Mutations in *CTC1*, encoding conserved telomere maintenance component 1, cause Coats plus

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Coats plus is a highly pleiotropic disorder particularly affecting the eye, brain, bone and gastrointestinal tract. Here, we show that Coats plus results from mutations in *CTC1*, encoding conserved telomere maintenance component 1, a member of the mammalian homolog of the yeast heterotrimeric CST telomeric capping complex. Consistent with the observation of shortened telomeres in an *Arabidopsis CTC1* mutant and the phenotypic overlap of Coats plus with the telomeric maintenance disorders comprising dyskeratosis congenita, we observed shortened telomeres in three individuals with Coats plus and an increase in spontaneous γH2AX-positive cells in cell lines derived from two affected individuals. CTC1 is also a subunit of the α-accessory factor (AAF) complex, stimulating the activity of DNA polymerase-α primase, the only enzyme known to initiate DNA replication in eukaryotic cells. Thus, CTC1 may have a function in DNA metabolism that is necessary for but not specific to telomeric integrity.

Telomeres comprise long TTAGGG nucleotide repeats and associated proteins located at the ends of chromosomes. Telomeres both protect the chromosome terminus from unwanted nuclease and DNA repair activities and provide a mechanism to compensate for the inability of DNA polymerase to replicate the 5′ end of a linear chromosome. Telomeric DNA is packaged by a core group of proteins that bind the DNA duplex and the 3′ G overhang on the chromosome terminus. The associated telomere proteins form a protective cap that prevents the chromosome end from eliciting a DNA damage response.

Although it is essential to sequester the DNA end from nuclease and DNA repair activities for much of the cell cycle, during S phase the telomere must be made accessible to telomerase and the DNA replication machinery[1].

The bone marrow failure syndrome dyskeratosis congenita represents a clinically and genetically heterogeneous collection of phenotypes arising from telomeric protein dysfunction. Dyskeratosis congenita–associated mutations have been identified in the genes encoding DKC1, TERC, TERT, NOP10, NHP2 and TCAB1, which belong to the telomerase holoenzyme responsible for maintaining telomere length, and a member of the shelterin protein complex TINF2, which is responsible for maintaining the structural integrity of the telomere. Thus, dyskeratosis congenita is a disorder of telomere maintenance and is associated with shortened telomeres[2,3].

Coats plus is a rare disorder of which the most characteristic features are retinal telangiectasia and exudates (Coats disease), a distinctive pattern of intracranial calcification with an associated leukodystrophy and brain cysts, osteopenia with a tendency to fractures and poor bone healing, and a high risk of life-limiting gastrointestinal bleeding and portal hypertension caused by the development of vasculature ectasias in the stomach, small intestine and liver[4–9] (**Fig. 1**). These extra-neurological problems differentiate Coats plus from Labrune syndrome (leukoencephalopathy with calcifications and cysts), in which affected individuals otherwise show an identical neuroradiological appearance[10]. Of note, some individuals with Coats plus develop sparse, graying hair, dystrophic nails and a normocytic anemia that might reflect a degree of bone marrow failure. These latter

Figure 1 Features of Coats plus. (**a**) Cranial axial computed tomography (CT) scan showing characteristic coarse and asymmetrically distributed calcification. Note the cyst in the left frontal cortex. (**b**) Cranial axial T2 magnetic resonance imaging (MRI) scan taken at similar level to **a**, highlighting the asymmetric, high signal of the deep and subcortical white matter. (**c**) X ray of knee illustrating mixed lytic and sclerotic lesions that are mainly metaphyseal. (**d**) Retinal photograph showing retinal microangiopathy. Scale bar, ~2.5 mm. (**e**) Endoscopy image showing areas of erythema, which represent vascular ectasia involving the gastric antrum. Scale bar, ~2.5 cm. The neuroradiological features of Labrune syndrome (leukoencephalopathy with calcifications and cysts) are identical to those in **a** and **b**.
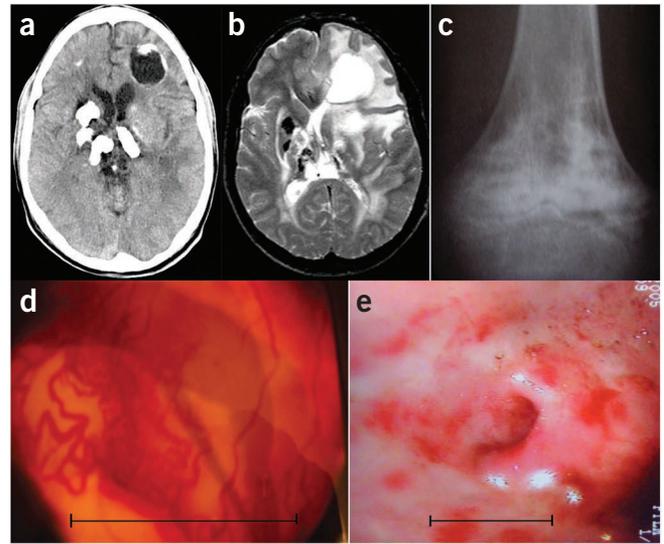
features are also observed in dyskeratosis congenita. Furthermore, individuals with Hoyeraal-Hreidarsson or Revesz syndrome, both of which are associated with telomeric shortening, can have intracranial calcification and, in the case of Revesz syndrome, an exudative retinopathy[2].

In view of reports of both male and female affected siblings, we considered it most probable that Coats plus was an autosomal recessive trait, although we noted that our collection of ten families fulfilling strict diagnostic criteria (characteristic intracranial calcification with white matter changes, exudative retinopathy with telangiectasia and fractures with poor bone healing and/or gastrointestinal vascular ectasia) included only one family in which the parents were known to be related. Because we were unable to define a disease-associated locus using SNP arrays (data not shown), we undertook whole-exome sequencing of two siblings (F335_P1 and F335_P2) and two additional unrelated individuals (F332 and F336) showing classical features of Coats plus (**Supplementary Table 1**). We performed whole-exome capture followed by massively parallel sequencing. Over 4.8 Gb of sequence was generated for each subject, such that >76% of the coding bases of the GENCODE-defined exome were represented by at least ten reads (**Supplementary Table 2**). We identified single-nucleotide substitutions and small insertion and/or deletion variants using our in-house variant calling pipeline. We analyzed the exome variant profiles under a model of a rare autosomal recessive disorder. Taking into account difficulties in identifying insertion and deletion variants, we looked for genes harboring at least one previously unobserved nonsynonymous or splice-site substitution or a coding insertion or deletion in the same gene in all four individuals, with the same variant(s) required

in the siblings examined. *CTC1* (encoding conserved telomere maintenance component 1) was highlighted as the only candidate gene when using this strategy. Sanger sequencing confirmed the variants in these affected individuals, and all parents tested showed appropriate heterozygosity for a single variant. In light of these data, we proceeded to sequence an additional nine affected individuals from seven families showing typical disease characteristics.

We identified biallelic *CTC1* variants that were likely to be pathogenic in the affected proband(s) from nine of the ten families with Coats plus that were sequenced (**Table 1**). We also identified two *CTC1* variants in a Norwegian individual (F319) having exudative retinopathy with a history of recurrent fractures but in whom cranial imaging had not been undertaken (and who therefore did not fulfill our initial inclusion criteria). In only one family with a prior clinical assignment of Coats plus were we unable to identify *CTC1* variants. In the ten families with presumed biallelic mutations, six harbored a nonsense lesion in combination with a missense variant, while the consequences of the splice-site variant seen in F273 and the in-frame deletions observed in F367 and F382 are currently unknown. The probands from F342 carried two missense variants (c.775G>A and c.2518C>T). Of the 14 distinct

Table 1 Ancestry, pedigree structure and sequence alterations in individuals with Coats plus

Family	Ancestry	Tested	Nucleotide alterations	Exons	Amino acid alterations	Parental consanguinity
F273	Egyptian	1A, M, F	Het. c.2831dupC | Het. c.3011+4A>C	17 | intron 18	p.His945Serfs*56 | splice donor[b]	No
F319	Norwegian	1A	Het. c.721C>T | Het. c.2923A>G	5 | 17	p.Gln241* | p.Arg975Gly	No
F332[a]	English	1A	Het. c.724_727delAAAG | Het. c.2959C>T	5 | 18	p.Lys242Leufs*41 | p.Arg987Trp	No
F335_P1[a]	Scottish	2A	Het. c.724_727delAAAG | Het. c.2959C>T	5 | 18	p.Lys242Leufs*41 | p.Arg987Trp	No
F335_P2[a]	Scottish	2A	Het. c.724_727delAAAG | Het. c.2959C>T	5 | 18	p.Lys242Leufs*41 | p.Arg987Trp	No
F336[a]	English	1A	Het. c.724_727delAAAG | Het. c.2611G>A	5 | 15	p.Lys242Leufs*41 | p.Val871Met	No
F339	Canadian	2A	–	–	–	Yes (third cousins)
F340	European-American	1A	Het. c.19C>T | Het. c.2959C>T	1| 18	p.Gln7* | p.Arg987Trp	No
F342_P1	English and Italian	2A, M, F	Het. c.775G>A | Het. c.2518C>T	5 | 15	p.Val259Met | p.Arg840Trp	No
F342_P2	English and Italian	2A, M, F	Het. c.775G>A | Het. c.2518C>T	5 | 15	p.Val259Met | p.Arg840Trp	No
F345	Swiss and French	1A	Het. c.724_727delAAAG | Het. c.1507G>C	5 | 9	p.Lys242Leufs*41 | p.Gly503Arg	No
F367	African and European	1A, 1U, M	Het. c.859C>T | Het. c.2954_2956delGTT	6 | 18	p.Arg287* | p.Cys985del	No
F382	Portuguese	1A, 1U, M, F	Het. c.2954_2956delGTT | Het. c.3586_3606del	18 | 23	p.Cys985del | p.Leu1196_Arg1202del	No

A, affected; U, unaffected; M, mother; F, father; Het., heterozygous.
[a]Used in the primary exome sequencing screen. [b]Predicted by SplicePort to significantly reduce the strength of the canonical donor site.

Figure 2 Schematic representation of the human *CTC1* gene. (**a**) *CTC1* spans 23,273 bp of genomic sequence on chromosome 17p13.1 (8,128,140–8,151,413). Neighboring genes are also shown. Tel., telomere; cen., centromere. (**b**) Position of identified mutations within the *CTC1* gene. Protein alterations are given in brackets.
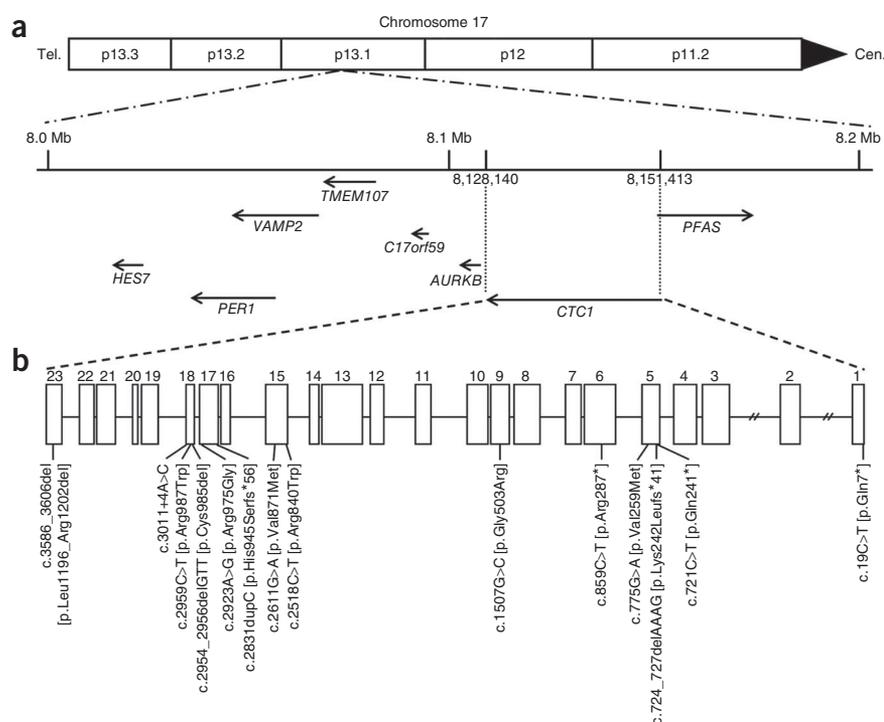
mutations observed, three were seen in more than one family (c.724_727del, four families; c.2954_2956del, two families; c.2959C>T, three families) (**Table 1**).

Reflecting apparently rapid evolutionary divergence, some telomeric proteins show poor interspecies conservation[11]. The human CTC1 protein sequence shares only 69% identity with mouse, 30% with zebrafish and 14% with *Arabidopsis*. Not unexpectedly, although the residues affected by nonsynonymous missense changes are well conserved across mammals, ClustalW alignment showed a relatively low level of residue conservation in other species (**Supplementary Fig. 1**). Related to this, 15 of the 20 *CTC1* mutations we observed occurred in one of four exons (**Fig. 2**). However, although we suspect that this clustering is of functional significance, it is difficult to model the domain structure of CTC1 because of the high degree of sequence divergence from other proteins[12]. Of note, none of the 14 distinct, putatively pathogenic *CTC1* variants have been annotated as polymorphisms in dbSNP. Moreover, 13 of these variants were not seen in 1,730 European-American and African-American subjects collated in the Exome Variant Server database, and the c.19C>T transition seen in F340 was recorded in only 1 of 1,497 European-Americans (the same ancestral background as the affected proband).

Cerebroretinal microangiopathy with calcifications and cysts (CRMCC) is a term coined to encompass the Coats plus and Labrune syndrome phenotypes, on the grounds that the neuroradiological characteristics of these two disorders are essentially identical[7,8]. Thus, we sequenced *CTC1* in a collection of probands from 21 families showing stereotypical intracranial calcification and white matter changes in the absence of extra-neurological features, but we were unable to identify any likely pathogenic variants (**Supplementary Table 3**).

Recently, it has been shown that CTC1 interacts with STN1 and TEN1 to form the mammalian homolog of the yeast heterotrimeric Cdc13-Stn1-Ten1 (CST) telomeric capping complex[13,14]. Consequently, we also undertook Sanger sequencing of *OBFC1* (*STN1*) and *TEN1* in our collection of individuals with Labrune syndrome and in the proband with Coats plus in whom we identified no *CTC1* mutations. Again, sequencing identified no variants of likely pathogenic significance (**Supplementary Tables 3** and **4**).

In view of the finding of shortened telomeres in an *Arabidopsis CTC1* mutant[13] and the phenotypic overlap between Coats plus and the telomeric maintenance disorders comprising dyskeratosis congenita, we measured telomere lengths[15] in F332, F367 and F382 at the ages of 20, 20 and 3 years, respectively. Samples were also available from the mother and heterozygous brother of F367 and from both parents of F332 and F382. Testing showed markedly shortened telomeres in lymphocytes and granulocytes from the peripheral blood

of F367 and F382 and telomeric lengths at the lower range of normal in the heterozygous relatives of these individuals (**Fig. 3**). In F332, telomere length was recorded on the first percentile for age in both lymphocytes and granulocytes, with parental telomere lengths also at the lower range of normal.

Considering the enrichment for *CTC1* variants in individuals with a predefined clinical and radiological diagnosis, we are confident that mutations in *CTC1* represent the major cause of Coats plus. The observation of a single family, F339, in whom we were unable to identify mutations suggests possible genetic heterogeneity or a phenocopy. Of note, the parents of this child are related as third cousins, but there was no evidence of homozygosity around any of the three CST protein–encoding genes (data not shown).

Although Coats plus is rare and inherited as an autosomal recessive trait, none of the ten mutation-positive families we identified are consanguineous. In keeping with this, all of the affected individuals in these families are compound heterozygotes for two different *CTC1* variants (with six of ten families harboring a nonsense and missense mutation in combination). This observation helps to explain our inability to define a disease locus using autozygosity mapping and leads us to speculate that biallelic null mutations might be incompatible with development, whereas homozygosity for (most) missense variants may be associated with a normal phenotype or a different pathogenic one.

We and others have noted the highly stereotyped neuroradiological features common to Coats plus and Labrune syndrome, leading to the introduction of the umbrella term CRMCC. However, a recent analysis of our cohort (data not shown) suggested that the two groups might be distinguished according to the presence or absence of extra-neurological features, as, in our experience, individuals without retinal abnormalities have never shown any skeletal or gastrointestinal manifestations over extended time periods. The genetic data presented here support this conclusion, indicating that Coats plus and Labrune syndrome are not allelic.

CTC1 comprises 23 exons and encodes a 134.5-kDa protein. Because of rapid evolutionary divergence, the human CST complex was only defined recently[13,14]. This led to the recognition that mammalian
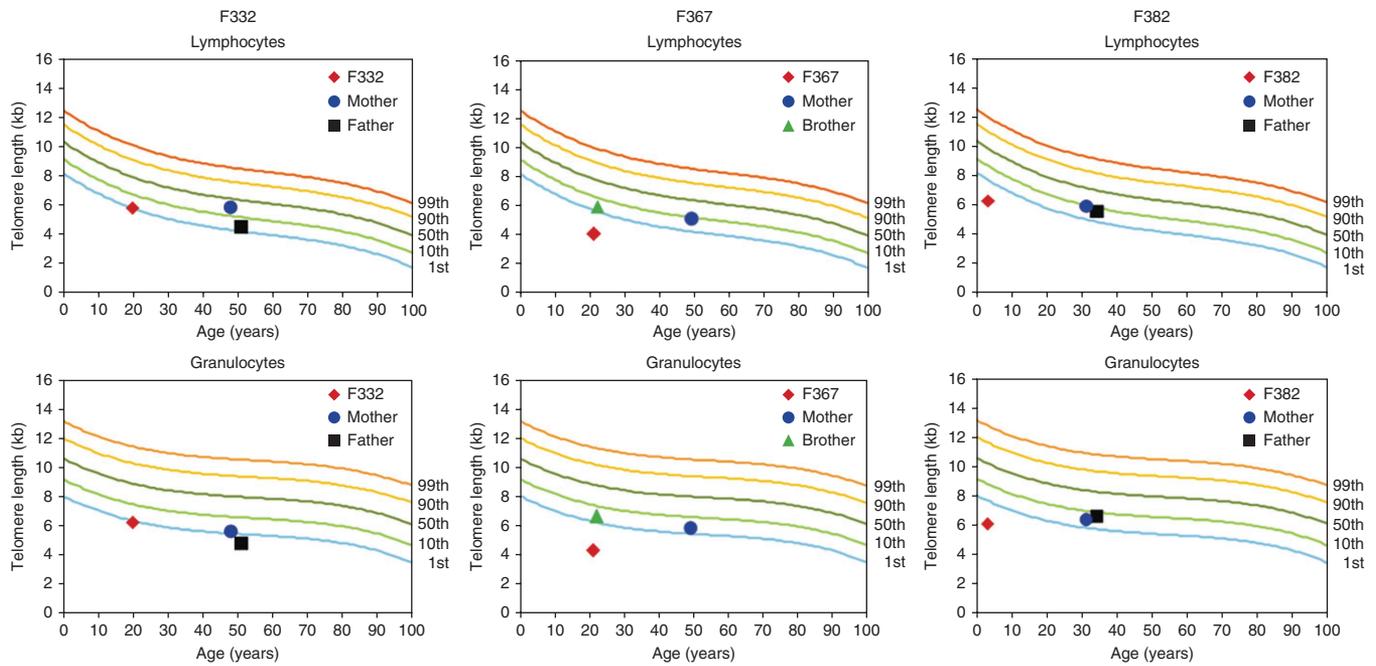
Figure 3 Telomere length analysis in subsets of leukocytes as measured by automated multicolor flow-FISH. Telomere lengths were assessed in lymphocytes and granulocytes from F332, F367 and F382 at the ages of 20, 20 and 3 years, respectively, and from unaffected family members harboring a single *CTC1* mutation. Telomere lengths were markedly shortened in two affected individuals (F367 and F382) and were at the lower range of normal in their heterozygous relatives. In F332, telomere length was recorded on the first percentile for age in both lymphocytes and granulocytes, with parental telomere lengths at the lower range of normal (being less than the first percentile in the father in granulocytes). The reference range for telomere length over age in percentiles was derived from telomere length analyses in lymphocytes and granulocytes from 400 healthy individuals.

CTC1 is identical to one subunit of α-accessory factor (AAF-132), whereas a second subunit of AAF (AAF-44, also known as OBFC1) corresponds to mammalian STN1 (ref. 16).

The AAF complex stimulates the activity of DNA polymerase-α primase, the only enzyme known to initiate DNA replication in eukaryotic cells[17]. AAF functions by binding single-stranded DNA (ssDNA) and enhancing DNA polymerase-α primase association with a DNA template. The finding that human CTC1-STN1 modulates DNA polymerase-α primase activity indicates that, as for budding yeast CST, mammalian CTC1-STN1 provides a link to the lagging strand replication machinery. Thus, it can be proposed that a conserved function of CST is to promote efficient priming of telomeric C-strand synthesis. In addition, given the role of Cdc13 in budding yeast, another conserved function of the CST complex could be to regulate telomerase. All of these processes may contribute to maintaining telomeric DNA integrity and could couple the conventional replication process to telomere-specific priming and telomerase-dependent elongation steps[18] (see **Supplementary Fig. 2**).

Experiments in budding yeast and *Arabidopsis* have identified a functional role for the CST complex in maintaining telomeric structural integrity, and our finding of shortened telomeres in individuals with *CTC1* mutations confirms an important role for the CST complex in mammalian telomere metabolism. However, the CST complex only partially localizes to telomeres and binds to ssDNA in a sequence-independent manner (through the oligonucleotide/oligosaccharide-binding (OB) fold domains predicted to be present in all three subunits)[13,14]. Of note, knockdown of *CTC1* in human cells resulted in an increase in the number of γH2AX foci not confined to telomeres[13]. Although we did not study chromosomal localization, we observed a significant increase in spontaneous γH2AX-positive cells in cell lines from the two affected individuals (F332 and F382) that we were able to examine ($P = 0.0001$ and 0.0007, respec-

tively), indicative of an ongoing DNA damage response (**Supplementary Fig. 3**). Consequently, it is possible that the CST complex may have a more general role in DNA replication and repair that is frequently required by but not specific to telomeres.

Our interest in Coats plus and Labrune syndrome derives from ongoing research into disorders associated with the presence of intracranial calcification. As such, our cohort is biased toward individuals with obvious neurological involvement. It is possible then that the phenotypic spectrum associated with *CTC1* mutations may be broader than that presented here. Of note, phenotypic overlap with dyskeratosis congenita was appreciated at the time of the first clinical description of Coats plus, leading us to speculate that cohorts of individuals with a diagnosis of dyskeratosis congenita may be enriched for pathogenic variants in *CTC1*, *OBFC1* (*STN1*) and/or *TEN1*.

URLs. National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/; SplicePort, http://spliceport.cs.umd.edu/; GENCODE, http://www.gencodegenes.org/; ClustalW, http://www.ebi.ac.uk/tools/msa/clustalw2/; Primer3Plus, http://www.primer3plus.com/.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

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ONLINE METHODS

Subject ascertainment. Subjects were ascertained internationally through colleagues in pediatric neurology and clinical genetics. Written informed consent was obtained for all participants. The study had full ethical approval from the Leeds Multicentre Research Ethics Committee (07/Q1206/7).

Exome sequencing. Genomic DNA was extracted from lymphocytes from affected individuals, parents and siblings by standard techniques. For whole-exome analysis, targeted enrichment and sequencing were performed on DNA extracted from peripheral blood from four individuals (F332, F335_P1, F335_P2 and F336). Enrichment was performed using the SureSelect Human All Exon Kit v.1 (Agilent) for the Applied Biosystems SOLiD system. Emulsion PCR (ePCR) was conducted on the resultant sample library, and products were then sequenced on a SOLiD 4 sequencer (Life Technologies). Sequence data were mapped using SOLiD Bioscope software (Life Technologies), with the hg18 human genome as a reference. SNPs were called using the diBayes tool in the BioScope software suite with the medium stringency setting and then filtered to remove those SNPs with less than 5× coverage. A total of 4.8 Gb of sequence mapped uniquely to the hg18 genome reference, with 76.4% of the targeted exome covered at tenfold or higher (**Supplementary Table 2**).

Sanger sequencing. Mutation analysis was performed by direct sequencing of purified genomic PCR products using the BigDye Terminator v3.1 cycle sequencer system (Applied Biosystems). Primers were designed for individual exons and intron boundaries of *CTC1*, *OBFC1* (*STN1*) and *TEN1* using Primer3Plus and the reference sequences of each gene (primer sequences are provided in **Supplementary Table 5**; details of experimental conditions are available on request).

Telomere length measurement. Telomere length was measured in white blood cell subsets by automated multicolor flow-FISH analysis[15] of granulocytes, CD45RA$^+$ lymphocytes (naive T cells), CD45RA$^-$ lymphocytes (memory T cells), CD20$^+$ lymphocytes (B cells), CD57$^+$ lymphocytes (NK/NKT cells) and total leukocytes, and compared with age-matched controls. 'Markedly short' was defined as less than the first percentile for age.

FACS for measurement of γH2AX positivity. Lymphoblastoid cells from two affected individuals (F332 and F382) and three control individuals were seeded at a density of 5×10^5 cells per well of a six-well plate (Sigma) and maintained at 37 °C overnight. Cells were pelleted and washed in PBS and fixed in 500 µl of 1× Fixation Solution (Millipore γH2AX Phosphorylation Assay Kit) for 20 min at 4 °C. Cells were pelleted and washed in 1 ml of PBS, pelleted again and resuspended in 50 µl of 1× permeabilizing solution (Millipore γH2AX Phosphorylation Assay Kit), and 3.5 µl of appropriate antibody was added (FITC-conjugated antibody to γH2AX or control FITC-conjugated rabbit IgG) and incubated at 4 °C for 20 min. We added 100 µl of 1× washing solution to each sample, and cells were pelleted and resuspended in 400 µl of PBS. Samples were analyzed using the BD Biosciences FACSCalibur using CellQuest Pro software, and data were analyzed using FlowJo.