

BRIEF REPORT

Biallelic variants in *DNA2* cause microcephalic primordial dwarfism

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Abstract

Microcephalic primordial dwarfism (MPD) is a group of rare single-gene disorders characterized by the extreme reduction in brain and body size from early development onwards. Proteins encoded by MPD-associated genes play important roles in fundamental cellular processes, notably genome replication and repair. Here we report the identification of four MPD individuals with biallelic variants in *DNA2*, which encodes an adenosine triphosphate (ATP)-dependent helicase/nuclease involved in DNA replication and repair. We demonstrate that the two intronic variants (c.1764-38_1764-37ins(53) and c.74+4A>C) found in these individuals substantially impair *DNA2* transcript splicing. Additionally, we identify a missense variant (c.1963A>G), affecting a residue of the ATP-dependent helicase domain that is highly conserved between humans and yeast, with the resulting substitution (p.Thr655Ala) predicted to directly impact ATP/ADP (adenosine diphosphate) binding by *DNA2*. Our findings support the pathogenicity of these variants as biallelic hypomorphic mutations, establishing *DNA2* as an MPD disease gene.

KEYWORDS

DNA repair, DNA replication, *DNA2*, growth, microcephalic primordial dwarfism

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1 | INTRODUCTION

Microcephalic primordial dwarfism (MPD) is an umbrella term for a group of rare monogenic disorders of extreme growth failure, characterised by marked microcephaly and short stature. MPD has been operationally defined in terms of both occipito-frontal circumference (OFC) and height being at least four standard deviations (*SD*) below the age- and sex-matched population mean (Faivre et al., 2002; Klingseisen & Jackson, 2011), although less restrictive criteria to encompass individuals with milder growth restriction have also been used (Shaheen et al., 2018). MPD encompasses several phenotypically distinct Mendelian disorders, such as Seckel syndrome (Majewski, Goecke, & Opitz, 1982; Seckel, 1960), microcephalic osteodysplastic primordial dwarfism Type 1 and Type 2 (Majewski, Ranke, Schinzel, & Opitz, 1982; Majewski, Stoeckenius, Kemperdick, & Opitz, 1982), and Meier-Gorlin syndrome (Gorlin, Cervenka, Moller, Horrobin, & Witkop, 1975). A phenotype continuum between primary microcephaly and MPD is also established for a number of genes (Shaheen et al., 2018; Verloes, Drunat, Gressens, & Passemard, 1993).

Proteins encoded by MPD disease genes participate in essential cellular processes, including DNA replication (Bicknell, Bongers, et al., 2011; Bicknell, Walker, et al., 2011; Burrage et al., 2015; Fenwick et al., 2016; Guernsey et al., 2011; Logan et al., 2018; Vetro et al., 2017), DNA damage response signalling and DNA repair (Harley et al., 2016; Murray et al., 2014, 2015; O'Driscoll, Ruiz-Perez, Woods, Jeggo, & Goodship, 2003; Ogi et al., 2012; Qvist et al., 2011; Reynolds et al., 2017). Collectively, variants in these genes are thought to cause disease by prolonging the cell cycle, with reduced cell proliferation resulting in a smaller number of cells throughout the body and brain, and therefore a smaller person (Klingseisen & Jackson, 2011).

The adenosine triphosphate (ATP)-dependent helicase/nuclease DNA2 is a multifunctional enzyme, involved in various aspects of DNA replication and repair, including Okazaki fragment maturation during lagging strand synthesis (Ayyagari, Gomes, Gordenin, & Burgers, 2003; Bae, Bae, Kim, & Seo, 2001; Gloor, Balakrishnan, Campbell, & Bambara, 2012), DNA end resection during double-strand break repair (Cejka et al., 2010; Karanja, Cox, Duxin, Stewart, & Campbell, 2012; Nimonkar et al., 2011; Niu et al., 2010; Sturzenegger et al., 2014), degradation of reversed replication forks to promote replication restart after genotoxic stress (Thangavel et al., 2015) and regulation of replication checkpoint activation (Duxin et al., 2012). Additionally, DNA2 has been implicated in mitochondrial DNA replication and repair (Duxin et al., 2009; Zheng et al., 2008). Most of the cellular functions of DNA2 have been attributed to its nuclease activity, whereas the role of DNA2 helicase activity long remained unclear. However, recently it was shown to act as an ATP-dependent translocase to promote rapid DNA degradation during DNA end resection (Levikova, Pinto, & Cejka, 2017; Miller et al., 2017). Unsurprisingly, because of its importance for genome replication and stability, DNA2 is essential for mammalian embryonic development (Lin et al., 2013). A homozygous intronic DNA2 variant

was previously reported as the likely causal variant for two related individuals diagnosed clinically with Seckel syndrome. This variant was shown to cause aberrant splicing and reduced DNA2 protein levels in patient cells, with cellular phenotypes rescued by transient wild-type DNA2 expression (Shaheen et al., 2014). Here, we report the identification of additional biallelic DNA2 (NM_001080449.2; MIM# 601810) variants in four unrelated MPD patients. Using cellular splicing assays and molecular modeling we provide evidence that these result in partial loss of function of the essential DNA replication/repair protein it encodes. Our work, alongside the findings of Shaheen et al. (2014), therefore establishes a causal link between DNA2 deficiency and impaired growth.

2 | RESULTS

From WES sequencing of 192 MPD patients without a molecular diagnosis, we identified three unrelated patients (P1, P3, and P4) with biallelic variants in DNA2 (Figure 1a and Table S1 and S2), with a fourth phenotypically similar patient (P2) identified through targeted resequencing of DNA2 in our cohort. No likely causative variants in other genes were evident in WES datasets from P1, P3, and P4 (Tables S3 and S4). The two novel intronic variants (NM_001080449.2:c.1764-38_1764-37ins(53) and NM_001080449.2:c.74+4A>C) and single missense variant (NM_001080449.2:c.1963A>G, p.Thr655Ala) were validated using Sanger sequencing (Figure 1b). Parents of the affected individuals were heterozygous for these variants, and segregation in unaffected siblings from P1 and P3 consistent with an autosomal recessive inherited disorder. None of the variants were present in the gnomAD (genome aggregation) database (Lek et al., 2016), establishing these alleles to be very infrequent in the general population, in keeping with a rare Mendelian disorder. All variants were submitted to the LOVD Global Variome database (<https://databases.lovd.nl/shared/genes/DNA2>).

The patients exhibited severe microcephaly with OFC ranging from -5.7 *SD* to -9.6 *SD*, as well as markedly reduced height, ranging from -4.6 *SD* to -11.1 *SD* (Table S1, Figure 1c). Three out of four patients with DNA2 variants were noted to have prominent, large upper incisors and a high frontal hairline, however a common facial gestalt was not otherwise apparent (Table S2, Figure 1d). No significant health problems were noted, aside from P3 who had severe thoracic kyphoscoliosis and recurrent chest infections (Table S2). All had normal intellectual development, with P3 and P4 now adolescents and P2 healthy when last met aged 46. Normal cognition, lack of a sloping forehead and proportionate OFC to height reduction led us to clinically classify these patients as MPD, rather than Seckel syndrome. Given the overlapping clinical phenotypes, we concluded that these variants were likely to be pathogenic, despite their predominantly intronic nature, and we next investigated the consequences of these variants on DNA2 messenger RNA (mRNA) splicing.

P1 and P2, not knowingly related, were homozygous for the same variant, a 53 bp insertion in the middle of the small (78 bp) intron 11

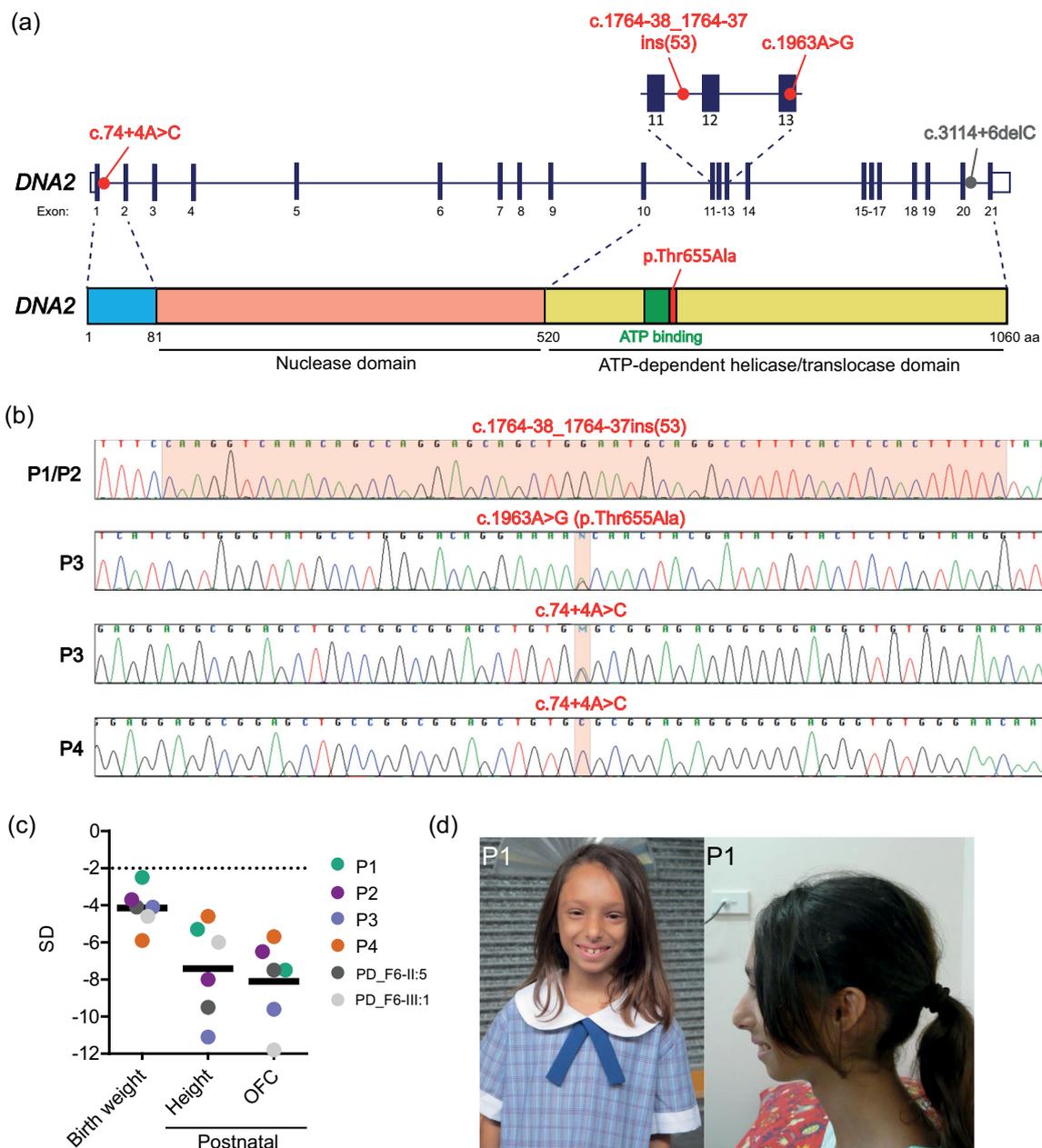


FIGURE 1 Identification of biallelic *DNA2* variants in microcephalic primordial dwarfism patients. (a) *DNA2* variants c.74+4A>C, c.1764-38_1764-37ins(53) and c.1963A>G (p.Thr655Ala, p.T655A) identified in four microcephalic primordial dwarfisms (MPD) patients are indicated in red on a schematic of the *DNA2* gene (NM_001080449.2), with the previously reported c.3114 + 6delC variant (Shaheen et al., 2014) shown in grey. Key domains and motifs are indicated on a cartoon model of the *DNA2* protein (NP_001073918) structure. (b) Sanger sequencing chromatograms demonstrating *DNA2* variants (marked in red) in MPD patients. (c) Affected individuals exhibit extreme reduction in birth weight, postnatal height, and occipito–frontal circumference (OFC), reflecting global growth failure of prenatal onset and extreme microcephaly (this study: P1–P4; Shaheen et al., 2014: PD_F6-II:5, PD_F6-III:1). Measurements plotted as Z-scores (standard deviation, SD, of measurement from population mean for age and sex). Black bars represent the mean. (d) Photographs of P1. Written consent to publish photographs was obtained from the family

of *DNA2* (c.1764-38_1764-37ins(53); Figure 1a and Table S1). SpliceSiteFinder-like, MaxEntScan, and Human Splicing Finder algorithms (Alamut Visual) predicted the creation of a new splice donor site after the first four bases of inserted sequence, suggesting that *DNA2* transcript splicing could be affected by this variant. As patient-derived cell lines were not available and attempts to generate a lymphoblastoid cell line from P1 peripheral blood leukocytes (PBLs)

were not successful, we used a minigene-based splicing assay to study the consequences and establish the pathogenicity of this variant. Minigene splicing reporters (Singh & Cooper, 2006) containing the genomic region covering *DNA2* exon 11 to exon 13 from a healthy control and P1 were constructed (Figure 2a). While the splicing control with a disrupted acceptor splice site of intron 11 (c.1764-1G>A) demonstrated complete abrogation of correct

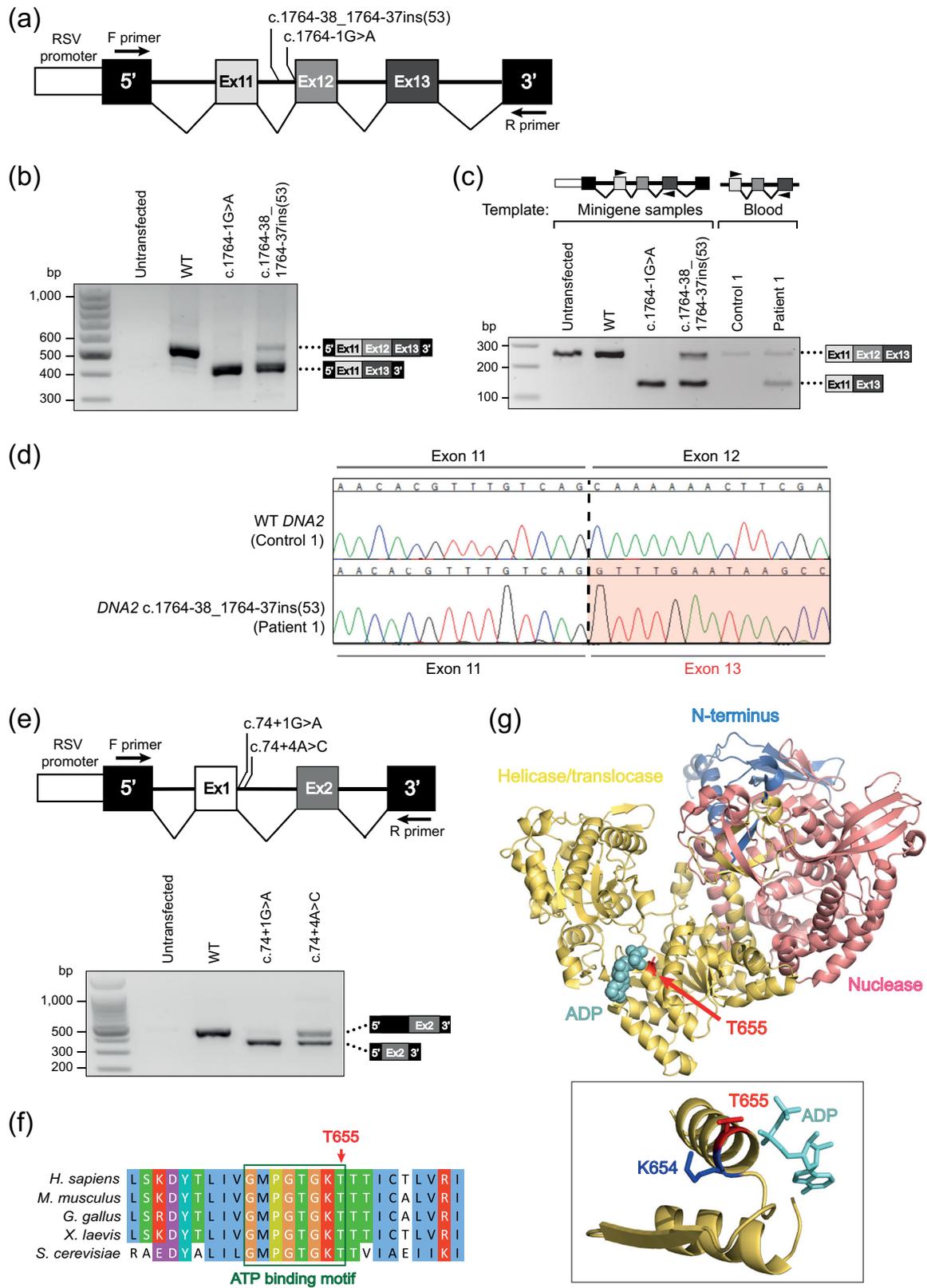


FIGURE 2 Continued.

splicing, the c.1764-38_1764-37ins(53) variant resulted in marked, but partial loss of correct splicing (Figure 2b). *DNA2* transcript analysis using RNA isolated from P1 PBLs also demonstrated altered splicing (Figure 2c), confirming that partial loss of correct *DNA2* splicing occurs as a result of this intronic variant. Capillary sequencing of reverse-transcription polymerase chain reaction (RT-PCR) products demonstrated correctly spliced mRNA for the wild-type control, with *DNA2* exons 11, 12, and 13 included (Figure 2b–d), while for P1, the majority contained full-length exons 11 and 13, but lacked exon 12 (Figure 2b–d), in keeping with the c.1764-38_1764-37ins(53) insertion causing substantial skipping of exon 12. Absence of exon 12 causes a frameshift leading to a premature termination codon in exon 13 (p.Ser588ArgfsTer4), which would promote degradation of such transcripts by nonsense-mediated decay and/or lead to the translation of a severely truncated protein missing the majority of the helicase domain, including the ATP binding site. Both would result in marked reduction of cellular levels of functional *DNA2* protein.

P4 was homozygous for a putative splice site variant in intron 1 (c.74+4A>C; Figure 1a and Table S1), predicted to be potentially deleterious (CADD score = 16.4). Furthermore, splice prediction analysis suggested that this variant would weaken the donor splice site of intron 1, and therefore result in aberrant splicing of *DNA2*. This was confirmed experimentally using another minigene assay (Figure 2e). Here, the c.74+4A>C mutant exhibited two RT-PCR products that corresponded to correctly spliced and incorrectly spliced transcript lacking canonical exon 1, respectively. Nevertheless, as a result of residual levels of correct splicing for both the c.74+4A>C and c.1764-38_1764-37ins(53) variants, low levels of wild-type transcript and protein would still be produced, in keeping

with *DNA2* function being essential for mammalian development (Lin et al., 2013).

Lastly, patient P3, who has the most severe reduction in height and the smallest head circumference (Table S1), was found to be compound heterozygous for the same c.74+4A>C variant *in trans* with a missense variant, c.1963A>G (p.Thr655Ala, p.T655A). P2 and P3 were phenotypically similar and not knowingly related, thus providing additional evidence to support pathogenicity of the c.74+4A>C variant. Likewise, there was strong evidence for the pathogenicity of the p.T655A substitution: the CADD score was 28.2, and given that threonine residue 655 is conserved to yeast and forms part of the ATP-binding motif of the *DNA2* helicase/translocase domain (Figures 1a, 2f), it was likely to be functionally critical. Furthermore, substitution of the neighboring lysine 654 residue (corresponding to lysine 671 in older literature based on previous nomenclature) abolishes *DNA2* ATPase activity, reducing the speed of ssDNA degradation (Levikova et al., 2017; Masuda-Sasa, Imamura, & Campbell, 2006). Therefore, substitution of the adjacent p.T655 might also be expected to affect hydrolysis of ATP, reducing the rate of translocation of *DNA2* along DNA, consequently diminishing nuclease-dependent degradation of ssDNA, and negatively impacting on DNA end resection. To investigate this possibility further, the crystal structure of mouse *DNA2* (80% overall sequence identity to human *DNA2*; 100% in the ATP-binding motif) bound to the ATP hydrolysis product, adenosine diphosphate (ADP; Zhou, Pourmal, & Pavletich, 2015) was examined.

Molecular modeling of the p.T656A variant (the mouse equivalent of human p.T655A) with FoldX (Guerois, Nielsen, & Serrano, 2002; Schymkowitz et al., 2005) predicted a negligible effect of the

FIGURE 2 Transcriptional and structural consequences of *DNA2* variants identified in MPD patients. (a) RHCglo minigene reporter constructs used in the minigene splicing assay to assess the effect of the *DNA2* c.1764-38_1764-37ins(53) variant. A positive control for splicing disruption was generated by introducing a point mutation, abolishing the acceptor splice site of *DNA2* intron 11. Arrows indicate primers used for RT-PCR analysis. (b) c.1764-38_1764-37ins(53) affects splicing of *DNA2* transcript. HeLa cells were transfected with minigene constructs, followed by RNA extraction, cDNA generation, and RT-PCR analysis to assess *DNA2* splicing patterns. RT-PCR products amplified from the HeLa cDNA of the minigene assay samples using F and R primers (see panel a) were separated by agarose gel electrophoresis. Sanger sequencing analysis of cloned PCR products revealed that the product with higher electrophoretic mobility represents transcript lacking exon 12, and the lower mobility product the correctly spliced transcript. (c) The splicing defect caused by *DNA2* c.1764-38_1764-37ins(53) is also detected in patient (P1) PBLs. PCR was performed using primers located in exon 11 and exon 13 of *DNA2* (indicated by short black arrows) products using cDNA generated from minigene assay samples and from RNA extracted from P1 peripheral blood. Agarose gel electrophoresis and Sanger sequencing analysis of PCR products recapitulated the previous findings. (d) Sanger electropherograms demonstrate that the c.1764-38_1764-37ins(53) variant results in skipping of *DNA2* exon 12. (e) Top: RHCglo minigene reporter constructs used in the minigene splicing assay to assess the effect of the *DNA2* c.74+4A>C variant. As a control, the c.74+1G>A point mutation was introduced, abolishing the acceptor splice site of *DNA2* intron 1. Bottom: *DNA2* c.74+4A>C variant affects splicing of *DNA2* transcript. PCR products amplified from HeLa cDNA of the minigene assay samples were separated by agarose gel electrophoresis. Sanger sequencing analysis of cloned PCR products revealed that the product with higher electrophoretic mobility represents transcript lacking exon 1 and the lower mobility product the correctly spliced transcript. (f) *DNA2* threonine 655, mutated in MPD patient P3, is a highly conserved residue in the ATP binding motif (boxed in green) within the helicase/translocase domain. Alignment generated and visualized using Jalview multiple sequence alignment software (Waterhouse, Procter, Martin, Clamp, & Barton, 2009). (g) *DNA2* p.K654 and p.T655 are important for ATP/ADP binding. Top: *DNA2* protein domains (N-terminus, nuclease and helicase/translocase) are represented in different colors (PDB ID: 5EAW; [Zhou et al., 2015]). ADP is shown in cyan spheres; p.T655, shown in red, indicates mouse *DNA2* p.T656 (the equivalent of human p.T655). Below: this threonine residue contacts ADP, similar to p.K654 (p.K655 in mouse, indicated in blue = p.K654 in human *DNA2*). Substitution of K654 abolishes *DNA2* ATPase activity. ADP, adenosine diphosphate; ATP, adenosine triphosphate; cDNA, complementary DNA; MPD, microcephalic primordial dwarfism; PBL, peripheral blood leukocytes; RT-PCR, reverse-transcription polymerase chain reaction

variant on intramolecular protein stability ($\Delta\Delta G = -0.15$ kcal/mol). However, the affected threonine residue forms substantial intermolecular contacts with ADP, burying 25.1 \AA^2 of solvent-accessible surface area, supporting modulation of this interaction as the basis for pathogenicity of this substitution. Notably, analysis with mCSM-lig (Pires, Blundell, & Ascher, 2016) predicted that the p.T656A variant weakens the interaction with ADP (by ~20%). This change in affinity would be expected to reduce DNA2 helicase activity and processivity.

However, as the DNA2 structure does not contain the magnesium divalent cation required for ATPase activity, we next examined homologous structures that contained both Mg^{2+} and ADP. For this we used crystal structures of human UPF1 (Chakrabarti et al., 2011) and *Saccharomyces cerevisiae* SEN1 helicases (Leonaite et al., 2017), both of which are highly homologous to DNA2 around the ATP-binding site, at the amino acid and structural level (Figure S1A,B). Here, a substantially stronger reduction in ADP binding affinity as a result of the threonine to alanine change was predicted (2.6- to 3.1-fold), likely due to contacts between the threonine residue and Mg^{2+} at the ATPase site (Figure S1B,C). Furthermore, molecular modelling of substitutions at the adjacent lysine residue predicted these to similarly weaken the interaction with ADP (Figure S1C). Notably, substitution of this lysine residue in DNA2 has previously been shown to abrogate ATPase activity (Levikova et al., 2017; Masuda-Sasa et al., 2006). Therefore, while structural modelling of mutational effects on protein-ligand interactions has limitations, this molecular modelling, in conjunction with the direct physical contact of this threonine residue with ADP/ Mg^{2+} , suggests that p.T655A negatively affects cellular DNA2 enzyme function in the same manner as p.K654E/R, reducing processivity during DNA end resection.

3 | DISCUSSION

Our identification of four unrelated patients with biallelic DNA2 variants provides strong genetic evidence for DNA2 as an MPD disease gene. As such, this substantively confirms the conclusions of Shaheen et al., who reported the homozygous c.3114+6delC intronic DNA2 variant, found within a region of homozygosity, as the likely cause of Seckel syndrome in an extended consanguineous Saudi Arabian family (Shaheen et al., 2014). The term "Seckel syndrome" has frequently been used to describe patients with a sloping forehead, prominent nose, and intellectual disability, alongside disproportionate microcephaly (Hall, Flora, Scott, Pauli, & Tanaka, 2004; Kalay et al., 2011; Majewski, Goecke, et al., 1982). Individuals from the originally reported family were reported as having "Seckel-like" facies (Shaheen et al., 2014); however, our cases do not have such an appearance. Consequently, we suggest employing the broader term "MPD" to categorize the phenotype of individuals with biallelic DNA2 variants.

Heterozygous missense variants in DNA2 have been associated with mitochondrial myopathy, as 'adult-onset autosomal dominant

progressive external ophthalmoplegia and mitochondrial DNA deletions type 6' (PEOA6; Ronchi et al., 2013). The level of DNA2 deficiency could account for the very different phenotypes. Marked impairment in cellular DNA2 activity in MPD, resulting from biallelic DNA2 variants, would be consistent with the necessity of adequate DNA2 protein levels during embryonic development, particularly during rapid cell proliferation, with a disruption in timely nuclear DNA replication/repair leading to fewer cells being generated, resulting in a smaller individual. In contrast, haploinsufficiency would be developmentally tolerated, but in the long term could result in mitochondrial DNA depletion and adult-onset myopathy.

However, a simple model of differing levels of deficiency is difficult to reconcile with the lack of any features associated with mitochondrial disease in any of the six individuals with biallelic variants in DNA2 (our study and Shaheen et al., 2014) or their carrier parents. This argues against a simple DNA2 "dosage-effect" model to account for distinct mitochondrial and growth phenotypes. Furthermore, heterozygous DNA2 truncating variants appear to be tolerated in the general population (gnomAD; Lek et al., 2016). Such population data seemingly runs counter to two case reports associating truncating variants with childhood myopathy (Chae et al., 2015; Phowthongkum & Sun, 2017), however, both studies failed to assess parental variant status or demonstrate mitochondrial DNA deletions, rendering their findings inconclusive. Therefore, as proposed by Shaheen et al., the PEOA6 DNA2 variants (Ronchi et al., 2013) may instead have an allele-specific effect. The biochemical findings of Ronchi et al were most consistent with decreased nuclease activity, whereas the p.T655A variant we identified in the helicase domain is expected to specifically impair end resection activity of DNA2 (Daley et al., 2017). This raises the possibility that differing functional consequences on this multifunctional enzyme could account for the different phenotypic outcomes in MPD and PEOA6. Notably, variants in RBBP8, which encodes CtIP, another DNA end resection factor, also lead to MPD (Qvist et al., 2011).

In conclusion, our findings in conjunction with the work of Shaheen et al., establish DNA2 as an MPD gene. Future studies will be important to establish the molecular and cellular basis for the differing phenotypes of PEOA6 DNA2 and MPD-DNA2, with ascertainment of further cases, derivation of patient cell lines and development of relevant animal models, important to distinguish between potential disease mechanisms.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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