Mutations in TOP3A Cause a Bloom Syndrome-like Disorder

Carol-Anne Martin,1,6 Kata Sarlós,2,6 Clare V. Logan,1,6 Roshan Singh Thakur,2,6 David A. Parry,1 Anna H. Bizard,2 Andrea Leitch,1 Louise Cleal,1 Nadia Shaukat Ali,3 Mohammed A. Al-Owain,4 William Allen,5 Janine Altmüller,6 Miriam Aza-Carmona,7,8 Bushra A.Y. Barakat,3 Jimena Barraza-García,7,8 Amber Begtrup,9 Massimo Bogliolo,8,10 Megan T. Cho,10 Jaime Cruz-Rojo,11 Hassan Ali Mundi Dhahahi,3 Nursel H. Elcioglu,11 GÖSgene,11 Granínne S. Gorman,14 Rebekah Jobling,15 Ian Keesterton,16 Yoshiihiyo Kishita,17 Masakazu Kohda,17 Polona Le Quesne Stabej,13 Asam Jassim Malallah,3 Peter Nürnberg,8 Akira Ohtake,18 Yasushi Okazaki,17 Roser Pujoil,8,10 Maria José Ramirez,8,10 Anya Revah-Politi,19 Masaru Shimuara,20 Paul Stevens,16 Robert W. Taylor,14 Lesley Turner,21 Hywel Williams,13 Carolyn Wilson,5 Gökhan Yigit,22 Laura Zahavich,15 Fowzan S. Alkuraya,23,27 Jordi Surralles,8,10,24,27 Alejandro Iglesias,25,27 Kei Murayama,20,27 Bernd Wollnik,22,27 Mehul Dattani,13,27 Karen E. Heath,7,8,27 Ian D. Hickson,2,6* and Andrew P. Jackson1,*

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 RMI1, 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

https://doi.org/10.1016/j.ajhg.2018.07.001.

*Correspondence: iandh@sund.ku.dk (I.D.H.), andrew.jackson@igmm.ed.ac.uk (A.P.J.)

© 2018 The Authors. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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. Mutations in BLM are typically biallelic loss-of-function mutations. 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. Dissolution of a dHJ has been proposed to explain the increase in SCEs in resolvases (SLX-MUS81 and GEN1 nucleases) can yield the alternative processing of dHJs by Holliday junction resolvases (SLX-MUS81 and GEN1 nucleases) can yield crossover events, and increased usage of this pathway has been proposed to explain the increase in SCEs in BLM-deficient cells. Crossover events between homologous chromosomes can lead to loss of heterozygosity (LOH), which can be detrimental to cell survival and contribute to increased cancer predisposition. Additionally, unresolved recombination intermediates can persist into mitosis, leading to chromosome bridges, and act as a source of genome instability.

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 AAAO8410).

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. For calculation of Z scores for growth measurements published for Bloom syndrome, 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. 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) TOP3α cDNA 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’-CCCTCGTCACAC GACTGTGCAGAAGGA-3’ (T3_Fs_FW) and 5’-TCTTTCGCACT GTCTGTACGGAGG-3’ (T3_Fs_RW).

Expression and Purification of TRR
The previously described plasmids encoding RM1 and RM2 and hTopolIIα were co-transformed into E. coli Rosetta 2 cells, and the complex was expressed. The cells were disrupted in buffer A (50 mM Tris-HCl [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-HCl [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 T812LfsTer101RR complex were performed in a similar manner. BLM was purified as described previously.

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

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’-CUUACCGCUGAUUCUUGA-3’ [sTopIIIα 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% Na2CO3H2O3, 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 stain 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 KCl) for 20 min at 37°C, fixed with methanol and acetic acid (3:1 vol/vol), and dropped onto slides. Samples were aged for at least 3 days and then stained with 1.56 μg/mL Hoechst 33258 (Sigma, B2883). Slides were irradiated in 2× SSC with ultraviolet C (356 nm) at 0.260 J/cm², washed with 2× SSC, and stained with 10% Giemsa in phosphate buffer.

**SCE analysis of peripheral-blood samples was performed according to standard methodology in a diagnostic lab setting.**

**Immunofluorescence and microscopy**
Mitotic abnormalities and G1-associated defects were analyzed as described previously. In brief, for mitotic analysis, cells were seeded onto 22 × 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 resuspended on 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-TopIIIα (1:1,000) 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 (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 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), 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 = 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...

---

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

<table>
<thead>
<tr>
<th>Family</th>
<th>Individual</th>
<th>Gene</th>
<th>Biallelic Nucleotide Mutations</th>
<th>Predicted Amino Acid Consequence(s)</th>
<th>Sex</th>
<th>Country of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>P1</td>
<td>TOP3A</td>
<td>c.[2718del];[2718del]</td>
<td>p.[Thr907LeufsTer101];[Thr907LeufsTer101]</td>
<td>female</td>
<td>USA (Czech and Irish ancestry)</td>
</tr>
<tr>
<td>P2</td>
<td>P2</td>
<td>TOP3A</td>
<td>c.[2271dup];[2271dup]</td>
<td>p.[Arg7S8LysfsTer3];[Arg7S8LysfsTer3]</td>
<td>female</td>
<td>United Arab Emirates</td>
</tr>
<tr>
<td>P3</td>
<td>P3</td>
<td>TOP3A</td>
<td>c.[2271dup];[2271dup]</td>
<td>p.[Arg7S8LysfsTer3];[Arg7S8LysfsTer3]</td>
<td>female</td>
<td>United Arab Emirates</td>
</tr>
<tr>
<td>P4</td>
<td>P4</td>
<td>TOP3A</td>
<td>c.[2271dup];[2271dup]</td>
<td>p.[Arg7S8LysfsTer3];[Arg7S8LysfsTer3]</td>
<td>female</td>
<td>United Arab Emirates</td>
</tr>
<tr>
<td>P5</td>
<td>P5</td>
<td>TOP3A</td>
<td>c.[527C&gt;T];[1072_1073dup]</td>
<td>p.[Ala340Val];[Thr359GlyfsTer17]</td>
<td>female</td>
<td>Japan</td>
</tr>
<tr>
<td>P6</td>
<td>P6</td>
<td>TOP3A</td>
<td>c.[2271dup];[2271dup]</td>
<td>p.[Arg7S8LysfsTer3];[Arg7S8LysfsTer3]</td>
<td>female</td>
<td>Syria</td>
</tr>
<tr>
<td>P7</td>
<td>P7</td>
<td>TOP3A</td>
<td>c.[2428del];[2428del]</td>
<td>p.[Ser810LeufsTer2];[Ser810LeufsTer2]</td>
<td>female</td>
<td>Spain</td>
</tr>
<tr>
<td>P8</td>
<td>P8</td>
<td>TOP3A</td>
<td>c.[2428del];[2428del]</td>
<td>p.[Ser810LeufsTer2];[Ser810LeufsTer2]</td>
<td>male</td>
<td>Spain</td>
</tr>
<tr>
<td>P9</td>
<td>P9</td>
<td>TOP3A</td>
<td>c.[2771dup];[2771dup]</td>
<td>p.[Arg7S8LysfsTer3];[Arg7S8LysfsTer3]</td>
<td>female</td>
<td>Saudi Arabia</td>
</tr>
<tr>
<td>P10</td>
<td>P10</td>
<td>TOP3A</td>
<td>c.[2771dup];[2771dup]</td>
<td>p.[Arg7S8LysfsTer3];[Arg7S8LysfsTer3]</td>
<td>female</td>
<td>Saudi Arabia</td>
</tr>
<tr>
<td>P11</td>
<td>P11</td>
<td>RMI1</td>
<td>c.[1255_1259del];[1255_1259del]</td>
<td>[Lys419LeufsTer5];[Lys419LeufsTer5]</td>
<td>female</td>
<td>Turkey</td>
</tr>
<tr>
<td>P12</td>
<td>P12</td>
<td>RMI1</td>
<td>c.[1255_1259del];[1255_1259del]</td>
<td>[Lys419LeufsTer5];[Lys419LeufsTer5]</td>
<td>female</td>
<td>Turkey</td>
</tr>
<tr>
<td>–</td>
<td>MC1</td>
<td>TOP3A</td>
<td>c.[298A&gt;G];[403C&gt;T]</td>
<td>p.[Met100Val];[Arg135Ter]</td>
<td>female</td>
<td>UK</td>
</tr>
</tbody>
</table>

Nomenclature is according to transcript GenBank: NM_004618.4 for TOP3A and GenBank: NM_004618.4 and NM_024945.2 for RMI1.
aClose to donor splice site of exon 10.
bPreviously reported individual with adult-onset mitochondrial disease.

---

The American Journal of Human Genetics 103, 221–231, August 2, 2018 223
Table 1.32 Whereas stature was normal (163 cm) in this adult-onset mitochondrial disorder (denoted as MC1 in Table 3 and Figure 1B).

Table 2. Growth Parameters of Individuals with TOP3A and RMI1 Mutations

<table>
<thead>
<tr>
<th>Individual</th>
<th>Mutated Gene</th>
<th>Gestational Age (Weeks)</th>
<th>Birth</th>
<th>Postnatal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutant Gene</td>
<td>Gestational Age (Weeks)</td>
<td>Weight (SD)</td>
<td>OFC (SD)</td>
</tr>
<tr>
<td>P1</td>
<td>TOP3A</td>
<td>37</td>
<td>1.35</td>
<td>27.5</td>
</tr>
<tr>
<td>P2</td>
<td>TOP3A</td>
<td>37</td>
<td>1.9</td>
<td>43</td>
</tr>
<tr>
<td>P3</td>
<td>TOP3A</td>
<td>35</td>
<td>2.2</td>
<td>31.8</td>
</tr>
<tr>
<td>P4</td>
<td>TOP3A</td>
<td>40</td>
<td>2.2</td>
<td>4.2</td>
</tr>
<tr>
<td>P5</td>
<td>TOP3A</td>
<td>35</td>
<td>1.91</td>
<td>34</td>
</tr>
<tr>
<td>P6</td>
<td>TOP3A</td>
<td>38</td>
<td>1.90</td>
<td>32.5</td>
</tr>
<tr>
<td>P7</td>
<td>TOP3A</td>
<td>34</td>
<td>1.41</td>
<td>28.5</td>
</tr>
<tr>
<td>P8</td>
<td>TOP3A</td>
<td>34</td>
<td>1.41</td>
<td>28.5</td>
</tr>
<tr>
<td>P9</td>
<td>TOP3A</td>
<td>40</td>
<td>2</td>
<td>3.4</td>
</tr>
<tr>
<td>P10</td>
<td>TOP3A</td>
<td>36</td>
<td>1.6</td>
<td>32</td>
</tr>
<tr>
<td>P11</td>
<td>RMI1</td>
<td>34</td>
<td>1.59</td>
<td>38</td>
</tr>
<tr>
<td>P12</td>
<td>RMI1</td>
<td>38</td>
<td>1.80</td>
<td>38</td>
</tr>
<tr>
<td>MC1c</td>
<td>TOP3A</td>
<td>40</td>
<td>2.2</td>
<td>38</td>
</tr>
</tbody>
</table>

Z scores are the standard deviation from population mean for age and sex. The following abbreviation is used: NA, not available.

*Adjusted for gestation.

Growth-hormone treatment from 4 years, 3 months of age to 5 years, 11 months of age showed no response.

Previously reported individual with adult-onset mitochondrial disease (ataxia and progressive external ophthalmoplegia).32

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 TopIIIz

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 TopIIIz in primary dermal fibroblast cell lines derived from P1, P7, and P8. Immunoblotting demonstrated that the amount of full-length TopIIIz 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 TopIIIz. 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 TopIIIz^{Thr907LeufsTer101} 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 TopIIIz^{WT}, the complex containing TopIIIz^{Thr907LeufsTer101} (hereafter referred to as TopIIIz^{P1}) 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 TopIIIz^{WT}, TopIIIz^{P1} 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 TopIIIz^{P1} 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, 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. Therefore, we performed detailed characterization of the mitotic consequences of TopIIIZ deficiency in primary fibroblasts from P1. DAPI staining demonstrated elevated amounts of chromatin bridges (control [C] = 0, parent = 0.3 ± 0.4, and P1 = 4 ± 0.6) and lagging chromatin 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 (C = 2.7 ± 0.3, parent = 13.1 ± 1.1, and P1 = 26.7 ± 3.8).

We then examined the post-mitotic consequences of the observed chromosome segregation errors by enriching for G1 cells (Figures 4C and 4D). Micronuclei often arise from chromosome segregation errors, 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,
Table 3. Clinical Phenotype of Individuals with TOP3A and RMI1 Mutations

<table>
<thead>
<tr>
<th>TOP3A</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>P7</th>
<th>P8</th>
<th>P9</th>
<th>P10</th>
<th>MC1</th>
<th>P11</th>
<th>P12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>5 months</td>
<td>3 years</td>
<td>8 years</td>
<td>10 years</td>
<td>15 years</td>
<td>19 months</td>
<td>3 years</td>
<td>7 years</td>
<td>11 years</td>
<td>4 years, 7 months</td>
<td>adult</td>
<td>7 years</td>
<td>13 years</td>
</tr>
<tr>
<td>Prenatal-onset growth restriction</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Elevated sister chromatid exchange</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>NA</td>
<td>yes</td>
<td>NA</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>NA</td>
<td>no</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Café-au-lait macules</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>NA</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Developmental delay</td>
<td>mild</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>NA</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Cancer</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>NA</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Decreased subcutaneous fat</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>NA</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Gastroesophageal reflux</td>
<td>yes</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>NA</td>
<td>no</td>
<td>NA</td>
<td>NA</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>NA</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Recurrent infections</td>
<td>yes</td>
<td>no</td>
<td>NA</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>NA</td>
<td>yes</td>
<td>yes</td>
<td>NA</td>
<td>no</td>
</tr>
<tr>
<td>Malar rash</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>NA</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes (severe, deceased)</td>
<td>yes (after heart transplant)</td>
<td>normal echocardiogram</td>
<td>NA</td>
<td>yes (asymptomatic)</td>
<td>HCM</td>
<td>no</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mitochondrial DNA depletion in muscle</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>N/A</td>
<td>yes (87%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>yes (&gt;80%)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Other</td>
<td>CDH, gastrosotmy</td>
<td>no</td>
<td>abnormal movements and tics</td>
<td>no</td>
<td>hearing loss, CMAMMA</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>microcytic anemia</td>
<td>no</td>
<td>PEO, ataxia</td>
</tr>
</tbody>
</table>

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.

*Expressive speech delay only.

1All individuals are younger than 15 years old; in Bloom syndrome, neoplasia typically manifests in early adulthood.

2Recurrent otitis media and tonsillitis, leading to tonsillectomy.

3Recurrent upper-respiratory-tract infections and oral thrush.

4Mild left-ventricle dilatation.

5Anemia due to beta-thalassemia trait.
and P1 = 7.5 ± 1.4). Elevated amounts of 53BP1 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 ± 0.5, parent = 4.5 ± 0.5, and P1 = 11 ± 1.7). Altogether, we conclude that impaired dHJ decatenation in TopIIIα-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,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*) in RMI1 in two affected cousins (P11 and P12) from a consanguineous Turkish family (F8; Table 1 and 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 × 10⁻⁵) 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.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 \textit{RMI2}; café-au lait macules were evident, but only one individual showed mild-growth impairment.\textsuperscript{38} Because RMI2 is not essential for BTR (BLM, TopIIIz, and RMII) enzymatic function,\textsuperscript{12} 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, TopIIIz, and RMII are conserved in all eukaryotes but RMI2 is absent in invertebrates and yeast.\textsuperscript{13}

Bloom syndrome is associated with a predisposition to both solid tumors and hematological malignancies.\textsuperscript{3} However, cancers have not been reported in our individuals with \textit{TOP3A} mutations. Nevertheless, all are still children, and because malignancy in Bloom syndrome typically manifests in early adulthood, \textit{TOP3A} mutations could also confer an increased risk of neoplasia.

Distinct from Bloom syndrome, some of our individuals with \textit{TOP3A} mutations have clinical features consistent with mitochondrial dysfunction, and several exhibit a dilated cardiomyopathy. Notably, an individual with chronic progressive external opthalmoplegia and cerebellar ataxia, along with a novel functional role for TopIIIz in the decatenation of mitochondrial DNA after its replication, has been recently reported\textsuperscript{32} (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 TopIIIz 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 TopIIIz in mitochondrial DNA replication.

Mechanistically, our cellular and biochemical studies suggest that the identified \textit{TOP3A} mutations reported here are severe hypomorphs, consistent with the established essential cellular and developmental functions of TopIIIz.\textsuperscript{34} Given that marked growth restriction is common in individuals with \textit{TOP3A} and \textit{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 hemicatenanes into mitosis leads to the formation of chromatin and ultrafine mitotic bridges that have previously been reported in Bloom syndrome\textsuperscript{19} and in individuals with microcephalic dwarfism and condensin mutations.\textsuperscript{39} The resulting genome instability arising from persistent chromatin bridges\textsuperscript{30,41} and micronuclei\textsuperscript{42} could therefore impair cell viability during development, causing microcephaly and global growth restriction. In summary, we demonstrate that mutations in \textit{TOP3A} cause microcephalic dwarfism with increased SCE, implicating the BTRR complex as a cause of growth disorders.

**Figure 3.** Individuals with \textit{TOP3A} Mutations Have More SCEs, Reflecting Chromatid Hyper-recombination

(A) Schematic of dHJ processing. dHJs are either dissolved by the BTRR complex or alternatively cleaved by structure-specific nucleases that lead to dHJ resolution.

(B and C) BrdU strand-specific labeling of sister chromatids shows that TOP3A cells have more SCEs than control and parent cells. (B) Representative images of P1 and parent fibroblast cell lines. (C) Quantification of SCEs in fibroblast cell lines (P1, P5, P7, and P8) or PHA-stimulated lymphocytes derived from peripheral-blood samples (P2 and P3). The median value was plotted with more than ten metaphase spreads counted per subject. Pairwise non-parametric Mann Whitney tests were performed against parental control cells. F1, F2, and F5 SCEs were scored in independent laboratories.
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.

Acknowledgments

We thank the families and clinicians for their participation, the Potentials Foundation and Walking with Giants Foundation, the Medical Research Council (MRC) Institute of Genetics & Molecular Medicine core sequencing service, and G. Stewart and D. Fitzpatrick for helpful discussions. This work was supported by funding from European Research Council 281847; MRC Human Genetics Unit U127580972; and Danish National Research Foundation DNRF115 (I.D.H.), a Danish Cancer Society postdoctoral fellowship (A10769 to R.S.T.); Ministerio de Economía y Competitividad (MINECO) SAF2015-66831-R and SAF2017-84646-R (K.E.H.); the Institutio Catalana de Recerca i Estudis Avancats Academia program, MINECO SAF2015-64152-R, the European Commission,
the Fondo Europeo de Desarrollo Regional “Una Manera de Hacer Europa,” and the Centro de Investigación Biomédica en Red de Enfermedades Raras, an initiative of the Instituto de Salud Carlos III (J.S.); Wellcome Centre for Mitochondrial Research 203105/Z/16/Z, MRC G0601943 and G0800674, and National Health Service Highly Specialised Service for Rare Mitochondrial Disorders of Adults and Children (R.W.T. and G.S.G.); German Federal Ministry of Education and Research 01GM1404, E-Rare network EuroMicro (01GM1404), and German Research Foundation SFB102 project D02 (B.W.); the Practical Research Project for Rare/Intractable Diseases (18ek0109273 and 18ek0109177) from the Japan Agency for Medical Research and Development (K.M, A.O, and Y.O); the Great Ormond Street Hospital Children’s Charity and NIHR Great Ormond Street Hospital Biomedical Research Centre (M.T.D.); and NIH National Center for Advancing Translational Sciences UL1TR001873 (A.R.P. and A.I.). The content and views expressed are solely the responsibility of the authors and not necessarily those of the NIH, NIHR, or Department of Health.

Declaration of Interests
M.C. and A.B. are employees of GeneDx Inc., a wholly owned subsidiary of OPKO Health Inc.

Received: April 30, 2018
Accepted: June 29, 2018
Published: July 26, 2018

Web Resources
GnomAD, http://gnomad.broadinstitute.org

References


