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Telomere dysfunction and chromothripsis

Aurélie Ernst[1], David T.W. Jones[2], Kendra K. Maass[3], Agata Rode[1], Katharina I. Deeg[4], Billy Michael Chelliah Jebaraj[5], Andrey Korshunov[6], Volker Hovestadt[1], Michael A. Tainsky[7], Kristian W. Pajtler[2], Sebastian Bender[2], Sebastian Brabetz[2], Susanne Gröbner[2], Marcel Kool[2], Frauke Devens[1], Jennifer Edelmann[5], Cindy Zhang[8], Pedro Castelo-Branco[8], Uri Tabori[8], David Malkin[9], Karsten Rippe[4], Stephan Stilgenbauer[5], Stefan M. Pfister[2], Marc Zapatka[1] and Peter Lichter[1]

[1] Division of Molecular Genetics, German Cancer Research Center (DKFZ), Heidelberg, Germany
[2] Division of Pediatric Neurooncology, German Cancer Research Center (DKFZ), Heidelberg, Germany
[3] Division Functional Architecture of the Cell, German Cancer Research Center (DKFZ), Heidelberg, Germany
[4] Genome Organization and Function, German Cancer Research Center (DKFZ), Heidelberg, Germany
[5] Department of Internal Medicine III, University of Ulm, Germany
[6] Department of Neuropathology University Hospital, Clinical Cooperation Unit Neuropathology, German Cancer Research Center (DKFZ), Heidelberg, Germany
[7] Department of Oncology, Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI
[8] Division of Pediatric Hematology-Oncology and the Labatt Brain Tumour Research Centre, The Hospital for Sick Children, Toronto, ON, Canada
[9] Division of Hematology/Oncology and Department of Pediatrics, The Hospital for Sick Children and Department of Pediatrics, University of Toronto, Toronto, ON, Canada

Chromothripsis is a recently discovered form of genomic instability, characterized by tens to hundreds of clustered DNA rearrangements resulting from a single dramatic event. Telomere dysfunction has been suggested to play a role in the initiation of this phenomenon, which occurs in a large number of tumor entities. Here, we show that telomere attrition can indeed lead to catastrophic genomic events, and that telomere patterns differ between cells analyzed before and after such genomic catastrophes. Telomere length and telomere stabilization mechanisms diverge between samples with and without chromothripsis in a given tumor subtype. Longitudinal analyses of the evolution of chromothriptic patterns identify either stable patterns between matched primary and relapsed tumors, or loss of the chromothriptic clone in the relapsed specimen. The absence of additional chromothriptic events occurring between the initial tumor and the relapsed tumor sample points to telomere stabilization after the initial chromothriptic event which prevents further shattering of the genome.

Chromothripsis is a recently discovered form of genome instability, whereby one or a few chromosomes are affected by tens to hundreds of clustered DNA rearrangements.[1,2] Localized chromosome shattering occurs as a one-time catastrophic genomic event, followed by inaccurate repair of the derivative fragments.[3,4] This phenomenon has been observed in 2–3% of all cancers, across various tumor types, and has been associated with poor prognosis in certain entities.[2,5] Chromothripsis is thought to promote, and in some cases perhaps even cause, cancer development, since it can lead to the loss of tumor suppressor genes, to the formation of oncogenic fusions and to oncogene amplification.[1,2] Several features in tumor cells have been linked to chromothripsis, which is presumed to be a context-dependent event. Notably, we reported an association of chromothripsis with *TP53* mutation in subsets of medulloblastoma and acute myeloid leukemia.[2] However, the precise genomic context, the trigger(s) for the DNA double-strand breaks as well as the mechanistic basis of the shattering and repair process are currently unknown. Several nonexclusive mechanisms have been proposed, among which are DNA damage in micronuclei,[6] premature chromosome condensation, breakage-fusion-bridge (BFB) cycles and telomere dysfunction.[2,7,8]

Telomeres are essential structures for genomic stability.[9,10] These specialized nucleoprotein complexes stabilize chromosome ends by protecting them from end-to-end fusions and DNA degradation. In humans, telomeres are comprized largely of (TTAGGG)n tandem repeats associated with capping proteins, which constitute the shelterin complex. Telomeric repeat sequences are added by the telomerase enzyme,

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Correspondence to: Peter Lichter, German Cancer Research Center (DKFZ), Division of Molecular Genetics, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany, Fax: +49 6221 42 4639,
E-mail: peter.lichter@dkfz-heidelberg.de

What's new?

Chromothripsis is characterized by extensive locally clustered genomic rearrangements, whereby chromosome shattering is followed by rejoining of the DNA fragments by error-prone repair mechanisms. The present study elaborates on a previously proposed role in the initiation of chromothripsis for telomere erosion and breakage-fusion-bridge (BFB) cycles, in which chromosomes repeatedly break and are rejoined. In cells lacking normal mechanisms for genome preservation, telomere attrition and BFB cycles induced chromothripsis. Subsequent activation of tumor-specific telomere maintenance mechanisms prevented further chromosomal shattering. The findings suggest that telomere maintenance pathways may represent therapeutic targets in chromothripsis-positive tumors.

which compensates for the loss of chromosome ends during cell division. In contrast to germline cells, most somatic cells lack telomerase expression.[11] As a result, telomeres in somatic cells become progressively shorter with each cell division. When they reach a critical length, telomeres are no longer protected by the shelterin complex, and are then recognized as DNA double-strand breaks that trigger the DNA damage response. As a consequence, the cells undergo replicative senescence and/or apoptosis as a biological barrier to prevent neoplastic transformation.[11] However, if protective mechanisms such as p53 function are compromized, cells may continue to proliferate; the further erosion of telomeres hinders their function of protecting the chromosome ends and eventually leads to chromosomal instability.[9] Unprotected or broken chromosome ends can fuse to other chromosomal extremities via nonhomologous end joining (NHEJ).[12] These fusions create di-centric chromosomes that eventually break during anaphase, when the two centromeres are pulled in opposite directions.[13] The BFB cycle then continues in the following cell cycle, when sister chromatids fuse after DNA replication. To overcome telomere shortening, cancer cells must regain the ability to maintain telomeres, either through the activation of telomerase activity or via the alternative lengthening of telomeres (ALT) pathway, which involves DNA recombination.[14] Telomere length, expression of the telomerase reverse transcriptase (*TERT*) and the presence of ALT are prognostic factors in several tumor entities.[15,16]

A link between telomere attrition, BFB cycles and chromothripsis was shown in the context of childhood acute lymphoblastic leukaemia with recurrent amplification of megabase regions of chromosome 21.[8] In individuals born with the rare constitutional Robertsonian translocation between chromosomes 15 and 21, amplification is initiated by a chromothripsis event involving the Robertsonian chromosome.[8] In sporadic iAMP21, BFB cycles typically represent the initiating event, often followed by chromothripsis.[8] We hypothesized that telomere dysfunction, followed by BFB cycles, can also be a precursor to chromothripsis in other tumor entities. We show that telomere erosion can lead to chromothripsis and that tumors of a given entity with and without chromothripsis differ in terms of their telomere length and telomere stabilization mechanisms.

Material and Methods

Whole-genome sequencing of fibroblasts from LFS patients

Purified DNA was quantified using the Qubit Broad Range double-stranded DNA assay (Life Technologies). Genomic DNA was sheared using an S2 Ultrasonicator (Covaris). Whole genome sequencing library preparations were performed according to the manufacturer's instructions (NEBNext, NEB). The quality of the libraries was assessed using a Bioanalyzer (Agilent). Sequencing was performed using the Illumina MiSeq platform. Read alignment was done with BWA[17] to the human hg19 reference genome. Copy number analysis was performed using library cn.mops in paired mode and applying DNAcopy for segmentation[18] with R (R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/). Raw reads have been submitted to the EGA genome phenome archive with restricted access. The use of these cultures for research was an IRB approved project from MD Anderson Cancer Center.

Tumor material and patient characteristics

Clinical samples and data were collected after receiving written informed consent according to protocols approved by the respective institutional review boards. Tumor samples of CLL patients were collected at the University of Ulm and were described in a previous study.[19] Ependymoma samples were collected from various institutions and were described previously.[20] Glioblastoma samples were collected and analyzed within the ICGC PedBrain consortium (Bender, Gronych, Hutter, Warnatz *et al*. in revision). Medulloblastoma samples were also collected and analyzed within the ICGC PedBrain consortium and were described previously.[21] At least 80% of tumor cell content was estimated in all tumor samples by H&E staining of cryosections of the piece from which nucleic acid extraction was performed. Diagnoses were confirmed by histopathological assessment by at least two independent pathologists. Clinical and patient characteristics are shown in Supporting Information Tables 1 and 2.

qPCR analysis of telomere length

Measurement of telomere length was carried out using a Q-PCR-based technique as previously described.[22,23]

Propensity score matching

In order to identify samples matching the chromothriptic CLL cases according to the clinical characteristics we used the library MatchIt within R (http://www.jstatsoft.org/v42/i08/).[24] We applied the method "nearest" to select four control samples not showing chromothripsis per affected case being best matched according to age, mutation status of *TP53*, *SF3B1* and *Notch1* as well as del 11q22.q23, trisomy 12q13, del 13q14 and del 17p13.

Analysis of public TCGA datasets

Public TCGA datasets were downloaded from the TCGA website (https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm) for bladder carcinoma (BLCA), cervical squamous cell carcinoma (CESC), colon adenocarcinoma (COAD), lung adenocarcinoma (LUAD), stomach adenocarcinoma (STAD), uterine corpus endometrioid carcinoma (UCEC), $n > 400$ cases for each tumor entity. *TERT* gene expression levels of tumor samples were extracted from normalized RNASeqV2 Level 3 data files. Analysis of copy-number alterations was performed using the Affymetrix Human SNP Array 6.0 'nocnv_hg19' Level 3 data files (containing only somatic alterations). *TERT* copy-number states were derived by extracting the mean intensity of the segment containing the *TERT* gene. To identify the number of breakpoints per chromosome for each sample, we first filtered segments that were supported by <100 probes, as these likely represent technical artifacts. We then counted the number of breakpoints for which consecutive segments had a mean intensity difference larger than 0.2. This number was then normalized for the respective chromosome length.

FISH

We selected a medulloblastoma patient-derived xenograft case with high-level amplification of the *TERT* locus and chromothripsis on chromosome 17. We designed a FISH probe to a region of chromosome 17 for which we expected >2 signals in this tumor because of the rearrangements associated with chromothripsis, based on the paired-end sequencing data. Two-color interphase FISH[25] was performed using a rhodamine-labeled probe (RP11-98205; chromosome 17) and a Cy5-labeled probe (TERT; 117B23). Pretreatment of slides, hybridization, posthybridization processing and signal detection were performed as described previously.[25] Samples showing sufficient FISH efficiency (>90% nuclei with signals) were evaluated by two independent investigators. Metaphase FISH for verifying clone-mapping position was performed using peripheral blood cell cultures of healthy donors as outlined previously. Amplifications presented as innumerable tight clusters of signals for the probe for *TERT*.

Telomere-specific fluorescent *in situ* hybridization

Telomere-specific FISH was performed using a FITC-labeled peptide nucleic acid telomere probe (Dako, Glostrup, Denmark) as previously described.[26] The ALT phenotype is characterized by a variable number of large, ultra-bright telomere DNA aggregates. Tumors containing ≥1% tumor cells displaying such aggregates were considered as ALT-positive.

Measurement of telomere length by terminal restriction fragment analysis

Telomere lengths were determined by a terminal restriction fragment (TRF) kit (Roche Diagnostics, Mannheim, Germany) as previously described.[27] DNA samples were digested with the restriction enzymes RsaI and HinfI and run on agarose gels. A biotinylated γ-DNA molecular weight marker was used as DNA length standard. The DNA samples were depurinated, denatured and transferred to a positively charged nylon membrane Hybond-N (Amersham Pharmacia Biotech, Little Chalfont, UK) by capillary blotting. The membrane was washed in saline–sodium citrate buffer. The blot was hybridized with a (TTAGGG)3 telomere probe and washed. Chemiluminescent detection was performed according to the Detection Kit (Roche Diagnostics, Basel, Switzerland). Detection was carried out on an X-ray Hyperfilm ECL. Calculation of the mean telomere length followed the manufacturer's instruction and formula (TeloTAGGG Telomere Length Assay, Roche). Alpha Ease FC version 6.0.2 was used to measure the band intensity or chemiluminescent signal.

TERT expression in ependymoma

Expression values for *TERT* for ependymoma cases of the RELA subgroup (which includes cases with chromothripsis) were based on Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays from a publically available dataset from a previous study of our group.[20] Gene expression data have been deposited at the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) and are accessible under accession number GSE64415.

TERT expression and breakpoint analysis in glioblastoma

Expression values for the *TERT* gene were from RNA sequencing analyses from a glioblastoma cohort analyzed within ICGC (Bender, Gronych, Hutter, Warnatz *et al.* in revision). Chromothripsis scoring and number of breakpoints per chromosome were based on whole-genome sequencing data from the same cohort of patients. Analysis of the stability of the chromothriptic patterns over time was performed based on copy-number plots derived from 450k array data and whole-genome sequencing data.

C-circle assay

The C-circle assay was performed as described previously.[28] Genomic DNA was quantified using a Qubit Fluorometer (Life Technologies). A 30 ng DNA was combined with 10 µl 2 × Φ29 Buffer, 7.5 U Φ29 DNA polymerase (both NEB), 0.2 mg/ml BSA, 0.1% (v/v) Tween 20, 1 mM each dATP, dGTP and dTTP and incubated for 8 hr at 30°C and then at 65°C for 20 min. Reactions without addition of polymerase

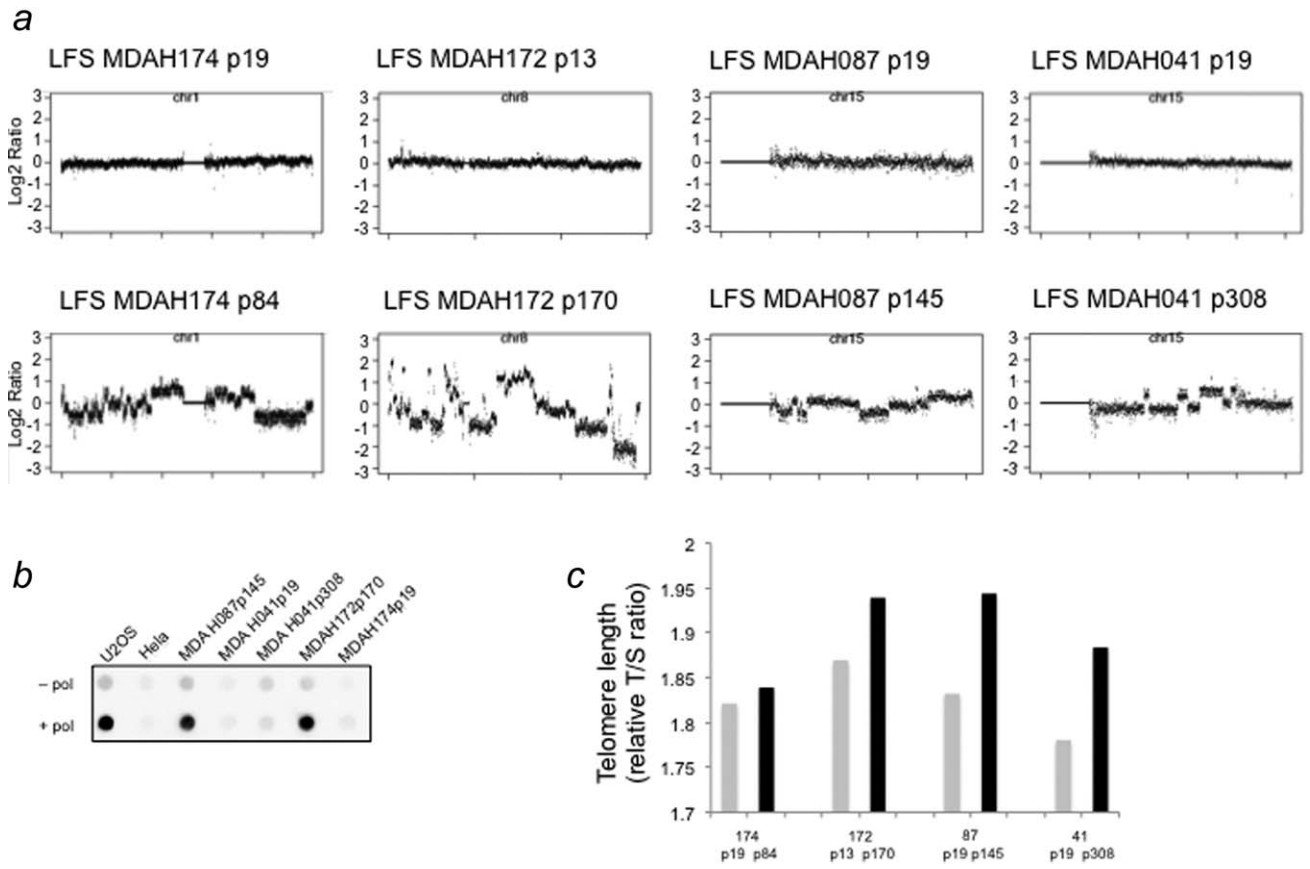

Figure 1. (*a*) Copy number plots from whole-genome sequencing analysis of fibroblast cultures from four LFS patients at early and late passage for each case. One chromosome harboring copy-number oscillations characteristic of catastrophic genomic events is shown in each case (the copy-number profiles for all chromosomes are shown in Supporting Information Figure 1).(*b*) C-circle levels in early and late passage fibroblasts from LFS patients. As references, ALT-negative HeLa cells and ALT-positive U2OS cells were used. For each sample, reactions without addition of polymerase (- pol) were included as controls. MDAH041 cells, which are ALT-negative even at the post-crisis stage, have been shown previously to have telomerase activity at late passages.[30](*c*) Analysis of telomere length by qPCR in fibroblasts from LFS patients. T/S ratios show the ratios between telomere repeat copy number and single copy gene copy number.

were included as controls. After adding 2 × SSC, the sample was dot-blotted onto a 2 ×-SSC-soaked Roti-Nylon plus membrane (pore size 0.45 μm, Carl Roth). The membrane was baked for 20 min at 120°C, hybridized and developed using the TeloTAGGG Telomere Length Assay Kit (Roche). Chemiluminescent signals were detected using a ChemiDoc MP imaging system (Bio-Rad).

Results

Telomere shortening can lead to chromothripsis

Healthy human fibroblasts eventually senesce in culture. Due to the absence of p53, however, fibroblasts from some patients affected by Li-Fraumeni syndrome (LFS)—who harbour heterozygous germline *TP53* mutations—can bypass this senescence, and continue to proliferate with further telomere shortening.[29] The cells acquire an altered morphology, show chromosomal anomalies and escape from the growth crisis.[29] We therefore hypothesized that telomere shortening in LFS fibroblasts might lead to chromothripsis, and that the cells might survive because of the compromized p53 damage response.

To detect potential chromothriptic events, we performed low-coverage whole-genome sequencing of matched early passage (p13–19) and late passage (p84–308) fibroblasts from four LFS patients. For each of the four postcrisis cultures, several chromosomes harboured copy-number gains and/or losses (Supporting Information Fig. 1). Interestingly, massive DNA rearrangements clustered on one chromosome and typical oscillations between two copy-number states characteristic of chromothripsis were also observed in all four postcrisis cultures (Fig. 1*a*). Fibroblasts from the same patients analyzed at early passages showed no or very minor copy-number aberrations (Supporting Information Figure 1).

Analysis of DNA C-circles, partially single-stranded circular DNA elements containing telomeric repeats that are reported to be specific markers of ALT activity,[28] demonstrated that two of three analyzed postcrisis cultures for which material was available showed activation of the ALT pathway, whereas the two analyzed early passage cultures

were ALT-negative (Fig. 1*b*). At late passages, most cultures remain negative for TERT and have long and heterogeneous telomeres, while a minority demonstrates strong *TERT* expression and short and stable telomere lengths.[30] In one of the four analyzed cultures (MDAH041), which was shown to be ALT-negative with the C-circle assay, telomerase activity has previously been reported at late passages.[30] The other three cultures were negative for TERT even at late passages, and this held true in independent postcrisis clones from the same culture.[30] Therefore, the ability to re-activate telomerase or to use the ALT pathway in order to bypass senescence may be strain-specific in fibroblasts derived from individuals with LFS,[30] or may be related to the type of underlying *TP53* mutation, which may confer differential dysfunction. Quantitative real-time PCR analysis showed that telomeres were longer in all four cultures at the late passages as compared to early passages (Fig. 1*c*). These findings suggest that in such cells, telomere attrition leads to catastrophic genomic events, followed by telomere re-stabilization. Based on these results, we hypothesized that telomere attrition might lead to chromothripsis and that telomere length might differ between tumors affected by chromothripsis and control cases without chromothripsis.

Telomere patterns differ between tumors affected by chromothripsis and control cases without chromothripsis

We measured telomere length by terminal restriction fragment (TRF) analysis in four medulloblastoma cases with chromothripsis and six cases without chromothripsis. All samples were obtained from patients prior to treatment. Three of the four medulloblastomas with chromothripsis were from patients with LFS and a Sonic Hedgehog (SHH) subgroup tumor. The fourth case was also from the SHH subgroup and harbored a somatic *TP53* mutation. The control group included two SHH medulloblastomas, 3 WNT medulloblastomas and one Group 4 medulloblastoma (all with wild-type *TP53* both in the tumor and germline, see Supporting Information Table 1). The medulloblastomas without chromothripsis showed shorter telomeres and less frequent activation of ALT as compared to the cases for which chromothripsis was detected (Fig. 2*a*, Supporting Information Fig. 2).

In order to compare telomere length between cases with and without chromothripsis in additional tumor entities, we measured telomere length by quantitative PCR in two further cohorts.

From a cohort of patients with ependymomas, which have previously been genomically characterized,[20] we identified eight cases with chromothripsis and nine control cases without chromothripsis from the same molecular subgroup (RELA fusion positive). None of the patients received chemo- or radiotherapy prior to the surgical removal of the tumor. Telomere length was significantly higher in cases with chromothripsis as compared to the cases without chromothripsis (Fig. 2*b*). *TP53* mutations are extremely rare in ependymoma,[31] but p53 accumulation is detected in >80% of RELA fusion positive ependymomas.[32] Alterations affecting other genes related to *TP53*, such as *CDKN2A* (which is frequently deleted in ependymomas, see Supporting Information Table 1) might therefore be associated with chromothripsis in this tumor entity.

In a cohort of patients with chronic lymphocytic leukemia (CLL) analyzed on Affymetrix 6.0 SNP arrays [19] (Edelmann *et al.* in preparation), we identified seven cases with chromothripsis and 26 control cases without chromothripsis, but with comparable clinical parameters (*e.g.* same age distribution, same status for nonchromothriptic chromosomal aberrations and mutations known to affect the prognosis of CLL patients, including the same proportions of mutations in *TP53,* see Supporting Information Table 1). Unlike in medulloblastoma and ependymoma, cancer cells had shorter telomeres in the cases with chromothripsis as compared to the cases without chromothripsis (Fig. 2*c*).

The underlying mechanisms linking telomere maintenance with chromothripsis therefore seems to be entity-specific, which prompted us to further investigate the putative role of telomere stabilization mechanisms in chromothripsis.

Telomere stabilization mechanisms in tumors with chromothripsis

In order to proliferate indefinitely, cancer cells must elongate their telomeres through telomerase up-regulation or by activation of the ALT pathway. In the particular case of chromothriptic cancer cells, it is presumed that the derivative chromosomes resulting from chromothripsis are likely stabilized in order to prevent further progressive chromosomal catastrophes that would be incompatible with cell survival.

In a cohort of ependymoma patients for whom we previously published the molecular classification,[20] *TERT* expression was higher in cases affected by chromothripsis as compared to cases without chromothripsis from the same molecular subgroup (Fig. 3*a*). Mutations in *ATRX* or *DAXX* have been associated with ALT activation, but no such mutation was detected in any of the tumors of the RELA subgroup using whole-exome sequencing. None of the samples was ALT positive as assessed by the C-circle assay (Supporting Information Fig. 3), suggesting that ALT might not represent a dominant mechanism of telomere maintenance in this subgroup of ependymomas. In line with these results, no ependymoma showed evidence of ALT activation in a previous study on 57 pediatric ependymomas.[33] Therefore, stabilization of the chromothripsis-derived chromosomes is likely achieved through the increased *TERT* expression seen in ependymoma. Interestingly, focal gains of 5p including the *TERT* locus have previously been reported in ependymoma.[34]

In glioblastoma, high-level amplification of the *TERT* gene locus and high *TERT* expression were associated with chromothriptic cases with particularly high numbers of breakpoints per chromosome (Figs. 3*b* and 3*c*). In addition, we also identified three glioblastoma cases affected by chromothripsis in which ALT was active (Fig. 3*d*), showing that

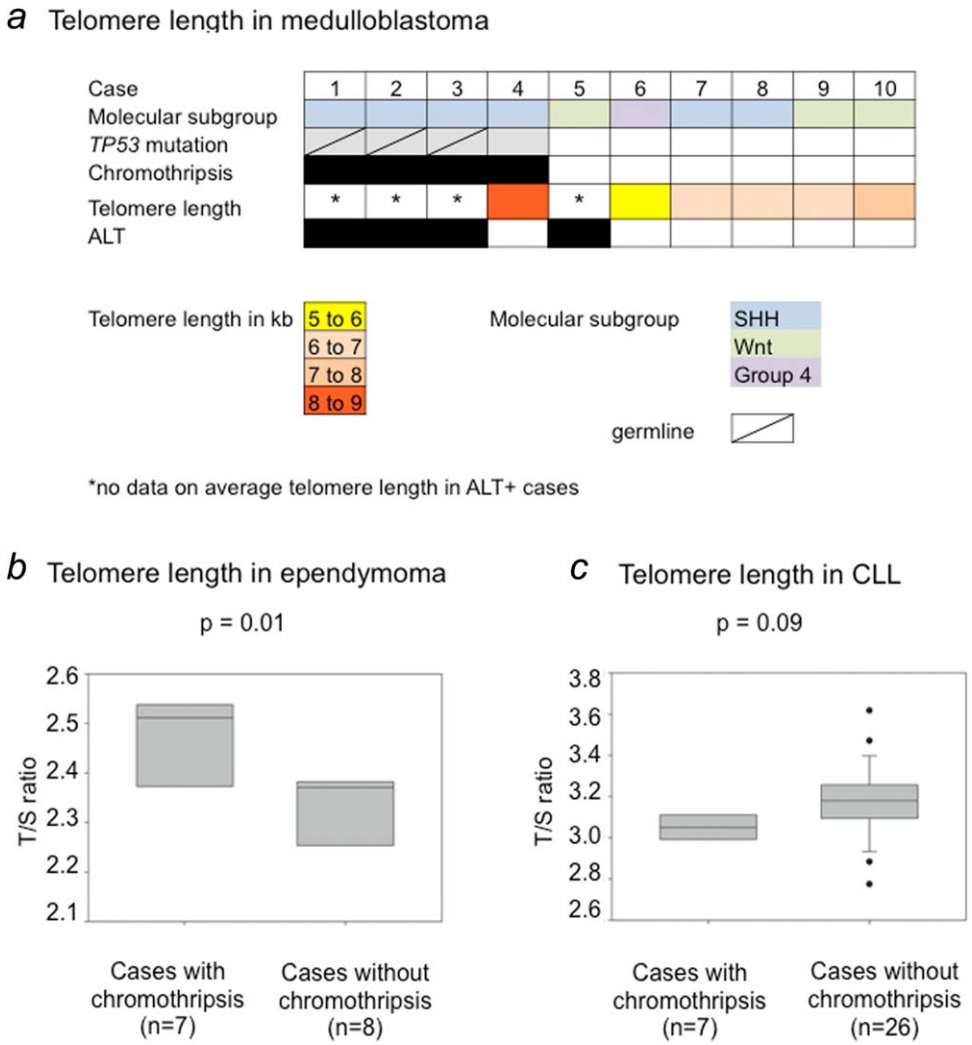

Figure 2. (*a*) TRF blot analysis showing telomere length in medulloblastoma specimens with chromothripsis (cases 1–4) or without chromothripsis (cases 6–10). Data on molecular subgroup classification of the cases, *TP53* status, telomere length and detection of ALT are presented. ALT, alternative lengthening of telomeres; SHH, Sonic hedgehog. The original TRF blot is shown as Supporting Information Figure 2.(*b*) Analysis of telomere length by qPCR in ependymoma. T/S ratios show the ratio between telomere repeat copy number and single copy gene copy number. $n = 15$ (7 cases with chromothripsis and 8 cases without chromothripsis). Average values for two independent experiments are shown.(*c*) Analysis of telomere length by qPCR in chronic lymphocytic leukemia (CLL). T/S ratios show the ratio between telomere repeat copy number and single copy gene copy number. $n = 33$ (7 cases with chromothripsis and 26 cases without chromothripsis). Unpaired *t*-tests were used to test for statistical significance. Two-tailed *p*-values are shown.

various stabilization mechanisms can play a role in glioblastomas affected by chromothripsis.

We then investigated potential differences in stabilization mechanisms between cases with and without chromothripsis in additional tumor entities outside the central nervous system. For this, we reanalyzed publicly available datasets of bladder, cervical, colon, lung, stomach and uterus cancer ($n > 400$ for each tumor entity). Tumors with higher copy-numbers of the *TERT* locus and with higher *TERT* expression showed more DNA breakpoints per chromosome (Figs. 3*e* and 3*f*), which may further point to a requirement for telomere stabilization subsequent to dramatic genomic rearrangement.

Based on our analysis of the fibroblast cultures from the LFS patients as well as glioblastoma tumor samples, it appears that telomere stabilization after chromothriptic events can be achieved either by telomerase activation or by ALT. We further assessed ALT activation in four SHH medulloblastomas from LFS patients and three additional SHH medulloblastomas from cases with somatic *TP53* mutations, all with chromothripsis. All seven cases were ALT-positive (one representative example is shown in Supporting Information Fig. 4). The prevalence of ALT in medulloblastomas with chromothripsis therefore seems to be much higher than the general prevalence, which was reported to be ~18% in anaplastic medulloblastomas (which is also the histological subtype typically seen in *TP53*-mutant SHH tumors).[35] *TERT* promoter mutations have been reported in ~83% of SHH medulloblastomas derived from adult patients and represent

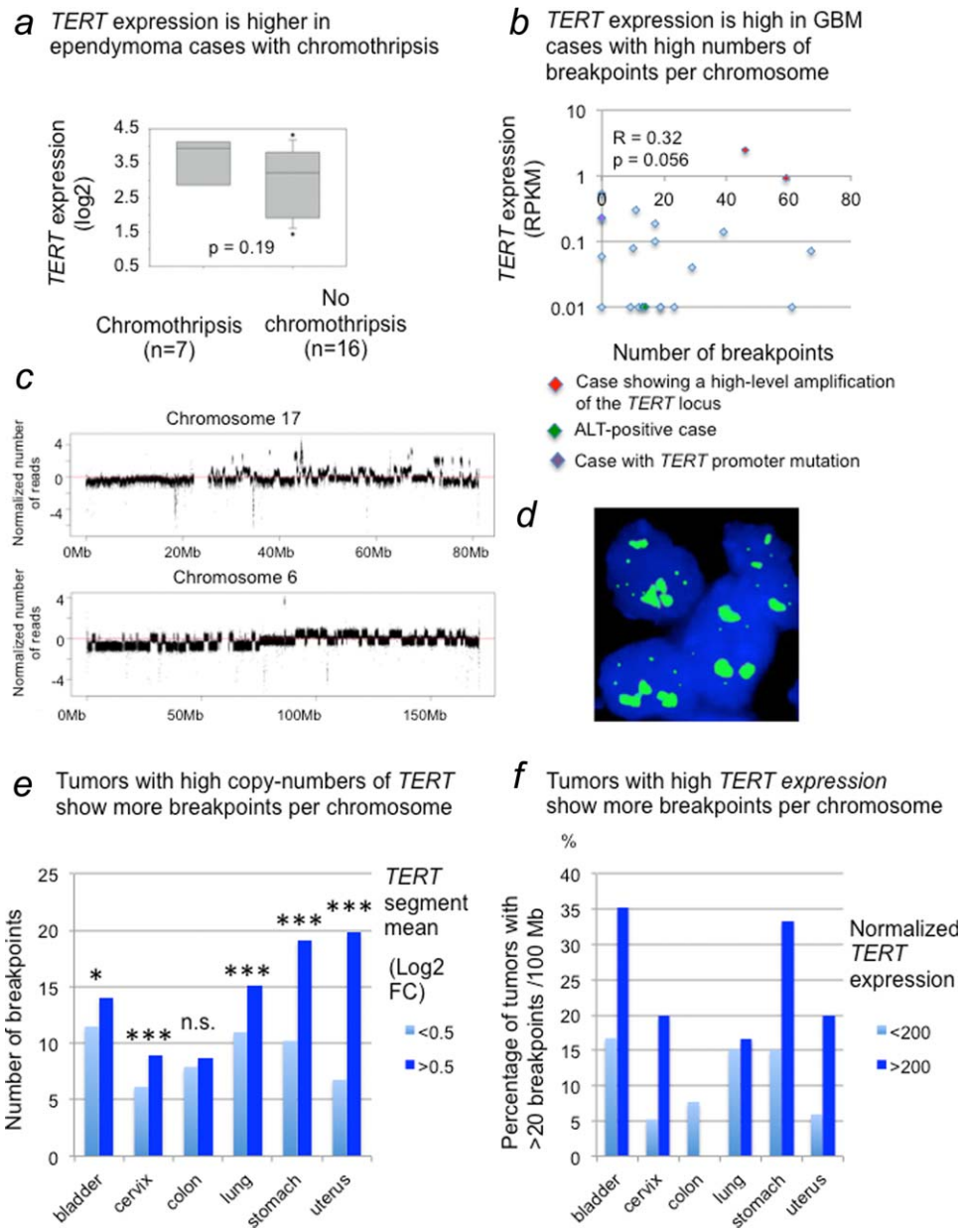

Figure 3. (*a*) *TERT* expression levels (log2 ratios) in ependymoma cases with or without chromothripsis. Unpaired *t* test was used to test for statistical significance.(*b*) *TERT* expression in glioblastoma (GBM) in relation to the highest number of breakpoints per chromosome for each case. Red dots show cases with high-level amplifications of the *TERT* locus. Purple dot shows a case with *TERT* promoter mutation. Green dots show cases for which ALT activation was shown. *R*, Pearson correlation coefficient. Transcript abundance is shown in reads per kilobase per million (RPKM). Information on alterations affecting the p53 pathway for all cases is shown in Supporting Information Table 1.(*c*) Copy-number plots of two glioblastoma cases with high-level amplifications of the *TERT* locus, showing chromothriptic patterns with high numbers of breakpoints on chromosome 17 and chromosome 6, respectively (cases depicted in red in Figure 3b).(*d*) Telomere FISH showing ALT activation in a glioblastoma specimen for which chromothripsis was detected (telomere probe in green).(*e*) Analysis of the relationship between the highest number of breakpoints per chromosome (normalized for chromosome size) for each case and copy number of *TERT* in six tumor entities of TCGA ($n > 400$ per entity). *, $p < 0.05$; ***, $p < 0.0005$. Unpaired *t*-tests were used to test for statistical significance within each entity.(*f*) Analysis of the relationship between the percentage of cases with high numbers of breakpoints per chromosome (normalized to chromosome size) and *TERT* expression in six tumor entities of TCGA.

an alternative mechanism.[36] Based on these results, we conclude that medulloblastomas with and without chromothripsis differ in terms of their telomere stabilization mechanisms.

To address the question of the stability of the chromothriptic patterns over time, we then compared primary and relapse tumors from the same patients (Supporting Information Table 2). In three glioblastoma cases for which paired samples were available, the rearrangements present in the initial sample were also detected in the relapse specimen (one representative example is shown in Figs. 4*ad*). In three

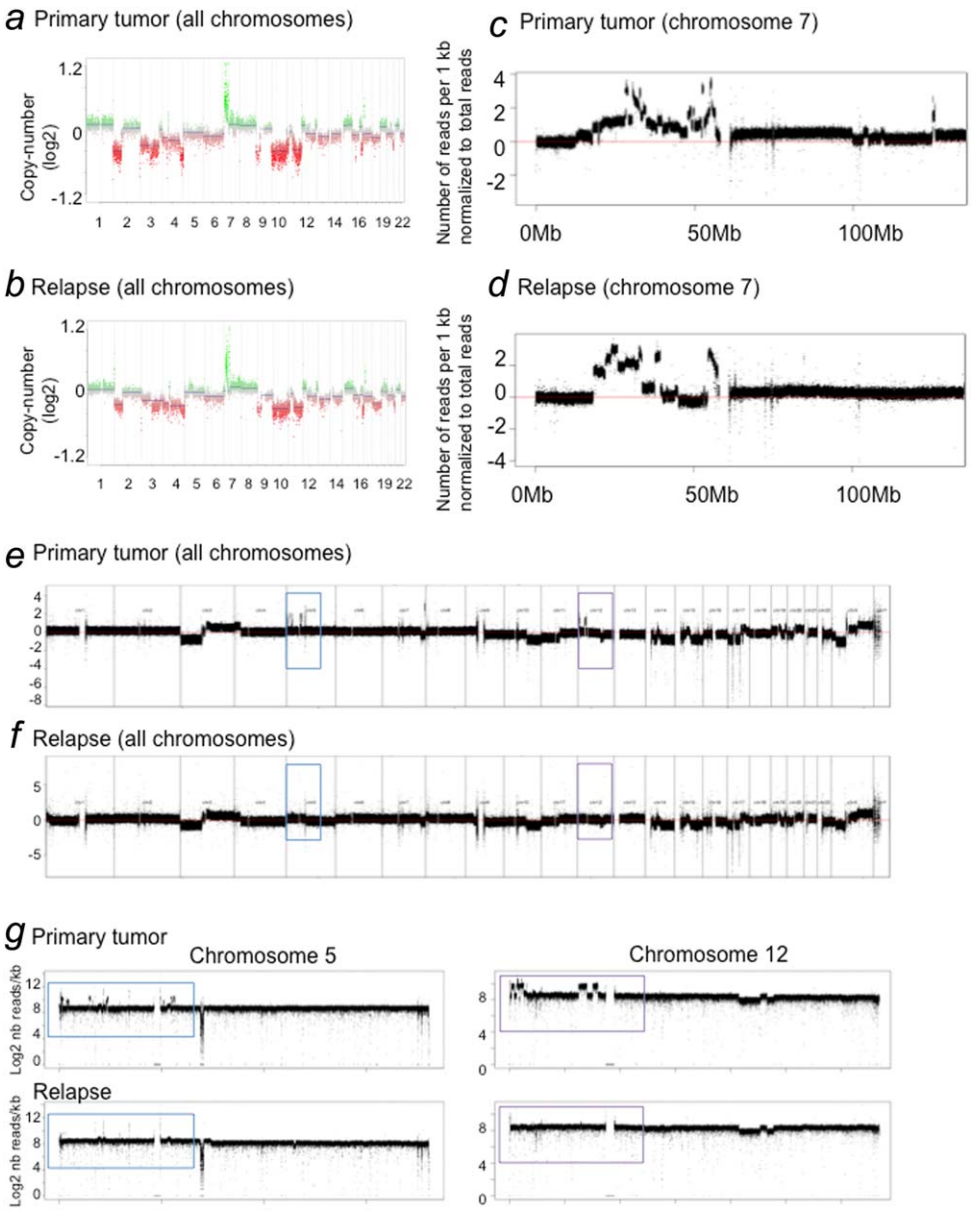

Figure 4. (*a–d*) Whole-genome copy-number plots of a glioblastoma case based on 450k data (left panels, a and b) and whole-genome sequencing data for chromosome 7 (right panels, c and d) showing the stability of the chromothriptic patterns between the primary tumor and the relapse specimens. **(e–g)** Copy-number plots of a medulloblastoma case from a LFS patient showing chromothripsis in the primary tumor but no chromothriptic pattern in the relapse sample. The two upper panels show the entire genome (e, f) and the lower panels (g) depict chromosomes for which chromothripsis was detected in the primary tumor.

ependymoma cases, the characteristic chromothriptic patterns also persisted (data not shown). In a SHH medulloblastoma of a LFS patient, the chromothriptic patterns detected in the primary tumor were not observed in the relapse sample despite a genome-wide sequencing depth of more than 30× (Figs. 4*eh* and Supporting Information Table 2). This finding is likely explained by the outgrowth of a recurrent clone lacking the derivative pieces and/or an increased sensitivity of the chromothriptic clone to therapy. Similarly, a CLL case for which matched primary and relapse samples were available showed chromothripsis in the initial sample but not in the relapse specimen (Supporting Information Table 2). From these longitudinal studies, we can conclude that the chromothriptic patterns are either stabilized, in which case the relapse specimens show very similar aberrations to the primary tumors, or they are lost by clonal selection in the relapse samples. Strikingly, we have not observed any case where there were additional chromothriptic events in the relapse as compared to the primary tumor. Therefore, telomere stabilization mechanisms are likely activated after the occurrence of chromothripsis, to prevent continued (and presumably therefore lethal) genomic catastrophe.

Reconstruction of the sequence of events

In order to test whether telomere stabilization mechanisms are activated after the occurrence of chromothripsis, we performed 2-color interphase FISH on a SHH medulloblastoma specimen from a patient with LFS showing a high-level amplification of the *TERT* locus and chromothripsis on chromosome 17. The observed patterns were identical in each cell, with no evidence for distinct subclones. The absence of a subpopulation of cells showing high-level amplification of the *TERT* locus but not additional copies of the locus on chromosome 17 confirms that telomere stabilization occurs either concomitant with, or more likely shortly after, the chromothriptic event (Supporting Information Fig. 5).

Discussion

Telomere dysfunction increases with the number of divisions a cell goes through. Compared to the cells of healthy subjects, these essential protective chromosome caps shorten faster in the cells of LFS patients, leading to an increased frequency of telomeric fusions in such cells.[37] The LFS fibroblast cultures analyzed in our study were previously described as showing high frequencies of dicentric chromosomes and telomere associations.[29] BFB cycles and erroneous centrosome segregation during mitosis may trigger chromosome fragmentation, with derivative DNA fragments being stitched back together through error-prone repair mechanisms. The impaired p53 damage response may allow some cells to survive such a genomic catastrophe. Therefore, our results provide a possible explanation for the high prevalence of chromothripsis in medulloblastomas of LFS patients. Activation of ALT seems to be exceptionally frequent in LFS-associated medulloblastoma, and may mark telomeres stabilized after chromothripsis.

We show an association between telomere dysfunction and chromothripsis not only in the context of LFS, but also independently of the *T53* mutation status, since only a minority of the analyzed CLL, glioblastoma and ependymoma cases had *TP53* mutations. In the cases where no germline or somatic *TP53* mutation was detected, other aberrations affecting the DNA damage response and/or potentially indirectly leading to p53 dysfunction (*e.g. CDKN2A/B* deletion) likely allowed the cell to avoid apoptosis despite telomere dysfunction.

The telomere patterns and stabilization mechanisms identified herein are specific to each tumor entity. For instance, chromothripsis is associated with increased telomere length in medulloblastoma and ependymoma (Figs. 2*a* and 2*b*), but with shorter telomeres in CLL (Fig. 2*c*) and in esophageal adenocarcinoma.[38] The persistence of very short telomeres may be a feature of cells in which ALT is active,[39] but these distinct patterns may also be explained by differences in telomerase activation capacity depending on the lineage of the tumor cell of origin.[40] Short and dysfunctional telomeres may limit normal stem cell proliferation and predispose to cancer by selection of stem cells with defective DNA damage responses that are prone to genome instability.[41]

Interestingly, analyses of primary and relapsed specimens from the same patients identified only two types of evolution for the chromothriptic patterns. We first observed cases where the clone showing chromothripsis in the primary tumor remained as a dominant clone in the relapse tumor, with very similar aberrations in both specimens. These cases highlight the role of the stabilization mechanisms and show minimal independent clonal evolution after the time of diagnosis. Such cases have been described in other tumor entities, such as in CLL in the first report on chromothripsis.[1] In other cases, in contrast, the clone with the derivative chromosome(s) was outgrown by other clones lacking the chromosome(s) affected by chromothripsis. Disappearance of a chromothriptic clone was also previously been reported in a case study in CLL.[42] Thus, in some instances, the rearrangements resulting from chromothripsis may be important for tumor initiation but not required for tumor maintenance.[1] In those cases where chromothripsis does not seem to drive later tumor evolution, it may be that altered genes on the derivative chromosome may somehow sensitize the cells to treatment. The fact that no case was detected with further chromothriptic events occurring between the diagnosis and the relapse highlights the importance of telomere stabilization mechanisms in preventing further rearrangements.

Several studies provided first experimental evidence for a potential link between chromothripsis and telomere dysfunction. Broken telomeres were shown to lead to the accumulation of polyploid cells.[43] The formation of polyploid cells due to dysfunctional telomeres and cytokinesis failure may explain the link between hyperploidy and chromothripsis.[44] In pancreatic carcinomas and osteosarcomas, telomeric dysfunction may trigger chromosomal fragmentation through persistent breakage-fusion bridge events.[45] Garsed and colleagues described the architecture and evolution of cancer-associated neochromosomes in liposarcoma.[46] In this very specific context, telomere attrition and BFB were not shown to act as a trigger for the initial chromothriptic events, but BFB cycles were implicated in the evolution of the chromothriptic-rearranged neochromosomes. In esophageal adenocarcinoma, telomere shortening was shown to be more prominent in cases bearing localized complex rearrangements,[38] and several cases therein showed evidence of both BFB and chromothripsis.[38] In hematopoietic malignancies, Campbell and colleagues suggested dicentric chromosome formation by telomeric erosion as a potential mechanism leading to chromothripsis in iAMP21.[8] Recently, Maciejowski and colleagues suggested a telomere-based mechanism for chromothripsis in cancer.[47]

Our study shows that telomere dysfunction and BFB cycles are a general mechanism leading to chromothripsis in several tumor entities. In the absence of functional barriers preserving genome integrity, telomere attrition followed by end-to-end fusion and subsequent breakage induces

chromothripsis. This is followed by the establishment of tumor entity-specific telomere maintenance mechanisms that "lock in" these changes and prevent further, lethal catastrophe. Our data therefore highlight that telomere maintenance mechanisms may represent a target for therapeutic intervention in chromothripsis-positive tumors.

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