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## Biallelic variants in *DNA2* cause microcephalic primordial dwarfism

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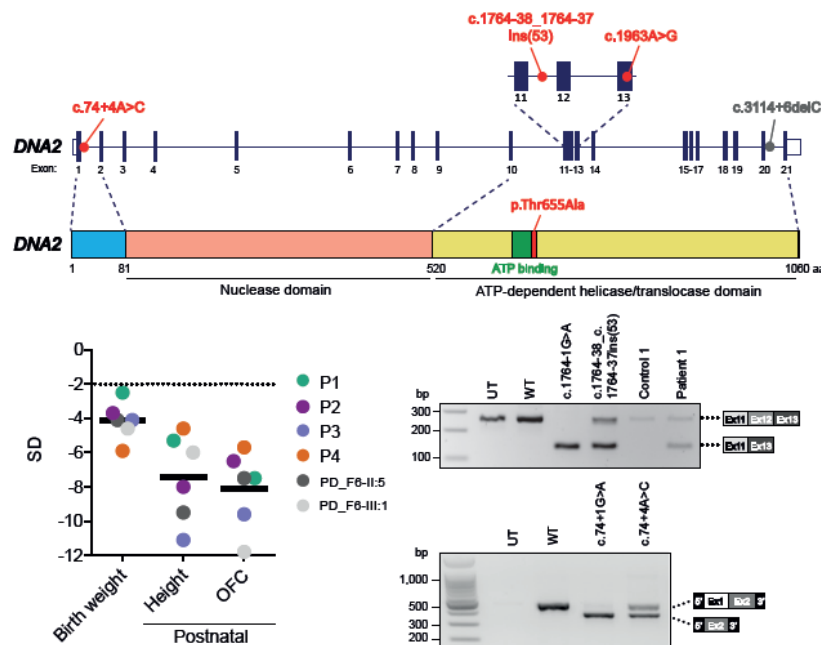
## **Abstract**

Microcephalic primordial dwarfism (MPD) is a group of rare single-gene disorders characterised by 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 identification of four MPD individuals with biallelic variants in *DNA2*, which encodes an 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 binding by *DNA2*. Our findings support pathogenicity

of these variants as biallelic hypomorphic mutations, establishing *DNA2* as an MPD-disease gene.

## Graphical Abstract

We report identification of biallelic *DNA2* variants in four unrelated individuals with Microcephalic primordial dwarfism (MPD). Using cellular splicing assays and molecular modelling we provide evidence that these variants result in partial loss of function of *DNA2*, an ATP-dependent helicase/nuclease with functions in DNA replication and repair, supporting their pathogenicity and establishing *DNA2* as an MPD-disease gene.



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

## Main text

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 4 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 (MCPH) 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; Murray et al., 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 ATP-dependent helicase/nuclease DNA2 is a multi-functional 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 wildtype 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 modelling 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.

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* (**Fig. 1A, Supp. Table S1 and S2**), with a fourth phenotypically similar patient (P2) identified through targeted re-sequencing of *DNA2* in our cohort. No likely causative variants in other genes were evident in WES datasets from P1, P3 and P4 (**Supp. Table 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 (**Fig. 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 (**Supp. Table S1, Fig. 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 (**Supp. Table S2, Fig. 1D**). No significant health problems were noted, aside from P3 who had severe thoracic kyphoscoliosis and recurrent chest infections (**Supp. 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* 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 of *DNA2* (c.1764-38\_1764-37ins(53), **Fig. 1A, Supp. 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 employed 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 (**Fig. 2A**). While the splicing control with a disrupted acceptor



splice site of intron 11 (c.1764-1G>A) demonstrated complete abrogation of correct splicing, the c.1764-38\_1764-37ins(53) variant resulted in marked, but partial loss of correct splicing (**Fig. 2B**). *DNA2* transcript analysis using RNA isolated from P1 PBLs also demonstrated altered splicing (**Fig. 2C**), confirming that partial loss of correct *DNA2* splicing occurs as a result of this intronic variant. Capillary sequencing of RT-PCR products demonstrated correctly spliced mRNA for the wild-type control, with *DNA2* exons 11, 12 and 13 included (**Fig. 2B-D**), while for P1, the majority contained full-length exons 11 and 13, but lacked exon 12 (**Fig. 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) (**Fig. 1A, Supp. 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 (**Fig. 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 wildtype 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 (**Supp. 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 (**Fig. 1A**, **Fig. 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, ADP, (Zhou, Pourmal, & Pavletich, 2015) was examined.

Molecular modelling 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 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 Å<sup>2</sup> 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<sup>2+</sup> 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 (**Supp. Fig. 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<sup>2+</sup> at the ATPase site (**Supp. Fig. S1B,C**). Furthermore, molecular modelling of substitutions at the adjacent lysine residue predicted these to similarly weaken the interaction with ADP (**Supp. Fig. 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<sup>2+</sup>, suggests that p.T655A negatively affects cellular DNA2 enzyme function in the same manner as p.K654E/R, reducing processivity during DNA end resection.

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 employed 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 categorise the phenotype of individuals with biallelic *DNA2* variants.

Heterozygous missense variants in *DNA2* have been associated with mitochondrial myopathy, with 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 phenotype from that described here. Severe depletion arising from biallelic variants impairing nuclear genome replication and DNA repair would then cause MPD. Marked impairment in cellular DNA2 activity in MPD,

resulting from both non-coding and coding *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 multi-functional 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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## References

- Ayyagari, R., Gomes, X. V., Gordenin, D. A., & Burgers, P. M. J. (2003). Okazaki fragment maturation in yeast: I. Distribution of functions between FEN1 and DNA2. *Journal of Biological Chemistry*, 278(3), 1618-1625. doi:10.1074/jbc.M209801200
- Bae, S. H., Bae, K. H., Kim, J. A., & Seo, Y. S. (2001). RPA governs endonuclease switching during processing of Okazaki fragments in eukaryotes. *Nature*, 412(6845), 456-461. doi:10.1038/35086609
- Bicknell, L. S., Bongers, E. M. H. F., Leitch, A., Brown, S., Schoots, J., Harley, M. E., . . . Jackson, A. P. (2011). Mutations in the pre-replication complex cause Meier-Gorlin syndrome. *Nature Genetics*, 43(4), 356-360. doi:10.1038/ng.775
- Bicknell, L. S., Walker, S., Klingseisen, A., Stiff, T., Leitch, A., Kerzendorfer, C., . . . Jeggo, P. A. (2011). Mutations in ORC1, encoding the largest subunit of the origin recognition complex, cause microcephalic primordial dwarfism resembling Meier-Gorlin syndrome. *Nature Genetics*, 43(4), 350-356. doi:10.1038/ng.776
- Burrage, L. C., Charng, W. L., Eldomery, M. K., Willer, J. R., Davis, E. E., Lugtenberg, D., . . . Yang, Y. (2015). De Novo GMNN Mutations Cause Autosomal-Dominant Primordial Dwarfism Associated with Meier-Gorlin Syndrome. *American Journal of Human Genetics*, 97(6), 904-913. doi:10.1016/j.ajhg.2015.11.006
- Cejka, P., Cannavo, E., Polaczek, P., Masuda-Sasa, T., Pokharel, S., Campbell, J. L., & Kowalczykowski, S. C. (2010). DNA end resection by Dna2-Sgs1-RPA and its stimulation by Top3-Rmi1 and Mre11-Rad50-Xrs2. *Nature*, 467(7311), 112-116. doi:10.1038/nature09355.DNA
- Chae, J. H., Vasta, V., Cho, A., Lim, B. C., Zhang, Q., Eun, S. H., & Hahn, S. H. (2015). Utility of next generation sequencing in genetic diagnosis of early onset neuromuscular disorders. *J Med Genet*, 52(3), 208-216. doi:10.1136/jmedgenet-2014-102819

- Chakrabarti, S., Jayachandran, U., Bonneau, F., Fiorini, F., Basquin, C., Domcke, S., . . . Conti, E. (2011). Molecular mechanisms for the RNA-dependent ATPase activity of Upf1 and its regulation by Upf2. *Mol Cell*, *41*(6), 693-703. doi:10.1016/j.molcel.2011.02.010
- Daley, J. M., Jimenez-Sainz, J., Wang, W., Miller, A. S., Xue, X., Nguyen, K. A., . . . Sung, P. (2017). Enhancement of BLM-DNA2-Mediated Long-Range DNA End Resection by CtIP. *Cell Rep*, *21*(2), 324-332. doi:10.1016/j.celrep.2017.09.048
- Duxin, J. P., Dao, B., Martinsson, P., Rajala, N., Guittat, L., Campbell, J. L., . . . Stewart, S. A. (2009). Human Dna2 Is a Nuclear and Mitochondrial DNA Maintenance Protein. *Molecular and Cellular Biology*, *29*(15), 4274-4282. doi:10.1128/MCB.01834-08
- Duxin, J. P., Moore, H. R., Sidorova, J., Karanja, K., Honaker, Y., Dao, B., . . . Stewart, S. A. (2012). Okazaki fragment processing-independent role for human Dna2 enzyme during DNA replication. *Journal of Biological Chemistry*, *287*(26), 21980-21991. doi:10.1074/jbc.M112.359018
- Faivre, L., Le Merrer, M., Lyonnet, S., Plauchu, H., Dagoneau, N., Campos-Xavier, A. B., . . . Cormier-Daire, V. (2002). Clinical and genetic heterogeneity of Seckel syndrome. *Am J Med Genet*, *112*(4), 379-383. doi:10.1002/ajmg.10677
- Fenwick, A. L., Kliszczak, M., Cooper, F., Murray, J., Sanchez-Pulido, L., Twigg, S. R. F., . . . Bicknell, L. S. (2016). Mutations in CDC45, Encoding an Essential Component of the Pre-initiation Complex, Cause Meier-Gorlin Syndrome and Craniosynostosis. *American Journal of Human Genetics*, *99*(1), 125-138. doi:10.1016/j.ajhg.2016.05.019
- Gloor, J. W., Balakrishnan, L., Campbell, J. L., & Bambara, R. A. (2012). Biochemical analyses indicate that binding and cleavage specificities define the ordered processing of human Okazaki fragments by Dna2 and FEN1. *Nucleic Acids Research*, *40*(14), 6774-6786. doi:10.1093/nar/gks388



- Gorlin, R. J., Cervenka, J., Moller, K., Horrobin, M., & Witkop, C. J. (1975). Malformation syndromes. A selected miscellany. *Birth defects original article series*, 11(2), 39-50.
- Guernsey, D. L., Matsuoka, M., Jiang, H., Evans, S., MacGillivray, C., Nightingale, M., . . . Samuels, M. E. (2011). Mutations in origin recognition complex gene ORC4 cause Meier-Gorlin syndrome. *Nature Genetics*, 43(4), 360-365. doi:10.1038/ng.777
- Guerois, R., Nielsen, J. E., & Serrano, L. (2002). Predicting changes in the stability of proteins and protein complexes: A study of more than 1000 mutations. *Journal of Molecular Biology*, 320(2), 369-387. doi:10.1016/S0022-2836(02)00442-4
- Hall, J. G., Flora, C., Scott, C. I., Pauli, R. M., & Tanaka, K. I. (2004). Majewski osteodysplastic primordial dwarfism type II (MOPD II): Natural history and clinical findings. *American Journal of Medical Genetics*, 130A(1), 55-72. doi:10.1002/ajmg.a.30203
- Harley, M. E., Murina, O., Leitch, A., Higgs, M. R., Bicknell, L. S., Yigit, G., . . . Jackson, A. P. (2016). TRAP promotes DNA damage response during genome replication and is mutated in primordial dwarfism. *Nat Genet*, 48(1), 36-43. doi:10.1038/ng.3451
- Kalay, E., Yigit, G., Aslan, Y., Brown, K. E., Pohl, E., Bicknell, L. S., . . . Wollnik, B. (2011). CEP152 is a genome maintenance protein disrupted in Seckel syndrome. *Nature Genetics*, 43(1), 23-26. doi:10.1038/ng.725
- Karanja, K. K., Cox, S. W., Duxin, J. P., Stewart, S. A., & Campbell, J. L. (2012). DNA2 and EXO1 in replication-coupled, homology-directed repair and in the interplay between HDR and the FA/BRCA network. *Cell Cycle*, 11(21), 3983-3996. doi:10.4161/cc.22215
- Klingseisen, A., & Jackson, A. P. (2011). Mechanisms and pathways of growth failure in primordial dwarfism. *Genes and Development*, 25(19), 2011-2024. doi:10.1101/gad.169037
- Lek, M., Karczewski, K. J., Minikel, E. V., Samocha, K. E., Banks, E., Fennell, T., . . . Exome Aggregation, C. (2016). Analysis of protein-coding genetic variation in 60,706 humans. *Nature*, 536(7616), 285-291. doi:10.1038/nature19057
- Leonaite, B., Han, Z., Basquin, J., Bonneau, F., Libri, D., Porrua, O., & Conti, E. (2017). Sen1 has unique structural features grafted on the architecture of the Upf1-like helicase family. *EMBO J*, 36(11), 1590-1604. doi:10.15252/embj.201696174
- Levikova, M., Pinto, C., & Cejka, P. (2017). The motor activity of DNA2 functions as an ssDNA translocase to promote DNA end resection. *Genes and Development*, 31(5), 493-502. doi:10.1101/gad.295196.116

- Lin, W., Sampathi, S., Dai, H., Liu, C., Zhou, M., Hu, J., . . . Shen, B. (2013). Mammalian DNA2 helicase/nuclease cleaves G-quadruplex DNA and is required for telomere integrity. *EMBO Journal*, *32*(10), 1425-1439. doi:10.1038/emboj.2013.88
- Logan, C. V., Murray, J. E., Parry, D. A., Robertson, A., Bellelli, R., Tarnauskaitė, Ž., . . . Jackson, A. P. (2018). DNA Polymerase Epsilon Deficiency Causes IMAGE Syndrome with Variable Immunodeficiency. *The American Journal of Human Genetics*. doi:10.1016/j.ajhg.2018.10.024
- Majewski, F., Goecke, T., & Opitz, J. M. (1982). Studies of microcephalic primordial dwarfism I: Approach to a delineation of the Seckel syndrome. *American Journal of Medical Genetics*, *12*(1), 7-21. doi:10.1002/ajmg.1320120103
- Majewski, F., Ranke, M., Schinzel, A., & Opitz, J. M. (1982). Studies of microcephalic primordial dwarfism II: The osteodysplastic type II of primordial dwarfism. *American Journal of Medical Genetics*, *12*(1), 23-35. doi:10.1002/ajmg.1320120104
- Majewski, F., Stoeckenius, M., Kemperdick, H., & Opitz, J. M. (1982). Studies of microcephalic primordial dwarfism III: An intrauterine dwarf with platyspondyly and anomalies of pelvis and clavicles—osteodysplastic primordial dwarfism type III. *American Journal of Medical Genetics*, *12*(1), 37-42. doi:10.1002/ajmg.1320120105
- Masuda-Sasa, T., Imamura, O., & Campbell, J. L. (2006). Biochemical analysis of human Dna2. *Nucleic Acids Res*, *34*(6), 1865-1875. doi:10.1093/nar/gkl070
- Miller, A. S., Daley, J. M., Pham, N. T., Niu, H., Xue, X., Ira, G., & Sung, P. (2017). A novel role of the Dna2 translocase function in DNA break resection. *Genes and Development*, *31*(5), 503-510. doi:10.1101/gad.295659.116
- Murray, J. E., Bicknell, L. S., Yigit, G., Duker, A. L., van Kogelenberg, M., Haghayegh, S., . . . Jackson, A. P. (2014). Extreme Growth Failure is a Common Presentation of Ligase IV Deficiency. *Human Mutation*, *35*(1), 76-85. doi:10.1002/humu.22461
- Murray, J. E., Van Der Burg, M., Ijspeert, H., Carroll, P., Wu, Q., Ochi, T., . . . Bicknell, L. S. (2015). Mutations in the NHEJ component XRCC4 cause primordial dwarfism. *American Journal of Human Genetics*, *96*(3), 412-424. doi:10.1016/j.ajhg.2015.01.013
- Nimonkar, A. V., Genschel, J., Kinoshita, E., Polaczek, P., Campbell, J. L., Wyman, C., . . . Kowalczykowski, S. C. (2011). BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA break repair. *Genes and Development*, *25*(4), 350-362. doi:10.1101/gad.2003811

- Niu, H., Chung, W.-h., Zhu, Z., Kwon, Y., Zhao, W., Chi, P., . . . Sung, P. (2010). Mechanism of the ATP-dependent DNA End Resection Machinery from *S. cerevisiae*. *Nature*, *467*(7311), 108-111. doi:10.1038/nature09318.
- O'Driscoll, M., Ruiz-Perez, V. L., Woods, C. G., Jeggo, P. A., & Goodship, J. A. (2003). A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. *Nature Genetics*, *33*(4), 497-501. doi:10.1038/ng1129
- Ogi, T., Walker, S., Stiff, T., Hobson, E., Limsirichaikul, S., Carpenter, G., . . . Jeggo, P. A. (2012). Identification of the First ATRIP-Deficient Patient and Novel Mutations in ATR Define a Clinical Spectrum for ATR-ATRIP Seckel Syndrome. *PLoS Genetics*, *8*(11). doi:10.1371/journal.pgen.1002945
- Phowthongkum, P., & Sun, A. (2017). Novel truncating variant in DNA2-related congenital onset myopathy and ptosis suggests genotype–phenotype correlation. *Neuromuscular Disorders*, *27*, 616-618. doi:10.1016/j.nmd.2017.03.013
- Pires, D. E. V., Blundell, T. L., & Ascher, D. B. (2016). MCSM-lig: Quantifying the effects of mutations on protein-small molecule affinity in genetic disease and emergence of drug resistance. *Scientific Reports*, *6*(March), 1-8. doi:10.1038/srep29575
- Qvist, P., Huertas, P., Jimeno, S., Nyegaard, M., Hassan, M. J., Jackson, S. P., & Borglum, A. D. (2011). CtIP Mutations Cause Seckel and Jawad Syndromes. *PLoS Genet*, *7*(10), e1002310. doi:10.1371/journal.pgen.1002310
- Reynolds, J. J., Bicknell, L. S., Carroll, P., Higgs, M. R., Shaheen, R., Murray, J. E., . . . Stewart, G. S. (2017). Mutations in DONSON disrupt replication fork stability and cause microcephalic dwarfism. *Nature Genetics*, *49*(4), 537-549. doi:10.1038/ng.3790
- Ronchi, D., Di Fonzo, A., Lin, W., Bordoni, A., Liu, C., Fassone, E., . . . Comi, G. P. (2013). Mutations in DNA2 link progressive myopathy to mitochondrial DNA instability. *American Journal of Human Genetics*, *92*(2), 293-300. doi:10.1016/j.ajhg.2012.12.014
- Schymkowitz, J., Borg, J., Stricher, F., Nys, R., Rousseau, F., & Serrano, L. (2005). The FoldX web server: an online force field. *Nucleic Acids Res*, *33*(Web Server issue), W382-388. doi:10.1093/nar/gki387
- Seckel, H. P. G. (1960). *Bird-headed dwarfs: studies in developmental anthropology including human proportions*: CC Thomas.
- Shaheen, R., Faqeih, E., Ansari, S., Abdel-Salam, G., Al-Hassnan, Z. N., Al-Shidi, T., . . . Alkuraya, F. S. (2014). Genomic analysis of primordial dwarfism reveals novel disease genes. *Genome Research*, *24*(2), 291-299.

- Shaheen, R., Maddirevula, S., Ewida, N., Alsahli, S., Abdel-Salam, G. M. H., Zaki, M. S., . . . Alkuraya, F. S. (2018). Genomic and phenotypic delineation of congenital microcephaly. *Genet Med*. doi:10.1038/s41436-018-0140-3
- Singh, G., & Cooper, T. A. (2006). Minigene reporter for identification and analysis of cis elements and trans factors affecting pre-mRNA splicing. *BioTechniques*, 41(2), 177-181. doi:10.2144/000112208
- Sturzenegger, A., Burdova, K., Kanagaraj, R., Levikova, M., Pinto, C., Cejka, P., & Janscak, P. (2014). DNA2 cooperates with the WRN and BLM RecQ helicases to mediate long-range DNA end resection in human cells. *Journal of Biological Chemistry*, 289(39), 27314-27326. doi:10.1074/jbc.M114.578823
- Thangavel, S., Berti, M., Levikova, M., Pinto, C., Gomathinayagam, S., Vujanovic, M., . . . Vindigni, A. (2015). DNA2 drives processing and restart of reversed replication forks in human cells. *Journal of Cell Biology*, 208(5), 545-562. doi:10.1083/jcb.201406100
- Verloes, A., Drunat, S., Gressens, P., & Passemard, S. (1993). Primary Autosomal Recessive Microcephalies and Seckel Syndrome Spectrum Disorders. In M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. H. Bean, K. Stephens, & A. Amemiya (Eds.), *GeneReviews((R))*. Seattle (WA).
- Vetro, A., Savasta, S., Russo Raucci, A., Cerqua, C., Sartori, G., Limongelli, I., . . . Zuffardi, O. (2017). MCM5: A new actor in the link between DNA replication and Meier-Gorlin syndrome. *European Journal of Human Genetics*, 25(5), 646-650. doi:10.1038/ejhg.2017.5
- Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M., & Barton, G. J. (2009). Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, 25(9), 1189-1191. doi:10.1093/bioinformatics/btp033
- Zheng, L., Zhou, M., Guo, Z., Lu, H., Qian, L., Dai, H., . . . Shen, B. (2008). Human DNA2 is a mitochondrial nuclease/helicase for efficient processing of DNA replication and repair intermediates. *Molecular Cell*, 32(3), 325-336. doi:10.1016/j.molcel.2008.09.024.
- Zhou, C., Pourmal, S., & Pavletich, N. P. (2015). Dna2 nuclease-helicase structure, mechanism and regulation by Rpa. *eLife*, 4, 1-19. doi:10.7554/eLife.09832

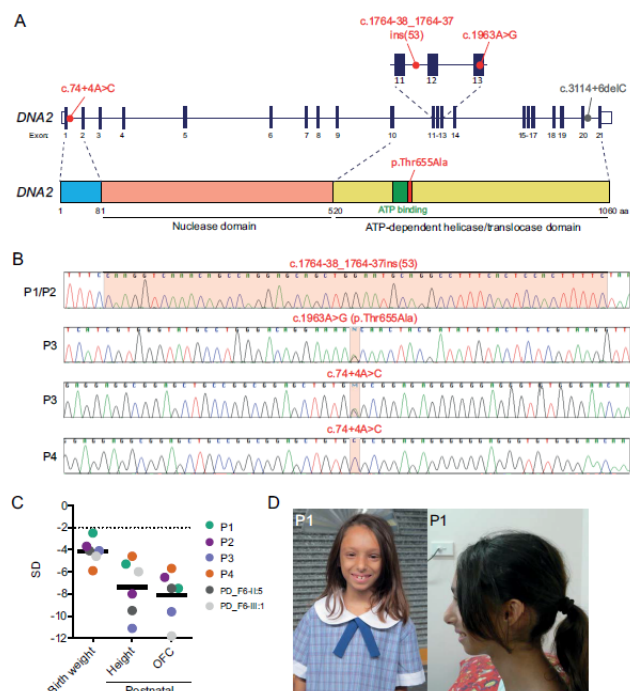
## Figure 1. Identification of biallelic *DNA2* variants in microcephalic primordial dwarfism patients.

**A:** *DNA2* variants c.74+4A>C, c.1764-38\_1764-37ins(53) and c.1963A>G (p.Thr655Ala, p.T655A) identified in four 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 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.



**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 visualised 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 colours (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.

