse ortholog, Bcas3, is 98% identical to BCAS3 and is essential for mouse development and angiogenesis. Homozygous Bcas3<sup>+/−</sup> knockout leads to embryonic lethality in mice. Mutant embryos are growth retarded and show defects in the morphology and vasculature of the head and heart. BCAS3 is well established as a critical protein regulating the cytoskeleton and vasculature of the head and heart. BCAS3 variants are found in breast cancer (hence, the previous gene name, breast carcinoma-amplified sequence 3), and high levels of BCAS3 were noted in tumor cells and blood vessels of different brain tumors, such as glioblastoma and hemangiopericytoma, as well as in brain abscesses. Genome-wide association studies (GWASs) have associated variants in BCAS3 with coronary artery disease and with gout. One previous study suggested rare homozygous missense variants in BCAS3 as candidate variants for autosomal recessive intellectual disability.

Here, we establish bi-allelic BCAS3 variants as a cause of autosomal recessive syndromic global developmental delay. We used comparative transcriptomics and proteomics data from probands’ fibroblasts to experimentally explore dysregulated pathways. While there are similarities to previous murine knockout studies on a global level, levels of several proposed key proteins, including CDC42 and Vimentin, were unchanged in probands’ fibroblasts. Furthermore, there were no signs of defective angiogenesis in any of the identified probands, and migration assays using fibroblasts’ primary fibroblasts did not show measurable defects in cell migration. We further explored the biological consequences of BCAS3 dysfunction by investigating the phenotypes of a Drosophila loss-of-function model and confirmed an essential role of nudnira during development independent of angiogenesis.

### Material and methods

#### Ethical approval

Written informed consent was obtained from the parents of the underage probands for diagnostic procedures and next-generation sequencing as well as for the publication of identifying facial images. This study was approved by the local institutional review board of the Medical Faculty of the University of Tübingen, Germany (vote 180/2010BO1).

#### Next-generation sequencing and analysis

Whole-exome or -genome sequencing was performed at different genetic institutes via next-generation sequencing techniques according to the local standard protocols. All variants were confirmed via Sanger sequencing with standard methods and chemicals (primer sequences are available upon request).

**Family 1 and family 6**

Exome sequencing of affected probands and data analysis were performed as previously described.

**Family 2**

Exome sequencing for two affected siblings (F2-II.2 and F2-II.3) was performed at the Institute of Medical Genetics and Applied Genomics in Tübingen as previously described. After excluding pathogenic or likely pathogenic variants in genes known to be associated with neurological or developmental disorders, variants were filtered for rare (gnomAD minor allele frequency < 0.1%) homozygous or compound heterozygous variants shared between the two affected siblings.

**Family 3**

Exome sequencing was performed for the index proband in a CLIA-certified and CAP- and ISO 15189-accredited laboratory (Blueprint Genetics) as previously described. Segregation of the identified variant was performed by Sanger sequencing in the Clinical Genomics Laboratory at IWK Health.

**Family 4**

Single-exome sequencing was performed on F4-II.1 as previously described.

**Family 5**

Exome sequencing was performed as previously described. The BCAS3 variant was identified by sharing candidate genes among collaborators.

**Family 7**

Informed consent was provided according to the Baylor-Hopkins Center for Mendelian Genomics Research Protocol (IRB number: H-29697). Exome sequencing for two affected siblings (F7-II.1 and F7-II.2) was performed as previously described.

**Family 8**

The affected proband and his unaffected parents were recruited to the 100K Genomes Project (100KGP), a national genome sequencing initiative approved by the Health Research Authority Committee East of England, Cambridge South (REC: 14/EE/1112). Library preparation was performed with TruSeq DNA PCR-Free Library Prep, and sequencing was performed on a HiSeq X instrument. Variants were called jointly (as a trio) with Platypus v.0.7.9.5 and then filtered with the Genomics England Tiering process. We used Manta<sup>2</sup> to detect structural variants and called the paternally inherited 311 kb deletion that was previously detected via chromosomal microarray analysis (OGT 8x60k with CytoSure Interpret v.3.4.3.).

**Family 9**

Trio whole-exome sequencing (WES) of the affected proband and her parents was performed and analyzed by GeneDX as previously described.

### Fibroblast cultivation

Human dermal fibroblasts were maintained at 37°C, 5% CO2, and 100% relative humidity in fibroblast medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) (Merck) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) in cell culture flasks. Cells were split via trypsinization and passaged into new flasks or seeded at a defined density for the Oris cell migration assay.

### Oris cell migration assay

Fibroblast migration was investigated with an Oris cell migration plate (Platypus Technologies). Control (four lines) and proband fibroblasts (two lines) were isolated and seeded at a density of 6 × 10⁴ cells per well into a 96-well Oris cell migration plate according to the manufacturer’s guidelines (5 wells per cell line). After a pre-incubation step of 24 h, stoppers were removed and further incubated for 30 h. Afterward, the cells were stained with ActinRed 555 (Sigma) according to the manufacturer’s instructions and imaged.
with an Operetta High Content Imaging System (Perkin Elmer). Using ImageJ, we calculated the covered area per well.

**Protein isolation and immunoblotting**

Upon reaching high confluence, primary fibroblasts were washed with cold PBS, scraped off in PBS, centrifuged at 800 g for 5 min, and frozen at −80°C. Pellets of primary fibroblasts were lysed in RIPA buffer (Sigma) containing protease inhibitors (Roche) for 45 min on a rotator at 4°C. Cell debris were pelleted at 15,800 g and 4°C for 30 min. The protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. 10 μg of protein was eluted in 5× pink buffer (Thermo Fisher Scientific) at 95°C. Samples were separated on 10% polyacrylamide gels and transferred onto a Hybond-P polyvinylidene difluoride (PVDF) membrane (Merck). Membranes were blocked in 5% milk in TBS-T and incubated overnight with the primary antibodies against rabbit α-BCAS3 (1:1,000, Ethyl laboratories), rabbit α-CDC42 (1:5,000, Abcam), rabbit α-Vimentin (1:5,000, Cell Signaling Technologies), and mouse α-GAPDH (1:20,000, Meridian Life Sciences) in Western Blocking Reagent (Roche) at 4°C, followed by three washes with TBS-T and incubation with HRP-conjugated secondary antibodies (Jackson Immunoresearch) for 1 h at room temperature. Proteins were visualized with the Immobilon Western chemiluminescent HRP substrate (Merck).

**RNA isolation and RNA sequencing**

For RNA extraction, fibroblasts from two primary proband fibroblast cell lines (F3-II.1 and F4-II.1) and two primary control fibroblast cell lines (CO-1, female, 24 years old and CO-2, male, 22 years old) were cultivated. RNA was isolated from three independently grown flasks for each cell line (biological replicates, 4×3 RNA samples) via the Qiagen RNeasy mini kit. RNA quality was assessed with the Agilent 2100 Bioanalyzer RNA Nano Kit (Agilent Technologies, Santa Clara, CA, United States). All samples had high RNA integrity numbers (RIN > 9). Using the NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs, Ipswich, MA, United States) with 100 ng of total RNA input for each sequencing library, we generated poly(A)−selected sequencing libraries according to the manufacturer’s manual. All libraries were sequenced on the Illumina NovaSeq 6000 platform in paired-end mode with 2 × 51 bp reads and at a depth of approximately 30 million clusters each. Library preparation and sequencing procedures were performed by the same individual, and a design aimed to minimize the introduction of technical batch effects was chosen.

We assessed the quality of raw RNA sequencing (RNA-seq) data in FASTQ files by using ReadQC (v.2019_11) to identify potential sequencing cycles with low average quality and base distribution bias. Reads were preprocessed with SeqPurge (v.2019_11) and aligned with STAR (v.2.7.3a), allowing spliced read alignment to the human reference genome (build GRCh37). Alignment quality was analyzed with MappingQC (ngs-bits v.2019_11) and visually inspected with the Broad Integrative Genome Viewer (v.2.7.0). On the basis of the Ensembl genome annotation (GRCh37, Ensembl release 97), we obtained read counts for all genes by using subread (v.2.0.0).

For gene expression analysis, we filtered raw gene read counts to retain only genes with at least 1 count per million (cpm) in at least half of the samples and normalized them by the trimmed mean of M values (TMM) procedure, leaving >13,000 genes for determining differential expression in each of the pairwise comparisons between affected and control samples. The analysis was performed with edgeR (v.3.26.8) with negative binomial distributions and gene-wise testing with generalized linear models.

For each proband, triplicate expression profiles from corresponding fibroblast cell lines were compared to the average of the bioinformatic control cell lines. In addition, the average of both proband triplicate sets was compared to the average of control triplicate sets (Table S2).

Gene expression changes are expressed as log2-fold changes and expression in the control group was used as a baseline. Significant genes with an adjusted p value (false discovery rate [FDR]) of less than 0.01 and an absolute log2-fold change of at least 1 are reported.

**NanoLC-MS/MS analysis and data processing**

Protein samples for liquid chromatography-mass spectrometry (nanoLC-MS/MS) were generated from the two primary proband fibroblast cell lines in biological replicates (F3-II.1 and F4-II.1 corresponding to label-free quantification [LFQ] intensity proband [pat]_1, 2, 3 and LFQ intensity pat_4, 5, 6 in Table S4) and compared to three primary control fibroblast cell lines (LFQ intensity control [ctrl]_1, 2, 3). Protein extracts were purified with SDS-PAGE (Invitrogen). Coomassie-stained gel pieces were excised and in-gel digested via trypsin as described previously.16 For desalting with C18 stage tips,17 extracted peptides were separated on an Easy-nLC 1200 system coupled to a Q Exactive HF mass spectrometer (both Thermo Fisher Scientific) as described previously18 with slight modifications. The peptide mixtures were separated via a 2-h segmented gradient from 10%–33%–50%–90% HPLC solvent B (80% acetonitrile in 0.1% formic acid) in HPLC solvent A (0.1% formic acid) at a flow rate of 200 nL/min. The 12 most intense precursor ions were sequentially fragmented in each scan cycle via higher energy collisional dissociation (HCD) fragmentation. In all measurements, sequenced precursor masses were excluded from further selection for 30 s. The target values for MS/MS fragmentation were 103 charges and 3 × 106 charges for the MS scan.

Acquired MS spectra were processed with the MaxQuant software package v.1.5.2.819 with an integrated Andromeda search engine.20 A database search was performed against a target-decoy Homo sapiens database obtained from UniProt, containing 96,817 protein entries and 286 commonly observed contaminants. Endoprotease trypsin was defined as protease with a maximum of two missed cleavages. Oxidation of methionine and N-terminal acetylation were specified as variable modifications, whereas carbamidomethylation on cysteine was set as a fixed modification. The initial maximum allowed mass tolerance was set to 4.5 parts per million (ppm) for precursor ions and 20 ppm for fragment ions. Peptide, protein, and modification site identifications were reported at an FDR of 0.01, estimated by the target/decoy approach.21 The LFQ algorithm was enabled, and matches between runs22,23 and LFQ protein intensities were used for relative protein quantification. Downstream bioinformatics analyses (two-sample t tests and volcano plots) were performed with the Perseus software package v.1.5.0.1S. Data were filtered for contaminants and reverse sequences and were only identified by site entries.

**Over-representation analysis**

Ontology and pathway analyses for MS data as well as RNA-seq data were performed with the WEB-based gene set analysis toolkit.
containing ampicillin were plated and incubated overnight at 37°C. Individual colonies were picked and inoculated overnight in liquid culture followed by plasmid purification. Purified DNA was sent to Bestgene (BestGene, CA, United States) for embryo microinjection.

**Drosophila husbandry and strains**

Both control and *rudhira* knockdown crosses were maintained in Instant *Drosophila* Medium (Carolina). Crosses were set up at 25°C or 29°C and kept in incubators with a 12-h day-night cycle. The following lines were utilized in this study: two UAS-RNAi lines against *rudhira*, UAS-*rudhira*-RNAi-a (Bloomington *Drosophila* Stock Center [BDSC] #51691, *rudhira* RNAi inserted on 3rd chromosome) and UAS-*rudhira*-RNAi-b (Vienna *Drosophila* Resource Center [VDRC ID dna9673]); GAL4 driver lines elav-Gal4 (BDSC #8765, BDSC #5144), D42-Gal4 (BDSC #8816), and Appl-Gal4 (from Aaron Voigt, Department of Neurology in RWTH Aachen University); control lines for *rudhira*-RNAi-a (BDSC #36304, TRIP RNAi background lines with attP40 site on 3rd chromosome); and UAS-GFP-RNAi (BDSC #9331, GFP RNAi inserted on 2nd chromosome) as second RNAi control line. To generate the transgenic UAS-*rudhira*-RNAi-b *Drosophila* line, we diluted the construct received from VDRC (1 μL of an ~50–100 ng/μL stock) by adding 10 μL of TE buffer and mixed thoroughly. DHSe2-competent cells were transformed with 2 μL from the diluted mixture. Agar plates containing ampicillin were plated and incubated overnight at 37°C. Individual colonies were picked and inoculated overnight in liquid culture followed by plasmid purification. Purified DNA was sent to Bestgene (BestGene, CA, United States) for embryo microinjection.

**Results**

**Bi-allelic loss-of-function variants in BCAS3 cause a syndromic neurodevelopmental disorder**

To unravel the genetic cause of a neurodevelopmental disorder, we independently investigated affected probands from the consanguineous families F1, F2, and F3 (Figure 1) using WES. The molecular diagnostic analysis did not show pathogenic or likely pathogenic variants in genes known to be associated with neurological or developmental disorders in any of these families. WES data were next screened for potential candidate variants, including heterozygous, compound heterozygous, and homozygous variants. Independently, in all three families, predicted homozygous loss-of-function variants in BCAS3 microtubule-associated cell migration factor (*BCAS3*) were identified, namely, the homozygous stop-gain variant c.73C>T (p.Gln25*) (GenBank: NM_001099432.3) in BCAS3 in F1 and the homozygous variants c.726T>G (p.Tyr242*) and c.1457C>G (p.Ser486*) in F2 and F3, respectively. All three variants were absent from in-house databases as well as from public databases (not present in gnomAD v.2.1.1 or gnomAD v.3). Furthermore, there were no homozygous BCAS3 loss-of-function variants present in gnomAD, and a low observed/expected (o/e) ratio of 0.31 (0.22–0.47) hints at an intolerance for BCAS3 loss-of-function variants.
convulsions and five probands had generalized tonic or tonic-clonic seizures. Only one of these probands had pharma-co-resistant epilepsy.

All probands were born with length, weight, and head circumferences within normal ranges. Eleven of 13 probands developed microcephaly (median \(-3.1\) SD), and 11 of 14 probands developed a short stature (median \(-2.6\) SD). Two probands were normocephalic at the time of examination (F3-II.1, \(-0.58\) SD and F3-II.2, \(-1.23\) SD); however, they may still develop mild microcephaly, as they were 5 years and 19 months of age, respectively, at the time of examination. Minor dysmorphic facial features were observed in most probands, including full lips (9/12) with an everted lower lip (15/15); a short philtrum (8/11); large malformed, wide-spaced, or mispositioned teeth (10/14); prominent (10/15) and curved (11/15) eyebrows; an open mouth (15/15); and a long face (13/15) (Figure 2B, Table 1). Another frequent feature was strabismus, present in ten of 15 probands. The results of cardiovascular examinations, including transthoracic echocardiography, were mostly unremarkable. An asymptomatic bicuspid aortic valve was detected in two probands. Brain magnetic resonance images (MRIs) of 13 probands revealed a thin dysgenic corpus callosum, mostly

---

**Figure 1. Pedigrees and genetic variants**

(A) Pedigrees of eight families segregating syndromic GDD. Filled black symbols indicate affected individuals. Variants in BCAS3 are presented below the pedigrees. Homozygous variants are presented as \(m/m\) in the pedigree. Compound heterozygous variants are presented with \(m_1\) (cyan) and \(m_2\) (orange). For F9, the phase of the BCAS3 variants was unclear.

(B) BCAS3 genomic and protein domain structures. Type and position of ten identified germline variants. Two additional variants from F9 were depicted in gray because the phase was not shown as in the other variants. Conservation across species is shown for the positions of the two compound heterozygous missense variants.
## Table 1. Clinical and genetic findings in individuals with bi-allelic BCA13 variants

| F1 II.1 | F1 II.2 | F2 II.1 | F2 II.2 | F2 II.3 | F2 II.7 | F3 II.1 | F3 II.2 | F4 II.1 | F5 II.1 | F5 II.2 | F6 II.1 | F6 II.2 | F7 II.1 | F7 II.2 | F8 II.1 | F9 II.1 |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| cDNA (GenBank: NM_001099432.3) | c.736C>T, hom | c.736C>T, hom | c.726T>G, hom | c.726T>G, hom | c.1487C>G, hom | c.1487C>G, hom | c.[790C>T] [792C>A] | c.2227C>T, hom | c.2227C>T, hom | c.1133delT, hom | c.1133delT, hom | p.500delT, hom | p.500delT, hom | c.2074+4, 2074+6delGA, intron3-gene deletion exons 13-21 | c.[576C>A]; [578C>T] (phase uncertain) |
| Protein (GenBank: NP_001092902.1) | p.Gln25*, hom | p.Gln25*, hom | p.Tyr242*, hom | p.Tyr242*, hom | p.Ser466*, hom | p.Ser466*, hom | p.[Pro667Leu]; [Gly771Arg] | p.Gln743*, hom | p.Gln743*, hom | p.Val1378 Ala1385*, hom | p.Val1378 Ala1385*, hom | p.Met177 Arg183* | p.Met177 Arg183* | p.[?]; [?] | p.[Cys192*]; [Gln111P] |
| Sex | male | female | female | male | female | female | female | male | female | female | female | male | female | female | male | female |
| Ethnic background | Iranian | Arab-Palestinian | Arab-Palestinian | European descent | Albanian | European descent | Arab-Palestinian | European descent | Arab-Palestinian | European descent | Arab-Palestinian | European descent | Iranian-Lor | European descent | European-Chinese |
| Age (years) at examination | 19 | 13 | 19 | 18 | 5 | 5 | 19 months | 8 | 15 | 11 | 11 | 6 | 7 | 6 | 18 | 14 |
| Current height (cm) | 160 (± 2.3 SD) | 152 (± 0.7 SD) | 136 (± 4.2 SD) | 142 (± 4.4 SD) | 95 (± 2.8 SD) | 114 (± 1.28 SD) | 78 (± 0.92 SD) | 109 (± 3.3 SD) | 140 (± 3.4 SD) | 129 (± 2.1 SD) | 127 (± 2.4 SD) | 90 (± 5.0 SD) | 110 (± 2.2 SD) | 96 (± 3.8 SD) | N/A | 147.5 (± 1.37 SD) |
| Current weight (kg) | 44 (± 3.0 SD) | 34 (± 1.7 SD) | 42 (± 8.0 SD) | 33 (± 3.9 SD) | 25 (± 1.9 SD) | 10 (± 1.39 SD) | 10 (± 1.11 SD) | 35 (± 1.5 SD) | 46 (± 0.9 SD) | 23 (± 6.3 SD) | 20 (± 3.1 SD) | 14 (± 2.7 SD) | 17 (± 2.1 SD) | 13 (± 3.2 SD) | 40.4 (± 3.0 SD) | N/A |
| Current head circumference (cm) | 51 (± 2.8 SD) | 49.5 (± 3.2 SD) | 50.0 (± 4.0 SD) | 52.2 (± 1.9 SD) | 46.5 (± 2.8 SD) | 49.5 (± 0.58 SD) | 43 (± 1.21 SD) | 43.0 (± 6.7 SD) | N/A | N/A | 48 (± 3.8 SD) | 47 (± 3.1 SD) | 47 (± 3.8 SD) | 46 (± 3.9 SD) | 51 (age 15 years, 2.6 SD) | 53 (± 0.80 SD) |
| Motor development | sitting at 18 months, walking at 36 months | sitting at 17 months, walking at 36 months | sitting at 17 months, walking at 36 months | sitting at 17 months, walking at 72 months | sitting at 15 months, walking at 30 months | sitting at 15 months, commando crawling 15 months | no sitting or walking | sitting at 10 months, walking at 33 months | sitting at 9 months, walking at 26 months | cannot walk | cannot walk | sitting at 1 year, walking at 4 years | sitting at 1 year, cannot walk | sitting at 6 months, walking at 36 months |
| Cognition at last follow-up | severe ID | severe ID | severe ID | severe ID | severe ID | severe ID | severe ID | severe ID (K2 = 45) | severe ID (K2 = 55) | severe ID | severe ID | severe ID | severe ID | severe ID |
| Speech at last follow-up | can speak, about ten words | can speak, about ten words | no speech | no speech | can speak, about five words | can speak, about five words | can speak, about ten words | can speak, about three words | can speak, about six words | can speak, about ten words | can speak, about nine words | can speak, about twelve words | |
| Epilepsy, seizure types, age of onset | no | no | no | no | no | one febrile convolution, 36 months | febrile convulsions about every 6 months, 29 months | no | GTCs, age of 4 months, pharmacoresistant | two tonic seizures, 5 years | no | tonic seizures, 10 years, pharmacoresistant | tonic seizures, 2 years, pharmacoresistant | three febrile seizures, 6 months | no | complex partial seizures, 1–2 spells/day |
| Neurological examination | hyperreflexia, lower limb spasticity, spastic gait | hyperreflexia, lower limb spasticity, spastic gait | hyperreflexia, lower limb spasticity, spastic gait | hyperreflexia, lower limb spasticity | hyperreflexia, lower limb spasticity, spastic gait | hyperreflexia, lower limb spasticity, dystonia, gait imbalance | hyperreflexia, lower limb spasticity, dystonia, mild contractures in knees, non-ambulatory | hyperreflexia, lower limb spasticity, dystonia, mild contractures in knees, non-ambulatory | hyperreflexia, lower limb spasticity, dystonia, mild contractures in knees, non-ambulatory | hyperreflexia, lower limb spasticity, dystonia, mild contractures in knees, non-ambulatory | hyperreflexia, lower limb spasticity, dystonia, mild contractures in knees, non-ambulatory | hyperreflexia, progressive spasticity, spastic quadriplegic | hyperreflexia, progressive spasticity, spastic quadriplegic | (Continued on next page)
### Table 1. Continued

| Dysmorphic facial features | F1-II.1 | F1-II.2 | F2-II.2 | F2-II.3 | F3-II.1 | F3-II.2 | F4-II.1 | F5-II.1 | F5-II.2 | F6-II.1 | F6-II.2 | F7-II.1 | F7-II.2 | F8-II.2 | F9-II.1 |
|----------------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, wide-spaced teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, large malpositioned teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, wide-spaced eyes, broad nasal bridge, full lips, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, large malpositioned teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, large malpositioned teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, large malpositioned teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, large malpositioned teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, large malpositioned teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, large malpositioned teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, large malpositioned teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, large malpositioned teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, large malpositioned teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, large malpositioned teeth, open mouth, long face** | **prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, long face** |

**Dysmorphic facial features**: prominent, full and curved eyebrows, synophrys, full lips, everted lower lip, short philtrum, wide-spaced teeth, open mouth, long face

**Instrumental examinations**

| Ophthalmologic findings | strabismus | strabismus | strabismus | strabismus | strabismus | strabismus | strabismus | normal | normal | normal | normal | normal | normal | normal |
|--------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-------|-------|-------|-------|-------|-------|-------|
| Cardio-vascular (e.g., echo results) | N/A | N/A | TTE: bicuspid aortic valve | TTE: bicuspid aortic valve | normal TTE | normal TTE | N/A | normal TTE | normal TTE | normal TTE | normal TTE | normal TTE | normal TTE | normal TTE |
| Age, brain imaging | 9 years, posterior thin corpus callosum, impaired myelination | 5 years, posterior thin corpus callosum, impaired myelination | 22 years, posterior thin corpus callosum, cerebral and cerebellar atrophy, normal MRA | 21 years, posterior thin corpus callosum, cerebellar and cerebellar atrophy, normal MRA | 7 years, posterior thin corpus callosum, hyperintensity of dentate nuclei on T2 | 14 months, posterior thin corpus callosum, delayed myelination, hypoplastic pons and cerebellum | 5 months, posterior thin corpus callosum, delayed myelination, impaired myelination | 9 years, posterior thin corpus callosum, delayed myelination, impaired myelination, VGAM | 4.5 years, posterior thin corpus callosum, cerebellar and cerebellar atrophy, delayed myelination, VGAM | 7 years, posterior thin corpus callosum, cerebellar and cerebellar atrophy, delayed myelination, VGAM | 4 years, posterior thin corpus callosum, cerebellar and cerebellar atrophy, delayed myelination | 6 years, posterior thin corpus callosum, cerebellar and cerebellar atrophy, delayed myelination | 7 years, posterior thin corpus callosum, cerebellar and cerebellar atrophy, delayed myelination | N/A |

**Abbreviations** are as follows: GTCS, generalized tonic-clonic seizure; TTE, transthoracic echocardiogram; MRA, magnetic resonance angiography; VGAM, vein of Galen aneurysmal malformation; ID, intellectual disability; GDD, global developmental delay.
Figure 2. Phenotypic features of probands with bi-allelic BCAS3 variants
(A) Prevalence of signs and symptoms in probands with bi-allelic BCAS3 variants. The numerator and denominator in brackets indicate the number of affected probands and the number of probands assessed for the respective feature, respectively.
(B) Facial photographs of 14 affected individuals with mild dysmorphic facial features, including full lips with an everted lower lip; short philtrum; mispositioned, wide-spaced large teeth; and synophrys. Furthermore, ptosis is present in F1-II.1, F2-II.2, and F2-II.3, and strabismus can be seen in several photographs except for in those of F4-II.1, F5-II.1, F7-II.1, F7-II.2, and F8-II.2.

(legend continued on next page)
affecting the splenium in all 13 probands. Furthermore, moderate cerebral and cerebellar atrophy (more pronounced in the older probands) and delayed myelination (observed in the younger probands) were present in most probands (Figure 2C, Table 1, Table S2). A vascular malformation, most likely a vein of Galen malformation, was identified in one proband (F5-II.2, Figure 2C). Otherwise, time-of-flight magnetic resonance angiography (TOF-MRA) in three probands did not reveal an abnormal cerebral blood vessel configuration.

In another additional proband, F9-II.1, two BCAS3 stop-gain variants (one paternal and the second de novo) were identified. Although the phase of the variants was not determined, we regarded a bi-allelic distribution most likely given the striking phenotypic similarities encompassing the characteristic core clinical features (Table 1).

**BCAS3 is absent in proband-derived fibroblasts**

To compare the protein levels of BCAS3, we performed immunoblotting analysis from fibroblasts of probands F3-II.1 and F4-II.1 and control cell lines. BCAS3 was undetectable in both proband-derived cell lines, while it was clearly detected in the control fibroblast lines (Figure 3A). Although we did not observe any full-size BCAS3, we cannot rule out the presence of a truncated protein in F3-II.1, as the homozygous stop-gain variant p.Ser486* lies upstream of the antibody detection site (position 870 to C terminus). More interestingly, BCAS3 was also not detectable in fibroblasts from F4-II.1, harboring two compound heterozygous missense variants (c.1700C>T [p.Pro567Leu] and c.1729G>A [p.Gly577Arg]). This indicates that the missense variants lead to a misfolded or unstable protein that is most likely degraded.

**Exploring dysregulated pathways in proband-derived fibroblasts**

Next, we examined two previously proposed interaction partners of BCAS3, namely, CDC42 and Vimentin, by using immunoblotting. Both were present at roughly estimated similar protein levels in proband fibroblasts and control fibroblast cell lines (Figure 3A). To further investigate the global impact of the bi-allelic BCAS3 variants on different pathways, we performed RNA-seq and nanoLC-MS/MS comparing the two proband cell lines with healthy control cell lines. The transcriptomic data showed a significant reduction in BCAS3 mRNA in F3-II.1 of -2.92 log fold change (logFC), indicating partial nonsense-mediated decay due to the homozygous nonsense variant, while the mRNA levels were not significantly reduced in F4-II.1 (-0.21 logFC).

With nanoLC-MS/MS, BCAS3 was not detectable in either proband cell line, consistent with the results from immunoblotting. However, BCAS3 was probably at the detection limit of this method, as nanoLC-MS/MS was able to detect BCAS3 in only two out of three controls, rendering immunoblotting the more reliable method for addressing this question. Next, we examined the correlation between transcriptomics and proteomics data. RNA-seq was able to detect mRNAs from 13,223 different genes. For 5,483 proteins, corresponding proteomics data from nanoLC-MS/MS were available. MS did not detect any proteins for which no RNA data were present. Expression changes with significant up-or downregulated genes at the mRNA level (FDR < 0.01) correlated very well with significant up- or downregulated proteins in MS (r = 0.89, p = 2.2 × 10^-16) (Figure S2A). The high correlation coefficient indicates high data quality and reliability. Similarly, proteins that could only be measured in controls (but not in probands) via MS were significantly downregulated in the RNA-seq data, while proteins that were only detectable in probands (but not in controls) via MS were upregulated in the RNA-seq data (Figure S2B). Following this proof of consistency, we analyzed expression of previously proposed key interaction partners of BCAS3, such as ACTB, CDC42, PTK2, PTK2B, TGFBR, VEGFA, VEGFR, and VIM. Substantial mRNA expression changes (>0.5 logFC) were only present in PTK2B (~1 logFC) and vimentin (0.52 logFC) (Table S5). Corresponding MS data were only present for a few of these proteins and did not show any substantial changes at the protein level. Specifically, for PTK2B, there were no MS data available, and vimentin was not significantly changed.

We next used WebGestalt for ORA of RNA-seq and MS data. From the RNA-seq data, 804 genes were significantly up- or downregulated (FDR < 0.01 and absolute |log FC| > 1.00, Table S3). The resulting ORA showed the following biological processes (Gene Ontology terms) as top hits (Figure 3B): “extracellular structure organization,” “angiogenesis,” and “epithelial cell proliferation.” In the nanoLC-MS/MS data, 59 proteins were significantly up- or downregulated (log Student’s t test p value ≤ -1.3 and absolute |log FC| > 1.00, Table S4). ORA resulted in “extracellular structure organization” as a top hit followed by “angiogenesis” and “actomyosin structure organization” with a considerable margin (Figure 3C).

**Migration of proband-derived fibroblasts is not affected**

Because the promotion of cell migration is supposed to be one primary function of BCAS3 and was impaired in the knockout mouse model,2 we investigated the migration of proband-derived fibroblasts. A cell migration assay using the two proband cell lines (F3-II.1 and F4-II.1) and four control fibroblast lines showed no impairment of migration in proband fibroblasts compared with control fibroblast lines (Figure 3D).

(C) Exemplary MRI of probands from families F2, F3, F5, and F9. Midsagittal T2/T1 showing a thin dysgenic corpus callosum (white arrow), especially of the posterior part (splenium). This is present in all available MRIs. Coronal TOF-MRA displaying normally developed large arterial blood vessels of the brain. A vascular malformation, most likely a vein of Galen malformation, was identified in one proband (F3-II.2) and is marked with a white asterisk on the T2 images.
rudhira silencing induces tissue-specific developmental defects in a Drosophila loss-of-function model

In mice, loss of Bcas3 causes embryos to die on embryonic day (E) 9.5.2 We used a Drosophila loss-of-function model to further investigate the role of rudhira, which shares 32% identity with human BCAS3, in tissue-specific development. Using the temperature-dependent UAS-Gal4 expression system,28,29 we expressed two different RNAi constructs against rudhira (BDSC #51691 and VDRC ID dna9673, hereafter referred to as rudhira-RNAi-a and rudhira-RNAi-b, respectively) under different neuronal Gal4 drivers (Figure 4A). Expression of rudhira-RNAi-a using pan-neuronal elav-Gal4 caused lethality at the embryonic stage at 25°C and 29°C, and expression of rudhira-RNAi-b at 25°C caused lethality at the larval/pupal stage. Using a second pan-neuronal driver, Appl-Gal4,30 it was possible to obtain adult flies at 25°C with both rudhira-RNAi lines, while at 29°C, expression of rudhira-RNAi-a led to paralyzed flies that died on the first day after eclosion. The difference between the two pan-neuronal drivers can possibly be explained by the onset and strength of driver expression. High-throughput RNA-seq expression patterns indicate that Appl is active at a later stage of development compared to Elav.31 Knockdown of rudhira in motor neurons (D42-Gal4) at 25°C caused lethality at the larval stage (rudhira-RNAi-a line) or at the larval/pupal stage (rudhira-RNAi-b line), while lethality was shifted to the embryonic stage with the rudhira-RNAi-a line at 29°C. The
shift in lethality at higher temperatures to an earlier time point is most likely explained by the temperature-dependent efficiency of transgene expression of the UAS-Gal4 system.

Gene dosage compensation in Drosophila adjusts the expression of X chromosome genes by doubling expression in males. Hence, the X chromosome neuronal driver Appl-Gal4 should be expressed at higher levels in hemizygous Appl-Gal4/Y males than in their heterozygous Appl-Gal4/+ female counterparts. By setting up crosses between Appl-Gal4 homozygous female virgins with UAS-rudhira-RNAi-a or UAS-rudhira-RNAi-b males, we hypothesized that there would be a higher female-to-male ratio in the knockdown group than in the control group. Indeed, at 25°C, male Appl-rudhira-RNAi-a (Appl-Gal4/Y;+;UAS-rudhira-RNAi-a/+;UAS-rudhira-RNAi-a/a) or male Appl-rudhira-RNAi-b flies were rarely observed, unlike in an Appl-control cross where males and females occurred at Mendelian ratios (Figure 4B). From a total of 402 offspring collected from the Appl-control cross (Appl-Gal4 homozygous female virgin flies crossed with TRiP background males), there were 156 females (51%) and 151 males (49%) in the F1 generation. In contrast, from a total of 143 offspring collected by crossing Appl-Gal4 homozygous female virgin flies with UAS-rudhira-RNAi-a males, 142 were females (99.3%) and 1 (0.7%) was male. Similarly, out of a total of 221 progeny with the rudhira-RNAi-b line, 204 Appl-rudhira-RNAi-b females (92.3%) and only 17 Appl-rudhira-RNAi-b males (7.7%) were observed. Detailed phenotyping of adult Appl-rudhira-RNAi-a flies obtained at 25°C revealed reduced longevity, severe locomotion defects and a wing phenotype (Figures S3D–S3G), whereas consistent with the late onset of Appl expression, no significant defects in larval locomotion or neuromuscular junction morphology were identified in Appl-rudhira-RNAi-a larvae (Figure S4). However, these findings need to be replicated in a second UAS-rudhira-RNAi line. Additional neuronal and nonneuronal Gal4 drivers caused lethality or tissue-specific phenotypes in the rudhira-RNAi-a line but were not tested in a second rudhira-RNAi line (Figure S3).

Taken together, experiments from the rudhira-RNAi-a line suggest that RNAi-mediated knockdown of rudhira causes developmental defects in neuronal and nonneuronal tissues in a dose-dependent manner. The lethality of pan-neuronal and motor-neuronal knockdown was confirmed with the rudhira-RNAi-b line.

Discussion

In this study, we provide evidence that bi-allelic variants of BCAS3 cause a syndromic neurodevelopmental disorder. We have described 15 probands from eight unrelated families with ten different bi-allelic germline variants of BCAS3. The associated syndromic phenotype comprises GDD with severe ID, microcephaly, a short stature, a movement disorder with pyramidal tract involvement, strabismus, and seizures in many cases. Furthermore, similar mild facial dysmorphic features including a long face, tooth abnormalities, full lips with an everted lower lip, and a short philtrum, were present in almost all examined probands. Neuroradiologically, in addition to different
degrees of myelination deficits in younger probands and mild to moderate global atrophy in older probands, all probands showed a dysgenic corpus callosum with isolated or pronounced involvement of the splenium. These syndromic features should help to identify and diagnose more probands with pathogenic or likely pathogenic bi-allelic BCAS3 variants.

Of note, in addition to the homozygous BCAS3 variant, family 7 was found to have a homozygous variant in HELZ (MIM: 606699, c.3322A>G [p.Ile1108Val] [GenBank: NM_014877]), which was published as a candidate DD/ID-associated gene as part of a large DD/ID cohort (identifier BAB4698). Mutation prediction tools, including PolyPhen, SIFT, and LRT, predicted the HELZ variant as benign, neutral, and tolerated, respectively. Additionally, the CADD score of the HELZ variant was 15.45. Given the obvious phenotypic similarity between affected individuals of family 7 and other subjects with BCAS3 variants in this manuscript and the low prediction scores of likelihood for damaging effects of the given HELZ variant (in contrast to the BCAS3 variant, which results in a frameshift at codon 177), we consider the identified BCAS3 variant as the major driver of the probands’ phenotype.

Based on the bi-allelic nonsense variants identified in most probands, we suspected the disease mechanism to be loss of function. By showing that BCAS3 was absent in both immunoblot and nanoLC-MS/MS analyses in fibroblasts from proband F4-II.1 carrying bi-allelic missense variants, we strengthened this hypothesis and confirmed the missense alleles as likely damaging and probably disease causing. Data from the knockout mouse model and similarities of the phenotypes between CDC42 (MIM: 116952)- and BCAS3-associated disease suggested a close regulatory interplay between BCAS3 and CDC42. Heterozygous missense variants in CDC42 have recently been associated with a heterogeneous developmental disorder that includes variable growth dysregulation, facial dysmorphism, and neurodevelopmental, immunological, and hematological abnormalities (MIM: 616737, Takenouchi-Ko-saki syndrome). Interestingly, RNA-seq and nanoLC-MS/MS data based on proband’s fibroblasts showed expression levels of CDC42 as well as of several other previously proposed interaction partners mostly unchanged. Nevertheless, the broad categories of pathways resulting from transcriptomics and proteomics ORA were similar to previous transcriptomics analyses from the Bcas3 knockout mouse model and “extracellular matrix organization” and “angiogenesis” were the most enriched and most significant hits. One major limitation of this analysis is the limited comparability of proband’s fibroblasts to the previously analyzed mouse yolk sac. Notably, fibroblasts and the presumably most disease-relevant cell line from the mouse model, endothelial cells, are derived from primitive mesenchyme.

The pathophysiological consequences of loss-of-function mutations in BCAS3 seem to be potentially different in mice and humans. Compared to phenotypes observed in the knockout mouse, diseases in humans resulting from a loss of BCAS3 were less severe. While knockout leads to disorganized vessels in the brain, yolk sac, cardiovascular malformation, and embryonic death in knockout mice, the probands reported herein were born at term with normal weight and without major maldevelopment. There is no clear evidence of cardiovascular malformations in humans. Only asymptomatic bicuspid aortic valves were detected by echocardiography in two probands. Interestingly, in one of the 13 examined MRIs, a vein of Galen malformation was identified. TOF-MRA images of three other probands revealed normally developed large blood vessels in the brain. In summary, cardiovascular or vascular malformations in the brain do not appear to be a relevant part of the human phenotype.

Similarly, cell migration does not seem to be equally affected in humans as in mice. Endothelial cell migration of Bcas3 knockout cells was strongly impaired and has been proposed to be one key component that contributes to impaired angiogenesis. Therefore, we would have expected probands’ fibroblast cell lines to show reduced cell migration in the migration assays. However, migration was not impaired in proband cells compared to in control cell lines, with the obvious limitation that human endothelial cell lines might behave differently. In summary, the phenotype in humans resulting from loss of BCAS3 is less severe than that in mice, and angiogenesis does not seem to be a major component of human pathogenesis.

The Drosophila loss-of-function model showed lethality in two different RNAi lines using pan-neuronal and motor-neuronal drivers. The adult flies that hatched under the X chromosome Appl driver were mostly female, indicating a dose dependency. Similarly, shifted time points of lethality under a higher temperature hints to a dose-dependent effect, although this was only explored in one RNAi line. Additional results showing specific phenotypes for different neuronal and nonneuronal drivers such as locomotion defects and abnormal wing postures for the Appl driver or the rough eye phenotype (using the eye-specific GMR driver) were based on the rudhira-RNAi-a line but must be interpreted with some caution as no investigation of a second RNAi line has been performed to confirm these findings.

Our data suggest an essential role for BCAS3/Rudhira in the development of neuronal cell populations that appear to be independent of angiogenesis and cell migration. We encourage follow-up studies based on neuronal cell models to explore the developmental biology and the human pathomechanisms of this Mendelian disorder.

Data and code availability

The data that support the findings of this study are available from the corresponding authors upon request. Human variants and phenotypes have been deposited in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar) under the submission name ClinVar: BCAS3_0001. The respective ClinVar accession numbers can be found in Table S1.
Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.ajhg.2021.04.024.

Consortia

The members of the Care4Rare Canada Consortium are Kym M. Boycott, Michael Brudno, Francois P. Bernier, Clara D. van Karnebeek, David A. Dyment, Kristin D. Kernohan, Michi A. Innes, Ryan E. Lamont, Jillian S. Parboosingh, Deborah A. Marshall, Christian R. Marshall, Roberto Mendoza-Londono, James J. Dowling, Robin Z. Hayeems, Bartha M. Knoppers, Anna M. Lehan, and Sara A. Mostafavi. The members of the Genomics England Research Consortium are John C. Ambrose, Prabhu Arumugam, Marta Bleda, Freya Boardman-Pretty, Christopher R. Bousted, Helen Brittain, Mark J. Caulfield, Georgia C. Chan, Greg Elgar, Tom Fowler, Adam Giess, Angela Hamblin, Shirley Henderson, Tim J.P. Hubbard, Rob Jackson, Louise J. Jones, Dalia Kasperaviciute, Melis Kayikci, Athanasios Kousathanas, Lea Lahnstein, Sarah E.A. Leigh, Ivonne U.S. Leong, Javier F. Lopez, Fiona Mal-eady-Crowe, Loukas Moutsianas, Michael Mueller, Nirupa Murugasu, Anna C. Need, Peter O’Donovan, Chris A. Odhams, Christine Patch, Mariana Buongermino Pereira, Daniel Perez-Gil, John Pullinger, Tahrima Rahim, Augustine Rendon, Tim Rogers, Kevin Savage, Kushmita Sawant, Richard H. Scott, Afshan Siddiq, Alexandre Sieghart, Samuel C. Smith, Alona Sosinsky, Alexander Stuckey, Melanie Tanguy, Ellen R.A. Thomas, Simon R. Thompson, Arianna Tucci, Matthew J. Welland, Eleanor Williams, Katarzyna Witkowska, Suzanne M. Wood, and Magdalena Zarowiecki.

Acknowledgments

We are grateful to the affected individuals and their families whose cooperation made this study possible. The study has been supported by the Deutsche Forschungsgemeinschaft (DFG, grant SCH0754/2-1 to L.S.) and funding of the NGS Competence Center Tübingen (INST 37/1049-1), the Solve-ND project (grant 779257 to H. Hengel, A.J., and L.S.), the US National Human Genome Research Institute (NHGRI) and National Heart Lung and Blood Institute (NHLBI) to the Baylor-Hopkins Center for Mendelian Genomics (BHCMG, U1M HG006542, J.R.L.), the US National Institute of Neurological Disorders and Stroke (NINDS) (R35NS105078 to J.R.L.), and the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre Programme and made possible through access to the data and findings generated by the 100,000 Genomes Project (managed by Genomics England Limited and funded by the NIHR and NHS England), by the Care4Rare Canada Consortium funded by Genome Canada and the Ontario Genomics Institute (OGI-147), the Canadian Institutes of Health Research, Ontario Research Fund, Genome Alberta, Genome British Columbia, Genome Quebec, and Children’s Hospital of Eastern Ontario Foundation, and by the NIH R01 (2R01NS058721-07A1). H. Hengel was supported by the fortune program of the University of Tübingen (grant #2554-0-0). H. Hengel and L.S. are members of the European Reference Network for Rare Neurological Diseases (project ID 739510). D.P. was supported by a Clinical Research Training Scholarship in Neuromuscular Disease partnered by the American Brain Foundation and Muscle Study Group and International Rett Syndrome Foundation (IRSt grant #3701-1). T.B.H. was supported by the DFG (project number 418081722). The families were collected as part of the SYNaPS Study Group collaboration funded by the Wellcome Trust and strategic award (Synaptopathies) funding (WT093205 MA and WT104033AIA).

Declaration of interests

J.R.L. has stock ownership in 23andMe, is a paid consultant for Regeneron Genetics Center, and is a co-inventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases, and bacterial genomic fingerprinting. The Department of Molecular and Human Genetics at Baylor College of Medicine receives revenue from clinical genetic testing conducted at Baylor Genetics (BG) Laboratories; J.R.L. is a member of the Scientific Advisory Board of BG Laboratories. A.B. is an employee of GeneDx. All other authors declare no competing interests.

Received: December 1, 2020
Accepted: April 29, 2021
Published: May 21, 2021

Web resources

ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
GenBank, https://www.ncbi.nlm.nih.gov/genbank/
gnomAD server, https://gnomad.broadinstitute.org
OMIM, https://omim.org/
WebGestalt, http://www.webgestalt.org/

References

1. Siva, K., Venu, P., Mahadevan, A., S.K., S., and Inamdar, M.S. (2007). Human BCAS3 expression in embryonic stem cells and vascular precursors suggests a role in human embryogenesis and tumor angiogenesis. PLoS ONE 2, e1202.
2. Shetty, R., Joshi, D., Jain, M., Vasudevan, M., Paul, J.C., Bhat, G., Banerjee, P., Abe, T., Kiyonari, H., VijayRaghavan, K., and Inamdar, M.S. (2018). Rudhira/BCAS3 is essential for mouse development and cardiovascular patterning. Sci. Rep. 8, 5632.
3. Bärlund, M., Monni, O., Kononen, J., Cornelison, R., Torhorst, J., Sauter, G., Kallioniemi, A.; and Kallioniemi OLLI-P (2000). Multiple genes at 17q23 undergo amplification and overexpression in breast cancer. Cancer Res. 60, 5340–5344.
4. Nikpay, M., Goel, A., Won, H.H., Hall, L.M., Willenborg, C., Kanoni, S., Saleheen, D., Kyriakou, T., Nelson, C.P., Hopewell, J.C., et al. (2015). A comprehensive, 1,000 Genomes-based genome-wide association meta-analysis of coronary artery disease. Nat. Genet. 47, 1121–1130.
5. Li, C., Li, Z., Liu, S., Wang, C., Han, L., Cui, L., Zhou, J., Zou, H., Liu, Z., Chen, J., et al. (2015). Genome-wide association analysis identifies three new risk loci for gout arthritis in Han Chinese. Nat. Commun. 6, 7041.
6. Hu, H., Kahrizi, K., Musante, L., Fattahi, Z., Herwig, R., Hosseini, M., Oppitz, C., Abedini, S.S., Suckow, V., Larti, F., et al. (2019). Genetics of intellectual disability in consanguineous families. Mol. Psychiatry 24, 1027–1039.
7. Makrythanasis, P., Maroofian, R., Stray-Pedersen, A., Musaev, D., Zaki, M.S., Mahmoud, I.G., Selim, L., Elbadawy, A., Jhangiani, S.N., Coban Akdemir, Z.H., et al. (2018). Biallelic variants in KLF14 cause intellectual disability with microcephaly. Eur. J. Hum. Genet. 26, 330–339.
8. Hengel, B., Buchert, R., Sturm, M., Haack, T.B., Schelling, Y., Mahajnah, M., Sharkia, R., Azem, A., Balousha, G., Ghanem, Z., et al. (2020). First-line exome sequencing in Palestinian and Israeli Arabs with neurological disorders is efficient and facilitates disease gene discovery. Eur. J. Hum. Genet. 28, 1034–1043.

9. Hawer, H., Mendelsohn, B.A., Mayer, K., Kung, A., Malhotra, A., Tupanan, S., Schleit, J., Brinkmann, U., and Schaffrath, R. (2020). Diphthamide-deficiency syndrome: a novel human developmental disorder and ribosomopathy. Eur. J. Hum. Genet. 28, 1497–1508.

10. Kremer, L.S., Bader, D.M., Mertes, C., Kopajtich, R., Pichler, G., Iuso, A., Haack, T.B., Graf, E., Schwarzmayer, T., Terrile, C., et al. (2017). Genetic diagnosis of Mendelian disorders via RNA sequencing. Nat. Commun. 8, 15824.

11. Monies, D., Abouelhoda, M., Al-Sayed, M., Alhassnan, Z., Alotaibi, M., Kayahi, H., Al-Owain, M., Shah, A., Rahbeeni, Z., Al-Muhaizea, M.A., et al. (2017). The landscape of genetic diseases in Saudi Arabia based on the first 1000 diagnostic panels and exomes. Hum. Genet. 136, 921–939.

12. Karaca, E., Harel, T., Pehlivan, D., Jhangiani, S.N., Gambin, T., Coban Akdemir, Z., Gonzaga-Jauregui, C., Erdin, S., Bayram, Y., Campbell, I.M., et al. (2015). Genes that Affect Brain Structure and Function Identified by Rare Variant Analyses of Mendelian Neurologic Disease. Neuron 88, 499–513.

13. Turnbull, C., Scott, R.H., Thomas, E., Jones, L., Murugaesu, N., Pretty, F.B., Halai, D., Baple, E., Craig, C., Hamblin, A., et al.; 100 000 Genomes Project (2018). The 100 000 Genomes Project: bringing whole genome sequencing to the NHS. BMJ 361, k1687.

14. Chen, X., Schulz-Trieglaff, O., Shaw, R., Barnes, B., Schlesinger, F., Källberg, T., Cox, A.J., Kruglyak, S., and Saunders, C.T. (2016). Manta: rapid detection of structural variants and indels for germline and cancer sequencing applications. Bioinformatics 32, 1220–1222.

15. Guillen Sacoto, M.J., Tchasovnikarova, I.A., Torti, E., Forster, C., Andrew, E.H., Anselm, I., Baranano, K.W., Briere, L.C., Cohen, J.S., Craig, W.J., et al.; Undiagnosed Diseases Network (2020). De Novo Variants in the ATPase Module of MORC2 Cause a Neurodevelopmental Disorder with Growth Retardation and Variable Craniofacial Dysmorphism. Am. J. Hum. Genet. 107, 352–363.

16. Borchert, N., Dieterich, C., Krug, K., Schütt, W., Jung, S., Nordheim, A., Sommer, R.J., and Macek, B. (2010). Proteogenomics of Pristionchus pacificus reveals distinct proteome structure of nematode models. Genome Res. 20, 837–846.

17. Rappsilber, J., Mann, M., and Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat. Protoc. 2, 1896–1906.

18. Kliza, K., Taumer, C., Pinzuti, I., Franz-Wachtel, M., Kunzelmenn, S., Stiegitz, B., Macek, B., and Husnjak, K. (2017). Internally tagged ubiquitin: a tool to identify linear polyubiquitin-modified proteins by mass spectrometry. Nat. Methods 14, 504–512.

19. Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372.

20. Cox, J., Neuhausen, N., Michalski, A., Scheltema, R.A., Olsen, J.V., and Mann, M. (2011). Andromeda: a peptide search engine integrated into the MaxQuant environment. J. Proteome Res. 10, 1794–1805.

21. Elias, J.E., and Gygi, S.P. (2007). Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat. Methods 4, 207–214.

22. Schwanhäusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., and Selbach, M. (2011). Global quantification of mammalian gene expression control. Nature 473, 337–342.

23. Luber, C.A., Cox, J., Lauterbach, H., Fancke, B., Selbach, M., Tschopp, J., Akira, S., Wiegmans, M., Hochrein, H., O’Keeffe, M., and Mann, M. (2010). Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. Immunity 32, 279–289.

24. Liao, Y., Wang, J., Jachning, E.J., Shi, Z., and Zhang, B. (2019). WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs. Nucleic Acids Res. 47 (W1), W199–W205.

25. Karczewski, K.J., Franchioli, L.C., Tiao, G., Cummings, B.B., Alföldi, J., Wang, Q., Collins, R.L., Larichchia, K.M., Ganna, A., Birnbaum, D.P., et al.; Genome Aggregation Database Consortium (2020). The mutational constraint spectrum quantified from variation in 141,456 humans. Nature 581, 434–443.

26. Sobreira, N., Schiettecatte, F., Valle, D., and Hamosh, A. (2015). GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. Hum. Mutat. 36, 928–930.

27. Song, X., Beck, C.R., Du, R., Campbell, I.M., Coban-Akdemir, Z., Gu, S., Breman, A.M., Stankiewicz, P., Ira, G., Shaw, C.A., and Lupski, J.R. (2018). Predicting human genes susceptible to genomic instability associated with Alu/Alu-mediated rearrangements. Genome Res. 28, 1228–1242.

28. Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

29. Dufy, J.B. (2002). GAL4 system in Drosophila: a fly geneticist’s Swiss army knife. Genesis 34, 1–15.

30. Torroja, L., Chu, H., Kotovska, I., and White, K. (1999). Neuronal overexpression of APPL1, the Drosophila homologue of the amyloid precursor protein (APP), disrupts axonal transport. Curr. Biol. 9, 489–492.

31. Gelbart, W.M., and Emmert, D.B. (2013). FlyBase High Throughput Expression Pattern Data. (FlyBase).

32. Breen, T.R., and Lucchesi, J.C. (1986). Analysis of the dosage compensation of a specific transcript in Drosophila melanogaster. Genetics 112, 483–491.

33. Cline, T.W., and Meyer, B.J. (1996). Vive la différence: males vs females in flies vs worms. Annu. Rev. Genet. 30, 637–702.

34. Martinelli, S., Krumbach, O.H.F., Pantaleoni, F., Coppola, S., Amin, E., Pannone, L., Nouri, K., Farina, L., Dvorsky, R., Lepri, E., et al.; University of Washington Center for Mendelian Genomics (2018). Functional Dysregulation of CDC42 Causes Diverse Developmental Phenotypes. Am. J. Hum. Genet. 102, 309–320.