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Mutations in TOP3A Cause a Bloom Syndrome-like Disorder

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Bloom syndrome, caused by biallelic mutations in BLM, is characterized by prenatal-onset growth deficiency, short stature, an erythematous photosensitive malar rash, and increased cancer predisposition. Diagnostically, a hallmark feature is the presence of increased sister chromatid exchanges (SCEs) on cytogenetic testing. Here, we describe biallelic mutations in TOP3A in ten individuals with prenatal-onset growth restriction and microcephaly. TOP3A encodes topoisomerase III alpha (TopIIIα), which binds to BLM as part of the BTRR complex, and promotes dissolution of double Holliday junctions arising during homologous recombination. We also identify a homozygous truncating variant in RM11, which encodes another component of the BTRR complex, in two individuals with microcephalic dwarfism. The TOP3A mutations substantially reduce cellular levels of TopIIIα, and consequently subjects’ cells demonstrate elevated rates of SCE. Unresolved DNA recombination and/or replication intermediates persist into mitosis, leading to chromosome segregation defects and genome instability that most likely explain the growth restriction seen in these subjects and in Bloom syndrome. Clinical features of mitochondrial dysfunction are evident in several individuals with biallelic TOP3A mutations, consistent with the recently reported additional function of TopIIIα in mitochondrial DNA decatenation. In summary, our findings establish TOP3A mutations as an additional cause of prenatal-onset short stature with increased cytogenetic SCEs and implicate the decatenation activity of the BTRR complex in their pathogenesis.

Introduction

Microcephalic primordial dwarfism (MPD) is used to collectively describe a heterogeneous group of disorders characterized by significant in utero and postnatal growth retardation alongside marked microcephaly.1 Bloom syndrome (MIM: 210900) is also associated with prenatal growth restriction, short stature, and microcephaly. It is distinguished by an erythematous sun-sensitive facial rash that can become evident during childhood.2 A predisposition to the development of cancer in early adulthood is also seen, and both solid tumors and hematological malignancies are a major cause of early death.3 Additionally, a key cytogenetic feature of Bloom syndrome is an increased number of sister chromatid exchanges (SCEs).4 Notably, when

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molecular testing is inconclusive, a finding of elevated SCEs is currently utilized for diagnostic confirmation of Bloom syndrome (see GeneReviews in Web Resources).

In 1995, Bloom syndrome was shown to be caused by mutations in BLM (MIM: 604610), which encodes a RecQ family DNA helicase.5 Mutations in BLM are typically biallelic loss-of-function mutations.5 BLM forms the BTRR complex with topoisomerase III alpha (TopIIIα) and RecQ-mediated genome instability proteins 1 and 2 (RM1 and RM2, respectively). Together, these proteins process double Holliday junctions (dHJs) that arise as a result of homologous-recombination-mediated repair of double-stranded DNA (dsDNA) breaks during DNA synthesis.2–13 The process of dHJ dissolution requires two steps. First, the BTRR complex promotes the convergent branch migration of the dHJ to create a hemicatenane intermediate, and then this structure is decatenated by TopIIIα in concert with RM1 and RM2.14,15 Dissolution of a dHJ by this mechanism can be completed without any potentially detrimental exchanges between genetic markers flanking the original site of homologous recombination. The alternative processing of dHJs by Holliday junction resolvases (SLX-MUS81 and GEN1 nucleases) can yield crossover events, and increased usage of this pathway can contribute to increased cancer predisposition.16 Crossover events between homologous chromosomes can lead to loss of heterozygosity (LOH),17 which can be detrimental to cell survival and contribute to increased cancer predisposition.18 Additionally, unresolved recombination intermediates can persist into mitosis, leading to chromosome bridges, and act as a source of genome instability.19

Here, we report the identification of pathogenic mutations in TOP3A (MIM: 601243) in ten individuals with Bloom syndrome-like phenotypic features and characterize the cellular consequences of these mutations.

**Material and Methods**

**Research Subjects**

Genomic DNA from the affected individuals and family members was extracted from peripheral blood by standard methods or obtained from saliva samples with Oragene collection kits according to the manufacturer's instructions. Informed consent was obtained from all participating families, and all procedures performed in studies involving human participants were in accordance with the Declaration of Helsinki. Research studies were approved by the Scottish multicenter Research Ethics Committee (05/MRE00/74), the Hospital Universitario La Paz Ethical Committee (PI-2630), the Great Ormond Street Hospital Research Ethics Committee (09/H0706/66), the University Medical Center Göttingen Ethics Committee (vote ref. 3/2/1), and the Institute for Genomic Medicine at Columbia University (protocol AAZ08410). Parents provided written consent for the publication of photographs of the affected individuals. For growth measurements, Z scores (standard deviations from population mean for age and sex) were calculated according to LMS (L, smooth curve; M, median; S, coefficient of variation) growth on the basis of British 1990 growth reference data.20 For calculation of Z scores for growth measurements published for Bloom syndrome,21 full-term gestation was assumed, and postnatal growth measurements were calculated from data provided for the 18- to 21-year age range.

**Exome Sequencing and Sanger Sequencing**

Exome sequencing and confirmatory capillary Sanger sequencing were performed according to standard methodologies as previously published.22,23 TOP3A and RM1 variants were annotated with GenBank: NM_004618.4 and NM_024945.2 reference sequences, respectively.

**Plasmid Construct and Protein Purification**

**Cloning of the Mutant hTopIIα Expression Vector**

A plasmid encoding the wild-type (WT) TOP3A cDNA24 was modified by the Quickchange XL Site-Directed Mutagenesis Kit (Agilent technologies) with the following primers to recapitulate the deletion and frameshift present in subject P1: 5’-CCCTCCGTACAC GACTGTCAGAGGGAGGAA-3’ (T3_FS_FW) and 5’-TCTTCTCTGCACA GTCGAGTAGAGGGAGG-3’ (T3_FS_RW).

**Expression and Purification of TRR**

The previously described plasmids encoding RM1 and RM213 and hTopollα20–21 were co-transformed into E. coli Rosetta 2 cells, and the complex was expressed. The cells were disrupted in buffer A (50 mM Tris-Cl [pH 7.5], 0.5 M NaCl, 10% glycerol, 0.1% IGEPAL, 2 mM β-mercaptoethanol, 40 mM imidazole, 1 mM PMSE, and protease inhibitor tablet [PI, EDTA-free, Roche] on ice before dounce homogenization and sonication. After the removal of cell debris by centrifugation, the lysate was affinity purified on a 5 mL HisTrap HP affinity column. The complex was further purified on a 5 mL HiTrap Heparin HP column in buffer B (50 mM Tris-Cl [pH 7.5], 10% glycerol, 0.1 mM EDTA, and 1 mM DTT) with a linear gradient of 200 mM to 1 M NaCl and then gel filtered on a 120 mL HiLoad 16/600 Superdex 200 column in buffer B containing 200 mM NaCl. Expression and purification of the mutant T Thr812LeufsTer101RR complex were performed in a similar manner. BLM was purified as described previously.25,26

**dHJ Dissolution**

The dHJ substrate construction and dissolution reactions were carried out as described previously.14

**Cell Culture**

Dermal fibroblasts were obtained by skin-punch biopsy and were maintained in amnioMAX C-100 complete medium (Life Technologies) in a 37°C incubator with 5% CO2 and 3% O2. siRNA oligonucleotides (siLUC: 5’-CUUACGGAGUACUUCGA-3’ [siTopolH] SMARTpool M-005279-01-0005, Dharmacon) were transfected into dermal fibroblasts with RNAiMAX (Life Technologies) according to the manufacturer’s instructions.

**Sister Chromatid Exchange Assay**

Dermal fibroblasts were treated with 10 μM BrdU for 48 hr followed by 0.5 μg/mL colcemid for 2 hr. Metaphases and nuclei were isolated in hypotonic buffer (0.25% KCl and 1% Na2CO3:H2O3), fixed with methanol and acetic acid (3:1 vol/vol), and dropped onto slides. Dried slides were rehydrated in PBS and then incubated with 2 μg/mL Hoechst 33342 in 2× saline sodium citrate (SSC) buffer (300 mM NaCl and 30 mM sodium citrate) for 15 min. Slides were then covered in 2× SSC buffer,
irradiated in ultraviolet A at 5,400 J/m², dehydrated in an ethanol series, and mounted in VECTASHIELD with DAPI.

**SCE Methodology for Analysis of P7 and P8**

Dermal fibroblasts were treated with 26.05 μM BrdU for 72 hr followed by 0.5 μg/mL colcemid for 5 hr and then harvested. Cells were resuspended in a pre-warmed hypotonic solution (0.051 M media for 30 min. Prometaphase cells were shaken off and reassociated defects, RO3306-treated cells were released into fresh media containing 0.2% Triton X-100 in PBS for 20 min. For G1-associated defects, RO3306 for 6 hr for the in

**Immunofluorescence and microscopy**

Mitotic abnormalities and G1-associated defects were analyzed as described previously. In brief, for mitotic analysis, cells were seeded onto 22 mm glass coverslips in 6-well plates. After 18 hr, cells were treated with 3.5 μM RO3306 for 6 hr for the induction of a late G2 arrest and were subsequently released into fresh media. After 45 min, cells were fixed with 4% paraformaldehyde containing 0.2% Triton X-100 in PBS for 20 min. For G1-associated defects, RO3306-treated cells were released into fresh media for 30 min. Prometaphase cells were shaken off and reseeded onto glass slides coated with poly-L-lysine. After 4–6 hr, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 10 min. Fixed cells were incubated with antibodies specific to cyclin A (Santa Cruz Biotechnology, sc-596), 53BP1 (Santa Cruz Biotechnology, sc-515841), and PICH.

**Immunoblotting**

Cells were lysed in 50 mM Tris-HCl (pH 8), 280 mM NaCl, 0.5% NP40, 0.2 mM EDTA, 0.2 mM EGTA, and 10% glycerol supplemented with a protease inhibitor tablet (Roche Life Science). Protein samples were run on a 4%–12% NuPAGE Bis-Tris precast gel (Life Technologies) and then immunoblotted with anti-Top1Hz raised against amino acids 652–1,001 (Proteintech, 14525-1-AP) and actin (Sigma, A2066).

**Results**

**Identification of Mutations in TOP3A**

In ongoing work to identify genes associated with microcephalic dwarfism, whole-exome sequencing (WES) was performed on subject P1, who had significant microcephaly and short stature (−5.7 and −4.4 standard deviations [SD], respectively). This identified the homozygous frameshift mutation c.2718del (p.[Thr907LeufsTer101]) (GenBank: NM_004618.4) in TOP3A in chromosomal region 17p11.2. After interrogation of other cohorts and clinical contacts made through GeneMatcher, we subsequently ascertained a further nine individuals from seven families affected by biallelic deleterious TOP3A variants that had also been discovered by WES (Table 1). All variants identified were validated by Sanger capillary sequencing, and all parents were confirmed to be heterozygous carriers. Aside from the c.2718del variant (minor allele frequency ¼ 0.00041%), none of the other variants were reported in GnomAD. Notably, four of the families from the United Arab Emirates, Syria, and Saudi Arabia (F2, F4, F6, and F7), were homozygous for the same variant.

**Prenatal-Onset Growth Restriction and Microcephaly in Individuals with TOP3A Mutations**

Prenatal growth restriction was evident from the substantially reduced birth weight in all individuals (mean weight

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**Table 1. Identified TOP3A and RMI1 Variants Associated with Microcephalic Dwarfism**

| Family | Individual | Gene | Biallelic Nucleotide Mutations | Predicted Amino Acid Consequence(s) | Sex | Country of Origin |
|--------|------------|------|-------------------------------|------------------------------------|-----|------------------|
| 1 P1   | TOP3A      | c.[2718del];[2718del] | p.[Thr907LeufsTer101];[Thr907LeufsTer101] | female | USA (Czech and Irish ancestry) |
| 2 P2   | TOP3A      | c.[2271dup];[2271dup] | p.[Arg758GlnfsTer3];[Arg758GlnfsTer3] | female | United Arab Emirates |
| 3     | TOP3A      | c.[2271dup];[2271dup] | p.[Arg758GlnfsTer3];[Arg758GlnfsTer3] | female | United Arab Emirates |
| 5     | TOP3A      | c.[527C>T][1072_1073dup] | p.[Ala176Val];[Tyr359GlyfsTer17] | female | Japan |
| 6 P6   | TOP3A      | c.[2271dup];[2271dup] | p.[Arg758GlnfsTer3];[Arg758GlnfsTer3] | female | Syria |
| 7 P7   | TOP3A      | c.[2428del];[2428del] | p.[Ser810LeufsTer2];[Ser810LeufsTer2] | female | Spain |
| 8 P8   | TOP3A      | c.[2428del];[2428del] | p.[Ser810LeufsTer2];[Ser810LeufsTer2] | male | Spain |
| 9 P9   | TOP3A      | c.[2771dup];[2771dup] | p.[Arg758GlnfsTer3];[Arg758GlnfsTer3] | male | Syria |
| 10 P10  | TOP3A    | c.[2771dup];[2771dup] | p.[Arg758GlnfsTer3];[Arg758GlnfsTer3] | female | Saudi Arabia |
| 11 P11  | RMI1     | c.[1255_1259del];[1255_1259del] | p.[Lys419LeufsTer5];[Lys419LeufsTer5] | female | Turkey |
| 12 P12  | RMI1     | c.[1255_1259del];[1255_1259del] | p.[Lys419LeufsTer5];[Lys419LeufsTer5] | female | Turkey |
| – MCI+  | TOP3A    | c.[298A>G];[403C>T] | p.[Met100Val];[Arg135Ter] | female | UK |

Nomenclature is according to transcript GenBank: NM_004618.4 for TOP3A and GenBank: NM_004618.4 and NM_024945.2 for RMI1.

*a*Close to donor splice site of exon 10.

*b*Previously reported individual with adult-onset mitochondrial disease.

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Table 1.32 Whereas stature was normal (163 cm) in this adult-onset mitochondrial disorder (denoted as MC1 in TOP3A Mutations)

Cardiomyopathy Is Present in Some Individuals with TOP3A Mutations

A single individual compound heterozygous for rare TOP3A variants has been reported recently with an adult-onset mitochondrial disorder (denoted as MC1 in Table 1).32 Whereas stature was normal (163 cm) in this adult female, several of the subjects reported here had clinical features that could also be attributable to mitochondrial dysfunction. P3 and P4 (family 2) had an additional diagnosis of dilated cardiomyopathy, which proved fatal in P4 at age 10 years. Another brother in this sibship died as a result of cardiomyopathy at the age of 13 years. Morphometric and other clinical information are not available for this individual. In P5 (family 3), a dilated cardiomyopathy was also reported, and skeletal muscle biopsy demonstrated 87% mitochondrial DNA depletion. P8 also had asymptomatic left ventricular dilatation noted on echocardiography, whereas P9 had hypertrophic cardiomyopathy.

Given the identification of multiple individuals with biallelic deleterious variants in TOP3A, alongside a consistent clinical phenotype across the cohort, we concluded that these mutations were likely to be pathogenic. We therefore pursued confirmatory functional studies by employing both biochemical and cell-biological approaches.

TOP3A Mutations Lead to Markedly Reduced Cellular Amounts of Enzyme TopIIIx

Most of the identified TOP3A mutations are predicted to prematurely truncate the encoded protein (Figure 2A) and hence are likely to have significant effects on cellular protein amounts. We therefore assessed the amounts of TopIIIx in primary dermal fibroblast cell lines derived from P1, P7, and P8. Immunoblotting demonstrated that the amount of full-length TopIIIx was substantially lower in total cell lysates from all three individuals than in cell lines from unrelated control individuals and parents (Figure 2B).

In most subjects, the frameshift mutations would be expected to result in nonsense-mediated decay of TOP3A transcripts, explaining the consequent loss of TopIIIx. However, the homozygous frameshift mutation in P1 (c.2718del [p.Thr907LeufsTer101]) is at the 3’ end of the gene and is not predicted to result in NMD; instead, it would result in a protein with a length similar to that of the WT enzyme. The frameshift did, however, result in abolition of the C-terminal zinc-finger domain, whose precise cellular function remains to be defined. We therefore
expressed recombinant TopIIIzThr907LeufsTer101 in *E. coli* and purified it to homogeneity as a complex with the co-expressed RM1 and RM2 in order to characterize it further. Notably, in contrast to the TRR complex containing TopIIIzWT, the complex containing TopIIIzThr907LeufsTer101 (hereafter referred to as TopIIIzP1) exhibited reduced stability during purification; lower yields and increased amounts of degraded products were evident on SDS-PAGE (Figure 2C). Nevertheless, when used in quantities equimolar to those of TopIIIzWT, TopIIIzP1 was proficient in a biochemical assay for dHJ dissolution when combined with other components of the BTRR complex. This indicates that this TopIIIz variant retains a near-normal level of single-stranded DNA (ssDNA) decatenation activity (Figures 2D–2F). Therefore, we concluded that the major consequence of the TopIIIzP1 variant, like the other truncating variants, is severe depletion of TopIIIz enzymatic activity in cells.

P5 was the only individual we identified to have an amino acid substitution, p.Ala176Val, that was present in trans with a frameshift mutation. This amino acid substitution is absent from GnomAD, and in silico analyses (MutationTaster, CADD, and SIFT) predict it to be deleterious. It is at a highly conserved residue (Figure S1) within the TOPA domain (Figure 2A) and would therefore be expected to be highly deleterious to enzymatic function.

Given the above results and that Top3a is a developmentally essential gene in mice, 34 we conclude that all of the identified mutations result in marked but most likely hypomorphic loss of function of TopIIIz as a result of a reduction in the cellular amount of the protein. Therefore, these mutations would be predicted to severely compromise the decatenation activity of the BTRR complex in dHJ dissolution in vivo. To assess this possibility, we next pursued cellular assays to assess SCE frequency.

**SCEs Are Markedly Elevated in Individuals with TOP3A Mutations**

dHJ can be processed by two pathways; first, dissolution by the BTRR complex yields non-crossover products only; second, resolution by endonucleases that cleave Holliday junctions generate both non-crossovers and crossover products that are visualized as SCEs (Figure 3A). To determine if dHJ dissolution is impaired in the cells of affected individuals, we assessed the frequency of SCEs. After BrdU incorporation, we performed differential sister chromatid staining on primary fibroblasts and PHA-stimulated peripheral blood leukocytes (Figures 3B and 3C). Cells from individuals with TOP3A mutations had substantially (3- to 6-fold) more SCEs, than cells from control individuals or heterozygous parents (p < 0.0001 for all affected individual cell lines; Figure 3C). Therefore, excessive crossover recombination was evident in all tested individuals with TOP3A mutations, indicating that diagnostic cytogenetic assessment of SCE levels is predictive of TOP3A mutations as well as BLM mutations.

**Chromosome Segregation Defects and Genome Instability Occur in Cells with TOP3A Mutations**

Impaired dHJ decatenation can result in persistently entangled sister chromatids that impede chromosome segregation at mitosis. 19 Therefore, we performed detailed characterization of the mitotic consequences of TopIIIz deficiency in primary fibroblasts from P1. DAPI staining demonstrated elevated amounts of chromatid bridges (control [C] = 0, parent = 0.3 ± 0.4, and P1 = 4 ± 0.6) and lagging chromatid and chromosomes (C = 0.9 ± 0.2, parent = 4 ± 0.7, and P1 = 9.2 ± 0.9) (Figures 4A and 4B), consistent with persisting chromosome entanglements. Additionally, increased numbers of ultrafine DNA bridges (UFBs) were revealed by immunostaining for PICH on DAPI-negative regions 19 (C = 2.7 ± 0.3, parent = 13.1 ± 1.1, and P1 = 26.7 ± 3.8).

We then examined the postmitotic consequences of the observed chromosome segregation errors by enriching for G1 cells (Figures 4C and 4D). Micronuclei often arise from chromosome segregation errors, 35 and analysis of DAPI-stained cells demonstrated substantially more micronucleated cells in the P1 fibroblast cell line than in control and parental cell lines (C = 0.4 ± 0.2, parent = 1.8 ± 0.5,
|                | TOP3A        |           |           |           |           |           |           |           |           |           |           | RMI1       |            |
|----------------|-------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|---|
|                | P1          | P2        | P3        | P4        | P5        | P6        | P7        | P8        | P9        | P10       | MC1       | P11        | P12        |
| Age            | 5 months    | 3 years   | 8 years   | 10 years  | 15 years  | 19 months | 3 years   | 7 years   | 11 years  | 4 years, 7 months | adult     | 7 years | 13 years |
| Prenatal-onset growth restriction | yes         | yes       | yes       | yes       | yes       | yes       | yes       | yes       | yes       | yes       | no        | yes       | yes        |
| Elevated sister chromatid exchange | yes         | yes       | yes       | NA        | yes       | NA        | yes       | yes       | yes       | NA        | no        | no        | NA         |
| Café-au-lait macules | yes         | yes       | yes       | yes       | yes       | no        | yes       | yes       | yes       | NA        | no        | no        | NA         |
| Developmental delay | mild       | no        | no        | no        | no        | mild<sup>a</sup> | no        | mild      | no        | mild      | NA        | mild      | no         |
| Cancer<sup>b</sup> | no          | no        | no        | no        | no        | no        | no        | no        | no        | NA        | no        | no        | no         |
| Decreased subcutaneous fat | no          | no        | no        | no        | no        | no        | yes       | no        | yes       | no        | NA        | no        | no         |
| Gastroesophageal reflux | yes       | NA        | NA        | NA        | no        | yes       | no        | NA        | no        | NA        | NA        | no        | no         |
| Diabetes mellitus | no          | no        | no        | no        | no        | no        | no        | no        | no        | no        | NA        | no        | no         |
| Recurrent infections | yes<sup>c</sup> | NA      | NA        | NA        | no        | no        | no        | NA        | yes       | yes<sup>d</sup> | NA        | no        | no         |
| Malar rash | no          | no        | no        | no        | no        | no        | no        | no        | no        | no        | NA        | no        | no         |
| Dilated cardiomyopathy | no          | no        | yes       | yes (severe, deceased) | yes (after heart transplant) | normal echocardiogram | NA | yes<sup>e</sup> (asymptomatic) | HCM | no        | NA        | NA        | NA         |
| Mitochondrial DNA depletion in muscle | NA          | NA        | NA        | N/A       | yes (87%) | NA        | NA        | NA        | NA        | yes (>80%) | NA        | NA        | NA         |
| Other | CDH, gastrosomy | no        | abnormal movements and tics | no | hearing loss, CMAMMA | no | no | no | microcytic anemia<sup>f</sup> | no | PEO, ataxia | no | no |

Abbreviations are as follows: CDH, Congenital dislocation of hip; CMAMMA, combined malonic and methylmalonic aciduria (MIM: 614265) due to ACSF3 (MIM: 614245) mutations; HCM, hypertrophic cardiomyopathy; NA, not available; and PEO, progressive external ophthalmoplegia.

<sup>a</sup>Expressive speech delay only.
<sup>b</sup>All individuals are younger than 15 years old; in Bloom syndrome, neoplasia typically manifests in early adulthood.
<sup>c</sup>Recurrent otitis media and tonsillitis, leading to tonsillectomy.
<sup>d</sup>Recurrent upper-respiratory-tract infections and oral thrush.
<sup>e</sup>Mild left-ventricle dilatation.
<sup>f</sup>Anemia due to beta-thalassemia trait.
and P1 = 7.5 ± 1.4). Elevated amounts of S3BP1 nuclear bodies were also seen in cyclin-A-negative (G1) cells, indicating the transmission of DNA damage from one cell cycle to the next36 (C = 0.8 ± 0.3, parent = 4.5 ± 0.5, and P1 = 11 ± 1.7). Altogether, we conclude that impaired dHJ decatenation in TopIII\textsubscript{a}-deficient cells results in both abnormal recombination and mitotic errors that lead to accumulation of DNA damage. Because chromatin bridges and UFBs are also present in individuals affected by Bloom syndrome,\textsuperscript{19} these findings are concordant with a shared disease mechanism.

### A Homozygous Truncating Variant in RMI1 Is Associated with Microcephalic Dwarfism

Finally, through WES we also identified the homozygous truncating variant c.1255_1259del (p.Lys419Leufs\(^*\)5) in RMI1 in two affected cousins (P11 and P12) from a consanguineous Turkish family (F8;\ Figure S3). Both individuals also had microcephalic dwarfism with a clinical phenotype and level of growth restriction similar to those of individuals with TOP3A mutations (Figure 1A and Tables 2 and 3).

### Discussion

Here, we report that biallelic mutations in TOP3A cause prenatal growth restriction, microcephaly, and short stature, a clinical phenotype overlapping that seen in Bloom syndrome. Furthermore, we have identified a rare RMI1 truncating variant (allele frequency = 1.1 × 10\textsuperscript{−3}) in two individuals. Given that no homozygous loss-of-function variants are reported in gnomAD, we expect this to be the cause of microcephalic dwarfism in individuals P11 and P12. However, it will be necessary to identify more individuals with other deleterious sequence variants to establish RMI1 as a disease-related gene.

Notably, individuals with TOP3A mutations exhibit elevated amounts of SCEs on cytogenetic testing. Although cell lines were not available from P11 and P12, we also predict that increased SCEs will occur in cells with the homozygous RMI1 p.Lys419Leufs\(^*\)5 variant given the important role of this protein in the BTRR complex.\textsuperscript{9,10,37} For many years, the diagnosis of Bloom syndrome rested on a demonstration of elevated SCEs, and this has continued to be employed in cases where molecular testing is inconclusive (see GeneReviews in Web Resources). Hence, analysis of other BTRR components is now warranted when elevated SCEs are detected. Indeed, given the clear phenotypic overlap between Bloom syndrome and individuals with TOP3A mutations, re-evaluation of individuals previously cytogenetically diagnosed with Bloom syndrome could be fruitful.
RMI2 is the fourth component of the BTRR complex. A single family in which two children were also found to have elevated SCEs was recently reported to have a homozygous deletion of RMI2; café-au lait macules were evident, but only one individual showed mild-growth impairment. Because RMI2 is not essential for BTR (BLM, TopIIIα, and RM1) enzymatic function, a more subtle cellular and developmental phenotype might therefore result. It also remains possible that the BTR complex has functions independent of RMI2 given that BLM, TopIIIα, and RM1 are conserved in all eukaryotes but RMI2 is absent in invertebrates and yeast.

Bloom syndrome is associated with a predisposition to both solid tumors and hematological malignancies. However, cancers have not been reported in our individuals with TOP3A mutations. Nevertheless, all are still children, and because malignancy in Bloom syndrome typically manifests in early adulthood, TOP3A mutations could also confer an increased risk of neoplasia.

Distinct from Bloom syndrome, some of our individuals with TOP3A mutations have clinical features consistent with mitochondrial dysfunction, and several exhibit a dilated cardiomyopathy. Notably, an individual with chronic progressive external ophthalmoplegia and cerebellar ataxia, along with a novel functional role for TopIIIα in the decatenation of mitochondrial DNA after its replication, has been recently reported (denoted as subject MC1). The compound-heterozygous mutations c.[298A>G];[403C>T], p.[Met100Val];[Arg135*] could have milder consequences than those reported in our cohort, and consistent with this, we found that MC1 does not exhibit short stature (Table 2) or elevated numbers of SCEs in a primary fibroblast line derived from this individual (Figure S2). Thus, it appears that the residual TopIIIα activity in MC1 is sufficient for the nuclear function of the BTRR complex in dHJ dissolution and for normal growth but could have become rate limiting for the role of TopIIIα in mitochondrial DNA replication.

Mechanistically, our cellular and biochemical studies suggest that the identified TOP3A mutations reported here are severe hypomorphs, consistent with the established essential cellular and developmental functions of TopIIIα. Given that marked growth restriction is common in individuals with TOP3A and BLM mutations, it seems likely that they have a shared pathogenic basis arising from reduced ssDNA decatenation activity of the BTRR complex. Persistence of unresolved hemi-catenanes into mitosis leads to the formation of chromatin and ultrafine mitotic bridges that have previously been reported in Bloom syndrome and in individuals with microcephalic dwarfism and condensin mutations. The resulting genome instability arising from persistent chromatin bridges and micronuclei could therefore impair cell viability during development, causing microcephaly and global growth restriction.

In summary, we demonstrate that mutations in TOP3A cause microcephalic dwarfism with increased SCE, implicating the BTRR complex as a cause of growth disorders.
Figure 4. DNA Catenanes Persist into Mitosis in Cells with TOP3A Mutations, Leading to Chromosome Segregation Defects and Genome Instability
(A and B) Chromosome segregation is impaired in TOP3A-deficient primary fibroblasts. (A) Representative images of chromatin bridges, lagging DNA (DAPI), and UFBs (detected by the presence of PICH and absence of DAPI stain). (B) Quantification of chromatin bridges, lagging DNA, and PICH-positive UFBs scored in control, parental, and P1 mitotic fibroblasts staged at anaphase B (experiments ≥ 3, n > 50 cells, error bars = SEM). To enrich for mitotic cells, we treated fibroblasts with R03306 for 6 hr and released and fixed them after 45 min.
(C) TOP3A P1 fibroblasts display significantly elevated amounts of micronuclei. Top: representative picture of control and P1 fibroblasts. Bottom: quantification of micronuclei containing interphase cells (experiments ≥ 3, n > 500, error bars = SEM). To enrich for G1 cells, we released R03306-treated cells into fresh media for 30 min and collected, re-seeded, and fixed prometaphase cells after 4–6 hr.
(D) P1 fibroblasts with TOP3A mutations display significantly elevated numbers of 53BP1 bodies in G1 nuclei. Top: representative images of 53BP1 foci (red) and DNA (DAPI). Bottom: quantification of cells with at least four 53BP1 foci in G1 nuclei (negative for cyclin A) (experiments ≥ 3, n > 500, error bars = SEM). Scale bar: 2 microns. Two-tailed t test was performed against parent cells.

Supplemental Data

Supplemental Data include three figures and one table and can be found with this article online at https://doi.org/10.1016/j.ajhg.2018.07.001.

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Declaration of Interests

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Web Resources

GenBank, https://www.ncbi.nlm.nih.gov/genbank/
GeneReviews, Sanz, M.M., German, J., and Cunniff, C. (1993).
Bloom’s Syndrome, https://www.ncbi.nlm.nih.gov/books/NBK1398/
GnomAD, http://gnomad.broadinstitute.org
OMIM, http://www.omim.org

References

1. Klingseisen, A., and Jackson, A.P. (2011). Mechanisms and pathways of growth failure in primordial dwarfism. Genes Dev. 25, 2011–2024.
2. Bloom, D. (1954). Congenital telangectatic erythema resembling lupus erythematosus in dwarfs; probably a syndrome entity. AMA Am. J. Dis. Child. 88, 754–758.
3. German, J. (1997). Bloom’s syndrome. XX. The first 100 cancers. Cancer Genet. Cytogenet. 93, 100–106.
4. Chaganti, R.S., Schonberg, S., and German, J. (1974). A manifold increase in sister chromatid exchanges in Bloom’s syndrome lymphocytes. Proc. Natl. Acad. Sci. USA 71, 4508–4512.
5. Ellis, N.A., Groden, J., Ye, T.Z., Straughen, J., Lennon, D.J., Ciocci, S., Proytcheva, M., and German, J. (1995). The Bloom’s syndrome gene product is homologous to RecQ helicases. Cell 83, 655–666.
6. German, J., Sanz, M.M., Ciocci, S., Ye, T.Z., and Ellis, N.A. (2007). Syndrome-causing mutations of the BLM gene in persons in the Bloom’s Syndrome Registry. Hum. Mutat. 28, 743–753.
7. Johnson, F.B., Lombard, D.B., Neff, N.F., Mastrangelo, M.A., Dewolf, W., Ellis, N.A., Marciniak, R.A., Yin, Y., Jaenisch, R., and Guarente, L. (2000). Association of the Bloom syndrome protein with topoisomerase IIalpha in somatic and meiotic cells. Cancer Res. 60, 1162–1167.
8. Wu, L., Davies, S.L., North, P.S., Goulaothic, H., Brou, J.F., Turley, H., Gatter, K.C., and Hickson, I.D. (2000). The Bloom’s syndrome gene product interacts with topoisomerase III. J. Biol. Chem. 275, 9636–9644.
9. Raynard, S., Bussen, W., and Sung, P. (2006). A double Holliday junction dissolvasome comprising BLM, topoisomerase IIalpha, and BLAP75. J. Biol. Chem. 281, 13861–13864.
10. Wu, L., Bachrati, C.Z., Ou, J., Xu, C., Yin, J., Chang, M., Wang, W., Li, L., Brown, G.W., and Hickson, I.D. (2006). BLAP75/ RMI1 promotes the BLM-dependent dissolution of homologous recombination intermediates. Proc. Natl. Acad. Sci. USA 103, 4068–4073.
11. Yin, J., Sobek, A., Xu, C., Meetei, A.R., Hoatlin, M., Li, L., and Wang, W. (2005). BLAP75, an essential component of Bloom’s syndrome protein complexes that maintain genome integrity. EMBO J. 24, 1465–1476.
12. Singh, T.R., Ali, A.M., Bussygina, V., Raynard, S., Fan, Q., Du, C.H., Andreason, P.R., Sung, P., and Meetei, A.R. (2008). BLAP18/RMI2, a novel OB-fold-containing protein, is an essential component of the Bloom helicase-double Holliday junction dissolvasome. Genes Dev. 22, 2856–2868.
13. Xu, D., Guo, R., Sobek, A., Bachrati, C.Z., Yang, J., Enomoto, T., Brown, G.W., Hoatlin, M.E., Hickson, I.D., and Wang, W. (2008). RMI, a new OB-fold complex essential for Bloom syndrome protein to maintain genome stability. Genes Dev. 22, 2843–2855.
14. Wu, L., and Hickson, I.D. (2003). The Bloom’s syndrome helicase complex suppresses crossing over during homologous recombination. Nature 426, 870–874.
15. Plank, J.L., Wu, J., and Hsieh, T.S. (2006). Topoisomerase IIalpha and Bloom’s helicase can resolve a mobile double Holliday junction substrate through convergent branch migration. Proc. Natl. Acad. Sci. USA 103, 11118–11123.
16. Wechsler, T., Newman, S., and West, S.C. (2011). Aberrant chromosome morphology in human cells defective for Holliday junction resolution. Nature 471, 642–646.
17. Ajima, J., Umezuz, K., and Maki, H. (2002). Elevated incidence of loss of heterozygosity (LOH) in an sgs1 mutant of Saccharomyces cerevisiae: roles of yeast RecQ helicase in suppression of aneuploidy, interchromosomal rearrangement, and the simultaneous incidence of both events during mitotic growth. Mutat. Res. 504, 157–172.
18. Luo, G., Santoro, I.M., McDaniel, L.D., Nishijima, I., Mills, M., Youssoufian, H., Vogel, H., Schultz, R.A., and Bradley, A. (2000). Cancer predisposition caused by elevated mitotic recombination in Bloom mice. Nat. Genet. 26, 424–429.
19. Chan, K.L., North, P.S., and Hickson, I.D. (2007). BLM is required for faithful chromosome segregation and its localization defines a class of ultrafine anaphase bridges. EMBO J. 26, 3397–3409.
20. Cole, T.J., Freeman, J.V., and Preece, M.A. (1998). British 1990 growth reference centiles for weight, height, body mass index and head circumference fitted by maximum penalized likelihood. Stat. Med. 17, 407–429.
21. Keller, C., Keller, K.R., Shew, S.B., and Plon, S.E. (1999). Growth deficiency and malnutrition in Bloom syndrome. J. Pediatr. 134, 472–479.
22. Murray, J.E., Bicknell, L.S., Yigit, G., Duker, A.L., van Kogelenberg, M., Haghayegh, S., Wieczorek, D., Kayserili, H., Albert, 230}
M.H., Wise, C.A., et al. (2014). Extreme growth failure is a common presentation of ligase IV deficiency. Hum. Mutat. 35, 76–85.

23. Tanaka, A.J., Cho, M.T., Millan, F., Juusola, J., Retterer, K., Joshi, C., Niyazov, D., Garnica, A., Gratz, E., Deardorff, M., et al. (2015). Mutations in SPATA5 Are Associated with Microcephaly, Intellectual Disability, Seizures, and Hearing Loss. Am. J. Hum. Genet. 97, 457–464.

24. Goulaouic, H., Roulon, T., Flamand, O., Grondard, L., Lavelle, F., and Riou, J.F. (1999). Purification and characterization of human DNA topoisomerase IIIalpha. Nucleic Acids Res. 27, 2443–2450.

25. Karow, J.K., Chakraverty, R.K., and Hickson, I.D. (1997). The Bloom’s syndrome gene product is a 3'-5' DNA helicase. J. Biol. Chem. 272, 30611–30614.

26. Henricksen, L.A., Umbricht, C.B., and Wold, M.S. (1994). Re­combina­tion replication protein A: expression, complex formation, and functional characterization. J. Biol. Chem. 269, 11121–11132.

27. Rooney D.E., ed. (2001). Human cytogenetics: Malignancy and acquired abnormalities, Third Edition (Oxford University Press).

28. Bizard, A.H., Nielsen, C.F., and Hickson, I.D. (2018). Detection of Ultrafine Anaphase Bridges. Methods Mol. Biol. 1672, 495–508.

29. Nicholls, T.J., Nadalutti, C.A., Motori, E., Sommerville, E.W., Gorman, G.S., Busu, S., Hoberg, E., Turnbull, D.M., Chinnery, P.F., Larsson, N.G., et al. (2018). Topoisomerase 3α is Required for Decatenation and Segregation of Human mtDNA. Mol. Cell 69, 9–23.e6.