The American Journal of Human Genetics, Volume *107*

Supplemental Data

De Novo and Inherited Variants in *GBF1* are Associated with Axonal Neuropathy Caused by Golgi Fragmentation

Natalia Mendoza-Ferreira, Mert Karakaya, Nur Cengiz, Danique Beijer, Karlla W. Brigatti, Claudia Gonzaga-Jauregui, Nico Fuhrmann, Irmgard Hölker, Maximilian P. Thelen, Sebastian Zetzsche, Roman Rombo, Erik G. Puffenberger, Peter De Jonghe, Tine Deconinck, Stephan Zuchner, Kevin A. Strauss, Vincent Carson, Bertold Schrank, Gilbert Wunderlich, Jonathan Baets, and Brunhilde Wirth

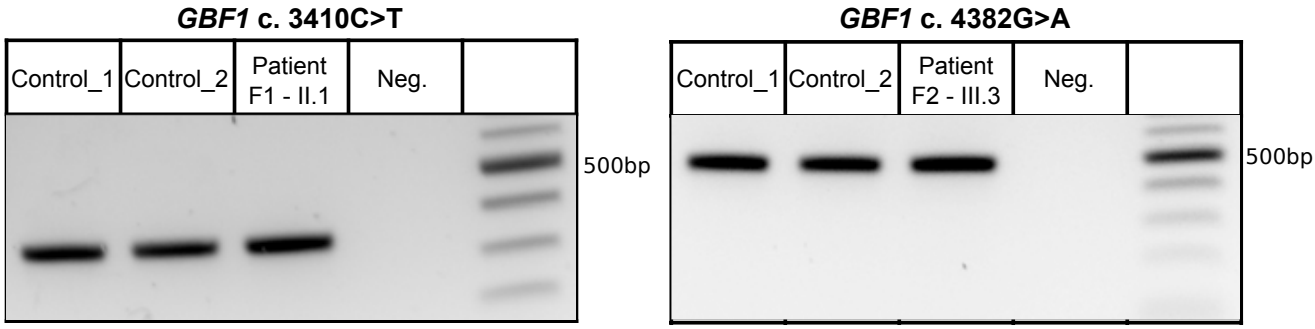

Figure S1. *GBF1* variants c.3410C>T and c.4382G>A do not affect splicing.

Determination of *GBF1* c.3410C>T and *GBF1* c.4382G>A transcripts by semi-quantitative RT-PCR. RNA was isolated from cultured fibroblast cell lines from *GBF1* index probands and control individuals. No additional PCR amplification products, indicating potential miss-splicing events, were observed.

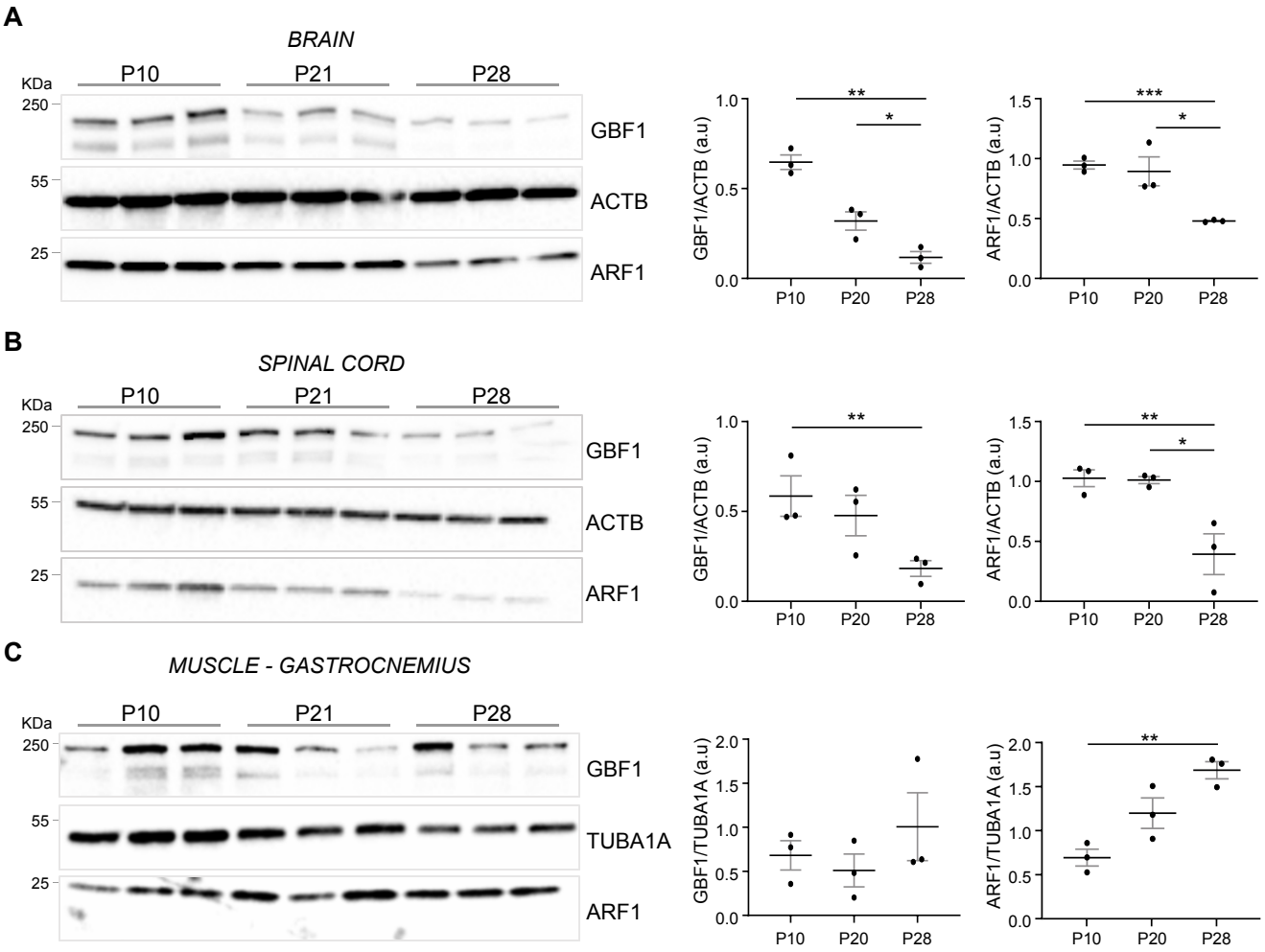

Figure S2. GBF1 levels in mouse brain, spinal cord and muscle.

Western blot analysis of brain **(A)**, spinal cord **(B)** and gastrocnemius muscle **(C)** from P10, P21, and P28 wild type mice. GBF1 and ARF1 levels were quantified by the ratio to the housekeepers ACTB or TUBA1A (for muscle). Blots quantifications of relative protein abundance are given in the graphs on the right. Bars show the mean ± SD from three independent samples. "*" denotes statistical significance (* $p \leq 0.05$, ** $p = 0.01$, and *** $p = 0.001$ unpaired two-tailed Student t test) between the different time points compared to P10. P= post-natal.

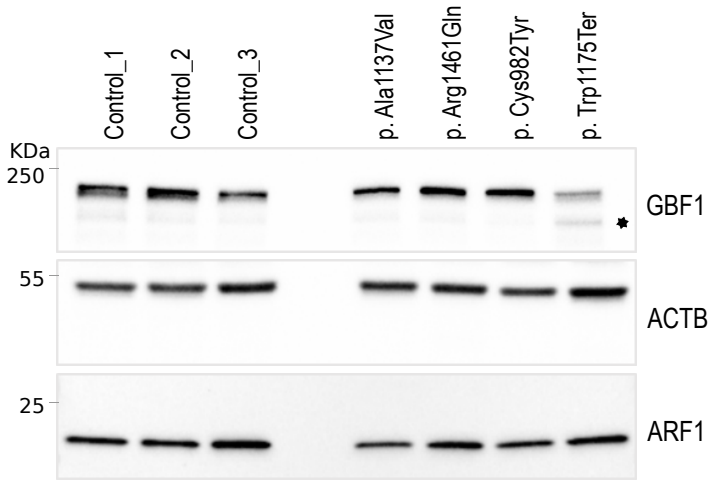

Figure S3. GBF1 levels in probands-derived fibroblasts.

Western blot analysis of total protein lysates isolated from fibroblast cell lines of three controls and the four probands. Blots were stained with antibodies against GBF1, ARF1, and the housekeeping protein ACTB to assure equal loading. * = potentially truncated version of GBF1

Application	Antibody / Dye	Dilution	Manufacturer	Catalogue number
Western blot	mouse α-GBF1 (25)	1:1000 o/n 4°C	Santa Cruz Biotechnology	sc-136240
	mouse α-ARF1 (ARFS 1A9/5)	1:500 o/n 4°C	Santa Cruz Biotechnology	sc-53168
	mouse α-Golgin 160 (C-8)	1:500 o/n 4°C	Santa Cruz Biotechnology	sc-374596
	mouse α-β-actin HRP conjugated	1:5000 1h / RT	Proteintech	HRP-60008
	mouse α-TUBA1A	1:1000 1h / RT	Sigma Aldrich	T9026
Cells / MNs Immunostaining	rabbit α-GBF1	1:100 o/n 4°C	Abcam	ab 86071
	mouse α-GM130	1:100 o/n 4°C	BD Transduction Laboratories	cat 610822
General Secondary antibodies / dyes	α-mouse-HRP	1:2500 1h / RT	Dianova	115-035-146
	α-rabbit-HRP	1:2500 1h / RT	Cell signaling	7074P2
	Phalloidin AlexaFluor 647	1:70 1h / RT	Thermo Fisher Scientific	A22287
	rabbit α-AlexaFluor-488	1:500 1h / RT	Thermo Fisher Scientific	A21206
	mouse α-AlexaFluor-488	1:500 1h / RT	Thermo Fisher Scientific	A10680
	mouse α-AlexaFluor-568	1:500 1h / RT	Thermo Fisher Scientific	A11031
	rabbit α-AlexaFluor-568	1:500 1h / RT	Thermo Fisher Scientific	A10042
	mouse α-AlexaFluor-647	1:500 1h / RT	Thermo Fisher Scientific	A31571

Table S1. Antibodies and dyes used in this study

Supplemental Materials and Methods

Ethics approval and informed consent

The study protocol for family 1 and 4 were approved by the University of Cologne Ethics Committee (Reference number: 13-022) and written informed consent for genetic analysis and clinical images from each studied subject were obtained.

Family 2 consented to genetic diagnostic follow-up by Antwerp University Hospital (UZA) and University of Antwerp (UA) and studies were approved under UZA/UA ethical committee 15/37/375.

Family 3 were consented to whole exome sequencing at Regeneron by the research team at the Clinic for Special Children under protocol 2008-095-CSC, which has IRB oversight through Lancaster General Hospital in Lancaster, Pennsylvania, USA.

All animal breedings and procedures were performed in accordance with the institutional animal care guidelines and the German animal welfare laws. They are approved under the reference numbers 84-02.04.2015.A378 and UniKoeln_Anzeige §4.18.002 of the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz NRW) state agency of North-Rhine-Westphalia.

Genetic analyses

Whole genome sequencing (WGS) was performed as previously described in.[1] Samples were prepared for WGS using Illumina´s TruSeq PCR-Free sample preparation kit (https://www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/truseq-dna-pcr-free.html). 1 µg of gDNA was fragmented to a mean target size of 350-450 bp using a Covaris E220 instrument, followed by end-repair, 3'-adenylation and ligation of indexed sequencing adaptors. The quality and concentration of all sequencing libraries was assessed using a LabChip GX instrument (Perkin Elmer), followed by further quality control using a MiSeq sequencer (Illumina) to obtain optimal cluster densities, insert size and library diversities. Sequencing libraries (one sample per lane) were hybridized to the surface of HiSeqX flowcells (v2 or v2.5) using the Illumina cBot™. Paired-end sequencing-by-synthesis (SBS) was performed on Illumina HiSeqX sequencers, using 2x150 cycles of incorporation and imaging. Real-time analysis involved conversion of image data to base-calling in real-time. All steps in the sample preparation and sequencing workflow were monitored using an in-house laboratory information management system (LIMS) with barcode tracking of all samples and reagents.

Whole exome sequencing was performed as previously described in.[2] Following the standard procedures of sample preparation, we used the Agilent v7 exome kit, which has a condensed target size of 37 Mb focusing on pure coding regions. The design has been optimized with respect to bait selection and boosting based on empirical experience coming from the earlier versions to improve coverage evenness. Library preparation was performed with 200 ng of DNA using the SureSelectXT Automated Target Enrichment for the Illumina paired-end multiplexed sequencing protocol, and the Agilent Bravo automated liquid handling platform. After validation (2200 TapeStation; Agilent Technologies, Santa Clara, CA) and quantification (Qubit System; Invitrogen, Waltham, MA), pools of libraries were generated and subsequently sequenced on an Illumina HiSeq4000 sequencing instrument using a paired-end 2 × 75 bp protocol resulting in this case in an average coverage of 80x with 91% of the target region covered at least 30x. For data analysis, we used a new version of our Varbank analysis tool (Varbank 2.0) (https://varbank. ccg.uni-koeln.de/varbank2/), which refers to the GRCh38/hg38 reference human genome and provides a number of new features at the online graphical user interface.

Culture and maintenance of probands-derived fibroblasts

Primary human fibroblasts were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% Fetal Bovine Serum (FBS, Biochrom), Penicillin/Streptomycin (Thermo Fisher Scientific), and Amphotericin B (Promocell). Cells were grown in 25cm or 75cm culture flasks and maintained in sterile incubators at 37°C, 95% relative humidity and 5% CO_2 atmosphere. All fibroblast lines were tested for mycoplasma contamination.

Antibodies used in this study

A detailed description of the antibodies used in this study (dilution, application, and manufacturer) is given in Table S1.

Semi-quantitative reverse transcription PCR (RT-PCR)

RNA was extracted from primary fibroblasts using the RNeasy kit (Qiagen), and concentrations were determined using the RiboGreen method (Life Technologies). 300ng of RNA were reverse transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen). Oligonucleotides were designed to flank *GBF1* exon 27 (FWD_Ex24_5′-CTACAGCGGGAAGAGACACC-3′ and REV_Ex28_5′-TGTGCCTGAACACAGAGGTG-3′) and exon 32 (FWD_Ex31_5′-GCTGTCCTTCATTGTGCGTG-3′ and REV_Ex33_5′-GAGGGTGCGAGAATCAGCTT-3′) in order to generate differently sized PCR products corresponding to potential alternate transcripts. PCR was performed with low cycle number (28) and 60°C annealing temperature).

Western blot

Probands-derived fibroblast were lysed in ice-cold RIPA buffer (SIGMA) with protease inhibitors (Complete Mini, Roche) for 30 minutes. After incubation, cells were centrifuged for 25 minutes, at maximum speed at 4°C. MNs were collected at the indicated time points (in vitro culture days DIV4, 12, and 20) and processed as described previously[4]. Dissected spinal cords and brains from P10 and P21 mice were snap-frozen and placed in homogenization tubes filled with ceramic beads (Peqlab). After the addition of ice-cold RIPA buffer with protease inhibitors, tissues were homogenized in the Precellys24 device (Peqlab) following a program of 25 seconds at 5500rpm. Immediately after, samples were sonicated for 10 minutes and centrifuged for 30 minutes at maximum speed at 4°C. If not immediately used, samples were stored at -80°C. Luminescent signal was detected using the detection reagents from Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) following the manufacturer´s instructions.

Fibroblasts immunostaining

40K cells grown on coverslips were washed three times with warm (37°C) PBS and fixed with 4% (w/v) paraformaldehyde (PFA). Fibroblasts were permeabilized in PBS-T (0.2% Tween-20) for 20 minutes and blocked for at least one hour in blocking solution (PBST + 5% BSA and 1% FCS). Primary antibodies were prepared in blocking solution and incubated overnight at 4°C. After three PBS-T changes, cells were incubated 1h at room temperature (RT) with different combinations of phalloidin and/or secondary antibodies diluted in blocking solution. After incubation, cells were washed three times with PBS-T and coverslips were rinsed in water prior mounting with ProLong mounting media with or without DAPI (Thermo Fisher Scientific). Information about antibodies and dilutions are given in Table S1.

Experiments with murine-derived cells or organs

All experiments involving mouse-derived cells and/or organs were approved by LANUV-NRW (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, reference number 84-02.04.2015.A378). Only wild-type (WT) FVB animals were used in this study.

Generation and immunostaining of primary murine motor neurons

For the generation of MNs, a pregnant mouse was euthanized and embryos at day 13.5 were removed from the uterus.[3] Embryos were sacrificed immediately by decapitation and the spinal cords were dissected as previously described[4]. Briefly, spinal cords were mechanically dissociated in 1% Trypsin (Worthington) with DNase I by pipetting. Isolated cells were seeded on Poly-D-Lysine (SIGMA) coated coverslips and grown in neuronal plating media consisting in DMEM supplemented with 5% Fetal Calf Serum (Biochrom), 0.6% Glucose (SIGMA).

Penicillin, Streptomycin, and amphotericin B. Cells were seeded in 12-well plates. 70K or 200K cells were seeded for imaging, or protein isolation, respectively. On the next day, the plating media was replaced for MN maintenance media consisting in Neurobasal medium (Thermo Fisher Scientific) supplemented with B27 (Thermo Fisher Scientific), 2 mM L-glutamine, Penicillin, Streptomycin, and Amphotericin B. In addition, the following growth factors were added: 50 ng/µl Ciliary Neurotrophic Factor (CNTF, Peprotech), 50 ng/µl Brain Derived Neurotrophic Factor (BDNF, Peprotech), and 50 ng/ul Glia Cell Line-derived Neurotrophic Factor (GDNF, Peprotech). MNs were maintained in sterile incubators at 37°C, 95% relative humidity and 5% CO_2 atmosphere. One-half of the culture media volume was refreshed every three days. 1µM Cytosine Arabinoside (AraC) was added to the media to eliminate dividing cells. MNs were fixed with 4% PFA supplemented with 4% sucrose (Sigma). Permeabilization, antibodies incubation, and mounting was performed as described for fibroblast immunostaining.

Microscopy and image analyses

Fluorescence images were acquired with a fully motorized microscope AxioImager M2 (Zeiss) equipped with an ApoTome.2 system mimicking confocality (Zeiss). NMJs images were acquired as z-stacks. Qualitative morphological analyses of Golgi morphology were based on GM130 staining. Precisely, the dispersion extent of the GM130 signal was categorized as: condensed or no fragmentation, intermediate fragmentation, and diffuse or extensive fragmentation. Golgi images were acquired with a 40X objective. More than 300 cells, representing three independent experimental replicates, were analyzed per fibroblast line. Fibroblast lines processing, image acquisition and quantification was performed blinded. For colocalization analysis, the Pearson colocalization coefficient was determined using the in-built colocalization tool of the ZEN software (Zeiss).

Statistical analyses

Statistical analyses were performed in GraphPad Prism 6 (GraphPad Software). Data are represented in percentage or as mean ± standard error of the mean/standard deviation (SEM/SD). Levels of statistical significance are given in GraphPad Prism 6 format where: * P = 0.05, ** P = 0.01, *** P = 0.001 and **** P = 0.0001. Specific statistical tests, sample size, and p values are indicated in the figure legends.

SUPPLEMENTAL REFERENCES

1. Karakaya, M., Storbeck, M., Strathmann, E.A., Delle Vedove, A., Holker, I., Altmueller, J., Naghiyeva, L., Schmitz-Steinkruger, L., Vezyroglou, K., Motameny, S., et al. (2018).

Targeted sequencing with expanded gene profile enables high diagnostic yield in non-5q-spinal muscular atrophies. Hum Mutat 39, 1284-1298.
2. Karakaya, M., Paketci, C., Altmueller, J., Thiele, H., Hoelker, I., Yis, U., and Wirth, B. (2019). Biallelic variant in AGTPBP1 causes infantile lower motor neuron degeneration and cerebellar atrophy. Am J Med Genet A 179, 1580-1584.
3. Hsieh-Li, H.M., Chang, J.G., Jong, Y.J., Wu, M.H., Wang, N.M., Tsai, C.H., and Li, H. (2000). A mouse model for spinal muscular atrophy. Nat Genet 24, 66-70.
4. Riessland, M., Kaczmarek, A., Schneider, S., Swoboda, K.J., Lohr, H., Bradler, C., Grysko, V., Dimitriadi, M., Hosseinibarkooie, S., Torres-Benito, L., et al. (2017). Neurocalcin Delta Suppression Protects against Spinal Muscular Atrophy in Humans and across Species by Restoring Impaired Endocytosis. Am J Hum Genet 100, 297-315.