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Supplemental Data

De Novo Variants in *LMNB1* Cause Pronounced Syndromic Microcephaly and Disruption of Nuclear Envelope Integrity

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Supplemental Note: Case Reports

Case 1

Individual 1 is the second child of healthy, unrelated Belgian parents. Pregnancy was uneventful, but prenatal ultrasound showed small head circumference. Birth parameters at term included a weight of 2610 g, length 46 cm and very small head circumference of 25 cm (-4.6 SD).

Brain MRI at ages 6 and 18 months showed besides the extreme microcephaly a small corpus callosum. Development of the child was delayed with independent walking at age 2.5 years and diagnosis of moderate intellectual delay and autism at the age of 4 years.

At the last clinical evaluation, age 9.5 years, his weight was 16 kg (-1.94 SD), length 122 cm (-2.7 SD), and head circumference 42 cm (-6.8 SD). He is still making slow progress, e.g. using few words and short sentences, being able to read individual characters and a few words, riding a tricycle. Clinical examination showed besides the microcephaly a mild hypertonia in hamstrings and triceps surae and mild hyperreflexia.

Case 2

Individual 2 is the first child of unrelated Belgian parents. She has an older paternal and maternal brother, both healthy. Pregnancy was uneventful, but prenatal ultrasound showed small head circumference. Birth parameters at term included a weight of 3460 g, length 51 cm and head circumference of 32 cm (-1.9 SD). She had a delayed development with independent walking at age 2 years and slow language acquisition. Re-examination at age 9 years and 4 months showed a mild to moderately delayed girl with microcephaly (-3.7 SD), and mild dysmorphic features. She follows special education. The microdeletion involving part of the *LMNB1* gene is inherited from her mother. The adult head circumference of the mother is 50.3 cm (- 2.7 SD). The mother followed normal school with extra support.

Case 3

Individual 3 is the fourth child of healthy, non-consanguineous Italian parents. The three sisters are healthy. Prenatal ultrasound showed microcephaly and amniocentesis was performed showing a normal fetal karyotype (46,XX). Delivery was via caesarean section at 35 weeks of gestation because

of previous sections and maternal pain. At birth, length and weight were 2190 g (24th centile) and 42 cm (4th centile), respectively, while head circumference was 28 cm (<3rd centile, -2.8 SD). Growth remained severely delayed for all parameters, with absolute microcephaly. Re-examination at age 5 years and 7 months showed a severely delayed girl with severe microcephaly. She has not achieved the sitting position, neither any language. Brain MRI at this age shows pachygyria and lissencephaly. Postnatal standard karyotype, and molecular karyotyping were both normal.

Case 4

Individual 4 was last evaluated at the age of 2 years and 8 months. Pregnancy history was significant for preterm labour at 28 weeks gestation, at which time microcephaly was noted. The mother was placed on bedrest and delivery was at 38-5/7 weeks. Head circumference at birth measured 29 cm (-4 SD). Work-up during and after the pregnancy for TORCH infections and Zika virus were negative. Seizure activity began within the first day of life. A brain MRI performed on day 2 of life showed a simplified gyral pattern and dysgenesis of the corpus callosum. Seizures have been relatively well controlled with anti-epileptic medications. Because of recurrent pneumonia, presumably due to aspiration, a G-tube was placed. Other medical history includes cortical visual impairment and chronic constipation. She presented with a significant developmental delay. At 2 years 8 months, she had good head control and was able to sit in a tripod position with support, swing her arms but not yet reach or grab, and she was making noises. Severe microcephaly continues to be noted while length is tracking slightly below the 1st percentile and weight is at the 14th percentile. Family history is unremarkable.

Cases 5, 6, 7

Individuals 5, 6 and 7 belong to the same Arabic sibship. Individual 5 is the youngest of healthy, unrelated parents. Pregnancy was uneventful. At 20 months of age, her weight was 6.210 kg (-4.5 SD), height: 77 cm (-2.4 SD), and head circumference 45 cm (-4.42 SD). At clinical examination, microcephaly, severe development delay with axial hypotonia, mild scoliosis, and lack of language and communication interest was noted. She has two older and equally affected brothers. Individual 6 is the firstborn of the couple. Prenatal ultrasound at PMA of 30 weeks showed a small head circumference. Delivery occurred via C-section because of foetal bradycardia. Weight at birth was 2.500 gr (-2.3 SD),

no other anthropometric birth parameters are available. He presented with severe microcephaly and seizure onset at 14 days of life. He gradually developed a spastic tetraparesis in association with severe axial hypotonia without control of the head. At clinical evaluation (when he was admitted to the genetic centre in Barcelona for a second opinion, age 11 years), his weight was 28 kg (-1.37 SD), height: 122 cm (-4,07 SD), BMI: 18.79 kg/m2 (-0.07 SD), and head circumference 40 cm (-10 SD). Besides the severe microcephaly, he showed long eyelashes, a high palate, gingival thickening and hypertrichosis. He has no language and motor dysfunction GMFCS level V. Currently, he has no longer epileptic seizures and antiepileptic treatment was stopped. Because of an aspiration pneumonia secondary to dysphagia and gastroesophageal reflux a G-tube was placed.

Brain MRI (11 years) showed a simplified gyral pattern, prominent extra-axial spaces, dilated ventricles related to the low volume of the brain. Cerebellum and brainstem were preserved. A metabolic work-up was negative. Molecular karyotype showed the presence of 280 kb duplication on chromosome 17p13.3, which is considered as a variant of unknown significance, not present in the other affected brother. A gene panel specific for infantile epileptic encephalopathy did not demonstrate pathogenic mutations.

The younger brother, individual 7, and the second child of this couple, showed a highly similar clinical course. At clinical evaluation (age 10 years), his weight was 29 kg (-0.79 SD), height: 125.34 cm (-2,50 SD), BMI 18.46 kg / m2 (0,15 SD), and head circumference 39 cm (-10 SD). He also has a severe developmental delay with spastic tetraparesis, scoliosis and no language. Brain imaging showed the same simplified gyral pattern, as seen in his older brother. He still has seizures that are well controlled with valproate, levetirazetam, and clobazam. Additional metabolic examinations and molecular karyotype were normal in this individual.

Supplemental Table 1

Summary of sequencing platforms and coverage in four unrelated families reported with a *LMNB1* variant

	Individual 1	**Individual 3**	**Individual 4**	**Individuals 5-7**
Sequencing laboratory	Center for Human Genetics, UZ Leuven, Belgium	TIGEM Institute, Pozzuoli, Naples, Italy	Greenwood Genetic Center, USA	Bio Array Alicante, Spain
Sequencing type	Trio exome sequencing + Sanger sequencing of *LMNB1* in proband and parents	Trio exome sequencing + Sanger sequencing of *LMNB1* in proband and parents	Trio exome sequencing + Sanger sequencing of *LMNB1* in proband and parents	Trio exome sequencing + Sanger sequencing of *LMNB1* in probands and parents
Capture and library construction	Roche SeqCap EZ Exome Enrichment v.3.0	Agilent SureSelect Clinical Research Exome v2	Agilent SureSelectXT Clinical Research Exome v2	Agilent SureSelect Clinical Research Exome v2
Sequencing platform	Illumina HiSeq2500	Illumina NextSeq500	Illumina NextSeq500	Illumina NovaSeq
Average depth of targeted bases	80X	125X	259X	125X
Percentage of bases covered >10x	94.0%	96.40%	97.0%	96.4%
Target of analysis	Whole exome	Whole exome	Whole exome	Custom gene panels: microcephaly (~800 genes), epilepsy (~1200 genes) and cerebral atrophy (~250 genes)

Overview of *de novo* variants identified by WES in individuals 1, 3-4

ID	**Gene**	**Chr**	**Genomic position**	**REF allele**	**ALT allele**	**HGVS**	**dbSNP**
Individual 1	*LMNB1*	5	126140563	C	G	NM_005573.3:ex2:c.455C>G:NP_005564.1:p.Ala152Gly	-
Individual 1	*RAD51C*	17	56774110	A	G	NM_058216.3:ex3:c.461A>G:NP_478123.1:p.Glu154Gly	rs758847241
Individual 3	*LMNB1*	5	126113297	A	G	NM_005573.3:ex1:c.97A>G:NP_005564.1:p.Lys33Glu	-
Individual 3	*TMEM218*	11	124967600	G	C	NM_001080546.2:ex5:c.250C>G:NP_001074015.1:p.Leu84Val	-
Individual 3	*CACNA1I*	22	40030701	C	T	NM_001003406.1:ex5:c.712C>T:NP_001003406.1:p.Arg238Cys	-
Individual 4	*LMNB1*	5	126113324	C	T	NM_005573.3:ex1:c.124C>T:NP_005564.1:p.Arg42Trp	-
Individual 4	*SEPT14*	7	55910834	T	G	NM_207366.2:ex5:c.372-13A>C	-
Individual 4	*RBM19*	12	114392971	C	T	NM_001146699.1:ex7:c.886G>A:NP_001140171.1:p.Val296Met	rs1409746316

Chr: chromosome; REF allele: reference allele; ALT allele: alternative allele;

Supplemental Table 2

Pathogenicity scores of missense and splice variant

Software	Variant & Score	Classification comment
PolyPhen2 (HumVar score)	p.Ala152Gly: 0.77 p.Lys33Glu: 1 p.Arg42Trp: 1 c.939+1G>A	Possibly damaging Probably damaging Probably damaging NA
PROVEAN	p.Ala152Gly: -3.67 p.Lys33Glu: -3.29 p.Arg42Trp: -6.67 c.939+1G>A	Deleterious Deleterious Deleterious NA
MutationTaster	p.Ala152Gly: 60 p.Lys33Glu: 56 p.Arg42Trp: 101 c.939+1G>A: NA	Disease causing Disease causing Disease causing Disease causing
GERP++	p.Ala152Gly: 5.15 p.Lys33Glu: 2.72 p.Arg42Trp: 2.72 c.939+1G>A: 5.84	Highly constrained Constrained Constrained Highly constrained
PhastCons100 (vertebrate)	p.Ala152Gly: 1 p.Lys33Glu: 1 p.Arg42Trp: 1 c.939+1G>A: 1	Conserved Conserved Conserved Conserved
dbscSNV **ADA score** **RF score**	c.939+1G>A: 0.99 c.939+1G>A: 0.938	Splice-altering Splice-altering
HSF 3.1	c.939+1G>A	Broken WT donor site

Pathogenicity predictions. A summary of the pathogenicity predictions and conservation scores for each missense and splice-site mutation generated using the following pathogenicity prediction software tools:

PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/)

PROVEAN (http://provean.jcvi.org/index.php)

MutationTaster (http://mutationtaster.org/)

GERP++ and PhastCons100 conservation scores have been retrieved from the corresponding tracks in the UCSC Genome Browser (https://genome.ucsc.edu/).

Splice-site predictions for the c.939+1G>A mutation have been generated using Human Splicing Finder (HSF) 3.1 software tool (http://www.umd.be/HSF/) or retrieved from the Ensembl Variant Effect Predictor tool (http://grch37.ensembl.org/Homo_sapiens/Tools/VEP).

Supplemental Materials and Methods

Cell culture: WT and *LMNB1$^{-/-}$* Hela cells (Canopy Biosciences, Saint Louis, MO) were cultured in DMEM (ThermoFisher; #10-017-CV) supplemented with 10% fetal bovine serum (VWR; #97068-085) and 1% penicillin/streptomycin (ThermoFisher; #30-002-CI) in a 5% CO_2 humidified tissue culture incubator. The *LMNB1$^{-/-}$* Hela cells, generated using CRISPR-Cas9 editing, contain a 2bp deletion in exon 1 on the first allele and a 2bp insertion in exon 1 on the second allele.

We derived lymphoblast cell lines of the probands with the p.Lys33Glu, p.Ala152Gly and p.Arg42Trp mutations, as well as from one healthy parent of proband p.Lys33Glu and a healthy parent of proband p.Arg45Trp. These parental lines served as controls. Control and proband lymphoblast cells were cultured in RPMI 1640 (ThermoFisher; #10-040-CV) supplemented with 10% fetal bovine serum (VWR; #97068-085), 1% antibiotic/antimycotic (Sigma A5955), L-Glutamine (Sigma G7513) in a 5% CO_2 humidified tissue culture incubator.

Transfection: Expression constructs for the different *LMNB1* variants were generated by site-directed mutagenesis. The cDNA sequence change for each variant is: p.Lys33Glu (c.97A>G), p.Lys33Thr (c.98A>G), p.Arg42Trp (c.124C>T), p.Ala152Gly (c.455C>G), and p.Arg29Trp (c.85C>T). 500,000 cells were plated per well in a 6 well dish 18h prior to transfection in growth media. The cells were rinsed with PBS followed by addition of 0.5 mL of OptiMEM (ThermoFisher; #31985062) and then transfected with 5µg of plasmid and 10µl Lipofectamine 2000 (ThermoFisher; #11668019) per well OptiMEM. After 5h, the media was replaced with 1 ml of OptiMEM and the cells collected 24 h post-transfection for analysis. For more facile comparison, we choose to use the protein variant (instead of the nucleotide change) when describing the HeLa transfection results in the main text.

Subcellular Fractionation: Cells were fractionated into nuclear and cytosolic fractions using the following protocol. Aliquots of the same cells were reserved in order to determine transfected protein input. All steps were performed on ice and centrifugation at 4°C. Subcellular fractionation buffer (SF buffer) containing 250 mM sucrose, 10 mM KCl, 20 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, and 1 mM EDTA, 1mM DTT, and protease inhibitor cocktail, was added to trypsinized cell pellets to generate homogenates. Homogenates were passed through a 25-gauge needle ten times before centrifugation at 8,000 rpm (10,000 x g) for 12 min resulting in a pellet containing nuclei, and a cytosolic supernatant fraction. RIPA buffer was added to the fractionated cellular components to solubilize the proteins and a BCA assay was performed to quantify total protein concentration in each fraction.

SDS-PAGE and Western Blot Analysis: Cells were released from the culture plates using trypsin and lysed in cold lysis buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate), with protease inhibitors (ThermoFisher; #A32955) and 2mM DTT. Following incubation for 30 min on ice, and vortexing for 20 sec, the cells were centrifuged at 20,000xg for 10 min at 4°C. Total protein was quantified in the supernatant using the Micro BCA Protein Assay (ThermoFisher; #23235). 60 µg of cell lysate per sample and 5 µl of 10x concentrated media was prepared in Lamelli buffer, incubated at 95°C for 5 min and chilled at 4°C. Samples were separated on an 8% SDS-PAGE gel. Protein was transferred to 0.45uM pore nitrocellulose (Abersham; #10600004) at 110 volts for 2h at 4°C in transfer buffer. The membrane was rinsed and stained with Ponceau S for normalization. The membranes were blocked with 5% milk/TBST for 1h at room temperature. LMNB1 western blots were incubated with HRP conjugated anti-rabbit antibody (Jackson ImmunoResearch; #111-035-144) at 1:1000 overnight at 4°C, washed 3x with TBST, and developed with SuperSignal West Pico PLUS ECL reagent (ThermoFisher; #34577). All images were captured on the Bio-Rad ChemiDoc MP

Imaging System (Bio-Rad; #12003154). Analysis was done with Image Lab Software (Bio-Rad #1709690, ver5.2.1).

Immunostaining and confocal microscopy: HeLa cells were seeded onto coverslips in a twelve well cell culture plate. After washing with Dulbecco's PBS (DPBS) containing calcium and magnesium, the cells were fixed to the slides with the addition of 3.7% formaldehyde for 10 mins followed by washing 4 times in DPBS containing calcium and magnesium. Permeabilization was achieved with the addition of 1 mL of 0.1% Triton-X in DPBS for 10 mins at room temperature and then wells were washed three times with DPBS containing calcium and magnesium. Fixed coverslips were blocked using 3% bovine serum albumin and incubation for 1 h at room temperature on a rotator followed by washing with DPBS. A rabbit monoclonal anti-lamin B1 antibody from Cell Signaling Technology (D9V6H; cat # 13435; 1:1000 dilution in WT and KO HeLa and 1:4000 in transfected KO cells) and mouse monoclonal anti-lamin A/C antibody from Santa Cruz Biotechnology (cat #: SC-7292; 1:1000 dilution) were added for 1 h at room temperature followed by incubation with an Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 568 anti-mouse IgG secondary antibody (Abcam, 1:500 dilution) in the dark for 1 hr. After washing, the coverslips were mounted with Prolong Gold^{TM} containing DAPI and visualized using an Olympus FV3000 confocal microscope. For quantification of the different nuclear and nuclear envelope phenotypes, at least one hundred cells were counted on slides from three independent experiments and the percentage of cells with each phenotype averaged. Statistical analysis was performed using a Dunnett's t test.

Polyploidy screening via Fluorescence in situ hybridization: Fixed cells were pre-treated for 10 min with 0.1 mg pepsin/ml (Sigma) in 0.01 mol/l HCl at 37°C followed by a 3 min wash in 1 x PBS. Post-fixation was performed by incubating the slides for 10 min in a 1% formaldehyde solution with 0.05 mol/l $MgCl_2$ and 1 x PBS at 4°C. Slides subsequently were washed in 1 x

PBS and dehydrated by ethanol series. Then 5µl of probe mixture was applied to the slide, covered with a coverslip (10mm x 10mm) and sealed with rubber cement. We used the probe mixtures: Séq uniques 13/21 (Cytocell, 13q14.2/21q22.13, Spectrum Green/Spectrum Orange), and CEPXY (Vysis, Xp11.1-q11.1 Alpha Satellite DNA/Yq12 Satellite III, Spectrum Green/Spectrum Orange). Nuclei and probe were denatured simultaneously on a hot plate at 75°C for 5 min. Hybridization was allowed to take place overnight in a humid chamber at 37°C. After hybridization, excess or non-specific bound probe was removed by subsequent washes in 0.4 x SSC/0.3% NP-40 (73°C for 1 min), 2 x SSC/0.1% NP-40 (room temperature for 1 min) and 2 x SSC (room temperature for 1 min) followed by dehydration through ethanol series. After drying, the slides were mounted in Vectashield anti-fading medium (Vector Laboratories, Peterborough, UK) containing 2.5 ng/ml 40,6-diamidino-2-phenylindole (DAPI; Boehringer Ingelheim GmbH, Germany). Nuclei were examined using an Axioplan 2 microscope (Zeiss NV, Zaventem).

Metaphase mitotic spindle analysis: Control and proband lymphoblast cells were cultured in RPMI 1640 (ThermoFisher; #10-040-CV) supplemented with 10% fetal bovine serum (VWR; #97068-085), 1% antibiotic/antimycotic (Sigma A5955), L-Glutamine (Sigma G7513) in a 5% CO_2 humidified tissue culture incubator. Next we used the protocol as described by Qian et al., 2017 [1]. For RO3306 washout experiments, cells were initially treated with 2mM thymidine for 24 h, washed with PBS, and released in fresh medium for 4 h prior to treatment with 9 µM RO3306. After 12 h, the cells were washed with PBS and incubated with fresh medium containing 10µM MG132 for 90 min.

Cells were transferred to a glass slide using the Tharmac CellSpin I (5’ 500rpm) and fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and blocked in 3% BSA/PBS. Cells were subsequently incubated with primary antibodies [alpha-Tubulin diluted at 1/1000 - Sigma-Aldrich, cat n° T6074 and gamma-Tubulin diluted at 1/500 – Abcam, cat n° ab11317] in

1.0% BSA/PBS for 2 h at room temperature and with secondary antibodies for 1 h at room temperature. DNA was stained with DAPI. Confocal images were acquired with a Leica TCS SPE laser-scanning confocal system mounted on a Leica DMI 4000B microscope, and equipped with a Leica ACS APO 63X 1.30NA oil DIC objective. For quantification, Z stack scans were performed through each cell (4-6 sections, 1 μm intervals), and analyzed using ImageJ software and the Z project 'sum slices' feature.

Supplemental Reference

1. Qian, J.,García-Gimeno, M.A., Beullens, M., Manzione, M., G., Igual, J.C., Heredia, M., Sanz, P., Gelens, L., Bollen, M. (2017). An Attachment-Independent Biochemical Timer of the Spindle Assembly Checkpoint. Mol. Cell *68*, 715-73.