De novo mutations in PLXND1 and 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, PLXND1 and REV3L in MBS patients. PLXND1 and 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 Plxnd1 and 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 PLXND1 and REV3L mutations are responsible for a proportion of MBS patients suggests that de novo mutations in other genes might account for other MBS patients.
Möbius syndrome (MBS) (MIM 157900) is a rare congenital cranial dysinnervation disorder characterized by non-progressive facial palsy and impairment of ocular abduction, due to paralysis or weakness of the facial (n7) and abducens (n6) nerves, and frequently other cranial nerves1–4. 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 misoprostol5 or cocaine6 exposure during pregnancy, has been hypothesized to lead to hindbrain hypoxia, resulting in cranial nerve dysfunction5. A limited number of familial cases with an atypical manifestation of MBS have been reported6–13. In addition, a founder mutation in HOX B1 has been associated with an autosomal recessive form of an MBS-like syndrome, with limited phenotypic overlap with typical MBS14. However, the vast majority of patients has a sporadic occurrence can be secondary to misoprostol7 or cocaine8 exposure during pregnancy, because biallelic mutations are associated with the unrelated Jeune and Mainzer-Saldino Syndromes in humans9,10. 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_001101357) (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_0029121) (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 screened 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 screening 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_4455GC>CA; 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 proteins14. 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) (Fig. 1; Supplementary Fig. 1; Supplementary Tables 2 and 3). The presence 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 mutations15. The amino acids that are affected by the missense variants in REV3L and PLXND1 are highly conserved throughout evolution (p.Glu387Gly (P11), p.Asn1895Lys (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 0.0001217 for REV3L (P value = 2.4 × 10−4) and 9.34e−85 for PLXND1 (P value = 1.8 × 10−8). 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
**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 immunoglobulin; 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 protein (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 Rev3l partner Rev7; ZF, zinc finger.

**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 dentritic cells. Sema3E/PlexinD1 signalling is involved in the development of two descending forebrain tracts, the corticofugal and the striatonigral tracts. To determine the effect of deficiency of **PLXND1** in central nervous system development, we compared morphologic structures of the embryonic brain of wild-type (wt), heterozygous and homozygous **PlexinD1** knockout mice at E16.5. Histological comparison of the three genotypes revealed consistent hypoplasia of the corpus callosum, anterior commissure and fasciculus retroflexus in **Plxnd1**−/− mice (Fig. 2a). These three brain structures are bundles of neural fibres that connect brain areas. Both the corpus callosum and the anterior commissure allow interhemispheric communication, whereas the fasciculus retroflexus links the forebrain with the midbrain.

**Plxnd1** mutant mice show a defect of neuronal migration. The facial nerve phenotype in **Plxnd1** heterozygous and homozygous mice was investigated. Analogous to observations in brain autopsies of an MBS patient, we observed a significant decrease in the number of motoneurons in the facial branchiomotor nucleus in homozygous mice as compared with wt mice (Unpaired t-test P value = 0.0049; Fig. 2b). The development of the cranial nerve undergoes a complex migration within the rodent hindbrain. 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. To trace the nature of the decreased neuronal density of the FBM, we carried out an immunohistochemical analysis using the motoneuron-specific molecular marker Isl1 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**−/− mutant 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.
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. 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. 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. 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[a]pyrene diolepoxiperoxidase (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). 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. 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 were also seen in patients P1 and P9, and which are commonly seen in other MBS patients (Supplementary Table 5). In addition, the vertebral defects in Plxnd1 mutant mice are reminiscent of the scoliosis in patient P1, and the
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.
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 PLXND1 mutations might 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 several 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 intraterine 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 BiosoScope 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

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**Figure 4 | Survival, replication of bulky DNA lesions and DNA damage signaling in Rev3l-mutated MEF lines.** (a) Survival of wt (+/+) and Rev3l-deficient (−/−) MEF lines after mock exposure, or after exposure to Benzo(a)pyrene diolepoxyde (BPDE). Rev3l heterozygous MEFs are slightly sensitive to BPDE indicating a more efficient response in parental lines. Error bars, s.e.m. (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 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.

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The approach for the six isolated cases was slightly different. For these, we applied a filtering scheme excluding variants observed in dbSNP (v138) and 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.

Mouse tissue preparation. Seven Pldnl1 wt, seven Pldnl1 heterozygous and seven Plxnd1 homozygous embryos at E16.5 (stage corroborated according to Theiler50) 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 agars embedded 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 Thelier50) and five wt and five Rev3l heterozygous P0 newborn pups were killed and fixed 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 Pldnl1 wt, seven Pldnl1 heterozygous and seven Plxnd1 homozygous embryos at E16.5, five Rev3l wt and five Rev3l heterozygous embryos at E16.5 (stage corroborated according to Thelier50) and five Rev3l wt and five Rev3l heterozygous P0 newborn pups were embedded in paraffin, sectioned and were stained with Nissl stain according to routine protocols52.

Immunohistochemistry. Two Pldnl1 wt, two Pldnl1 heterozygous and two Plxnd1 homozygous embryos were embedded in agar and processed for immunohistochemistry with the Ila-1 antibody (catalogue number 80779510; Invitrogen, Carlsbad, CA). We used the Ila-1 antibody to detect the number of motoneurons in the developing spinal cord. Immunohistochemistry was performed on E16.5 embryos (Genbank ID NM_002912; CCDC160, NM_015662; SPO11, NM_012444 and IFT172, NM_015662).

Quantitative PCR (QPCR) analysis. SYBR green-based real-time QPCR expression analysis was performed on an Applied Biosystem Fast 7500 System machine. Primer sequences around exon 10 were designed using Primer3 software (http://frodo.wi.mit.edu/) encompassing the candidate variants. To include 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_015103) and CCDC160 (Genbank ID NM_015662). Sequences of primer pairs 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 (Clonetech, 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 PRIS 3730 DNA Analyzer (Applera 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 interest 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 immortalization57.

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 Biosystem 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. GUSB 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 threshold cycles (Ct) values were within the range of cDNA 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 method53. 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.

Reverse transcriptase–PCR. cDNA from patient 1 EBV-LCLs and one control were used to amplify REV3L. The first pair of primers was designed surrounding the canonical splice-site mutation (c.1096+1G>A) in exon 10, and a control pair of primers was designed within exon 14 (see Supplementary Table 6).

Mouse breeding. Pldnl1 heterozygous mice were maintained on a C57BL/6 background and mated to generate Pldnl1 knockout and heterozygous embryos (male and female) for analysis. Male and female newborns were harvested from timed matings. Noon was designated E0.5. Embryos were genotyped as described52. All Pldnl1 mouse work was performed according to the University of Pennsylvania animal care guidelines.

C57BL6 wt, Rev3l heterozygous and Rev3l-deficient mice and embryos were described before36. C57BL6/E16.5 Rev3l heterozygous mice were mated to generate male and female embryos and newborns for analysis. Rev3l heterozygous and wt mouse embryos (E16.5) were derived from timed matings. Noon was designated E0.5. Embryos were genotyped as described52. Mice were treated using Federation of Laboratory Animal Science Association (FLASA) standards and mouse experiments were performed under permit DEC 11108 from the animal committee of the Leiden University Medical Center.

Mutation analysis. Primer sequences were designed using Primer3 software (http://frodo.wi.mit.edu/) and used to identify mutations by NGS of all exons of the candidate genes. A total of 100,000 reads were obtained, of which 42,319 were unique. In total, 20,203 reads were mapped, corresponding to a coverage of ≥4×, and 8,732 high-quality reads were found that mapped to a single position. A total of 8,371 variants were identified at ≥2× depth, of which 3,486 were unique. From the remaining variants, 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 common synonymous variants, as well as variants present in ≥2% of the 5,031 exomes. Manual inspection of mapped reads excluded likely false positive, variants. After Sanger sequencing, only two variants were validated de novo mutations in one of the trios.

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Survival assay. Rev3L survival assays were conducted with Rev3L+/−, Rev3L+/+ and Rev3L−/− MEFs. In each well of a six-well plate, 0.5 × 10^4 exponentially growing cells were seeded in MEF medium (DMEM supplemented with 10% fetal calf serum, glutamates and antibiotics) and cultured overnight. Then, the cells were exposed to 0–300 nM BPDE (Biochemical Institute for Environmental Carcinogens, Grosshadendorf, Germany) that was dissolved in anhydrous tetrahydrofuran (THF, Sigma-Aldrich) for 60 min in serum-free medium at 37°C. The cells were trypsinized and seeded in MEF medium at densities in 9-cm dishes. 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 replicons using the Alkaline DNA unwinding assay was determined following seeding of 5 × 10^4 exponentially growing cells per well in a 24-well plate and culturing overnight in MEF medium at 37°C. The cells were mocked 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 pulse labelled with [3H]thymidine (2 μCi/ml; 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 replicons was calculated by the following equation: (radioactivity in dsDNA/total radioactivity) × 100%. The data were obtained with single Rev3L+/− and Rev3L−/− MEF lines and two Rev3L−/− MEF lines.

Western blotting. In each well of a six-well plate, 2.5 × 10^4 exponentially growing cells were seeded in MEF medium and cultured overnight. Then, the cells were mocked 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 Laemmli buffer. The proteins were separated by SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (Hybond-C extrastack, Merk, Darmstadt, Germany). 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. 2348; dilution 1:4,800; phosphorylated Rpa32 (at serines 4/8; rabbit polyclonal; cat. no. A300-245A; dilution 1:1,000), total Rpa (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 data24. 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 (*), P values smaller than 0.01 (**), or 0.001 (***). were considered highly significant.

References
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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.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.


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