Human RTEL1 deficiency causes Hoyeraal–Hreidarsson syndrome with short telomeres and genome instability

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Hoyeraal–Hreidarsson syndrome (HHS), a severe variant of dyskeratosis congenita (DC), is characterized by early onset bone marrow failure, immunodeficiency and developmental defects. Several factors involved in telomere length maintenance and/or protection are defective in HHS/DC, underlining the relationship between telomere dysfunction and these diseases. By combining whole-genome linkage analysis and exome sequencing, we identified compound heterozygous RTEL1 (regulator of telomere elongation helicase 1) mutations in three patients with HHS from two unrelated families. RTEL1 is a DNA helicase that participates in DNA replication, DNA repair and telomere integrity. We show that, in addition to short telomeres, RTEL1-deficient cells from patients exhibit hallmarks of genome instability, including spontaneous DNA damage, anaphase bridges and telomeric aberrations. Collectively, these results identify RTEL1 as a novel HHS-causing gene and highlight its role as a genomic caretaker in humans.

INTRODUCTION

Telomeres represent the end of linear chromosomes and are constituted of specific repetitive sequences (TTAGGG in mammals). The repeated telomeric motif serves as a platform for specific factors (the shelterin complex) which protect telomeres from degradation and fusion (1). Telomeres may adopt a lariat structure whereby the 3' G-overhang invades the double-stranded telomere to form a loop (the T-loop), a structure thought to be necessary to mask the chromosome extremity from DNA repair factors (2). The G-rich and repetitive sequence of telomeres can promote other complex structures such as G-quadruplex DNA (G4), which represent barriers to the DNA replication machinery (3). Hence, DNA replication through telomere, which is mainly unidirectional (4), requires the activity of several factors to overcome these topological obstacles. In addition, because of DNA processing and incomplete DNA replication inherent to telomere structure, each

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replicative cell cycle leads to telomere shortening. In some cells, this phenomenon is counteracted by the reverse transcriptase activity of a ribonucleoprotein complex, the telomerase, which processively adds TTAGGG repeats at telomeres. Defects in telomere protection or length maintenance result in telomere dysfunction, senescence, apoptosis or genomic instability (1). The Hoyeraal–Hreidarsson syndrome (HHS) represents a rare and severe variant of the bone marrow failure disease dyskeratosis congenita (DC) (5). HHS is principally characterized by intra-uterine growth retardation, early onset bone marrow failure, immunodeficiency, microcephaly and cerebellar hypoplasia (6–8). In most of the cases, HHS patients succumb in their first decade to combined immunodeficiency resulting from their drastic bone marrow failure. The syndromic presentation of HHS likely originates from a defect of cells with high turnover such as the hematopoietic system. Although the unifying features of HHS/DC is an increased frequency of dysfunctional telomeres resulting in premature senescence, the origin of this phenotype is heterogeneous and can stem either from an accelerated telomere shortening (as observed in Dyskerin, TERC, TERT, TIN2 and TCAB1 deficiencies) or from altered replication/capping of telomeres with overall normal length (9,10). Although our knowledge on the HHS/DC etiology has recently expanded through the discovery of several HHS/DC-causing genes (9,11–14), several HHS/DC cases remain molecularly uncharacterized. We here report the identification of three patients with HHS caused by inherited RTEL1 deficiency. RTEL1 (regulator of telomere elongation helicase 1; OMIM 608833) is an ATP-dependent DNA helicase that was initially identified in mice as a dominant telomere length regulator (15). The clinical features of the patients as well as the study of the RTEL1-deficient cells provide new insights into the multiple functions of RTEL1 as a genomic caretaker in humans.

### RESULTS

#### Patients’ clinical features

We investigated three patients (P1, P2 and P3) with similar clinical features, born to two unrelated non-consanguineous families (P1 and P2 were siblings). They all suffered from intrauterine growth retardation diagnosed between 19 and 21 weeks of gestation, which led to a premature fetal extraction. At birth, they presented with major hypotrophy (height and weight <2nd percentile according to age) and overt microcephaly (<2nd percentile) (Table 1). Cerebral magnetic resonance imaging, performed because of cerebellar ataxia or developmental delay, revealed cerebellar atrophy in all patients. Patient 2 also had a corpus callosum dysmorphism (Table 1). Cerebral magnetic resonance imaging, performed because of cerebellar ataxia or developmental delay, revealed cerebellar atrophy in all patients. Patient 2 also had a corpus callosum dysmorphism (Table 1). The immunologic phenotype included lymphopenia predominantly on B cells and progressive hypogammaglobulinemia (patient 2 had a complete agammaglobulinemia at 6 months of age) requiring intravenous immunoglobulin substitution (Supplementary Material, Table S1). All patients progressively developed a pancytopenia, with bone marrow aspiration showing bone marrow hypoplasia requiring blood and platelet transfusions (Supplementary Material, Table S1). Patients 1 and 3 developed gastrointestinal features—mainly diarrhea and oral leukoplakia. Colonic biopsy performed on patient 1 showed a non-specific lymphoplasmocytic infiltrate with chorionic edema but no mucosal ulceration. Patients 1 and 2 died from invasive pulmonary aspergillosis at 4 years and 18 months of age, respectively. Patient 3 is 5 years old and is still alive. The association of intrauterine growth retardation, microcephaly, cerebellar hypoplasia, immunodeficiency including B
lymphopenia, and progressive pancytopenia, found in P1, P2 and P3, are prototypical clinical features of HHS (6–8).

**Patients’ cells exhibit abnormally short telomeres**

As impaired telomere length maintenance is known to cause HHS (16), we measured telomere length in patients’ whole blood cells. All three patients exhibited abnormally short telomeres (estimated at 6.6, 7 and 5.6 kb, respectively) compared with their parents, their healthy brothers (Fig. 1A) and pediatric controls (10). Because we failed to establish a fibroblast cell line from P3’s skin biopsy, analyses reported hereafter were performed in activated T lymphocytes (T-blasts) from P3 and in fibroblasts from P1 and P2. Quantitative FISH (Q-FISH)
confirmed the telomere length reduction in P1’s and P2’s primary fibroblasts, and in P3’s T-blasts (P < 0.0001; Fig. 1B and C). However, in vitro telomerase activity was unaffected as shown in P3’s T-blasts using a TRAP assay (17) (Fig. 1D).

 Patients’ cells exhibit genome instability and telomere aberrations

Signs of altered DNA replication were found in patients’ cells since the number of spontaneous DNA damages, materialized by 53BP1 foci, was increased (P < 0.0001) (18) (Fig. 2A and B). In addition, P1’s and P2’s primary fibroblasts exhibited increased telomere dysfunction-induced foci (TIFs (19)) revealed by the co-localization of 53BP1 with a telomere probe (Fig. 2C and D) and accelerated cellular senescence (Supplementary Material, Fig. S1) (P < 0.0001). Of note, P3’s T-blasts with four or more TIFs were not found (Fig. 2D). However, anaphase bridges (with telomeric signals), a hallmark of abnormal mitotic events associated with genome instability, were found in P3’s T-blasts but not in control (Fig. 2E). Consistently, P1’s and P2’s fibroblasts also exhibited a significant increased frequency of anaphase bridges (P < 0.001) (Fig. 2F and G). In order to know whether the genome instability found in patients’ cells also affected telomeres, we performed telomeric FISH analysis (Fig. 3A). Cells from all three patients exhibited an increase in sister telomere loss (Fig. 3C and D) and in terminal deletion (Fig. 3D and J), whereas hallmarks of telomere fragility such as multiple telomeric signals (MTS), interstitial telomeric signal (ITS) or sister telomere fusions were observed very infrequently. Remarkably, chromosome orientation FISH (CO-FISH), which allows to distinguish between telomere strands replicated by the leading or the lagging mechanisms, confirmed an increase in terminal deletion (Fig. 3K) and in telomere sister loss (Fig. 3L) in all three patients (P < 0.0001). This analysis also indicated that telomere losses predominantly affected the leading strand (Fig. 3F and L). An augmentation of homologous recombination events between sister chromatids at telomere (P < 0.0001), known as telomere sister chromatid exchange (T-SCE), was also observed in P3’s T-blasts and in SV40-transformed fibroblasts from P2 (Fig. 3H and M). Overall, cells from the three patients exhibited the common features of spontaneous DNA damages, short telomeres, telomere instability and anaphase bridges, evoking global genome instability.

Patients carry compound heterozygous RTEL1 mutations

To identify the molecular origin of the short telomere syndrome in these patients, we combined whole-genome linkage analysis with whole-exome sequencing. First, we hypothesized that the HHS in P1 and P2 (who are siblings and from a non-consanguineous family) was with an autosomal-recessive inheritance, since in family 1, two children were affected while the parents were healthy and exhibited normal telomere length (ruling out an autosomal-dominant inheritance with disease anticipation or de novo mutation) (Fig. 4A). We, thus, performed genome-wide linkage analysis in family 1 in order to find the haplotypes specifically shared by the patients P1 and P2 and different from the healthy brother. We next performed whole-exome sequencing analysis with DNA extracted from patient 2. We reasoned that the pathogenic variants identified in P2 would have to be located in one of the genetic region determined by genome-wide linkage analysis (Fig. 4B). In addition, given the rarity and the severity of the disease, we also suspected that the pathogenic gene variants would not be listed in the public SNP databases (dbSNPs, EVS and 1000 Genome) or in our in-house database. Lastly, owing to the likely autosomal-recessive inheritance of the disease, we filtered the results and retained only genes harboring a single homozygous variant or compound heterozygous variants. After these filtering procedures, RTEL1 (regulator of telomere elongation helicase 1) appeared to be the only gene meeting the above-mentioned criteria (Fig. 4B). Given RTEL1’s known role in telomere length regulation and genome stability, we considered that the RTEL1 variants identified in P2 might cause the latter’s HHS.

The same whole-exome sequencing approach and analysis was performed on DNA from patient P3. The analysis for P3 led to the identification of 51 genes with homozygous or compound heterozygous variants not listed in the databases (Fig. 4B). Strikingly, RTEL1 was also identified by this approach. Given the similar clinical and cellular phenotypes observed in patients P1, P2 and P3, we consider that RTEL1 is the strongest candidate for patient P3.

Physiological alternative splicing events generate two main RTEL1 variants in human cells: variant 2 (Ensembl: ENST00000370018; Uniprot: Q9NZ71.2; 1219 amino acids) and variant 6 (Ensembl: ENST00000360203; Uniprot: Q9NZ71.6; 1300 amino acids), which differ in their C-terminal parts (Fig. 5A and Supplementary Material, Fig. S2). P1 and P2 carried a heterozygous missense mutation in exon 24 (used by both RTEL1 variants) at position c.2097C>G which results in the p.I699M amino acid substitution and a heterozygous missense mutation in exon 34B (specific of the RTEL1 variant 6) at position c.3730T>C which leads to the p.C1244R amino acid substitution (Fig. 5A). Sanger sequencing confirmed that P1 and P2 carried both RTEL1 mutations, whereas the healthy brother had wild-type sequence (Fig. 4B). The c.3730T>C and the c.2097C>G mutations were inherited from the father and the mother, respectively (Fig. 4B). P3 carried a heterozygous mutation in exon 25 (c.2233G>A) which results in the p.V745M amino acid substitution (present in both RTEL1 variants) and a G-to-A substitution at position +5 of intron 24 (IVS24 +5G>A) used by both RTEL1 variants (Fig. 5A). Sanger sequencing confirmed the c.2233G>A and IVS24 +5G>A mutations in P3 and indicated that they were inherited from the father and the mother, respectively, although absent in the healthy brother (Fig. 4C). The IVS24 +5G>A mutation, located at the vicinity of the exon 24, may affect the splicing and expression of RTEL1 (a hypothesis supported by western blot analysis, see what follows). The three missense mutations, absent from the mutation databases, have a high score in Polyphen 2 analysis (p.V745M: 0.967; p.I699M: 1; p.C1244R: 0.998), suggesting that they may be highly deleterious for RTEL1 function.

Structural impact of the RTEL1 mutations identified in patients

RTEL1 belongs, as XPD (xeroderma pigmentosum, also known as ERCC2), FANCI (Fanconi anemia) and ChlR1 (Warsaw breakage syndrome), to the subclass of FeS cluster-containing
Figure 2. Analysis of spontaneous 53BP1 foci, TIFs and anaphase bridges in patients’ cells. (A) Representative image of spontaneous 53BP1 foci detected in T-blasts from control and patient P3. (B) Quantitative analysis of cells exhibiting six or more 53BP1 foci per nucleus. (C) Representative pictures of nuclei from control and P2’s primary fibroblasts showing that a part of 53BP1 foci colocalized with a telomeric FISH probe (Telo-Cy3) and therefore represent TIFs. (D) The percentages of cells from controls and from the patients with four or more TIFs are indicated. (E) A representative image of anaphase bridge detected in P3’s T-blasts. DNA is labeled by DAPI and telomeres detected by FISH (Telo-Cy3 probe). The telomeric signal located in the anaphase bridge is zoomed in the inset. (F) Two representative images of anaphase bridges detected in P1’s SV40-transformed fibroblasts by DAPI staining. (G) The percentages of anaphase bridges detected in SV40-transformed fibroblasts from control and patients P1 and P2 are indicated. The cell passage numbers are indicated in brackets.
Figure 3. Telomeric aberrations detected in patients' cells by FISH and CO-FISH. (A) A representative image of telomeric FISH performed on a metaphase spread. Representative images of a normal chromosome (B), a chromosome with a sister loss (C) and a chromosome with terminal deletion (D) detected by telomeric FISH. A representative image of normal chromosomes (E), a chromosome with a sister loss of the leading strand (F), a chromosome with terminal deletion (G) and T-SCE (H) detected by telomeric CO-FISH. Quantification of telomere sister losses (I), and terminal deletions (J) detected by FISH in patients' cells and appropriate controls. For FISH, SV40-transformed fibroblasts were at passage numbers 6 for control and 5 for the patients. Quantification of terminal deletions (K), telomere sister losses affecting the leading or the lagging strand (L) and T-SCE (M) detected by CO-FISH in patients' cells and controls. For CO-FISH, SV40-transformed fibroblasts were at passage numbers 15 for control and 5 for the patients.
Figure 4. Identification of RTEL1 mutations in HHS patients. (A) Pedigrees of families 1 and 2. (B) Filters used to analyze the whole-exome sequencing data and identified RTEL1 variants. (C) Inheritance of the RTEL1 variants identified in family 1 analyzed by Sanger sequencing. (D) Inheritance of the RTEL1 variants identified in family 2 analyzed by Sanger sequencing.
Figure 5. Structural consequences of RTEL1 mutations. (A) Schematic structure of the pre-mRNA RTEL1 coding for isoform 2 (1219 amino acids) and isoform 6 (1300 amino acids). The nature and localization of the mutations identified in P1, P2 and P3 are stated by arrows. (B) I699 and V745 are located in the catalytic core of RTEL1. Sequence alignment of the C-terminal end of the RTEL1 catalytic core with other members of the SF2 family. The XPD (ERCC2) amino acids, which are mutated in XP, CS and TTD, are shown in yellow. (C and D) View of the experimental 3D structure of *Thermoplasma acidophilum* XPD helicase (pdb 2vsf) at the HD1/HD2 interface. The side chains of I516 and I605, which correspond to I699 and V745 in human RTEL1, are shown, together with those of amino acids, mutated in XP/CS and TTD. (E) The C-terminal extension, specific for the RTEL1 1300 variant, contains a RING finger, in which C1244 is one of the zinc-binding amino acids.
DNA helicases within the SF2 DEAH subfamily (20,21). I699 (mutated in P1 and P2) and V745 (mutated in P3) are located in the catalytic core of RTEL1 that extends from amino acid 1 to 760 and comprises, as XPD, two Rad51/RecA-like helicase domains HD1 and HD2, as well as an FeS domain (22,23) (Fig. 5B). I699 is located at the HD1/HD2 interface, which forms a composite ATP-binding site (Fig. 5C and D) and is predicted to play a critical role for the conformation/stability of the ATP-binding site or the dynamics associated with the conformational switch upon ATP hydrolysis (22,23). Remarkably, mutations in the analogous region of XPD (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) (Fig. 5B). V745 (mutated in P3) is included in a C-terminal extension of the HD2 domain and likely participates to the inner core of an alpha-helix (Fig. 5B and Supplementary Material, Fig. S3) which is predicted to influence the stability of this structure. This extension in SF2 family helicases is involved in protein interactions (20). Thus, the p.V745M substitution probably affects interaction of RTEL1 with putative partner(s). Remarkably, mutations in the analogous region of XPD were also identified in TTD patients (Fig. 5B) (22). C1244 (mutated in P1 and P2) is located in the additional C-terminal region of RTEL1 isoform 6, and is included in a globular domain containing eight other cysteine residues (Supplementary Material, Fig. S3). This domain is conserved in the RTEL1 family and presents significant similarities with RING fingers, several of which functioning as, or interacting with, E3 ubiquitin ligases (Fig. 5E). RING finger motifs (Fig. 5E) coordinate two zinc atoms in a cross-brace arrangement through six cysteines (Fig. 5E and F). The p.C1244R mutation would thus preclude zinc binding in the first zinc-binding site, and have a dramatic impact on the functional properties of the RING finger domain of RTEL1, which remains to be characterized (Fig. 5E and F). Importantly, western blot analysis consistently revealed that the RTEL1 expression was slightly reduced in P1’s and P2’s fibroblasts and sharply decreased in P3’s T-blasts. This indicates that the RTEL1 mutations found in P3 strongly impact on the RTEL1 expression and/or stability (Fig. 5G).

**DISCUSSION**

By combining wide genome linkage analysis with whole-exome sequencing approaches, we identified compound heterozygous **RTEL1** mutations in three HHS patients from two unrelated families. The three patients exhibited similar clinical features and cellular phenotype principally characterized by telomere length reduction, telomeric aberrations and genomic instability. Unfortunately, the extreme cellular toxicity of RTEL1 overexpression and the cellular fragility of patients’ cells precluded functional complementation [Supplementary Material, Fig. S4, (24–26)]. Since complete loss of Rtel1 function in mice is embryonic lethal, the mutations we identified in patients likely cause hypomorphic, although severe, RTEL1 deficiency. Two of the four RTEL1 mutations (p.I699M and p.V745M) affect residues of the catalytic core domain of RTEL1. This suggests that these mutations may have a strong impact on RTEL1 activity and/or stability. The deleterious consequences of p.I699M and p.V745M substitutions are further supported by the fact that mutations located in the analogous domain of XPD cause CS, XP and TTD (22). We also identified a p.C1244R mutation located in a hitherto uncharacterized RING domain specific of the isoform 6 of RTEL1. Given the structural similarity of the RTEL1 RING domain to that of BMI-1, it can be proposed that this domain participates to hetero- or homo- dimerization or to ubiquitin transfer. If so, the partners and/or targets, as well as the consequences of such interactions on cellular metabolism and/or genome stability, remain to be characterized.

The cellular phenotype of RTEL1 deficiency in P1, P2 and P3 is strikingly similar and consists in the shortening of telomere and the increase in spontaneous 53BP1 foci, anaphase bridges, sister telomere losses and terminal deletions. However, the patients’ cell phenotype suggests an overall genome instability likely caused by defective DNA replication and repair; it is worth noting that it differs from other HHS patients who present with fragile telomere phenotype characterized by MTS, ITS and telomere fusion (9,27). In addition to the increased telomeric aberrations, RTEL1-deficient cells exhibit short telomeres. It has recently been proposed that telomeres from murine Rtel1-deficient cells were unable to be elongated by the telomerase complex (24). Thus, the telomere length reduction observed in RTEL1-deficient patients could stem from an inability of the active telomerase complex to reach the telomeres. However, it is important to note that the clinical presentation, as well as the telomere length reduction found in young human RTEL1-deficient patients, is more severe than observed in DC caused by a defect of a telomerase component [e.g. in TERT and TERC deficiencies (28)]. This suggests that the clinical, cellular and telomeric features found in RTEL1 deficiency do not rely solely on impaired telomerase activity at telomeres. Unlike primary fibroblast from P1 and P2, no increase in TIFs was noticed in T-blasts from P3. This apparent discrepancy between fibroblasts and T-blasts has previously been reported in TERT-deficient cells and could be the consequence of a differential capacity of cells to tolerate the accumulation of DNA lesions (29). The finding that rare RTEL1 polymorphisms are associated with glioma (30) further supports the concept that RTEL1 is required to ensure genome integrity. Because of early death of HHS patients due to severe immunodeficiency, they rarely develop cancer. However, one can envisage that parents who carry a heterozygous **RTEL1** mutation may be at a high cancer risk, a possibility that deserves a careful follow-up to detect the possible occurrence of an oncogenic process. Of note, **RTEL1** mutations were recently independently reported in three other cases of DC/HHS (25).
Overall, our study identifies RTEL1 as a novel HHS-causing gene and provides evidence that RTEL1 warrants genome stability, telomere length maintenance and integrity in humans. Although several recent studies (mainly performed in the nematode Caenorhabditis elegans and in mice) have reported on RTEL1 functions in DNA repair, DNA replication, meiosis and telomere protection (21,24,26,31,32), many questions remain unsolved. In addition, the phenotype of patients’ cells suggests that RTEL1 deficiency could lead to fusions, sister entanglements, replication block or unresolved homologous recombination events. Future studies using RTEL1-deficient cells from patients should help clarify the multiple roles of RTEL1 acting as a genomic caretaker in humans. Our findings also suggest that less severe mutations in RTEL1 may lead to cancer predisposition or other bone-marrow failure syndromes such as DC.

**MATERIALS AND METHODS**

**Patients and cells**

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density centrifugation from blood samples. Phytohemagglutinin (PHA)-induced T-cell blasts were obtained by stimulating PBMCs for 3 days with 6.25 μg/ml of PHA (Sigma) and then subsequently cultured for 5–15 days in RPMI-1640 supplemented with 10% human AB serum and 100 U/ml IL-2. Informed consent for our study was obtained from the families in accordance with the Declaration of Helsinki. This study was also approved by the Institutional Review Board (INSERM). Control fibroblasts were from skin biopsies from pediatric healthy donors (no difference in phenotype was noticed in the function of gender).

**GWAS genotyping**

Genotyping using Illumina Human Linkage-24 BeadChip was carried out according to standard methodology. In brief, each sample (200 ng) was whole-genome-amplified, fragmented, precipitated and resuspended in appropriate hybridization buffer. Denatured samples were hybridized on prepared Human Linkage-24 BeadChips for a minimum of 16 h at 48°C. After hybridization, the BeadChips were processed for the single-base extension reaction as well as staining and imaging on an Iscan (Illumina) and an Autoloader 2 (Illumina). Normalized bead intensity data obtained for each sample were loaded into the Illumina GenomeStudio software 2011.1, which converted fluorescence intensities into SNP genotypes.

**Whole-exome sequencing**

Standard manufacturer protocols were used to perform target capture with the Illumina TruSeq exome enrichment kit and sequencing of 100 bp paired-end reads on Illumina HiSeq. We generated ~10 Gb of sequence for each subject such that 90% of the coding bases of the exome defined by the consensus coding sequence project were covered by at least 10 reads. We removed adaptor sequences and quality-trimmed reads using the Fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and then used a custom script to ensure that only read pairs with both mates present were subsequently used. Reads were aligned to hg19 with BWA31, and duplicate reads were marked using Picard (http://picard.sourceforge.net/) and excluded from downstream analyses. Single-nucleotide variants and short insertions and deletions (indels) were called using samtools (http://samtools.sourceforge.net/) pileup and varFilter32 with the base alignment quality adjustment disabled and were then quality-filtered to require at least 20% of reads supporting the variant call. Variants were annotated using both ANNOVAR33 and custom scripts to identify whether they affected protein-coding sequence and whether they had previously been seen in dbSNP131, the 1000 Genomes data set (October 2011) or in ~160 exomes previously sequenced at our center. A variant called in the patient was considered to be a candidate mutation if it was not reported in dbSNP131 or the 1000 Genomes data set (October 2011).

**Immunofluorescence and antibodies**

Immunofluorescence experiments were performed as previously described (9). Mouse monoclonal anti-KU70 was from Neomarkers. Anti-53BP1 was from Novus Biological, and Alexa488 goat F(Ab’)2 secondary antibodies were from Molecular Probes.

**Anti-hRTEL1 antibody production**

Rabbit antibodies against purified C-terminal RTEL1 (last 750 amino acids) were prepared by AGRO-BIO (France). Immune sera from two rabbits were obtained and specific antibodies were affinity-purified on NHS-activated sepharose columns covalently bound to C-terminal RTEL1. Antibody elution was carried out at acidic pH and antibody solutions were pH-neutralized.

**FISH, CO-FISH and image capture**

FISH analysis was conducted as previously described (9). CO-FISH and image capture were performed as previously described (33).

**Telomeric restriction fragment and TRAP assay**

Measurement of the lengths of the terminal restriction fragments was performed by Southern blotting as described in Touzot et al. (9). TRAP assay was performed as described in Kim et al. (17).

**Statistical analysis**

For telomere aberrations observed by FISH and CO-FISH, statistical analysis was conducted as previously described (9). Telomere aberrations of each condition were compared considering the total number of chromatid ends.
SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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