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De novo mutations in \textit{PLXND1} and \textit{REV3L} cause Möbius syndrome

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Möbius syndrome (MBS) is a neurological disorder that is characterized by paralysis of the facial nerves and variable other congenital anomalies. The aetiology of this syndrome has been enigmatic since the initial descriptions by von Graefe in 1880 and by Möbius in 1888, and it has been debated for decades whether MBS has a genetic or a non-genetic aetiology. Here, we report de novo mutations affecting two genes, \textit{PLXND1} and \textit{REV3L} in MBS patients. \textit{PLXND1} and \textit{REV3L} represent totally unrelated pathways involved in hindbrain development: neural migration and DNA translesion synthesis, essential for the replication of endogenously damaged DNA, respectively. Interestingly, analysis of \textit{Plxnd1} and \textit{Rev3l} mutant mice shows that disruption of these separate pathways converge at the facial branchiomotor nucleus, affecting either motoneuron migration or proliferation. The finding that \textit{PLXND1} and \textit{REV3L} mutations are responsible for a proportion of MBS patients suggests that de novo mutations in other genes might account for other MBS patients.
Mobius syndrome (MBS) (MIM 157900) is a rare congenital cranial dysinnervation disorder characterized by non-progressive facial palsy and impairment of ocular abdution, due to paralysis or weakness of the facial (n.7) and abducens (n.6) nerves, and frequently other cranial nerves. Both intrauterine environmental factors and genetic causes have been proposed for the aetiology and pathogenesis of MBS. A disruption of blood vessel migration during development, which can be secondary to misoprostol or cocaine exposure during pregnancy, has been hypothesized to lead to hindbrain hypoxia, resulting in cranial nerve dysfunction. A limited number of familial cases with an atypical manifestation of MBS have been reported. In addition, a founder mutation in HOXB1 has been associated with an autosomal recessive form of an MBS-like syndrome, with limited phenotypic overlap with typical MBS. However, the vast majority of patients has a sporadic occurrence with a very low recurrence risk for siblings. This is consistent with either the involvement of environmental factors or with dominant de novo mutations as the underlying mechanism.

The broad spectrum of neuropathological and neuroradiological findings suggests that MBS is due to a developmental defect of the entire rhombencephalon, rather than an isolated cranial nerve disruption. This is in contrast to hereditary congenital facial palsy (HCFP), which is characterized by the isolated dysfunction of the facial nerve. Two HCFP loci have been identified: HCFP1 (MIM 601471) at chromosome 3q21-q22 (ref. 18) and HCFP2 (MIM 604185) at chromosome 10q21.3-q22.1 (ref. 19). Extensive studies have been conducted to explain the HCFP phenotype and to pave the way for genetic studies in MBS, but analysis of genes in the HCFP1 and HCFP2 loci, the HCFP phenotype and to pave the way for genetic studies in MBS patients, has so far not identified any causative mutations in HCFP and in MBS patients.

In this study we undertake exome sequencing of two parent trios and six isolated patients with classical MBS to investigate the possibility of an underlying genetic cause. We identify de novo mutations in five different genes. For two of these genes, PLXND1 and REV3L, additional de novo mutations in other MBS patients are identified. The causality of de novo PLXND1 and REV3L mutations for the neuropathological features of MBS is further supported by analysis of the respective knockout mice. Strikingly, for both heterozygous mutants we observe hypoplasia of the facial branchiomotor nucleus, which is consistent with the facial nerve weakness in MBS patients. Together, the present data establish de novo mutations as a cause for MBS, providing a rationale for exome sequencing in patient–parent trios to identify de novo mutations in other genes underlying MBS.

**Results**

**Identification of de novo mutations by exome sequencing.** We excluded all variants inherited from either parent in the two trios under the hypothesis of de novo mutations as the underlying cause of MBS (Supplementary Table 1). Two de novo variants were detected in patient 1 (P1) (Fig. 1a; reported previously), one in the SPO11 gene (c.712T>G; p.Cys238Arg; NM_012444) and the other in the PLXND1 gene (c.6585C>A; p.Asn1859Lys; NM_015103) (Fig. 1a,b; Supplementary Fig. 1; Supplementary Tables 2 and 3). SPO11 is involved in meiotic recombination, and targeted disruption of this gene in mice results in fertility problems, but no features related to MBS. The occurrence of a de novo mutation in PLXND1 was more consistent with a role for this gene in MBS, given that PLXND1 was previously considered a strong candidate for HCFP1 and MBS.

Trio-exome sequencing in patient 2 did not reveal any de novo variant. A different analytical approach was taken for the six patients with isolated MBS and this was to Sanger sequence all candidate variants identified in the exome studies (Supplementary Tables 1 and 4). Sanger sequencing confirmed de novo variants, identified by exome sequencing, in IFT172, CCDC160 and REV3L, in three unrelated patients. The de novo missense mutation in the IFT172 gene (NM_015662) identified in patient 3 (Fig. 1; Supplementary Tables 2 and 3) is unlikely to be causative for the MBS phenotype, because biallelic mutations are associated with the unrelated Jeune and Mainzer-Saldino Syndromes in humans. The de novo variant in the X-chromosomal CCDC160 gene of patient 4 (P4) (a male) is a c.501delA frameshift mutation in exon 2 (NM_00110357) (Supplementary Fig. 1; Supplementary Tables 2 and 3). Patient 5 (P5; Fig. 1c) carries a de novo variant in REV3L, c.1096+1G>A, affecting the canonical donor splice site in exon 10 (NM_002912) (Fig. 1d; Supplementary Fig. 1, Supplementary Tables 2 and 3). Aberrant splicing products were identified by quantitative reverse transcriptase–PCR analysis of REV3L messenger RNA obtained from Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCLs) of patient P5. Sequencing of these products revealed aberrant REV3L transcripts lacking either exon 9 or exons 9 and 10, in addition to decreased levels of normal REV3L transcript (Supplementary Fig. 2).

**Additional de novo mutations in the PLXND1 and REV3L.** Next, we sequenced CCDC160, PLXND1 and REV3L by Sanger sequencing in a cohort of 103 MBS patients, including some HCFP patients who were not previously tested for PLXND1 (ref. 21). The sequencing identified four additional de novo mutations in unrelated patients, two affecting PLXND1 and two affecting REV3L (Fig. 1; Supplementary Fig. 1; Supplementary Tables 2 and 3). The PLXND1 mutations were identified in patients P9 (c.4454_4455G>C; p.Arg1485Pro) and P10 (c.3018C>T; p.Leu1006Leu). The mutation in P9 affects a conserved arginine residue in the GTPase-activating protein domain of the protein. The silent mutation in P10 is at a leucine residue in the IPT/TIG domain (immunoglobulin-like fold, plexins, transcription factors/transcription factor immunoglobulin) that is characteristic for plexin proteins. The two additional de novo mutations in REV3L were identified in patients P11 and P12. A de novo missense mutation c.1160A>G was identified in patient P11, predicting an amino-acid substitution p.Glu387Gly. In addition, patient P12 carried a nonsense mutation (c.2662A>G; p.Thr888*), which is predicted to result in a loss-of-function allele.

The absence of PLXND1, REV3L and CCDC160 truncating variants in NHLBI Exome Sequencing Project (ESP; http://evs.gs.washington.edu/EVS/) and a low residual variant intolerance score for these genes of 1.30 (4.88th percentile), −2.1 (1.55th percentile) and 0.014 (54.98th percentile), respectively, are consistent with PLXND1, REV3L and CCDC160 being intolerant to loss-of-function mutations. The amino acids that are affected by the missense variants in REV3L and PLXND1 are highly conserved throughout evolution (p.Glu387Gly (P11), p.Asn1859Lys (P1), p.Arg1485Pro (P9) and p.Leu1006Leu (P10)) (Supplementary Fig. 3).

The probability of identifying multiple de novo mutations in the same gene in a cohort of 103 individuals was calculated using the Poisson test and is 0.000127 for REV3L (P value = 2.4 × 10⁻⁴) and 9.34 × 10⁻⁵ for PLXND1 (P value = 1.8 × 10⁻⁵). The presence of a mutation in the other allele in these patients was excluded by sequencing the coding exons of PLXND1 and REV3L. Screening of CCDC160 gene in our cohort of male MBS patients did not reveal additional de novo mutations.

**PLXND1 deficiency affects neural fibres’ structures.** PLXND1 encodes a protein of 521 amino acids and is a member of the plexin family of proteins. Plexins bind to semaphorins (Semas), a
large family of extracellular, secreted and membrane proteins.25–27. PLXND1 is expressed in the vascular system and in specific structures of the central nervous system, such as the cranial and spinal ganglia, the cortical plate, the external granular layer of the cerebellum, the striatum and in dendritic cells.28–30. PLXND1 is involved in the development of the cranial nerve undergoes a complex migration within the rodent hindbrain (Fig. 2c). Between E10.5 and E14.5, neurons in rhombomere 4 (r4) start migrating tangentially along the ventral midline, reaching r5 and r6. Then they migrate dorsally until the alar/basal plate boundary and begin to migrate radially to their final destination in the pial surface of r6 (refs 33–35). To trace the nature of the decreased neuronal density of the FBM, we carried out an immunohistochemical analysis using the motoneuron-specific molecular marker Islet-1 in brains from E16.5 embryos (Fig. 2d). In line with this migration pattern, all FBM motoneurons reached r6 at E16.5 in wt mice (Fig. 2d). In sharp contrast, in all of the Plxnd1−/− and Plxnd1−/− mice, a subpopulation of motoneurons was still located in r4 and r5 at E16.5. Collectively, these results support a causative role of a defect in motoneuron migration caused by a heterozygous Plxnd1 mutation akin to the de novo PLXND1 mutations in MBS patients P1, P9 and P10.

Figure 1 | Clinical features of Möbius syndrome patients and de novo mutations in PLXND1 and REV3L. (a) Clinical features of the patients carrying the mutations in PLXND1 (P1, P9 and P10). Patient P1 shows an impairment of ocular abduction in a relaxed facial position; she is not able to smile and she cannot close her eyes completely. Patient P9 in a relaxed facial position presents left upper facial paralysis. Patient P10 showing bilateral facial paralysis, inability to close his mouth and lagophthalmos. (b) Genomic structure of the PLXND1 gene and the PLXND1 protein structure and the position of de novo mutations. The different protein domains are depicted. GAP, GTPase-activating protein; IPT/TIG, immunoglobulin-like fold, plexins, transcription factors/transcription factor immunoglobin; D1-PBM, PDZ-binding motif; MET, mesenchymal–epithelial transition. (c) Clinical features of the patients carrying the mutations in REV3L (P5, P11 and P12). Patient P5 in a relaxed position, showing the characteristic inability of MBS patients to smile in the second photograph. Both eyes show incomplete closure. There is agenesia of the left pectoralis muscle (Poland variant) and absence of digits from the left hand. Patient P11 has both eyes fixed in straight position and a complete deficiency of both abduction and adduction. There is a complete inability to follow objects laterally. Furthermore, a bilateral facial nerve palsy is present producing a lagophthalmos on eye closure in both eyes, and oral rim asymmetry with an inability to smile. Patient P12 in a relaxed facial position. The ability to smile of P12 has been improved following plastic surgery at age of 13 years. Inappropriate closure of both eyes is still present. (d) Schematic structure of human REV3L gene (left) and REV3L protein (right). MBS-associated mutations detected in three patients (P5, P11 and P12) are indicated. The coloured bands represent the known domains of the protein. Rev7-binding domain, site of binding of the heterodimeric Rev31 partner Rev7, ZF, zinc finger.
**Rev3l heterozygous mice show hypoplasia of neural structures.** The human REV3L protein acts as the catalytic subunit of DNA polymerase ζ, a key protein in replication of endogenously or exogenously damaged DNA\(^36\). Consistent with the phenotypes of the Rev3l heterozygous mice, in situ hybridization data show that Rev3l is highly expressed in the developing embryonic brain around mid-gestation (Supplementary Fig. 4). Biallelic inactivation of Rev3l in mice leads to mid-term embryonic lethality. Rev3l-deficient mouse embryos display pleiotropic morphological abnormalities, but are invariably growth-retarded and display massive apoptosis, notably of the brain\(^36\). Analysis of the subarachnoid space at E16.5 in Rev3l\(^+/−/−\) mice revealed a highly significant increase in subarachnoid volume (t-test, \(P = 0.0047\); Fig. 3a). In addition, Rev3l heterozygous mice at P0 showed significantly reduced hindbrain volumes as compared with wt (t-test, \(P = 0.015\); Fig. 3b). We then analysed the facial motor nucleus at P0 (Fig. 3c,d). In contrast to the Plxnd1 mutant mice, there was no evidence for a motoneuron migration defect in the Rev3l heterozygous embryos. Nevertheless, there was a strong decrease in the number of motoneurons in Rev3l heterozygous embryos (t-test, \(P < 0.0001\); Fig. 3d). These data provide strong support for hypoplasia of the motor nucleus consequent to a heterozygous Rev3l mutation, similar as the REV3L mutation in MBS patients P5, P11 and P12.

**Rev3l mutant MEFs displayed increased DNA damage.** The essential role of Rev3l in replication of damaged DNA has been extensively studied in vitro and in Rev3l-deficient cells and mice\(^36\)--\(^39\). We wondered whether the neuronal defects in MBS patients and Rev3l heterozygous mice reflect partial replication defects at DNA carrying endogenous DNA lesions. To this aim, we analysed responses of immortalized Rev3l mutant mouse embryonic fibroblasts (MEFs) to Benzo[\(α\)]pyrene dioleloxipoxide (BPDE), an agent that induces DNA damage that mimics bulky endogenous nucleotide lesions. Rev3l heterozygous MEFs displayed a slight, but significant, decrease in cell survival (Fig. 4a). To substantiate the effect of haploinsufficiency, we measured replication of BPDE-damaged DNA in these cells and found that Rev3l heterozygous cells displayed a slight but significant defect, suggesting the presence of replication stress (Fig. 4b, right panel). Since replication stress induces DNA damage-signalling cascades that initiate cellular senescence or apoptosis, we measured phosphorylation of the signalling proteins Rpa, Chk1 and H2AX (Fig. 4c)\(^40\). Remarkably, the Rev3l heterozygous cells displayed a level of DNA damage signalling that was almost as high as in Rev3l-deficient cells. These data suggest that, in Rev3l heterozygous mice, marginally under-replicated endogenous DNA lesions induce strong DNA responses, contributing to the observed neuronal phenotypes.

**Discussion**

We have identified de novo mutations in PLXND1 and REV3L in six unrelated sporadic MBS patients. The neuropathological alterations and other clinical findings in MBS patients correlate to those in the Plxnd1 and Rev3l mutant models. Thus, hypoplasia of the facial motor nucleus that was observed in both mouse models has also been documented in MBS patients\(^5\)--\(^16\). In addition, several developmental anomalies that were previously reported in Plxnd1 knockout mice are mirrored in patients carrying a PLXND1 mutation, as well as other MBS patients. For example, craniofacial bone abnormalities observed in Plxnd1 knockout mice\(^41\) were also seen in patients P1 and P9, and which are commonly seen in other MBS patients\(^17\) (Supplementary Table 5). In addition, the vertebral defects in Plxnd1 mutant mice are reminiscent of the scoliosis in patient P1, and the

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**Figure 2 Plxnd1 mouse brain characterization.** (a) Paraffin sagittal sections of embryonic mouse brains processed with Nissl staining at E16.5. These two pictures are medial sections of a view of the corpus callosum (CC), the anterior commissure (ac) and the fasciculus retroflexus (rf). These three structures appear hypoplastic in Plxnd1\(^+/−/−\) mice. Scale bars, 500 μm. (b) Graphic representation of the number of motoneurons in facial motor nucleus in wt, heterozygous and homozygous Plxnd1 knockout (mean ± s.d.). Unpaired t-test was used to calculate the \(P\) value = 0.0049 (\(N = 5\)). (c) Schematic representation of the facial nerve migratory process along the hindbrain. The motoneuron migratory pathway is indicated with a red arrow. The rhombomeres (r3–r11) boundaries are marked with a line. Cb, cerebellum; FBM, facial motor nucleus; SpC, spinal cord. The upper part in (d) shows the immunohistochemical staining of facial FBM motoneurons with anti-Islet-1 antibody (dark brown). The rhombomere units (r3–r7) are marked with a dashed line. Black arrows point the motoneurons migration across the rhombomeres in the heterozygous and the homozygous Plxnd1 knockout mouse. The area of the facial motor nucleus is marked by the square. Scale bar, 300 μm. A detailed view of the facial motor nucleus and motoneurons migration is shown in the lower part of d. Motoneurons appear outside the facial nucleus along the migratory path of the facial nerve in both the heterozygous and the homozygous genotypes. Scale bar, 100 μm. All reported \(P\) values tested were calculated by the unpaired t-test using Graphpad software.
Haemorrhages seen in Plxnd1 mutant mouse resemble the facial haemangiomas seen in patient P1 (Supplementary Table 5). A possible causative de novo mutation was also identified in CCDC160. No PLXND1-coding mutations were identified in HCFP1 families, although their genetic defect maps to the PLXND1 locus, suggesting the presence of regulatory mutations affecting the spatiotemporal expression of PLXND1 during embryonic brain development.

The clinical features of patients with heterozygous PLXND1 and REV3L mutations are highly variable with no obvious genotype–phenotype correlations. While the variable phenotype of patients P1, P5 and P9–P11 are consistent with the clinical criteria for MBS, patient P12, who was identified as having a heterozygous nonsense mutation in REV3L, only had bilateral facial paralysis and no weakness of the abducens nerves, suggestive of the restricted HCFP phenotype (Fig. 1; Supplementary Table 5). Thus, our data show that MBS and HCFP can be allelic conditions. In contrast to P12, the phenotype of patient P5, who was identified as having a heterozygous loss-of-function splice-site mutation in REV3L, presented with a wide range of features in addition to the cranial nerve palsies, including absence of digits on the left hand, Poland anomaly, hearing loss and cardiac defect. This variability is highly reminiscent of the variable phenotype of completely Rev3l-deficient embryos. Rev3l deficiency may result in a stochastic ablation of cell lineages during embryonic development.

**Figure 3 | Morphological analysis of the brains of Rev3l embryonic and newborn mice.** (a) Example of a sagittal section of the embryonic mouse head at E16.5 stage used for subarachnoid volume rendering, processed with Nissl staining. The subarachnoid space is shown in green. Scale bar, 1 mm. Right: subarachnoid volume rendering measures of both genotypes. In all Rev3l heterozygous embryos the subarachnoid space is significantly increased as compared with wt embryos (Unpaired t-test P value = 0.0047, N = 5). Scale bar, 500 μm (b) Example of one of the hindbrain sagittal sections at P0 used for hindbrain volume rendering. We measured the volume of five wt and five heterozygous mice. The hindbrain boundaries are indicated by dashed lines. Scale bar, 500 μm. Right: Rev3l heterozygous mice show a significant decrease of hindbrain volume (Unpaired t-test P value = 0.015, N = 5).

(c) Lateral hindbrain sections at the P0 stage of a view of the facial motor nucleus, inside the square. Scale bar, 300 μm. (d) Higher magnification of the facial motor nucleus showing paucity of Rev3l heterozygous motoneurons. Scale bar, 100 μm. Right: quantification of motoneurons in the facial motor nucleus showing paucity of Rev3l heterozygous motoneurons. Scale bar, 100 μm. Right: quantification of motoneurons in the facial motor nucleus, in each side of the hindbrain, in five wt and five heterozygous P0 mice (mean ± s.d.). The difference is statistically highly significant (Unpaired t-test P value < 0.0001, N = 10). We do not show both genotypes because there are no visible differences between them. 5N, trigeminal nucleus; 7N, facial nerve; Cb, cerebellum; HB, hindbrain; Is, isthmus; r1–r11, rhombomeres r1 to r11; Sb, subarachnoid space; Sk, skull; SpC, spinal cord. A P value smaller than 0.05 was considered to be statistically significant (*). P values smaller than 0.01 (**) or 0.001 (***) were considered highly significant.
Figure 4 | Survival, replication of bulky DNA lesions and DNA damage signaling in Rev3l-mutated MEF lines. (a) Survival of wt (+/+), Rev3l heterozygous (+/-) and Rev3l-deficient (-/-) MEF lines after mock exposure, or after exposure to Benzo(a)pyrene diolepoxide (BPDE). Rev3l heterozygous MEFs are slightly sensitive to BPDE indicating haploinsufficiency. Experiments were performed in triplicate. Error bars, s.e.m. The asterisks indicate a significant difference between the wt and the heterozygous lines (unpaired t-test, P<0.05). (b) Representation of replication fork progression after exposure to BPDE, or mock exposure. Rev3l heterozygous MEFs display normal fork progression at undamaged DNA (mock treated, left panel), but a marginal defect in replication of bulky lesion-containing DNA (BPDE, right panel). Error bars, s.e.m. (c) Immunoblots to detect the phosphorylation of DNA damage-signalling markers upon treatment with BPDE. γ-H2AX: phosphorylated histone H2AX. RpaS4/8-P: 32 kDa subunit of single-stranded DNA-binding protein Rpa, phosphorylated at Ser 4 and/or Ser 8. Total levels of Rpa are somewhat variable consequent to slight differences in proliferation rate between the lines. Chk1S354-P: Checkpoint kinase-1, phosphorylated at Ser 354. β-actin: loading control. Rev3l-heterozygous MEFs display DNA damage signalling that is almost as strong as Rev3l-deficient MEFs.

development. On the basis of these and our current data, it is conceivable that the spectrum of MBS symptoms reflects the stochastic loss of neuronal precursors, caused by replicative stress at endogenous DNA lesions and consequent DNA damage responses, during embryonic development. Likewise, the variable clinical presentation that is seen for MBS could be due to similar stochastic disruptions of motoneuron migration to the FBM nucleus.

Exome sequencing has detected causative de novo mutations in two (or three including the CCDC160 mutation) in the initial cohort of eight MBS patients. This result is of general interest, as previous studies have suggested previously that vascular disruptions during the first trimester of pregnancy rather than genetic mechanisms are the leading cause of MBS. While vascular disruptions caused by various teratogens can explain the MBS phenotype in some patients, our current data indicate that exome sequencing in parent–patient trios is a powerful approach for identifying causative mutations in MBS. It is to be expected that whole-genome sequencing will deliver an even higher yield when focusing on de novo mutations in coding and noncoding regions. That only three de novo mutations were identified in PLXND1 and three in REV3L in our cohort suggests a low frequency of mutations in these genes as a cause of MBS, implying genetic heterogeneity and intrauterine insults as causative events in other MBS patients. We expect that the implementation of whole-exome sequencing and whole-genome sequencing of parent–patient trios in a diagnostic setting will further dissect the aetiology of MBS.

Methods

Patients. We identified eight isolated Möbius patients in this study. They were the only affected individuals in their families. These patients (patients 1–8) were selected from a cohort of patients with the pathognomonic signs of MBS: facial and abducens palsy. Clinical details of these patients and other patients carrying de novo mutations (P9, P10, P11 and P12) are given in the Supplementary Table 5 and in Fig. 1a. Informed consent was obtained from all the subjects involved in this study, and all procedures were in accordance with the ethics of the World Medical Association Declaration of Helsinki. Ethical approval for the reported studies was obtained from the Medical Ethical Committee Arnhem-Nijmegen, from the University of Parma, the University of Istanbul and University Hospital LA FE, Valencia.

Whole-exome sequencing. Exome sequencing was performed on DNA from peripheral blood, using the SureSelect Human All Exon v2 50 Mb Kit (Agilent, Santa Clara, CA) followed by multiplexed analysis on a SOLiD 4 System sequencing slide (Life Technologies, Carlsbad, CA) as described previously. Colour space reads were mapped to the hg19 reference genome with the SOLiD BisoScope version 1.3 software, which utilizes an iterative mapping approach. Sequence analyses from all the patients are summarized in Supplementary Table 4. Statistical single-nucleotide variants were subsequently called by the Di-Bayes algorithm with high call stringency. Called variants were combined and annotated with a custom
analysis pipeline. The total number of variants identified in each patient are summarized in Supplementary Table 1. Of these variants, some were located in the exonic sequences or located in canonical splice sites, defined as the dinucleotides up- and downstream of exonic sequence (Supplementary Table 1).

Under the hypothesis of de novo mutations as the underlying cause of MBS syndrome, we excluded all variants inherited from either parent in the two trios (Supplementary Table 1). From the remaining variants in these two patients, we excluded synonymous variants, as well as variants present in (single-nucleotide polymorphism database) dsSNP (v138) or in our in-house-sequenced exome database (5,031 exomes), and variants in noncoding regions with the exception of canonical splice-site sequences. Manual inspection of mapped reads excluded additional, likely false positive, variants. After Sanger sequencing, only two variants were validated de novo mutations in one of the trios.

The approach for the six isolated cases was slightly different. For these, we applied a filtering scheme excluding variants observed in dsSNP (v138) and in our in-house database (5,031 exomes) (Supplementary Table 1). We further analysed all truncating variants, missense variants with a PhyloP score > 2.5 or those affecting genes with a possible involvement in MBS, as suggested by the gene ontology term or mouse mutant phenotype. Candidate variants were validated by Sanger sequencing, and parental DNAs were tested for de novo occurrence of the respective variant (Supplementary Tables 2 and 3).

The reference gene sequences referred to in this study are available in the RefSeq database under the following accession code: PLXND1, NM_015130; REV3L, NM_002912; CCDC160, NM_001101357; SPO11, NM_012444 and IFT172, NM_015662.

Mutation analysis. Primer sequences were designed using Primer3 software (http://frodo.wi.mit.edu/) encompassing the candidate variants. To exclude additional changes in either REV3L, PLXND1 or CCDC160, we designed primers for the amplification of all exons and intron–exon boundaries of REV3L (Genbank ID NM_002912), PLXND1 (Genbank ID NM_015130) and CCDC160 (Genbank ID NM_001101357). Sequences of primers are shown in Supplementary Table 6. PCR was performed on 50 ng of genomic DNA with Taq DNA polymerase (Invitrogen, Carlsbad, CA). A nucleofast 96 PCR plate (Clontech, Mountain View, CA) was used to purify the PCR amplicons, according to the manufacturer’s protocol. Sequence analysis was performed with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM 3730 DNA Analyzer (Applied Corp, Foster City, CA). PCR products were sequenced by Sanger sequencing and analysed using Vector NTI software (Life Technologies).

Cell culture. EBV-LCL cells from patient P5 and five controls were cultured to a density of 0.5 × 10^6 cells per ml. After incubation for 4 h at 37 °C, cells were harvested by centrifugation at 200g for 5 min at room temperature, washed once with PBS, pelleted by centrifugation at 200g for 5 min at room temperature and snap frozen in liquid nitrogen. MEFs were obtained from E13.5-day-old embryos of interested Rev3l−/− mice. The embryos were finely minced, trypsinized and the resulting MEFs were cultured in DMEM supplemented with 10% fetal calf serum and antibiotics until spontaneous immortalization47.

First-strand synthesis. EBV-LCL cells were harvested by centrifugation at 6,000g at room temperature. RNA isolation was performed using the NucleoSpin RNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocols. RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s protocol. Complementary DNA (cDNA) was purified with NucleoSpin extract II (Qiagen) according to the manufacturer’s protocol.

Quantitative PCR (QPCR) analysis. SYBR green-based real-time QPCR expression analysis was performed on an Applied Biosystems Fast 7500 System machine. Primer sequences around exon 10 were designed using Primer3 software (http://frodo.wi.mit.edu/) (Supplementary Table 6). Primers encompassed at least one boundary between two exons. GUSβ was used as a reference gene48. QPCR quantifications were performed in duplicate on the equivalent of 5 μg of total RNA from the first-strand synthesis and included a water control. Experimental three replicates (Ct) values were within the range of CDA/ΔCDA dilutions used to validate the primers. The melt curves of all PCR products showed a single PCR product. All water controls were negative. Differences in expression of the REV3L alleles were calculated by the comparative Ct or 2−ΔΔCt method59. The P value was derived from the standard score (Z-value) as compared with the normal distribution of the five controls. We used an α level of 0.05, because only one gene was assessed.

Influence of parental origin. Mouse breeding. Plxnd1 heterozygous mice were maintained on a C57BL/6 background and mated to generate Plxnd1 knockout and heterozygous embryos (male and female). Plxnd1 null and wt mouse embryos (E16.5) were harvested from timed matings. Noon was designated E0.5. Embryos were genotyped as described52. All Plxnd1 mouse work was performed according to the University of Pennsylvania animal care guidelines.

C57BL/6 wt, Rev3l heterozygous and Rev3l-deficient mice and embryos were described before36. C57BL6/16 Rev3l heterozygous mice were generated to mate to generate male and female embryos and newborn mice for analysis. Rev3l heterozygous and wt mouse embryos (E16.5) were derived from timed matings. Noon was designated E0.5. Embryos were genotyped as described36. Mice were treated using Federation of Animal Science Associations (FELASA) standards and mouse experiments were performed under permit DEC 11108 from the animal committee of the Leiden University Medical Center.

Mouse tissue preparation. Seven Plxnd1 wt, seven Plxnd1 heterozygous and seven Plxnd1 homozygous embryos at E16.5 (stage corroborated according to Thelberg29) were killed and brains were dissected in PBS at room temperature and fixed in 4% paraformaldehyde at 4 °C overnight. The next day, brains were washed in PBS, dehydrated in ethanol and stored at −20 °C. Brains were processed for paraffin and agarsore embedding and sectioned into 16- and 90-μm slices using a microtome and vibratome, respectively.

We analysed two different stages to compare phenotypic aberrations. Five wt and five Rev3l heterozygous embryos at E16.5 (stage corroborated according to Thelberg29) and five wt and five Rev3l heterozygous P0 newborns were killed and frozen by immersion in phosphate-buffered 4% paraformaldehyde (0.1 M; pH 7.4) at 4 °C for 24 h. Fixed brains and heads were embedded in paraffin and they were sectioned into 16-μm slices using a microtome.

Tissue staining. Seven Plxnd1 wt, seven Plxnd1 heterozygous and seven Plxnd1 homozygous embryos at E16.5, five Rev3l wt and five Rev3l heterozygous embryos at E16.5 (stage corroborated according to Thelberg29) and five Rev3l wt and five Rev3l heterozygous P0 newborns were embedded in paraffin, sectioned and were stained with Nissl stain according to routine protocols52.

Immunohistochemistry. Two Plxnd1 wt, two Plxnd1 heterozygous and two Plxnd1 homozygous embryos were embedded in agarose and processed for immunohistochemistry with the lact-1 antibody (catalogue number 80500, obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City). Sections were washed in PBS and then treated with 0.1% hydrogen peroxide in PBS for 1 h in the dark to inactivate endogenous peroxidase activity. After several rinses in PBS (with 0.2% Triton X-100), sections were blocked with 0.5% goat serum, 0.2% BSA and 0.2% Triton X-100 (Sigma, St Louis, MO, USA) in PBS for 4 h and then incubated overnight at 4 °C with monoclonal antibody anti-lact-1 (1:50 in blocking solution). Subsequently, sections were incubated with biotinylated goat anti-mouse secondary antibody (1:300, 2 h incubation; BA-9200, Vector Laboratories, Burlingame, CA, USA), and then with avidin–biotin-peroxidase complex (2,000, 2 h incubation; Vectastain-ABC kit; Vector Laboratories). All antibodies were diluted in the same blocking solution as the primary antibody. The histochemical detection of the peroxidase activity was carried out using 0.03% diaminobenzidine and 0.005% H2O2. After immunoreaction, the sections were mounted, dehydrated and then coverslipped with Eukitt (Fluka, Buchs, Switzerland).

Quantification of motoneurons. We counted the total number of motoneurons presents in the facial motor nucleus in paraffin sagittal sections processed with Nissl staining. We used five Plxnd1 wt, five Plxnd1 heterozygous and five Plxnd1 homozygous mice at E16.5 and five Rev3l wt and five Rev3l heterozygous P0 newborns, and we counted separately the number of motoneurons from the right and left hindbrain sides (around 12 sections each side) (20–25 slices total per side). For the counting we used the open-source ImageJ Cell Counter software52. Differences in the number of motoneurons between the two groups of mice and the wt mice were calculated by a comparative t-test, using Graphpad Software (http://graphpad.com/quickcalc/ttest1c.htm).

Brain structures volume rendering. We performed volume rendering of image stacks on serial light microscopic sections of 16 μm using the open-source image) TrakEM2 (ref. 53). For the volume rendering of the subarachnoid space around 100 sagittal slices of each of the five Rev3l wt and five Rev3l heterozygous mice at E16.5 were used. This embryonic stage was selected because the calcification of the cranial bones is not completed yet allowing the sectioning. For hindbrain volumes comparison, around 80 slices of each of the five Rev3l wt and five Rev3l heterozygous brains at P0 were used. After alignment of all images from each brain respectively, we highlighted the areas of interest in each section to measure their volume. The brain were delineated according to the Allen Developing Mouse Brain Atlas from rhombomere r1 until r11, including the cerebellum.
Survival assay. Rev3L survival assays were conducted with Rev3L+/−, Rev3L−/− and Rev3L+/+ MEFs. In each well of a six-well plate, 0.5 × 10⁶ exponentially growing cells were seeded in MEF medium (DMEM supplemented with 10% fetal calf serum, glutamx and antibiotics) and cultured overnight. Then, the cells were exposed to 0–300 nM BPDE (Biochemical Institute for Environmental Carcinogens, Grosshandorf, Germany) that was dissolved in anhydrous tetrahydrofuran (THF, Sigma-Aldrich) for 60 min in serum-free medium at 37°C. The cells were trypan blue stained and seeded in MEF medium at 10⁵ cells/dish. After culturing for 12–14 days at 37°C, cell clones were fixed, stained with methylene blue and the number of cell clones was counted (n = 3). The number of clones after mock treatment was set at 100%. The data were obtained with two and three independent Rev3L+/− and Rev3L+/+ MEF lines, respectively, and one Rev3L−/− MEF line.

Replication progression assay. Progression of replications using the Alkaline DNA unwinding assay was determined following seeding of 5 × 10⁴ exponentially growing cells per well in a 24-well plate and culturing overnight in MEF medium at 37°C. The cells were mock treated with the solvent THF or exposed to 500 nM BPDE in serum-free medium for 15 min at 37°C. The medium was removed, cells were washed once with 150 mM NaCl and radio-labeled with [3H]thymidine (2 μCi mL⁻¹; 76 Ci mmol⁻¹) in MEF medium for 15 min at 37°C. MEF medium with radioactive thymidine was removed and the cells were cultured in MEF medium supplemented with 10 μM cold thymidine. At different times after pulse labeling, the local unwinding of DNA ends of elongating replication forks, the separation of single-stranded DNA and double-stranded DNA (dsDNA) using hydroxylapatite columns and the determination of radioactivity in the fractions containing single-stranded DNA and dsDNA was performed (n = 3). The percent of maturation of replications was calculated by the following equation: (radioactivity in dsDNA/total radioactivity) × 100%. The data were obtained with single Rev3+/− and Rev3+/+ MEF lines and two Rev3−/− MEF lines.

Western blotting. In each well of a six-well plate, 2.5 × 10⁵ exponentially growing cells were seeded in MEF medium and cultured overnight. Then, the cells were mock treated with the solvent THF or exposed to 500 nM BPDE for 15 min in serum-free medium at 37°C. After rinsing the cells with PBS, cells were cultured in MEF medium. At different times after treatment, up to 12 h, cells were lysed using RIPA buffer. Lysates were clarified by centrifugation at 20,000 g for 15 min at 4°C. Forty micrograms per lane of total protein was separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C-extra, Amersham Biosciences). The membranes were incubated overnight at 4°C with antibodies specific for γH2AX (S129) (mouse monoclonal; Millipore; cat. no. 05-0636; dilution 1:1,000), phosphorylated Chk1 (at serine 345; rabbit polyclonal; Cell Signaling; cat. no. 9531S; dilution 1:1,000), phosphorylated Rpa2 (at serines 4/8; rabbit polyclonal; Millipore; cat. no. AB-2024-SA; dilution 1:1,000), total Rpa2 (rat polyclonal; Cell Signaling; cat. no. 2208; dilution 1:1,000), and β-actin (mouse monoclonal; Oncogene; cat. no. C010; dilution 1:20,000). After incubation with appropriate secondary antibodies conjugated to peroxidase (Bio-Rad), proteins were visualized using enhanced chemoluminescence detection. Uncropped scans with indicated size markers are shown in Supplementary Fig. 5.

Statistical analysis. The probability of finding two mutations in a cohort of 103 in each gene was calculated by the Poisson test. We took into account the presence of two alleles and estimated gene mutation rates based on recent exome sequencing data34. Moreover, a conservative Bonferroni correction was done for multiple testing.

Statistical significance of differences in motoneuron counts between the wt mice and the mutant mice were determined by the unpaired t-test. The significance of differences of the hindbrain volume and subarachnoid space between the Rev3L heterozygous and the wt mice was calculated by the comparative t-test. Significance in sensitivities of MEF lines to BPDE and in replication fork progression after exposure to BPDE was determined using the unpaired t-test, α = 0.05. A P value smaller than 0.05 was considered to be statistically significant (*), and a P value smaller than 0.01 (**) or 0.001 (***) were considered highly significant.

References

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Author contributions


Additional information

Accession numbers: The novel exome sequencing data generated in this study has been deposited at the European Genome-phenome Archive [http://www.ebi.ac.uk/ega/] under accession numbers EGAS00001001250.

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