Biallelic Mutations in DNM1L are Associated with a Slowly Progressive Infantile Encephalopathy

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ABSTRACT: Mitochondria are highly dynamic organelles, undergoing continuous fission and fusion, and mitochondrial dynamics is important for several cellular functions. DNM1L is the most important mediator of mitochondrial fission, with a role also in peroxisome division. Few reports of patients with genetic defects in DNM1L have been published, most of them describing de novo dominant mutations. We identified compound heterozygous DNM1L variants in two brothers presenting with an infantile slowly progressive neurological impairment. One variant was a frame-shift mutation, the other was a missense change, the pathogenicity of which was validated in a yeast model. Fluorescence microscopy revealed abnormally elongated mitochondria and aberrant peroxisomes in mutant fibroblasts, indicating impaired fission of these organelles. In conclusion, we described a recessive disease caused by DNM1L mutations, with a clinical phenotype resembling mitochondrial disorders but without any biochemical features typical of these syndromes (lactic acidosis, respiratory chain complex deficiency) or indicating a peroxisomal disorder.

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KEY WORDS: DNM1L; mitochondrial dynamics; mitochondrial fission; mitochondrial disorders; peroxisome

Mitochondria are subcellular organelles that create a highly dynamic network, resulting from the balance of fusion and fission processes. Mitochondrial dynamics plays a central role in several aspects of mitochondrial life and death, being important for regulation of mitochondrial biogenesis, mitophagy, apoptosis, interaction with endoplasmic reticulum, and many other functions [Archer, 2013]. Thus, it is not unexpected that impairment of mitochondrial dynamics may lead to human disorders, and in fact it has been implicated in many neurodegenerative diseases such as Parkinson’s and Huntington’s diseases [Itoh et al., 2013], but also in tumors [Boland et al., 2013] or other pathological conditions [Archer, 2013; Dorn, 2013]. Although in several cases altered mitodynamics processes are part of complex pathogenic mechanisms, there are some monogenetic forms affecting key players of this system [Burtt et al., 2015]. Among these, the two most common syndromes are caused by mutations in nuclear genes coding for proteins with a role in mitochondrial fusion: Charcot–Marie– Tooth disease type 2A caused by mutations in MFN2 (MIM #608507), encoding mitofusin 2, a GTPase of the mitochondrial outer membrane that joins together adjacent mitochondria; and optic atrophy caused by mutations in OPA1 (MIM #605290), encoding a GTPase that mediates fusion of the mitochondrial inner membrane. Regarding fission factors, there are only two reports describing biallelic mutations in MFF (MIM #614783), coding the mitochondrial fission factor, found in subjects with infantile Leigh-like encephalopathy [Shamseldin et al., 2012; Koch et al., 2016] and an increasing number of papers reporting dominant mutations in DNM1L (Dynamin-1-like, MIM #603850), [Waterham et al., 2007; Vanstone et al., 2016; Sheffer et al., 2016; Fahrm et al., 2016] and a single family with a lethal disorder due to recessive DNM1L mutations [Yoon et al., 2016]. In addition, there are other genetic disorders that have been associated with disturbed mitochondrial dynamics [Burtt et al., 2015]: optic atrophy with premature catacaacts, caused by mutations in OPA3 (MIM #606580), encoding a protein with a still undefined function but involved in regulating the shape of mitochondria; and Charcot–Marie–Tooth disease type 2K caused by mutations in GDAP1 (MIM #606598), encoding a mitochondrial protein that regulates mitochondrial dynamics, critical for the proper function of myelinated peripheral nerves.

We describe here two brothers with psychomotor delay, ocular and cerebellar involvement, carrying compound heterozygous mutations in DNM1L. The corresponding protein DNM1L (also known as DRP1, Dynamin-Related Protein 1), is a member of the dynamin superfamily of GTPases, which mediate membrane remodeling during a variety of cellular processes. It has the typical structure of the dynamin-related proteins, with a large GTPase domain, a middle domain, and a GTPase effector domain (GED). DNM1L is a cytosolic protein that translocates to organelle membranes when activated, then oligomerizes into ring structures that wrap around the scission site and cut the membrane through a GTP-dependent mechanism [Heymann and Hinshaw, 2009]. In addition to mitochondrial fission, DNM1L also contributes to the division of peroxisomes [Li and Gould, 2003].

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Detailed methods for biochemical assays, molecular genetics, structural and protein analyses, fluorescence microscopy, and functional studies in yeast (Supp. Table S1) are reported as online supporting information. The study was approved by the Ethical Committee of the Besta Neurological Institute, Milan, Italy, in agreement with the Declaration of Helsinki. Written informed consent was obtained from the parents of the patients.

Patients are two siblings, second (II–2) and third (II–3) children of non-consanguineous parents (Fig. 1A). Family history was reported unremarkable; an older brother (II–1) was in good health.

Patient 1 (P1, II–2), now 16 years old, was born after uncomplicated pregnancy and delivery; the neonatal period was normal. His early development was referred normal, but after 1 year of age psychomotor delay became evident and he was unable to walk alone. At 2 years, strabismus was noticed. During childhood, mild cognitive impairment became evident. First evaluation was performed at 3 years of age: he needs assistance to walk and presented ataxia and hyper-reflexia at the lower limbs. Electroencephalography, electroretinogram (ERG), visual and brainstem auditory evoked potentials, and cerebral MRI were normal. He was first evaluated in our institute at 6 years of age. Neurological examination showed mild progression of neurological signs: he presented oculomotor apraxia, dysarthria, dysmetria, pyramidal signs and mild cognitive impairment, and he needed assistance to walk because of spastic–ataxic gait. MRI disclosed bilateral minor T2-hyperintensities in the subthalamic nucleus. MR spectroscopy was not performed. Blood routine exams, amino acids, lactate and pyruvate serum levels, as well as urinary organic acids were normal. Suspecting a mitochondrial disease, a musculo-cutaneous biopsy was performed: an isolated parastomal myopathy was documented, whereas all mitochondrial respiratory chain (MRC) complex activities were normal in muscle (Supp. Table S2). Treatment with coenzyme Q10 and riboflavin was started but no clinical benefits were observed. Instrumental exams performed 1 year later (7 years of age) were stable, except for MRI, which was normal, and visual evoked potential that showed bilateral increased latency; fundus oculi and ERG were confirmed normal. The family refused further medical imaging examinations. The last clinical evaluation was performed at 16 years of age, and the patient was stable. Only starting from 12 years of age, the biochemical assays showed elevated lactate (3,551–3,358 µmol/l, n.v. 580–2,100 µmol/l) and pyruvate (190–175 µmol/l, n.v. 40–140 µmol/l) serum levels. Very long chain fatty acids, phytic, and pristanic acid plasma levels, performed at 16 years of age, were normal.

Patient 2 (P2, II–3), now 3 years old, is the younger brother, born after uncomplicated pregnancy and delivery. His early development was referred normal, but after 1 year of age, psychomotor delay became evident and he was unable to walk and walk alone. Cognitive development was referred normal. At 14 months, strabismus became evident. Neurological examination at 3 years of age confirmed psychomotor delay and showed association of cerebellar and pyramidal signs: bilateral upper limb dysmetria, mild dysarthria, trunk titubation, diffuse hyper-reflexia; autonomous gait was not achieved, and walking was possible only with support. Routine exams (fasting glucose, electrolytes, triglycerides, cholesterol), amino acids, very long chain fatty acids, lactate, and pyruvate, phytic, and pristanic acid plasma levels were normal.

Previous molecular analyses of ATM (ataxia-telangiectasia mutated, MIM #607585) and APTX (apraxin, MIM #606350) genes were negative. The DNA of P1 was subjected to targeted resequencing of 94 nuclear genes associated with mitochondrial disorders; 385 nucleotide variants were found, 25 of them with a frequency <1% in public databases and predicted to cause missense or nonsense changes, frame-shifts or to affect splicing. Since the pedigree was suggestive for a recessive trait, we looked at genes with a homoyzogous variant or two heterozygous variants: this filtering procedure identified a single entry, DNM1L (NM_012062.4), where a transition c.106A→G (chr12:32854352) and a small deletion c.346_347delGA (chr12:32861135-32861136) were present. The first variant is predicted to cause an amino acid change p.Ser36Gly (NP_036192; Supp. Fig. S1A), affecting a highly conserved residue (Supp. Table S3) and with high scores of pathogenicity, according to different bioinformatics tools (Supp. Table S4). Ser36 lies close to the β-phosphate group of GDP. Structural analysis indicated that the hydroxyl group of the serine side chain can form a hydrogen bond with the oxygen of the carbonyl group of Thr33, suggesting an architectural role for Ser36. However, according to the structure resolved by Kishida and Sugio (http://www.rcsb.org/pdb/explore/explore.do?structureId=3W6P), it is possible that the hydroxyl group of Ser36, which together with Ser35, Gly37, and Lys38 forms a tight loop around the β-phosphate [Wenger et al., 2013], can make a moderate H-bond with the oxygen of the β-phosphate group of GDP, suggesting a potential role of Ser36 in contributing to the binding of GTP/GDP. Independently from the specific role of the hydroxyl group of the Ser36 side chain, substitution with glycine inhibits the formation of the H-bond with Thr33 or with the β-phosphate group of GTP/GDP (Supp. Fig. S1B).

The deletion of two nucleotides is expected to cause a shift in the reading frame with the introduction of a premature stop codon (p.Glu116Lysfs*6) and the loss of about 85% of the protein sequence, including the GED, the middle domain and most of the GTase domain (Supp. Fig. S1A). By Sanger sequencing, the two variants were detected also in the affected brother P2, whereas the father was heterozygous for the missense change and the father was heterozygous for the deletion (Fig. 1A).

Because no residual material was available from muscle biopsy, to evaluate the effect of the identified DNM1L variants on transcript and protein, we studied fibroblasts from P1. Using cDNA obtained from P1 RNA, we amplified the DNM1L transcript; its sequencing revealed almost exclusive expression of the maternal allele, whereas the expression of the paternal allele, carrying the deletion c.346_347delGA, was strongly reduced (Fig. 1B), possibly because of nonsense-mediated mRNA decay.

Accordingly, the total amount of DNM1L protein detected by Western blot analysis was reduced in P1 fibroblasts compared with controls (Fig. 1C); these findings suggest the almost exclusive expression of the mutant protein with the missense change p.Ser36Gly in the patient’s samples.

Finally, in total DNA extracted from fibroblasts, we quantified the amount of mtDNA by quantitative PCR. In P1, the mtDNA content was partly but significantly lower than controls (~50% of the mean control value) (Fig. 1D).

Given the role of DNM1L in regulation of the dynamics of mitochondria and peroxisomes, we performed microscopy fluorescence studies on P1 fibroblasts and analyzed these organelles by ad hoc imaging software. First, we stained mitochondria with a specific dye (Mitotracker red). Using cells cultured in normal glucose medium, we observed a similar filamentous mitochondrial network in mutant and control fibroblasts, although the shape factor of mitochondria was slightly lower in mutant cells than controls, indicating a more elongated network. At higher magnification, the network of P1 cells showed a “chain-like” structure, not observed in controls (Fig. 2A). The immunofluorescence staining with the specific antibody against DNM1L showed a strongly reduced signal in P1 cells compared with controls, in accordance with Western blot experiments; no ev-
Figure 1. Molecular and protein studies in patients with DNM1L mutations. A: Pedigree of the family, with segregation of the identified DNM1L variants. Black symbols indicate affected subjects. B: Electropherograms of the regions containing the DNM1L mutations identified in genomic DNA from patient 1 (gDNA, upper panels) and in complementary DNA, retro-transcribed by RNA obtained from patient’s fibroblasts (cDNA, lower panels). C: Immunoblot analysis of total lysates from control (CT1, CT2, and CT3) and patient’s (P1) fibroblasts using α-DRP1, α-HSP60, α-VDAC1, and α-GAPDH antibodies. The latter was used as loading control; VDAC1 and HSP60, mitochondrial proteins, are an index of mitochondrial content in each sample. D: mtDNA levels assessed by quantitative PCR in fibroblasts from P1 and three control subjects (CT). The mean value of the mtDNA/nDNA ratio obtained in the control subjects was set as 1. Two different mtDNA amplicons were used (blue and red bars). Error bars represent SDs.

ident or exclusive co-localization of DNM1L with mitochondria was observed, indicating that residual protein remained mainly cytoplasmic (Supp. Fig. S2). We also cultured cells in glucose-free galactose medium, a condition that forces cells to use the oxidative phosphorylation (OXPHOS) system and usually causes elongation of the mitochondrial network in control cells, whereas it leads cells with MRC defects to mitochondrial fragmentation. Even in this case, the mitochondrial network of mutant fibroblasts remained filamentous as in controls. In order to highlight any mitochondrial fission defect in P1 cells, we treated fibroblasts with different stressors (an uncoupling agent, FCCP, and an oxidant, H₂O₂) or by serum starvation: as expected, these stresses led to increased fragmentation of the mitochondrial network in control cells but the effect was less evident or absent in mutant cells, suggesting impairment of the mitochondrial fission machinery in the latter (Supp. Fig. S3).

Then, DNM1L was co-transfected with a GFP targeted to mitochondria in order to identify cells expressing the mitochondrial fission protein. The transient overexpression of wild-type DNM1L in P1 fibroblasts caused the fragmentation of the mitochondrial network, as seen in control cells (Supp. Fig. S4). This finding, pointing out that the impaired fission process detected in mutant cells can be restored if wild-type DNM1L is present, confirmed that dysfunctional DNM1L is the cause of the observed phenotype.

Finally, we used an antibody against a peroxisomal protein, PMP70, to visualize peroxisomes. In contrast with the highly diffused punctated staining observed in control cells, in P1 cells, organelles were larger and less uniformly distributed into the cytoplasm; moreover, they often presented as "dashes" rather than dots (Fig. 2B and Supp. Fig. S5A). Accordingly, the morphometric analysis showed reduced shape factor and increased area in the patient’s cells compared to controls (Supp. Fig. S5B).

To assess the pathogenicity of the p.Ser36Gly DNM1L variant, complementation studies were performed in a S. cerevisiae strain lacking DNM1, hereafter referred to as Δdnm1. DNM1 is the yeast ortholog of human DNM1L and the human p.Ser36 residue is conserved between the two species, corresponding in yeast to p.Ser39 (Supp. Table S3). Another change involving a neighboring residue, p.Lys38Ala, although not found in patients, has been reported to affect the function of DNM1, acting as a dominant negative mutation [Bourne et al., 1991]. In human DNM1L, and likely in the yeast
Dnm1p, human Lys38 (or yeast Lys41) is involved in an electrostatic interaction with the α- and β-phosphate of GTP/GDP (Supp. Fig. S1) [Wenger et al., 2013]. Thus, substitution of Lys38 with alanine strongly decreases the ability of the protein to bind GTP and completely inhibits the GTPase activity [Zhu et al., 2004].

Both mutant alleles, dnm1S39G and dnm1K41A, were constructed in order to compare the phenotype between the two mutations in the yeast model. The DNM1 null mutant, dnm1Δ, was transformed either with the wild-type DNM1, dnm1S39G or dnm1K41A alleles, under the endogenous DNM1 promoter, as well as with the empty plasmid. To test the possible effects on mitochondrial function, we first evaluated the oxidative growth by spot assay analysis on synthetic complete medium supplemented with either glucose or glycerol, at 28°C and 37°C. The oxidative growth of the dnm1Δ/dnm1S39G mutant was partially affected compared with the DNM1 wild-type strain, both at 28°C and 37°C, whereas the growth of the dnm1Δ/dnm1K41A mutant strain (Supp. Fig. 6A) was similar to the strain dnm1Δ. To further investigate the OXPHOS defect, the oxygen consumption was measured at 37°C, where a more severe phenotype was observed. According to the growth phenotype, the oxygen consumption rate of the dnm1Δ/dnm1S39G mutant was 30% lower than that of the wild-type strain, whereas the oxygen consumption rate of dnm1K41A and of the dnm1 null strain was decreased by 50% (Supp. Fig. 6B).

Then, because partial complex IV deficiency was observed in P1 fibroblasts, we measured COX activity, which was 15%–20% lower in the dnm1Δ/dnm1S39G mutant strain compared to the wild-type strain (Supp. Fig. 6C).

In the absence of the Dnm1 protein, cells are deficient in the fission process and display a network-like or linear mitochondrial morphology [Bleazard et al., 1999; Bernhardt et al., 2015]. To study this phenotype in the pathological mutant, we constructed a Δdnm1 strain which carries both a plasmid-encoding a mitochondrial targeted GFP, to allow visualization of the mitochondrial morphology (Supp. Fig. S7), and either DNM1 or dnm1S39G alleles or the empty plasmid. As expected, all the cells of the dnm1Δ strain showed a linear (≈60%) or network-like (≈40%) morphotype. Almost all the cells of the wild-type DNM1 strain (≈98%) showed a filamentous morphotype, which is typical of a wild-type strain. The strain harboring the dnm1S39G allele showed an intermediate phenotype, with a filamentous, linear or network-like morphotype of ≈15%, ≈50%, and ≈35%, respectively (Supp. Fig. 6D). To further confirm the fission defects, cells were treated with sodium azide, which causes fragmentation of mitochondria when the mitochondrial fission machinery is properly functioning. In most of the wild-type DNM1 cells, the mitochondrial network became fragmented upon treatment with this stressor whereas in the Δdnm1 strain the
original mitochondrial network was maintained in more than 90% of the cells (Supp. Fig. 6E). In the dnm1Δ mutant strain, the frequency of cells showing fragmented mitochondria was similar to the null strain.

Since mutations in several nuclear genes encoding for proteins involved in mitochondrial dynamics, such as MGM1 (the homologous gene of human OPAL1) [Nolli et al., 2015; Sesaki et al., 2003], Fzo1 (hMfn1) [Rapaport et al., 1998], and DNM1 itself [Bernhardt et al., 2015], affect mtDNA stability in yeast, we investigated whether the dnm1 mutations determine an increase of mtDNA extended mutability. We measured the frequency of petite, that is, clones which are unable to grow on an oxidative carbon source due to large deletions or lack of mtDNA. Deletion of DNM1 as well as the expression of dnm1Δ resulted in a significant increase of petite frequency (~50%–60%) compared with the wild-type strain, whereas expression of dnm1Δ902 partially increased the mtDNA instability (~25% at 28°C and ~40% at 37°C), which is, however, much higher than the DNM1 wild-type strain (~2% and ~8%, respectively) (Supp. Fig. 8A).

Finally, we tested whether the mutation Ser39Gly has a dominant or recessive effect. First, we observed that in the diploid hemizygous DNM1/dnm1Δ strain the petite frequency was double that of the homozygous wild-type strain, indicating haploinsufficiency. The petite frequency of the heteroallelic DNM1/dmn1Δ/dnm1Δ902 strain was intermediate, suggesting that the mutation is recessive due to partial loss of function. In contrast, the petite frequency of the heteroallelic DNM1/dmn1Δ/dnm1Δ902 was higher than that of the hemizygous strain (Supp. Fig. 8B), indicating that Lys41Ala, as previously reported [Frank et al., 2001], is partially dominant.

Although the biological function of DNM1L/DRP1 in mitochondrial and peroxisomal fission has been studied extensively, its associated human disease spectrum is only beginning to be clarified. Few patients with de novo dominant mutations in DNM1L have been reported [Waterham et al., 2007; Vanstone et al., 2016; Sheffer et al., 2016; Fahrer et al., 2016], and only one family with recessive nonsense DNM1L mutations [Yoon et al., 2016]. We identified and validated biallelic mutations in DNM1L in two brothers classified as having a mitochondrial disorder. The p.(Glu116Lysfs*6) allele appears to be a null allele, according to its (i) strongly reduced RNA expression and (ii) abolition of ~85% of DNM1L's coding frame, including about half of the catalytic GTPase domain and other functionally-essential domains. The p.Ser39Gly yeast allele (corresponding to p.Ser36Gly in humans) is hypomorphic, retaining partial activity; moreover, it behaves as a recessive trait. The parents (1-1, I-2) carried each a heterozygous DNM1L mutation and were completely healthy; the same applies for the parents of the recently reported siblings with recessive DNM1L mutations [Yoon et al., 2016], confirming recessive inheritance in these two families. Drp1 deletion in mice was embryonic lethal [Ishihara et al., 2009]. The analysis performed in yeast indicated that the dnm1 null mutant, although viable, shows a severe OXPHOS defect, whereas the missense mutation p.Ser36Gly is hypomorphic; this could explain the milder phenotype observed in our patients compared with the mouse model and to the recently published siblings with two nonsense DNM1L mutations [Yoon et al., 2016].

The first reported heterozygous dominant DNM1L mutation was associated with early onset lethal disease characterized by failure to thrive, hyporeactivity, hypotonia and optic atrophy, demyelination and abnormal cortical pattern at MRI, and death in the first month of age [Waterham et al., 2007]. A similar lethal disease was also reported in the recessive cases with complete deficiency of DNM1L [Yoon et al., 2016]. Additional cases with a de novo DNM1L mutation have been described, characterized by milder phenotypes: developmental delay, refractory epilepsy, normal brain MRI, and prolonged survival [Vanstone et al., 2016]; childhood-onset epileptic encephalopathy with diffuse cerebral atrophy [Fahrer et al., 2016] or postnatal microcephaly, developmental delay, and pain insensitivity [Sheffer et al., 2016]. In contrast, the patients we described presented a slowly progressive neurological disease, characterized by mild cognitive impairment, cerebellar and pyramidal signs and ocular involvement. In P1, MRI disclosed transient alteration at profound grey matter levels but was normal at following controls. Recessive versus dominant mutations, and hypomorphic alleles versus nonsense mutations or variants with different gain of function effects could partly explain the observed different phenotypes.

While the clinical and radiological presentation of our patients was suggestive for a mitochondrial disorder, the biochemical analyses were not so informative. Lactic acidosis was not observed in P2 (now 3 years old) and was evident in P1 only at 12 years. Notably, lactate levels were also normal in the recently published DNM1L mutant cases [Vanstone et al., 2016; Yoon et al., 2016, Fahrer et al., 2016], and elevated lactate is an inconstant feature in patients with mutations in MFF, another mitochondrial fission factor [Koch et al., 2016]. The activities of the MRC complexes were normal in P1 muscle, and only a partial deficiency of complex IV was detected in P1 fibroblasts. Likewise, in most of the previously reported DNM1L cases and in MFF mutant subjects, there was no defect in the MRC, suggesting that fission impairment does not impact strongly the OXPHOS system [Waterham et al. 2007; Vanstone et al., 2016; Koch et al., 2016; Yoon et al., 2016]. Interestingly, decreased complex IV activity has been recently reported in a DNM1L-mutant case [Sheffer et al., 2016]. In addition to unspecific biochemical data on mitochondrial involvement, we have no clue about a peroxisomal dysfunction; plasma phytic and pristanic acid concentrations, typically increased in subjects with peroxisomal disorders [Koch et al., 2003], were normal in our patients. Once again, this study confirms the usefulness of NGS for identification of the molecular defect in patients with very rare disorders, where knowledge of the clinical phenotype is inevitably limited.

Given its function, impairment of DNM1L is expected to cause a defect of mitochondrial and peroxisomal fission. Although not evident from the routine laboratory analyses, we were able to show impairment of these organelles in mutant fibroblasts by fluorescence microscopy studies. Interestingly, patients with mutations in either DNM1L or MFF showed a fission defect in both mitochondria and peroxisomes, highlighting a common mechanism for division of these organelles [Koch et al., 2003, 2016]. Although it is debated whether they are evolutionarily related, several functional similarities are emerging, indicating links of peroxisomes to mitochondria [Mohanty and McBride, 2013]. A broad range of cellular functions are dependent on dynamics of the mitochondrial network, including segregation and maintenance of mtDNA [Okamoto and Shaw, 2005]. Interestingly, in P1 fibroblasts we observed a reduced amount of mtDNA compared with controls. This result parallelsizes with the findings in yeast, where the presence of the mutation Ser39Gly was associated with an increase in cytoplasmic petite colonies, a phenotype that reflects deletion or large scale deletions of mtDNA [Lodi et al., 2015].

An NGS-based screening of genes associated with mitochondrial disorders identified compound heterozygous DNM1L mutations in two brothers presenting an infantile slowly progressive mitochondrial encephalopathy. Thus, DNM1L mutations have to be considered in cases with recessive mitochondrial diseases. Although we demonstrated aberrant peroxisomes in patients’ cells, we did not find clear signs or symptoms suggesting a peroxisomal disorder; nevertheless, we cannot exclude that the clinical presentation in our
patients may have been worsened by the additional defect in peroxisomal fission. Our report suggests a wider clinical picture related to DNM1L mutations, as suggested by the increasing number of DNM1L-mutant cases with peculiar phenotypes; all this information will lead to better define the spectrum of this disease.

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