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TOP3A amplification and ATRX inactivation are mutually exclusive events in pediatric osteosarcomas using ALT

Alexandre de Nonneville1,2,3,4,* Sébastien Salas5,*, François Bertucci3,4,*, Alexander P Sobinoff6, José Adélaïde3, Arnaud Guille3,*, Pascal Finetti3,*, Jane R Noble2, Dimitri Churikov1,*, Max Chaffanet3,*, Elise Lavit5, Hilda A Pickett6,*, Corinne Bouvier7, Daniel Birnbaum3,*, Roger R Reddel2,** & Vincent Géli1,***

Abstract

In some types of cancer, telomere length is maintained by the alternative lengthening of telomeres (ALT) mechanism. In many ALT cancers, the α-thalassemia/mental retardation syndrome X-linked (ATRX) gene is mutated leading to the conclusion that the ATRX complex represses ALT. Here, we report that most high-grade pediatric osteosarcomas maintain their telomeres by ALT, and that the majority of these ALT tumors are ATRX wild-type (wt) and instead carry an amplified 17p11.2 chromosomal region containing TOP3A. We found that TOP3A was overexpressed in the ALT-positive ATRX-wt tumors consistent with its amplification. We demonstrated the functional significance of these results by showing that TOP3A overexpression in ALT cancer cells countered ATRX-mediated ALT inhibition and that TOP3A knockdown disrupted the ALT phenotype in ATRX-wt cells. Moreover, we report that TOP3A is required for proper BLM localization and promotes ALT DNA synthesis in ALT cell lines. Collectively, our results identify TOP3A as a major ALT player and potential therapeutic target.

Keywords alternative lengthening of telomeres; ATRX; osteosarcomas; telomeres; TOP3A

Subject Categories Cancer; Chromatin, Transcription & Genomics; Musculoskeletal System

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Introduction

The vast majority of human cancers upregulate telomerase, while the others rely on a mechanism called alternative lengthening of telomeres (ALT) based on homologous recombination (HR)-mediated DNA replication (Sobinoff & Pickett, 2020). The choice between telomerase and ALT seems dependent on the cell-of-origin of the tumor (Lafferty-Whyte et al., 2009; Claude & Decottignies, 2020). Tumors originating from epithelial cells endowed with low telomerase activity frequently upregulate telomerase through mutations in the TERT promoter (Chiba et al., 2017) or genomic rearrangements (Peifer et al., 2015). In tumors of mesenchymal or neuroectodermal origin, activation of TERT expression is less common and these tumors exhibit a high frequency of ALT activation (Kent et al., 2020).

Alternative lengthening of telomeres is characterized by specific features including heterogeneous telomere length, partial clustering of telomeric DNA in promyelocytic leukemia (PML) bodies, partially double-stranded circles with an intact C-strand (C-circles), and telomere sister chromatid exchanges (Yeager et al., 1999; Cesare & Griffith, 2004; Londoño-Vallejo et al., 2004; Henson et al., 2009), although this last feature cannot be assessed directly on tumor tissue. It is accepted that tumors that acquire the ALT phenotype are prone to replication stress, which when unresolved may lead to fork collapse and as a consequence to double-strand breaks (DSBs). ALT uses these breaks to initiate break-induced telomere synthesis by RAD51-dependent and RAD52-dependent mechanisms (Verma et al., 2019; Zhang et al., 2019; Hoang & O’Sullivan, 2020). Multiple...
genomic repair pathways converge at telomeres to promote replication fork restart and telomere break repair (Sobinoff et al., 2017). Disruption of telomere chromatin may be at the root of replicative stress (Jiang et al., 2011; O’Sullivan et al., 2014). Recent results indicate that telomeres can be also elongated during mitosis (mitotic DNA synthesis) in ALT-associated PML body (APB)-like foci to counteract replication stress (Garcia-Exposito et al., 2016; Min et al., 2017; Özer & Hickson, 2018).

The α-thalassemia/mental retardation syndrome X-linked (ATRX) protein is a SWI/SNF2-like chromatin remodeler that deposits histone H3.3 at pericentric heterochromatin and telomeres (Goldberg et al., 2010; Wong et al., 2010). ATRX, or the death domain-associated protein (DAXX) gene which encodes a protein that forms a heterodimeric complex with ATRX, is frequently inactivated by mutation in tumors with ALT (Heaphy et al., 2011; Lovejoy et al., 2012). Because ATRX limits replication stress (Clunes et al., 2013; Leung et al., 2013; Huh et al., 2016) and telomeres are prone to replication stress (Sieir et al., 2009), loss of ATRX likely results in telomeric DSBs that initiate homology-directed repair at telomeres (Hoang et al., 2020; Sobinoff & Pickett, 2020). In human tumors, ATRX was described as associated with mutations in the ATRX pathway (Heaphy et al., 2011). This is well established in ALT-positive gliomas in which the IDH1R132H mutation is associated with ATRX loss (Nguyen et al., 2013; Ohba et al., 2020). ATRX loss has been proposed as a marker of ALT (Sieverling et al., 2020), but the view that ATRX/DAXX loss is essentially equivalent to the presence of ALT activity may apply only to specific types of tumors, in specific genomic or epigenetic contexts, and may divert attention from the need to identify alternative molecular actors involved in this telomere maintenance mechanism (TMM; Barthel et al., 2017; Brosnan-Cashman et al., 2018; Graham et al., 2019; de Nonneville & Reddel, 2021).

High-grade osteosarcomas are highly aggressive malignant bone tumors that mainly occur in children and adolescents. Their therapeutic management includes neoadjuvant chemotherapy, which in pediatric contexts consists of the combination of high-dose methotrexate, ifosfamide, and VP16, followed by surgery, and then adjuvant chemotherapy (Le Deley et al., 2007). When metastatic, the 5-year survival rate of these tumors is less than 30%, indicating the critical need for new treatment strategies (Sayles et al., 2019). Osteosarcomas exhibit a high frequency of ALT activation (Ulaner et al., 2003; Sanders et al., 2004; Henson et al., 2005). Strikingly, previous reports showed that ATRX mutation and/or loss of protein expression is detectable in only 30% of ALT-positive osteosarcomas (Chen et al., 2014; Liao et al., 2015). This discrepancy between a high level of ALT and a low proportion of ATRX inactivation led us to hypothesize that ATRX alteration may not be the only alteration responsible for the ALT mechanism in osteosarcoma.

We have here analyzed the TMM in 22 non-metastatic, non-pre-treated, high-grade pediatric osteosarcomas. We identified TOP3A amplification and overexpression as a mutually exclusive event with ATRX inactivation, and validated TOP3A as an ALT regulator in the ATRX wild-type (wt) setting. We also identified a number of genes for which mutation, amplification, or loss of copy number are closely associated with ALT, revealing new insights into the mechanisms by which telomeres are maintained. Moreover, our data suggest that ALT-positive osteosarcomas may represent different entities in term of therapeutic opportunities.

Results

High frequency of ALT in high-grade osteosarcomas

We characterized a series of high-grade osteosarcomas from 22 non-metastatic and non-pre-treated pediatric patients whose clinicopathological characteristics are described in Table 1. Using the presence of circular partially single-stranded extrachromosomal C-rich telomeric repeat sequences (C-Circles) as an ALT marker, we found that 16 of the 22 high-grade osteosarcomas were ALT positive (Fig 1A). The C-circle assay-based classification was consistent with the presence or absence of APBs in ALT-positive and -negative tumors, respectively (Fig 1B shows a representative photomicrograph; APB analysis was done on 14/22 tumors due to limited availability of tumor material). To explore telomere length distribution in the tumor samples, we further used a telomere shortest length assay (TeSLA), which allows the evaluation of the size of individual telomeres in the bulk population of telomeres (Lai et al., 2017). In ALT-positive tumors, telomere length was more heterogeneous with an increase in the median telomeric fragment size consistent with the general organization of ALT telomeres (Cesare & Reddel, 2010; Fig 1C-E). Thus, by using three different criteria, we were able to unambiguously determine that 73% of our series of high-grade osteosarcomas maintained their telomeres by ALT.

Compared to patients with ALT-negative tumors, patients with ALT-positive tumors had no significant differences regarding age at diagnosis, sex, tumor size, response to neoadjuvant chemotherapy, relapse rate, or deaths (Table 2). With a median follow-up of 126.2 months, 7 out of 16 patients with an ALT-positive tumor, and 1 out of 6 patients with an ALT-negative tumor relapsed. The 5-year Disease-Free Survival (DFS) was 55.6% in ALT-positive tumors vs. 83.3% in ALT-negative tumors, but the difference was not significant likely because of the small cohort size ($P = 0.319$; Log-rank test; Fig EV1A). When the cohort was stratified according to the pathological response to neoadjuvant chemotherapy, poor responders, whether ALT positive or ALT negative, experienced poor DFS outcomes, whereas in the good responders group, only ALT-positive patients relapsed with 64.8% 5-year DFS vs. 100% in ALT-negative patients ($P = 0.205$; Log-rank test; Fig EV1B).

ALT-positive high-grade osteosarcomas mostly express wt ATRX

We next applied targeted next-generation sequencing (tNGS) to the 22 tumors using a panel of 756 genes (Table EV1). With an average sequencing depth of 994×, we identified in the whole series a total of 288 mutations in 200 different genes the potential function of which in telomere maintenance is indicated in Table EV2. We found 244 single-nucleotide variants (non-synonymous and stop-gains), 25 indels, and 1 splice-site mutation (Table EV2). The median mutational burden per tumor (5.46) was higher in ALT-positive tumors than in ALT-negative tumors, but the difference was not significant (Fig EV2).

tNGS revealed recurrent genetic alterations (7/22) in TP53 as previously reported (Chen et al., 2014; Kovac et al., 2015; Bousquet et al., 2016), with 6 out of the 7 mutated tumors being in the ALT-positive group (Fig 2A, Table EV2). Overall, 13 genes were found mutated in at least three tumors (TP53, ATRX, ATAD2, ERCC2, PAMA35B, KMT2D, PCH15, PRX, RB1, REV3L, TET2, TPR, and
TSC2, the functions of which are described in Table EV1). Strik-
ingly, only 4 out of the 16 ALT-positive tumors had an
ATRX mutation and 1 of the 16 had an ATRX deep deletion (Fig 2A; see also the array-comparative genomic hybridization [aCGH] data). More-
over, none of the tumors exhibited a mutation in the gene encoding
the ATRX-associated protein DAXX. Consistent with the tNGS
results, immunohistochemistry analysis of 14/22 available tumors
confirmed ATRX and DAXX protein expression in those identified
by DNA sequencing as wt for ATRX and DAXX (Fig 2B). We
concluded that 69% (11/16) of the ALT-positive tumors displayed a
seemingly unaltered ATRX/DAXX complex. Our results reveal that a
majority of pediatric osteosarcomas were able to maintain their
telomeres by ALT while expressing wt ATRX.

Many of the mutated genes were functionally related (Fig 2C
and Table EV1). For instance, in addition to ATRX, several play a
role in replication, replication stress response, or DNA repair
(MUS81, ERCC2, SMARCAD1, SMARCAL1, POLA1, POLE, POLD1,
REV3L, and RAD54L). Along the same line, although the

Table 1. Clinico-pathological characteristics of 22 pediatric osteosarcomas.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age at diagnosis</th>
<th>Topography</th>
<th>Histology</th>
<th>Tumor size (mm)</th>
<th>Pathological response to neoadjuvant chemotherapy</th>
<th>ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>16</td>
<td>Proximal femoral diaphysis</td>
<td>Osteoblastic and chondroblastic</td>
<td>22</td>
<td>Good</td>
<td>ALT neg</td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
<td>Mid-tibial diaphysis</td>
<td>Chondroblastic</td>
<td></td>
<td>Good</td>
<td>ALT neg</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>Proximal tibial diaphysis</td>
<td>Conventional</td>
<td>76</td>
<td>Good</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>Tibial metaphysis</td>
<td>Conventional</td>
<td>82</td>
<td>Good</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>Femoral diaphysis</td>
<td></td>
<td></td>
<td>Good</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>Proximal femoral metaphysis</td>
<td>Osteogenic</td>
<td>110</td>
<td>Good</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>Distal femoral diaphysis</td>
<td>Osteoblastic and chondroblastic</td>
<td>180</td>
<td>Good</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>Distal femoral diaphysis</td>
<td>Osteoblastic and chondroblastic</td>
<td>34</td>
<td>Good</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>Proximal humeral diaphysis</td>
<td></td>
<td>174</td>
<td>Good</td>
<td>ALT neg</td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>Distal femoral metaphysis</td>
<td>Osteoblastic</td>
<td>140</td>
<td>Good</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Male</td>
<td>17</td>
<td>Proximal humeral diaphysis</td>
<td>Osteoblastic and chondroblastic</td>
<td>150</td>
<td>Good</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Male</td>
<td>10</td>
<td>Proximal tibial metaphysis</td>
<td>Osteogenic</td>
<td>95</td>
<td>Good</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>Proximal humeral diaphysis</td>
<td>Osteoblastic</td>
<td>155</td>
<td>Good</td>
<td>ALT neg</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>Distal femoral diaphysis</td>
<td>Osteoblastic</td>
<td></td>
<td>Poor</td>
<td>ALT neg</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>Distal femoral diaphysis</td>
<td>Osteoblastic</td>
<td>75</td>
<td>Poor</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
<td>Proximal tibial diaphysis</td>
<td>Osteoblastic</td>
<td>112</td>
<td>Poor</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Male</td>
<td>19</td>
<td>Distal femoral metaphysis</td>
<td></td>
<td>100</td>
<td>Poor</td>
<td>ALT neg</td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
<td>Femoral diaphysis</td>
<td>Osteoblastic</td>
<td></td>
<td>Poor</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Male</td>
<td>15</td>
<td>Distal femoral diaphysis</td>
<td>Osteogenic</td>
<td>80</td>
<td>Poor</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>Distal femoral diaphysis</td>
<td>Chondroblastic</td>
<td>90</td>
<td>Poor</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>Proximal tibial diaphysis</td>
<td>Chondroblastic</td>
<td>100</td>
<td>Poor</td>
<td>ALT pos</td>
</tr>
<tr>
<td>Male</td>
<td>17</td>
<td>Distal femoral diaphysis</td>
<td>Chondroblastic</td>
<td>210</td>
<td>Poor</td>
<td>ALT pos</td>
</tr>
</tbody>
</table>

ALT, alternative lengthening of telomeres.

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Figure 1.
frequency of mutation for each individual gene was low, 16 of the 22 tumors were mutated in genes associated with histone lysine methylation (KMT2B, KMT2C, KMT2D, DOT1, NSD1, KDM4A, and TET2) or nucleosome remodeling (SMARCAD1; Fig 2C and Table EV1). Although most of these genes were not previously reported to be altered in osteosarcoma including pediatric forms (Chen et al., 2014; Kovac et al., 2015; Bousquet et al., 2016; Sayles et al., 2019), and although the mutations detected were not functionally validated, our results suggest that the epigenetic landscape in these pediatric tumors might be frequently altered (see Discussion).

We also sequenced canonical histone genes of the HIST1 cluster that are frequently mutated in sarcomas (Nacev et al., 2019; Flaus et al., 2021). We found histone mutations in HIST1H1B, HIST1H1C, HIST1H1D, HIST1H1E, HIST1H1F, HIST1H2A, HIST1H2AM, HIST1H2B, HIST1H3A, HIST1H3B, HIST1H4A, and HIST1H4L, potentially expanding the landscape of oncohistone mutations in osteosarcoma (Table EV2 and Fig EV3; Nacev et al., 2020). Of note, 11/13 of the histone mutations including two mutations in HIST1H3A and HIST1H4H that modify their C-terminal region were found in ALT-positive tumors suggesting that these different mutations may contribute to a favorable environment for ALT activity (see Discussion).

**TOP3A amplification and ATRX mutation are exclusive genomic events in ALT-positive high-grade osteosarcoma**

We next determined the genomic rearrangements of the 22 tumors. DNA copy number profiling using aCGH showed many regions of frequent gains and losses. Globally, ALT-positive tumors appeared more rearranged than ALT-negative tumors, but the difference was not significant, likely due to the low number of ALT-negative tumors (Fig EV2B). Four regions (6p21.1, 8q24.21, 17p11.2, and 19q12) were amplified with high frequency in the tumors, while two were frequently deleted (6q14.3 and 11q14.1; Fig 3A and Table EV2).

---

**Table 2. Correlation between alternative lengthening of telomeres (ALT) status and clinicopathological characteristics.**

<table>
<thead>
<tr>
<th>Correlation</th>
<th>All</th>
<th>ALT-negative</th>
<th>ALT-positive</th>
<th>P-value (Khi²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
<td>55%</td>
<td>3</td>
<td>50%</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>45%</td>
<td>3</td>
<td>50%</td>
</tr>
<tr>
<td>Radiological size in mm (mean, Std. Deviation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>110.3, 49.7</td>
<td>77.8, 61.9</td>
<td>119.6, 43.9</td>
<td></td>
<td>0.142</td>
</tr>
<tr>
<td>Pathological response to neoadjuvant chemotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor responder</td>
<td>9</td>
<td>41%</td>
<td>2</td>
<td>33%</td>
</tr>
<tr>
<td>Good responder</td>
<td>11</td>
<td>59%</td>
<td>4</td>
<td>67%</td>
</tr>
<tr>
<td>Metastatic relapse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>14</td>
<td>64%</td>
<td>5</td>
<td>83%</td>
</tr>
<tr>
<td>Yes</td>
<td>8</td>
<td>36%</td>
<td>1</td>
<td>17%</td>
</tr>
<tr>
<td>Death</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>16</td>
<td>73%</td>
<td>5</td>
<td>83%</td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>27%</td>
<td>1</td>
<td>17%</td>
</tr>
</tbody>
</table>
Figure 2. α-thalassemia/mental retardation syndrome X-linked wild-type (ATRX-wt) in expressed in alternative lengthening of telomeres (ALT)-positive high-grade osteosarcomas.

A Oncoprint graph showing the distribution of mutations (green rectangles) and copy number variations (red rectangle, amplification; blue rectangle, copy loss) in all samples. Tumors are distributed according to ALT positivity (ALT+ or ALT−) and ATRX status (ATRX-mutated or ATRX-wt).

B Tissue sections of two representative high-grade osteosarcomas with hematoxylin eosin saffron (HES) staining (left panel), anti-ATRX (HPA064684) immunochemistry (middle panel), and anti-DAXX (HPA008736) immunochemistry (right panel), showing ATRX protein expression in two ALT-positive samples. In the top panel, intratumoral ATRX expression is negative (positive control osteoclasts are shown). In the bottom panel, intratumoral ATRX expression is high. Scale bars are 250 μm.

C STRING network showing mutated gene-clusters with related functions.
Figure 3.
We classified the most relevant genes that were either amplified (red bar) or deleted (blue bar) in the tumors and mainly considered the genes for which an amplification or a deletion occurred in at least three tumors (see Fig 2A). Within the gained regions, ATAD2, MYC, RUNX2, RQOL4, ERBB2, PDGFRα, and CCNE1 were recurrently observed in the tumors and appear to be potential drivers, confirming previous studies in osteosarcoma (Chen et al., 2014; Kovac et al., 2015; Both et al., 2016; Bousquet et al., 2016; Sayles et al., 2019; Suehara et al., 2019; Guimarães et al., 2021).

Within the 17p11 amplified region, previously described to be frequently amplified in osteosarcomas (Forus et al., 1995; Tarkkanen et al., 1995; van Dartel et al., 2002, 2004; Bayani et al., 2003; Henriksen et al., 2003; Squire et al., 2003; Lau et al., 2004; Both et al., 2016), the TOP3A gene was always present in the amplon, that is, all amplifications included TOP3A, among a number of other genes (Fig 3B). TOP3A caught our attention since it has an essential role in ALT (Tsai et al., 2003; Temime-Smaali et al., 2008; Sobinoff et al., 2017; Pan et al., 2019; Loe et al., 2020; see below). Strikingly, TOP3A was amplified only in the ALT-positive ATRX-wt tumors and not in ALT-positive ATRX-mutated/deleted (ATRX-mut) tumors (71 vs. 0%; Fig 2A).

Interestingly, we found that in most tumors in which TOP3A is amplified (4,001, 4,078, 4,591, 4,665, and 5,136), the TP53 gene was at the edge of the TOP3A amplification (Table EV3). This is particularly notable in tumors 4,001, 4,078, and 5,136, as opposed to tumors 2,254, 3,103, 4,464, and 8,299 where the copy number alteration (CNA) profile of the tumors seems likely that these amplifications result in breakage-induced inactivation of at least one of the two alleles of TP53.

We also observed that MMS21 (which cooperates with the SMCS5/6 complex in DNA repair) and NBS1 (from the MRE11/RAD50/NBS1 DNA repair complex), which both contribute to maintain telomere length by ALT (Compton et al., 2007; Potts & Yu, 2007; Zhong et al., 2007; Barroso-González et al., 2019), were amplified with high frequency in the tumors (Fig 2A). However, in contrast to TOP3A and NBS1, MMS21 amplification was also observed at high frequency in ALT-negative tumors, suggesting that it does not have a specific role in the maintenance of ALT telomeres in pediatric osteosarcomas. Conversely, copy number losses of SMARCAL1 and XRCC5/KU80 were recurrently observed in ALT-positive tumors, suggesting that depletion of these proteins may promote ALT (see Discussion).

Overall, these results suggest that amplification of TOP3A could be a new hallmark of the ALT-positive/ATRX-wt tumors.

**TOP3A is upregulated in ALT-positive ATRX-wt osteosarcomas**

Next, we analyzed the transcriptomic profiles of the tumor samples (12 had good quality RNA) to highlight the differentially expressed genes (DEGs) between 9 ALT-positive and 3 ALT-negative tumors (Fig 4 and Table EV3). As shown in Fig 4A, the most upregulated genes in the ALT-positive tumors were CBRS, COP3, MAGE6, and TOP3A, and a large number of histone genes from the HIST1 cluster (HIST1H2AI, HIST1H3I, HIST1H1B, and HIST1H3B). Consistent with the amplification of the gene, TOP3A mRNA was overexpressed in the ALT-positive tumors overall and in the ALT-positive ATRX-wt tumors but not in the ALT-negative or ALT-positive ATRX-mutated tumors (Fig 4B), strengthening the point that TOP3A overexpression and ATRX mutation are exclusive genomic events in ALT-positive osteosarcomas. The fact that several histone genes were overexpressed in ALT-positive tumors compared to ALT-negative tumors suggests that overexpression of histone genes may favor ALT in pediatric osteosarcomas (Fig EV4).

Globally, ontology analysis by Gene Set Enrichment Analysis (GSEA) in ALT-positive versus ALT-negative tumors revealed an enrichment in the expression of classical pathways related to chromosome duplication and transmission (Fig 4C and Table EV4). The prominence of DNA replication pathways in ALT-positive tumors in
the absence of any evidence of increased replication rate may reflect an increased need for restarting of stalled replication forks (Zhang & Zou, 2020). Conversely, ALT-negative tumors were particularly enriched in genes related to the FGFR2-associated signaling pathways that may be associated with telomerase reactivation in pediatric osteosarcoma (Greenfield et al., 2020).

**Figure 4.**

TOP3A expression in ALT-positive ATRX-wt and ALT-positive ATRX-mutated cell lines tumors as a validation of the tumor results

One of the main discoveries of our study is that TOP3A amplification and ATRX mutation are mutually exclusive genomic events in ALT-positive high-grade osteosarcoma. However, in ALT-positive/
Figure 5.
ATRX-wt tumors, even if wt ATRX is normally expressed, the possibility exists that the ATRX protein might be non-functional. We therefore sought to validate the results obtained from the tumors. We analyzed nine ALT-positive human osteosarcoma-derived cell lines (CAL72, HuO9, KPD, ZK58, NOS1, SaOS2, U2OS, G292, and NY; Lovejoy et al., 2012; Flynn et al., 2015). Sequencing data from the Sanger and Broad Institutes indicated that none of these cancer cell lines displayed a mutation in ATRX or DAXX (Tate et al., 2019), except NOS1 that had a homozygous loss of the ATRX genes (Loo et al., 2010). By Western blot, five out nine of these cell lines did not produce ATRX full-length protein, while four cell lines (KPD, ZK-58, G292, and NY) maintained ATRX protein expression (Fig 5A). We measured TOP3A mRNA expression in these nine osteosarcoma cell lines by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and found that its expression was lower in the ALT-positive ATRX-wt cell lines compared to in the ALT-positive ATRX-mutated cell lines, except NOS1 that had a homozygous loss of the ATRX genes (Loo et al., 2010). We next sought to determine the importance of TOP3A in ALT-positive cell lines that were either ATRX-wt or ATRX-mutated. To do this, we did a TOP3A knockdown (KD) in different ALT-positive cell lines described above and analyzed its impact on ALT hallmarks (Fig EV4B). In the five ALT-positive ATRX-wt cell lines, TOP3A KD decreased C-circles levels to different levels reflecting a disruption of the ALT phenotype. In contrast, TOP3A KD did not compromise C-circle levels in ALT-positive ATRX-mutated cell lines (Fig 6A). Interestingly, TOP3A KD did not affect APB frequency and mean telomere intensity in APBs in U2OS transfected with pCMV-ATRX (Fig 5F). Thus, TOP3A overexpression countered the ATRX-mediated ALT inhibition, strengthening the results obtained with the clinical samples.

**TOP3A knockdown disrupts ALT phenotype and increases telomeric DNA damage**

We next sought to determine the importance of TOP3A in ALT-positive ATRX-wt cells. To do this, we did a TOP3A knockdown (KD) in different ALT-positive cell lines described above and analyzed its impact on ALT hallmarks (Fig EV4B). In the five ALT-positive ATRX-wt cell lines, TOP3A KD decreased C-circles levels to different levels reflecting a disruption of the ALT phenotype. In contrast, TOP3A KD did not compromise C-circle levels in ALT-positive ATRX-mutated cell lines (Fig 6A). Interestingly, TOP3A KD did not affect APB frequency and mean telomere intensity in APBs in U2OS transfected with pCMV-ATRX (Fig 5F). Thus, TOP3A overexpression disrupted the ATRX-mediated ALT inhibition, strengthening the results obtained with the clinical samples.

**TOP3A is required for proper BLM localization and promotes ALT DNA synthesis**

Interestingly, our results indicate that TOP3A depletion particularly affects telomere maintenance in the ALT-positive ATRX-wt NY cell
Figure 6.
line. Because NY cells overexpress TOP3A, we hypothesized that this may lead to more BLM localization at telomeric sequences. Although not significant via Mann–Whitney, we observed an increase in the intensity of BLM at telomeres in the NY cells compared to U2OS cells (Fig 7A, right and left panels). We also found that TOP3A depletion clearly affects BLM localization at APBs in both cell lines (Fig 7A). Given this result, we measured ALT DNA synthesis (Sobinoff et al., 2017; Zhang et al., 2019) in the NY and UO2S cell lines (Fig 7B). We found that the NY cell line had a higher number of cells with EdU foci in APBs and that TOP3A depletion compromised ALT DNA synthesis in both cell lines (Fig 7B, right and left panels). Taken together, these results indicate that increased levels of TOP3A in NY cells correlate with increased levels of BLM at ALT telomeres and that TOP3A promotes ALT DNA synthesis. These results may explain the greater dependence of NY cells on TOP3A.

Discussion

Although ALT is the TMM commonly utilized by osteosarcomas, many of these tumors have wt ATRX, a gene which is frequently mutated in ALT-positive cancers (Ulaner et al., 2003; Sanders et al., 2004; Henson et al., 2005; Chen et al., 2014; Liau et al., 2015). The contrast with other types of sarcomas is illustrated by a study of ALT phenotype and ATRX status in 519 sarcoma samples which found loss of ATRX expression in approximately 50% of ALT-positive leiomyosarcomas, undifferentiated pleomorphic sarcomas, and pleomorphic liposarcomas, whereas ATRX mutation and loss of expression was observed in only 30% of osteosarcomas (Liau et al., 2015). We show here that another mutational event is characteristic of ATRX-wt osteosarcomas.

We found that ALT tumors carried either an ATRX mutation or an amplification of the 17p11.2 region as two mutually exclusive events. In this chromosomal region, we identified TOP3A amplification as the major genomic feature of the ALT tumors expressing wt ATRX. Gene expression analyses revealed that TOP3A was overexpressed in the ALT-positive ATRX-wt tumors compared to either ALT-positive ATRX-mutated or ALT-negative tumors.

Amplification of the 17p11.2 region has been previously reported in osteosarcomas with a frequency ranging from 20 to 78% suggesting the presence of one or more oncogenes in this region (Forus et al., 1995; Tarkkanen et al., 1995; van Dartel et al., 2002, 2004; Bayani et al., 2003; Henriksen et al., 2003; Squire et al., 2003; Lau et al., 2004; Both et al., 2016). Of note, overexpression of genes belonging to the amplified region (COPS3, PMP22, G4D4, ARCHAP4, TOP3A, SHMT1, and RASD1) was studied in osteosarcoma cell lines (Both et al., 2016). While TOP3A, SHMT1, and RASD1 overexpression resulted in increased proliferation, ARCHAP4, COPS3, and PMP22 overexpression had a stimulatory effect on migration and invasion of the cells. COP3 and PMP2 overexpression additionally improved the ability of the cells to form new colonies (Both et al., 2016). However, the contribution of this region was not studied with respect to telomere maintenance previously. Here, we have shown that TOP3A amplification and overexpression is an alternative to ATRX inactivation, and the most frequent genetic event identified in ALT-positive high-grade pediatric osteosarcoma.

The hypothesis that TOP3A overexpression was instrumental in maintaining ALT in ATRX-wt cells was confirmed by in vitro experiments showing first that TOP3A overexpression could restore ALT features in ALT-positive ATRX-mutated cell lines transfected with wt ATRX and second that TOP3A KD disrupted the ALT phenotype with a concomitant increase in telomeric DNA damage in ALT-positive ATRX-wt cells. TOP3A is a type I DNA topoisomerase operating within the BLM-TOP3A-RIM1 complex (known as the BTR complex) that suppresses crossing-over during HR (Wu & Hickson, 2003). TOP3A was previously shown to be localized at ALT telomeres and to be required to maintain ALT telomeres (Tsai et al., 2006; Temime-Smaali et al., 2008). TOP3A was further shown to promote POL3-dependent telomere synthesis through a mechanism analogous to break-induced replication (Dilley et al., 2016; Sobinoff et al., 2017; Lu et al., 2019). The central role of the BTR complex in ALT was further underscored by the recent discovery...
that its localization at telomere ends is essential for ALT activity (Loe et al., 2020; Zhang et al., 2021). Here we have shown that TOP3A depletion affects BLM localization at APBs and compromises ALT DNA synthesis in U2OS and NY cell lines that are ATRX mutated and ATRX-wt, respectively. However, we found that the NY cell line has more BLM at APBs and higher levels of ALT DNA
synthesis consistent with the fact that NY cells overexpress TOP3A and are more sensitive to TOP3A depletion. TOP3A has also been described to have a role in the processing of ultra-fine bridges that connect the separating sister DNA molecules during anaphase at specific unreplicated genomic loci, including telomeres (Sarloš et al., 2018), suggesting that TOP3A plays a role in mitotic DNA synthesis (MiDAS; Ozer & Hickson, 2018). Moreover, recent results indicated that reconstituted PML bodies consisting of polySUMO/polySIM condensates targeting telomeres were able to cluster telomeres and generate ALT telomeres through a process mediated by BLM and RAD52 (Min et al., 2019; Zhang et al., 2019, 2020).

Collectively, these studies reveal that BIR, MiDAS, and ALT share molecular processes in which TOP3A is a major actor (Epum & Haber, 2021). We speculate that in tumors undergoing replication stress, TOP3A overexpression promotes these molecular processes, accounting for its overexpression being a critical contributor to ALT activity in tumors.

Strikingly, we observed that amplification of the 17p11.2 region may both amplify TOP3A and disrupt at least one allele of TP53 in most ATRX-wt tumors. Analysis of the TP53 status in the tumors carrying an amplified 17p11.2 region raises the interesting hypothesis that the 17p11 amplification could do “double duty,” on the one hand amplifying TOP3A and on the other hand inactivating TP53.

Beside TOP3A amplification, our study revealed alterations in other genes that have been implicated in ALT. NBS1, the depletion of which results in inhibition of ALT in human cell lines (Compton et al., 2007; Zhong et al., 2007), was amplified in ALT-positive tumors. siP100, which sequesters the MRN complex thereby suppressing ALT (Jiang et al., 2005), had a loss of copy number. These results highlight the importance of the MRN complex in the formation of ALT telomeres in these pediatric tumors. Loss of KU86 may also play a role in ALT, as suggested by several studies (Zellinger et al., 2007; Wang et al., 2009; Yu et al., 2015). Of note, we did not find alterations in SLX4IP that was reported to antagonize BLM activity during ALT Maintenance (Panier et al., 2019).

We found mutations in genes that organize the structure of chromatin. Although not previously reported in osteosarcomas, mutations in KMT2B/MLL2, KMT2C/MLL3, or KMT2D/MLL4 that catalyze mono- or di-methylation of H3K4 (Hyun et al., 2017) are frequently observed in many types of cancer (Rao & Dou, 2015; Fagan & Dingwall, 2019). Interestingly, it was recently reported in mouse embryonic stem cells deleted for ATRX, TP53, and TERT that inhibiting KDM4B/JMJD2B (which demethylates H3K9 and H3K36) drives ALT activation (Udagawa et al., 2021). Consistent with this observation, we found that among the five ALT-positive ATRX-mutated tumors, two carried mutations in TP53 and KDM4A/JMJD2A (E23K and K463Q; that also demethylates H3K9 and H3K36), suggesting that simultaneous inactivation of TP53, ATRX, and KDM4A/JMJD2A may also drive ALT in pediatric osteosarcoma.

In pediatric high-grade glioma, the G34R mutation in H3.3 triggers ALT, irrespective of the ATRX status. In contrast, only a subset of H3.3-K27M tumors activate ALT (Minasi et al., 2021). This result is to be compared with a recurrent amplification of 17p11.2 targeting TOP3A and mutually exclusive with ATRX deletion/mutations that has been identified in diffuse intrinsic pontine glioma with a H3.3-K27M mutation (Mackay et al., 2017). Together with our data, these results suggest that under conditions that reprogram the epigenetic landscape, ATRX inactivation or TOP3A amplification may represent two independent ways of promoting ALT.

We found that of the 13 tumors displaying mutations in histones genes of the HIST1 cluster, 11 were ALT positive irrespective of their ATRX status. Two mutations (in HIST1H3A and HIST1H4H) either introduced a frameshift or modified the C-terminal region known to be crucial for the interaction with the histone chaperone ASFI (Natsumet al., 2007), the depletion of which leads to the rapid induction of ALT in primary human lung fibroblasts (O’Sullivan et al., 2014). In addition, we observed that many histone genes of the HIST1 cluster were upregulated in ALT-positive tumors compared to ALT-negative tumors. We favor the idea that overexpression of canonical histone genes in the ALT-positive tumors might create an imbalance between canonical histones and histone variants (Maya Miles et al., 2018), which may generate replication stress promoting ALT.

Finally, the analysis of clinical data revealed that, among patients with good response to neoadjuvant chemotherapy, ALT positivity of the pre-treatment biopsy was associated with worse DFS than ALT negativity (4/9 events vs. 0/4 at 126.2 of median follow-up, respectively). This observation, which needs to be validated in a larger cohort, might suggest the relevance of studying TMM as a biomarker in high-grade pediatric osteosarcomas to reassign ALT-positive good responders as candidates for alternative adjuvant therapy after surgery. To date, there is no prognostic marker able to differentiate good responders from poor responders at diagnosis. This result is consistent with many other studies showing that, with the notable exception of glioblastomas, ALT positivity is associated with unfavorable clinical outcome of most tumor types (Henson & Reddel, 2010).

In conclusion, our study identified a number of genomic features associated with ALT, providing new targetable proteins and potential therapeutic opportunities for osteosarcoma. The results emphasize the potential interest in developing TOP3A inhibitors for treatment of ALT-positive ATRX-wt osteosarcomas.

Materials and Methods
Patient cohorts and tumor samples
Frozen tumor samples of 22 high-grade osteosarcomas from pediatric patients treated at Timone Hospital (Marseille, France) were retrospectively obtained. At the time of these diagnostic biopsies, all patients had strictly localized diseases and were naïve of any pre-treatment. Diagnosis of osteosarcoma was made by an expert pathologist of the French Sarcoma Group and all specimens were conventional high-grade osteosarcomas. Normal tissue was obtained from surgical resection and after neo-adjuvant chemotherapy for 21 patients. All patients or their legal guardian provided written informed consent (also see Ethics Declaration below).

Ethics declaration
This study was approved by the Aix-Marseille University Research Ethics Committee. Tumor material was obtained from the Timone Hospital biobank (Tumorothèque et Banque de Muscles, Hôpital de la Timone—Registration no. AC-2018-3105 at the Ministry of Higher Education, Research and Innovation). Signed and written formal consent was obtained from all patients or their legal guardian. The
For each sample, the genomic profile was established by using aCGH DNA copy number profiling. For this study were provided by the computing facilities DISC (Datacenter IT and Scientific Computing) of the Centre de Recherche en Cancérologie de Marseille.

DNA copy number profiling

For each sample, the genomic profile was established by using aCGH onto high-resolution 4 × 180 K CGH-microarrays (SurePrint G3-Human CGH-Microarray, Agilent Technologies). Human female DNA was used as reference (G152A, Promega, Madison, WI, USA). Both experimental and analytic methods have been previously described (Adélaide et al., 2007). All probes for aCGH were mapped according to the Genome Reference Consortium Human Build 37 (CGCh37/Hg19; https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/). We used two different threshold values (log2 ratio > 0.2 and >0.5) to distinguish low- (gain/loss) from high-amplification/deletion) level CNA, respectively. Percentage of altered genome was the number of probes above the threshold divided by the total number of probes for autosomal chromosomes. To identify the overall altered regions, we used the GISTIC2.0 algorithm with alteration thresholds set to 0.15 and with a corrected threshold probability (q < 0.25) to define a statistically relevant region.

Transcriptomic analyses

DNA microarrays were used to define the transcriptional profiles of 12 tumor samples with a RIN ranging from 8.1 to 9.9. Experiments were done as recommended by the manufacturer (Affymetrix, Thermo Fisher) from 100 ng of total RNA for each sample using the GeneChip™ WT PLUS Reagent Kit and the Affymetrix GeneChip™ HuGene 2.0 ST arrays. Expression data were normalized by RMA with the nonparametric quantile algorithm in R using Bioconductor and associated packages (version 3.5.2; http://www.r-project.org/). Supervised analysis comparing the expression profiles between sample classes was done using a moderated t-test with empirical Bayes statistic included in the limma R package (version 3.38.3). Significantly, DEGs were defined by the following thresholds: P-value < 0.05 and fold change FC >2x. Ontology analysis was done by the GSEA algorithm with the Reactome pathway database included in the MSigDB (version 7.2) 101. GSEA was done with 1000 gene-set permutations as parameters and significantly DEG sets were defined by the following thresholds: P-value < 0.05 and q-value < 0.25.

Immunohistochemistry

Immunohistochemistry was performed using ATRX (1:500, Sigma Aldrich) and DAXX (1500, Sigma Aldrich) antibodies. The immunohistochemical protocol included deparaffinization, hydration, antigen retrieval, primary antibody incubation, and detection and visualization as per the manufacturer’s instructions.

Telomere shortest length assay

The TeSLA method was performed as described (Lai et al., 2017). Briefly, 50 ng of genomic DNA was added to a final volume of 20 μl ligation buffer containing 1,000 units of T4 DNA ligase (New England Biolabs), 1× Cut Smart Buffer (New England Biolabs), 1 mM ATP, and 1 nM of TeSLA telorettes (TeSLA Telo 1–6) and incubated at 35°C for 16 h followed by heat inactivation at 65°C for 10 min. After ligation, genomic DNA was digested using a set of restriction enzymes (2 U each of CviAII, BfaI, NdeI, and MseI, New England Biolabs) and then treated with 1 U of Shrimp Alkaline Phosphatase (rsAP, New England Biolabs) at 37°C for 60 min in a final volume of 50 μl. This mixture was subsequently heat inactivated at 80°C for 20 min and 10 μl of sample was added to 10 μl of adapter ligation.
mix (1 µM AT adapter, 1 µM TA adapter, 1 mM ATP, 1× Cut Smart Buffer and 2,000 units of T4 DNA Ligase) and incubated at 16°C for 16 h. After adapter ligation, the sample was heat inactivated at 65°C for 10 min and subsequently diluted to a concentration of 15 pg DNA/µl (1:25 dilution). For each sample analyzed, we performed eight independent PCRs (94°C for 2 min followed by 26 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 15 min) using a total of 25 µl mix containing 30 pg DNA, 2.5 U FailSafe enzyme (Epicenter), 1× FailSafe buffer H (Epicenter), and 250 nM primers (adapter and TeSLA TP). PCR products were electrophoresed on a 0.85% agarose gel (1.5 V/cm for 19 h). The gels were dried for 75 min at 60°C, denatured in 0.5 M NaOH/1.5 M NaCl for 1 h, and neutralized in 0.5 M Tris–HCl (pH 8.0)/1.5 M NaCl for 1 h. Gels were then rinsed in 2× SSC and prehybridized in Church buffer (250 mM sodium phosphate buffer, pH 7.2, 7% [wt/vol] SDS, 1% [wt/vol] BSA fraction V grade, and 1 mM EDTA) for 2 h at 37°C. Finally, gels were hybridized overnight with a γ-[32P]-ATP-labeled (TTAGGG)3 oligonucleotide probe, washed three times in 0.1× SSC for 15 min at 37°C, and exposed to a PhosphorImager screen overnight. Images were analyzed using MATLAB-based software to detect and annotate the size of telomere bands including the percentage of shortest telomeres and average telomere lengths.

**Cell culture and cell lines**

All cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum in a humidified incubator at 37°C with 5% CO2. HCT116, CAL72, HuO9, KPD, ZK58, NOS1, SaOS2, U2OS, G292, and NY cell lines were obtained from the American Type Culture Collection. IIICF cell lines are in vitro-immortalized breast fibroblast cells that were established from an individual with Li–Fraumeni syndrome (Maclean et al., 1994; Rogan et al., 1995; Bryan et al., 1997). Cell lines were authenticated by 16-locus short-tandem-repeat profiling and tested for mycoplasma contamination by CellBank Australia (Children’s Medical Research Institute, Westmead, NSW, Australia).

**Transfection**

An ATRX expression vector, pCMV6-Entry-ATRX, and the empty vector pCMV6-Entry were obtained from OriGene. Transfection with each vector was performed using FuGENE reagent (Promega). Cells were harvested at 72 h post-transfection and analyzed as described. Stable TOP3A-overexpressing U2OS cell lines were generated as follows: cells were seeded at 2 × 10⁵ per 6-well plates and reverse transfected with 1 µg DNA of either pcDNA-TOP3A or pcDNA-Empty plasmid (courtesy of JF Riou), using FuGENE-6 reagent (Promega) according to the manufacturer’s instructions. G418 (400 µg/ml) was added after 24 h for selection over 1 month. Cells were continuously cultured in G418 to ensure overexpression. Protein overexpression was confirmed via Western blot analysis.

**Western blot**

Cells were collected and lysed in radioimmunoprecipitation assay buffer. Proteins were resolved by electrophoresis using a Tris-acetate gel or 4–12% Bis-Tris gel (Invitrogen). Transferred membranes were blocked in 5% milk and incubated with primary antibody overnight. Antibodies were used against ATRX (Sigma HPA064684, SC10078, and SC1540; 1:800 dilution), DAXX (Sigma HPA008736; 1:800 dilution), Vinculin (TS191; 1:600 dilution), γ-tubulin (Sigma, TS192; 1:1,000 dilution), and TOP3A (proteintech 14,525-1-AP; 1:600 dilution).

**RNA interference**

Two TOP3A Silencer Select siRNAs were designed and synthesized by Life Technologies (s14311 & s224746), and Silencer Select RNAi Negative Control #2 (#4390847) was used as the control siRNA. Cell suspensions were transfected at 20–50% confluence with Lipofectamine RNAiMAX (Life Technologies) at a final siRNA concentration of 30 nM. Culture medium was changed after 48 h and cells were harvested for analysis 72 h post-transfection. KD efficiency was validated by western blot analysis.

**Quantitative reverse transcription PCR**

KD experiments using siRNA were verified by qRT–PCR. Total RNA was isolated using the RNeasy mini kit (Qiagen) and DNase-treated to remove genomic DNA. Reverse transcription was performed with 2 µg isolated RNA, 500 ng oligo(dT)15 primer, 40 U RNasin, 0.5 mM dNTPs, and 20 U M-MLV-Reverse Transcriptase (Promega). Quantitative PCR was performed using SYBR Green qPCR Master Mix (Roche) according to the manufacturer’s instructions. Primer sequences are supplied as supplementary data (Table EV4). PCRs were performed on cDNA equivalent to 100 ng total RNA and carried out for 40 amplification cycles, followed by melt curve analysis. For each sample, a replicate omitting the reverse transcription step was included as a negative control. Primer PCR efficiencies were calculated by standard curve. Real-time data were analyzed using the ΔΔC(t) method, with GAPDH as the reference gene, and expressed as fold-change relative to the appropriate control (±SEM).

**Immunofluorescence and fluorescence in-situ hybridization**

Cells were grown on cover slips. Slides were subjected to pre-extraction by incubation in KCM permeabilization solution (120 mM KCl, 20 mM NaCl, 10 mM Tris, 0.1% (v/v) Triton X-100) for 10 min. Slides were then washed in PBS, fixed at room temperature for 10 min in PBS with 4% (v/v) formaldehyde. For tumor samples, 4 µm paraffin sections were cut and dewaxed. Slides were rehydrated then microwave heated to 120°C in 90% glycerol, 10 mmol/l Tris (pH 10.5), 1 mmol/l EDTA, and maintained at 110–120°C for 15 min. The slides were cooled and rinsed in PBS. All subsequent treatments of cell lines and paraffin sections were identical. Cells were blocked with 100 µg/ml DNase-free RNase A (Sigma) in antibody-dilution buffer (20 mM Tris–HCl, pH 7.5, 2% (v/v) BSA, 0.2% (v/v) fish gelatin, 150 mM NaCl, 0.1% (v/v) Triton X-100, and 0.1% (w/v) sodium azide) for 1 h at room temperature. Antibodies were used against PML (Santa Cruz, sc-966; Goat; 1:400 dilution), γ-H2AX (Merck Millipore, Mouse; 1:1,000 dilution), BLM (Bethyl Laboratories, A300-110A; Rabbit; 1:500 dilution), and TOP3A (Temime-Smaali et al., 2008; Rabbit; 1:1,000 dilution). Slides were incubated with primary antibody diluted in antibody-dilution...
buffer for 1 h at room temperature, washed in phosphate-buffered saline-Tween-20, and incubated with appropriate AlexaFluor secondary antibodies diluted in antibody-dilution buffer for 1 h at room temperature in a humidified chamber. Subsequent telomere FISH was performed. Slides were washed in PBST and fixed for 10 min in PBS with 4% (v/v) formaldehyde at room temperature. Slides were then subjected to a graded ethanol series (70% (v/v) for 3 min, 90% (v/v) for 2 min, and 100% for 2 min) and allowed to air-dry in the dark. Dehydrated slides were then overlaid with 0.3 µg/ml TAMRA-OO-(CCCTAA)₃ or Alexa 488-OO-(CCCTAA)₃ telomeric PNA probe (Panagene) in PNA hybridization solution (70% (v/v) deionized formamide, 0.25% (v/v) NEN blocking reagent (Perkin-Elmer), 10 mM Tris–HCl, pH 7.5, 4 mM Na₂HPO₄, 0.5 mM citric acid, and 1.25 mM MgCl₂), denatured at 80°C for 3 min, and hybridized at room temperature overnight. Slides were washed in PNA wash A and then in PNA wash B for 15 min each. 4′,6-diamidino-2-phenylindole (DAPI) was added at 50 ng/ml to the final wash. Finally, slides were rinsed briefly in deionized water and mounted in DABCO anti-fade mounting media.

**ATSA assay (ALT telomere DNA synthesis in APBs)**

To visualize DNA synthesis at telomeres, cells were treated with 10 µM of RO-3306 for 18 h for G2 synchronization. G2 synchronized cells were then pulsed with 10 µM EdU for 1 h. Cells were permeabilized in KCM permeabilization solution (120 mM KCl, 20 mM NaCl, 10 mM Tris, 0.1% (v/v) Triton X-100) for 10 min, then fixed with 4% formaldehyde PBS solution for 10 min. The Click-iT® Alexa Fluor 647 azide reaction (Invitrogen) was then performed according to the manufacturer’s instructions. Indirect immunofluorescence for PML and telomere-FISH were then performed according to the relevant methods section.

**Automated image analysis**

ZEN microscopy images (.czi) were processed into extended projections of z-stacks using ZEN desk 2011 software (Zeiss) and imported into Cellprofiler v2.1.1 (29) for analysis. The DAPI channel was used to mask individual nuclei as primary objects. Foci within each segmented nucleus were identified using an intensity threshold-based mask. Any given object was considered to be overlapping another object when at least 80% of the first object’s area was enclosed within the area of a second object.

**Data availability**

The datasets and computer code produced in this paper are available at the ArrayExpress database with the following accession number: RNA Affymetrix GeneChip: E-MTAB-11743 (Title: Alternative Lengthening of Telomeres (ALT) and ATRX inactivation in high-grade pediatric osteosarcomas. Permanent link: https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-11743); CGH: E-MTAB-11747 (Title: Genome profiling by comparative genomic hybridization (CGH) in pediatric osteosarcoma. Permanent link: https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-11747).

**The paper explained**

**Problem**

To maintain their telomeres, most human cancer cells upregulate telomerase, while the rest uses a mechanism based on HR-mediated DNA replication ALT. High-grade osteosarcomas are highly aggressive bone tumors that mainly occur in children and adolescents, with limited treatment options. Strikingly, osteosarcomas exhibit a high frequency of ALT. In contrast to many other tumors, ATRX, a chromatin remodeler, is mutated in only 30% of ALT-positive osteosarcomas, indicating that these may not be the only alterations responsible for ALT.

**Results**

We characterized non-metastatic, non-pre-treated, high-grade pediatric osteosarcomas and uncovered that more than 70% of these tumors maintained their telomeres via the ALT pathway. In the ALT tumors, TOP3A gene amplification and overexpression was more prevalent than ATRX loss-of-function. Because ATRX mutation and TOP3A overexpression were mutually exclusive, overexpression of TOP3A appeared as an alternative mechanism driving ALT in pediatric osteosarcoma. In ALT-positive human osteosarcoma-derived cell lines, TOP3 overexpression also correlated with the presence of ATRX. Moreover, TOP3A was required to localize the BLM helicase to telomeres and promote ALT-associated DNA synthesis in ALT osteosarcoma cell lines. Several genes whose alteration is associated with ALT in pediatric osteosarcoma were additionally identified, revealing new mechanistic insights into telomere maintenance in these tumors. Overall, these results identify TOP3A or TOP3A downstream signaling as a potential therapeutic target.

**Impact**

Amplification of TOP3A is a new hallmark of the ALT mechanism in tumors that are otherwise wt for ATRX. The discovery that TOP3A amplification/overexpression is mutually exclusive with ATRX mutation constitutes a strong argument for the involvement of TOP3A in onco genesis. Our work therefore positions TOP3A action at telomeres as a prognostic marker and a new potential therapeutic target in ALT-associated pediatric osteosarcomas.

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**Author contributions**

Alexandre de Nonneville: Conceptualization; formal analysis; validation; supervision; investigation; visualization; methodology; writing – original draft.

Discriminative analysis; supervision; funding acquisition; validation; investigation; writing – review and editing. Vincent Géli: Conceptualization; formal analysis; supervision; funding acquisition; validation; investigation; writing – original draft; project administration.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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