Mutations in the telomerase component NHP2 cause the premature ageing syndrome dyskeratosis congenita

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Dyskeratosis congenita is a premature aging syndrome characterized by muco-cutaneous features and a range of other abnormalities, including early greying, dental loss, osteoporosis, and malignancy. Dyskeratosis congenita cells age prematurely and have very short telomeres. Patients have mutations in genes that encode components of the telomerase complex (dyskerin, TERC, TERT, and NOP10), important in the maintenance of telomeres. Many dyskeratosis congenita patients remain uncharacterized. Here, we describe the analysis of two other proteins, NHP2 and GAR1, that together with dyskerin and NOP10 are key components of telomerase and small nucleolar ribonucleoprotein (snoRNP) complexes. We have identified previously uncharacterized NHP2 mutations that can cause autosomal recessive dyskeratosis congenita but have not found any GAR1 mutations. Patients with NHP2 mutations, in common with patients bearing dyskerin and NOP10 mutations had short telomeres and low TERC levels. SiRNA-mediated knockdown of NHP2 in human cells led to low TERC levels, but this reduction was not observed after GAR1 knockdown. These findings suggest that, in human cells, GAR1 has a different impact on the accumulation of TERC compared with dyskerin, NOP10, and NHP2. Most of the mutations so far identified in patients with classical dyskeratosis congenita impact either directly or indirectly on the stability of RNAs. In keeping with this effect, patients with dyskerin, NOP10, and now NHP2 mutations have all been shown to have low levels of telomerase RNA in their peripheral blood, providing direct evidence of their role in telomere maintenance in humans.

GAR1 | bone marrow failure | telomeres

Dyskeratosis congenita is a multisystem premature aging syndrome characterized by muco-cutaneous features, bone marrow failure, and a range of other abnormalities, including early greying, dental loss, osteoporosis, and malignancy (1, 2). It is genetically heterogeneous, with X-linked, autosomal dominant and autosomal recessive subtypes. The gene mutated in the X-linked form, *DKC1*, encodes a highly conserved nucleolar protein, called dyskerin (3), that plays an essential role in ribosome biogenesis and pre-mRNA splicing (4), converting specific uridine residues to pseudouridine during the maturation of ribosomal and spliceosomal RNAs (4, 5). This function is performed in concert with three other proteins (NOP10, NHP2, and GAR1), as well as an H/ACA small nucleolar (sno) RNA molecule that acts as a guide for this RNP complex (6, 7). Defective pseudouridylation activity has been observed in mice with reduced *Dkc1* expression (8) and mouse embryonic stem cells with *Dkc1* missense mutations (9), but to date there is no evidence of a similar effect in cells from dyskeratosis congenita patients (10, 11).

Dyskerin, GAR1, NHP2, and NOP10 also form part of the telomerase RNP complex (7, 10, 12) [supporting information (SI) Fig. S1] responsible for maintaining the protective repeat sequences at the ends of each chromosome (13, 14). Dyskeratosis congenita patients have very short telomeres (10, 15), and some have been shown to have reduced levels of TERC (the RNA component of telomerase) (16, 17). It has been suggested therefore that dyskeratosis congenita may primarily be a disorder of telomere maintenance. The most compelling evidence supporting this view is that autosomal dominant dyskeratosis congenita can result from TERC mutations (18), which cause a reduction in telomerase activity and give rise to disease via haploinsufficiency (19–21). Heterozygous missense mutations in the reverse transcriptase component of telomerase (TERT) that abolish telomerase activity have been reported in two large pedigrees with dyskeratosis congenita-like features (22, 23). In other families, heterozygous *TERT* missense mutations do not clearly segregate with the disease (24), but may be acting as risk factors for aplastic anemia (25). Further evidence that dyskeratosis congenita is a disorder of telomere maintenance comes from the recent observation in a small number of patients that the disease may result from mutations in a component of shelterin (TIN2), a telomere-specific protein complex (26).

We hope to gain further insight into the role of telomerase deficiency in human disease through the identification of the gene(s) responsible for the uncharacterized autosomal recessive form of dyskeratosis congenita. Homozygosity mapping in consanguineous families has indicated that this form of the disease is genetically heterogeneous. Affected individuals in one family have been shown to be homozygous for a missense mutation to a highly conserved amino acid in NOP10 that is associated with reduced levels of TERC and very short telomeres (17). This finding prompted us to review the mutation status of the genes encoding two other proteins of the telomerase–snoRNP core complex in dyskeratosis congenita patients.

Results

We have analyzed the *GAR1* and *NHP2* genes in 117 unrelated and genetically uncharacterized dyskeratosis congenita patients. A total of seven and nine different sequence changes have been identified in the *GAR1* and *NHP2* genes, respectively (Table S1). Six of the changes in *GAR1* are intronic and one is a silent polymorphism in the coding sequence; none are predicted to disrupt the GAR1 protein. Five of the changes in the *NHP2* gene

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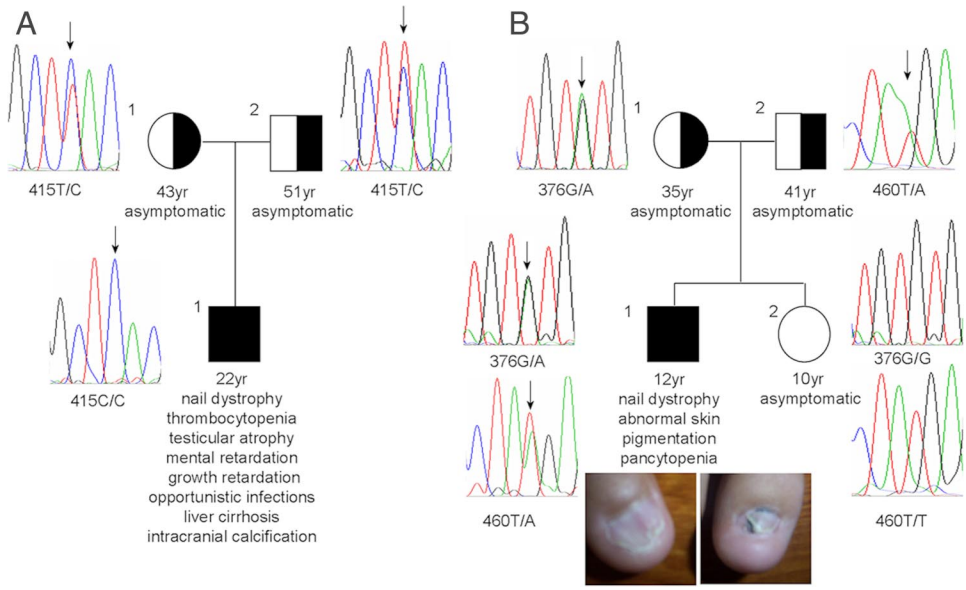

Fig. 1. *NHP2* mutations cause autosomal recessive dyskeratosis congenita. Sequencing traces from within the *NHP2* gene are shown next to each family member. Mutations are indicated by arrows and specified below each panel. The age in years and abnormalities observed are given below each individual, with nail dystrophy illustrated in one case. (*A*) Family DCR096. (*B*) Family DCR177.

are intronic and do not seem to disrupt bases essential for correct splicing. A missense mutation (c.352G>A, p.Ala118Thr) was identified in *NHP2* in one of the control subjects: this mutation has been previously reported as a polymorphism, present in 2/94 samples from black individuals (25). The remaining three sequence changes are private mutations that disrupt the NHP2 protein; they were not seen in a screen of 282 control samples of mixed ethnic origin (25), or in an additional 98 healthy individuals of Turkish origin that we have investigated.

The first of these private *NHP2* mutations, c.415T>C (p.Tyr139His) was identified as a homozygous change in a sporadic male case of dyskeratosis congenita. This young Turkish man had nail dystrophy, thrombocytopenia, testicular atrophy, opportunistic infections, growth and mental retardation, liver cirrhosis, and intracranial calcification. His parents were asymptomatic and heterozygous for this mutation (Fig. 1) and are not reported to be related. Two other coding mutations were identified in an unrelated Turkish male who was a compound heterozygote for a missense mutation (c.376 G>A; p.Val126Met) and a stop codon mutation (c.460T>A, p.X154ArgextX*52). The latter is predicted to give rise to a markedly elongated protein, with 51 aa being added to the C terminus of the protein, which normally consists of 153 aa (Fig. 2). The affected boy presented with dyskeratosis congenita at the age of 12 years, with the classical mucocutaneous triad of nail dystrophy (Fig. 1*B*), leucoplakia, and reticulate skin pigmentation. He developed peripheral pancytopenia because of progressive bone marrow failure; no other somatic abnormalities were reported. Both parents were heterozygotes (mother, p.Val126Met; father, p.X154ArgextX*52) and asymptomatic, as was a younger sister who has a normal genotype (Fig. 1*B*). Taken together, these results indicate that the classical form of dyskeratosis congenita, displaying features of premature aging, can be caused by biallelic mutations in *NHP2*.

To determine whether the *NHP2* mutations had any effect on telomere maintenance, telomere lengths were measured. In both patients with biallelic *NHP2* mutations telomeres were very short (Fig. 3*A*). Age-adjusted telomere length measurements show that the patients lie outside the normal range and are comparable with patients with dyskerin mutations (Fig. 3*B*). Parents heterozygous for the c.376G>A and c.460T>A mutations have telomere lengths that are short but within the normal range, whereas the unaffected normal sibling has normal telomere length. One of the parents heterozygous for the c.415T>C mutation has normal telomere length, whereas the other parent with this genotype has short telomeres, but again this length is within the normal range (Fig. 3*B*).

As well as having shortened telomeres, patients with dyskeratosis congenita have also been shown in some cases (with dyskerin, TERC, and NOP10 mutations) to have reduced TERC levels (16–17). Like dyskerin and NOP10, NHP2 associates with

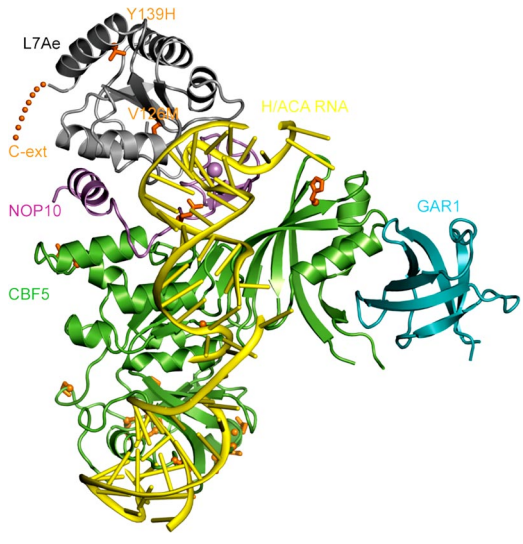

Fig. 2. Location of amino acid substitutions in NHP2. Mutations of NHP2 in dyskeratosis congenita patients (along with mutations of dyskerin and NOP10) are mapped onto the crystal structure of *Pyrococcus furiosus* H/ACA RNP. Mutated residues are shown as orange Cα spheres and side chains of the *P. furiosus* structure. Dots represent the C-terminal extension caused by the stop codon mutation. The three NHP2 mutations are labeled. L7Ae (equivalent to NHP2) is colored gray, NOP10 is purple, Cbf5 (equivalent to dyskerin) is green, GAR1 is blue, and the H/ACA RNA is yellow. The figure was prepared by using PyMOL (DeLano Scientific LLC) by Keqiong Ye.

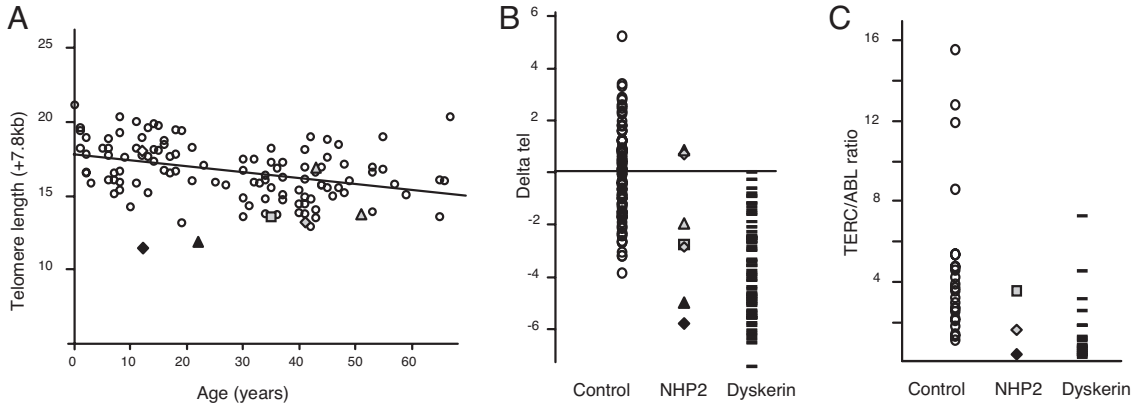

Fig. 3. NHP2 mutations result in short telomeres and reduced TERC levels. (*A*) Telomere length measurements in 112 healthy control subjects (open circles) are plotted against age with a line of best fit. Members of families in which NHP2 mutations are segregating are shown as gray triangles (Y139H heterozygotes), a black triangle (Y139H homozygote), gray square (V126M heterozygote), gray diamond (X154R heterozygote), black diamond (V126M/X154R, compound heterozygote), and open diamond (normal sibling). (*B*) Age-adjusted telomere lengths (delta tel, the difference between the observed telomere length and the length expected from the line of best fit drawn in panel a) for healthy control subjects (open circles, $n = 112$), NHP2 family members (as in *A*), and patients with dyskerin mutations (dashes, $n = 67$). (*C*) TERC levels, expressed as a TERC/ABL ratio, in healthy control individuals (open circles, $n = 24$), NHP2 family members (as in *A*), and patients with dyskerin mutations (dashes, $n = 27$).

TERC, which suggested that telomere maintenance may also be affected through reduction in TERC levels in patients with biallelic *NHP2* mutations. In one of the cases presented here, where the appropriate material is available, we see that the TERC level is lower than that observed in healthy control subjects and comparable with levels seen in patients with dyskerin mutations (Fig. 3*C*). The TERC levels in both heterozygous parents lie within the normal range.

To demonstrate that the reduction in TERC levels observed in patient material could arise as a consequence of the *NHP2* mutations, *NHP2* was knocked down in HeLa cells by using siRNAs against *NHP2*. Two independent siRNAs were highly effective, reducing *NHP2* mRNA levels by >95% (Fig. 4*A*). This knockdown translated into significantly reduced levels of TERC at 48 and 72 h after transfection (Fig. 4*B*). Interestingly, siRNA-mediated knockdown of *GAR1* in HeLa cells was not associated with reduced TERC levels (Fig. 4 *C* and *D*). To investigate the pathogenicity of the identified mutations, we have expressed mutant and wild-type *NHP2* in HeLa cells in which the endogenous *NHP2* is depleted. These experiments

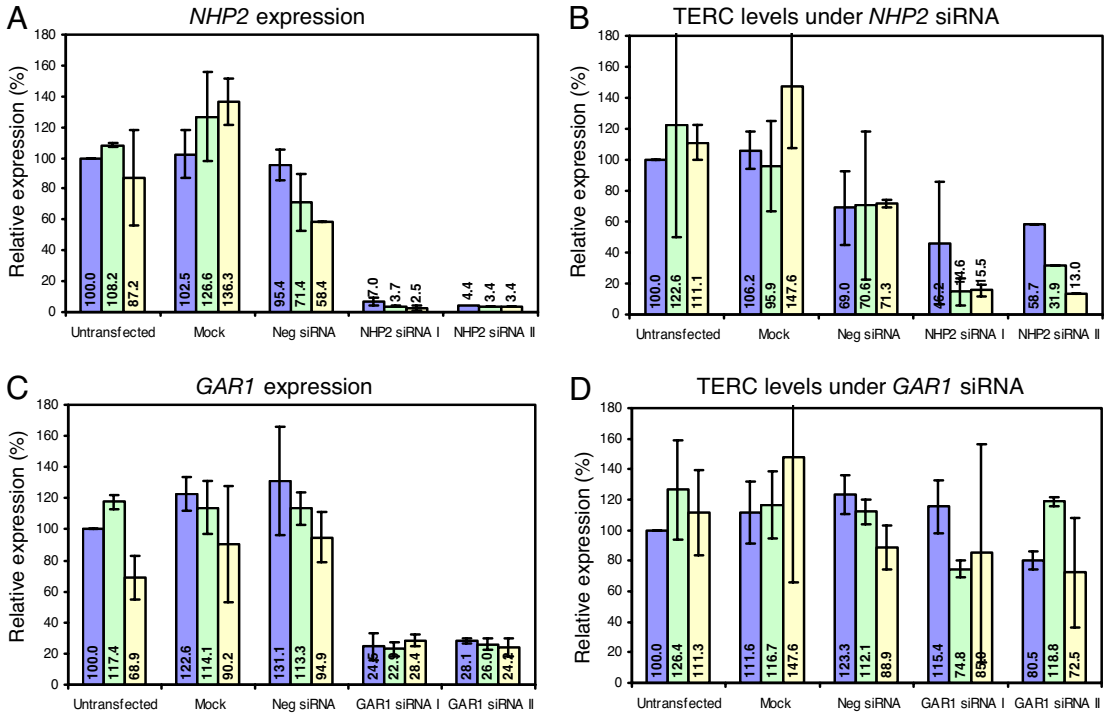

Fig. 4. Depletion of *NHP2* but not *GAR1* results in reduced TERC levels. The amount of *NHP2*, *GAR1*, and TERC, measured by QRT-PCR and normalized to ABL, is expressed relative to the level seen in the untransfected cells at the 24 h time point. The different manipulations are indicated below each panel. Each bar shows the mean of two experiments: blue bars, 24 h; green bars, 48 h; yellow bars, 72 h. (*A*) *NHP2* expression. (*B*) TERC levels under manipulation with *NHP2* siRNAs. (*C*) *GAR1* expression. (*D*) TERC levels under manipulation with *GAR1* siRNAs.

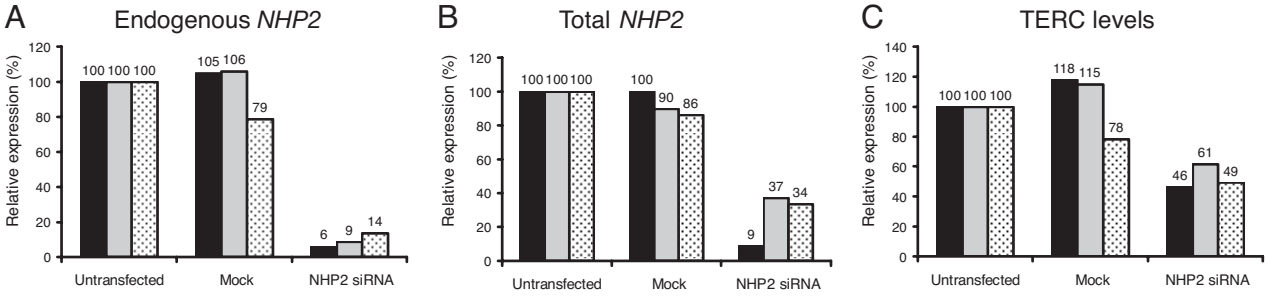

Fig. 5. Wild-type, but not mutant, *NHP2* can increase TERC accumulation. Expression levels of endogenous *NHP2* (*A*), total *NHP2* (*B*), and accumulation of TERC (*C*) are shown in HeLa cell lines into which either an empty vector (black bars), wild-type NHP2 (gray bars), or c.415T>C mutant NHP2 (dotted bars) have been stably transfected. Endogenous and total *NHP2* are distinguished by using different TaqMan probes specific to either the 3′ UTR (endogenous only) or to the coding sequence (total) of *NHP2*. The effect of *NHP2* siRNA, which is specific to the endogenous transcript, is shown relative to the untransfected cells as indicated beneath each panel.

suggest that expression of wild type *NHP2* can increase TERC accumulation compared with cells with exogenous mutant *NHP2* (Fig. 5).

Discussion

In this study, we show that biallelic mutations in the telomerase-snoRNP component NHP2 cause dyskeratosis congenita. Heterozygous carriers of these mutations are asymptomatic and this pattern of inheritance therefore represents an autosomal recessive form of the disease. It has previously been shown that mutations in other components of this ribonucleoprotein complex can give rise to the same disease phenotype. Dyskerin mutations, being X-linked, give rise to the disease in the hemizygous state whereas TERC mutations have been shown to cause dyskeratosis congenita through haploinsufficiency. Heterozygous mutations in the reverse transcriptase component of telomerase (TERT) are associated with a more variable phenotype (23–25, 27, 28), but have also been shown to cause a disease that resembles dyskeratosis congenita (22). More recently, homozygous mutations in TERT have been shown to cause the more severe form of dyskeratosis congenita (29, 30), and one family has been described in which the autosomal recessive inheritance of a missense mutation in NOP10 gives rise to a classical dyskeratosis congenita phenotype (17).

We can therefore now begin to see a pattern emerging indicating that mutations to the protein components that stabilize the telomerase and snoRNP complexes (dyskerin, NOP10 and NHP2) act only in a recessive form. We suggest that, when individuals possess only mutant alleles, the disease may present in the first generation. It is certainly true of the hemizygous *DKC1* mutations causing X-linked dyskeratosis congenita that often appear *de novo*. This presentation is in contrast to the telomerase-specific components (TERC and TERT), which can cause the disease in a heterozygous state, through haploinsufficiency, with variable penetrance and associated with disease anticipation. There is a suggestion that the heterozygotes for both *NOP10* mutations (17) and the *NHP2* mutations have a tendency toward shorter telomere lengths. This shortening is not the case for carriers of *DKC1* mutations, who have normal telomere lengths (T.V., unpublished data). This observation may not be surprising considering that the process of X-chromosome inactivation allows for the selection of cells that express only the normal *DKC1* allele in the peripheral blood of these heterozygotes. Recently described mutations in the shelterin component TIN2 seem to represent a different mechanism, whereby *de novo* heterozygous mutations can cause severe disease in the first generation (26).

NHP2 is an RNA binding protein, but the specificity for the binding of H/ACA snoRNAs by NHP2 comes through its association with dyskerin via the small intermediate protein NOP10 (7). The recently solved structure of the complete H/ACA snoRNP particle in archaea shows how these proteins form a triangular structure with the catalytic domain of dyskerin (Cbf5) at the center, and the three corners being formed by the PUA domain of dyskerin, NHP2 (L7ae in archaea) and Gar1 (Fig. 2) (31). Nop10 is sandwiched between dyskerin and Nhp2. The cognate RNA binds Cbf5, Nop10 and Nhp2 directly, whereas the Gar1 corner makes no contact with the RNA. Most of the amino acid substitutions that have been identified in dyskerin map to the PUA domain or the N-terminal extension of the protein that seems to wrap around this domain (32, 33). It is suggested that these substitutions could weaken the interaction between dyskerin and its cognate RNAs, leading to a decreased accumulation of the RNAs in the cell (31). The same is likely to be true of the residue Arg-34 in NOP10, which is mutated in autosomal recessive dyskeratosis congenita (17) as this residue has been shown to bind the RNA directly, right at its point of contact with dyskerin (31).

We now observe missense mutations in the third RNA-binding component of the complex, NHP2. One of these (Tyr139His) causes significant disruption to a highly conserved amino acid, which lies toward the C-terminal end of the protein. Another mutation gives rise to a large aberrant C-terminal extension to the protein, which would interfere with its ability to bind the other proteins as well as the cognate RNA (Fig. 2). However, an extended search for mutations in the non-RNA binding component, GAR1 (31, 32), has failed to reveal any amino acid substitution in this protein in dyskeratosis congenita patients. We also observe that, whereas knockdown of dyskerin (34), NOP10 (17), and now NHP2 leads to reduced TERC levels in human cells, it is not the case for GAR1; this observation suggests that, in human cells, GAR1 has a different impact on the accumulation of TERC compared with dyskerin, NOP10, and NHP2.

In yeast, accumulation of H/ACA snoRNPs depends upon the integrity of Nhp2p, which together with Cbf5p (dyskerin) and Nop10p constitute the core protein components of these particles (35, 36). It has been shown that depletion of Cbf5, Nop10, and Nhp2, but not Gar1, leads to destabilization of H/ACA RNAs and that, whereas the presence of Cbf5p, Nhp2p, and Nop10p is required for the accumulation of human TERC, Gar1p is not required (37). Similarly, the stepwise assembly of mammalian H/ACA ribonucleoparticles requires the core proteins dyskerin, NOP10, and NHP2 to be recruited to the site of transcription where they directly associate with the nascent H/ACA snoRNA, whereas GAR1 associates separately at a later stage by replacing the protein NAF1 from the already assembled H/ACA–snoRNP complex (7, 38).

Taken together, these results suggest that most of the telomerase mutations so far identified in patients with classical dyskeratosis congenita impact either directly or indirectly on the stability of RNA. In keeping with this impact, patients with dyskerin, NOP10, and now NHP2 mutations have all been shown to have low levels of telomerase RNA in their peripheral blood, providing direct evidence of their role in telomere maintenance in humans.

Methods

The Dyskeratosis Congenita Registry. All of the patients included in this study had been enrolled in the Dyskeratosis Congenita Registry initially at the Hammersmith Hospital and were relocated to Barts and the London School of Medicine and Dentistry (2006). The clinical criteria used for entry into this registry have been described in ref. 39. In addition to patients with the classical diagnostic mucocutaneous triad of dyskeratosis congenita, we have included patients who present with one or more of these features if in addition they have bone marrow failure and two of the other somatic abnormalities associated with this disease or if they have at least four of the features associated with the severe variant of this disease, previously recognized as the Hoyeraal–Hreidarsson syndrome. Patients presenting solely with aplastic anemia were not included: a large survey of this disease did not reveal pathogenic mutations in the core proteins of the snoRNP complex (25). All samples were obtained with informed consent and with the approval of our local ethics committee.

Mutation Screening. The coding sequence of the *GAR1* and *NHP2* genes, comprising seven and four exons, respectively, was amplified in standard PCRs (ABgene) from genomic DNA prepared from peripheral blood using DNA extraction kits (Gentra). Sequences of primers used in these PCRs are given in Table S2. The resulting fragments were screened for mutation by denaturing high-performance liquid chromatography on the WAVE DNA fragment analysis system (Transgenomic). Before analysis, each fragment was denatured at 95°C for 5 min and cooled slowly either with or without the mixing in of an equal amount of DNA amplified from a normal healthy control subject.

Telomere Length Measurement. As described in ref. 18, telomere lengths were determined in genomic DNA extracted from whole blood by Southern blot analysis using a subtelomeric probe (pTelBam8) from the long arm of chromosome 7, which includes ≈7.8 kb subtelomeric DNA. The size of peak signal intensity was determined by using the UVP Gelpro software and compared with values obtained from a set of 110 normal individuals.

Measurement of TERC Levels. RNA was prepared from whole blood using a QIAamp RNA blood mini kit (QIAGEN Germany). After DNase treatment, random hexamers (pdN6, Amersham Pharmacia) were used to prime cDNA synthesis in standard reactions. Expression of *TERC* and *ABL* were determined by quantitative real-time PCR on the ABI PRISM 7700 sequence detection system (Applied Biosystems) using primers and probes that have been described in ref. 17. Twenty-five-microliter reactions were performed by using TaqMan Universal PCR master mix, 2 μl of cDNA with 300 nM TERC primers and 200 nM TERC probe or 100 nM ABL primers and 300 nM ABL probe. As described elsewhere, standard curves were generated from dilutions of a recombinant TERC plasmid or control RNA samples prepared from the K562 cell line, and the levels of *TERC* expression were normalized to the level of *ABL* expression to obtain a *TERC/ABL* ratio for all samples.

siRNA Analysis. All siRNA experiments were performed on HeLa cells cultured at 37°C and 5% CO_2 in DMEM (Cambrex), supplemented with L-glutamine, 10% FCS, and penicillin/streptomycin antibiotics. Cells were seeded into six-well plates at a density of 2×10^5 per well 24 h before treatment. Transfections were carried out by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were transfected with two predesigned siRNAs to *NHP2* or *GAR1* (Ambion) at 50 nM, a predesigned negative control siRNA (Ambion) at 30 nM and a mock. Cells were harvested at 24-, 48-, and 72-h time points, and RNA was extracted by using an RNeasy mini kit (Qiagen GmbH). Quantitation of RNA levels was performed as described above, except that the delta Ct method was used to calculate relative expression levels, which were then compared with untreated control cells.

NHP2 Rescue. *NHP2* expression constructs and stable cell lines were prepared as described in ref. 17. Briefly, the coding sequence of the *NHP2* gene was amplified from cDNA, cloned into the expression vector pIRES2-EGFP (Clontech), and mutated to introduce the c.415T>C mutation. Stably transfected HeLa clones were pooled for use in transient siRNA transfection studies as described above. Cells from each line (with the empty vector, wild-type, or mutant *NHP2*) were transfected with siRNA against *NHP2*, and RNA was extracted after 48 h for *NHP2* and TERC analysis. It is important to note that the siRNA targets the 3′ UTR of the *NHP2* mRNA. This UTR is absent from the plasmid-derived transcript, resulting in siRNA depletion of the endogenous *NHP2* only.

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